

# ***Interactive comment on “Identification of lower-order inositol phosphates (IP<sub>5</sub> and IP<sub>4</sub>) in soil extracts as determined by hypobromite oxidation and solution <sup>31</sup>P NMR spectroscopy” by Jolanda E. Reusser et al.***

## **Anonymous Referee #1**

Received and published: 25 January 2020

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 <sup>31</sup>P 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

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studies based on resistance to bromination, this is the first direct identification by solution  $^{31}\text{P}$  NMR. I recommend publication, but ask the authors to consider the following comments in their revision.

1. 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.

2. 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.

3. It has been claimed that inositol phosphates account for a negligible amount of soil

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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.

4. 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.

5. 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.

Line-by-line comments:

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.

Line 17: shouldn't the 'broad signal' be destroyed by hypobromite oxidation?

Line 20: I understood that one of the myo-IP5 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 de-

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tected in two of the soils here. Could the authors comment on this?

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.

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

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.

Line 97: it’s not clear why these four soils were chosen for study – perhaps add a brief explanation.

Line 118: This sentence seems redundant if the method was the same. Delete?

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.

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 unbromi-

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nated extracts.

Line 262: What could the broad signal possibly be, in brominated extracts?

Line 225: comma instead of period. The persistence of some phosphodiesteres suggests incomplete oxidation.

Line 276: this depends on how spectra were deconvoluted – see point above.

Line 278: It's interesting to see evidence for the two conformers of neo-IP6. 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?

Line 283: Aren't lower-order esters destroyed by bromination?

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 tetraakisphosphates resist bromination).

Line 311: 6 in subscript.

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.

Line 404: also along the Haast chronosequence: Turner, B. L., A. Wells, and L. M. Condon. 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.

Line 418: see above. I think the concentrations on the brominated extracts should be

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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.

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?

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.

Table 3 – you could combine this table with Table 1 to streamline display items.

Table 4 – indicate that the broad peak also represents phosphomonoesters.

Table 5 – I think it's fairly safe to assume that the chiro-IP6 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-IP6 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.

Table S1 – this indicates a considerable proportion of the phosphorus has been lost

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during the bromination procedure.

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