Plant and Soil

Milling plant and soil material in plastic tubes over-estimates carbon and under-estimates nitrogen concentrations

--Manuscript Draft--

Manuscript Number:	PLSO-D-12-01064R1
Full Title:	Milling plant and soil material in plastic tubes over-estimates carbon and under-estimates nitrogen concentrations
Article Type:	Manuscript
Keywords:	Carbon, Fourier-transform infrared spectroscopy, grinding, microcentrifuge tubes, milling, nitrogen.
Corresponding Author:	Stuart W. Smith University of Aberdeen Aberdeen, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Aberdeen
Corresponding Author's Secondary Institution:	
First Author:	Stuart W. Smith
First Author Secondary Information:	
Order of Authors:	Stuart W. Smith
	A. H. Jean Robertson
	Andrew A. Meharg
	Robin J. Pakeman
	David Johnson
	Sarah J. Woodin
	René van der Wal
Order of Authors Secondary Information:	
Abstract:	Background and aims Milling of plant and soil material in plastic tubes, such as microcentrifuge tubes, over-estimates carbon (C) and under-estimates nitrogen (N) concentrations due to the introduction of polypropylene into milled samples, as identified using Fourier-transform infra-red spectroscopy. Methods and results This study compares C and N concentrations of roots and soil milled in microcentrifuge tubes versus stainless steel containers, demonstrating that a longer milling time, greater milling intensity, smaller sample size and inclusion of abrasive sample material all increase polypropylene contamination from plastic tubes leading to overestimation of C concentrations by up to 8 % (0.08 g g-1). Conclusions Erroneous estimations of C and N, and other analytes, must be assumed after milling in plastic tubes and milling methods should be adapted to minimise such error.
Response to Reviewers:	Plant and Soil: reviewers comments "Milling plant and soil material in plastic tubes over-estimates carbon and under-estimates nitrogen concentrations" Reviewer #1: Line 80: Explanation of the experimental design is a little ambiguous here. How many samples were analysed under each treatment? - this is not explicit in the text. Whilst it is possible to glean it from the table, some reference to the level of replication would be helpful in the methods. Line 81 now reads: Twenty samples were milled per tube type and ten samples for stainless steel jars at randomly selected intervals for each treatment. For the sample

size treatment, three additional samples (< 20 mg) were milled to aid the statistical analysis.

Line 89:
The authors cite a version of the R statistical environment that is 3 years out of date. Whilst it is unlikely that the core features used in this article will have changed in that time, it is advisable to keep the software up to date, given that it is free and open source.

Agree with the reviewer, although as stated, it would not affect the outcome of the statistics used in this manuscript.

Table 1:
The table caption states 'Statistics for %C vs sample size are based on sample weights <26.5 mg only.' The methods state that sample sizes of 10 - 60 mg were used in the experiment. No explanation is offered as to why the sample size has been limited in the text. The authors should include an explanation of the basis by which this dataset was limited.

Table 1: I have removed ‘Statistics for %C vs sample size are based on sample weights <26.5 mg only’ from the table caption and added a footnote:
bStatistics relate to the linear model for both stainless steel and microcentrifuge tube combined with sample sizes <26.5mg only, whilst the predicted lines are for the full range of sample sizes.

Figure 2C:
The non-linear model appears to relate to samples < 26.5 mg.If so, it is slightly misleading to present the complete data for samples 10 - 60 mg, but to include a model fit which refers to only part of that data, without making explicit reference to it either in the figure caption or the plot itself. This feeds into the previous question as to why the authors chose to limit the data in the first place. There are likely perfectly good reasons, but they should be explained.

The text in the methods now includes (LN 97):
However, for sample size the interaction could not be determined due to differences in linearity (linear for stainless steel; non-linear for microcentrifuge tube). In order to compare the strength of the interaction term for sample size on %C with the other milling treatments (milling time and intensity) a single linear model was used on sample sizes <26.5 mg, a threshold below which a linear relationship was displayed.

Reviewer #2: This article aims to compare C and N analyses of plant and soil material that had been previously milled using stainless steel jars or microcentrifuge tubes, to detect potential contamination from the polypropylene tubes that could alter the analytical results. The article is well presented and give good recommendations to reduce or avoid sample contamination. However I think some little details should be clarified before being accepted for publication.

Introduction
Line 56: Maybe, you could give more examples (2-3 references is enough) of studies using microcentrifuge tubes for milling, showing that this method is really a common practice.

There are now 3 references:
(Warren and Adams 2004; Salvo-Chirnside et al. 2011; Nadeem et al. 2012)

Lines 60-61: The increase of C concentration from 44.56% to 50.02% is not a 5.45% increase, but an increase of 5.45 percentage points. It equals to an increase of about 12%. Find the same mistake for the reduction of N content.

Altered to to include term percentage points:
However, during an investigation of C loss from root litter we found that the average C concentration was 5.45 percentage points higher (50.02 vs 44.56 %C), and N concentration 0.072 percentage points lower (0.912 vs 0.985 %N), in roots milled in

	microcentrifuge tubes as compared to steel jars (Figure 1).’ Material and methods It is not clear whether you used only one sample per treatment combination (milling time, sample size and milling intensity) or there were some replicates. Line 81 now reads: Twenty samples were milled per tube type and ten samples for stainless steel jars at randomly selected intervals for each treatment. For the sample size treatment, three additional samples (< 20 mg) were milled to aid the statistical analysis. Figures Check the figure order. The figure 3 appears to be the last one, while the previous one is the figure 4. Figure input order was incorrect and has been altered.

Stuart Smith
Institute of Biological and Environmental Sciences
University of Aberdeen
Cruickshank Building
St. Machar Drive
Aberdeen
AB24 3UU
UK
s.w.smith@abdn.ac.uk
Tel: (+44) 01224 272273

1/11/2012

Dear Editors,

I wish to submit the following paper to Plant and Soil:

Milling plant and soil material in plastic tubes over-estimates carbon and under-estimates nitrogen concentrations

Stuart W. Smith, A. H. Jean Robertson, Robin J. Pakeman, René van der Wal, Sarah Woodin, Andrew A. Meharg, David Johnson

Accurate analysis of carbon (C) and nitrogen (N) content of plants and soils is crucial for assessing how climate and land-use change affects the global biogeochemical cycle. This is partly achieved by milling samples to homogenise material prior to chemical analysis. Milling of plant and soil material in plastic tubes, such as microcentrifuge tubes, over-estimates C and under-estimates N concentrations, due to the introduction of plastic, a polypropylene mixture, into milled samples, identified using Fourier-transform infra-red spectroscopy. In this study we compare C and N concentrations of roots and soil milled in microcentrifuge tubes versus stainless steel containers, demonstrating that a longer milling time, greater milling intensity, smaller sample size and inclusion of abrasive sample material all increase polypropylene contamination from plastic tubes leading to overestimation of C concentrations of up to 8% (0.08 g/g^{-1}) and on average 0.074 % (0.74 mg/g^{-1}) lower N concentrations. Use of erroneous C and N concentrations could have large implications when calculating element budgets as well as having impacts in many areas of biological science.

I hope you will find this contribution suitable for publication and look forward to hearing from you.

Yours faithfully,

Stuart Smith

Plant and Soil: reviewers comments "Milling plant and soil material in plastic tubes over-estimates carbon and under-estimates nitrogen concentrations"

Reviewer #1: Line 80:
Explanation of the experimental design is a little ambiguous here. How many samples were analysed under each treatment? - this is not explicit in the text. Whilst it is possible to glean it from the table, some reference to the level of replication would be helpful in the methods.

Line 81 now reads: Twenty samples were milled per tube type and ten samples for stainless steel jars at randomly selected intervals for each treatment. For the sample size treatment, three additional samples (< 20 mg) were milled to aid the statistical analysis.

Line 89:
The authors cite a version of the R statistical environment that is 3 years out of date. Whilst it is unlikely that the core features used in this article will have changed in that time, it is advisable to keep the software up to date, given that it is free and open source.

Agree with the reviewer, although as stated, it would not affect the outcome of the statistics used in this manuscript.

Table 1:
The table caption states 'Statistics for %C vs sample size are based on sample weights <26.5 mg only.' The methods state that sample sizes of 10 - 60 mg were used in the experiment. No explanation is offered as to why the sample size has been limited in the text. The authors should include an explanation of the basis by which this dataset was limited.

Table 1: I have removed 'Statistics for %C vs sample size are based on sample weights <26.5 mg only' from the table caption and added a footnote:
[b]Statistics relate to the linear model for both stainless steel and microcentrifuge tube combined with sample sizes <26.5mg only, whilst the predicted lines are for the full range of sample sizes.

Figure 2C:
The non-linear model appears to relate to samples < 26.5 mg.If so, it is slightly misleading to present the complete data for samples 10 - 60 mg, but to include a model fit which refers to only part of that data, without making explicit reference to it either in the figure caption or the plot itself. This feeds into the previous question as to why the authors chose to limit the data in the first place. There are likely perfectly good reasons, but they should be explained.

The text in the methods now includes (LN 97):
However, for sample size the interaction could not be determined due to differences in linearity (linear for stainless steel; non-linear for microcentrifuge tube). In order to compare the strength of the interaction term for sample size on %C with the other milling treatments (milling time and intensity) a single linear model was used on sample sizes <26.5 mg, a threshold below which a linear relationship was displayed.

Reviewer #2: This article aims to compare C and N analyses of plant and soil material that had been previously milled using stainless steel jars or microcentrifuge tubes, to detect potential contamination from the polypropylene tubes that could alter the analytical results.

The article is well presented and give good recommendations to reduce or avoid sample contamination. However I think some little details should be clarified before being accepted for publication.

Introduction
Line 56: Maybe, you could give more examples (2-3 references is enough) of studies using microcentrifuge tubes for milling, showing that this method is really a common practice.

There are now 3 references:
(Warren and Adams 2004; Salvo-Chirnside et al. 2011; Nadeem et al. 2012)

Lines 60-61: The increase of C concentration from 44.56% to 50.02% is not a 5.45% increase, but an increase of 5.45 percentage points. It equals to an increase of about 12%. Find the same mistake for the reduction of N content.

Altered to to include term percentage points:
However, during an investigation of C loss from root litter we found that the average C concentration was 5.45 percentage points higher (50.02 vs 44.56 %C), and N concentration 0.072 percentage points lower (0.912 vs 0.985 %N), in roots milled in microcentrifuge tubes as compared to steel jars (Figure 1).'

Material and methods
It is not clear whether you used only one sample per treatment combination (milling time, sample size and milling intensity) or there were some replicates.

Line 81 now reads:
Twenty samples were milled per tube type and ten samples for stainless steel jars at randomly selected intervals for each treatment. For the sample size treatment, three additional samples (< 20 mg) were milled to aid the statistical analysis.

Figures
Check the figure order. The figure 3 appears to be the last one, while the previous one is the figure 4.

Figure input order was incorrect and has been altered.

*Revised version including track changes

Click here to download Revised version including track changes: MicrocentrifugetubeCcontaminationPLANTSOILcorrections[1].docx

Milling plant and soil material in plastic tubes over-estimates carbon and under-estimates nitrogen concentrations

Stuart W. Smith[a,b,c*], A. H. Jean Robertson[c], Andrew A. Meharg[a], Robin J. Pakeman[c], David Johnson[a] , Sarah J. Woodin[a], René van der Wal[b]

[a]IBES, University of Aberdeen, St Machar Drive, Aberdeen AB24 3UU, UK.

[b]ACES, University of Aberdeen, St Machar Drive, Aberdeen AB24 3UU,UK.

[c]The James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH, UK.

* Corresponding author. E-mail address: s.w.smith@abdn.ac.uk (S.W. Smith).

Abstract

Background and aims Milling of plant and soil material in plastic tubes, such as microcentrifuge tubes, over-estimates carbon (C) and under-estimates nitrogen (N) concentrations due to the introduction of polypropylene into milled samples, as identified using Fourier-transform infra-red spectroscopy. *Methods and results* This study compares C and N concentrations of roots and soil milled in microcentrifuge tubes versus stainless steel containers, demonstrating that a longer milling time, greater milling intensity, smaller sample size and inclusion of abrasive sample material all increase polypropylene contamination from plastic tubes leading to overestimation of C concentrations by up to 8 % (0.08 g g^{-1}). *Conclusions* Erroneous estimations of C and N, and other analytes, must be assumed after milling in plastic tubes and milling methods should be adapted to minimise such error.

Keywords: Carbon, Fourier-transform infrared spectroscopy, grinding, microcentrifuge tubes, milling, nitrogen.

Introduction

Analysis of the carbon (C) and nitrogen (N) contents of plants and soils is crucial for assessing how climate and land-use change affect global biogeochemical cycles (Guo and Gilford 2002; Bellamy et al. 2005; Powlson et al. 2011). Estimation of ecosystem C and N stores, inputs and losses depends upon accurate determination of C and N concentrations in ecological materials. Automated elemental analysis has become ubiquitous for C and N determination, due to its accuracy and reliability (Kalembas and Jenkinson, 1973; Soon and Abboud 1991; Lal et al. 2001). This type of analysis, based on dry oxidation, only requires a 2–20 mg sub-sample and thus precision is dependent on sample homogeneity (Jimenez and Ladha 1993). Homogenisation is achieved through milling, often in stainless steel grinding jars containing stainless steel balls, with mills typically processing 1–3 samples at once (Allen 1989). However, significant time can be saved in preparation of large sets of samples using microcentrifuge tubes with stainless steel balls, with tens of samples being processed simultaneously (Warren and Adams 2004; Salvo-Chirnside et al. 2011; Nadeem et al. 2012). Milling in microcentrifuge tubes avoids the loss of material which occurs through cleaning of steel grinding jars between each use and is thus ideal for small quantities of plant and soil material (e.g. roots, decomposed litter, soil fauna). The use of disposable containers also minimizes any cross-sample contamination. However, during an investigation of C loss from root litter we found that the average C concentration was 5.45 percentage points higher (50.02 vs 44.56 %C), and N concentration 0.072 percentage points lower (0.912 vs 0.985 %N), in roots milled in microcentrifuge tubes as compared to steel jars (Figure 1). The additional C was identified, using Fourier-transform infrared (FTIR) spectroscopy, to be atactic-polypropylene ($(C_{15}H_{30})_n$) and some copolymers, originating from the microcentrifuge tubes. Abraded polypropylene also increased sample mass with N free material, thus reducing

67 the overall N concentration. This study aimed to define milling procedures that would
68 preclude plastic contamination, investigating the effects of milling time, intensity and sample
69 size on measured C and N concentrations of roots and soils milled in microcentrifuge tubes
70 and in steel jars. Material milled by both methods was tested for polypropylene by FTIR
71 spectroscopy.

72

73 **Materials and Methods**

74 Two microcentrifuge types were investigated; reaction vial safe-lock 2 ml Retsch® tubes
75 (81.7 %C) and 2 ml Alpha laboratories microcentrifuge tubes (80.8 %C), compared to 10 ml
76 stainless steel jars (0.95 %C). Dried, finely chopped *Molinia caerulea* roots (diameter
77 0.35±0.07 mm) and two soil types, an organic iron-podzol (31.5 % sand, 61.5 % silt, 7.0 %
78 clay) and a sandy-loam (70.2 % sand , 14.8 % silt, 15 % clay) were milled on a Retsch®
79 Mixer Mill MM 400 (Retsch®, Germany) , testing the effects of three variables
80 ("treatments"): milling time (1-15 mins); milling intensity (15–30 Hz); sample size (10–60
81 mg). Twenty samples were milled per tube type and ten samples for stainless steel jars at
82 randomly selected intervals for each treatment. For the sample size treatment, three additional
83 samples (< 20 mg) were milled to aid the statistical analysis. One parameter was altered at a
84 time; the others remained constant at 10 mins, 30 Hz or 30 mg of root per tube. Only three
85 soil sample sizes were investigated (10.9, 28.5, 59.3 mg). Root particle size was not small
86 enough for CN analysis after 10 mins of milling in microcentrifuge tubes, so all samples were
87 re-milled in steel jars for 1 minute at 30 Hz. After milling, a 5 mg sub-sample was taken for
88 elemental analysis (Carlo-Erba NA 1500 Series 2, USA). Contamination of milled roots was
89 tested on a Bruker Vertex 70 Spectrometer (Bruker Optics, Ettlingen, Germany) comparing
90 milled samples to microcentrifuge shavings (methods as in Artz et al. 2008). Treatment

effects on percent C and N were analysed using linear models (except sample size on %C milled in microcentrifuge tubes which was fitted with a non-linear exponential function) in R (version 2.10.1, R Development Core Team, 2009). There was no significant difference between the plastic Alpha and Retsch tubes in any treatment ($P>0.05$), so these were grouped for statistical analysis. Difference between the change in C and N concentration with treatment in microcentrifuge tubes and in stainless steel jars is indicated by the interaction term of the model. However, for sample size the interaction could not be determined due to differences in linearity (linear for stainless steel; non-linear for microcentrifuge tube). In order to compare the strength of the interaction term for sample size on %C with the other milling treatments (milling time and intensity) a single linear model was used on sample sizes <26.5 mg, a threshold below which a linear relationship was displayed.

Results and Discussion

Carbon concentrations in microcentrifuge milled roots increased with increasing milling time and intensity, whilst %C of steel milled roots remained unchanged across both treatments (interaction terms in Table 1; Figure 2A, 2B). Milling a small quantity of sample (≤20 mg) produced the greatest polypropylene-derived C contamination (up to 8 %C or 0.08 g g^{-1}) of all the treatments, due to greater abrasion between the ball and tubes (Table 1; Figure 2C). Polypropylene was identified in all roots milled in microcentrifuge tubes, but not in steel milled samples (Figure 3). Milling small samples in microcentrifuge tubes should be avoided and milling time and intensity should be reduced, yet this can prevent particle size being sufficiently small for C and N analysis. Although not tested here, non-spherical balls could be used to dissipate the intensity of contact between ball and microcentrifuge tube (Salvo-Chirnside et al. 2011); however, cones require more energy to achieve the same degree of

115 homogenization as spherical balls (Herbst and Lo 1989; Lameck et al. 2006), potentially
116 resulting in similar contamination.

117

118 Carbon contamination was greater for soil than plant material, and greater for sandy-loam soil
119 than for organic iron-podzol (Figure 4), likely due to less organic matter and greater sand
120 content resulting in more abrasion of the tubes. The risk of polypropylene C contamination is
121 likely to be greater when milling abrasive material and this needs further investigation. It
122 should be noted, however, that the quantities of soil milled were small (30 mg of soil
123 occupied <1 % of a tube compared to ~22 % for roots) and, as contamination decreased with
124 increasing sample size, this may be less of a problem with much larger samples.

125

126 Milled roots had a significantly lower average N concentration (across all treatments) in
127 microcentrifuge tubes (0.621 %N) compared to steel jars (0.695 %N) (Table 1; Figure 2; D,
128 E, F). The lower N content corresponds with our initial observations (Figure 1) and is
129 assumed to be due to dilution of N by the addition of plastic to the milled sample mass.
130 Unlike root C, there was no significant interaction between milling method and milling time,
131 intensity, or sample size (Table 1). This is due to variability in %N, which was much greater
132 than variability in %C, even in steel milled samples. This likely reflects natural %N variation
133 within perennial root tissues of different ages (Robinson and Rorison 1988) and/or greater
134 analytical error at the low root N concentrations. Soil N concentrations were lower for
135 organic-iron podzol samples milled in microcentrifuge tubes compared to steel jars. This
136 effect was not detected in sandy-loam soil, despite its increased %C, due to low N
137 concentrations (~0.05 %) being poorly detected on the elemental analyser (data not shown).

138

Conclusions

Milling small amounts of soil and plant material in microcentrifuge tubes risks over-estimating %C and potentially under-estimating %N. To reduce sample contamination, milling of material in microcentrifuge tubes should be kept to as short duration, as low intensity and as large a sample size as possible for achieving the required particle size. Alternatively, the use of plastic tubes in milling should be avoided as, without quantification on a case-by-case basis, C contamination must be assumed. Any other analyte will be underestimated in samples milled in plastic containers that are abraded during milling. Use of erroneous C, N and other analyte concentrations could have large implications for calculation of element budgets and, indeed, for any biological studies involving elemental analysis.

Acknowledgements

We thank Ken Cruickshank, Angela Main, Rachael Hill, Elaine Runge and Marcel Junker for their skilled technical assistance. SWS was funded by a Biotechnology and Biological Sciences Research Council studentship.

References

Allen SE (1989) Chemical Analysis of Ecological Materials. 2nd ed., Blackwell Scientific, Cambridge, UK.

160 Artz RE, Chapman SJ, Robertson AHJ, Potts JM, Laggoun-De´farge JM, Gogo S, Comont L,
161 Disnar JR, Francez AJ (2008) FTIR spectroscopy can be used as a screening tool for organic
162 matter quality in regenerating cutover peatlands. Soil Biol Biochem 40: 515–527.

163

164 Bellamy PH, Loveland PJ, Bradley RI, Lark RM, Kirk GJD (2005) Carbon losses from all
165 soils across England and Wales 1978–2003. Nature 437: 245–248.

166

167 Guo LB, Gifford RM (2002) Soil carbon stocks and land use change: a meta-analysis. Glob
168 Change Biol 8:345-360.

169

170 Herbst JA, Lo YC (1989) Grinding efficiency with balls or cones as media. Int J Miner
171 Process 26, 141-151.

172

173 Jimenez RR, Ladha JK (1993) Automated elemental analysis: A rapid and reliable but
174 expensive measurement of total carbon and nitrogen in plant and soil samples. Comm Soil
175 Sci Plan 24: 1897-1924

176

177 Kalembasa SJ, Jenkinson DS (1973) Comparative study of titrimetric and gravimetric
178 methods for the determination of organic carbon in soil. J Sci Food Agri 24: 1085-1090

179

Lal R, Kimble JM, Follett RF, Stewart BA (2001). Assessment Methods for Soil Carbon. CRC Press, Boca Raton, FL, USA.

Lameck NS, Kiangi KK, Moys MH (2006) Effects of grinding media shapes on load behaviour and mill power in a dry ball mill. Miner Eng 19: 1357–1361.

Nadeem M, Mollier A, Morel C, Vives A, Prud'homme L, Pellerin S (2012) Maize (Zea mays L.) endogenous seed phosphorus remobilization is not influenced by exogeneous phosphorus availability during germination and early growth stages. Plant Soil 357: 13 -24.

Powlson DS, Whitmore AP, Goulding WT (2011) Soil carbon sequestration to mitigate climate change: a critical re-examination to identify the true and the false, Eur J Soil Sci 62: 42 – 55.

R Development Core Team (2009) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Robinson D, Rorison IH (1988) Plasticity in grass species in relation to nitrogen supply. Funct Ecol 2, 249-257.

Salvo-Chirnside E, Kane S, Kerr LE (2011) Protocol: high throughput silica-based purification of RNA from Arabidopsis seedlings in a 96-well. Plant Methods 7: 40.

Soon YK, Abboud S (1991) A comparison of some methods for soil organic carbon determination. Comm Soil Sci Plan 22: 943 – 954.

Warren CR, Adams MA (2004) What determines rates of photosynthesis per unit nitrogen in Eucalyptus seedlings? Funct Plant Biol 31: 1169 – 1178.

Figures

Figure 1. The shift in absolute percent carbon (**A**) and nitrogen (**B**) in partially decomposed root litter attributed to milling samples in microcentrifuge tubes. C and N difference values represent stainless steel jar milled material subtracted from microcentrifuge milled material. Dotted lines represent no difference due to milling method. Milling in microcentrifuge tubes significantly increased %C (paired t-test: $t_{309} = 33.798$, $p < 0.001$) and decreased %N (paired t-test: $t_{309} = -8.757$ $p < 0.001$) compared to stainless steel milled samples.

Figure 2. Carbon concentrations (%) in *Molinia caerulea* roots for different milling times (**A**), intensities (**B**) and sample sizes (**C**), and nitrogen concentrations (%) for the same treatments (**D**, **E**, **F**, respectively). Microcentrifuge tube milled samples are black closed symbols with a black solid line for the linear and non-linear model fit (Alpha and Retsch tubes combined); stainless steel jar milled samples are open circles with a dashed line. Milling time was not significant for percent N (D) so no line has been fitted.

Figure 3. FTIR spectra within the 2800 to 3000 (cm^{-1}) wavenumber region; the CH stretching region diagnostic of atactic-polypropylene. Spectra for 10 mg of *M. caerulea* roots milled in stainless steel

jars (solid black line) and Restch microcentrifuge tube (dashed line) and 60 mg of *M. caerulea* roots milled in Retsch microcentrifuge (dotted line) compared to a sample of plastic from a Retsch microcentrifuge tube (dashed and dotted line).

Figure 4. Carbon concentrations (%) in sandy-loam (A) and organic iron-podzol (B) soil for different sample sizes milled. Microcentrifuge tube milled samples are dark-grey bars and stainless steel jar milled samples are light-grey bars. Mean ± 1 SE, n = 3.

table
Click here to download table: Microtube_stats_table_correctionsFINAL.docx

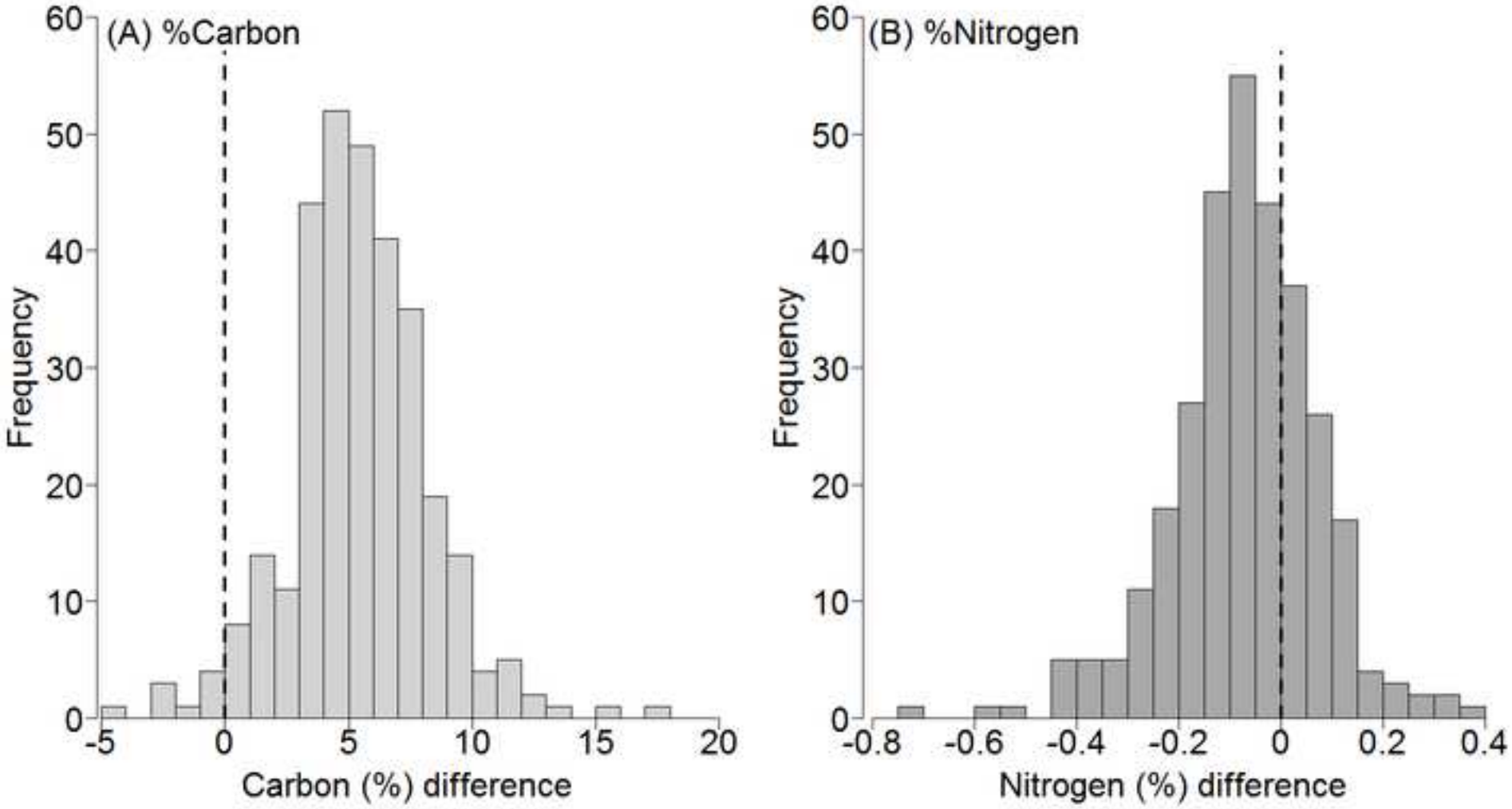
Table 1. Fitted lines for carbon (C) and nitrogen (N) concentrations of roots milled in microcentrifuge tubes and stainless steel jars for each 'treatment' (milling time, milling intensity, sample size).

Element	Treatment	Milling method	Predicted line	Treatment[a]	Milling Method	Treatment × milling method
Carbon	Time	Steel	y=45.79+0.04x	$F_{1,46}$= 37.61***	$F_{1,46}$= 39.70***	$F_{1,46}$= 6.26*
		Eppendorf	y=46.31+0.20x			
	Intensity	Steel	y=45.65+0.03x	$F_{1,46}$= 47.74***	$F_{1,46}$= 16.44***	$F_{1,46}$= 9.17**
		Eppendorf	y=43.28+0.24x			
	Sample Size	Steel	y=46.00+0.04x	$F_{1,25}$= 15.48***[b]	$F_{1,25}$= 39.56***	$F_{1,25}$= 5.47*
		Eppendorf	y=47.98(1+ exp (-0.18x))			
Nitrogen	Time	Steel	y=0.68+0.01x	$F_{1,46}$= 3.03 ns	$F_{1,46}$= 8.49**	$F_{1,46}$= 0.41 ns
		Eppendorf	y=0.60+0.01x			
	Intensity	Steel	y=0.6+0.24x	$F_{1,46}$= 6.6*	$F_{1,46}$= 9.94**	$F_{1,46}$= 2.46 ns
		Eppendorf	y=0.79-0.01x			
	Sample size	Steel	y=0.67+0.01x	$F_{1,55}$= 8.09**	$F_{1,55}$= 10.55**	$F_{1,55}$= 1.31 ns
		Eppendorf	y=0.54+0.01x			

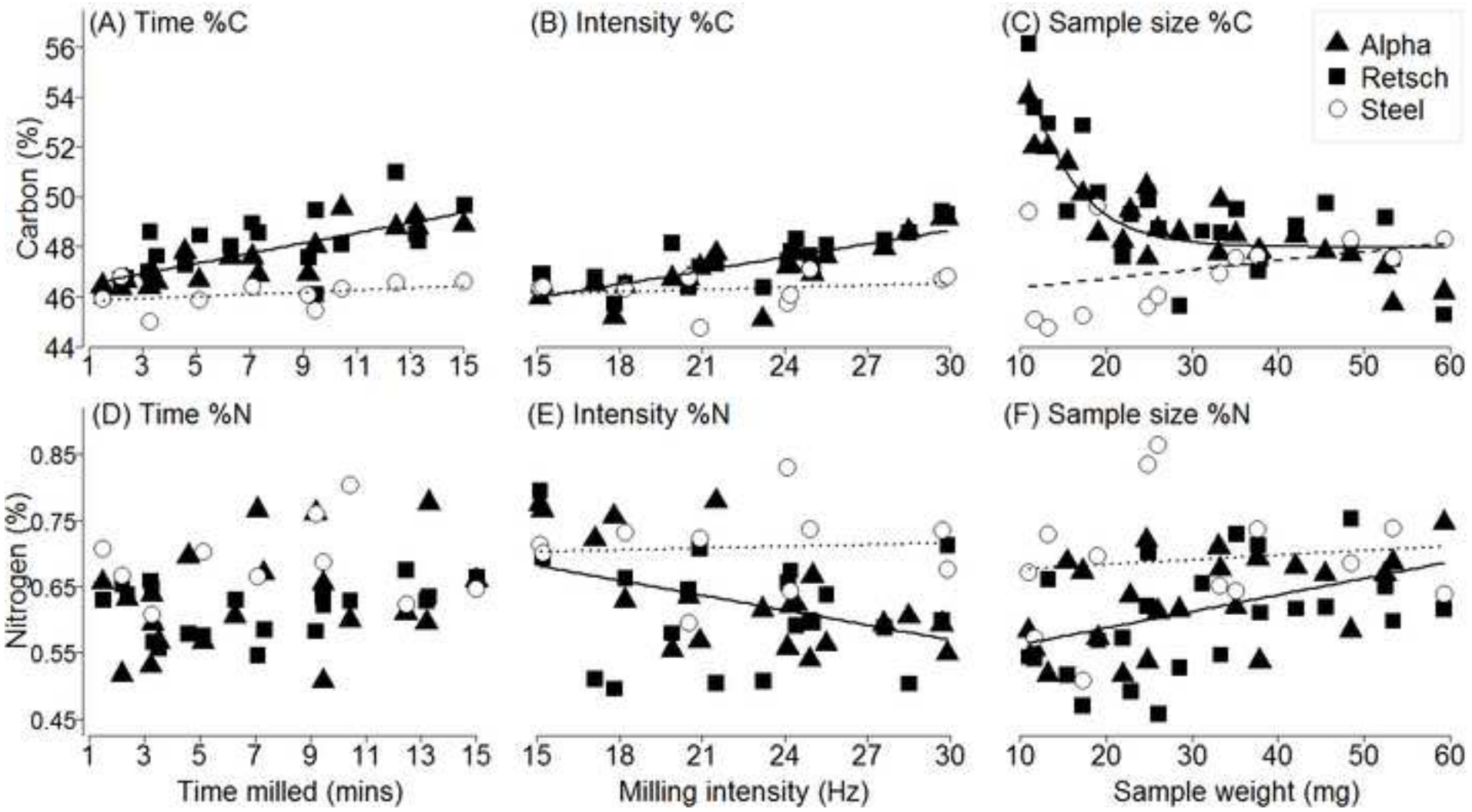
[a]Significance of each factor in the model (treatment, milling method and treatment × milling method interaction) are denoted by ns not significant, * p<0.05, ** p<0.01, *** p<0.001.

[b]Statistics relate to the linear model for both stainless steel and microcentrifuge tube combined with sample sizes <26.5mg only, whilst the predicted lines are for the full range of sample sizes.

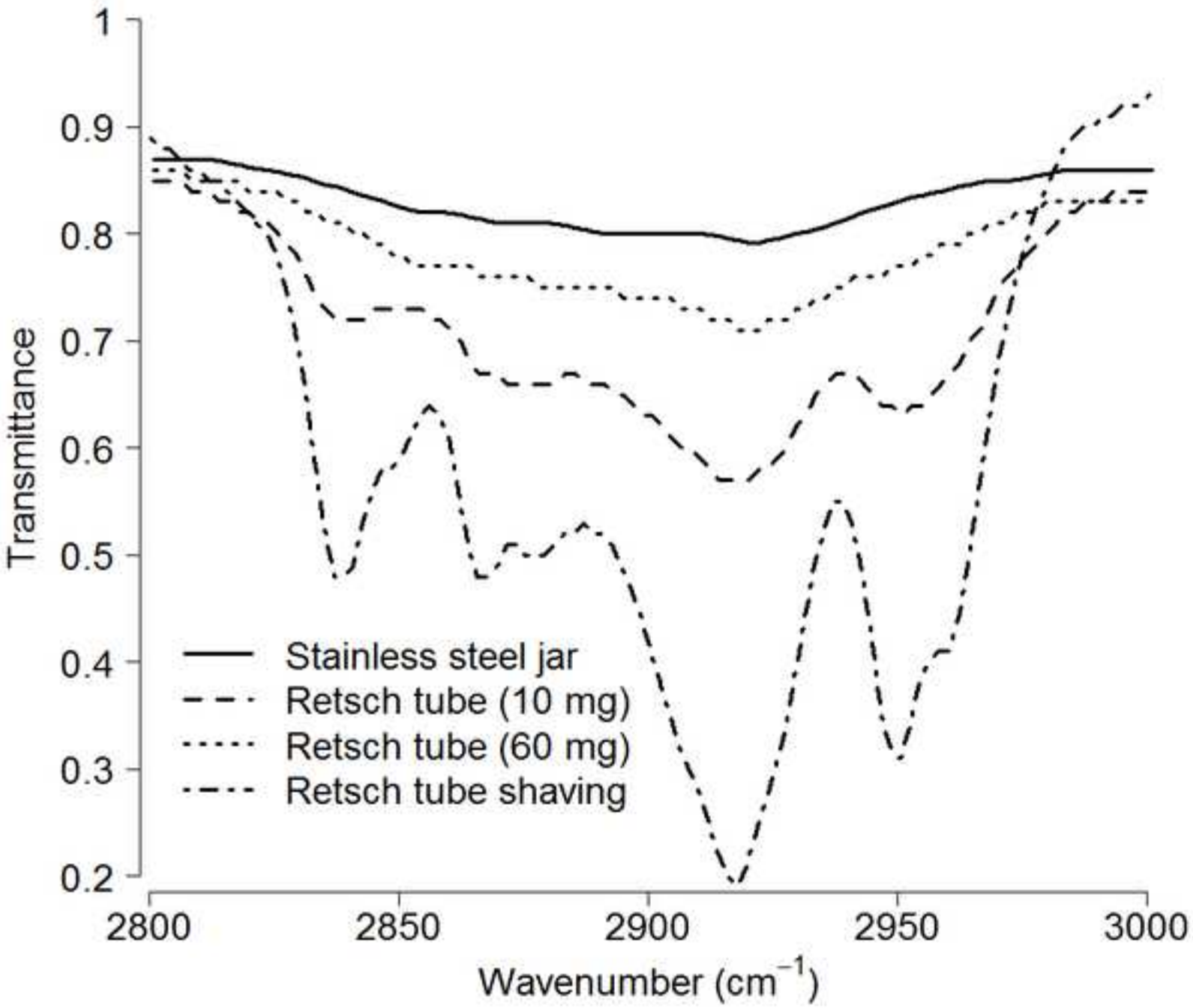
line figure
Click here to download high resolution image



line figure
Click here to download high resolution image



line figure
Click here to download high resolution image



line figure
Click here to download high resolution image