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_{3-1}. We have made this clearer in the body text.

We inserted the sentence (Lines 298-301): <u>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.</u>

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 occurs.

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). 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.
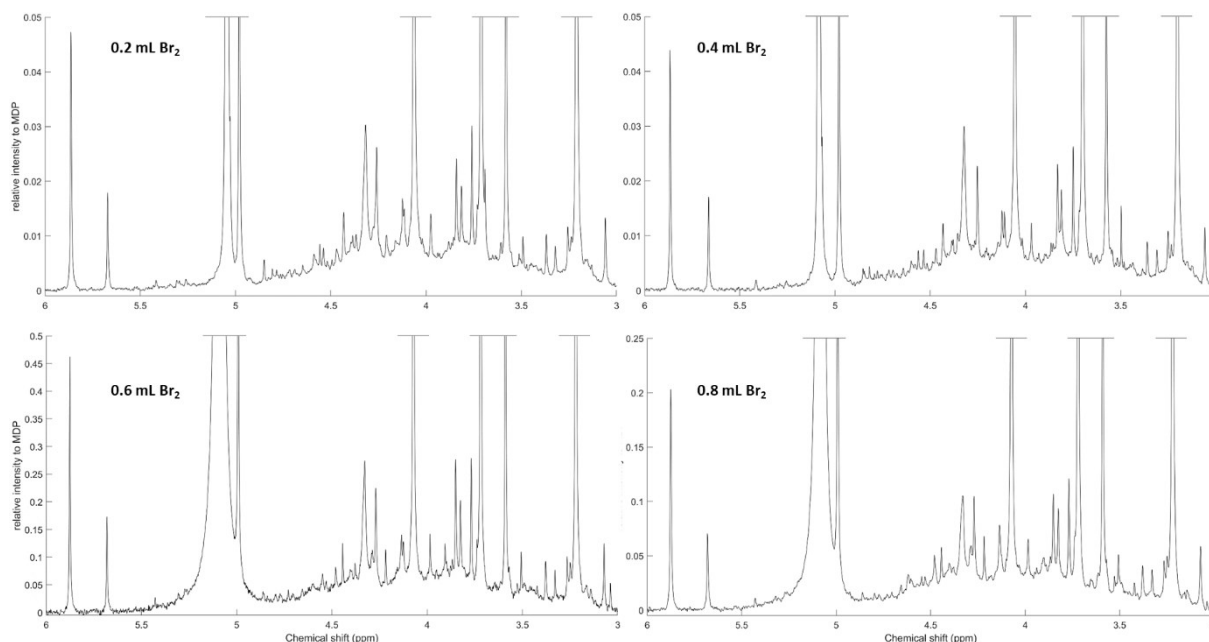
Figure 1. Solution ^{31}P nuclear magnetic resonance (NMR) spectra (500 MHz) of the orthophosphate and phosphomonoester region of hypobromite oxidised 0.25 M NaOH + 0.05 M EDTA Gleysol extract, using 0.2 mL, 0.4 mL, 0.6 mL and 0.8 mL Br_2 in the hypobromite oxidation procedure. Signal intensities were 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 131-133: 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 exaggerate 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 195-203): 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).

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 396-402: 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 103-104): 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 126-127: 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 409-411): <u>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.</u>

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:

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), <u>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.</u> 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 458-461: <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 'in phosphomonoester region'.

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 D-*chiro*-IP$_6$ 2-eq/4-ax.

We reported in the Result section 3.3, lines 290-293: *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).

To make this clearer in the Discussion section, we inserted (Lines 369-371): 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.

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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