

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

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4 Stuart W. Smith^{a,b,c*}, A. H. Jean Robertson^c, Andrew A. Meharg^a, Robin J. Pakeman^c, David
5 Johnson^a, Sarah J. Woodin^a, René van der Wal^b

6 ^aIBES, University of Aberdeen, St Machar Drive, Aberdeen AB24 3UU, UK.

7 ^bACES, University of Aberdeen, St Machar Drive, Aberdeen AB24 3UU,UK.

8 ^cThe James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH, UK.

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

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21 **Abstract**

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

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

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43 **Introduction**

44 Analysis of the carbon (C) and nitrogen (N) contents of plants and soils is crucial for
45 assessing how climate and land-use change affect global biogeochemical cycles (Guo and
46 Gilford 2002; Bellamy et al. 2005; Powlson et al. 2011). Estimation of ecosystem C and N
47 stores, inputs and losses depends upon accurate determination of C and N concentrations in
48 ecological materials. Automated elemental analysis has become ubiquitous for C and N
49 determination, due to its accuracy and reliability (Kalembas and Jenkinson, 1973; Soon and
50 Abboud 1991; Lal et al. 2001). This type of analysis, based on dry oxidation, only requires a
51 2–20 mg sub-sample and thus precision is dependent on sample homogeneity (Jimenez and
52 Ladha 1993). Homogenisation is achieved through milling, often in stainless steel grinding
53 jars containing stainless steel balls, with mills typically processing 1–3 samples at once
54 (Allen 1989). However, significant time can be saved in preparation of large sets of samples
55 using microcentrifuge tubes with stainless steel balls, with tens of samples being processed
56 simultaneously (Warren and Adams 2004; Salvo-Chirnside et al. 2011; Nadeem et al. 2012).
57 Milling in microcentrifuge tubes avoids the loss of material which occurs through cleaning of
58 steel grinding jars between each use and is thus ideal for small quantities of plant and soil
59 material (e.g. roots, decomposed litter, soil fauna). The use of disposable containers also
60 minimizes any cross-sample contamination. However, during an investigation of C loss from
61 root litter we found that the average C concentration was 5.45 percentage points higher
62 (50.02 vs 44.56 %C), and N concentration 0.072 percentage points lower (0.912 vs 0.985
63 %N), in roots milled in microcentrifuge tubes as compared to steel jars (Figure 1). The
64 additional C was identified, using Fourier-transform infrared (FTIR) spectroscopy, to be
65 atactic-polypropylene ((C₁₅H₃₀)_n) and some copolymers, originating from the microcentrifuge
66 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

91 effects on percent C and N were analysed using linear models (except sample size on %C
92 milled in microcentrifuge tubes which was fitted with a non-linear exponential function) in R
93 (version 2.10.1, R Development Core Team, 2009). There was no significant difference
94 between the plastic Alpha and Retsch tubes in any treatment ($P>0.05$), so these were grouped
95 for statistical analysis. Difference between the change in C and N concentration with
96 treatment in microcentrifuge tubes and in stainless steel jars is indicated by the interaction
97 term of the model. However, for sample size the interaction could not be determined due to
98 differences in linearity (linear for stainless steel; non-linear for microcentrifuge tube). In
99 order to compare the strength of the interaction term for sample size on %C with the other
100 milling treatments (milling time and intensity) a single linear model was used on sample sizes
101 <26.5 mg, a threshold below which a linear relationship was displayed.

102

103 **Results and Discussion**

104 Carbon concentrations in microcentrifuge milled roots increased with increasing milling time
105 and intensity, whilst %C of steel milled roots remained unchanged across both treatments
106 (interaction terms in Table 1; Figure 2A, 2B). Milling a small quantity of sample (≤ 20 mg)
107 produced the greatest polypropylene-derived C contamination (up to 8 %C or 0.08 g g^{-1}) of
108 all the treatments, due to greater abrasion between the ball and tubes (Table 1; Figure 2C).
109 Polypropylene was identified in all roots milled in microcentrifuge tubes, but not in steel
110 milled samples (Figure 3). Milling small samples in microcentrifuge tubes should be avoided
111 and milling time and intensity should be reduced, yet this can prevent particle size being
112 sufficiently small for C and N analysis. Although not tested here, non-spherical balls could be
113 used to dissipate the intensity of contact between ball and microcentrifuge tube (Salvo-
114 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.

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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.

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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).

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139 **Conclusions**

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

150

151 **Acknowledgements**

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

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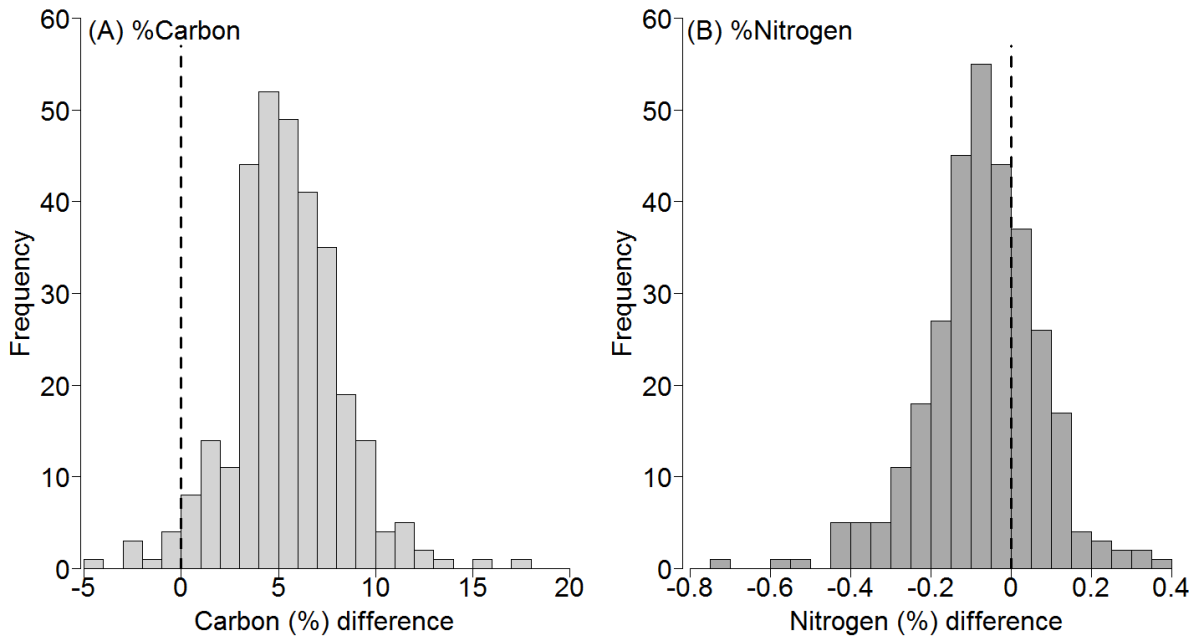
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224 **Figures**



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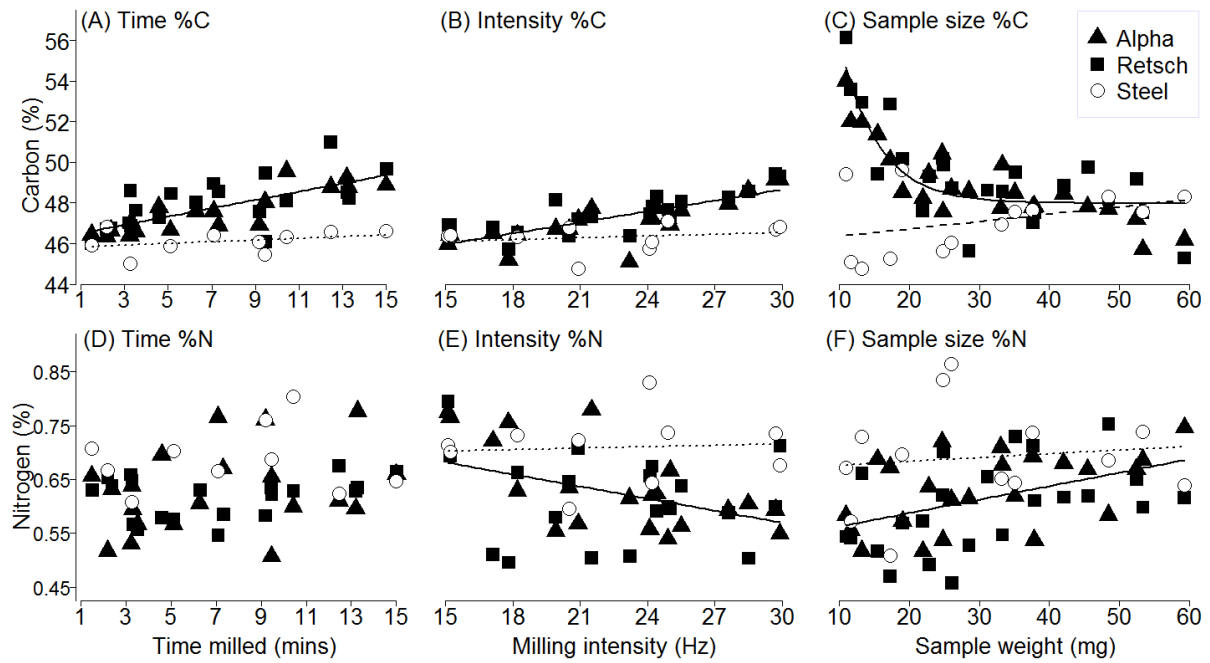
226 **Figure 1.** The shift in absolute percent carbon (A) and nitrogen (B) in partially decomposed root litter
227 attributed to milling samples in microcentrifuge tubes. C and N difference values represent stainless
228 steel jar milled material subtracted from microcentrifuge milled material. Dotted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

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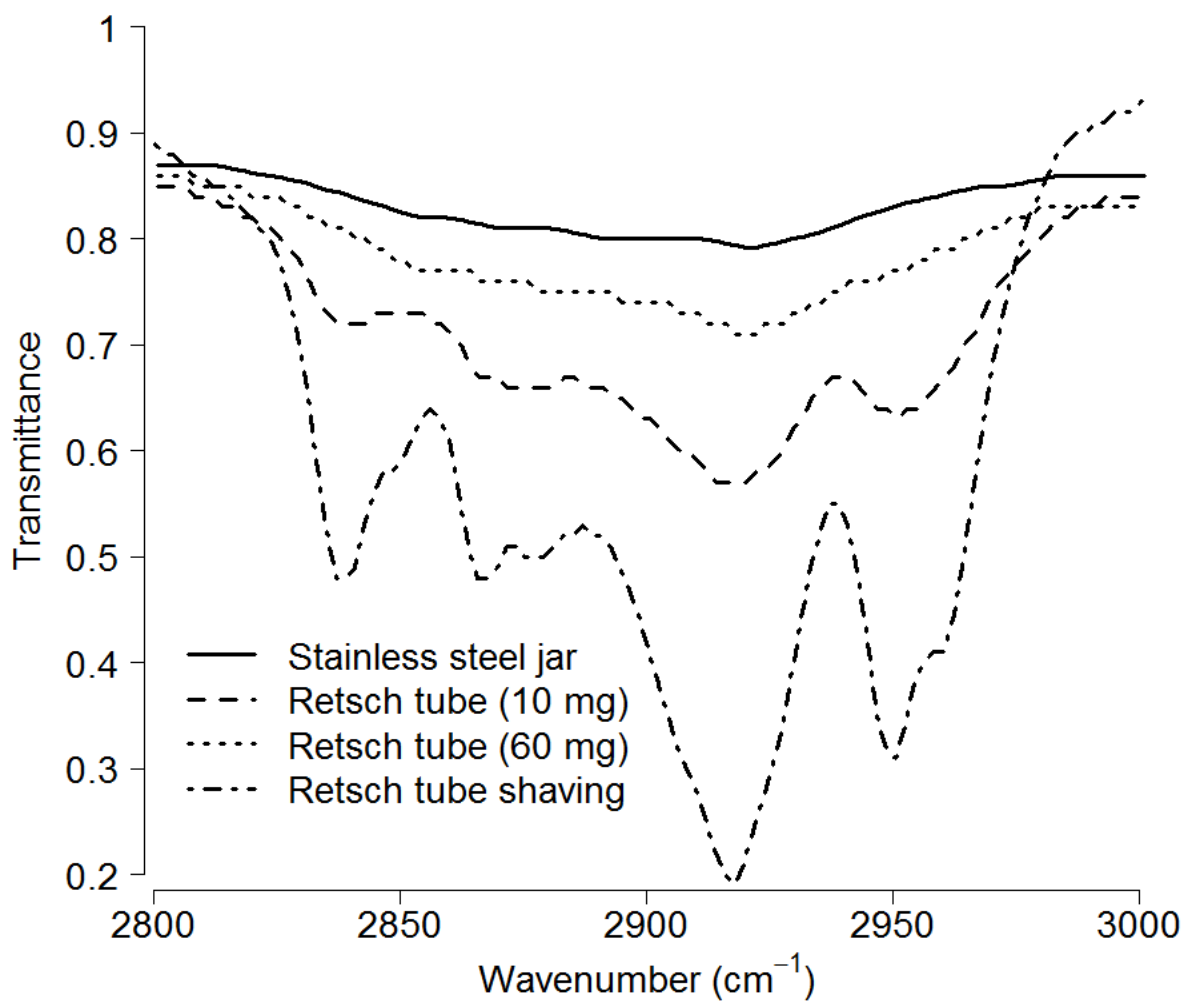
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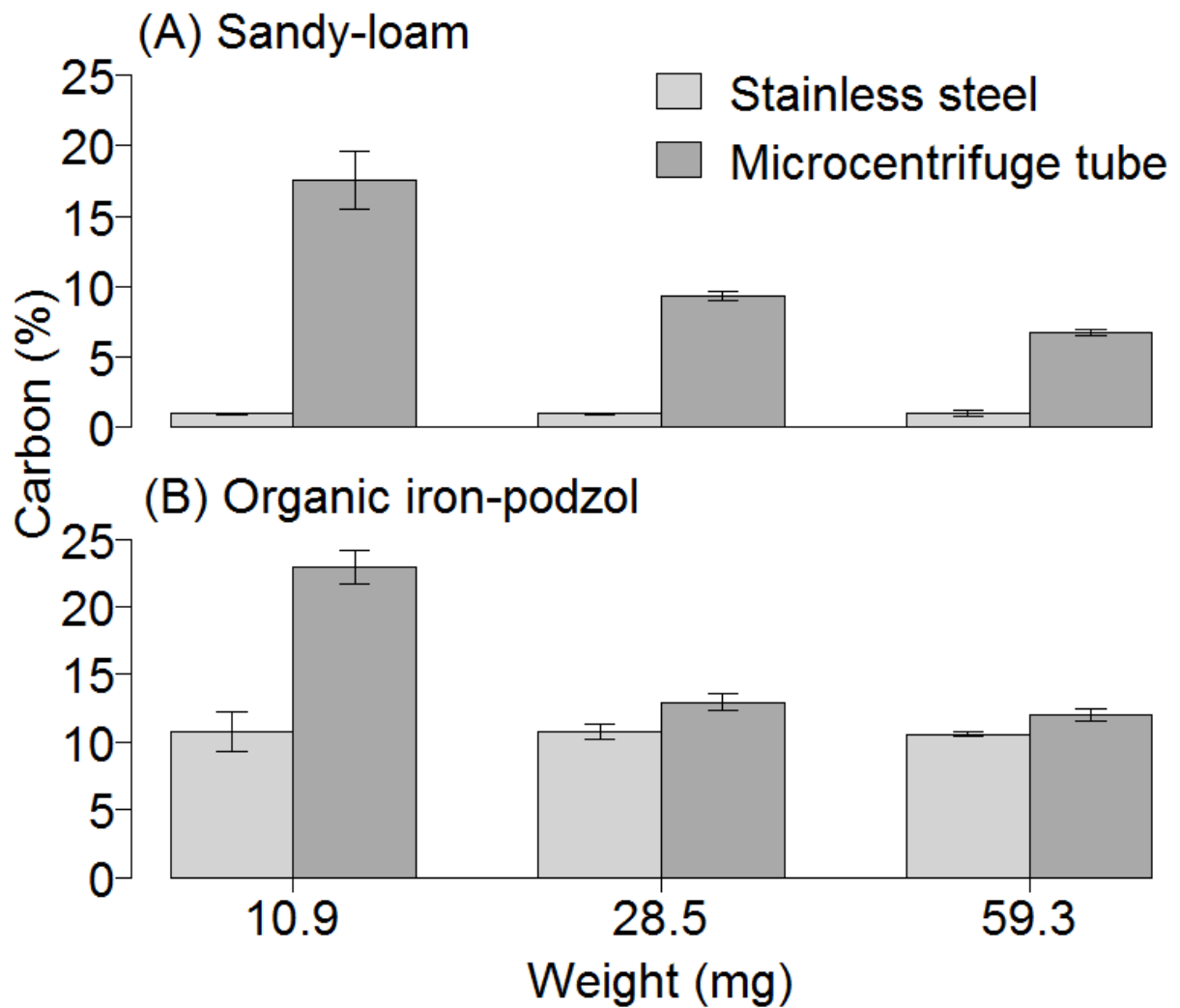
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237 **Figure 2.** Carbon concentrations (%) in *Molinia caerulea* roots for different milling times (A),
 238 intensities (B) and sample sizes (C), and nitrogen concentrations (%) for the same treatments (D, E,
 239 F, respectively). Microcentrifuge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



243

244 **Figure 3.** FTIR spectra within the 2800 to 3000 (cm⁻¹) wavenumber region; the CH stretching region
 245 diagnostic of atactic-polypropylene. Spectra for 10 mg of *M. caerulea* roots milled in stainless steel
 246 jars (solid black line) and Restch microcentrifuge tube (dashed line) and 60 mg of *M. caerulea* roots
 247 milled in Retsch microcentrifuge (dotted line) compared to a sample of plastic from a Retsch
 248 microcentrifuge tube (dashed and dotted line).



249

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

253

254 **Table 1.** Fitted lines for carbon (C) and nitrogen (N) concentrations of roots milled in microcentrifuge tubes and stainless steel jars for each
 255 ‘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	$y=0.54+0.01x$			

256 ^aSignificance of each factor in the model (treatment, milling method and treatment × milling method interaction) are denoted by ns not
 257 significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

258 ^bStatistics relate to the linear model for both stainless steel and microcentrifuge tube combined with sample sizes <26.5mg only, whilst the
 259 predicted lines are for the full range of sample sizes.

260