REVIEWER REPORT 1

Comment 1
I am happy to see this study on the inositol phosphate stereoisomers in soils, particularly the lower-order esters. The inositol phosphates are a quantitatively important and ecologically interesting group of phosphorus compounds in soils, but much remains unknown. This study uses hypobromite oxidation and solution 31P NMR spectroscopy to identify inositol phosphate stereoisomers in four soils. The spectroscopic work is of high quality. The presence of the higher-order stereoisomers is well-established, but this work identifies several lower-order esters in various stereoisomeric forms. Although these have been reported previously by chromatography, and inferred in NMR studies based on resistance to bromination, this is the first direct identification by solution 31P NMR. I recommend publication, but ask the authors to consider the following comments in their revision.

Response 1
We thank the reviewer for the positive comments.

Comment 2
Hypobromite oxidation destroys organic matter except the inositol phosphates, but this statement seems true only for the higher-order esters. The hexaphosphates definitely resist bromination (e.g.Turner et al. (2012)). However, it seems that earlier papers on the method suggested at least partial decomposition of the pentakisphosphates and complete decomposition of other esters. If these compounds persisted here, particularly the tetrakisphosphates, this suggests the possibility that oxidation was incomplete (see below). Did the authors test the resistance of the target compounds to bromination? If not, it might be worth adding a statement about the extent to which the lower esters are expected to resist bromination.

Response 2
The main reaction pathway of the hypobromite oxidation procedure is the oxidation of organic matter and not its bromination. Our study is based on existing publications using hypobromite oxidation to isolate IPs. However, the action of hypobromite oxidation on each IP species, and also on 'organic matter', has not been clearly determined. The resistance of IP to hypobromite oxidation is considered to be due to increased steric hindrance and the high charge density of the organic molecule. Hence, the resistance of lower order IP to hypobromite oxidation decreases with decreasing number of phosphate groups bound to the molecule. We agree with the reviewer that it is possible some IP_4 was partially oxidised to $IP_{3\text{-}1}$. We have made this clearer in the body text.

We inserted the sentence (Lines 301-304): This could possibly be due to the partial dephosphorylation of *myo*-IP_4 during the hypobromite oxidation procedure. The reason of the reduced resistance of lower-order IP to hypobromite oxidation compared to IP_{5+6} might be due to their reduced steric hindrance and charge density, as less phosphate groups are bound to the inositol ring.

Lastly, we note that Irving and Cosgrove (1981) reported inositol hexa- and penta-kisphosphates were resistant to hypobromite oxidation. Furthermore, in the current study, several peaks assigned to hexa- and pentakisphosphates in the hypobromite oxidised extracts were also present in the untreated extracts. Whilst the absolute concentration of these IPs may be questioned, we provide supporting evidence for their presence, which can be easily identified using solution ^{31}P NMR spectroscopy.

Comment 3
There appears to be a couple of problems with the bromination procedure here.
First, it appears that there was incomplete oxidation, with persistence of some diesters,

phosphonates, inositol tetrakisphosphates, and the broad signal (assuming it represents high molecular weight organic matter). Second, and as discussed by the authors, there appears to have been considerable loss of phosphorus during bromination, perhaps through precipitation, as indicated by a loss of orthophosphate, pyrophosphate, and the inositol hexakisphosphates. Inorganic phosphate should increase markedly following bromination, as organic phosphates are destroyed and converted to inorganic orthophosphate. This isn't a problem for identification, but represents a problem for the quantification of compounds in the brominated extracts, at least if these values are to represent concentrations of the identified forms in the original soil. Given the precipitation issue, the concentrations in brominated extracts should probably be considered unreliable, and it'd be better to give quantitative values only from those signals identified in the unbrominated extracts. Data from the brominated extracts are of course still useful as qualitative identifications.

Response 3

The ratio of soil extract to bromine used in previous studies were 50 (Turner et al., 2012), 25 (Turner and Richardson, 2004), 20 to 10 (Turner, 2020), and 10 (Almeida et al., 2018). Consequently, the ratio of volume of soil extract to bromine used in the current study (16.7) is similar and at the higher end of that reported in previous studies. Nevertheless, we carried out a pilot study to test different soil extract to bromine ratios on spectral quality in the Gleysol soil, which had the highest organic matter content among the soils analysed in the current study: ratios covered 50.0, 25.0, 16.7, and 12.5. Solution ^{31}P NMR spectroscopy on the hypobromite oxidised soil extracts revealed the overall peak diversity and intensity was highest for the 16.7 ratio (i.e. 0.6 mL Br_2 addition) (see Figure 1). Furthermore, we added a *myo*-IP_6 standard of known concentration to the Gleysol extract prior to hypobromite oxidation at the aforementioned ratios. These results showed that the recovery of added *myo*-IP_6 was highest (38%) for the 16.7 ratio compared to the 25.0 ratio (31%) or 12.5 ratio (32%). Of course, a problem with continuing to decrease the ratio of soil extract to bromine is that further oxidation of IP may occur.

Unfortunately, previous studies have not reported quality assurance/control data for the ratio of soil extract to bromine. Nevertheless, solution ^{31}P NMR spectra on hypobromite oxidised extracts in previous studies appear to show a broad signal in the phosphomonoester region based on a visual assessment: see Figure 3 in Turner et al. (2012) and Figure 3 in Turner and Richardson (2004). The authors did not include an underlying broad signal in their spectral deconvolution process. However, the study of Reusser et al. (2020a) showed that the inclusion of a broad signal in the phosphomonoester region is important for accurate quantification of the overlying sharp signals (i.e. *myo*-IP_6).

The persistence of on average half the organic P compounds as part of the broad signal in the phosphomonoester region highlights their chemical stability. Please also see Response 8 for more information.

The majority of NMR signals in the phosphodiester and phosphonate regions were removed following hypobromite oxidation. The small presence of some phosphodiesters or phosphonates in the Cambisol or Gleysol soils was interesting, but their identity is unclear. It is possible that a portion of these compounds may be protected from oxidation due to their complexation with other organic molecules and metals.

We consider the quantification of lower order IP in soil extracts following hypobromite oxidation to be a conservative estimation. This was stated in the body text (lines 435-441 of the initial manuscript). Whilst the reviewer is correct that some lower-order IP may have been oxidised, these extracts also have the advantage of reduced signal overlap, which facilitates peak assignment and spectral fitting.

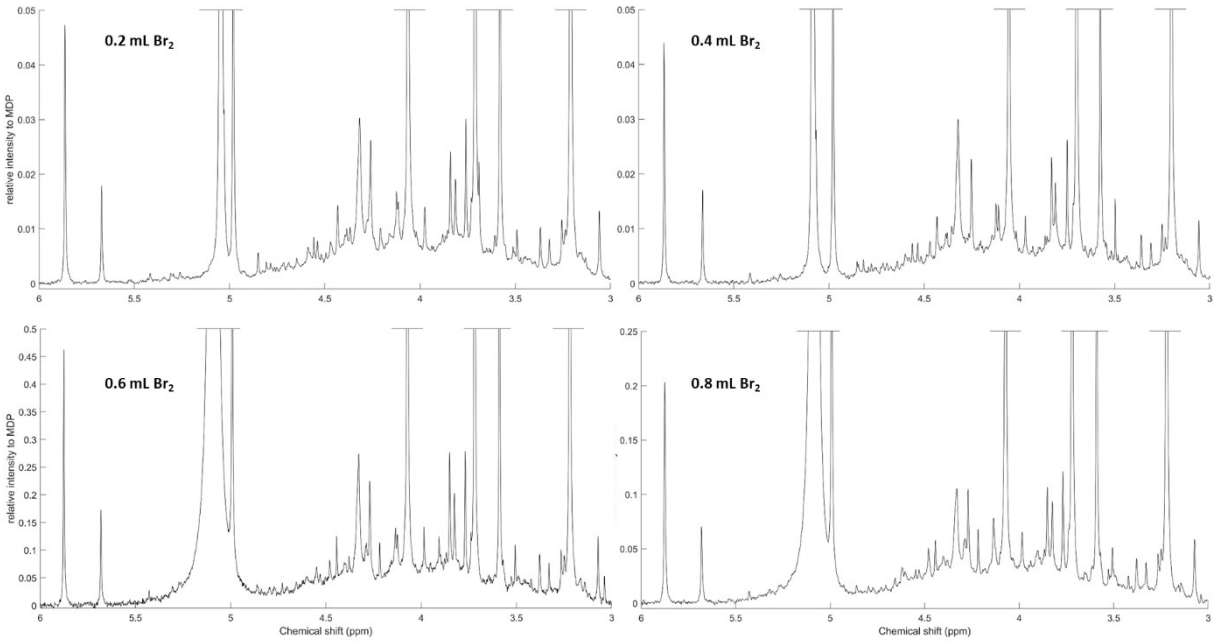

Figure 1. Solution ^{31}P nuclear magnetic resonance (NMR) spectra (500 MHz) of the orthophosphate and phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Gleysol extract, using 0.2 mL, 0.4 mL, 0.6 mL and 0.8 mL Br_2 in the hypobromite oxidation procedure. Signal intensities were normalised to the MDP peak (intensity of 1 on y-axes).

We added Figure 1 to the Supporting Information (Figure SI9), referring to it in the body text:
Line 134-136: The optimal volume of Br_2 for oxidation was assessed in a previous pilot study using 0.2, 0.4, 0.6 and 0.8 mL Br_2 volumes, and then observing differences in their NMR spectral features (Figure SI9).

Comment 4
It has been claimed that inositol phosphates account for a negligible amount of soil organic phosphorus and that their importance in the soil has been over-emphasized in the literature. This argument was made sufficiently strongly by one group that a prominent mycorrhizal ecologist, now sadly deceased, rewrote the section on inositol phosphate utilization by ectomycorrhizal fungi in her influential textbook. The authors might consider mentioning this in the discussion section, given the relatively large concentrations of inositol phosphates they detected in their soils.

Response 4
It depends on the soil, some soils contain a relatively high proportion of organic P as phytate, others not. We think that the reviewer refers to Smith et al. (2008). In this textbook, the study of Smernik and Dougherty (2007) was cited, who reported that phytate concentrations comprised less than 5% of total organic P in Australian soils. In our study, IP comprised between 1% and 18% of the total pool of organic P in European soils. On the point of IP utilisation by ectomycorrhizal fungi, we do not believe our study addresses this aspect as even low concentrations of phytate could be considered important depending on turnover, and we would therefore prefer not to comment.

Comment 5
The 'broad signal' is supposed to consist of high molecular weight organic compounds. These should be destroyed by hypobromite oxidation. If not, this suggests that either (1) the oxidation was incomplete, or (2) the broad signal is caused by something else other than high molecular weight compounds. The authors might comment on this.

Response 5
Correct, a portion of phosphomonoesters as part of the 'broad signal' has been found with apparent high molecular size (McLaren et al., 2015; McLaren et al., 2019) and appears to be associated with soil organic matter (McLaren et al 2020). However, as discussed in Response 2, factors increasing the resistance to hypobromite oxidation are steric hindrance and high charge density of an organic compound. Consequently, the action of hypobromite oxidation on phosphomonoesters exhibiting a broad NMR signal is unknown as their structure is undefined.

In general, sugars and ribonucleotides can most certainly be destroyed by hypobromite oxidation. However, there could be molecules with high molecular weight which would not be oxidised, e.g. highly resistant organic pesticides. To test this, one would need to carry out hypobromite oxidation on known compounds of high molecular weight present in soil and evaluate their resistance. Unfortunately, the composition of high molecular weight material in soil is not fully understood. We do not believe that hypobromite oxidation was incomplete based on details provided in Response 3, and that Br_2 was present in excess and soil extracts were kept at reflux after Br_2 addition. Furthermore, we note that based on a visual assessment a broad signal was also present in soil extracts following hypobromite oxidation in previous studies (Turner and Richardson, 2004; Turner et al., 2012).

Since a broad signal was observed in the NMR spectra on hypobromite oxidised extracts, we wanted to understand its structural composition. We carried out transverse relaxation (T_2) experiments in order to determine if the broad signal itself was comprised of (i) a series of neighbouring sharp peaks, which would likely arise from small molecules such as IP, or (ii) one (or a few) broad peak(s), which would likely arise from complex structures of 'higher' molecular weight (Bloembergen et al .1948). Our results support the latter, which suggest the remaining NMR signal as part of the broad signal is comprised of molecules with larger apparent molecular size than IP. Please also see Response 27.

In our study, we also propose a third option, namely that the complex structure of the compounds as part of the broad signal following hypobromite oxidation is due to their 'protection' via metals or configuration which enhances steric hindrance. Please also see Response 8.

Comment 6
Related to the broad signal, I think it would be worth explaining a little more about the deconvolution procedure used here. Some recent studies appear to have deconvoluted from the baseline to the top of the peaks in the monoester region, which is certain to overestimate the proportion of each signal. This might in turn exagerate differences between signals in brominated unbrominated extracts, given that the 'broad signal' appears to be reduced by bromination.

Response 6
The reviewer is correct. Studies that carry out spectral deconvolution by fitting sharp peaks from the peak maxima to the baseline will likely overestimate the proportion of sharp peaks. This was demonstrated in a recent study, which found that fitting a broad signal was needed for accurate quantification of organic P compounds (e.g. *myo*-IP_6) (Reusser et al., 2020b). In the current study, spectral deconvolution fitting was carried out with an underlying broad signal in the phosphomonoester region, as described in Reusser et al. (2020b). Briefly, we carried out scripts containing a non-linear optimization algorithm in MATLAB® R2017a (The MathWorks, Inc.) and fitted visually identifiable peaks by constraining their line-widths at half height as well as the lower and upper boundary of the peak positions. The sharp signals of high intensity (e.g. orthophosphate) and the broad peak were fitted using a Lorentzian lineshape, whereas sharp signals of low intensity were fitted using a Gaussian lineshape. We have made this clearer in the body text.

Inserted (Line 198-206): Due to overlapping peaks in the orthophosphate and phosphomonoester region, spectral deconvolution fitting (SDF) was applied as described in Reusser et al. (2020b). In brief, the SDF procedure involved the fitting of an underlying broad signal, based on the approach of Bünemann et al. (2008) and McLaren et al. (2019). We carried out the SDF with a non-linear optimisation algorithm in MATLAB® R2017a (The MathWorks, Inc.) and fitted visually identifiable peaks by constraining their line-widths at half height as well as the lower and upper boundary of the peak positions along with an underlying broad signal in the phosphomonoester region. The sharp signals of high intensity (e.g. orthophosphate) and the broad peak were fitted using Lorentzian lineshapes, whereas sharp signals of low intensity were fitted using Gaussian lineshapes.

Line-by-line comments

Comment 7 (Line 12)
most studies have identified inositol phosphates by NMR in recent decades, not chromatography. Perhaps you refer specifically to lower esters, in which case perhaps state this at the start of the sentence.

Response 7
We made this clearer in the text:

Changed from (Lines 10-12): This is because their quantification typically requires a series of chemical extractions, including hypobromite oxidation to isolate inositol phosphates, followed by chromatographic separation.

Changed to (Lines 10-12): This is because the quantification of lower-order IP typically requires a series of chemical extractions, including hypobromite oxidation to isolate IP, followed by chromatographic separation.

Comment 8 (Line 17)
shouldn't the 'broad signal' be destroyed by hypobromite oxidation?

Response 8
Please see Response 5. In addition, IP are considered to resist hypobromite oxidation due to steric hindrance and high charge density. The structural configuration and exact chemical nature of the compounds causing the broad signal in the phosphomonoester region is not known. Studies have shown that these compounds are of complex structure, apparent high molecular weight and resistant to enzymatic hydrolysis (Jarosch et al., 2015; McLaren et al., 2015; McLaren et al., 2019). Hence, as the chemical structure is unknown, its resistance to hypobromite oxidation could not be evaluated in advance. Nevertheless, our study shows that on average half of the organic P as part of the broad signal was oxidised following hypobromite oxidation. The remaining broad signal which is resistant to hypobromite oxidation suggests complex structures of high chemical stability. This has been stated in the manuscript (lines 462-464 in the initial manuscript).

Comment 9 (Line 20)
I understood that one of the myo-IP_5 forms (myo-inositol-1,3,4,5,6) is supposed to be rare in nature and therefore unlikely to occur in soils. This is because phytases cleave phosphates other than the C-2 phosphate, often leaving myo-inositol-2-phosphate as the final product. It's therefore a surprise to see this compound detected in two of the soils here. Could the authors comment on this?

Response 9
myo-(1,3,4,5,6)-IP_5 was reportedly measured as the thermal decomposition product of a phytate standard (Doolette and Smernik, 2018). It is possible that *myo*-IP_6 undergoes transformation via abiotic means to *myo*-(1,3,4,5,6)-IP_5, which could then be adsorbed by soil

constituents. Alternatively, *myo*-(1,3,4,5,6)-IP_5 could have been added biologically. For example, Stephens and Irvine (1990) report *myo*-(1,3,4,5,6)-IP_5 as an intermediate in the synthesis of IP_6 from *myo*-IP in the cellular slime mould *Dictyostelium*. In addition, Sun et al. (2017) report *myo*-(1,3,4,5,6)-IP_5 to occur as part of a possible minor pathway in the degradation of *myo*-IP_6 by *Aspergillus niger* phytase and acid phosphatase from potato. Later, Sun and Jaisi (2018) reported the presence of *myo*-(1,3,4,5,6)-IP_5 in different animal feeds and manures. We have revised the manuscript accordingly:
Lines 399-405: It is possible that an abiotic transformation of *myo*-IP_6 to *myo*-(1,3,4,5,6)-IP_5 occurs, which could then be adsorbed by soil constituents. Stephens and Irvine (1990) reported *myo*-(1,3,4,5,6)-IP_5 as an intermediate in the synthesis of IP_6 from *myo*-IP in the cellular slime mould *Dictyostelium*. Therefore, *myo*-(1,3,4,5,6)-IP_5 could have been biologically added to the soil. Furthermore, *myo*-(1,3,4,5,6)-IP_5 was present in different animal feeds and manures (Sun and Jaisi, 2018). Sun et al. (2017) reported *myo*-(1,3,4,5,6)-IP_5 and *myo*-(1,2,4,5,6)-IP_5 as intermediates in the minor, resp. major pathways of Aspergillus niger phytase and acid phosphatase (potato) phytate degradation.

Comment 10 (Line 43)
this is only partially correct – pigs are monogastrics, but phytate is still hydrolyzed during passage through the animal – probably in the hindgut – so pig manure tends to contain little phytate. See for example: Leytem, A. B., B. L. Turner, and P. A. Thacker. 2004. Phosphorus composition of manure from swine fed low-phytate grains: Evidence for hydrolysis in the animal. Journal of Environmental Quality 33:2380-2383. Turner, B. L., and A. B. Leytem. 2004. Phosphorus compounds in sequential extracts of animal manures: chemical speciation and a novel fractionation procedure. Environmental Science and Technology 38:6101-6108.

Response 10
We agree that the study of Leytem et al. (2004) indicates that phytate can be hydrolysed during passage through the animal. However, the authors did not measure lower order IP in their samples. Therefore, it is not known if a complete hydrolysis of phytate occurred or if IP_6 was hydrolysed to IP_5. We added this to the manuscript along with referring to transgenic pigs:

Changed from (lines 42-44): However, the addition of *myo*-IP_6 to soil can also occur via manure input because monogastric animals are incapable of digesting *myo*-IP_6 without the addition of phytases to their diets (Leytem and Maguire, 2007; Turner et al., 2007).

Changed to (lines 42-46): However, the addition of *myo*-IP_6 to soil can also occur via manure input because monogastric animals are mostly incapable of digesting *myo*-IP_6 without the addition of phytases to their diets (Leytem and Maguire, 2007; Turner et al., 2007). An exception to this are pigs, which were found to at least partially digest phytate (Leytem et al., 2004), and transgenic pigs expressing salivary phytase (Golovan et al., 2001; Zhang et al., 2018).

Comment 11 (Line 76)
perhaps add ‘and a chelating agent’ – the EDTA is important in the single-step extraction.

Response 11
Agreed, we added ‘and a chelating agent’.

Comment 12 (Line 80)
this was presumably the case in Turner and Richardson 2004, who presented chemical shifts of lower scyllo-IP esters, but did not detect the corresponding signals in NMR spectra of soil extracts.

Response 12
The author's assessment of the study by Turner and Richardson (2004) may be correct, which is discussed using their more recent study (Turner et al., 2012) in the following section. Other possible reasons are a low signal-to-noise ratio of their NMR spectra using their experimental procedure, or a focus on IP_6 rather than lower-order IP. We would prefer not to speculate in the manuscript, and have not made any changes.

Comment 13 (Line 97)
it's not clear why these four soils were chosen for study – perhaps add a brief explanation.

Response 13
Agreed, we inserted the sentence (Lines 106-107): The four soil samples were chosen from a larger collection based on their diverse concentration of P_{org} and composition of the phosphomonoester region in NMR spectra (Reusser et al., 2020b).

Comment 14 (Line 118)
This sentence seems redundant if the method was the same. Delete?

Response 14
Agreed, we have deleted the sentence.

Comment 15 (Line 121)
Turner recently published the hypobromite method as a chapter in the new book on inositol phosphate methods, which might be appropriate to cite here: Turner, B. L. 2020. Isolation of inositol hexakisphosphate from soils by alkaline extraction and hypobromite oxidation. Pages 39-46 in G. J. Miller, ed. Inositol Phosphates: Methods and Protocols. Springer US, New York, NY.

Response 15
Our study was carried out before the publication of Turner (2020), but is based on the method described in Turner et al. (2012). We have revised the text as follows:

Lines 129-130: The hypobromite oxidation procedure was similar to that reported in Turner (2020).

Comment 16 (Line 190 and 221)
Please provide more information on the deconvolution procedure.
Some recent studies appear to have deconvoluted from the baseline to the top of the peaks in the monoester region, which is certain to overestimate the proportion of each signal. This might in turn lead to differences between signals in brominated unbrominated extracts.

Response 16
Please see Response 6.

Comment 17 (Line 262)
What could the broad signal possibly be, in brominated extracts?

Response 17
Please also see Response 8. Furthermore, we speculate that it is a mixture of organic P compounds of complex structure, what could cause steric hindrance, and compounds that contain metal bridges and/or high charge densities, which hinder hypobromite oxidation.

Comment 18 (Line 225)
comma instead of period. The persistence of some phosphodiesters suggests

incomplete oxidation.

Response 18
We could not find the relevant text that the reviewer is referring to at Line 225 (or elsewhere in the manuscript). We are happy to review this upon advice on the location of the text.

Comment 19 (Line 276)
this depends on how spectra were deconvoluted – see point above.

Response 19
Please see Response 6.

Comment 20 (Line 278)
It's interesting to see evidence for the two conformers of *neo*-IP_6. The proportion of the two conformers is definitely related to pH – is it possible that pH was <12 in the extracts, promoting the presence of the two forms?

Response 20
Yes, indeed. However, we dissolved the freeze-dried material in 600 µL of 0.25 M NaOH solution, which was spiked with 25 µL of NaOD. We did not measure the pH of the final extract for NMR analysis but the minimal change in the chemical shift of the orthophosphate peak and its location compared to the four *myo*-IP_6 peaks suggest that the pH was above 12 (Crouse et al., 2000).

Comment 21 (Line 283)
Aren't lower-order esters destroyed by bromination?

Response 21
Please see Response 2.

Comment 22 (Line 292)
Turner and Richardson 2004 reported signals for two different scyllo-IP4 compounds. Signals from these were not identified in brominated soil extracts, but resolution was not as high as in this study. It looks like only a single scyllo-IP4 isomer was assessed here, so perhaps scyllo-IP4 is underestimated (assuming that the other scyllo-IP4 isomer occurs in soils, and that the tetrakisphosphates resist bromination).

Response 22
The reviewer is correct. Obtaining additional standards may increase the detection and amount of lower-order IP in soil extracts. Unfortunately, we were only able to test one *scyllo*-IP_4 isomer. This is partly due to limited time and resources, and the rarity of lower-order IP standards. We have revised the manuscript:

Insert (Lines 412-414): Turner and Richardson (2004) reported NMR-signals for two other *scyllo*-IP_4 isomers, which could not be tested for in this study due to the lack of available standards.

Comment 23 (Line 311)
6 in subscript.

Response 23
Corrected.

Comment 24 (Line 327)
orthophosphate should increase following bromination, as organic phosphates are converted to inorganic orthophosphate. This indicates precipitation or loss

of phosphates in some other way during the bromination procedure.

Response 24
During the hypobromite oxidation, phosphates are precipitated with barium acetate, washed with ethanol and then re-dissolved with ion exchange resins. During these processes, a loss of both, IP and orthophosphate presumably occurs, which we highlight in the manuscript (Lines 436-441 in the initial manuscript):

Since the main cause of resistance of IP to hypobromite oxidation is that of steric hindrance, which generally decreases with decreasing phosphorylation state and conformation of the phosphate groups (axial vs. equatorial), we assume that low recoveries of added *myo*-IP_6 is due to losses of precipitated P_{org} compounds during the precipitation and dissolution steps. This is supported by the decrease in the concentration of orthophosphate following hypobromite oxidation compared to untreated extracts. Therefore, quantities of IP as reported in the current study should be considered as conservative.

Comment 25 (Line 404)
also along the Haast chronosequence: Turner, B. L., A.Wells, and L. M. Condron. 2014. Soil organic phosphorus transformations along a coastal dune chronosequence under New Zealand temperate rain forest. Biogeochemistry 121:595-611. The Baker study on the Franz Josef involved the same sites as Turner et al. 2007, so the separate statement on the Baker study could probably be deleted and the citation rolled into with the others.

Response 25
Agreed, we have inserted this citation.

Comment 26 (Line 418)
see above. I think the concentrations on the brominated extracts should be considered unreliable, given the apparent loss of phosphorus during the procedure.
It'd probably be better to focus on quantitative values from comparable signals in the unbrominated extracts, and give information from the brominated extracts as qualitative identifications.

Response 26
For this reason, we showed both, the concentrations of organic P compounds before and after hypobromite oxidation (Table 4, Table SI1). However, peaks in the phosphomonoester region of untreated extracts have greater overlap, which can affect the accurate quantification of peaks belonging to lower-order IP. Hence, we used the hypobromite oxidation method, which was designed to isolate the IP fraction of soils (Cosgrove and Irving, 1980). Please also see Response 3.

Comment 27 (Line 434)
My impression is that the complexity of the monoester region means that deconvolution of all signals could easily account for the apparent broad signal. How does the possibility of more than one compound affect the accuracy of the deconvolution based on a single broad signal?

Response 27
Indeed, the findings of McLaren et al. (2019) and our study suggest that the broad signal itself is comprised of several components. These components are taken into account by including the broad signal into the spectral deconvolution fitting procedure (Lines 455-458 in the manuscript). We carried out the T_2 relaxation experiment in order to determine if the broad signal itself was comprised of a series of sharp peaks (i.e. inhomogeneous broadening) derived from small molecules, or perhaps a single (or few) peak (i.e. homogeneous broadening) derived from large and polymeric molecules (Schmidt-Rohr and

Spiess, 1994; McLaren et al., 2019). Furthermore, the transverse relaxation time is inversely related to the molecular size, i.e. larger molecules exhibiting shorter T_2 times than smaller molecules (Bloembergen et al., 1948; Claridge, 2016). As our results show, the T_2 times of the broad signal is significantly shorter compared to the ones of the IP, showing that it is not comprised of many sharp signals as IP but rather few broader signals generated by larger molecules or associations of molecules.

Comment 28 (Line 436)
This paragraph is awkward. First, the broad signal is supposedly made up of high molecular weight organic matter, which should be destroyed by bromination. Second, whether the compound forming the broad signal (or compounds, if they exist) occur in the soil is open to question – most scientists working on soil organic matter now accept that much of the high molecular weight material in alkaline soil extracts is formed as an artifact of the extraction procedure. Finally, the statement that the broad signal didn't change after 62 years of cropping seems to indicate precisely the opposite interpretation to that of the authors – that it demonstrates its importance in the soil P cycle. If it's so stable that it never changes, that suggests to me that it's actually fairly unimportant, at least ecologically or agronomically.

Response 28
Our hypobromite oxidised NMR spectra showed both, sharp signals and an underlying broad signal fitted with the spectral deconvolution fitting procedure. Because of that, we wanted to test if the broad signal was comprised of many sharp signals generated by small molecules (e.g. IP) or if other, larger molecules were causing the broad signal as reported in McLaren et al. (2015). To test this, we used a 'spin-echo' experiment to determine the transverse relaxation (T_2) times of the phosphomonoesters. Our results show that the T_2 times of compounds causing the broad signal were different to those of the IP. Therefore, the former are behaving as molecules of apparent high molecular size. Consequently, this broad signal must be taken into account when carrying spectral deconvolution fitting.

The mechanisms for the formation of this phosphomonoester(s) as part of the broad signal are not known. We are not aware of any evidence that shows the broad signal to be an artefact, or that they are formed during the extraction procedure. Our current model appears to be consistent with the organic matter literature. Nebbioso and Piccolo (2011) reported that high molecular weight material of organic matter in soil is an association of smaller organic molecules. These associations however would still cause a broad signal in the phosphomonoester region of soil extracts and could be a reason that some organic molecules containing P are protected from hypobromite oxidation. We have made this clearer in the body text.

Insert Line 461-464: <u>Nebbioso and Piccolo (2011) reported that high molecular weight material of organic matter in soil results from the association of smaller organic molecules. We suggest that these associations would still cause a broad signal in the phosphomonoester region of soil extracts and could be a reason that some organic molecules containing P are protected from hypobromite oxidation.</u>

We consider the compounds causing the broad signal to be important because of two reasons: 1) it exhibits a P pool of considerable amount and unknown structure, whose mobility and potential plant availability (e.g. with certain management strategies) are not known and; 2) the concentrations of more readily available organic P compounds may have been overestimated in the past by attributing the peaks of IP and the broad peak to nucleotides and phospholipid hydrolysis products. Please also see Response 8.

Comment 29 (Table 3)
you could combine this table with Table 1 to streamline display items.

Response 29
We would prefer not to combine these two tables as Table 1 shows general soil properties not measured in this study and Table 3 focuses on P concentrations based on methods presented in the M&M section. Therefore, we consider Table 3 to be better suited in the Results section.

Comment 30 (Table 4)
indicate that the broad peak also represents phosphomonoesters.

Response 30
Agreed, we added '<u>in phosphomonoester region</u>'.

Comment 31 (Table 5)
I think it's fairly safe to assume that the *chiro*-IP_6 is the D form, given that L-*chiro*-inositol has never been detected in phosphorylated form in nature. Also it's interesting to see from this table that the *neo*+D-*chiro*-IP_6 and the majority of the lower order esters were detected only in two of the four soils. I didn't get this impression from reading the text.

Response 31
Agreed, we have changed *chiro*-IP_6 2-eq/4-ax to <u>D-*chiro*-IP_6 2-eq/4-ax</u>.

We reported in the Result section 3.3, lines 290-293 (initial manuscript): *neo*-IP_6 was identified in the the 2-equatorial/4-axial and 4-equatorial/2-axial conformations, and c*hiro*-IP_6 in the 2-equatorial/4-axial confirmation, of the oxidised extracts in the Cambisol and Gleysol, <u>but were absent in the Ferralsol and the Vertisol</u> (Fig. SI4 and SI5 in the Supporting Information).

To make this clearer in the Discussion section, we inserted (Lines 372-374): In the current study, both conformations could be identified <u>in two of the four soil extracts</u>, which is likely due to improved spectral resolution and sensitivity.

Comment 32 (Table S1)
this indicates a considerable proportion of the phosphorus has been lost during the bromination procedure.

Response 32
Please see Responses 3, 24 and 26.

REFERENCES

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REVIEWER REPORT 2

Comment 1
The objective of this manuscript was to characterize and quantify inositol phosphates(IP) in soil extracts following hypobromite oxidation using ^{31}P nuclear magnetic resonance (P NMR) spectroscopy. This is a very technical paper with respect to the chemical methods utilized. Given that the mandate of this journal is: “interactions between the biological, chemical, and physical processes in terrestrial or extra-terrestrial life with the geosphere, hydrosphere, and atmosphere. The objective of the journal is to cut across the boundaries of established sciences and achieve an interdisciplinary view of these interactions” (from the journal website)”, this paper does not seem like a good fit for the journal. While the authors identified a wide range of different P compounds in their four soil samples, no attempt was made to relate these compounds back to broader biological, chemical or physical processes within these soils. As such, it will not be of interest to the majority of Biogeosciences readers, as currently written, and will likely be overlooked by the scientists who would be interested in such a technical paper. In my opinion, this would be a better fit in either an environmental chemistry journal or in the chemistry section of a soil science journal. Thus, in my opinion the authors should withdraw this paper from this journal and submit it to another journal that better fits the paper’s focus. If the authors choose not to do this, then they must significantly revise the manuscript to keep it within the journal’s scope, to clearly demonstrate the significance of these identified P compounds to P cycling in these soils, and to P cycling more broadly.

Response 1
Inositol phosphates are a very important component of the P cycle in both agricultural and environmental contexts. Indeed, several recent reviews have highlighted a stagnation of advancing our knowledge of the P cycle to address global challenges due to a lack of knowledge on organic P (George et al., 2018; Haygarth et al., 2018; McLaren et al., 2020). Our study provides new information on the chemical nature of a multitude of organic P species, which is essential to understand processes relating to their flux in nature and their function in the soil system. Furthermore, the production/accumulation as well as the hydrolysis of IP to lower order IP, involves the cycling of P and C in soil.

We used a novel approach of combining chemical extraction, hypobromite oxidation, and multiple NMR techniques to better understand the chemical composition of soil organic P. Furthermore, we strongly believe that our publication will be of great interest to a broad audience, including scientists working in agriculture, environment, sediments and waters. Lastly, we highlight that our study is the first to report the existence of 11 inositol phosphate species using direct spectroscopic evidence, and also provide new insight on the chemical and structural composition of ‘complex’ phosphomonoesters.

We added some sentences to the introduction and the conclusion section in order to highlight the importance of our study, please see Response 5.

We also thank the reviewer for their positive comment of the paper later in their review (see Comment 28).

Comment 2
Abstract: As written, the abstract make it clear that this is chemistry methods paper, not a biogeochemical study, because the results and conclusions highlighted in the abstract indication only that the authors were able to identify these peaks, but make no reference to their relative importance in the studied soils and to P cycling in these and other soils. This supports my point above that this is not an appropriate journal for this paper as currently written.

Response 2
In addition to Response 1 of Reviewer 2, we highlight the discussion in the body text on the importance and implications of our results (lines 24-26 in the Abstract, lines 358-362, 382-383, 391-402, 432-437, 465-471 in the Discussion section and lines 492-500 in the Conclusion section). Please also see Response 5.

Comment 3
In addition the abstract needs to be more carefully edited, as it is awkwardly written in places. For example, lines 14-15: "include the A horizon of a Ferrasol from Columbia, of a Cambisol from Switzerland, of a Gleysol from Switzerland and of a Cambisol from Germany" should be "include A horizons from a Ferrosol(Columbia), a Cambisol and a Gleysol froom Switzerland, and a Cambisol from Germany".

Response 3
Agreed, we have reworded the sentence:

Changed from (lines 14-15): Soil samples analysed include the A horizon of a Ferralsol from Colombia, of a Cambisol from Switzerland, of a Gleysol from Switzerland and of a Cambisol from Germany.

Changed to (lines 14-15): Soil samples analysed include A horizons from a Ferralsol (Colombia), a Cambisol and a Gleysol from Switzerland, and a Cambisol from Germany.

Comment 4
And why is the phrase "(using solution 31P NMR spectroscopy)" included inline 19, given that the method was given in line 13?

Response 4
We have deleted "(using solution 31P NMR spectroscopy)".

Comment 5
Introduction: The introduction provides a good overview of the chemical methodology for extracting and characterizing IP in soil, as would be expected for a chemical methods paper. It gives a very brief overview of the factors generally controlling IP in soils, but doesn't give much information about why there is a need to specifically characterize all of these different IP forms. What insights into soil P cycling would we gain from identifying these compounds that we don't already have by from the IP compounds we can already identify?

Response 5
Please see Response 1 of Reviewer 2. In addition, the majority of NMR studies have identified a small selection of compounds in the phosphomonoester region of NMR spectra on soil extracts (McLaren et al 2020). These are typically four IP_6 compounds, α- and β-glycerophosphate, and some RNA mononucleotides. Consequently, most studies have focused on the cycling of IP_6, which is considered relatively stable in soil. In the current study, we report up to 70 sharp signals in the phosphomonoester region of NMR spectra on soil extracts following hypobromite oxidation, which is considerably more than that typically reported in the literature. We could identify on average 48% of peaks in this region as arising from inositol phosphates, however, it is likely a much greater proportion of these sharp peaks will be due to inositol phosphates due to their resistance to hypobromite oxidation.

The majority of organic P studies have focused on the cycling of IP_6, particularly of *myo*-IP_6 (McLaren et al. 2020). We show that there is a much greater diversity of organic P compounds than previously thought, and second that they appear to be predominately lower-order inositol phosphates. This has major consequences to our understanding of P cycling, given the

different mechanisms and compounds involved than previously thought and their unknown function in the soil system. We have made this clearer in the introduction and the conclusion:

Insert Lines 95-101: We hypothesise that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of a wide variety of IP. This would have major consequences to our understanding of P cycling in terrestrial (and aquatic) ecosystems, as much more organic P compounds and mechanisms would be involved than previously thought. Furthermore, a better understanding of these organic P compounds in soil would also help improve strategies to increase their biological utilisation, which may reduce the amount of fertiliser needed in agricultural systems and thus influence the transfer of P to aquatic/marine ecosystems.

Insert Lines 496-500: Our study highlights the great diversity and abundance of IP in soils and therefore their importance in terrestrial P cycles. Further research on the mechanisms and processes involved in the cycling of this wide variety of IP in soil will have implications on our understanding of organic P turnover as well as plant availability, and possibly help improve fertiliser strategies in agricultural systems.

Comment 6
And what information would be expected from analyzing them in different soils?

Response 6
A diverse set of soils provides the opportunity to identify a greater array of organic P species than what might be present in only one soil. We refer to this in Lines 106-107 in the manuscript: The four soil samples were chosen from a larger collection based on their diverse concentration of P_{org} and composition of the phosphomonoester region in NMR spectra (Reusser et al., 2020).

The diversity of soil properties may also reveal different relative contributions of organic P species than that present in a particular soil type.

Comment 7
And the hypothesis seems to be something that was tacked on at the end, and doesn't make a lot of sense: "We hypothesize that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of IP". This again emphasizes that this is a chemical methods paper only.

Response 7
Please see Response 5 of Reviewer 2 with the following addition to the manuscript:

Insert Lines 95-101: We hypothesise that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of a wide variety of IP. This would have major consequences to our understanding of P cycling in terrestrial (and aquatic) ecosystems, as much more organic P compounds and mechanisms would be involved than previously thought. Furthermore, a better understanding of these organic P compounds in soil would also help improve strategies to increase their biological utilisation, which may reduce the amount of fertiliser needed in agricultural systems and thus influence the transfer of P to aquatic/marine ecosystems.

In addition, in a recent paper we obtained high-resolution NMR spectra that exhibited a plethora of sharp peaks and an underlying broad peak in the phosphomonoester region on

soil extracts (Reusser et al 2020). This suggested a much greater diversity of organic P species than previously thought. The identity of these sharp peaks was largely unknown and could not be attributed to the limited number of RNA mononucleotides and two glycerophosphates often reported in the literature. Furthermore, a review of the literature from the 1950s to 1970s indicated some studies report the presence of lower-order inositol phosphates in soil extracts using chromatographic approaches. Consequently, we hypothesised that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of inositol phosphates. If the majority of sharp peaks disappeared following hypobromite oxidation, then this would indicate that the sharp signals were due to non-inositol phosphate compounds. We combined previously published methods to test this hypothesis, but did not seek to advance or test the efficacy of these methods as is typically done in a 'methods' paper.

Comment 8
Other points in the Introduction: l. 35: "Riley Andrew et al., 2006)" why is the authors first name included (Andrew M. Riley is the first author of the paper)? This should be "Riley et al., 2006". And the listing in the References (l. 641-644) contains the first names of other authors of this paper. "Shears Stephen, B" should be "Shears, SB", and "Potter Barry VL" should be "Potter BVL". The correct names are very obvious when reading the manuscript, so I'm not sure why they are incorrect here.

Response 8
Agreed, we changed the reference accordingly. This error occurred because of a formatting issue in the EndNote library.
Reference entry changed to (lines 688-691): Riley, A. M., Trusselle, M., Kuad, P., Borkovec, M., Cho, J., Choi, J. H., Qian, X., Shears, S. B., Spiess, B., and Potter, B. V. L.: *scyllo*-Inositol pentakisphosphate as an analogue of *myo*-inositol 1,3,4,5,6-pentakisphosphate: Chemical synthesis, physicochemistry and biological applications, ChemBioChem, 7, 1114-1122, 10.1002/cbic.200600037, 2006.

Comment 9
l. 39 and elsewhere in the text: when citing a list of references, it is conventional to list them in order from oldest to most recent.

Response 9
We have updated the reference list.

Comment 10
l. 87: "was resistant" should be "were resistant", because it modified "signals", which is plural.

Response 10
Corrected.

Comment 11
Methods: As written, there is far too much technical information (e.g. about the transverse relaxation experiments), which will not be of any interest to the majority of readers of this journal.

Response 11
We are happy to reduce this if requested by the Editor. However, the approach is not well known outside of the NMR and organic P communities, and the additional information may be useful for understanding and for reproducibility in future experiments.

Comment 12
And other important information seems to be missing. See specific points listed below. Also, I believe that Turner has published a new paper of the hypobromite oxidation method. How does the method used compare to that method.

Response 12
We carried out the hypobromite oxidation procedure based on the method of Turner et al. (2012), and prior to the publication of Turner (2020). Briefly, Turner et al (2020) suggest taking a 10 mL aliquot of soil extract, adding 2 g of NaOH, and then adding 0.5 mL of bromine. This is slightly different to that reported in Turner et al (2012). In the current study, we similarly take a 10 mL aliquot of soil extract, but add 1 mL of 10 M NaOH, and add 0.6 mL of bromine. Please see Response 3 of Reviewer 1.

Insert Lines 129-130 in the manuscript: The hypobromite oxidation procedure is similar to that reported in Turner (2020).

Comment 13
l. 117: Please provide information on the total volume of extractant used and the total volume of filtrate produced, to help the reader put the hypobromite oxidation experiments into context. In line 121, it indicates that "10 mL of the filtrate was used". What proportion of the total filtrate is this – 10% or 100%?

Response 13
We used 25% of the total filtrate for the hypobromite oxidation. We have made this clearer in the manuscript:

Inserted (lines 124-126): Concentrations of organic P for NMR analysis were carried out using the NaOH-EDTA extraction technique of Cade-Menun et al. (2002) at a soil to solution ratio of 1:10, i.e. extracting 4 g of soil with 40 mL of extractant.

Changed from (lines 121-123): Briefly, 10 mL of the filtrate was placed in a three necked round bottom flask equipped with a septum, a condenser, a magnetic stir bar and thermometer (through a claisen adapter with N_2 adapter).

Changed to (lines 130-132): Briefly, 10 mL of the NaOH-EDTA filtrate (section 2.2) was placed in a three necked round bottom flask equipped with a septum, a condenser, a magnetic stir bar and thermometer (through a claisen adapter with N_2 adapter).

Comment 14
l. 144-145: This sentence is awkwardly written. Change "...in solution is that of molybdate unreactive P (MUP), which is considered to be largely that of organic P" to "in solution is molybdate unreactive P (MUP), which is predominantly organic P for these samples"

Response 14
Agreed.

Changed from (lines 144-145): The difference in concentrations of total P and MRP in solution is that of molybdate unreactive P (MUP), which is considered to be largely that of organic P.

Changed to (lines 153-154): The difference in concentrations of total P and MRP in solution is molybdate unreactive P (MUP), which is predominantly organic P for these samples.

Comment 15
l. 146-147: "a duplicate sample of the Cambisol and the Gleysol was spiked" should be "duplicate samples of the Cambisol and Gleysol were spiked"

Response 15
Corrected.

Comment 16
l. 161-162: The inclusion of the Vestergren et al. 2012 paper here confused me. This group left their samples to sit overnight because they used a sulfide treatment to remove paramagnetic ions. Was this also done for the current study? If so, then please describe the sulfide treatment more clearly. If not, then it would be better to replace this reference with one that is more appropriate.

Response 16
Vestergren et al. (2012) report in their body text: “Extraction of soils with NaOH/EDTA is known to hydrolyze several forms of phosphodiesters. This is considered an unavoidable drawback of the method, but it has been pointed out that it does not exclude deriving the original P composition when hydrolysis products can be traced back.[21] Therefore, when a hydrolysis product is observed, it must be determined what fraction of the compound was originally present in the soil, versus formed during extraction.[19] Whereas the longer sample preparation time for sulfide treatment increases hydrolysis (Figure S3 of the Supporting Information), the 2D methodology is very well suited to trace observed compounds back to their precursors”. The citation of Vestergren et al. (2012) in our manuscript refers to their findings in the Supporting Information (Figure 3). The authors present NMR spectra and report that more hydrolysis of phosphodiesters are due to the “longer exposure to high pH”, and that the ‘resting’ time of the extracts in the study was 18-20 hours at room temperature. The authors note in their study the mechanism of alkaline hydrolysis of organic P compounds to their hydrolysis products and the necessity of a reaction period lasting several hours for sufficient hydrolysis. Therefore, we think that the reference (Line 172) is appropriate and we would prefer to not replace it.

Comment 17
l. 193-195: Something seems to be missing here for the measurement of N observability. Using Ptot ICP-OES only makes sense if the entire sample after freeze-drying was used for the NMR analysis. However, that does not seem to be the case for this study. While it appears that the total mass of lyophilized material was used for the brominated samples (l. 167-168), a set mass (120 g) of the non-brominated lyophilized material was used, with no indication of how much of the total lyophilized material this represents. The proportion of total mass used must be factored into the equation to correctly determine NMR observability. This would also explain the differences in observability reported in the supplementary information (SI) for the brominated and unbrominated samples.

Response 17
P_{tot} NMR and P_{tot} ICP-OES refer to the P concentrations in mg P per kg soil measured in the extracts. Hence, the analysed P contents in the extracts were back-calculated to the original concentrations in the soil, including any partitioning in the extraction, freeze-drying and re-dissolving processes. We made this clearer in the text by inserting the units of the two parameters.

Insert (lines 209-211): ,where P_{tot} NMR refers to the total P content <u>in mg P/kg_{soil}</u> detected in the soil extracts using solution ^{31}P NMR spectroscopy and P_{tot} ICP-OES refers to the total P concentration <u>in mg P/kg_{soil}</u> measured in the soil extracts prior to freeze-drying using ICP-OES.

Comment 18
l. 206-225: There is no need to include this much detail about the transverse relaxation papers. As noted above, the majority of readers of this paper in this journal will not be interested in these details. In addition, this appears to be a repeat of what was done for the

McLaren et al. 2019 study. As such, all that is needed is to cite the previous publication. If the authors really thing this much detail is needed, it could be included in the SI.

Response 18
Please see Response 11.
Comment 19
L. 226-233: Why are methods for statistical analyses reported here, when no results of statistical analysis are included in the Results, Discussion or SI?

Response 19
We report in our studies average values as well as standard deviations. Furthermore, we carried out the one-way ANOVA with subsequent multi comparison of mean values using the Tukey's significance honestly significant difference procedure to determine whether the T_2 of the broad peak was significantly different from the IP peaks. The result of this statistical analysis is reported in the text (lines 330-332 of the initial manuscript): The average (n=4) T_2 times of the broad peak was significantly different than that of *scyllo*- and *myo*-IP_6 ($p < 0.05$).

Comment 20
Results: 1. Please provide spectra showing the entire spectrum for each brominated and unbrominated sample, scaled to allow the reader to see the full height of orthophosphate and the relative heights of other peaks compared to orthophosphate. All of the spectra currently in the manuscript show the monoester region only, with the orthophosphate peak truncated. This is needed to get a full sense of all the peaks for each sample, especially for the brominated samples.

Response 20
The main reaction was oxidation, not bromination of the samples. The aim of our study was the identification of IP, whose peaks appear in the phosphomonoester region. Hence, our spectra focus on the phosphomonoester region, which is also where the majority (> 99%) of NMR signals are located. We are unsure why the inclusion of the whole spectrum would add to the information already provided in Table 4. Nevertheless, we are willing to add the spectra of the Gleysol and Cambisol (Figure 2), where considerable amounts of phosphodiesters were measured before hypobromite oxidation, to the supporting information.

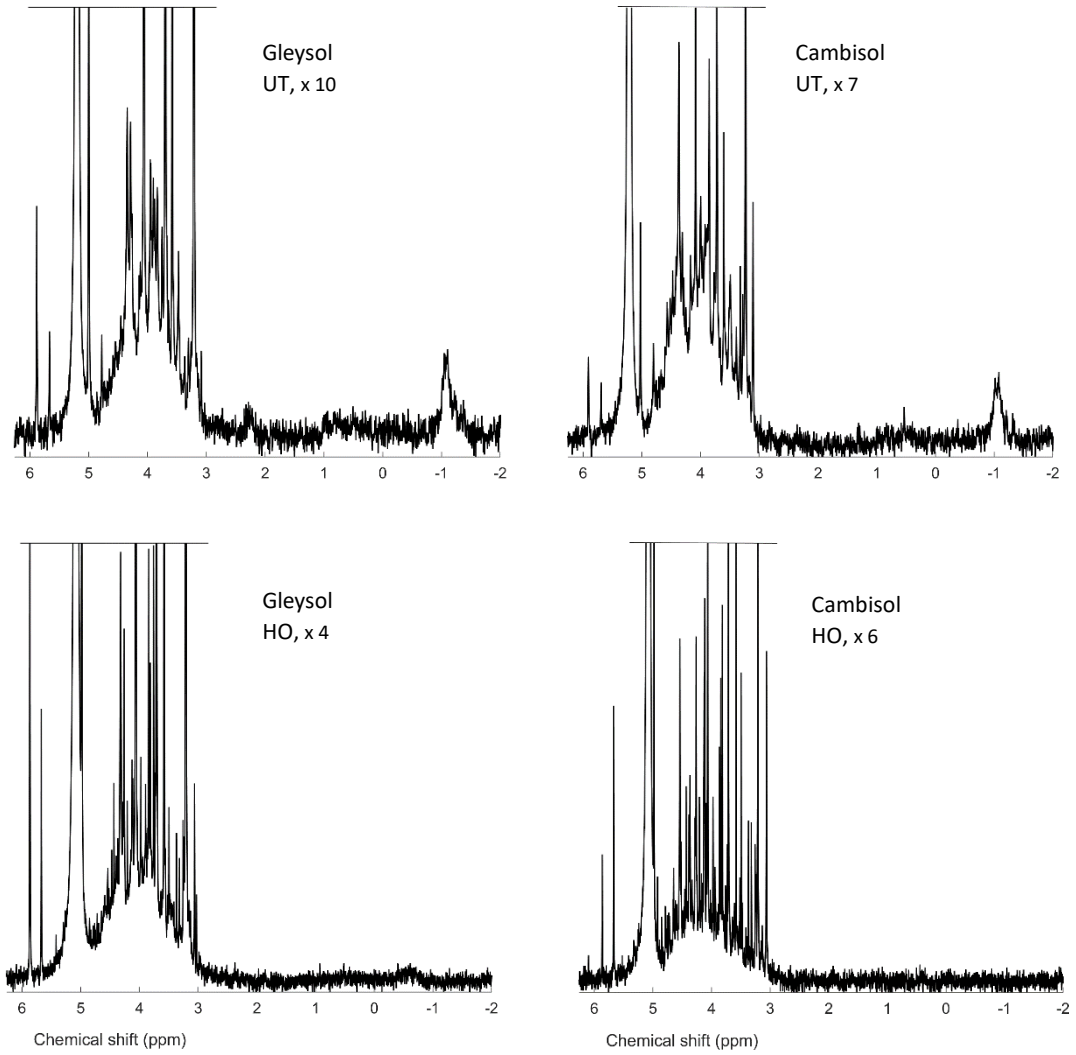

Figure 2. Solution ^{31}P nuclear magnetic resonance (NMR) spectra (500 MHz) of the orthophosphate, phosphomonoester and phosphodiester region on untreated (UT, on top) and hypobromite oxidised (HO, below) 0.25 M NaOH + 0.05 M EDTA soil extracts of the Gleysol (right) and Cambisol (left). Signal intensities were normalised to the MDP peak intensity. The vertical axes were increased for improved visibility of spectral features, as indicated by a factor.

Comment 21

The usefulness of the spectra shown in Fig. 3 are not clear. I am used to looking at NMR spectra, and I found these confusing, as with the exception of the Gleysol the red lines show little but noise. Again, this would be appropriate for a chemical methods paper, emphasizing that this is not the best journal for this study.

Response 21

The aim of the transverse relaxation (T_2) experiments was to determine if the underlying broad signal itself is caused by sharp peaks of IP or if another compound of larger structure than IP resisted hypobromite oxidation (Please also see Response 28, Reviewer 1). The red line of Figure 3 does not show a normal NMR soil spectrum but the result of the T_2 experiment with the longest spin-echo delay for each hypobromite oxidised soil sample. The spectra in black with the shortest spin-echo delay can be interpreted as a “normal” NMR soil spectra. We applied increasing spin-echo delays and acquired the resulting spectra for each step. However, due to visibility reasons, we only show the results of the shortest (black) and longest spin-echo delay (red). This presentation is normal for T_2 experiments (Claridge, 2016; Li et al., 2018a).

Figure 3 shows that the sharp peaks of IP after a spin-echo delay of 80*τ are still present (red line). In contrast, the broad peak partially disappears along with the orthophosphate peak, showing nothing else than noise. This highlights that the broad peak and orthophosphate peak are not of the same chemical composition as the rest of the sharp peaks, as it would not be visible only in the black spectra. As the T_2 are inversely related to a compound's molecular size, our results support the findings of Jarosch et al. (2015) and McLaren et al. (2015b); McLaren et al. (2019) that the compounds causing the broad signal are of larger molecular size than IP.

Comment 22
I am concerned that the authors report signals for non-IP compounds in their brominated spectra. In my experience with this technique, if there are any peaks for non-IP compounds, that suggests that the oxidation was incomplete. And that in turn raises questions about the authors' assignment of peaks in the brominated samples. How confident are the authors that all of the peaks were present in their soils prior to extraction and hypobromite oxidation? Isn't it possible that bromination degraded some high IPs (e.g. IP_6) to lower IPs (IP_5 and IP_4)? The recovery of the added *myo*-IP_6 was only 20 and 47%, which suggests it may have been degraded.

Response 22
According to the method, inositol hexakisphosphates and pentakisphosphates are stable to hypobromite oxidation, please see Response 2 of Reviewer 1. We tested the oxidation efficacy in a pilot study (Response 3, Reviewer 1). Furthermore, bromine was added in excess. If not all organic P species have been oxidised, this suggests that they are stable to hypobromite oxidation, highlighting their chemical stability. The losses occurred most certainly during the precipitation and re-dissolving procedure and not because of degradation. Please also see Response 24 of Reviewer 1. Furthermore, we identified inositol pentakisphosphates in untreated extracts, lines 312-320.

Comment 23
l. 255: change "Although," to "However,"

Response 23
Corrected.

Comment 24
l. 273: "A detailed view of the phosphomonoester region of spiked extracts is shown" should be "Detailed views of the phosphomonoester regions of spiked samples are shown"

Response 24
Agreed.

Changed from (lines 273-274): A detailed view of the phosphomonoester region of spiked extracts is shown in Fig. SI1 to SI5 of the Supporting Information.

Changed to (lines 289-290): Detailed views of the phosphomonoester regions of spiked samples are shown in Fig. SI1 to SI5 of the Supporting Information.

Comment 25
l. 306-316: I do not see the need to include any of this information about spin-echo analysis of selected P compounds in the current paper, as it will not be of any interest to the majority of readers of this paper in this journal.

Response 25
The 'spin-echo' analysis was carried out to provide evidence that there were other compounds different to IP resistant to hypobromite oxidation. Without these results, one

could assume that the broad signal itself could be comprised of sharp peaks caused by IP. Please also see Response 27, Review 1.

Comment 26
Discussion: The P-NMR literature cited in this section seems biased to papers by the Smernik group. I have concerns about this because that group prepared their samples for NMR differently from most other groups, and from what was done for the current study. As such, results from that group may not be directly comparable here.

Response 26
We are unsure what the reviewer means by their comment regarding citations. Citations are primarily used to support the claims of the authors made in the body text. If the reviewer believes we have incorrectly used a citation when supporting a claim, then we are happy to make corrections. Unfortunately, the reviewer has not provided any evidence to support her or his claim.

We are unsure as well what the reviewer means by this comment regarding NMR sample preparation. A comparison of methods for preparing NMR samples by Dr Ronald Smernik (e.g. Smernik and Dougherty (2007)) and that reported in the current study, clearly shows a large difference in sample preparation. Both of these methods also slightly differ to other groups using NMR approaches (Cade-Menun and Liu, 2014). Indeed, our approach is based on the studies of Vincent et al. (2013) and Spain et al. (2018), which is optimised to the high-resolution NMR spectrometers we have access to.

Lastly, we note that McLaren et al. (2019) is the only study reporting transverse-relaxation (T_2) experiments for organic P compounds in soil mineral samples. In addition, studies by Smernik et al. have also done much work on identifying lower-order IP in plant samples using solution ^{31}P NMR spectroscopy.

Comment 27
In addition, it shows an unfamiliarity with the broader P-NMR literature, which is of concern.

Response 27
Please see Response 26. In addition, we are unsure why the reviewer has made this assertion given the recent review paper on the chemical nature of soil organic P by two of the co-authors (McLaren et al. (2020)). Of course, it is possible that we may have made an error and have missed a relevant study. In this case, we would be happy to make corrections and strengthen the claims already made in the text. Unfortunately, the reviewer has not provided any details where a publication might have been missed or incorrectly cited.

Comment 28
In general, however, I think the authors have done a reasonable job of trying to relate these P compounds to the literature and to the soils, which would be suitable to this journal. However, they should note the overall small proportion of total P that some of these compounds comprise. Are compounds in such low concentrations really an integral component of P cycling.

Response 28
We thank the reviewer for the positive comment.

For example, water extractable inorganic P can be very small in terms of concentration but rather important in terms of function. In addition, we note that total IP comprised up to 18% of total P_{org} in hypobromite oxidised extracts and compounds causing the broad signal on average 23% of total P_{org} in untreated extracts. In our opinion, these organic P pool should not be neglected. Furthermore, ratios of IP_6 to IP_5 could provide a tool for assessing stability

of IP in soil systems, please see Response 2. water extractable inorganic P can be very small in terms of concentration but rather important functionally.

Comment 29
And in my opinion, section 4.3 is not appropriate for this journal and would not be of interest to the majority of readers, and so should be cut.

Response 29
This section refers to the structural composition and possible stability of compounds causing the broad signal in soil, which has implications to our understanding of soil organic matter and 'legacy' P in agroecosystems. Lines 462-468 in the manuscript: Since a portion of the broad signal is resistant to hypobromite oxidation, this suggests the organic P is complex and in the form of polymeric structures. The chemical resistance of the broad signal to hypobromite oxidation may also indicate a high stability in soil (Jarosch et al., 2015). Annaheim et al. (2015) found that concentrations of the broad signal remained unchanged between three different organic fertiliser strategies after 62 years of cropping. In contrast, the organic P compounds annually added with the fertilisers were completely transformed or lost in the slightly acidic topsoil of the field trial. Please also see Response 28, Reviewer 1.

Inserted Lines 461-464 in the manuscript: Nebbioso and Piccolo (2011) reported that high molecular weight material of organic matter in soil is an association of smaller organic molecules. These associations however would still cause a broad signal in the phosphomonoester region of soil extracts and could be a reason that some organic molecules containing P are protected from hypobromite oxidation. The large proportion of the broad signal in the total organic P pool demonstrates its importance in the soil P cycle.

Comment 30
l. 322-324: Other studies have looked at what was not extracted by NaOH-EDTA, including with acid extraction after NaOH-EDTA or with solid-state P-NMR. See for example studies by He et al. These would be more appropriate to cite here than McLaren et al., 2015a

Response 30
It is unclear which particular study by He et al the reviewer is referring to. McLaren et al. (2015a) determined the total concentrations of soil P using X-ray fluorescence spectroscopy, which was similarly the case here. The authors then compared these measures with that of aqua regia digestion, the ignition-H_2SO_4 and NaOH-EDTA extraction techniques, and also the summation of P fractions from a sequential chemical fractionation procedure based on Hedley et al. (1982). The authors report that the native soil of their study contained a fraction of strongly-held mineral P that was neither acid nor alkali extractable. They also considered the XRF method to be the most reliable for quantifying concentrations of total P in soil, which was similar to the summation of P fractions by sequential chemical fractionation. Furthermore, the authors provide supporting evidence that a relatively small portion of alkaline soluble organic P was not extracted by NaOH-EDTA.

We report in our study (lines 337-340 of the initial manuscript): On average, 44 % of total P (as measured with XRF) was extracted by NaOH-EDTA, which is consistent with previous studies (Turner, 2008; Li et al., 2018b; McLaren et al., 2019). The non-extractable pool of P is likely to comprise of inorganic P as part of insoluble mineral phases, but could also contain some organic P (McLaren et al., 2015a). Hence, we refer to the pool of P not extracted by NaOH-EDTA but measured by XRF. Therefore, we consider the publication of McLaren et al. (2015a) as the most suiable in this context.

The reviewer could be referring to He et al. (2007). Here the authors reported that P recoveries in NaOH-EDTA extracts of poultry manure were lower compared to extracts of

dairy manure. The authors attributed this lower recovery to the higher Ca content in the poultry manure. Increased Ca in the poultry manure may have resulted in less soluble forms of P that were not extracted with NaOH-EDTA. By using an additional extraction step (1 M HCl) following the NaOH-EDTA step, the authors were able to recover the remaining P from the poultry manure. Furthermore, solution ^{31}P NMR spectra of the HCl extract revealed that the majority of P was present as orthophosphate and to a lesser extent phytate. However, the study of He et al. (2007) was carried out on manure samples and are not relevant to soil samples.

Comment 31
l. 333-334: “This will result in the production of carbon dioxide and simple organic acids” This sentence does not seem to be relevant here. How is this related to P?

Response 31
It relates to what happens to the organic molecules containing phosphate as functional group. It gives more detail on what actually happens to the organic molecules during the hypobromite oxidation procedure. We reworded the sentence to make this clearer.

Changed from (lines 333-334): This will result in the production of carbon dioxide and simple organic acids.

Changed to (lines 352-354): The products of hypobromite oxidation are most probably carbon dioxide, simple organic acids from the oxidative cleavage of the phosphoesters and orthophosphate.

Comment 32
l. 340-342: If the authors had not shown peaks other than monoesters and orthophosphate, I might agree with them that the peaks in the monoester region are all IP. However, it is clear from the results they have shown that they did not have complete oxidation of all P compounds. So how can they be confident that they only have IP in the monoester region? This must be addressed.

Response 32
Please see Responses 3 and 5 of Reviewer 1.

Comment 33
l. 348-350: I’m confused by the some of the papers cited here. Why are studies that did not use chromatography cited here to make a point about chromatography. Please rephrase, or remove the non-chromatography references.

Response 33
It appears the reviewer has misread the sentence. We provide two different citation groups for studies involving chromatography and NMR spectroscopy (see below).

Lines 365-367 of the initial manuscript: The detection of *myo*-, *scyllo*-, *chiro*, and *neo*-IP_6 in untreated and hypobromite oxidised soil extracts is consistent with previous studies using chromatography (Irving and Cosgrove, 1982; Almeida et al., 2018) and NMR (Turner and Richardson, 2004; McLaren et al., 2015b; Jarosch et al., 2015; Vincent et al., 2013; Doolette et al., 2011a).

Comment 34
l. 356-363: As noted above, the authors did not have compete oxidation of all non-IP compounds in their extracts. So how can they be certain that this peak at 4.36 is an IP compound and not α-glycerol. In addition, other groups have reported a peak that sits very close to α-glycerol, and have urged caution about identifying this peak without spiking. This

emphasizes a need for a broader review of the literature than just papers from the Smernik group.

Response 34
We can confirm that bromine was present in excess and that soil extracts were kept at reflux following bromine addition. Furthermore, the volume of bromine added relative to the aliquot of soil extract was similar or greater in our study compared to that in previous studies (Turner et al 2012; Turner & Richardson 2004). Please see Responses 3 and 5 of Reviewer 1.

Unfortunately, the reviewer has not provided the reference to support his or her claim. We are not aware of any study that has identified another organic P species at the chemical shift at or near that of α-glycerophosphate. Nevertheless, in the current study, the assignment of α-glycerophosphate was based on spiking experiments in untreated soil extracts. Following hypobromite oxidation, this peak disappeared, revealing two peaks belonging to IP. This then provided strong evidence that the peak originally assigned to α-glycerophosphate was in fact due to an IP.

The assignment of one of the aforementioned peaks in hypobromite extracts was confirmed by spiking experiments with *neo*-IP_6 in the 2-equatorial/4-axial conformation. This resulted in the increased peak intensity at 4.37 ppm (C2,5) and its corresponding peak at 4.11 ppm (C1,3,4,6), which occurred at the known peak ratio of 4:2 for *neo*-IP_6 in the 2-equatorial/4-axial conformation, see Figure SI4 with the spiking results. Consequently, our results highlight the need for caution when assigning the α-glycerophosphate peak based on spiking experiments alone with α-glycerophosphate in untreated soil extracts. We would recommend that spiking with *neo*-IP_6 would also occur. We have revised the text, lines 379-383: Whilst a peak at δ 4.36 ppm would be assigned to α-glycerophosphate based on spiking experiments in the untreated extracts of the Cambisol and the Gleysol, hypobromite oxidation revealed the presence of the 2-equatorial/4-axial C2,5 peak of *neo*-IP_6 at δ 4.37 ppm, and also an unidentified peak at δ 4.36 ppm in the Cambisol. Therefore, the assignment and concentration of α-glycerophosphate may be unreliable in some soils of previous studies.

Comment 35
l. 370: change “extracts, which the” to “extracts, of which the”

Response 35
Corrected.

Comment 36
l. 383: add spaces between the numbers and words here: “1axial” should be “1 axial” or “1-axial”, etc.

Response 36
Agreed.

Changed from (line 383): the 1axial/5equatorial and 5axial/1 equatorial forms of *myo*-(1,2,3,4,6)-IP_5 are in a dynamic equilibrium,
Changed to (lines 407-408): the 1-axial/5-equatorial and 5-axial/1-equatorial forms of *myo*-(1,2,3,4,6)-IP_5 are in a dynamic equilibrium,

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REVIEWER REPORT 3

Comment 1
I made a careful reading of referee's comments, authors responses and modifications made in the manuscript. My conclusion is that the authors addressed most of the referee's comments in a adequate way. I agree with the referee 2 that the manuscript looks like very technical but a better knowledge of P species and O chemical transformations in soils could surely improve our ability to model and forecast the cycling of this element.

Response 1
We thank the reviewer for the positive feedback.

Comment 2
I identified some referre's comments for which there was an author's response but it was difficult to understand how the authors have improved their text accordingly: comments 6, 7, 12, 16, 29. The authors should explicitly refer to lines in the manuscript.

Response 2
We acknowledge that this could have been clearer. Consequently, we have edited the responses to Reviewer 2 in regards to the aforementioned comments, and have included line numbers. Revisions to the responses are highlighted below with an underline.

Reviewer 2, Comment 6
And what information would be expected from analyzing them in different soils?

Response 6
A diverse set of soils provides the opportunity to identify a greater array of organic P species than what might be present in only one soil. We refer to this in Lines 106-107 in the manuscript: The four soil samples were chosen from a larger collection based on their diverse concentration of P_{org} and composition of the phosphomonoester region in NMR spectra (Reusser et al., 2020).

The diversity of soil properties may also reveal different relative contributions of organic P species than that present in a particular soil type.

Reviewer 2, Comment 7
And the hypothesis seems to be something that was tacked on at the end, and doesn't make a lot of sense: "We hypothesize that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of IP". This again emphasizes that this is a chemical methods paper only.

Response 7
Please see Response 5 of Reviewer 2 with the following addition to the manuscript:

Insert Lines 95-101: We hypothesise that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of a wide variety of IP. This would have major consequences to our understanding of P cycling in terrestrial (and aquatic) ecosystems, as much more organic P compounds and mechanisms would be involved than previously thought. Furthermore, a better understanding of these organic P compounds in soil would also help improve strategies to increase their biological utilisation, which may reduce the amount of fertiliser needed in agricultural systems and thus influence the transfer of P to aquatic/marine ecosystems.

In addition, in a recent paper we obtained high-resolution NMR spectra that exhibited a plethora of sharp peaks and an underlying broad peak in the phosphomonoester region on soil extracts (Reusser et al 2020). This suggested a much greater diversity of organic P species than previously thought. The identity of these sharp peaks was largely unknown and could not be attributed to the limited number of RNA mononucleotides and two glycerophosphates often reported in the literature. Furthermore, a review of the literature from the 1950s to 1970s indicated some studies report the presence of lower-order inositol phosphates in soil extracts using chromatographic approaches. Consequently, we hypothesised that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of inositol phosphates. If the majority of sharp peaks disappeared following hypobromite oxidation, then this would indicate that the sharp signals were due to non-inositol phosphate compounds. We combined previously published methods to test this hypothesis, but did not seek to advance or test the efficacy of these methods as is typically done in a 'methods' paper.

Reviewer 2, Comment 12
And other important information seems to be missing. See specific points listed below. Also, I believe that Turner has published a new paper of the hypobromite oxidation method. How does the method used compare to that method.

Response 12
We carried out the hypobromite oxidation procedure based on the method of Turner et al. (2012), and prior to the publication of Turner (2020). Briefly, Turner et al (2020) suggest taking a 10 mL aliquot of soil extract, adding 2 g of NaOH, and then adding 0.5 mL of bromine. This is slightly different to that reported in Turner et al (2012). In the current study, we similarly take a 10 mL aliquot of soil extract, but add 1 mL of 10 M NaOH, and add 0.6 mL of bromine. Please see Response 3 of Reviewer 1.

Insert Lines 129-130 in the manuscript: The hypobromite oxidation procedure is similar to that reported in Turner (2020).

Reviewer 2, Comment 16
l. 161-162: The inclusion of the Vestergren et al. 2012 paper here confused me. This group left their samples to sit overnight because they used a sulfide treatment to remove paramagnetic ions. Was this also done for the current study? If so, then please describe the sulfide treatment more clearly. If not, then it would be better to replace this reference with one that is more appropriate.

Response 16
Vestergren et al. (2012) report in their body text: "Extraction of soils with NaOH/EDTA is known to hydrolyze several forms of phosphodiesters. This is considered an unavoidable drawback of the method, but it has been pointed out that it does not exclude deriving the original P composition when hydrolysis products can be traced back.[21] Therefore, when a hydrolysis product is observed, it must be determined what fraction of the compound was originally present in the soil, versus formed during extraction.[19] Whereas the longer sample preparation time for sulfide treatment increases hydrolysis (Figure S3 of the Supporting Information), the 2D methodology is very well suited to trace observed compounds back to their precursors". The citation of Vestergren et al. (2012) in our manuscript refers to their findings in the Supporting Information (Figure 3). The authors present NMR spectra and report that more hydrolysis of phosphodiesters are due to the "longer exposure to high pH", and that the 'resting' time of the extracts in the study was 18-20 hours at room temperature. The authors note in their study the mechanism of alkaline hydrolysis of organic P compounds to their hydrolysis products and the necessity of a reaction period lasting several hours for sufficient hydrolysis. Therefore, we think that the reference (Line 172) is appropriate and we would prefer to not replace it.

Reviewer 2, Comment 29
And in my opinion, section 4.3 is not appropriate for this journal and would not be of interest to the majority of readers, and so should be cut.

Response 29
This section refers to the structural composition and possible stability of compounds causing the broad signal in soil, which has implications to our understanding of soil organic matter and 'legacy' P in agroecosystems. Lines 462-468 in the manuscript: Since a portion of the broad signal is resistant to hypobromite oxidation, this suggests the organic P is complex and in the form of polymeric structures. The chemical resistance of the broad signal to hypobromite oxidation may also indicate a high stability in soil (Jarosch et al., 2015). Annaheim et al. (2015) found that concentrations of the broad signal remained unchanged between three different organic fertiliser strategies after 62 years of cropping. In contrast, the organic P compounds annually added with the fertilisers were completely transformed or lost in the slightly acidic topsoil of the field trial. Please also see Response 28, Reviewer 1.

Inserted Lines 461-464 in the manuscript: Nebbioso and Piccolo (2011) reported that high molecular weight material of organic matter in soil is an association of smaller organic molecules. These associations however would still cause a broad signal in the phosphomonoester region of soil extracts and could be a reason that some organic molecules containing P are protected from hypobromite oxidation. The large proportion of the broad signal in the total organic P pool demonstrates its importance in the soil P cycle.

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Identification of lower-order inositol phosphates (IP_5 and IP_4) in soil extracts as determined by hypobromite oxidation and solution ^{31}P NMR spectroscopy

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Abstract. Inositol phosphates (IP) are a major pool of identifiable organic phosphorus (P) in soil. However, insight on their distribution and cycling in soil remains limited, particularly of lower-order IP (IP_5 and IP_4). This is because the quantification of lower-order IP typically requires a series of chemical extractions, including hypobromite oxidation to isolate IP, followed by chromatographic separation. Here, for the first time, we identify the chemical nature of organic P in four soil extracts following hypobromite oxidation using solution ^{31}P NMR spectroscopy and transverse relaxation (T_2) experiments. Soil samples analysed include A horizons from a Ferralsol (Colombia), a Cambisol and a Gleysol from Switzerland, and a Cambisol from Germany. Solution ^{31}P NMR spectra of the phosphomonoester region on soil extracts following hypobromite oxidation revealed an increase in the number of sharp signals (up to 70), and an on average 2-fold decrease in the concentration of the broad signal compared to the untreated soil extracts. We identified the presence of four stereoisomers of IP_6, four stereoisomers of IP_5, and *scyllo*-IP_4. We also identified for the first time two isomers of *myo*-IP_5 in soil extracts: *myo*-(1,2,4,5,6)-IP_5 and *myo*-(1,3,4,5,6)-IP_5. Concentrations of total IP ranged from 1.4 to 159.3 mg P/kg_{soil} across all soils, of which between 9 % and 50 % were comprised of lower-order IP. Furthermore, we found that the T_2 times, which are considered to be inversely related to the tumbling of a molecule in solution and hence its molecular size, were significantly shorter for the underlying broad signal compared to the sharp signals (IP_6) in soil extracts following hypobromite oxidation. In summary, we demonstrate the presence of a plethora of organic P compounds in soil extracts, largely attributed to IP of various order, and provide new insight on the chemical stability of complex forms of organic P associated with soil organic matter.

1 Introduction

Inositol phosphates (IP) are found widely in nature and are important for cellular function in living organisms. They are found in eukaryotic cells where they operate in ion-regulation processes, as signalling or P storage compounds (Irvine and Schell, 2001). The basic structure of IP consists of a carbon ring (cyclohexanehexol) with one to six phosphorylated centers (IP_{1-6}) and up to nine stereoisomers (Angyal, 1963; Cosgrove and Irving, 1980). An important IP found in nature is *myo*-IP_6, which is used as a P storage compound in plant seeds. Another important species of IP is that of *myo*-(1,3,4,5,6)-IP_5, which is present in most eukaryotic cells at concentrations ranging from 15 to 50 µM (Riley et al., 2006). Species of IP_{1-3} are present in phospholipids such as phosphatidylinositol diphosphates and are an essential structural component of the cell membrane system (Strickland, 1973; Cosgrove and Irving, 1980).

Inositol phosphates have been reported to comprise more than 50 % of total organic phosphorus (P_{org}) in some soils (Cosgrove and Irving, 1980; McDowell and Stewart, 2006; Turner, 2007). Four stereoisomers of IP have been detected in soils, with the *myo* stereoisomer being the most abundant (56 %), followed by *scyllo* (33 %), *neo* and D-*chiro* (11 %) (Cosgrove and Irving, 1980; Turner et al., 2012). The largest input of *myo*-IP_6 to the soil occurs via the addition of plant seeds (Turner et al., 2002). However, the addition of *myo*-IP_6 to soil can also occur via manure input because monogastric animals are mostly incapable of digesting *myo*-IP_6 without the addition of phytases to their diets (Leytem et al., 2004; Leytem and Maguire, 2007; Turner et al., 2007b). An exception to this are pigs, which were found to at least partially digest phytate (Leytem et al., 2004), and transgenic pigs expressing salivary phytase (Golovan et al., 2001; Zhang et al., 2018). The accumulation of *myo*-IP_6 in soil occurs due to the negative charge of the deprotonated phosphate groups, which can coordinate to the charged surfaces of Fe- and Al-(hydro)-oxides (Anderson et al., 1974; Ognalaga et al., 1994), clay minerals (Goring and Bartholomew, 1951) and soil organic matter (SOM) (McKercher and Anderson, 1989), or form insoluble precipitates with cations (Celi and Barberis, 2007). These processes lead to the stabilisation of IP in soil resulting in its accumulation and reduced bioavailability (Turner et al., 2002). In contrast, the sources and mechanisms controlling the flux of *scyllo*-, *neo*- and D-*chiro*-IP_6 in soil remain unknown but are thought to involve epimerization of the *myo* stereoisomer (L'Annunziata, 1975).

Chromatographic separation of alkaline soil extracts revealed the presence of four stereoisomers of IP_6 and lower-order IP_{1-5} (Halstead and Anderson, 1970; Anderson and Malcolm, 1974; Cosgrove and Irving, 1980; Irving and Cosgrove, 1982). Irving and Cosgrove (1981) used hypobromite oxidation prior to chromatography to isolate the IP fraction in alkaline soils. The basis of this approach is that IP are considered to be highly resistant to hypobromite oxidation, whereas other organic compounds (e.g. phospholipids and nucleic acids) will undergo oxidation (Dyer and Wrenshall, 1941; Turner and Richardson, 2004). The resistance of IP to hypobromite oxidation is thought to be due to the high charge density and steric hindrance, which is caused by the chair conformation of the molecule and the bound phosphate groups, with the P in its highest oxidation state. Hypobromite oxidation of inositol (without phosphate groups) mainly results in the formation of inososes, which have an intact carbon ring (Fatiadi, 1968). Fatiadi (1968) considered that the oxidation of bromine with inositol is stereospecific and comparable to catalytic or bacterial oxidants.

A limitation of chromatographic separation of alkaline extracts is that there is a mixture of unknown organic compounds that can co-elute with IP, and result in an overestimation of IP concentrations (Irving and Cosgrove, 1981). However, this can also occur for IP, and historically, studies often reported the combined concentration of IP_6 and IP_5 due to a lack of differentiation in their elution times (McKercher and Anderson, 1968b). More recently,

Almeida et al. (2018) investigated how cover crops might mobilize soil IP using hypobromite oxidation on NaOH-EDTA extracts followed by chromatographic separation. The authors found that pools of *myo*-IP_6 and 'unidentified IP' accounted for 30 % of the total extractable pool of P and hypothesised that the 'unidentified IP' pool consists solely of lower-order *myo*-IP. Pools of lower order IP_{1-5} comprise on average 17 % of the total pool of IP in soil and account for an important pool of soil organic P in terrestrial ecosystems (Anderson and Malcolm, 1974; Cosgrove and Irving, 1980; Turner et al., 2002; Turner, 2007).

Since the 1980s, solution ^{31}P nuclear magnetic resonance spectroscopy (NMR) has been the most commonly used technique to characterise the chemical nature of organic P in soil extracts (Newman and Tate, 1980; Cade-Menun and Liu, 2014). An advantage of this technique is the simultaneous detection of all forms of organic P that come into solution, which is brought about by a single step extraction with alkali and a chelating agent (Cade-Menun and Preston, 1996). However, a limitation of the technique has been the loss of information on the diversity and amount of soil IP compared to that typically obtained prior to 1980 (Smith and Clark, 1951; Anderson, 1955; Cosgrove, 1963). To date, solution ^{31}P NMR spectroscopy on soil extracts has only reported concentrations of *myo*-, *scyllo*-, *chiro*- and *neo*-IP_6. The fact that lower-order IP were not reported in studies using NMR spectroscopy might be due to overlap of peaks in the phosphomonoester region, which makes peak assignment of specific compounds difficult (Doolette et al., 2009).

Turner et al. (2012) carried out hypobromite oxidation prior to solution ^{31}P NMR analysis of alkaline soil extracts to isolate the IP fraction. This had the advantage of reducing the number of NMR signal in the phosphomonoester region and consequently the overlap of peaks. The authors demonstrated the presence of *neo*- and *chiro*-IP_6 in NMR spectra via spiking of hypobromite oxidised extracts. Interestingly, the authors also reported the presence of NMR signals in the phosphomonoester region that could not be assigned to IP_6 and were resistant to hypobromite oxidation. They were not able to attribute the NMR signals to any specific P compounds, but hypothesised based on their resistance to hypobromite oxidation that they were due to lower-order IP.

The aim of this study was to identify and quantify IP in soil extracts following hypobromite oxidation using solution ^{31}P NMR spectroscopy. In addition, the structural composition of phosphomonoesters in soil extracts following hypobromite oxidation was probed using solution ^{31}P NMR spectroscopy and transverse relaxation experiments. We hypothesise that a large portion of sharp peaks in the phosphomonoester region of untreated soil extracts would be resistant to hypobromite oxidation, which would indicate the presence of a wide variety of IP. This would have major consequences to our understanding of P cycling in terrestrial (and aquatic) ecosystems, as much more organic P compounds and mechanisms would be involved than previously thought. Furthermore, a better understanding of these organic P compounds in soil would also help improve strategies to increase their biological utilisation, which may reduce the amount of fertiliser needed in agricultural systems and thus influence the transfer of P to aquatic/marine ecosystems.

2 Experimental section

2.1 Soil collection and preparation

Soil samples were collected from the upper horizon of the profile at four diverse sites. These include a Ferralsol from Colombia, a Vertisol from Australia, a Cambisol from Germany, and a Gleysol from Switzerland (FAO, 2014). The four soil samples were chosen from a larger collection based on their diverse concentration of P_{org} and composition of the phosphomonoester region in NMR spectra (Reusser et al., 2020). Background information and

some chemical properties of the soils are reported in Table 1. Briefly, the Ferralsol was collected from an improved grassland in 1997 at the Carimagua Research Station's long-term Culticore field experiment in Columbia (Bühler et al., 2003). The Vertisol was collected from an arable field in 2018 located in southern Queensland. The site had been under native shrubland prior to 1992. The Cambisol was collected from a beech forest in 2014, and is part of the "SPP 1685 – Ecosystem Nutrition" project (Bünemann et al., 2016; Lang et al., 2017). The Gleysol was collected from the peaty top soil layer of a drained marshland in 2017, which has been under grassland for at least 20 years.

Soil samples were passed through a 5 mm sieve and dried at 60°C for 5 days, except for the Ferralsol (sieved <2 mm) and the Vertisol (ground <2 mm), which were received dried. Total concentrations of C and N in soils were obtained using combustion of 50 mg ground soil (to powder) weighed into tin foil capsules (vario PYRO cube®, Elementar Analysesysteme GmbH). Soil pH was measured in H_2O with a soil to solution ratio of 1:2.5 (w/w) using a glass electrode.

[Suggested location Table 1]

2.2 Soil phosphorus analyses

Total concentrations of soil P were carried out by X-ray fluorescence spectroscopy (SPECTRO XEPOS ED-XRF, AMETEK®) using 4.0 g of ground to powder soil sample mixed with 0.9 g of wax (CEREOX Licowax, FLUXANA®). The XRF instrument was calibrated using commercially available reference soils. Concentrations of organic P for NMR analysis were carried out using the NaOH-EDTA extraction technique of Cade-Menun et al. (2002) at a soil to solution ratio of 1:10, i.e. extracting 4 g of soil with 40 mL of extractant.

2.3 Hypobromite oxidation

Hypobromite oxidation of NaOH-EDTA soil filtrates was carried out based on a modified version of the method described in Suzumura and Kamatani (1993) and Turner et al. (2012). The hypobromite oxidation procedure is similar to that reported in Turner (2020). Briefly, 10 mL of the NaOH-EDTA filtrate (section 2.2) was placed in a three necked round bottom flask equipped with a septum, a condenser, a magnetic stir bar and thermometer (through a claisen adapter with N_2 adapter). After the addition of 1 mL 10 M aqueous NaOH and vigorous stirring, an aliquot of 0.6 mL Br_2 (which was cooled prior to use) was added, resulting in an exothermic reaction where some of the soil extracts nearly boiled. The optimal volume of Br_2 for oxidation was assessed in a previous pilot study using 0.2, 0.4, 0.6 and 0.8 mL Br_2 volumes, and then observing differences in their NMR spectral features (Figure SI9). The reaction was heated to 100 °C within 10 min and kept at reflux for an additional 5 min. After cooling to room temperature, the solution was acidified with 2 mL of 6 M aqueous HCl solution in order to obtain a pH < 3, which was confirmed with a pH test strip. The acidified solution was reheated to 100 °C for 5 min under a stream of nitrogen to vaporise any excess bromine. The pH of the solution was gradually increased to 8.5 using 10 M aqueous NaOH solution. After dilution with 10 mL of H_2O, 5 mL 50 % (w/w) ethanol and 10 mL 10 % (w/w) barium acetate solution was added to the solution in order to precipitate any IP (Turner et al., 2012). The solution was then heated and boiled for 10 min and allowed to cool down overnight. The solution was subsequently transferred to a 50 mL centrifuge tube and a 10 mL aliquot of 50 % (w/w) ethanol was added, manually shaken, and centrifuged at 1500 g for 15 min. The supernatant was removed and a 15 mL aliquot of 50 % (w/w) ethanol was added to the precipitate, shaken, and then centrifuged again as before. The supernatant was removed and the process repeated once more to further purify the pool of IP. Afterwards, the precipitate was transferred with 20

147 mL of H_2O into a 100 mL beaker that contained a 20 mL volume (equating to a mass of 15 g) of Amberlite® IR-
148 120 cation exchange resin beads in the H^+ form (Sigma-Aldrich, product no. 06428). The suspension was stirred
149 for 15 min and then passed through a Whatman no. 42 filter paper. A 9 mL aliquot of the filtrate was frozen at
150 – 80 °C and then lyophilised prior to NMR analysis. This resulted in 18 - 26 mg of lyophilised material across all
151 soils. Concentrations of total P in solutions were obtained using inductively coupled plasma-optical emission
152 spectrometry (ICP-OES). Concentrations of molybdate reactive P (MRP) were obtained using the malachite green
153 method of Ohno and Zibilske (1991). The difference in concentrations of total P and MRP in solution is molybdate
154 unreactive P (MUP), which is predominantly organic P for these samples. To assess the of effect hypobromite
155 oxidation on the stability of an IP_6, duplicate samples of the Cambisol and the Gleysol were spiked with 0.1 mL
156 of a 11 mM *myo*-IP_6 standard. The recovery of the added *myo*-IP_6 following hypobromite oxidation was calculated
157 using Eq. (1):

158 $$Spike\ recovery\ (\%) = \frac{C_{spiked}\left(\frac{mg}{L}\right) - C_{unspiked}\left(\frac{mg}{L}\right)}{C_{standard\ added}\left(\frac{mg}{L}\right)}, \quad (1)$$

159 where C_{spiked} and $C_{unspiked}$ are the concentrations of *myo*-IP_6 in NaOH-EDTA extracts following hypobromite
160 oxidation of the spiked and unspiked samples, respectively. $C_{standard\ added}$ is the concentration of the added *myo*-IP_6
161 within the standard. As ^{31}P NMR spectroscopy of the standard revealed impurities, the concentration of *myo*-IP_6
162 in the standard was calculated based on the ^{31}P NMR spectrum.

163 **2.4 Sample preparation for solution ^{31}P NMR spectroscopy**

164 The lyophilised material of the untreated soil extracts was prepared for solution ^{31}P NMR spectroscopy based on
165 a modification of the methods of Vincent et al. (2013) and Spain et al. (2018). Briefly, 120 mg of lyophilised
166 material was taken and dissolved in 600 µL of 0.25 M NaOH-0.05 M Na_2EDTA solution (ratio of 1:5). However,
167 for the Cambisol sample, this ratio resulted in a NMR spectrum that exhibited significant line broadening.
168 Therefore, this was repeated on a duplicate sample but at a smaller lyophilised material to solution ratio (ratio of
169 1:7.5), as suggested in Cade-Menun and Liu (2014), which resolved the issue of poor spectral quality. The
170 suspension was stored overnight to allow for complete hydrolysis of phospholipids and RNA (Doolette et al., 2009;
171 Vestergren et al., 2012), which was then centrifuged at 10621 g for 15 min. A 500 µL aliquot of the supernatant
172 was taken, which was subsequently spiked with a 25 µL aliquot of a 0.03 M methylenediphosphonic acid standard
173 made in D_2O (Sigma-Aldrich, product no. M9508) and a 25 µL aliquot of sodium deuteroxide at 40 % (w/w) in
174 D_2O (Sigma-Aldrich, product no. 372072). The solution was then mixed and transferred to a 5 mm diameter NMR
175 tube.
176 A similar procedure was used for the soil extracts that had undergone hypobromite oxidation, except the total mass
177 of lyophilised material (18 - 26 mg) was dissolved with 600 µL of a 0.25 M NaOH-0.05 M Na_2EDTA solution.
178 However, for the Cambisol sample, the NMR spectrum exhibited considerable line-broadening, and an additional
179 400 µL aliquot of NaOH-EDTA solution was added to the NMR tube, mixed, and then returned to the NMR
180 spectrometer. This resolved the issue of poor spectral quality.

181 **2.5 Solution ^{31}P NMR spectroscopy**

182 Solution ^{31}P NMR analyses were carried out on all untreated and hypobromite oxidised soil extracts at the NMR
183 facility of the Laboratory of Inorganic Chemistry (Hönggerberg, ETH Zürich). All spectra were obtained with a
184 Bruker AVANCE III MD 500 MHz NMR spectrometer equipped with a cryogenic probe (CryoProbe™ Prodigy)

(Bruker Corporation; Billerica, MA). The ^{31}P frequency for this NMR spectrometer was 202.5 MHz and gated broadband proton decoupling with a 90° pulse of 12 µs was applied. Spectral resolution under these conditions for ^{31}P was < 1 Hz. Longitudinal relaxation (T_1) times were determined for each sample with an inversion recovery experiment (Vold et al., 1968). This resulted in recycle delays ranging from 8.7 to 30.0 sec for the untreated extracts and 7.8 to 38.0 sec for the hypobromite oxidised soil extracts. The number of scans for the untreated extracts was set to 1024 or 4096, depending on the signal to noise ratio of the obtained spectrum. All hypobromite oxidised spectra were acquired with 3700 to 4096 scans.

2.6 Processing of NMR spectra

All NMR spectra were processed with Fourier transformation, phase correction, and baseline adjustment within the TopSpin® software environment (Version 3.5 pl 7, Bruker Corporation; Billerica, MA). Line broadening was set to 0.6 Hz. Quantification of NMR signals involved obtaining the integrals of the following regions: 1) up to four phosphonates (δ 19.8 to 16.4 ppm); 2) the added MDP (δ 17.0 to 15.8ppm) including its two carbon satellite peaks; 3) the combined orthophosphate and phosphomonoester region (δ 6.0 to 3.0 ppm); 4) up to four phosphodiesters (δ 2.5 to -3.0 ppm), and 5) pyrophosphate (δ -4.8 to -5.4 ppm). Due to overlapping peaks in the orthophosphate and phosphomonoester region, spectral deconvolution fitting (SDF) was applied as described in Reusser et al. (2020). In brief, the SDF procedure involved the fitting of an underlying broad signal, based on the approach of Bünemann et al. (2008) and McLaren et al. (2019). We carried out the SDF with a non-linear optimisation algorithm in MATLAB® R2017a (The MathWorks, Inc.) and fitted visually identifiable peaks by constraining their line-widths at half height as well as the lower and upper boundary of the peak positions along with an underlying broad signal in the phosphomonoester region. The sharp signals of high intensity (e.g. orthophosphate) and the broad peak were fitted using Lorentzian lineshapes, whereas sharp signals of low intensity were fitted using Gaussian lineshapes. The NMR observability of total P (P_{tot}) in NaOH-EDTA extracts was calculated using Eq. (2) (Dougherty et al., 2005; Doolette et al., 2011b):

$$NMR\ observability\ (\%) = \frac{P_{tot}\ NMR}{P_{tot}\ ICP-OES} * 100\ \%\ , \quad (2)$$

where P_{tot} NMR refers to the total P content in mg P/kg_{soil} detected in the soil extracts using solution ^{31}P NMR spectroscopy and P_{tot} ICP-OES refers to the total P concentration in mg P/kg_{soil} measured in the soil extracts prior to freeze-drying using ICP-OES.

2.7 Spiking experiments

To identify the presence of IP in hypobromite oxidised extracts, samples were spiked with a range of standards and then analysed again using NMR spectroscopy. This involved the addition of 5 to 20 µL aliquots of an IP standard solution directly into the NMR tube, which was then sealed with parafilm, manually shaken, and then allowed to settle prior to NMR analysis. Each sample extract was consecutively spiked with no more than four IP standards. The NMR spectra of soil extracts after spiking were overlaid with the NMR spectra of unspiked soil extracts to identify the presence of IP across all soil samples. This comparison of NMR spectra was possible due to negligible changes in the chemical shifts of peaks among soil samples. The IP standards used in this study are listed in Table 2.

[Suggested location Table 2]

2.8 Transverse relaxation (T_2) experiments

Due to the presence of sharp and broad signals in the phosphomonoester region of NMR spectra on hypobromite oxidised soil extracts, transverse relaxation (T_2) experiments were carried out to probe their structural composition. The transverse relaxation (originally spin-spin relaxation) describes the loss of magnetisation in the x-y plane. This loss occurs due to magnetic field differences in the sample, arising either by instrumentally caused magnetic field inhomogeneities or by local magnetic fields in the sample caused by intramolecular and intermolecular interactions (Claridge, 2016). Generally, small, rapidly tumbling molecules exhibit longer T_2 relaxation times compared to large, slowly tumbling molecules (McLaren et al., 2019).

Briefly, solution ^{31}P NMR spectroscopy with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (Meiboom and Gill, 1958) was carried out on all hypobromite oxidised soil extracts, as described in McLaren et al. (2019). This involved a constant spin-echo delay (τ) of 5 ms, which was repeated for a total of eight iterations (spin-echo periods of 5, 50, 100, 150, 200, 250, 300, and 400 ms). A total of 4096 scans and a recycle delay of 4.75 sec was used for all iterations. Transverse relaxation times for the aforementioned integral ranges were calculated using Eq. (3) within the TopSpin® software environment. Due to overlapping peaks in the orthophosphate and phosphomonoester region, spectral deconvolution was carried out to partition the NMR signal, as described in McLaren et al. (2019). The T_2 times of the partitioned NMR signals were calculated using Eq. (3) within RStudio© (version 1.1.442):

$$M(t) = M_0 * e^{(-t*{T_2}^{-1})}, \quad (3)$$

where M refers to the net magnetisation derived from the average angular momentum in the x–y plane, τ refers to the spin-echo delay in milliseconds (ms), and T_2 refers to the transverse relaxation time (ms).

2.9 Statistical analyses and graphics

Statistical analyses were carried out using Microsoft® Excel 2016 and MATLAB R2017a (©The MathWorks, Inc.). Graphics were created with Microsoft® Excel 2016 and MATLAB R2017a (©The MathWorks, Inc.). Solution (1D) ^{31}P NMR spectra were normalised to the peak intensity of MDP (δ 16.46 ppm). Spectra from the T_2 experiments were normalised to the peak intensity of *scyllo*-IP_6 (δ 3.22 ppm).

A one-way ANOVA was carried out in MATLAB R2017a (©The MathWorks, Inc.) with a subsequent multi comparison of mean values using the Tukey's honestly significant difference procedure based on the studentised range distribution (Hochberg and Tamhane, 1987; Milliken and Johnson, 2009).

3 Results

3.1 Phosphorus concentrations in soil extracts

Concentrations of total soil P as determined by XRF ranged from 320 to 3841 mg P/kg_{soil} across all soils (Table 3). Concentrations of total P as estimated by the NaOH-EDTA extraction technique ranged from 160 to 1850 mg P/kg_{soil}, which comprised 28 to 51 % of the total soil P as determined by XRF. Pools of organic P comprised 28 to 72 % of the total P in NaOH-EDTA untreated soil extracts.

Concentrations of total P in NaOH-EDTA soil extracts following hypobromite oxidation ranged from 77 to 578 mg P/kg_{soil} (Table 3), which accounted for 31 to 48 % (on average 38 %) of the total P originally present in the extracts. Similarly, pools of organic P in NaOH-EDTA extracts following hypobromite oxidation were lower, comprising 22 to 48 % (on average 36 %) of that originally present in untreated NaOH-EDTA extracts across all soils.

[Suggested location Table 3]

3.2 Solution ^{31}P NMR spectra of hypobromite oxidised soil extracts

The most prominent signal in the NMR spectra of untreated NaOH-EDTA soil extracts was that of orthophosphate at δ 5.25 (±0.25) ppm, followed by the phosphomonoester region ranging from δ 6.0 to 3.0 ppm (Fig. 1). There were also some minor signals due to pyrophosphate δ -5.06 (±0.19) ppm (all soils), phosphodiesters ranging from δ 2.5 to -2.4 ppm (not detected in the Vertisol), and phosphonates (not including the added MDP) at δ 19.8, 19.2 and 18.3 ppm (not detected in the Gleysol). However, these compounds comprised less than 8 % of the total NMR signal.

Following hypobromite oxidation of NaOH-EDTA extracts, the most prominent NMR signals were found in the orthophosphate (65 % of total NMR signal) and phosphomonoester (35 % of total NMR signal) region across all soils (Fig. 1). Phosphodiesters and pyrophosphates were removed following hypobromite oxidation in the Ferralsol, the Vertisol and the Cambisol (DE). However, some signal remained in the Gleysol at low concentrations (0.4 % of the total NMR signal). Phosphonates were removed following hypobromite oxidation in the Ferralsol and the Vertisol, but a total of five sharp peaks in the phosphonate region were detected (δ 19.59, 18.58, 17.27 and 9.25 ppm) in the Cambisol. These peaks comprised 0.6 % of the total NMR signal.

The phosphomonoester region of NMR spectra on untreated NaOH-EDTA extracts exhibited two main features: 1) the presence of a broad signal centered at around δ 4.1 (±0.1) ppm with an average line-width at half height of 256.12 Hz; and 2) the presence of between 19 and 34 sharp signals. This was similarly the case on hypobromite oxidised extracts, except there was a decrease in the intensity of the broad signal and a change in the distribution and intensity of sharp signals. For the Cambisol and Gleysol, the number of sharp signals in the phosphomonoester region approximately doubled (to 40 and 70 sharp signals, respectively) following hypobromite oxidation. In contrast, less than half of the sharp signals remained in the Ferralsol following hypobromite oxidation (i.e. 14 of the 30 peaks originally present in the untreated extract), whereas one peak was removed following hypobromite oxidation in the Vertisol. There was little change (0.23 ppm) in the chemical shifts of peaks between the untreated and hypobromite oxidised extracts.

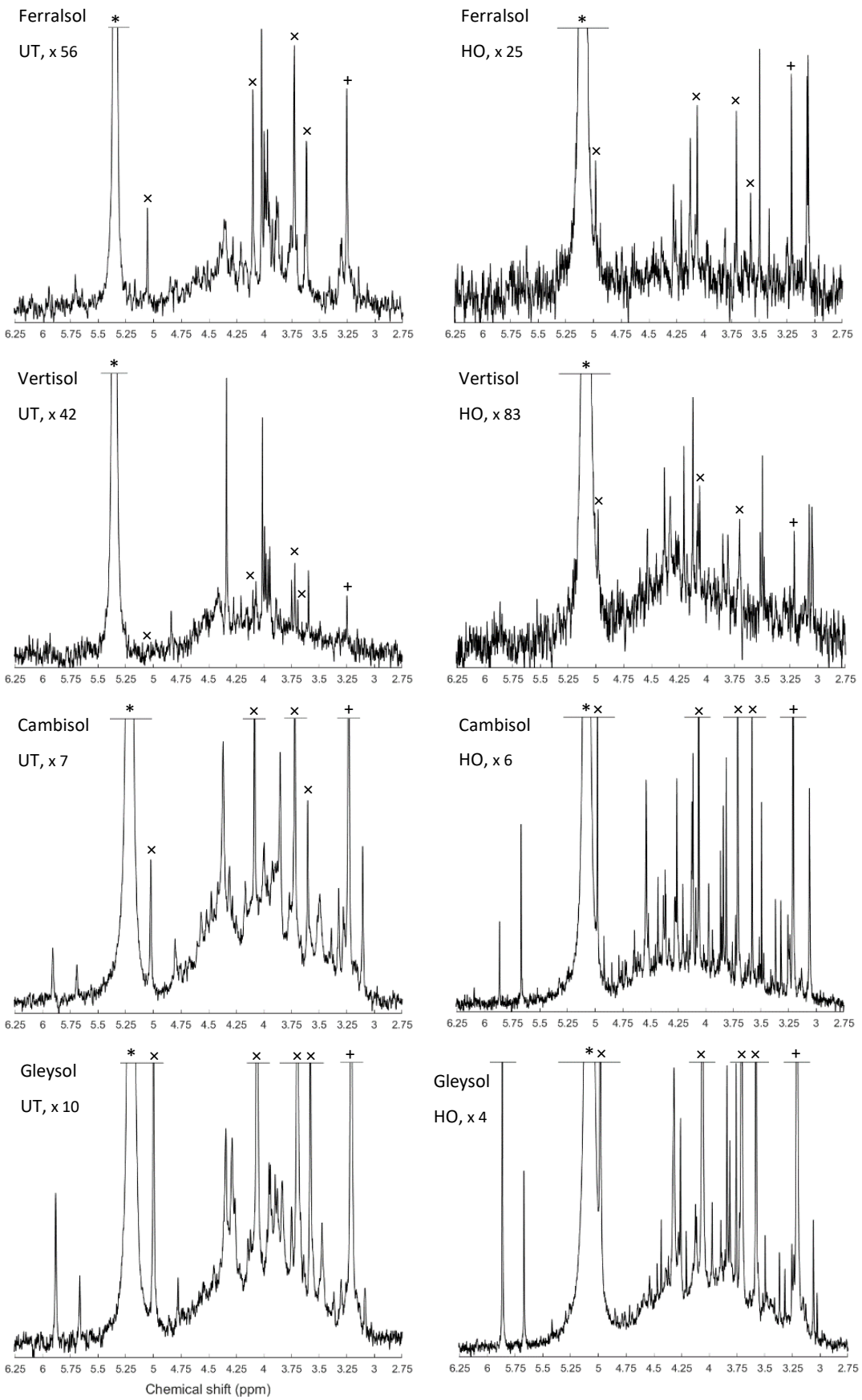

Figure 1. Solution ^{31}P nuclear magnetic resonance (NMR) spectra (500 MHz) of the orthophosphate and phosphomonoester region on untreated (UT) and hypobromite oxidised (HO) 0.25 M NaOH + 0.05 M EDTA soil extracts (Ferralsol, Vertisol, Cambisol and Gleysol). Signal intensities were normalised to the MDP peak intensity. The vertical axes were increased for improved visibility of spectral features, as indicated by a factor. The orthophosphate peak is marked with an asterisk. The symbol '×' marks the four individual peaks of *myo*-IP$_6$ and '+' the peak of *scyllo*-IP$_6$.

[Suggested location Table 4]

3.3 Identification and quantification of inositol phosphates (IP_6, IP_5 and IP_4) in soil extracts

Detailed views of the phosphomonoester regions of spiked samples are shown in Fig. SI1 to SI5 of the Supporting Information. The number of identified sharp peaks in the phosphomonoester region ranged from 7 (Vertisol) to 33 (Gleysol). *myo*- and *scyllo*-IP_6 were identified in the hypobromite oxidised extracts of all soils (Table 5). On average, 72 % of *myo*-IP_6 and 56 % of *scyllo*-IP_6 present in the untreated extracts remained in the hypobromite oxidised extracts (Table SI1 in the Supporting Information). *neo*-IP_6 was identified in the the 2-equatorial/4-axial and 4-equatorial/2-axial conformations, and c*hiro*-IP_6 in the 2-equatorial/4-axial confirmation, of the oxidised extracts in the Cambisol and Gleysol, but were absent in the Ferralsol and the Vertisol (Fig. SI4 and SI5 in the Supporting Information).

The *myo*, *scyllo*, *chiro* and *neo* stereoisomers of IP_5 were identified in various hypobromite oxidised extracts (Table 5). Two isomers of *myo*-IP_5 were identified in some extracts, which included *myo*-(1,2,4,5,6)-IP_5 and *myo*-(1,3,4,5,6)-IP_5. In addition, *scyllo*-IP_4 was detected in all soils except that of the Vertisol. There was insufficient evidence for the presence of *myo*-IP_4 in these soil samples, as only one of the two peaks of this compound was present in the NMR spectra of untreated extracts. This could possibly be due to the partial dephosphorylation of *myo*-IP_4 during the hypobromite oxidation procedure. The reason of the reduced resistance of lower order IP to hypobromite oxidation compared to IP_{5+6} might be due to their reduced steric hindrance and charge density, as less phosphate groups are bound to the inositol ring.

Concentrations of total IP ranged from 1.4 to 159.3 mg P/kg_{soil} across all soils, which comprised between 1 % (Vertisol) and 18 % (Gleysol) of the organic P in untreated NaOH-EDTA extracts (Table 3). Pools of IP_6 were the most abundant form of IP, which ranged from 0.9 to 144.8 mg P/kg_{soil} across all soils (Table 5). The proportion of IP_6 stereoisomers across all soils were in the order of *myo* (61 %, SD=12), *scyllo* (29 %, SD=3), *chiro* (6 %, SD=8) and *neo* (4 %, SD=5). Similarly, the *myo* and *scyllo* stereoisomer were also the most predominant forms of IP_5, but comprised between 83 % (Cambisol) and 100 % (Ferralsol and Vertisol) of total IP_5 (Table 5). Trace amounts of *scyllo*-IP_4 were also detected in three of the four soils. The ratio of total IP_6 to IP_5 differed across all soils (Fig. 2).

[Suggested location Table 5]

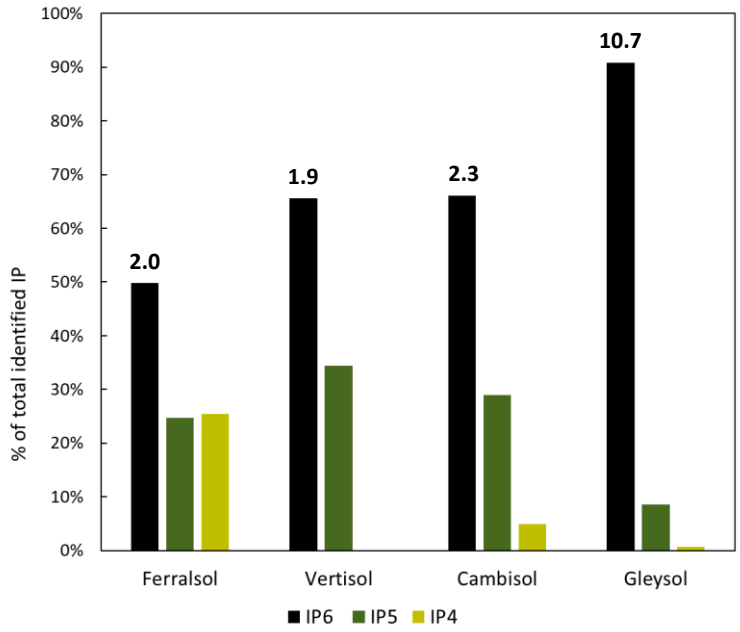

Figure 2. The proportion of total identifiable pools of inositol hexakisphosphates (IP_6), -pentakisphosphates (IP_5) or –tetrakisphosphates (IP_4) to that of the total pool of identifiable IP, as determined by solution ^{31}P NMR spectroscopy on four soil extracts (Ferralsol, Vertisol, Cambisol and Gleysol) following hypobromite oxidation. Values located above the IP_6 bar are the ratio of total identifiable IP_6 to that of IP_5 in each soil sample.

If sharp peaks arising from IP were identified in the NMR spectra on hypobromite oxidised extracts, a comparison was made with that of their corresponding untreated extracts. The sharp peaks of all stereoisomers of IP_6 were present in the untreated extracts. The five peaks of *myo*-(1,2,4,5,6)-IP_5 and the three peaks of *scyllo*-IP_5 were also identified. However, it was not possible to clearly identify other IP_5 compounds in untreated extracts due to overlapping signals. In the Gleysol, all three peaks of *scyllo*-IP_5 were detected, but only two of the possible five peaks could be clearly assigned to *myo*-(1,2,4,5,6)-IP_5. In the Ferralsol, both peaks of *scyllo*-IP_4 were present in the untreated extract, but only two of the three possible peaks could be assigned to *scyllo*-IP_5. In the Vertisol, no IP_5 was identified. Concentrations of IP in untreated extracts assessed by spectral deconvolution fitting were generally double than that measured in hypobromite oxidised extracts. Recoveries of added *myo*-IP_6 in the Gleysol and Cambisol following hypobromite oxidation were 47 % and 20 %, respectively.

3.4 Spin-echo analysis of selected P compounds

Due to the presence of sharp and broad signals in hypobromite oxidised soil extracts, the structural composition of phosphomonoesters was probed. A comparison of the NMR spectra at the lowest (1*τ) and highest (80*τ) pulse delays revealed a fast decaying broad signal for all hypobromite oxidised soil extracts, which was particularly evident in the Gleysol (Fig. 3). Calculated T_2 times of all IP_6 stereoisomers were longer than that of the broad signal (Table 6). The T_2 times of *scyllo*-IP_6 (on average 175.8 ms, SD=49.7) were generally the longest of all stereoisomers of IP_6. The T_2 time of the orthophosphate peak was the shortest, which was on average 11.5 ms (SD=4.9).

The average (n=4) T_2 times of the broad peak was significantly different than that of *scyllo*- and *myo*-IP_6 ($p < 0.05$). Significant differences in the T_2-times of *neo*- and D-*chiro*-IP_6 were not tested, as these compounds were not detected in the Ferralsol and the Vertisol.

336 [Suggested location Table 6]

337

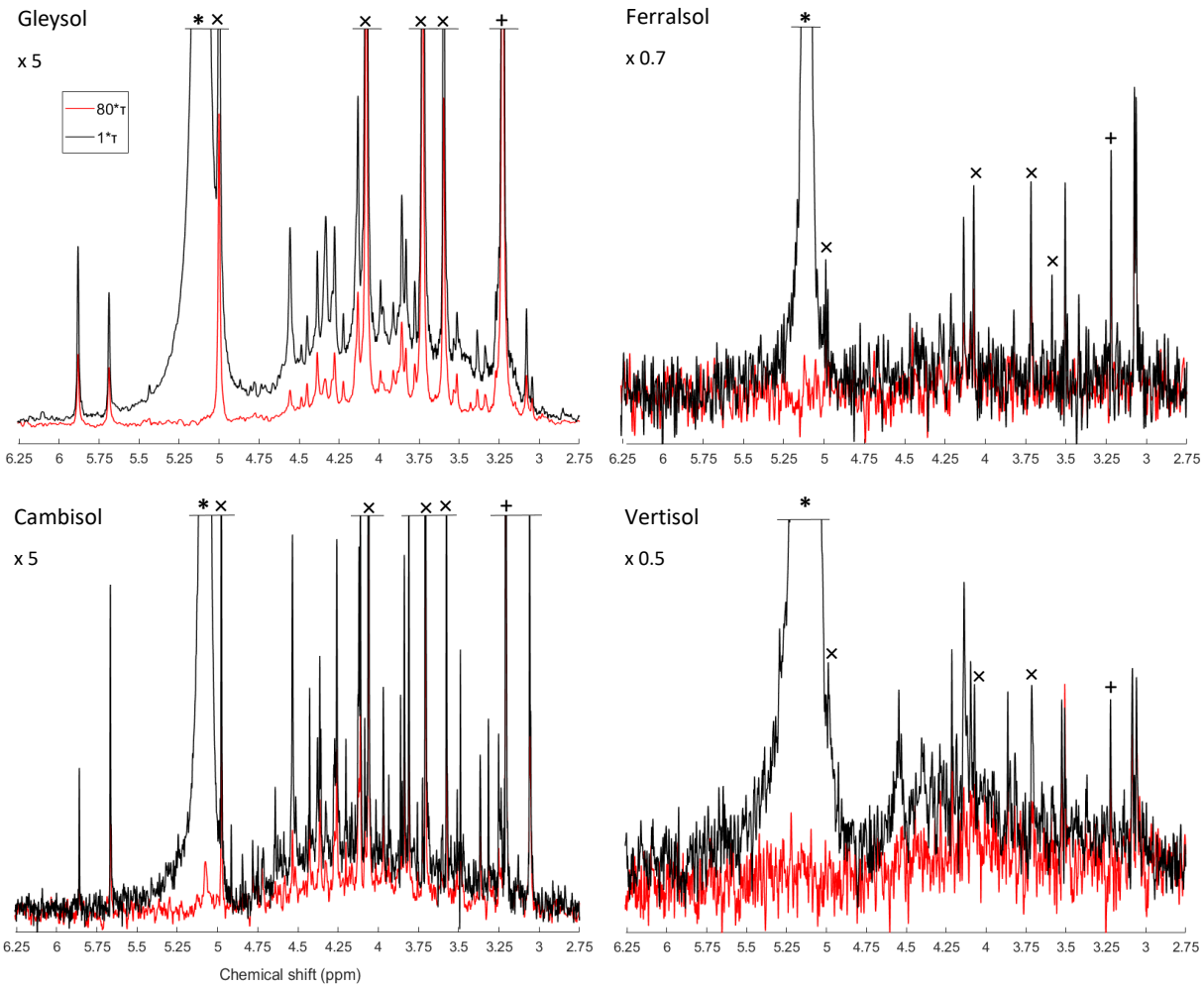

Figure 3. Solution ^{31}P NMR spectra of hypobromite oxidised soil extracts acquired with a CPMG pulse sequence with 1*τ (black) and 80*τ (red) spin-echo delays. The orthophosphate (*), *scyllo*-IP$_6$ (+) and *myo*-IP$_6$ peaks (×) are marked accordingly. Spectra were normalised to the maximum *scyllo*-IP$_6$ peak intensity in the 1*τ spectrum for each soil. The vertical axes were increased/decreased for better visualisation by an indicated factor.

338 # 4 Discussion

339 ## 4.1 Pools of phosphorus in untreated and hypobromite oxidised soil extracts

340 On average, 44 % of total P (as measured with XRF) was extracted by NaOH-EDTA, which is consistent with
341 previous studies (Turner, 2008; Li et al., 2018; McLaren et al., 2019). The non-extractable pool of P is likely to
342 comprise of inorganic P as part of insoluble mineral phases, but could also contain some organic P (McLaren et
343 al., 2015a). Nevertheless, the NaOH-EDTA extraction technique is considered to be a measure of total organic P
344 in soil, which can be subsequently characterised by solution ^{31}P NMR spectroscopy (Cade-Menun and Preston,
345 1996).

346 Hypobromite oxidation resulted in a decrease in the concentration of inorganic and organic P in NaOH-EDTA
347 extracts across all soils. The decrease of organic P is consistent with previous studies (Turner and Richardson,
348 2004; Turner et al., 2012; Almeida et al., 2018). However, Almeida et al. (2018) reported an overall increase in
349 the concentration of inorganic P following hypobromite oxidation, which the authors proposed to be caused by the

degradation of organic P forms not resistant to hypobromite oxidation. A decrease in the concentration of organic P in NaOH-EDTA extracts following hypobromite oxidation was expected based on the oxidation of organic molecules containing P. The products of hypobromite oxidation are most probably carbon dioxide, simple organic acids from the oxidative cleavage of the phosphoesters and orthophosphate (Irving and Cosgrove, 1981; Sharma, 2013).

Overall, hypobromite oxidation of NaOH-EDTA soil extracts resulted in a considerable increase in the number of sharp peaks and a decrease in the broad underlying peak in the phosphomonoester region compared to that of untreated soil extracts. This was particularly the case for the Cambisol and the Gleysol, which had high concentrations of extractable organic P. Since the broad peak is thought to be closely associated with the SOM (Dougherty et al., 2007; Bünemann et al., 2008; McLaren et al., 2015b), its decrease in soil extracts following hypobromite oxidation is consistent with that observed for other organic compounds (Turner et al., 2012). Our results indicate that the majority of sharp peaks present in the phosphomonoester region of untreated soil extracts are stable to hypobromite oxidation, and are therefore likely to be IP.

Across all soils, 5 to 15 peaks in the phosphomonoester region were removed following hypobromite oxidation compared to those in untreated extracts, which are likely due to the oxidation of: α-and β-glycerophosphate (Doolette et al., 2009; McLaren et al., 2015b), RNA mononucleotides (8 peaks) (Vincent et al., 2013), glucose 6-phosphate, phosphocholine, glucose 1-phosphate, or phosphorylethanolamine (Cade-Menun, 2015).

4.2 Phosphorus assignments of sharp peaks in hypobromite oxidised extracts

The detection of *myo-, scyllo-*, *chiro*, and *neo*-IP_6 in untreated and hypobromite oxidised soil extracts is consistent with previous studies using chromatography (Irving and Cosgrove, 1982; Almeida et al., 2018) and NMR (Turner and Richardson, 2004; Doolette et al., 2011a; Vincent et al., 2013; Jarosch et al., 2015; McLaren et al., 2015b). Turner et al. (2012) suggested that hypobromite oxidised extracts only contained *neo*-IP_6 in the 4-equatorial/2-axial conformation due to the absence of signals from the 2-equatorial/4-axial conformation. In the current study, both conformations could be identified in two of the four soil extracts, which is likely due to improved spectral resolution and sensitivity. The relative abundances of the four identified stereoisomers of IP_6 in soil extracts were similar to previous studies (Irving and Cosgrove, 1982; Turner et al., 2012).

Several studies have shown overlap of peaks relating to RNA mononucleotides and that of α-and β-glycerophosphate, which are the alkaline hydrolysis products of RNA and phospholipids, respectively. However, in the current study, several sharp peaks were present in hypobromite oxidised extracts which are in the chemical shift range of RNA mononucleotides and α-and β-glycerophosphate. Whilst a peak at δ 4.36 ppm would be assigned to α-glycerophosphate based on spiking experiments in the untreated extracts of the Cambisol and the Gleysol, hypobromite oxidation revealed the presence of the 2-equatorial/4-axial C2,5 peak of *neo*-IP_6 at δ 4.37 ppm, and also an unidentified peak at δ 4.36 ppm in the Cambisol. Therefore, the assignment and concentration of α-glycerophosphate may be unreliable in some soils of previous studies.

For the first time, we identified lower-order IP (IP_5 and IP_4) in soil extracts using solution ^{31}P NMR spectroscopy. Smith and Clark (1951) were the first to suggest the presence of IP_5 in soil extracts using anion-exchange chromatography, which was later confirmed (Anderson, 1955; Cosgrove, 1963; McKercher and Anderson, 1968b). Halstead and Anderson (1970) reported the presence of all four stereoisomers (*myo*, *scyllo*, *neo* and *chiro*) in the lower ester fractions (IP_2-IP_4) as well as the higher ester fractions (IP_5, IP_6) isolated from soil, with the *myo* stereoisomer being the main form in all fractions. In the current study, all four stereoisomers of IP_5 could be

detected in the hypobromite oxidised soil extracts, of which the *myo* and *scyllo* stereoisomers were the most abundant. The relative abundances of IP_5 stereoisomers are consistent with the findings of Irving and Cosgrove (1982) using gas-liquid chromatography on the combined $IP_6 + IP_5$ fraction. The detection of all four stereoisomers of IP_5 in NMR spectra provides direct spectroscopic evidence for their existence in soil extracts.

In addition to the four stereoisomers of IP_5, we were able to identify the presence of two isomers of *myo*-IP_5 in the Cambisol and Gleysol, i.e. *myo*-(1,2,4,5,6)-IP_5 and *myo*-(1,3,4,5,6)-IP_5. These two isomers have not yet been detected in soil extracts. A distinction of different *myo*-IP_5 isomers was not reported in earlier studies using chromatographic separation. In non-soil extracts, *myo*-(1,2,4,5,6)-IP_5 was detected by Doolette and Smernik (2016) in grapevine canes, and *myo*-(1,3,4,5,6)-IP_5 as the thermal decomposition product of a phytate standard (Doolette and Smernik, 2018). It is possible that an abiotic transformation of *myo*-IP_6 to *myo*-(1,3,4,5,6)-IP_5 occurs, which could then be adsorbed by soil constituents. Stephens and Irvine (1990) reported *myo*-(1,3,4,5,6)-IP_5 as an intermediate in the synthesis of IP_6 from *myo*-IP in the cellular slime mould *Dictyostelium*. Therefore, *myo*-(1,3,4,5,6)-IP_5 could have been biologically added to the soil. Furthermore, *myo*-(1,3,4,5,6)-IP_5 was present in different animal feeds and manures (Sun and Jaisi, 2018). Sun et al. (2017) reported *myo*-(1,3,4,5,6)-IP_5 and *myo*-(1,2,4,5,6)-IP_5 as intermediates in the minor, resp. major pathways of Aspergillus niger phytase and acid phosphatase (potato) phytate degradation. The presence of *myo*-(1,2,3,4,6)-IP_5 could not be confirmed as NMR analyses on the compound itself exhibited a broad NMR signal (Fig. SI7 in the Supporting Information). This is because in solutions with a pH of 9.5 or above, the 1-axial/5-equatorial and 5-axial/1-equatorial forms of *myo*-(1,2,3,4,6)-IP_5 are in a dynamic equilibrium, which can cause broadening (Volkmann et al., 2002). According to Turner and Richardson (2004) and Chung et al. (1999), the two identified *scyllo*-IP_4 peaks (signal pattern 2:2) can be attributed to the *scyllo*-(1,2,3,4)-IP_4 isomer. However, the two peaks of *scyllo*-IP_4 overlapped in the Cambisol and Gleysol with the peak at the furthest upfield chemical shift of *myo*-(1,2,4,5,6)-IP_5 at δ 3.25 ppm, and with the peak at the furthest downfield chemical shift of *myo*-(1,3,4,5,6)-IP_5 at δ 4.12 ppm. Turner and Richardson (2004) reported NMR-signals for two other *scyllo*-IP_4 isomers, which could not be tested for in this study due to the lack of available standards.

Whilst on average 48 % of the sharp peaks in the phosphomonoester region of soil extracts following hypobromite oxidation could be attributed to IP_6, IP_5 and *scyllo*-IP_4, the identity of many sharp peaks remain unknown. An unidentified peak at δ 4.33 ppm is present in all soil samples except in the Ferralsol, with concentrations of up to 10 mg P/kg_{soil} (Cambisol). Other unidentified peaks at δ 3.49, 3.86, 4.20 and 3.91 ppm were detected in all soils, with concentrations ranging from 1 to 2 mg P/kg_{soil}. Interestingly, two peaks upfield of *scyllo*-IP_6 became more prominent (at δ 3.08, 3.05 ppm) following hypobromite oxidation, which was particularly the case in the Vertisol soil. The diversity of organic P species in the Vertisol soil appears to be much greater than previously reported (McLaren et al., 2014). We hypothesise that many of these unidentified peaks arise from other isomers of *myo*- and *scyllo*-IP_5, based on the higher abundance of their IP_6 counterparts.

The ratio of IP_6 to lower-order IP varied across soils, which ranged in decreasing order: Gleysol ≫ Cambisol > Vertisol > Ferralsol. McKercher and Anderson (1968a) found a higher ratio of IP_6 to IP_5 in some Scottish soils (ratio 1.8 to 4.6) compared to some Canadian soils (0.9 to 2.4). The authors attributed this difference to the greater stabilization of IP_6 relative to lower esters in the Scottish soils, possible due to climatic reasons or effects of different soil properties. In a subsequent study, McKercher and Anderson (1968b) observed increased IP contents with increasing total organic P content. Studies of organic P speciation along chronosequences found that *myo*-IP_6 concentrations declined in older soils (McDowell et al., 2007; Turner et al., 2007a; Turner et al., 2014). Similarly,

Baker (1976) found that the $IP_6 + IP_5$ concentrations in the Franz Josef chronosequence increased until 1000 years, followed by a rapid decline. In our soil samples, the highest IP_6 to IP_5 ratio was found in the soil with the highest SOM content, suggesting a possible stabilization of IP_6 due to association with SOM (Borie et al., 1989; Makarov et al., 1997). In contrast, the Ferralsol sample containing high amounts of Fe and Al showed the smallest IP_6 to IP_5 ratio, even though IP_6 is known to strongly adsorb to sesquioxides (Anderson and Arlidge, 1962; Anderson et al., 1974). However, the production, input and mineralisation rates of IP_6 and IP_5 are not known for our soil samples. Further research is needed to understand the mechanisms controlling the flux of lower-order IP in soil.

In the Ferralsol and the Cambisol, there was an overall decrease in the concentration of IP_6 and IP_5 following hypobromite oxidation compared to the untreated extracts. Since the main cause of resistance of IP to hypobromite oxidation is that of steric hindrance, which generally decreases with decreasing phosphorylation state and conformation of the phosphate groups (axial vs. equatorial), we assume that low recoveries of added *myo*-IP_6 is due to losses of precipitated P_{org} compounds during the precipitation and dissolution steps. This is supported by the decrease in the concentration of orthophosphate following hypobromite oxidation compared to untreated extracts. Therefore, quantities of IP as reported in the current study should be considered as conservative.

4.3 Structural composition of phosphomonoesters in hypobromite oxidised soil extracts

The NMR spectra on hypobromite oxidised soil extracts revealed the presence of sharp and broad signals in the phosphomonoester region. Transverse relaxation experiments revealed a rapid decay of the broad signal compared to the sharp peaks of IP_6, which support the hypothesis that the compounds causing the broad signal arise from P compounds other than IP. These findings are consistent with that of McLaren et al. (2019), who probed the structural composition of phosphomonoesters in untreated soil extracts. Overall, measured T_2 times in the current study on hypobromite oxidised extracts were markedly longer compared to that on untreated extracts reported in McLaren et al. (2019). This could be due to removal of other organic compounds by hypobromite oxidation in the matrix and therefore a decrease in the viscosity of the sample. This would result in an overall faster tumbling of the molecules and hence an increased T_2 relaxation time. As reported by McLaren et al. (2019), calculations of the broad signal's linewidth based on the T_2 times were considerably lower compared to that of the standard deconvolution fitting (SDF). When applying the same calculations to our samples, the linewidth of the broad signal at half height is on average 5.2 Hz based on the T_2 times. In contrast, the linewidths acquired from the SDF average to 256.1 Hz. McLaren et al. (2019) suggested that the broad signal is itself comprised of more than one compound. Our results are consistent with this view and therefore it is likely that the main cause of the broad signal is a diversity of P molecules of differing chemical environments within this region, rather than the slow tumbling of just one macromolecule. Nebbioso and Piccolo (2011) reported that high molecular weight material of organic matter in soil is an association of smaller organic molecules. We suggest that these associations would still cause a broad signal in the phosphomonoester region of soil extracts and could be a reason that some organic molecules containing P are protected from hypobromite oxidation.

Since a portion of the broad signal is resistant to hypobromite oxidation, this suggests the organic P is complex and in the form of polymeric structures. The chemical resistance of the broad signal to hypobromite oxidation may also indicate a high stability in soil (Jarosch et al., 2015). Annaheim et al. (2015) found that concentrations of the broad signal remained unchanged between three different organic fertiliser strategies after 62 years of cropping. In contrast, the organic P compounds annually added with the fertilisers were completely transformed or lost in

the slightly acidic topsoil of the field trial. The large proportion of the broad signal in the total organic P pool demonstrates its importance in the soil P cycle.

Unexpectedly, the transverse relaxation times of orthophosphate were shorter than that of the broad signal. This was similarly the case in an untreated NaOH-EDTA extract of a forest soil with the same origin as the Cambisol as reported in McLaren et al. (2019). The authors hypothesised that this might be due to the sample matrix (i.e. high concentration of metals and organic matter). Whilst these factors are likely to affect T_2 times, they do not appear to be the main cause as the hypobromite oxidised extracts in the current study contained low concentrations of organic matter and metals as a consequence of the isolation procedure. The fast decay of orthophosphate was found across all four soil extracts with a diverse array of organic P concentrations and compositions of organic P in the phosphomonoester region. Therefore, another possible explanation could be a matrix effect or an association with large organic P compounds causing the broad signal (McLaren et al., 2019). It is known that dynamic intramolecular processes as ring inversion and intermolecular processes such as binding of small-molecule ligands to macromolecules can cause a broadening or a doubling of resonances (Claridge, 2016). When the smaller molecule is bound to the larger molecule, it experiences slower tumbling in the solution and hence a shorter T_2 time. It is possible that a chemical exchange of the orthophosphate with a compound in the matrix or an organic P molecule could result in the short T_2 time of the orthophosphate peak. We carried out a T_2 experiment on a pure solution of monopotassium phosphate (described in the Supporting Information), in which the matrix effects should be considerably reduced compared to the soil extracts. We found that the T_2 time of orthophosphate (203 ms) in the pure solution was considerably longer than that reported in soil extracts following hypobromite oxidation.

5 Conclusion

Inositol phosphates are an important pool of organic P in soil, but information on the mechanisms controlling their flux in soil remain limited due in part to an inability to detect them using solution ^{31}P NMR spectroscopy. For the first time, we identified six different lower-order IP in the solution ^{31}P NMR spectra on soil extracts. Solution ^{31}P NMR spectra on hypobromite oxidised extracts revealed the presence of up to 70 sharp peaks, which about 50 % could be identified. Our results indicate that the majority of the sharp peaks in solution ^{31}P NMR soil spectra were resistant to hypobromite oxidation, and therefore suggest the presence of diverse IP. Our study highlights the great diversity and abundance of IP in soils and therefore their importance in terrestrial P cycles. Further research on the mechanisms and processes involved in the cycling of this wide variety of IP in soil will have implications on our understanding of organic P turnover as well as plant availability, and possibly help improve fertiliser strategies in agricultural systems.

Furthermore, we provide new insight on the large pool of phosphomonoesters represented by the broad signal, of which a considerably portion was resistant to hypobromite oxidation. Further research is needed to understand the chemical composition of the broad signal, and the mechanisms controlling its flux in terrestrial ecosystems.

Data availability

All data presented in this study and the Supplement is also available by request from the corresponding author.

Author contribution

The experimental design was planned by JR, TM, DZ, RV and EF. The experiments were carried out by JR under supervision of TM, DZ and RV. RV provided the MATLAB code for the standard deconvolution fitting of the NMR spectra. The data was processed, analysed and interpreted by JR with support from TM, DZ and RV. JR prepared the manuscript with contributions from all co-authors.

Conflicts of interest

The authors declare that they have no conflict of interest.

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752 **Table 1. General characteristics of soil samples used in this study.**

Soil type	-	Ferralsol	Vertisol	Cambisol	Gleysol
Country	-	Colombia	Australia	Germany	Switzerland
Coordinates sampling site	-	4°30' N / 71°19' W	27°52' S / 151°37' E	50°21’ N / 9°55’ E	47°05' N / 8°06' E
Elevation	m ASL	150	402	800	612
Sampling depth	cm	0-20	0-15	0-7	0-10
Year of sampling	year	1997	2017	2014	2017
Land use	-	Pasture	Arable field	Forest	Pasture
C_{tot}	g C/kg_{soil}	26.7	23.9	90.3	148.3
N_{tot}	g N/kg_{soil}	1.7	1.9	6.6	10.9
pH in H_2O	-	3.6	6.1	3.6	5.0

753

754 **Table 2. Standard solutions used for the spiking experiment of the hypobromite oxidised soil extracts. All standards**
755 **were dissolved in 0.25 M NaOH and 0.05 M Na_2EDTA.**

Standard	Product number	Company/origin	Concentration of standard in NaOH-EDTA (mg/mL)
myo-IP_6	P5681	Merck (Sigma-Aldrich)	8.10
L-*chiro*-IP_6	Collection of Dr Max Tate		2.39
D-*chiro*-IP_6	CAY-9002341	Cayman Chemical	2.00
neo-IP_6	Collection of Dr Dennis Cosgrove, made up in 15 mM HCl		4.62
D-*myo*-(1,2,4,5,6)-IP_5	CAY-10008452-1	Cayman Chemical	2.00
myo-(1,2,3,4,6)-IP_5	93987	Merck (Sigma-Aldrich)	2.00
D-*myo*-(1,3,4,5,6)-IP_5	CAY-10009851-1	Cayman Chemical	2.00
D-*myo*-(1,2,3,5,6)-IP_5	CAY-10008453-1	Cayman Chemical	2.00
scyllo-IP_5	Collection of Dr Dennis Cosgrove		2.64
L-*chiro*-IP_5	Collection of Dr Dennis Cosgrove		2.24
neo-IP_5	Collection of Dr Dennis Cosgrove		2.45
myo-IP_4	Collection of Dr Dennis Cosgrove		2.76
scyllo-IP_4	Collection of Dr Dennis Cosgrove		2.41
neo-IP_4	Collection of Dr Dennis Cosgrove		2.33

756

757 **Table 3. Concentrations of total P as measured by XRF and 0.25 M NaOH + 0.05 M EDTA extractable P before and**
758 **after hypobromite oxidation of soil extracts. Concentrations of total P in NaOH-EDTA extracts were determined by**
759 **ICP-OES, whereas that of molybdate reactive P (MRP) was determined by the malachite green method of Ohno and**
760 **Zibilske (1991). Concentrations of molybdate unreactive P (MUP) were calculated as the difference between total P and**
761 **MRP.**

Measure		Ferralsol	Vertisol	Cambisol	Gleysol
XRF	P_{tot} (mg P/kg_{soil})	320	1726	3841	2913
NaOH-EDTA extractable P (untreated)	P_{tot} (mg P/kg_{soil})	160	484	1850	1490
	MRP (mg P/kg_{soil})	67	351	525	610
	MUP (P_{org}) (mg P/kg_{soil})	93	133	1326	880
NaOH-EDTA extractable P (hypobromite oxidised)	P_{tot} (mg P/kg_{soil})	77	158	580	578
	MRP (mg P/kg_{soil})	32	111	283	231
	MUP (P_{org}) (mg P/kg_{soil})	45	47	297	348

762

763 **Table 4. Concentrations (mg P/kg$_{soil}$) of P compounds in solution ^{31}P NMR spectra of 0.25 M NaOH + 0.05 M EDTA**
764 **soil extracts (Ferralsol, Vertisol, Cambisol and Gleysol) before and after hypobromite oxidation (HO). Quantification**
765 **was based on spectral integration and deconvolution fitting. The proportion of P detected in hypobromite oxidised**
766 **extracts compared to that in untreated extracts is provided in brackets.**

Phosphorus class		Ferralsol	Vertisol	Cambisol	Gleysol
Phosphonates	before HO	1.0	2.6	14.5	-
	after HO	-	-	3.0 (21)	0.2
Orthophosphate	before HO	54.8	221.4	434.3	368.3
	after HO	32.0 (58)	116.6 (53)	329.3 (76)	243.4 (66)
Phosphomonoester	before HO	36.3	39.1	501.1	399.2
	after HO	12.7 (35)	24.2 (62)	210.3 (42)	292.1 (73)
Broad peak in phosphomonoester region	before HO	21.6	30.9	305.8	216.7
	after HO	8.3 (39)	19.3 (63)	99.2 (32)	108.4 (50)
Phosphodiester	before HO	5.1	-	28.2	26.9
	after HO	-	-	-	2.0 (8)
Pyrophosphate	before HO	1.9	1.8	12.9	23.9
	after HO	-	-	-	-

767

768 **Table 5. Concentrations of identified inositol phosphates (IP) in hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA**
769 **soil extracts (Ferralsol, Vertisol, Cambisol and Gleysol). Concentrations were calculated from solution ^{31}P NMR spectra**
770 **using spectral deconvolution fitting including an underlying broad signal. When no concentration is given, the IP**
771 **compound was not detected in the respective soil extract. Chemical shift positions are based on the NMR spectrum of**
772 **the Cambisol extract (Fig. SI8 in the Supporting Information). Peak positions varied up to +0.018 ppm (Gleysol).**
773 **Conformation equatorial (eq) and axial (ax) according to Turner et al. (2012).**

Phosphorus compound	Chemical shift δ ppm	Concentrations (mg P/kg$_{soil}$)			
		Ferralsol	Vertisol	Cambisol	Gleysol
***myo*-IP$_6$**	4.97, 4.06, 3.70, 3.57	1.1	0.6	26.3	85.0
***scyllo*-IP$_6$**	3.20	0.4	0.3	15.6	41.1
***neo*-IP$_6$ 4-eq/2-ax**	5.86, 3.75	-	-	1.4	8.8
***neo*-IP$_6$ 2-eq/4-ax**	4.36, 4.11	-	-	4.0	1.3
D-*chiro*-IP$_6$ 2-eq/4-ax	5.66, 4.25, 3.83	-	-	9.4	8.6
***myo*-(1,2,4,5,6)-IP$_5$**	4.42, 3.97, 3.72, 3.36, 3.25	-	-	7.0	4.1
***myo*-(1,3,4,5,6)-IP$_5$**	4.12, 3.60, 3.23	-	-	2.8	1.3
***scyllo*-IP$_5$**	3.81, 3.31, 3.05	0.7	0.5	10.8	6.1
***neo*-IP$_5$**	4.64, 4.27, 4.01, 3.87, 3.13	-	-	3.3	2.1
***chiro*-IP$_5$**	4.61, 3.39	-	-	0.9	-
***scyllo*-(1,2,3,4)-IP$_4$**	4.12, 3.25	0.8	-	4.3	1.0
Total IP		3.0	1.4	85.9	159.3

774

775 **Table 6. Transversal relaxation times (T_2) of various P species in the orthophosphate and phosphomonoester regions as**
776 **determined by solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy and a Carr-Purcell- Meiboom-Gill**
777 **(CPMG) pulse sequence on hypobromite oxidised soil extracts.**

Phosphorus compound	T_2 [ms]			778
	Ferralsol	**Vertisol**	**Cambisol**	**Gleysol**
***myo*-IP_6**	163	140	139	121
***scyllo*-IP_6**	250	155	154	144
***neo*-IP_6**	-	-	203	102
***D-chiro*-IP_6**	-	-	108	132
orthophosphate	14	9	17	6
broad peak	44	69	89	62

SUPPORTING INFORMATION

NMR observability. Measures of NMR observability were calculated for the untreated and the hypobromite oxidised extracts of all soils. Measures of NMR observability refer to the percentage of total P detected using NMR compared to that by ICP-OES. For the untreated soil extracts, measures of NMR observability ranged from 52 % (Gleysol) to 89 % (Ferralsol), with an average NMR observability of 66 %. For the hypobromite oxidised extracts, measures of NMR observability ranged from 58 % (Ferralsol) to 94 % (Cambisol), with an average value of 83 %.

Inositol hexakisphosphate concentrations before and after hypobromite oxidation.

Table SI1. Concentrations of inositol hexakisphosphates in 0.25 M NaOH + 0.05 M EDTA soil extracts before and after hypobromite oxidation (HO). Quantification was based on spectral integration and deconvolution fitting of solution ^{31}P NMR spectra. The proportion of P (%) detected in hypobromite oxidised extracts compared to that in untreated extracts is provided in brackets.

Concentrations (mg P/kg$_{soil}$)		Ferralsol	Vertisol	Cambisol	Gleysol
***myo*-IP$_6$**	before HO	4.4	0.6	46.2	90.4
	after HO	1.1 (25)	0.6 (111)	26.3 (57)	85.0 (94)
***scyllo*-IP$_6$**	before HO	2.5	0.4	34.9	42.6
	after HO	0.4 (14)	0.3 (68)	15.6 (45)	41.1 (97)
***neo*-IP$_6$ 4-eq/2-ax**	before HO	-	-	4.2	7.0
	after HO	-	-	1.4 (33)	8.8 (126)
D-*chiro*-IP$_6$	before HO	-	-	7.2	6.7
	after HO	-	-	9.4 (130)	8.6 (128)

Solution ^{31}P NMR spectra of spiked hypobromite oxidised soil extracts

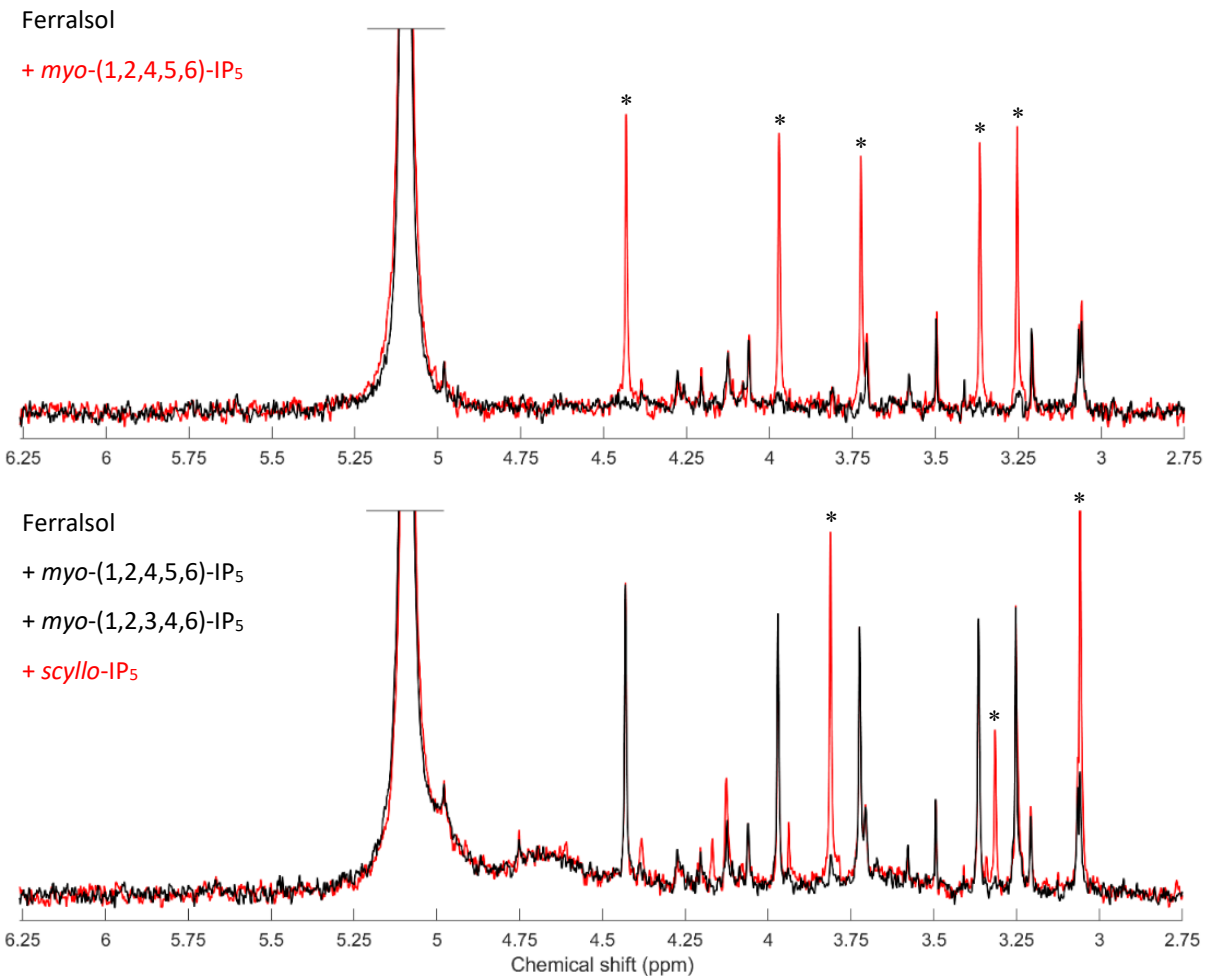

Figure SI1. Solution ^{31}P NMR spectra of the orthophosphate and phosphomonoester region on Ferralsol extract following hypobromite oxidation (black trace), and also that following a spike with an IP standard (red trace). Peaks assigned to the IP standard marked with *.

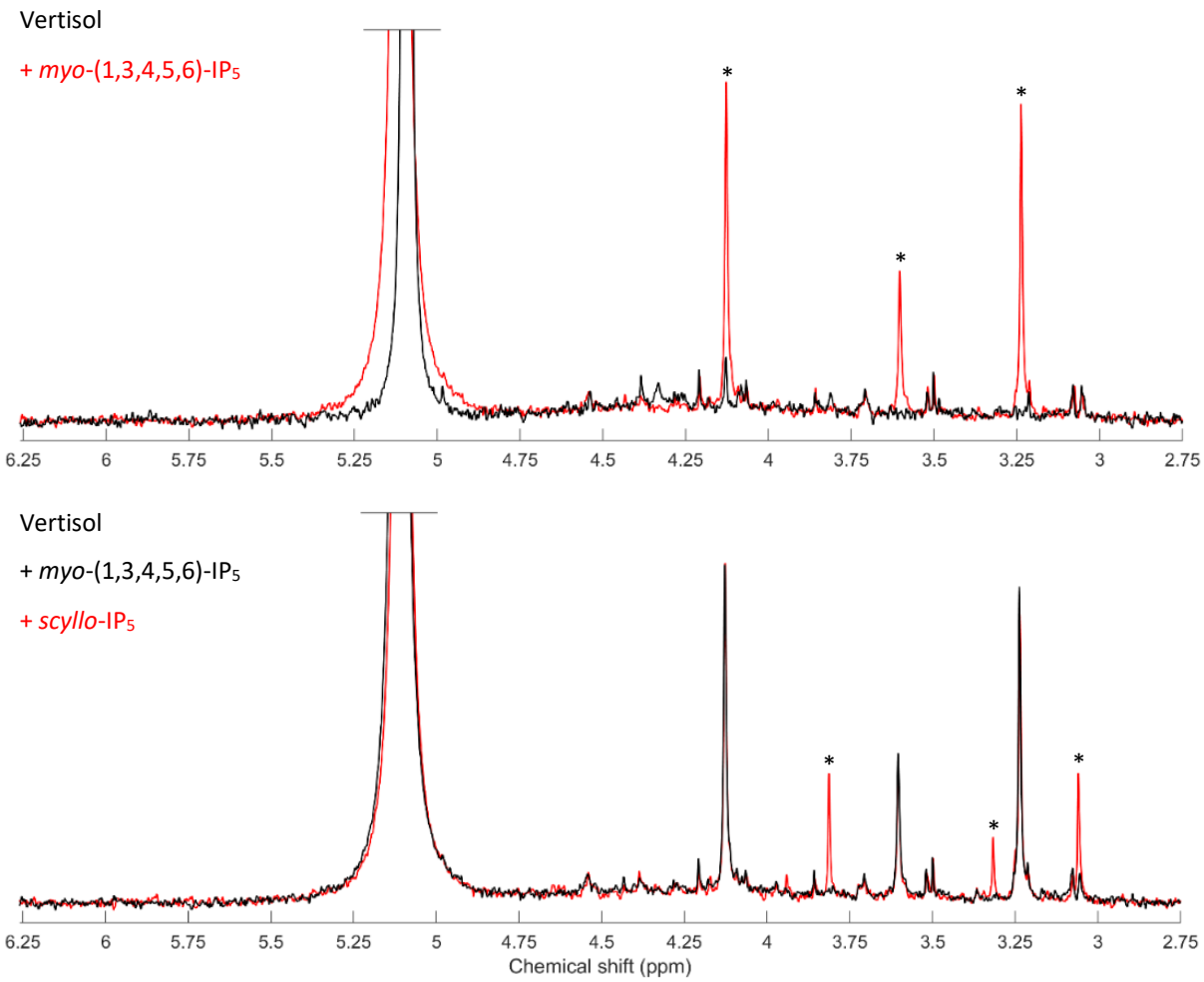

Figure SI2. Solution ^{31}P NMR spectra of phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Vertisol extract. Spiked spectrum with indicated standard in red. Peaks assigned to standard marked with *.

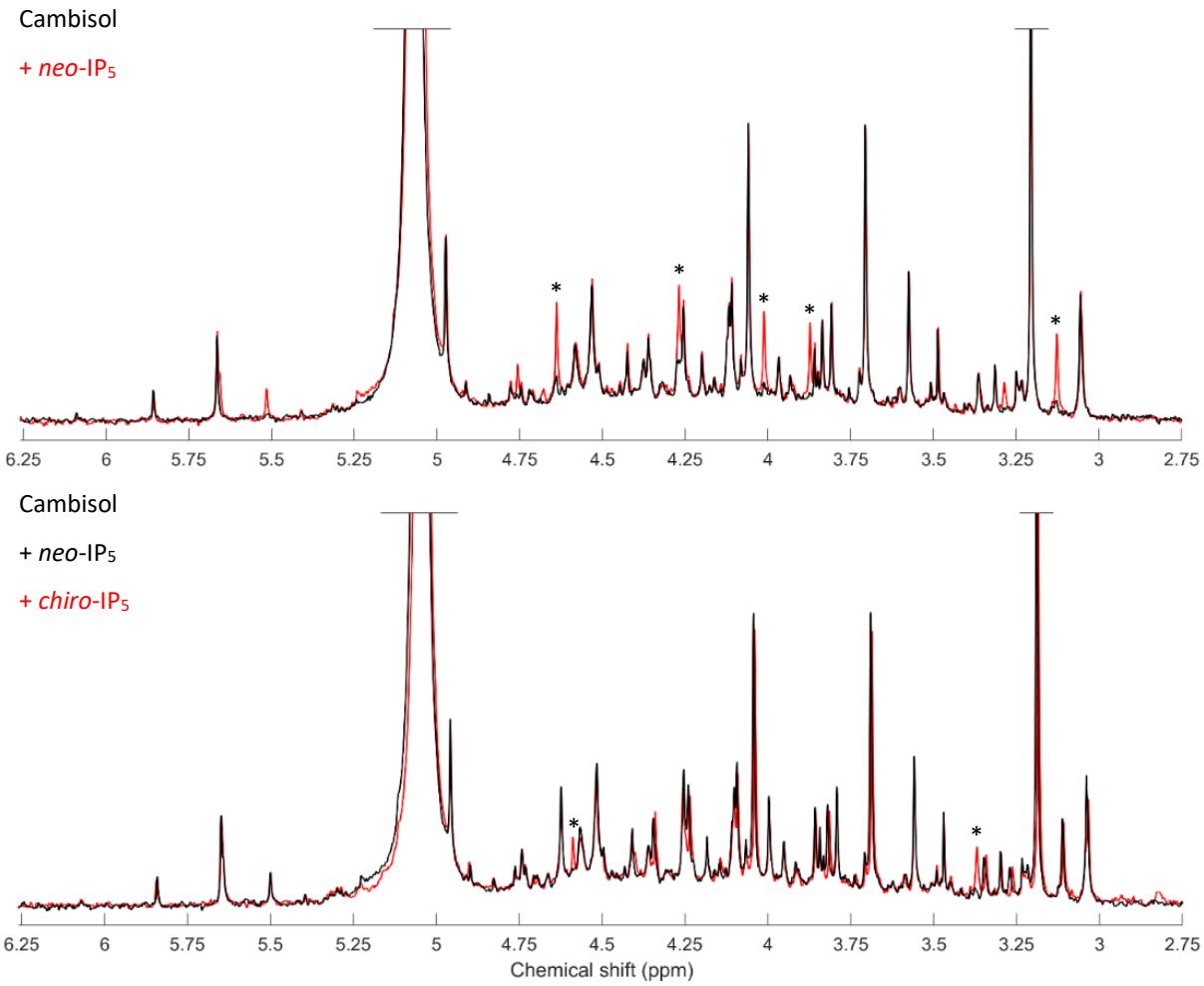

Figure SI3. Solution ^{31}P NMR spectra of phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Cambisol extract. Spiked spectrum with indicated standard in red. Peaks assigned to standard marked with *.

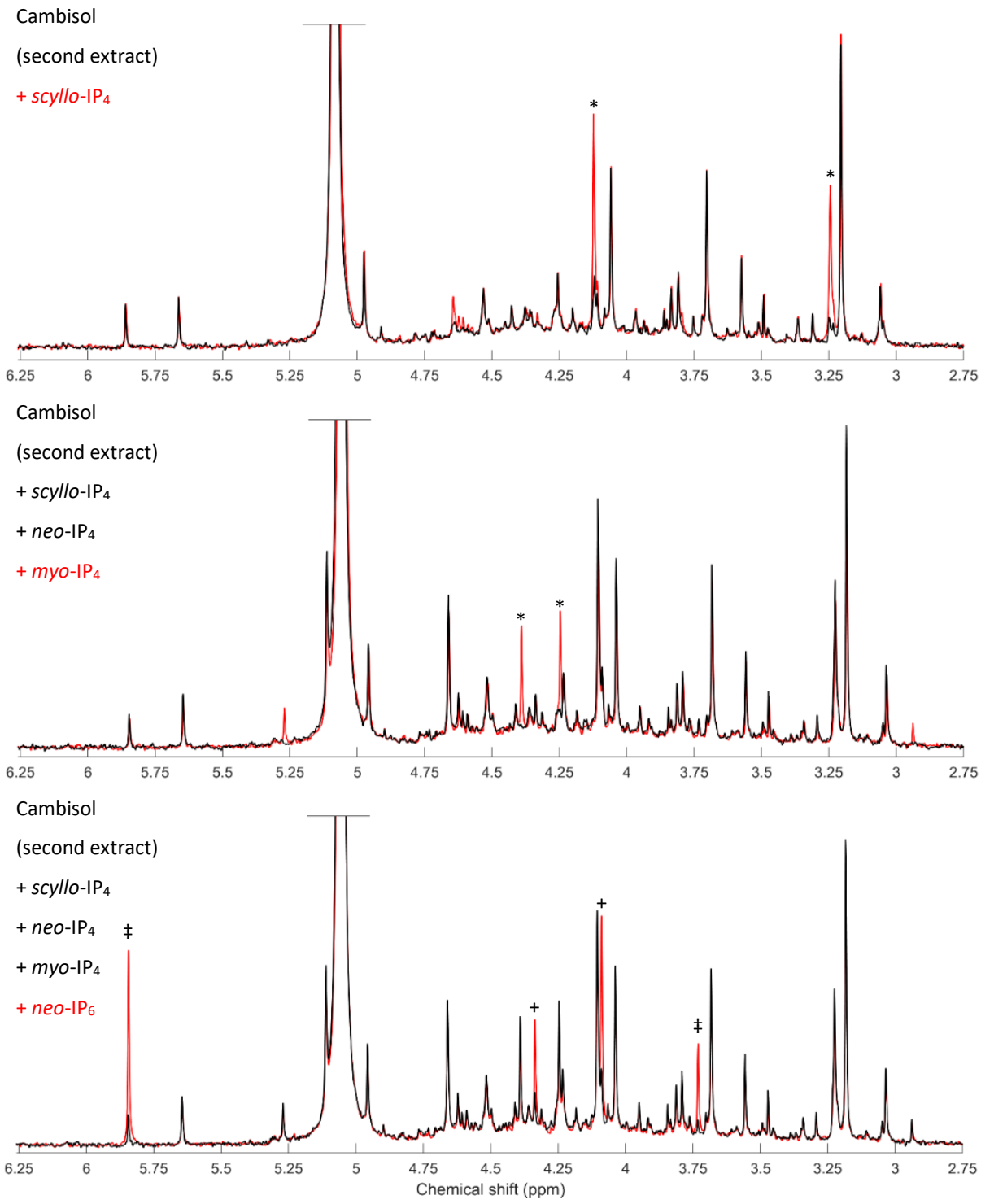

Figure SI4. Solution ^{31}P NMR spectra of phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Cambisol extract. Spiked spectrum with indicated standard in red. Peaks assigned to 4-equatorial/2-axial conformation marked with ‡, peaks assigned to 2-equatorial/4-axial conformation marked with +.

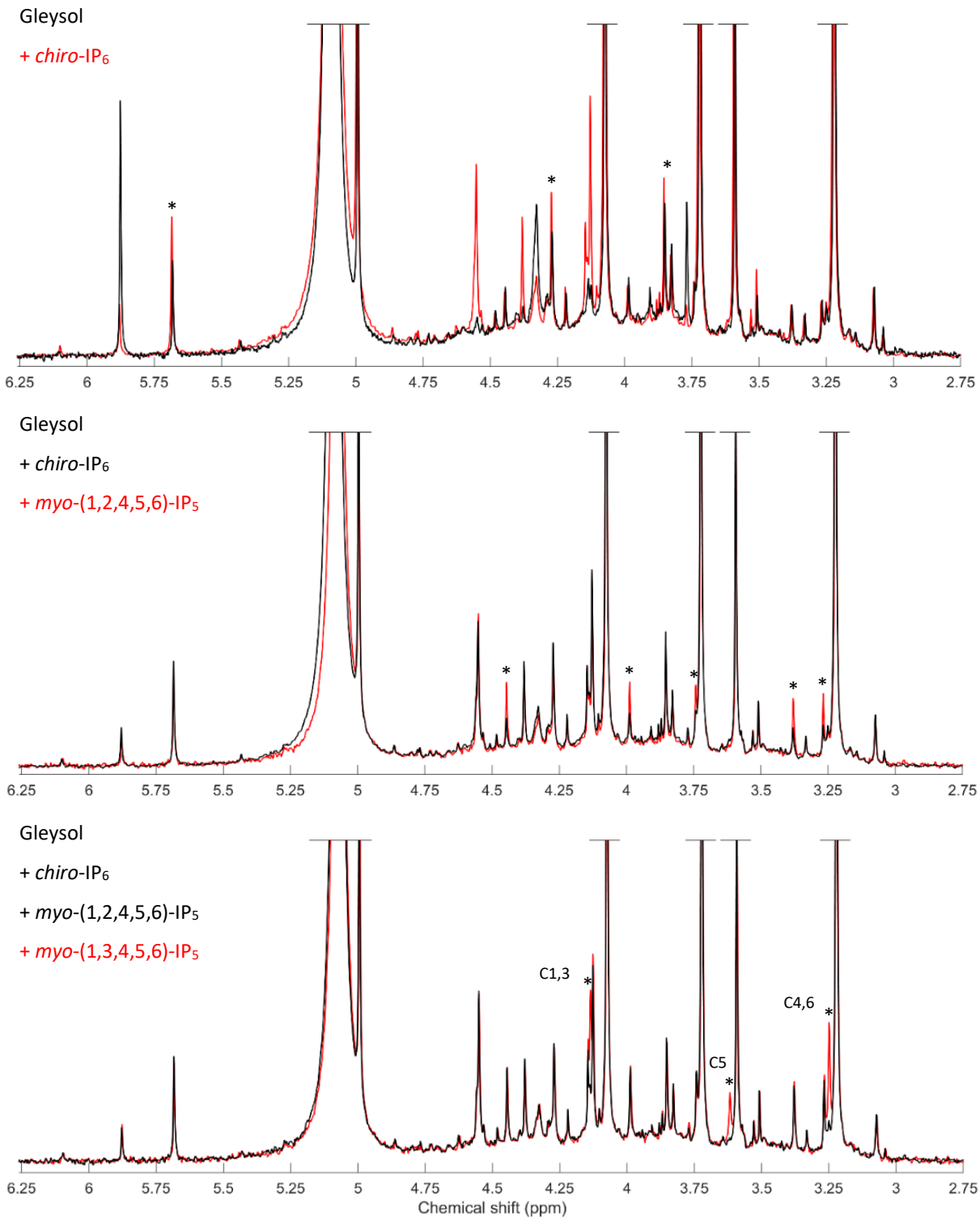

Figure SI5. Solution ^{31}P NMR spectra of phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Gleysol extract. Spiked spectrum with indicated standard in red. Peaks assigned to standard marked with *. For *myo*-(1,3,4,5,6)-IP$_5$, the respective phosphorylated carbon nuclei of the inositol have been marked based on the ^{31}P NMR spectrum prediction of the program Mnova 11.0.4 (©Mestrelab Research).

Transverse relaxation time of an orthophosphate solution. The analysis of a 0.25 M NaOH + 0.05 M EDTA solution containing 910 mg KH_2PO_4/L resulted in a single orthophosphate peak in the NMR spectrum (δ 5.09 ppm) with a linewidth at peak half height of 0.56 Hz. Transverse relaxation experiments were carried out (similar to that previously described) on the solution, which resulted in a T_2 time of 203 ms for orthophosphate.

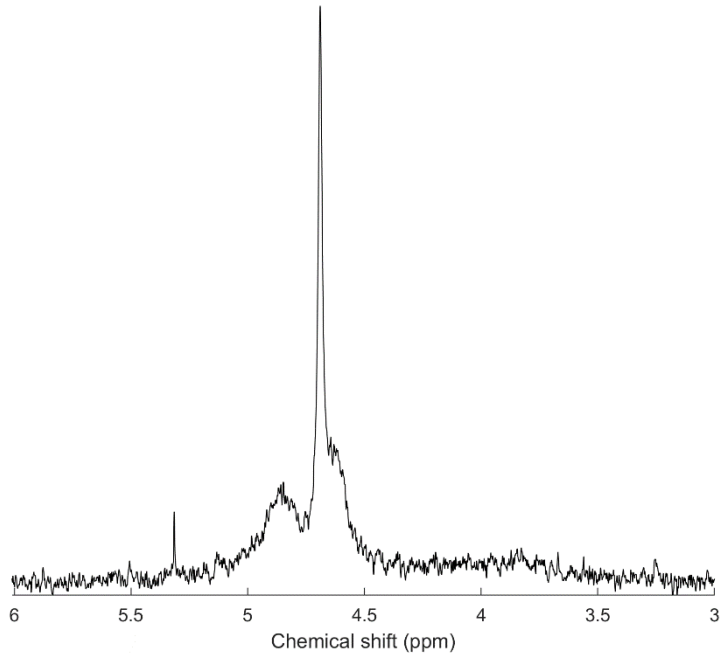

Figure SI7. Solution ^{31}P NMR spectrum of phosphomonoester region of purchased *myo*-(1,2,3,4,6)-IP_5 standard dissolved in 0.25 M NaOH + 0.05 M EDTA.

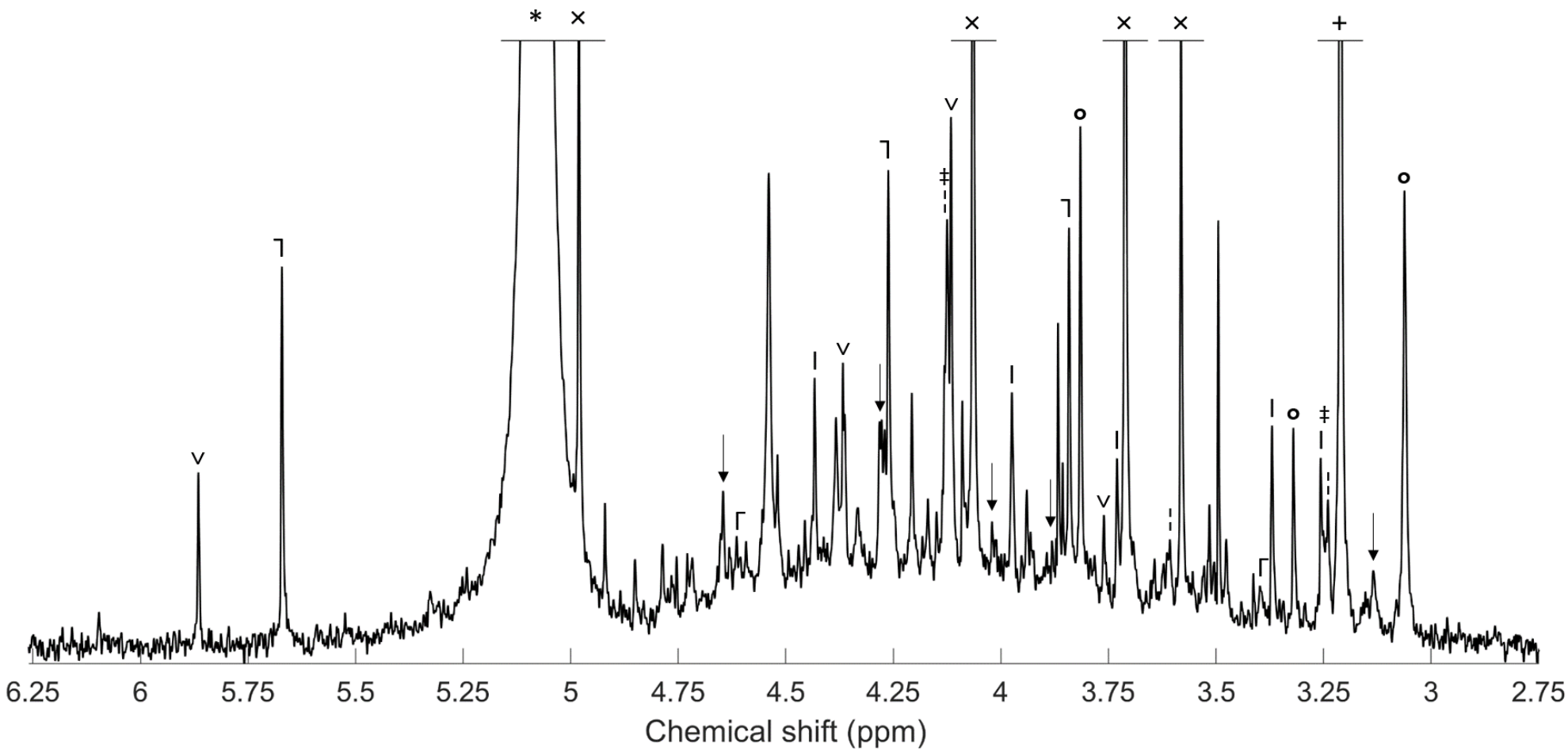

Figure SI8. Solution ^{31}P NMR spectra of phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Cambisol extract. All identified peaks are marked: orthophosphate (*), *myo*-IP$_6$ (×), *scyllo*-IP$_6$ (+), *neo*-IP$_6$ (ᐯ), *chiro*-IP$_6$ (⅂), *myo*-(1,2,4,5,6)-IP$_5$ (ǀ), *myo*-(1,3,4,5,6)-IP$_5$ (¦), *scyllo*-IP$_5$ (°), *neo*-IP$_5$ (↓), *chiro*-IP$_5$ (Γ), *scyllo*-(1,2,3,4)-IP$_4$ (‡). The chemical shifts in ppm of all identified peaks are listed in Table 5.

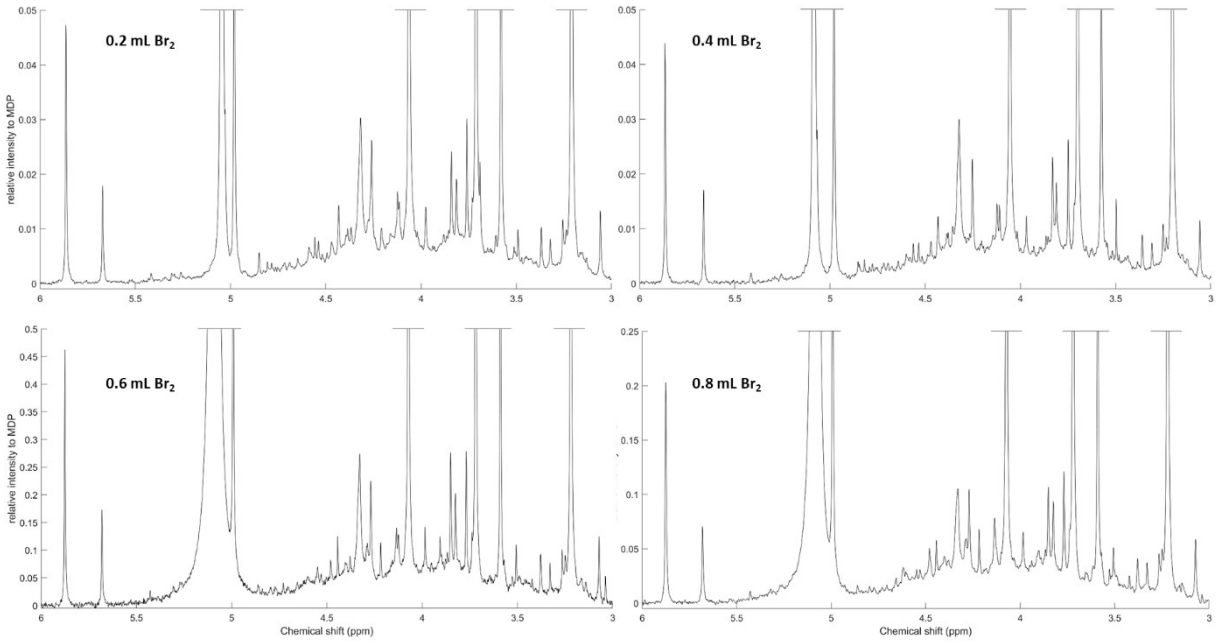

Figure SI9. Solution ^{31}P nuclear magnetic resonance (NMR) spectra (500 MHz) of the orthophosphate and phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Gleysol extract, using 0.2 mL, 0.4 mL, 0.6 mL and 0.8 mL Br_2 in the hypobromite oxidation procedure. Signal intensities were normalised to the MDP peak (intensity of 1 on y-axes).

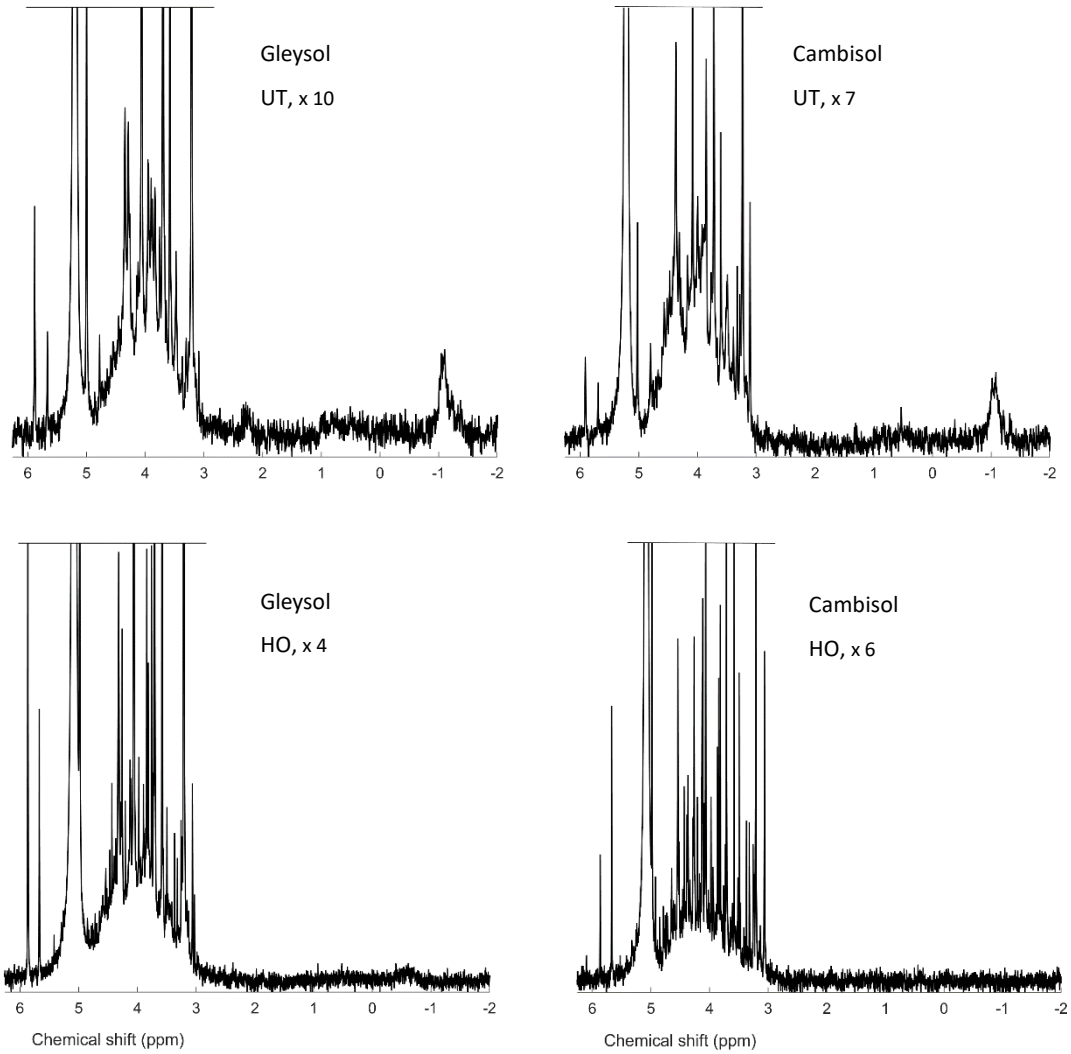

Figure SI10. Solution ^{31}P nuclear magnetic resonance (NMR) spectra (500 MHz) of the orthophosphate, phosphomonoester and phosphodiester region on untreated (UT, on top) and hypobromite oxidised (HO, below) 0.25 M NaOH + 0.05 M EDTA soil extracts of the Gleysol (right) and Cambisol (left). Signal intensities were normalised to the MDP peak intensity. The vertical axes were increased for improved visibility of spectral features, as indicated by a factor.