



Manure for millet: Grain $\delta^{15}\text{N}$ values as indicators of prehistoric cropping intensity of *Panicum miliaceum* and *Setaria italica*

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ABSTRACT

Broomcorn and foxtail millet are the only major domesticated plants indigenous to prehistoric Eurasia to rely on the C_4 photosynthetic pathway. Here we study the impact of animal manure (AM) on broomcorn millet (*Panicum miliaceum*) and foxtail millet (*Setaria italica*), grown in the Askov Long-Term Experiment, using unmanured soil and soil dressed with phosphorus plus potassium (PK) as reference treatments. Animal manure had a marked effect on yields and on the $\delta^{15}\text{N}$ values of grains. For broomcorn millets grown on manured soil, the average $\delta^{15}\text{N}$ value was 5.8‰. When grown on unmanured and PK-treated soils, the $\delta^{15}\text{N}$ values were 0.4‰ and 0.2‰, respectively. For foxtail millet the $\delta^{15}\text{N}$ values also differed between grains from unmanured (-1.0‰), PK (0.7‰) and manured (6.3‰) treatments. Thus, when compared to unmanured soil, the offset due to manure was 7.3‰ for *Setaria* and 5.3‰ for the two *Panicum* varieties. In accordance with previous studies on C_3 crops, our study suggests that $\delta^{15}\text{N}$ values in charred millet grains recovered from archaeological sites could provide a robust indicator of prehistoric manuring intensity.

1. Introduction

Broomcorn (common) millet (*Panicum miliaceum* L.) and foxtail millet (*Setaria italica* (L.), P.Beauv.) are warm-season annuals relying on C_4 -type photosynthesis and adapted to subtropical as well as temperate climate zones. Millets are gluten-free and enriched in the essential amino acids leucine, isoleucine and methionine, providing a higher protein quality than e.g. wheat (Kalinova and Moudry 2006). Compared with traditional C_3 -type cereals (such as wheat, rye, barley and oats) millets have a shorter growing season and lower requirements for water and plant nutrients (Cavers and Kane 2016), allowing them to be grown on less fertile soils. Broomcorn and foxtail millet emerged as early crops in northern China by the 6th millennium BC (Liu et al., 2009; Zhao 2011). Archaeobotanical evidence and stable isotope analyses combined suggest that millets were an important dietary staple for both humans and domestic animals (e.g. pigs) in northern China during the Neolithic (Pechenkina et al., 2005; Barton et al., 2009; Lu et al., 2009; Liu et al., 2012; Chen et al., 2016; Dong et al., 2021; Zhang et al., 2021). Studies based directly on ^{14}C -dated millet grains show that broomcorn millet expanded from East Asia to Europe by the mid-2nd millennium BC (Filipovic et al., 2020), and a similar timeframe is apparent for foxtail

millet in Europe (Zohary et al., 2012: 72). Stable isotope analysis registers a corresponding impact of C_4 foods on human diets in central Europe (Pospieszny et al., 2021).

Despite the rapidly growing evidence of the dietary role of millet, the intensity of millet management in early farming remains largely unknown. Jones et al. (2021) discuss issues that may have motivated and facilitated crop domestication and the origins of agriculture, and consider a range of plant characteristics that were potentially important in the domestication of wild plants. They summarized that rapid germination and early growth rate may have been particularly advantageous characteristics in the selection of wild species as successful crops. One additional factor could be selection of wild plants that show beneficial response to application of animal manure and other plant nutrient-containing organic wastes. It is well documented that addition of animal manure may increase crop yields by a factor 3 to 5 above yields obtained on unmanured soils (Christensen et al., 2019). With regard to millet management, the role of animal manure in the intensification of prehistoric agriculture remains obscure, although palaeodietary reconstructions suggest that domesticated pigs probably played a major role (Pechenkina et al., 2005; Barton et al., 2009; Chen et al., 2016; Dong et al., 2021). Wang et al. (2018) presented measurements of

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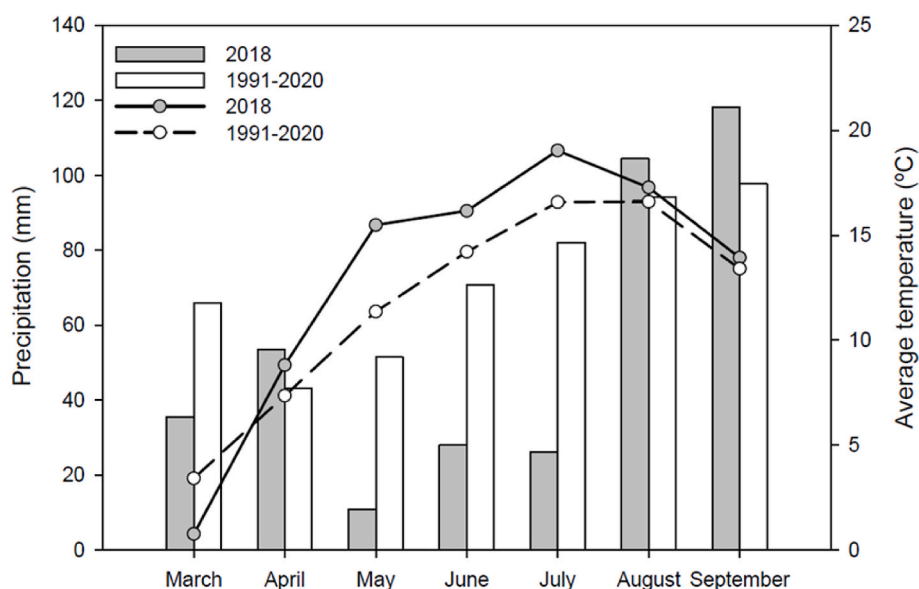


Fig. 1. Accumulated precipitation in 1.5 m height (bars) and monthly average air temperature in 2 m height (lines) from March to September 2018 and from 1991 to 2020 (long-term average). All records are from the weather station at the Askov Experimental Station.

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in archaeological charred grains that suggested use

of animal manure as a growth-promoting factor for millet grown during 5500 to 3500 cal BP in the Baishui Valley in North China. However, the lack of unmanured reference data prevented estimation of manuring intensity.

Previous studies based on well-controlled long-term field experiments involving manured and unmanured soil plots have successfully quantified the impact of manuring on C and N isotope ratios in modern and ancient cereal types (Bogaard et al., 2007; Fraser et al., 2011; Kanstrup et al., 2011). Here we apply a similar approach to quantify the impact of animal manure (AM) on growth parameters and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in grains of broomcorn millet and foxtail millet, using millets grown in long-term unmanured soil plots and in plots amended exclusively with mineral phosphorus (P) plus potassium (K) as reference treatments. Soil dressed with P and K were included to simulate millet behavior on soils exposed to slash-and-burn regime. The study employed the Askov long-term experiments established in 1894. This experiment represents a unique research platform with large gradients in fertility built up over more than 125 years of contrasting nutrient treatments. Testing the response of millets to soil nutrient levels under field conditions ensures realistic growth conditions and access to physiologically mature grain and straw. In the 2018 growing season, we established mini-plots in three replicated plots of each treatment (unmanured, PK and AM) with millets grown to physiological maturity. We determined harvest yields of straw and grain, grain weight, and total-C, total-N, C and N isotope ratios in grains.

2. Materials and methods

For this study, we grew two varieties of broomcorn millet (*Panicum miliaceum* L., var. Kornberger and var. Mammoth), here termed *Panicum-K* and *Panicum-M*, and one variety of foxtail millet (*Setaria italica* (L) P. Beauv.) in three treatments of the Askov Long-Term Experiments on Animal Manure and Mineral Fertilizers (Askov-LTE). We were able to obtain sufficient seeding material for two varieties of broomcorn millet and for one variety of foxtail millet.

2.1. The Askov LTE

The Askov-LTE was established in 1894 at Askov Experimental Station, South Denmark (55°28'N, 09°06'E), a site characterized by a mean annual precipitation and temperature of 945 mm and 8.4 °C,

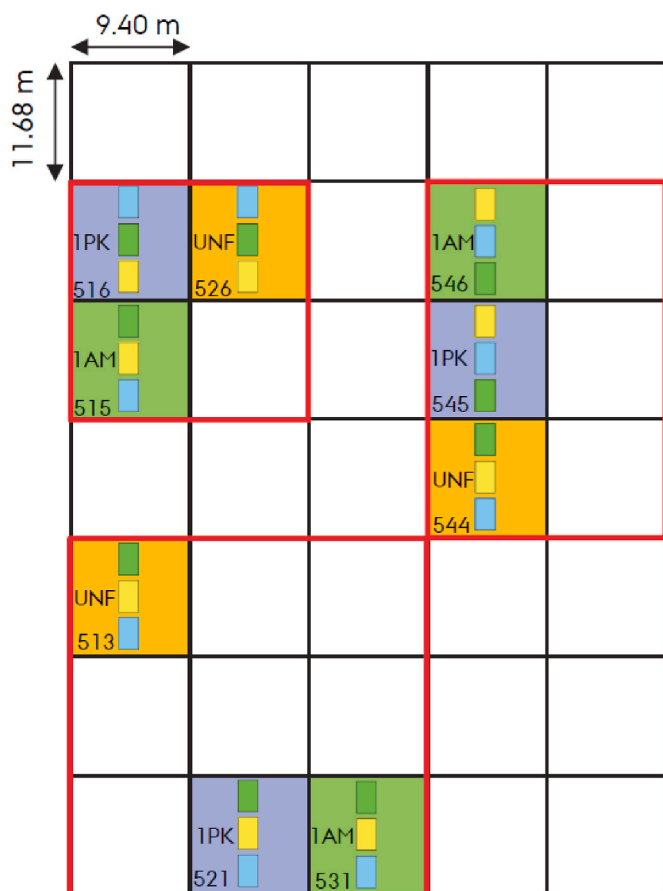


Fig. 2. The experimental layout and distribution of sub-plots for millets. The treatment plots (110 m²) are UNF, 1 PK and 1 AM. The millet sub-plots are *Setaria* (yellow), *Panicum-K* (green) and *Panicum-M* (blue). Thick red lines delineate the three blocks adopted in statistical analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



Fig. 3. The millet grown in the B5-field of the Askov Long-Term Experiment in the summer 2018 (see Fig. 2 for position of plots involved in this study). The photos show millet sub-plots shortly after germination (upper left), broomcorn millet (*Panicum*, upper right) and foxtail millet (*Setaria*, lower left) close to harvest time. Lower right shows harvest of foxtail millet. A net covered the sub-plots with millets during the growing period to protect the crops against bird damage.

respectively (1981–2019 averages). The soil is a light sandy loam with 11% clay, 13% silt and 76% sand and classifies as Alfisol (Typic Hapludalf) according to USDA Soil Taxonomy. The experiment includes four fields (blocks), termed B2– B3-, B4- and B5-field, that grow a four-course rotation of winter cereals, row crops, spring cereals and a grass-clover crop used for cutting. For a given year, each field supports one crop only but encompasses replicate plots kept without manures or fertilizers (UNF) and plots receiving different rates of animal manure (AM) and different combinations and rates of nitrogen (N), phosphorus (P) and potassium (K) in mineral fertilizers. The current study was embedded in the B5-field using the treatments UNF (since 1894), 1AM (since 1894) and 1 PK (since 1935). The size of individual treatment plots is 110 m².

Since 1973, the 1 PK plots have received 20 kg P ha⁻¹ and 83 kg K ha⁻¹ in superphosphate and potassium chloride, while the 1AM plots received 100 kg total-N ha⁻¹, 18 kg P ha⁻¹ and 90 kg K ha⁻¹ in cattle slurry, corresponding to 25 t ha⁻¹ (annual mean across the four crops in the rotation). Throughout the experimental period, cattle manure has been used with cattle slurry (stored 4–9 months in slurry tanks) being applied since 1973. Christensen et al. (2019) provide a detailed description of the Askov-LTE.

2.2. Millet cultivation

On May 7, 2018, we planted millets in three sub-plots (each 2.6 m²) established in the center of each of the three replicate plots of the UNF, 1 PK and 1AM treatments. The plot area surrounding the sub-plots grew winter wheat, established in the autumn 2017. Following laboratory germination tests of the seed materials, the seeding rates applied corresponded to 10, 38 and 29 kg seed ha⁻¹ for *Setaria*, *Panicum*-K and *Panicum*-M in order to obtain a final crop density of 250 plants m². The rate of nutrients added in 1AM for this crop was 153, 26 and 138 kg ha⁻¹ of total-N, P and K, respectively. The rates in the 1 PK treatment were 30 kg P ha⁻¹ and 120 kg K ha⁻¹. Since millets are sensitive to drought in the germination and early growth phase, we applied 31 mm of irrigation during May. Crop protection included hand weeding and insecticides while a coarse-meshed net covered the millet sub-plots throughout the

Table 1

Soil characteristics. Soil pH (0.01 M CaCl₂) was 6.5. Plant available P is extracted by 0.5 M NaHCO₃ while available K is extracted by 0.5 M NH₄OAc. From Kanstrup et al. (2011) and Cong et al. (2019). NA = data not available.

Treatment	Soil C (mg C g ⁻¹ soil)	Soil N (mg N g ⁻¹ soil)	Soil δ ¹³ C (‰)	Soil δ ¹⁵ N (‰)	Available P (mg P kg ⁻¹ soil)	Available K (mg K kg ⁻¹ soil)
UNF	9.5	0.87	-27.2	4.9	3.7	23.7
1PK	10.8	1.1	-27.2	5.1	NA	NA
1AM	12.1	1.12	-27.3 ^a	6.8 ^a	10.7	65.3

^a From 1½AM.

growing period to protect against bird damage. Fig. 1 shows monthly temperatures and precipitation for the 2018 growth period and the average for the period 1991–2020 while Fig. 2 outlines the experimental layout and position of the nutrient treatments and the millet subplots. Fig. 3 shows the millet grown in the B5-field in 2018, and Table 1 presents relevant soil C and N concentrations, and associated δ¹³C and δ¹⁵N values.

Harvest of *Setaria* took place on 7 August while harvest of *Panicum* sub-plots took place on 20 August. At this stage, grains in the upper half of the head were fully mature and prone to shattering. The harvested material was left to dry indoors before being separated into grain and straw. Subsequently, the grain fractions were dehusked and cleaned.

2.3. Analyses

Grain and straw fractions were dried at 55 °C for 24 h to determine dry matter yields. Grain weight was determined as the average mass of 1000 dehusked grains. Subsamples of 2.5 g dried grains were ball milled and 4–5 mg of each sample packed into tin (Sn) capsules and analyzed by high temperature dry combustion for total-C, total-N, and for the stable isotopes of ¹³C and ¹⁵N at UC Davis Stable Isotope Facility (Davis, CA, USA). A PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd.

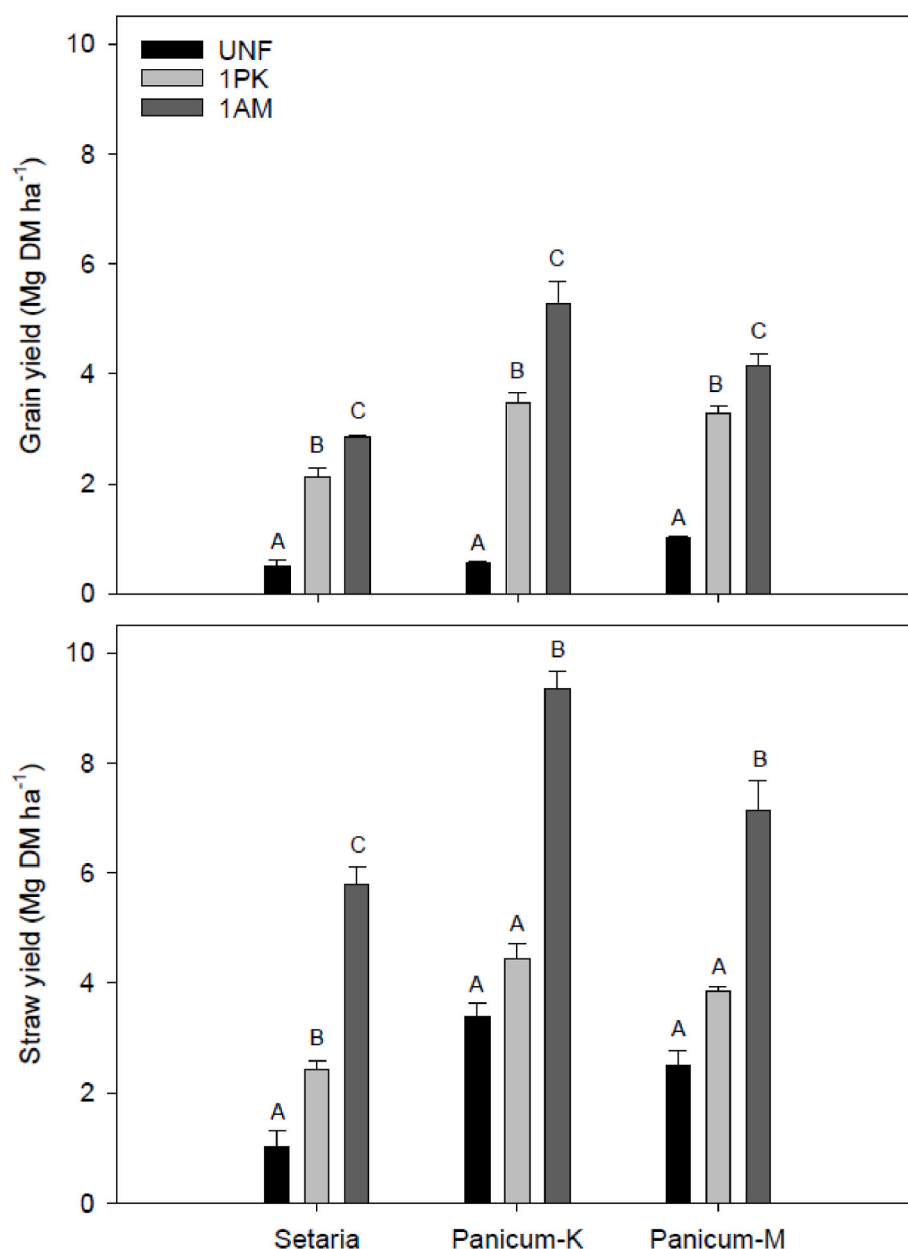


Fig. 4. Grain and straw dry matter (DM) yield (Mg ha⁻¹) of *Setaria*, *Panicum-K* and *Panicum-M* grown on unfertilized (UNF), PK amended (1 PK) and animal manured (1AM) plots of the Askov-LTE. Bars indicate mean values \pm 1 standard error ($n = 3$). Within millet varieties, letters denote statistical significance at $P < 0.05$.

Cheshire, UK) was used for analyses, with ¹³C and ¹⁵N isotope abundance being expressed in delta units (δ ‰) relative to the international standards VPDB (Vienna Pee Dee Belemnite) and Air, respectively.

2.4. Experimental design and statistics

The experimental design was a two-factorial split-plot design with 3 replicates and 27 sub-plots in total. Nutrient treatment was the main plot factor (NT: UNF, 1 PK and 1AM) while sub-plots were millet species (MS: *Setaria*, *Panicum-K* and *Panicum-M*). The statistical analysis relied on the R-project software package Version 3.4.0 (R Foundation for Statistical Computing). Linear mixed effect models tested the significance of NT and MS and their interaction on grain yield, quality and isotope parameters using the *lmer* function of the *lme4* package. Fixed effects were NT and MS while block was set as random effect. The significance of NT, MS and their interaction were assessed by analysis of variance (ANOVA) Type III. The criterion used for statistical significance was $P < 0.05$.

When NT, MS or the interaction between them were significant, a post hoc comparison was performed (Tukey HSD test) using the estimated marginal means (*emmeans*) function implemented in the R *emmeans* package.

3. Results

[Supplementary Table 1](#) reports all of the millet data. The grain yields of *Setaria* and the two *Panicum* varieties grown on plots dressed with animal manure were significantly larger than yields obtained on unmanured soil (Fig. 4). While yield levels on 1 PK and 1AM plots were higher for *Panicum* than for *Setaria*, yields did not differ for millets grown in unmanured treatments. The largest effect of manure appeared for *Panicum-K* with grain yields increasing almost ten-fold (0.55 Mg ha⁻¹ on UNF soil and 5.27 Mg ha⁻¹ on 1AM soil). However, the 1 PK-amended plots also supported grain yields that were significantly above those obtained on unmanured soil. The yield of straw followed the same

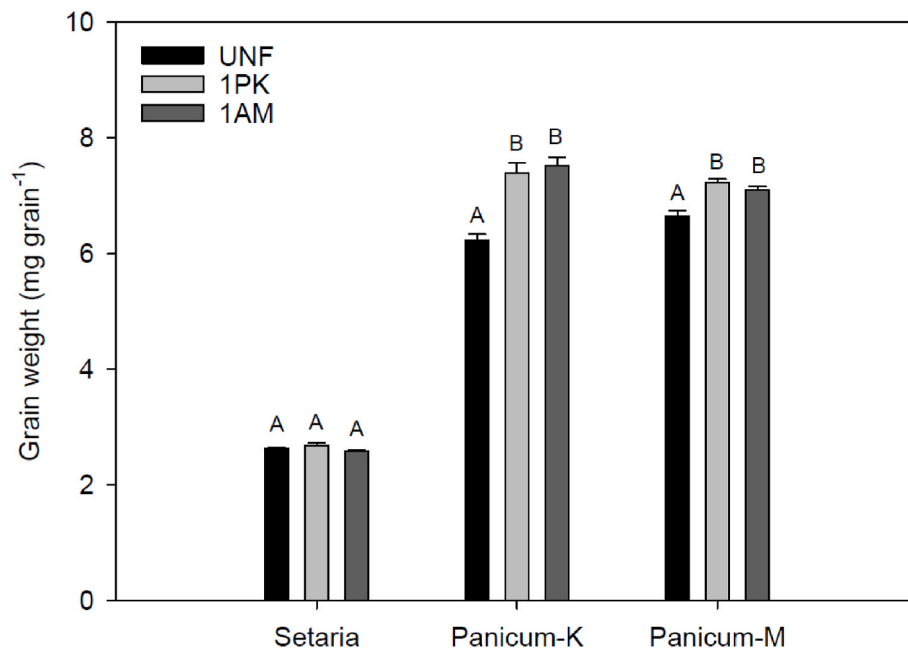


Fig. 5. Millet grain weight (mg grain⁻¹) based on the average weight of 1000 grains. See Fig. 4 for further legends.

general pattern as seen for grain yields but differences between unmanured and 1 PK treated soil were smaller and not statistically significant. For unmanured soil, the two *Panicum* varieties yielded more straw than *Setaria*.

Average grain weights were smaller for *Setaria* (2.6 mg grain⁻¹) than for *Panicum* and not affected by nutrient regime (Fig. 5). Grain weights of *Panicum-K* and *Panicum-M* grown on unmanured soil was slightly smaller (6.4 mg grain⁻¹) than weights of grain from plots with 1 PK and 1AM; these two treatments showed similar grain weights (7.4 mg grain⁻¹).

Concentration of C in grains was similar for all three millets and not affected by nutrient regime (Fig. 6). For *Setaria*, the N concentration was higher for grains harvested on unmanured plots (1.13% N) while grain N did not differ between 1 PK and 1AM treatments (0.97% N). Concentration of N in grains from the two *Panicum* varieties grown on 1AM and unmanured soil did not differ (0.98% N), whereas grains from the 1 PK treatment had smaller N concentrations (0.72% N).

The $\delta^{13}\text{C}$ values of grain ranged from -12.7‰ to -13.6‰ (Fig. 7). For *Panicum-M*, there was no effect of nutrient regime whereas grains of *Setaria* and *Panicum-K* grown on unmanured soil had lower $\delta^{13}\text{C}$ values than grains originating from soil receiving animal manure.

Animal manure had a marked effect on the $\delta^{15}\text{N}$ signature of millet grains (Fig. 7). For the two *Panicum* varieties grown on manured soil, the average $\delta^{15}\text{N}$ value was 5.8‰. When grown on unmanured and PK soils, the $\delta^{15}\text{N}$ values did not differ significantly (0.4‰ and 0.2‰, respectively). For *Setaria*, the $\delta^{15}\text{N}$ value differed significantly between grains from unmanured (-1.0‰), 1 PK (0.7‰) and manured (6.3‰) treatments. Thus, when compared to unmanured soil, the offset due to manure was 7.3‰ for *Setaria* and 5.3‰ for the two *Panicum* varieties.

4. Discussion

Animal manure application significantly increased yields of grain and straw whereas effects on grain weights were small, although significant for the two varieties of broomcorn millet. In a previous study of modern and ancient cereal types with C₃-type photosynthesis and grown in the Askov-LTE, we observed a similar positive effect of manure on grain and straw yields (Kanstrup et al., 2011). In contrast to these C₃-cereals, millets grown on 1 PK treated soils yielded significantly more than millets grown in unmanured soil. By including the 1 PK treatment,

we intended to simulate soil fertility following a slash-and-burn regime. During burning, most of the N contained in the cleared biomass becomes lost in gaseous forms or locked-up in the charred biomass while the soil retains mineral cations including P and K (Juo and Manu, 1996; Certini 2005). However, studies on the effect of wildfires in contemporary ecosystems suggest an impact on the N isotope ratio in plants grown on fire-affected soil (Szpak, 2014). Apparently, millets perform better than C₃-cereals on soils low in N, a beneficial plant trait that could have played a role for an early domestication of this crop (cf. Jones et al., 2021). Thus, differences in grain yields between 1 PK and 1AM were relatively small for *Setaria* and *Panicum-M* (0.7 and 0.9 Mg ha⁻¹, respectively) while the difference was somewhat higher for *Panicum-K* (1.8 Mg ha⁻¹).

The driving forces behind crop domestication of wild plants and the early development of ancient agriculture remain debated (Jones et al., 2021). One driver could be the observation that plants growing on soils exposed to animal manure (or other nutrient-containing waste) increased productivity compared to grains gathered from other sites. The use of manure as a growth promoting measure is associated with the intensification of early agriculture and facilitated by a parallel domestication of animals. Availability of sufficient manure for field application would depend on domesticated animals periodically kept in enclosures outside or in stables, e.g. to protect the herd from wild carnivores. Bakels (1997) considers that manuring was intimately associated with the intensification of agricultural activities, and ventures that the practice of confining animals relates to the very purpose of obtaining animal manure. Bogaard (2005) developed this argument further to frame the early integration of cultivation and herding in western Asia and Europe. Compared to collecting dung from grazing animals dispersed over large areas, periodic confinement of animals in small enclosures or folds would clearly facilitate collection of sufficient quantities of manure needed for systematic manuring.

Compared with unmanured soil, animal manure application raised the grain $\delta^{15}\text{N}$ values of millet by 5.3–7.3‰ whereas $\delta^{15}\text{N}$ was only little affected by addition of PK. Previous studies on C₃-cereals grown on manured plots in long-term field experiments show an increase in grain $\delta^{15}\text{N}$ values ranging from 4 to 9‰ (Bogaard et al., 2007; Fraser et al., 2011; Kanstrup et al., 2011). In the present study, we applied only one level of animal manure. The impact on grain $\delta^{15}\text{N}$ values depends on manuring level and frequency, but the effect of manure deposition on

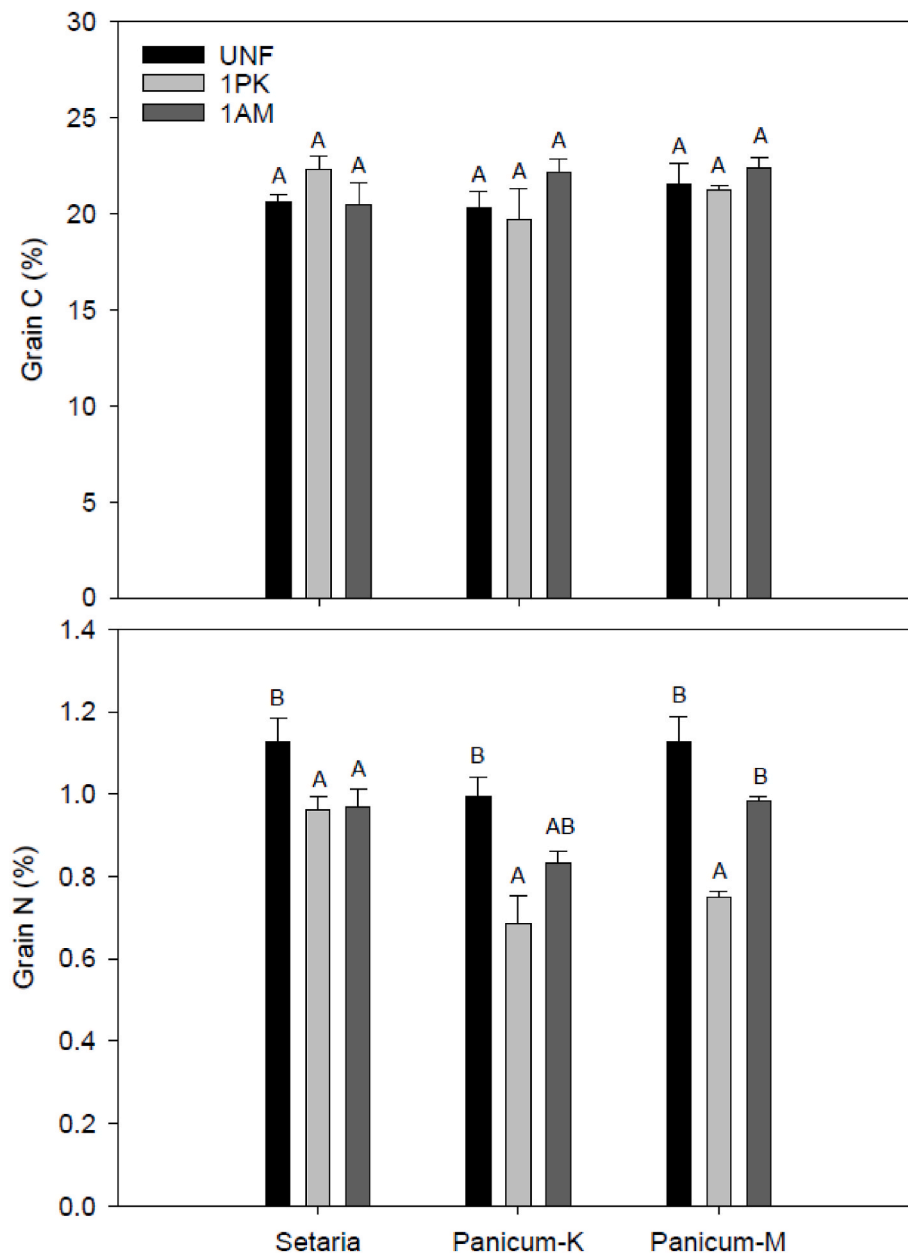


Fig. 6. The concentrations of C and N (%) in millet grains. See Fig. 4 for further legends.

grain $\delta^{15}\text{N}$ values is traceable long after manuring has ceased (Bogaard et al., 2007; Fraser et al., 2011). Studies of archaeological sites in Greenland and Kenya show that manure deposition can be traced for centuries to millennia by analysis of ^{15}N enrichment in soil and even in modern plants growing on previously manured soil (Commisso and Nelson, 2006, 2010; Shahack-Gross et al., 2008).

Conventional methods applied to detect ancient manuring include analysis of soils for P fractions and multi-element analysis (e.g. Nielsen and Kristiansen 2014) and biomarkers such as coprostanols (e.g. Bull et al., 1999). However, soils of ancient fields are seldom preserved, and may subsequently undergo changes that distort the soil's original properties, preventing an unambiguous interpretation of data. In contrast, analyses for N isotope composition of ^{14}C -dated archaeobotanical charred plant remains (e.g. cereal grains) provide a promising direct source of information on prehistoric manuring practice. The charring process in some studies is found to have no effect on grain $\delta^{15}\text{N}$ values (Aguilera et al., 2008; Kanstrup et al., 2012) while other studies observe a modest and predictable offset of $\sim +0.3\text{‰}$ (Fraser et al., 2013;

Nitsch et al., 2015).

While manuring had little effect on the $\delta^{13}\text{C}$ values of millet grains, the impact of manure on $\delta^{15}\text{N}$ values comes with serious implications for palaeodietary studies deriving prehistoric millet consumption from analysis of bone collagen from archaeological sites (Barton et al., 2009; Atahan et al., 2014; Pospieszny et al., 2021). The use of $\delta^{15}\text{N}$ values for collagen extracted from bone materials has been widely used to reconstruct human diets, including the relative contributions from plant and animal sources. The conventional approach has been to assume an enrichment from one trophic level (e.g. vegetation) to the next level (e.g. herbivores including humans) of 3–5‰. Dürrwächter et al. (2006) and Hedges and Reynard (2007) discussed the uncritical use of this approach in detail, and Bogaard et al. (2007) illustrated the archaeological implications of manure-induced increase in cereal grain $\delta^{15}\text{N}$ for reconstruction of prehistoric diet using results obtained for cereals grown in field experiments with long-term unmanured and animal manured soils. The present study confirms that intensive manuring similarly affects millets, the increase in the grain $\delta^{15}\text{N}$ values corresponding to around a

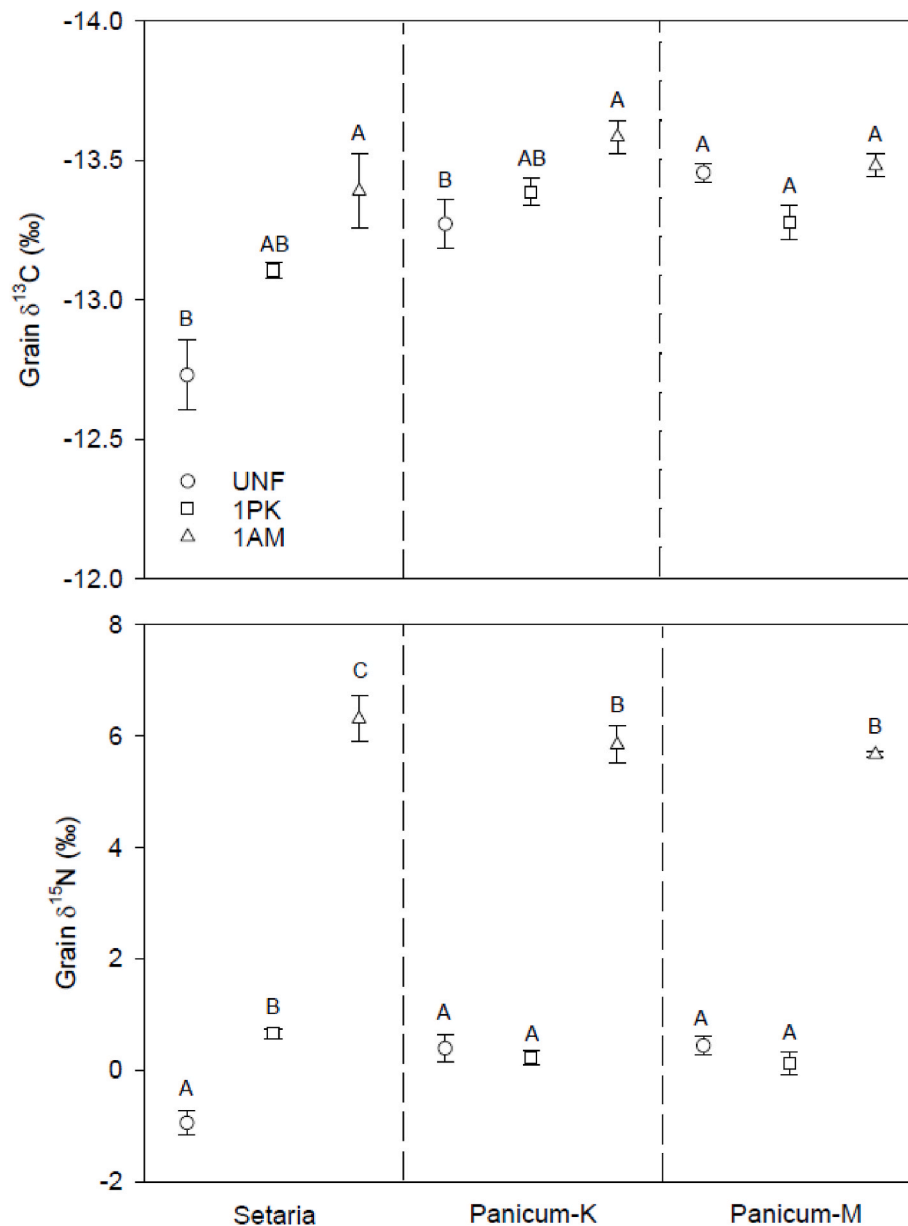


Fig. 7. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (‰) in millet grains. See Fig. 4 for further legends.

trophic level.

Analyses of N isotopic composition of charred C_3 -cereal grains from archaeological sites show great variation in $\delta^{15}\text{N}$ values. Stable N isotope determinations of 99 cereal samples (dating 2400–5900 cal BC) from 13 sites across Europe showed $\delta^{15}\text{N}$ values ranging from 0.1 to 7‰ (Bogaard et al., 2013). Kanstrup et al. (2014) examined 72 samples of charred cereal grains dating 0 to 3900 BC and collected from sites across Denmark. In this study, the $\delta^{15}\text{N}$ values varied from 1.5 to 8‰. Wang et al. (2018) analyzed charred grains of millets retrieved from three Chinese archaeological sites dating 3500 to 5500 BP and found $\delta^{15}\text{N}$ values ranging from 3.9 to 6.6‰ for foxtail millet and from 3.3 to 6.6‰ for broomcorn millet. At some sites, low $\delta^{15}\text{N}$ values reflecting crops grown on unmanured soils may occur along with higher values indicating crops grown with manure. This implies variable manuring practices with cereals grown with different manuring intensity and perhaps used for different purposes. For example, Kanstrup et al. (2014) found two sets of grain samples from naked barley within the same house structure but with different $\delta^{15}\text{N}$ values (2.4 and 4.7‰). The wide difference in $\delta^{15}\text{N}$ values of samples from the same house could potentially

have originated from different fields with different manuring strategies or from different harvests and subsequently kept separate to serve different purposes e.g. seeding material for the next season or different consumption aspects. Clearly, intra-site variation in $\delta^{15}\text{N}$ values of grains deserves greater attention when evaluating prehistoric manuring intensity and the development of ancient agrarian societies.

5. Conclusions

Our field trials have confirmed the significant impact of intensive manuring on yields and grain $\delta^{15}\text{N}$ values of broomcorn and foxtail millets. These results have important implications for the interpretation of archaeobotanical stable isotope values as well as palaeodietary analysis of humans, fauna and plants in ancient Eurasian ecosystems. An emerging hypothesis is that in northern China, as in western Eurasia, early farming systems that incorporated animal husbandry alongside cultivation fostered synergistic relationships between crops and livestock. The assessment of manuring through stable isotope analysis of archaeobotanical grain assemblages offers a useful index of these

ecological relationships and their variation through space and time.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jas.2022.105554>.

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