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Mid-season prediction of grain protein content to guide nitrogen management in milling wheat

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CONTENTS

1. Abstract

It is standard practice for UK milling wheat farmers to add extra Nitrogen (N) fertiliser late in the growing season to enhance grain protein content, without knowing whether this is necessary or not. Protein synthesis in grain depends on N supply from the soil and remobilisation of N from the leaves during the grain filling period, but also on other factors like sunlight, temperature and moisture. In this project, a recently developed new protein prediction indicator was tested to see how effective it was in predicting grain protein content and what was the best time for sampling. This new indicator is based on signals in the roots that regulate N uptake into the plant and gives an accurate assessment of the N status of the crop at the time of sampling.

Between 2019 – 2021, 10 field experiments were carried out on breadmaking varieties of wheat testing the effect of differing amounts of extra N applied at either GS 31 (early stem extension), GS 37 (late stem extension) or GS 70 (start of grain filling) compared to the normal rate of N applied for yield. Roots were sampled 3 times during the growing season and analysed for regulatory signals involved in N uptake. From the results of the root extractions, a protein prediction was made and compared to actual grain protein content at harvest.

The later the crops were sampled in the season, the more accurate the prediction of grain protein content. The sampling time was critical for the success of the prediction. Sampling at early stem extension was not effective as it was too soon after the main N fertiliser application which resulted in an overestimation of the protein prediction. Sampling during a drought period resulted in an underestimation of the protein prediction because N uptake was limited due to lack of moisture. It was therefore advised to sample late May/early June if a solid fertiliser was used for the late N application or from the 1st of June onwards if a foliar N was applied.

When the protein prediction was <13%, applying late N significantly improved the chances of achieving milling specification by 4-12 times depending on when the crop was sampled. When the protein prediction was ≥13%, 80% of samples gained a financial benefit by not wasting fertiliser when it was not needed. The largest financial benefit was gained where samples identified by the protein prediction test achieved milling specification without applying extra N. This compared to only 50% of samples gaining a financial benefit when late N was applied regardless of the outcome of the test, with 50% wasting money on fertiliser that was not required.

Overall, the protein prediction test was shown to be an effective tool to aid farmers in their decision processes on whether to apply late N or not. Reducing fertiliser use has positive effects on the environment by reducing N leaching, N_2O emissions from denitrification and CO_2 emissions from the manufacturing process. The protein prediction test can play an important role in this.

2. Introduction

Every year between the end of May and the beginning of July, milling wheat farmers in the UK will apply an extra 40 kg N/ha to their crop without knowing whether this is necessary or not. It is done as an insurance to enhance the protein in the grain, since millers require a protein content of ≥13% for which a financial premium is paid (NABIM, 2016). High protein levels enhance the quality of flour: their structures form bonds that increase the elasticity and extensibility of the dough (Kettlewell et al., 1998) and result in larger loaf volumes (Finney et al., 1957). The premium paid for grain with protein levels ≥13% varies with availability: the more farmers achieve milling specification, the lower the premium. However, this can change rapidly. For example, in 2021 only 20% of milling wheat achieved milling standard due to adverse weather conditions and premiums rose from £17.20 per tonne of wheat in August to £40.70 by November (Davies, 2021). The rapid rise was caused by global trends and a tight domestic supply. When this occurs, millers will take grain with protein levels <13%, up to 12%, but the premium will be reduced.

The excessive use of nitrogen and its associated risk of polluting waterways by N leaching and polluting the atmosphere by volatilisation of urea has long been a concern (Dampney et al., 1995). It is therefore important that farmers apply the right amount of fertiliser at the right time to optimise the nitrogen use efficiency of their crops and minimise polluting the environment. This has been recognised by government who have set up a framework for the reduction of nitrogen fertiliser input in their 25-year environment plan (HM Government, 2018). The steep rise in fertiliser prices since 2021 has focused attention on improving practices that no longer make economic sense, like adding "just a little bit extra" fertiliser as an insurance. Clearly, a method to predict grain N content during the growing season would enable farmers to target their late N applications better and this was identified as a future R&D requirement by the HGCA more than 20 years ago: "An effective prediction method would reduce costs to UK quality wheat growers and minimise any undesirable environmental effect arising from unnecessary nitrogen use. Successful development of this prediction method would enable more confident use of foliar N by growers and allow more precise decisions of if, and how much, extra N is needed by individual crops in order to meet a specific market requirement for grain protein concentration" (Turley et al., 2001).

2.1. Indicators for predicting grain protein

Over the last 30 years, several indicators have been suggested to be suitable predictors of grain protein content. These include total N in flag leaves (Sylvester-Bradley, 1990; Tindall et al., 1995; Sexton et al., 2006), chlorophyll measurement of the flag leaves using a SPAD meter (Lopez-Bellido et al., 2004; Debaeke et al., 2006; Monostori et al., 2016), near infrared reflectance spectroscopy (NIR) of developing ears (Bhandari, 2000; Weightman et al., 2011) or remote sensing using satellite imaging (Zhao et al., 2005).

There are difficulties with all of them. Total N in flag leaves increases rapidly at fertiliser applications below 200 kg N/ha but then reaches a maximum at around 4% (Yara, 2005). To predict protein, it is necessary to be able to measure differences in the 200 – 300 kg N range since most farmers will apply at least 200 kg N/ha for yield, which is exactly the range where there are few changes in leaf N%. Furthermore, from anthesis onwards total N in leaves decreases due to remobilisation (Gregory et al., 1981) so critical values change depending on the growth stage at which the leaves were sampled. Therefore, although it is possible to determine critical values for leaf N% from individual experiments that predict protein for that specific year, it is difficult to extrapolate these values to different sites and seasons.

The problems with measuring chlorophyll as an indicator of protein N are the same as those associated with using leaf N%, which is not surprising since there is a close correlation between chlorophyll and leaf N% (Lopez-Bellido et al., 2004). It was found that SPAD readings could predict protein N accurately where N was a limiting factor but could not be used when wheat was over-fertilised (Debaeke et al., 2006). Although SPAD meter measurements are promoted as a quick and easy alternative to measuring total N in leaves, it takes at least 30 leaves per plot to measure an accurate average (Lopez-Bellido et al., 2004). Chlorophyll levels are not consistent between sites and years and influenced not just by N but also by crop variety, other nutrients like sulphur and crop diseases. There is significant interaction between seasons and SPAD readings, where the same fertiliser applications result in different SPAD readings in different years (Monostori et al., 2016).

An excellent relationship with R^2 = 0.88 was found between protein prediction using NIR measurements of immature wheat ears and the actual harvest protein (Bhandari, 2000). Unfortunately, due to relatively small number of samples, the calibration curve to determine the algorithm to predict protein was based on the same sample set. When this technique was used on a wider range of samples in different years and with different varieties, the R^2 was reduced to 0.45, particularly when it was used on commercial samples (Weightman et al., 2011). Prediction of protein using NIR was possible where N was limiting (Apan et al., 2006) but generally using the technique failed to predict protein accurately (Freeman et al., 2003).

The advantage of satellite remote sensing is that it can provide large-scale coverage of site-specific properties of crops in real time at relatively low cost but it is very difficult to do so quantitatively (Zhao et al., 2005). The main challenges are associated with retrieval of surface reflectance and removal of the influence of aerosols for each image pixel. Excellent correlations between predicted and actual protein using satellite imaging have been reported (Øvergaard et al., 2010) but these were achieved by splitting the experimental field of 160 plots in half, with 80 plots used as the calibration set to predict protein at the other 80 plots. It is debatable whether this technique would work as well on different sites and over seasons as it would require an extensive level of calibration.

2.2. Factors influencing protein synthesis

One of the reasons why it is so difficult to predict protein is that protein synthesis in the grain is the result of several factors which interact with each other in a complex way (Figure 1). It is well established that nitrogen supply is one of the main drivers of protein synthesis (Sylvester-Bradley, 1990). N is an essential element without which proteins cannot be formed, because N is part of the amine group (NH_3) in amino acids. Amino acids consist of an amine group and a carboxylic acid (COH_2) connected together with a carbon. They are the base unit of protein since proteins consist of long chains of hundreds or thousands of amino acids. In plants, the N for amino acids is provided by N uptake from the soil but the carbon skeleton of the amino acids is produced by photosynthesis. If either N supply or photosynthesis are limited, protein synthesis will be reduced.

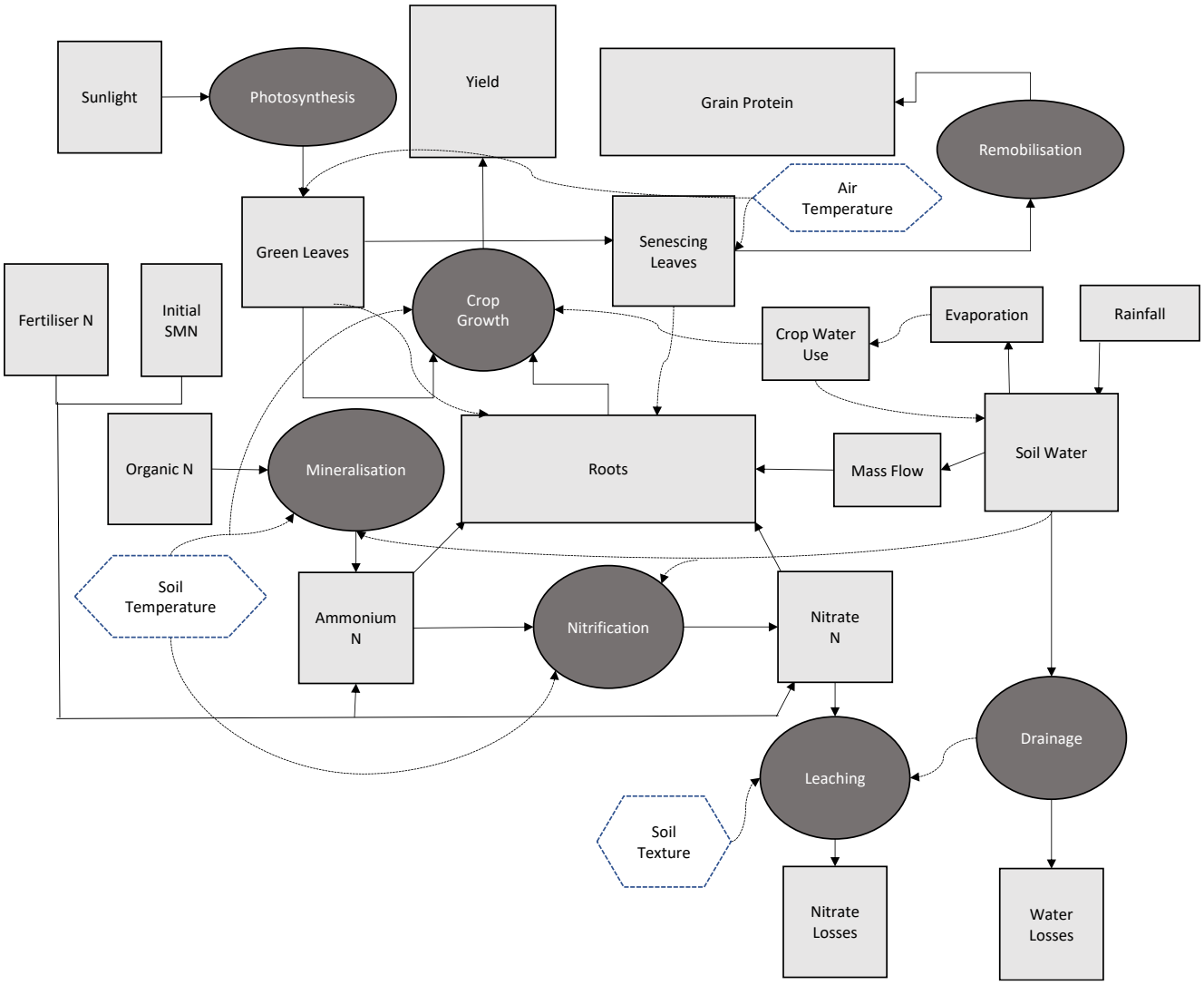

Figure 1. A schematic representation of the flow of Nitrogen between various pools in the soil and plant resulting in yield and protein. Circles represent processes that influence the flow of N. Physical flows of N are denoted by solid lines and flows of information are denoted by broken lines. The figure has been adapted and extended from Addiscott and Whitmore (1987).

To increase protein synthesis in the grain, it is important to optimise both N supply and photosynthesis. N supply is provided from the soil as mineralised N (SMN) and by applying N fertilisers. N supply in the soil depends on organic matter levels driving mineralisation and soil water governing drainage and leaching. Mineralisation depends on soil temperature. Soil water depends on rainfall, evaporation and drainage which is related to soil texture (Addiscott and Whitmore, 1987). There may be sufficient N supply in the soil but it needs to be taken up by the crop to be effective. N crop uptake depends on rooting depth, crop growth and mass flow, which is the process of transpiration by the leaves driving the movement of water in the soil towards the roots by creating a water gradient. Although there are many complex interactions that will influence N use efficiency, it is relatively straightforward for farmers to control N supply by the application of N fertilisers.

In contrast, it is much more difficult for farmers to enhance photosynthesis. Photosynthesis depends on sunlight to provide energy and the amount of chlorophyl in leaves to convert this energy for carbon assimilation. Higher photosynthesis rates are associated with larger leaf surfaces and higher chlorophyll content to better intercept light. Additional N supply has been shown to increase the amount, per leaf area, of chlorophyll and activities of enzymes involved with photosynthesis (Lawlor et al., 1987a) so in that aspect photosynthesis can be enhanced by increasing N supply. However, the amount of sunlight depends on the weather conditions during crop growth and is out of the farmer's control. Temperature also has an effect on photosynthesis (Lawlor et al., 1988): leaves stay green for longer at lower temperatures which enables them to continue carbon assimilation for longer. At higher temperatures, leaves will senesce sooner and N will be remobilised from the leaves to the grain. This mechanism explains also why it is very difficult to get both high yield and high protein because increasing yield and increasing protein are mutually exclusive processes: either leaves stay green for longer which enhances yield or leaves senesce resulting in N remobilisation into the grain and hence increased protein (Barraclough et al., 2010). Most indicators used to predict protein are based on the N status of the crop at the time of sampling and can determine whether there is sufficient N or not, but are unable to distinguish whether this will result in high yields or high protein. This is another disadvantage of the indicators currently being used.

2.3. A new method to predict grain protein content

In order to circumvent the problems associated with the current protein prediction indicators in use, a new method has been developed by Hill Court Farm Research, which is an environmental and agricultural research laboratory that also measures commercial samples for the farming industry to help farmers with their fertiliser decisions. The approach was to look at plant physiological processes that change in response to N supply. A first attempt was to look at the accumulation of sugars in leaves; when N is deficient, protein synthesis is hampered resulting in an increase of sugars like glucose and fructose in the leaves (Marschner, 2002). Although good results could be obtained for individual field experiments, the actual concentrations of sugars accumulated depended on the rate

of photosynthesis at the time of sampling. When all leaves were sampled at the same time during a single field experiment with various applications of N, a good relationship between N supply and sugar accumulation was observed. However, comparing different field experiments that had been sampled at different times and seasons, there was no consistency in the response and unfortunately this idea had to be abandoned.

It was therefore decided to look at N uptake in the roots. The reason for this is that roots are the gateway between N in the soil and N in the plant: a process that is tightly regulated. There are at least three different types of N transporters in the roots (Crawford and Glass, 1998): 1) a transporter with high affinity for N so that it is very efficient at extremely low soil N concentrations but with a low maximum uptake capacity. This transporter is always switched on, whether N is present in the soil or not; 2) a similar high-affinity transporter but this transporter becomes active only when N is present in the soil. Its maximum uptake capacity is still low but higher than the first one; and 3) a low-affinity transporter that operates at high N supply in the soil and which has a high N uptake capacity. This transporter contributes to the bulk of N uptake in crops commercially grown in the presence of fertiliser.

N transporters are not just regulated by N supply in the soil, but also by signals from within the plant. The exact nature of these signals is still unknown but has been suggested to include cycling of amino acids between shoots and roots (Plett et al., 2018). Regulatory signals can operate either through positive or negative feedback. Positive feedback is driven by demand: if there is a need for more N in the plant, transporters are upregulated to increase uptake. Negative feedback is driven by satiation: when there is sufficient N and demand has been satisfied, N transporters in the roots are downregulated and N uptake is reduced. It was shown that N uptake is mainly regulated by a negative feedback mechanism (Imsande and Touraine, 1994). By measuring these regulatory root signals, it was shown that they only appeared when the growth demand was satisfied and leaf N was above 4% (Blake-Kalff and Blake, 2022). Furthermore, by relating the root signals to protein content at harvest, it was possible to calculate an algorithm which could be used to predict protein levels and decide whether it was necessary to apply late N.

The aim of this study was to validate and extend previous findings in order to determine how well this new protein prediction method worked under different conditions. Key objectives were to determine:

1) Is the test effective in predicting protein on different sites and under various climatic conditions?
2) What sampling times give the best protein prediction?
3) What are the risk factors and cost benefits to farmers using the new method?
4) Is this method an improvement on current methods used by commercial farmers?

3. Materials and methods

3.1. Field Set Up

During 2019, 2020 and 2021, an identical field experiment was set up at 10 different sites across the country. On each side, 3 varieties of winter wheat (*Triticum aestivum*, v Zyatt, Skyfall & Siskin) were grown at 8 different N treatments as shown in Table 1. There were 3 replicates per treatment per site. The base rate of N fertiliser was determined each spring by measuring the soil Nitrogen Supply (SNS) using the CF Nmin test which comprises soil mineral N (SMN) and Additionally Available N (AAN): a measure of N that will become available through mineralisation and taken up by the crop during the growing season. An estimate of spring crop N was taken at the time of sampling by counting tiller numbers per m^2. Fertiliser applications for each site and dates of application are shown in Appendix 8.1. More details about the experimental setup have been described by Morris et al. (2022).

Table 1. Fertiliser Regime for each treatment.

Treatment	Nitrogen Fertiliser Regime	Extra N applied above Base Rate (kg/ha)
T1	No N applied	0
T2	Base rate N, enough to get maximum yield	0
T3	As T2, + 40 Kg N/ha as solid at GS 32	40
T4	As T2, + 40 Kg N/ha as solid at GS 37	40
T5	As T2, + 40 Kg N/ha as foliar at GS 70	40
T6	As T2, + 40 Kg N/ha as solid at GS 32, + 40 Kg N/ha as solid at GS 37	80
T7	As T2, + 40 Kg N/ha as solid at GS 37, + 40 kg N/ha as foliar at GS 70	80
T8	As T2, + 40 Kg N/ha as solid at GS 32, + 40 Kg N/ha as solid at GS 37, + 40 kg N/ha as foliar at GS 70	120

3.2. Weather Data

Daily rainfall, temperature and radiation were collected from the local weather stations of each site for the relevant time period. Thermal time was calculated by accumulative addition of the average daily temperature from the drilling date. Only days with an average temperature >0 ^{0}C were included. The different stages of wheat growth against thermal time were derived from the wheat growth guide (AHDB, 2021).

3.3. Crop Sampling

Crops were sampled at GS 32 (early stem extension), GS 39 (late stem extension) and GS 70 (milk development of the grain). Growth stages were determined according to Zadoks et al. (1974). Sampling dates for each site are shown in Appendix 8.2. At each sampling date, the soil around 5 plants per replicated plot was loosened with a fork and the whole plant including all tillers and crown roots was pulled up, put in a bag and sent to Hill Court Farm Research.

3.4. Processing of Roots

Plants were processed immediately upon arrival. Stems were cut about 2 cm above the crown roots and as much soil as possible was removed. The cut-off roots were slightly wetted, gently brushed and then all soil was washed off by holding them for about 20 seconds between two opposing high-pressure water jets. The rapidity of this process kept the contact with water to a minimum preventing osmosis of nutrients out of the roots. After cleaning, the roots were patted dry with kitchen paper, the last bit of stem was removed with scissors and the cleaned roots were dried O/N at 80 ^{0}C. Dried roots were milled to a fine powder in a Judge JEA86 Coffee Grinder.

3.5. Analyses

SMN and AAN were measured according to the CF Nmin® method (Patent GB2471288, 2012). Roots were extracted and analysed according to Blake-Kalff and Blake (2022). Protein N at harvest was determined by NIRS (Morris et al., 2022). Results were statistically analysed using Sigmaplot 14.5 or by using the Chi-square Test.

3.6. Crop N Uptake Calculations

Crop N at a specific thermal time was calculated using the following formula (Whitmore and Addiscott, 1987): $Y = (A^{1/n} + e^{-kx})^{-n}$, where Y = Crop N; A = Maximum Crop N, n = 1.5, k = rate constant which determines the shape of the curve and point of inflexion, and x = Thermal time. A was calculated as follows: A = (Grain Yield x DW) x grain N + (DW x Grain Yield/HI x (1-HI)) x straw N), where DW = 0.85; HI = Harvest Index at 0.51; grain N was measured; straw N was estimated at 0.3% for nil-N plots and 0.7% for fertilised plots. The rate constant k was determined by setting crop N uptake at 70% of A at GS 39 (AHDB, 2021). Crop N uptake rates for thermal time intervals were determined by (crop N_{end} – Crop N_{start})/interval days.

3.7. Calculations of Net Gain or Loss

The following parameters were used to calculate a net loss or gain following a decision based on the prediction. Cost of fertiliser: £2/kg N, so £80/ha for an application of 40 kg N. Cost of fertiliser spreading: £13.40/ha. Milling premium: protein >13%: £30/tonne, protein <13%: £0/tonne. Average

Wheat Yield: 9 t/ha. Average field: 10 ha. The loss/gain was calculated as Proportion of samples x Milling Premium x Fertiliser Cost (including spreading costs). Fertiliser Cost was negative when applied or positive when not applied. For example, where fertiliser was applied and 49% of samples achieved milling specification: 0.49 x (£270 - £93.40) = net gain of £86.53/ha and 0.51 x (£0 - £93.40) = - £47.63 loss where milling specification was not achieved. The cost of the test was not incorporated in the calculations because all samples were tested. However, the current price is £32 per field so works out as £3.20/ha.

4. Results

4.1. Weather data

To be able to compare the growth of wheat over 3 different seasons at 10 different sites, the rainfall and radiation were plotted against thermal time (Figure 2). The top panel was modified from the Wheat Growth Guide (AHDB, 2021) to show how thermal time related to certain stages of wheat development. The 3 solid vertical lines across all panels indicate the thermal time related to the start of ear formation, the start of flowering and the start of grain filling. The triangles indicate the fertiliser applications for each site in relation to thermal time. The stars indicate when the crops were sampled.

The length of the graph from left to right was an indication of the temperature during the growing season: the coldest years were the most contracted whereas the warmest years were the most extended. Similarly, the width of a month on the graph was a measure of its temperature with narrow months being cold and wide months being warm. For example, at Agrii 2021 April was very narrow, indicating it was very cold; thermal time increased by only 186 ^{o}C, which was equivalent to an average daily temperature of 6.2 ^{o}C. In comparison, in 2019, the average daily temperature at the Agrii site was 8.3 ^{o}C and in 2020 10.6 ^{o}C. Similarly, the narrow month of April at SRUC 2021 had a daily average temperature of only 4.7 ^{o}C, compared to 7.6 ^{o}C in 2019 and 7.9 ^{o}C in 2020.

The daily rainfall was indicated by the black peaks and troughs. There was a big difference in rainfall between years and sites and large variations between months. For example, at Agrii 2021, there was only 0.6 mm rainfall in April, but 71.7 mm fell in May. At SRUC 2021, only 13.5 mm rain fell in April, followed by 128.3 mm in May up to the 26th, followed by 15 days of no rain at all. In 2019, all sites experienced a dry April, but June was quite wet. In 2020, there was some rain in April but no rain at all in May at the Agrii site, whereas the SRUC site was very dry in April followed by average rainfall in May.

The radiation measurements gave an indication of sunlight availability during the growing season. Unfortunately, at the Agrii sites only a maximum radiation per day was recorded instead of total daily radiation like at the other sites. Also, the recorder broke down at the end of July 2019 and no data

were available for August. Measurements at SRUC 2019 seemed extremely low and were probably due to a recording error rather than being realistic. There was no consistency over the years in how the radiation was measured between different sites, some years it was in MJ/m_2 whereas in other years it was in W/m_2. Although this made it difficult for direct comparisons, it is still possible to draw conclusions from the changing patterns. For example, in 2021 there was a big drop in radiation at all sites compared to previous years during the grain filling period, particularly at NIAB Sutton Scotney.

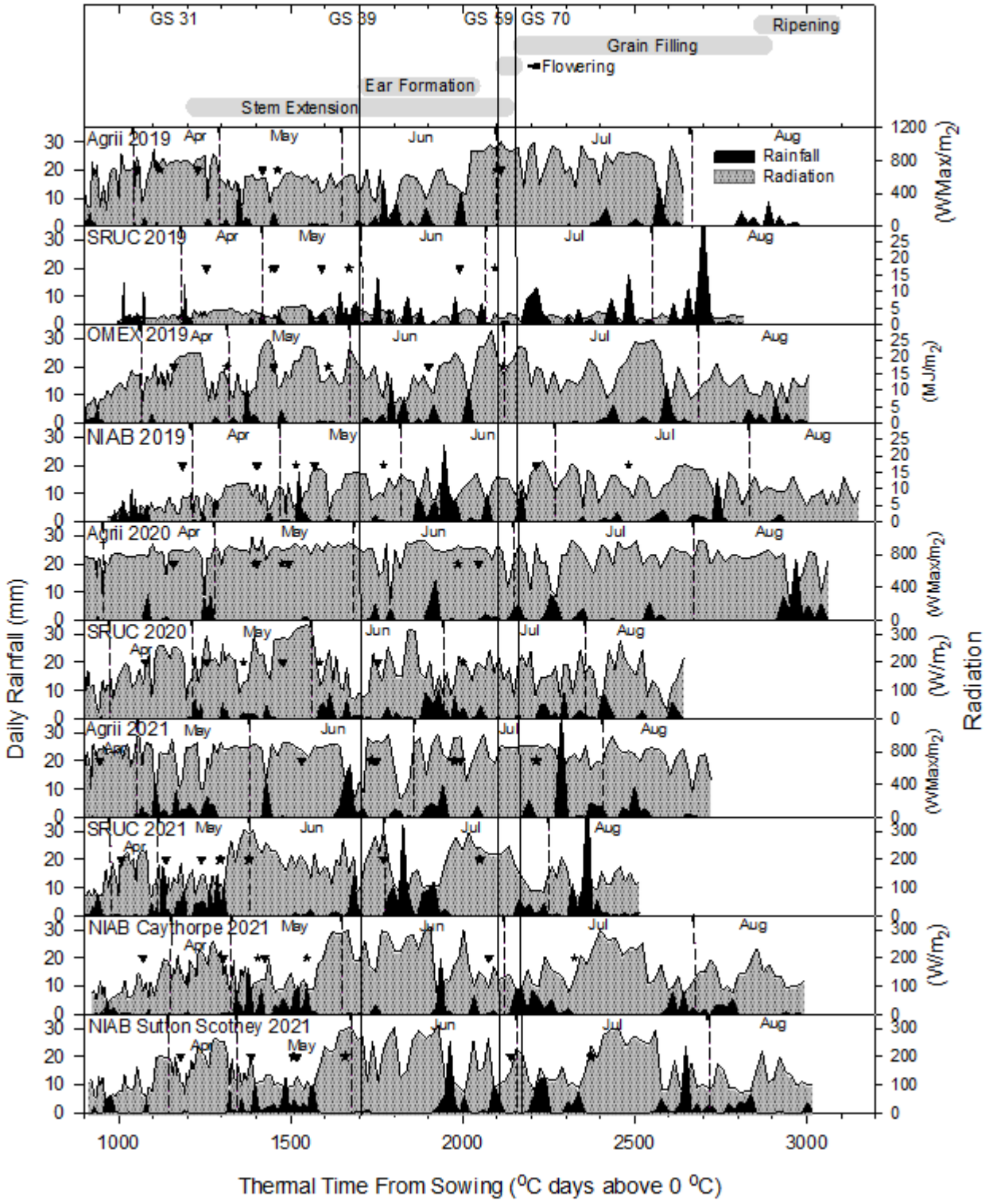

Figure 2: Daily rainfall (black areas) and radiation (grey areas) against thermal time for each site. (▼) Fertiliser Application; (★) Crop Sampling.

4.2. Root extracts in relation to Soil Nitrogen Supply

Root extracts increased with increasing soil and fertiliser N supply at all growth stages (Figure 3). Only three treatments were sampled at GS 32, which were T1) no N applied; T2) N applied to achieve yield and T3) an extra 40 kg N/ha applied on top of T2. Unfortunately, at SRUC 2019, Agrii 2019 and Agrii 2020 the plots were sampled before the T3 treatments was added so at these sites T2 and T3 received equal amounts of N. The highest extracts were measured at SRUC 2021, followed by NIAB Sutton Scotney 2021 and Agrii 2021, whereas the lowest extracts were measured on all 4 sites in 2019. At the lowest sites the extracts increased 3-7 times compared to the nil-N treatments whereas at the highest sites they increased 12 to 60 times compared to the nil-N treatment.

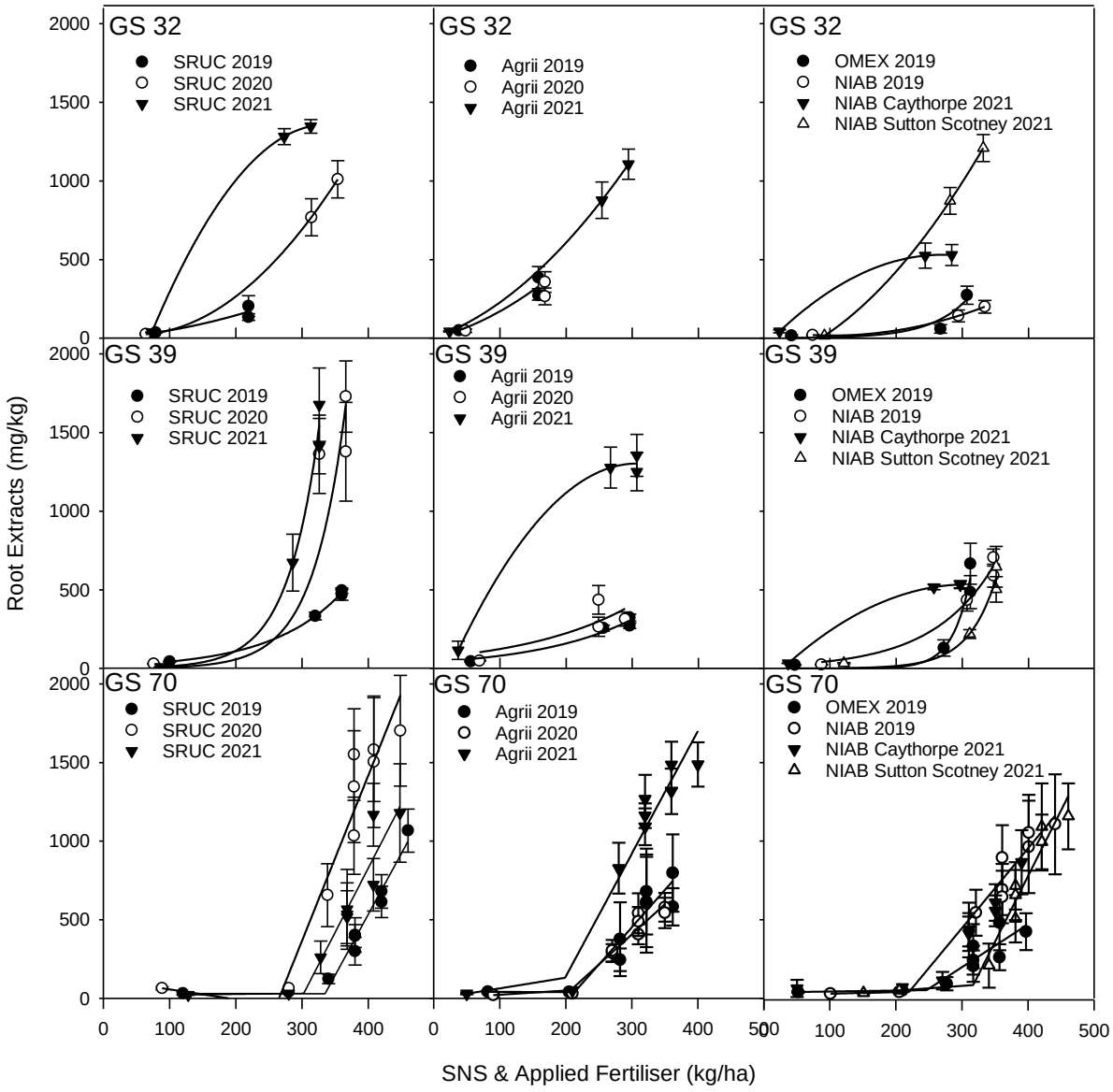

Figure 3: The relationship between root extracts and soil N supply plus applied fertiliser for each site sampled at either GS 32, GS 39, or GS 70.

At GS 39, apart from the 3 treatments mentioned for GS32, a fourth treatment (T4) was sampled which was the same as T3, but with the extra 40 kg N/ha applied at GS 37 instead of GS 31. The interval between adding T4 and sampling the crop varied between sites from 4 days at Agrii 2019 to 16 days at NIAB 2019. The increase in root extracts compared to Nil-N varied between 5 to 75-fold for T2, 6 to 157-fold for T3 and 3 to 8-fold for T4. The lowest increases were measured at SRUC 2019, Agrii 2019 & Agrii 2020 whereas the highest increases were measured at SRUC 2020, SRUC 2021 & Agrii 2021.

AT GS 70, all 8 treatments were sampled which gave a better range of SNS plus applied fertiliser from which to deduct a curve. At all sites, the extracts were lowest at T1 and T2, after which there was a linear increase with increasing N supply. The slopes of the linear increase varied between sites and could be separated into 3 different groups; 1) At Agrii 2019, OMEX 2019, NIAB 2019 & SRUC 2020, the slope was around 2 mg root extract/kg N; 2) At Agrii 2020, Agrii 2021 & NIAB Caythorpe 2021, the slope was around 4 mg root extract/kg N; and 3) At SRUC 2019, SRUC 2021 & NIAB Sutton Scotney 2021, the slope was around 8 mg root extract/kg N. The increase in root extracts compared to Nil-N varied between 2 to 28-fold for T2, 5 to 43-fold for T3, T4 & T5, 6 to 52-fold for T6 & T7 and 10 to 53-fold for T8.

The timing of fertiliser application differed between sites and years, so the effect of the length of interval between fertiliser application and crop sampling on the amount of root extracts was determined for T3 and T4 at GS 39 (Figure 4a). The fertiliser application for both T3 and T4 was 40 kg/ha but the timing of the application differed by about 14 days. The specific effect of the application of 40 kg/ha was measured by determining the difference in root extract amounts at T3 or T4 to those at T2 which did not receive 40 kg N/ha. When the interval between application and sampling was below 14 days at T4, there was a linear relationship between length of the interval and change in root extracts in 7 of the 9 sites with R^2 of 0.81. The two outlier sites were Agrii 2021, which was a very backward crop sampled 4-6 weeks later than the other sites, and NIAB 2019 which had the longest interval at 16 days. There was no T4 measurement for Agrii 2019 because the fertiliser was applied after the crop had been sampled. When the interval was longer than 14 days, in 9 out of 10 sites after T3 application there was no relationship between change in root extracts and length of the interval (R^2 = 0.001). The one outlier was Agrii 2021 which had an interval of only 6 days, which appeared to fit in with the T4 regression line.

It was possible to model N uptake in relation to thermal time using the equation proposed by Whitmore and Addiscott (1987) and hence determine a daily N uptake rate. There was a good

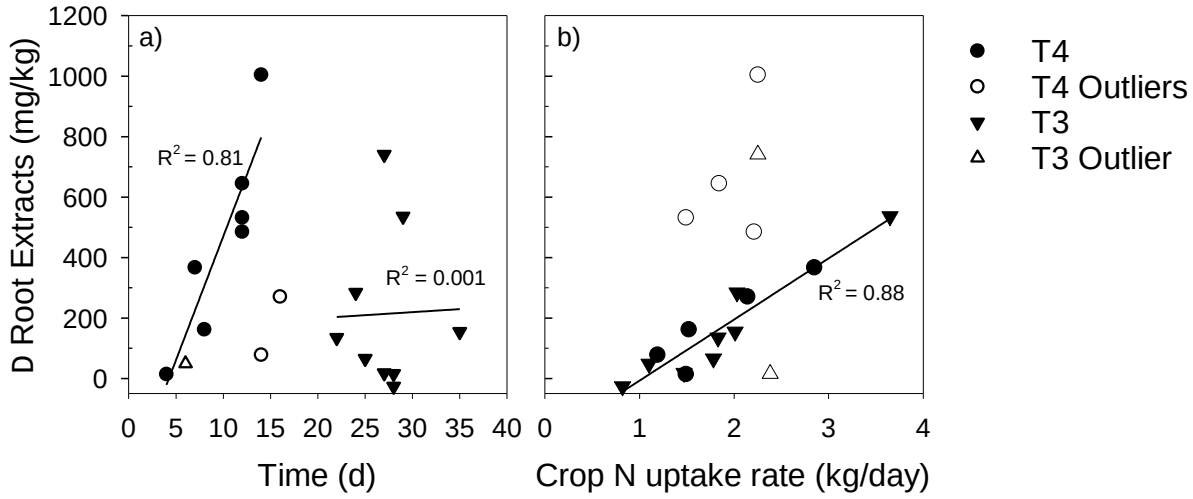

Figure 4: a) The relationship between the length of interval from fertiliser application to sampling against the difference in root extracts between T3 or T4 and T2. b) The relationship between daily crop N uptake rates and the difference in root extracts between T3 or T4 and T2.

relationship between crop N uptake rate and the increase in root extracts at T3 compared to T2 (R^2 = 0.90) in 8 out of the 10 sites, with SRUC20 and SRUC21 as outliers (Figure 4b). Combining T3 & T4 showed a good relationship in 12 out of 18 sites (R^2 = 0.88), with SRUC 20 (T3), SRUC 21 (T3&T4), NIAB Caythorpe 21 (T4), NIAB Sutton Scotney 21 (T4) and OMEX 19 (T4) as outliers.

4.3. Protein Prediction

The root extracts were used to calculate a protein prediction as described by Blake-Kalff and Blake (2022). There was a significant relationship ($p<0.0001$) between the predicted protein and grain protein content measured at harvest at all growth stages (Figure 5). In 2019, the standard errors of the fitted curve for GS 32, GS 39 and GS 70 were 1.45, 1.13 and 1.02 respectively. The large scatter at GS 32 was partly caused by contamination of the roots with soil which skewed the results. This was solved once the cleaning procedure of the roots was improved during 2019. The prediction was furthest away from the 1:1 line at protein predictions < 11%, particularly at the nil-N treatment. At GS 32, the prediction was underestimated at the NIAB and OMEX sites. At GS 39, the trend line was close to the 1:1 line. The prediction was underestimated at the NIAB site. At GS 70, again the trend line was very close to the 1:1 line. Generally, the fit improved with higher protein predictions although there was a lot of scatter in the data.

In 2020, the standard errors for the fitted curves were 0.75, 0.70 and 0.61 for GS 32, GS 39 and GS 70 respectively. These were nearly half compared to 2019, but that was probably partly due to the fact that the number of sites were halved as well. The protein levels at the SRUC site were at all

growth stages predicted to be higher than those at the Agrii site, which was confirmed by the grain protein content measured at harvest. At both sites, the protein prediction was slightly overestimated compared to grain protein.

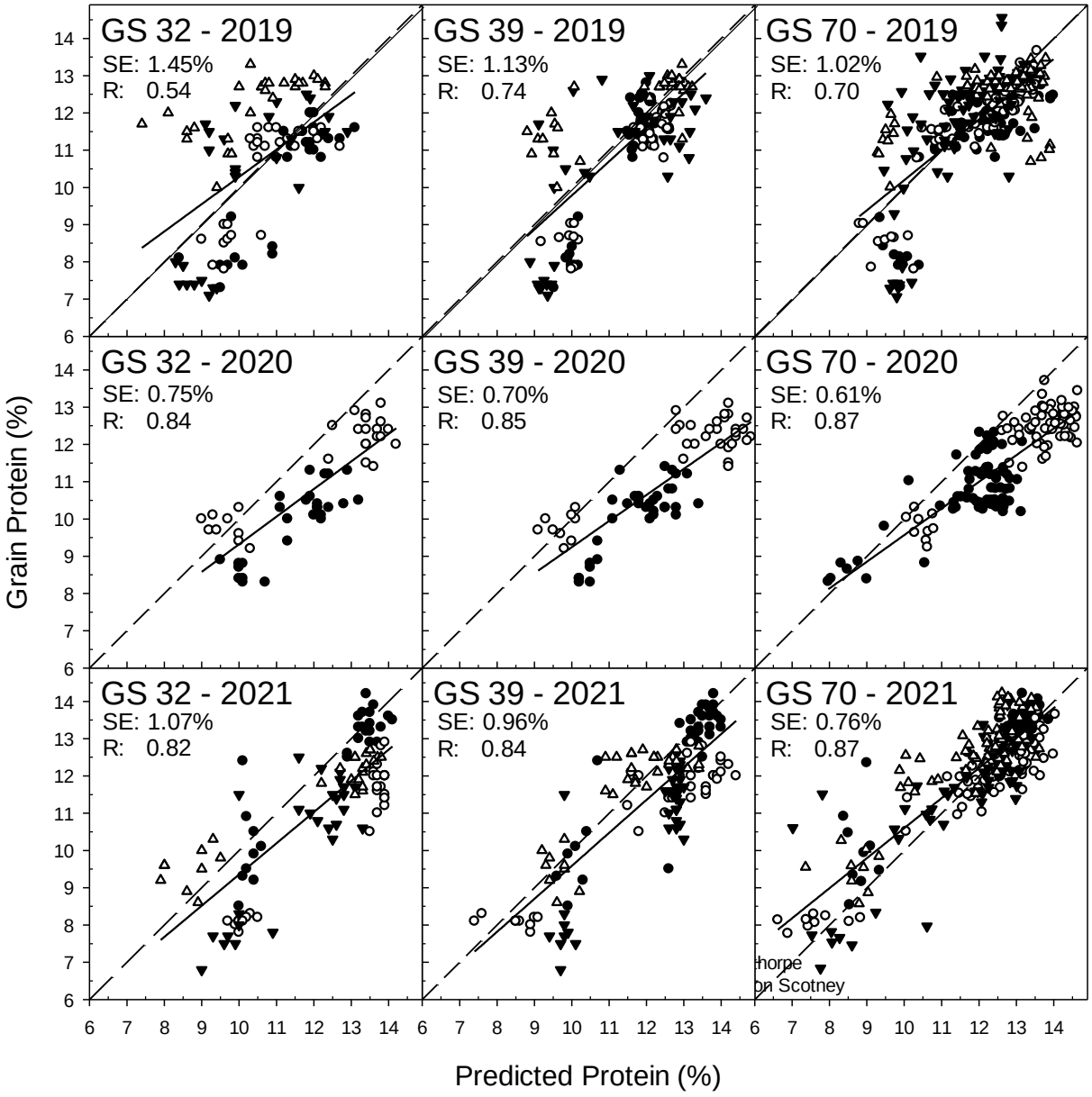

Figure 5: The relationship between protein predicted from root extracts at GS 32, GS 39 & GS 70 and grain protein content at harvest for 10 different sites harvested in 2019, 2020 & 2021. The 1:1 line is represented by the dotted line. SE = Standard Error, R = Correlation Efficient, p<0.0001

In 2021, the standard errors of the fitted curves were 1.07, 0.96 and 0.76 for GS 32, GS 39 and GS 70 respectively. At GS 32, the protein prediction was slightly overestimated at SRUC and NIAB Caythorpe sites. At GS 39, the fitted curve was close to the 1:1 line, particularly at higher protein predictions. The protein prediction was slightly underestimated at NIAB Sutton Scotney and slightly

overestimated at NIAB Caythorpe and SRUC. The protein prediction at the Agrii site very tightly followed the 1:1 line. At GS 70, the trend line converged towards the 1:1 line at higher protein levels, particularly at protein predictions >12%. Overall, there was a better protein prediction the later the plots were sampled within the season. Also, there was an improvement between seasons with better protein predictions in later years. The latter was probably due to the development of methods throughout the project and better sampling techniques.

4.4. Cost Benefit assessment of the protein prediction test

The trial setup gave an opportunity to compare the prediction against different scenarios and then to calculate a probability for each outcome as well as the cost gain or loss for each outcome. The protein prediction test has two outcomes: 1) the result is <13% at which the advice to farmers would be to apply extra N to achieve milling specification; or 2) the result is ≥13% at which the advice would be not to apply more N because milling specification should be achieved without. By comparing the actual protein at harvest from different treatments, it was possible to disentangle what would have happened if the farmer chose to follow the advice or decided to ignore it. For example, at GS 32 the prediction of T2 (fertilised for yield) and the actual protein of T2 represented the scenario that the farmer did not apply extra N, whereas the actual protein achieved in T5 (foliar application of 40 kg N) represented the scenario that the farmer applied late N. Similarly, we could compare harvest protein levels from T3 (+ 40 kg) as the No extra N scenario to T7 (+ 40 kg N + 40 kg foliar N) as the extra N scenario. To calculate monetary gain or loss, fertiliser was assumed to be £2/kg N, spreading costs were estimated at £13.40/ha and the milling premium at £30.00. The loss of premium was based on a worst-case scenario of losing all of it when protein was <13%. In reality, the milling premium decreases between 12.9% and 12.3% depending on the availability of high specification milling wheat. When excess N was applied to the samples where the protein prediction was ≥13%, only 49% of the samples achieved milling specification and it was therefore concluded that in 51% of the samples N was not the limiting factor for achieving protein >13%.

The majority of samples were predicted to be below 13% protein and this proportion increased from 61.6% at GS 32, to 69.5% at GS 39 and 71.4% at GS 70. This was opposite to the actual protein measured at harvest, where the proportion of samples <13% decreased from 78.8% at GS 32, to 76.8% at GS 39 and 74.5% at GS 70. At GS 32 there appeared to be an overestimation of the proportion of samples achieving protein <13%, but by GS 70 the predicted and the actual proportions were quite similar.

When the protein prediction was <13%, the recommendation to farmers would be to apply late N fertiliser. If this advice was ignored, only a small proportion of samples achieved actual protein levels ≥13% (Figure 6). This proportion was similar regardless of the time of sampling with only 5%,4% and 3% of the samples at GS 32, GS 39 and GS 70 achieving ≥13% protein, respectively.

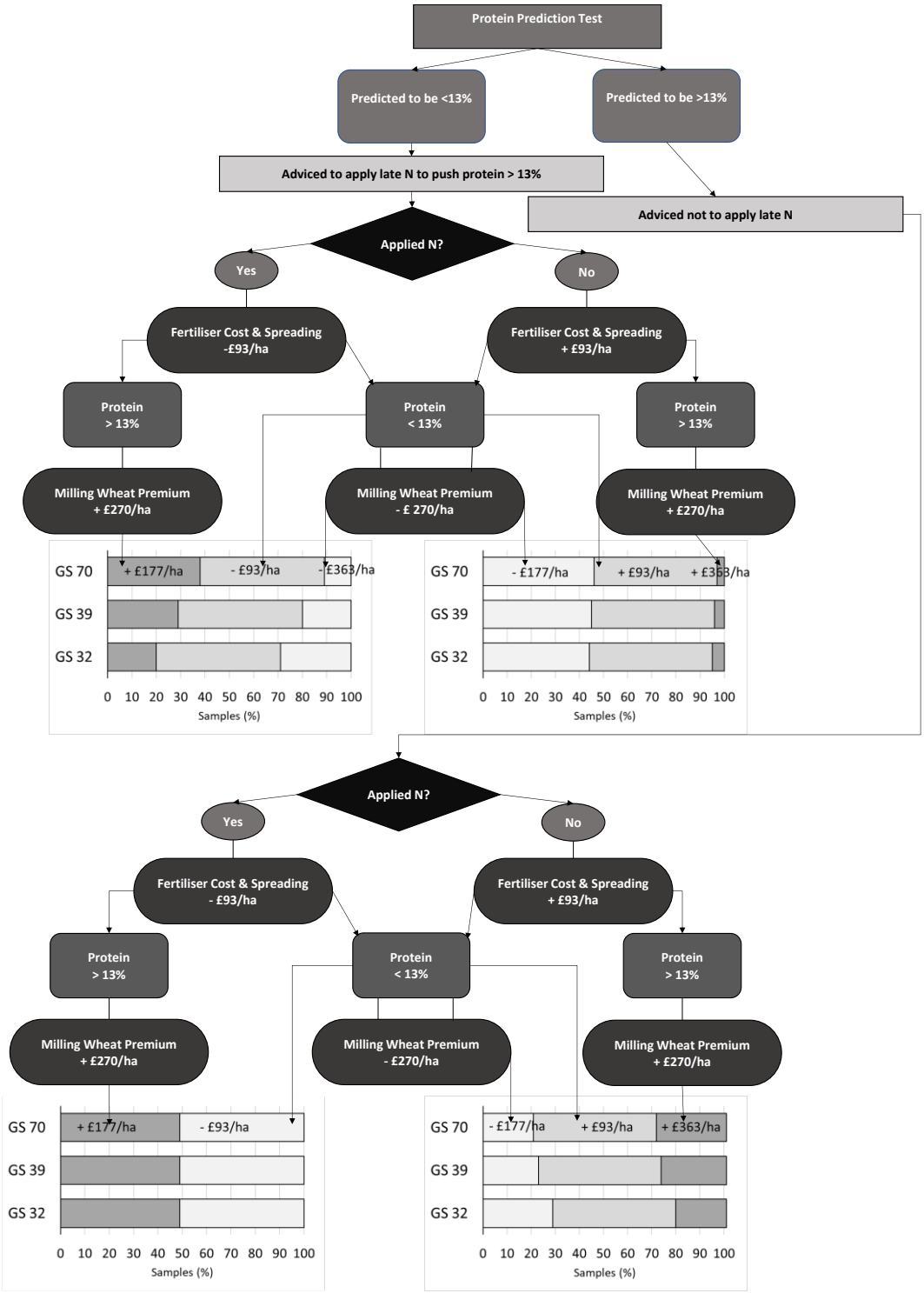

Figure 6. Flow chart describing possible scenarios and net loss/gain following a protein prediction test taken at GS 32, GS 39 and GS 70.

Although there was a large cost benefit of £363/ha where protein was achieved ≥13%, the chances of it happening was only between 3 to 5 fields out of 100. In contrast, taking into account that 51% of the samples would not have gained milling premium regardless of how much N had been applied, the chance of losing £177/ha was very high with 44 - 46 fields out of 100 being at risk.

It was significantly ($p<0.001$) better to follow the advice of applying late N when the prediction was <13%. The proportion of samples achieving milling specification increased from 20% at GS 32, to 29% at GS 39 and 38% at GS 70. Compared to not applying late N, this represents an increase of 4 to 12.5x in the number of fields achieving protein ≥13% with an associated cost benefit of £177/ha. There was still a substantial probability that protein levels would not be achieved, which decreased when the protein prediction test was taken later in the season: at GS 32, 29 out of 100 fields were at risk of losing £363/ha; at GS 39, it was 20 out of 100 fields and at GS 70 it was 11 out of 100 fields. The other 51 out of 100 fields lost £93/ha on applying fertiliser that was wasted.

When the test predicted protein levels ≥13%, farmers were advised not to apply late N as it would not be necessary. If this advice was ignored and late N was applied, only 49% of field achieved milling specification and 51% did not. Applying N when it was not needed roughly gave a 50:50 chance of either gaining £177/ha if milling specification was achieved or otherwise losing £93/ha when milling specification was not reached. It was significantly ($P<0.001$) better to adhere to the advice and not apply late N when the test results predicted protein to be ≥13%. This was because the protein prediction test was able to pinpoint those samples where N was sufficient and not the cause of low protein levels. In this scenario applying more N would be wasteful and without the desired effect of lifting the protein content above 13%. So, in 51% of the samples, the milling premium would not be reached but £93/ha was gained by not wasting fertiliser. A significant proportion of samples achieved protein levels >13% without adding late N, gaining £363/ha. This proportion increased with later sampling times: from 20% at GS 32, to 27% at GS 39 and 29% at GS 70. Overall, by following the advice of the test 71, 78 and 80 out of 100 fields sampled at GS 32, GS 39 and GS 70, respectively, achieved a cost benefit: a substantial risk reduction compared to ignoring the advice and always applying N as an insurance.

It is important to understand why the protein prediction was overestimated in a small proportion of samples where the application of late N would have achieved milling specification. At GS 32, 15 out of the 21 samples where this occurred came from NIAB Sutton Scotney 2021. At this site, there was no rain after fertiliser was applied in April followed by a lot of rain after fertiliser was applied in May. Out of the remaining 5 samples, 4 samples achieved protein ≥12.7% so would still have qualified for a partial premium. At GS 39, 7 out of 20 samples where the protein prediction was overestimated were from SRUC 2020. This site had no rain at all in April, followed by some rain in May. It rained on the day of sampling. The spring was cold and radiation was lower during July compared to 2021

(Figure 2). There was no rain after the T4 application until the day of sampling which affected 4 out of the 7 samples that were overestimated. Overall, out of the 20 samples 35% achieved protein levels ≥12.8% and 55% made ≥12.5% protein. At GS 70, 6 out of 20 samples where the prediction was overestimated came from NIAB 2019 and 6 from SRUC 2020. As mentioned above, at SRUC 2020 there was a period of lower radiation in July. At NIAB 2019, there was a lot of rain during June but a spell of drought after the foliar application. Overall, out of 19 samples that did not make milling specification, 37% achieved protein ≥12.8% and 68% ≥12.5%.

5. Discussion

5.1. Improvement on current indicators

There was an important difference in the response of root extracts to N supply compared to measures currently being used like the analysis of total N in flag leaf or taking SPAD readings using a chlorophyll meter. Whilst all three methods show an increase with increasing N supply, the leaf N or SPAD rises quickly from nil N and then reaches a plateau at N supply concentrations over 200 kg N/ha (Lopez-Bellido et al., 2004). Given that milling wheat requires around 280 kg N/ ha for yield plus potentially another 40 kg N/ha to boost protein (AHDB, 2021), the ranges of N supply at which leaf N or SPAD are measured for protein prediction fall at this plateau. For example, Lopez-Bellido et al. (2004) showed that SPAD readings only increased from 45 at 200 kg N/ha to 50 at 300 kg N/ha: an increase of only 10%. Similarly, in a classic N response curve shown by Yara (2005), the total N in the flag leaves increased from 4.3% to 4.5% in the 200 – 300 kg N/ha range: a 5% increase which is barely discernible from normal field variation. In contrast, the root extracts were hardly measurable at low concentrations, but then increased linearly with increasing N supply. Therefore, there was a clear difference between a supply of 200 kg N/ha with typical values around 50 – 100 mg/kg root extract and a supply of 300 kg N/ha with typical values between 1000 – 2000 mg/kg root extract. This massively improved the sensitivity of the protein prediction and reduced the impact of in-field variation.

5.2. Best Sampling Times

The variation in root extracts at different sites and years in response to N supply appeared to reflect the complexity of N uptake. This was not just governed by the amount of N in the soil or added as fertiliser but also by other factors like the dissolution of fertiliser, the movement of N through the soil and uptake into the crop. The timing of the sampling of root extracts to predict protein at harvest was therefore critical to the success of the prediction. Particularly at GS 32, there were too many other factors interfering due to the long time between sampling and harvest. Overall, however, the three main reasons for an inaccurate protein prediction were: 1) sampling during a drought; 2) sampling too soon after the main fertiliser application; or 3) sampling too soon after rainfall following a period of drought.

5.2.1. Sampling during a period of drought

Rainfall patterns in the UK appear to have changed due to climate change, with an increase in the prevalence of dry periods during springtime. In 2019, there was little rain between mid-March to the end of April which coincided with the lowest root extracts at GS 32 compared to the other years. In 2020, in the South of the UK, there was no rainfall during May and the root extracts at the Agrii site, located in the South East, were very low, particularly at GS 39. In contrast, the highest root extracts were found in 2021, when the spring was quite wet with a considerable amount of rainfall during May. It is unlikely that lack of rain hampered the dissolution of ammonium nitrate fertiliser, as even at soil moisture as low as 7% applied fertiliser dissolves within 3 hours (Oldham et al., 1997). However, it has been shown that the mobility of ions is greatly reduced when the soil dries out (Day et al., 1978). Particularly, N uptake in the crop is affected since the main mechanism for delivering N from the soil to the root surfaces is mass flow, which is the movement of dissolved nutrients into the crop as the plant absorbs water by transpiration. Mass flow decreases rapidly when soil moisture is reduced (Barraclough, 1986a). The low root extracts accurately reflected the low N uptake at the time of sampling due to lack of water, but it was not possible to correctly predict the harvest protein based on these results because the situation changed as soon as it rained after sampling.

5.2.2. Sampling too soon after applying fertiliser

The root extracts increased linearly with time for about 12 days after application of fertilisers. The application of fertiliser has been shown to increase the nitrate concentration in soil solution peaking after about 7 days, before returning to a steady-state equilibrium at about 14 days (Barraclough, 1986b). Sampling the crops within this timeframe appeared to overestimate the protein prediction, especially when there was sufficient rainfall to facilitate N uptake. Once the soil nitrate solution returned to an equilibrium, which was at a higher level after fertiliser application than before, the roots extracts were linearly related to the daily crop N uptake rate. This is probably not surprising as the roots facilitate N uptake into the rest of the plant, but it shows that the root extracts were a good indicator of the N status of the plant at the time of sampling.

5.2.3. Sampling too soon after rainfall following a period of drought

Once it rained after a period of drought, there could be a sudden increase in N in the soil solution which caused an overestimation of the protein prediction. For example, at GS 32, most of the overestimated samples came from one site: NIAB Sutton Scotney 2021. At this site, the rainfall in April after the 2nd fertiliser application of 80 kg N/ha was only 0.67 mm/day and it is likely that there was little crop N uptake so most of that application remained in the soil. Then after the 3rd fertiliser application of either 50 (T2) or 90 kg N/ha (T3), rainfall increased 6-fold to 3.87 mm/day. Probably this caused a flush of N uptake into the crop not just of the 3rd application but also of the remainder of the 2nd fertiliser application that was still left in the soil, resulting in high root extracts. Although this correctly reflected the N status of the crop at the time of sampling, it caused an overestimation of the

prediction. At GS 39 and GS 70, the sudden flush of N uptake had returned to equilibrium and the prediction no longer suggested protein levels >13% would be achieved without extra N. The lesson learnt from this was that after a prolonged dry period followed by substantial rainfall, there should be at least 14 days between fertiliser application and crop sampling, but preferably 21 days if possible.

5.2.4. Best sampling time for optimised protein prediction

It was clear from the results that the later the crop was sampled, the better the prediction. This confirmed similar observations by Lopez-Bellido et al. (2004), who stated that the closer a prognostic test is performed in relation to the event to be predicted, the greater the predictive power of the test. The best predictions were achieved at GS 70, at which it is still practically possible to apply late foliar N. However, this is too late for applying solid fertiliser. So, if solid fertiliser is used as the late N application, it is important not to be too early as the result will be overestimated as stated in section 5.2.2 and not too late as lack of moisture might prevent it from being taken up. The best sampling time using solid fertiliser is therefore the last week of May or the first week of June. The best sampling time using foliar N is from the first of June onwards. The downside of measuring SPAD readings late in the season is leaf senescence (Lopez-Bellido et al., 2004), which increases the variability of the results. Root extracts should not be affected by this and therefore can be sampled later than SPAD readings.

5.3. Accuracy of the protein prediction

Protein synthesis in grain depends not only on N supply but also on photosynthesis to provide the carbon skeletons for amino acids as well as the energy to drive the assimilation processes (Lawlor et al., 1987b). Therefore, when protein levels at harvest are below 13%, it is not known whether this is due to lack of N supply or due to inadequate photosynthesis. Because protein prediction is influenced only by N status of the crop, but harvest grain protein by N status *and* photosynthesis, it is not possible to get a perfect 1:1 relationship between the two. Despite that, there was a clear relationship between predicted and harvested protein, with a much tighter curve, compared to when protein N was predicted by NIR measurement of ears at the milky ripe stage (Weightman et al., 2011). The protein prediction test is designed to work best at protein levels >12%, since that is the range where most farmers growing milling wheat commercially will start from. At lower levels there may be more variation, usually for reasons explained in section 5.2, but it doesn't matter whether the protein prediction is 9% or 11%; both would get the same advice to apply late N.

Currently the default setting for milling wheat farmers is to apply extra N late in the season as an insurance to achieve milling specification, whether it is needed or not. The results showed that at best, only half the samples achieved milling specification, but also that half did not and lost money on fertiliser as a result. The 3-fold increase in fertiliser prices since 2021 make this policy of applying N as a routine considerably less cost effective. By using the protein prediction test, it was easier for

farmers to make the right decision. When the advice from the test was to apply fertiliser, only a very small percentage (<5%) achieved milling specification without extra N suggesting the test was correct in 95% of the samples. Reasons for a low prediction, but still making milling specification without N, could be drought at the time of sampling which restricted mass flow and reduced crop N uptake (Barraclough, 1986b) followed by rainfall after sampling. Other reasons could be late mineralisation of N from organic matter, particularly when the spring was colder than usual like in 2021.

When the advice from the test was not to apply late N, this decision was correct in nearly 80% of samples; a significant improvement on only getting it right in 50% of the samples where late N was applied as routine. This did not mean milling specification was always achieved, because meteorological conditions such as temperature, sunshine and rainfall during the grain filling period have a massive impact on protein content (Pan et al 2006). However, in those cases it was clear from the protein prediction test that N was not the limiting factor and that adding more N would be a waste of money. It will always remain a gamble because farmers cannot control the weather, but the odds using the test become more favourable. Furthermore, reducing the applications of late N where the test predicts it is not necessary to do so, will have a positive effect on the environment with less N leaching and less emissions.

5.4. Future R&D

Measuring root extracts is an accurate method to assess the N status of the crop at the time of sampling. This can be used throughout the growing season to study the effects of certain climatic conditions on N supply and uptake into the plant. Also, it will enable to distinguish the effects of N supply on protein synthesis from the effects of other climatic conditions, thus gaining a better understanding of which conditions facilitate high protein content in grain. The protein prediction test can be improved by introducing a modelling factor taking into account 1) amount of applied fertiliser, 2) length of interval between fertiliser application and sampling, 3) specific weather conditions during the interval like rainfall and sunlight.

5.5. Conclusion

In conclusion, root extracts were a more sensitive and therefore a better indicator of N status of the crops than SPAD readings, NIRs or measuring total N in flag leaves. Measuring root extracts appeared to give a reasonable prediction of protein as long as the roots were sampled between GS 39 and GS 70, not too close to the main fertiliser applications and not during periods of drought. Overall, the protein prediction test was shown to be an effective tool to aid farmers in their decision processes on whether to apply late N or not. Reducing fertiliser use has positive effects on the environment by reducing leaching and CO_2 emissions and the protein prediction test can play a key role in this.

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8. Appendix

8.1. Fertiliser Applications per site (amounts & dates)

Year	Site	SNS (kg/ha)	Fertiliser Application (kg/ha)					
			A	**B**	**C1**	**C2**	**D**	**E**
2019	Agrii	74	60	60	80	120	40	40
2019	SRUC	120	60	95	70	110	40	40
2019	OMEX	53	60	80	80	80	40	40
2019	NIAB	100	60	80	80	120	40	40
2020	Agrii	90	60	60	60	100	40	40
2020	SRUC	89	60	80	110	150	40	40
2021	Agrii	50	60	80	90	130	40	40
2021	SRUC	98	60	80	60	100	40	40
2021	NIAB Caythorpe	51	60	80	80	120	40	40
2021	NIAB Sutton Scotney	151	60	80	50	90	40	40

Explanation of Codes:

A = 1st fertiliser application

B = 2nd fertiliser application

C1 = 3rd fertiliser application at GS 32 (standard amount)

C2 = 3rd fertiliser application at GS 32 (standard amount + 40 kg N/ha)

D = 3rd or 4th fertiliser application at GS 37

E = Foliar fertiliser application at GS 70

Year	Site	Date of Fertiliser Application				
		A	**B**	**C**	**D**	**E**
2019	Agrii	27/02/2019	04/04/2019	24/04/2019	15/05/2019	02/07/2019
2019	SRUC	18/03/2019	16/04/2019	07/05/2019	21/05/2019	26/06/2019
2019	OMEX	NA	17/04/2019	17/04/2019	16/05/2020	19/06/2019
2019	NIAB	18/03/2019	29/03/2019	24/04/2019	13/05/2019	28/06/2019
2020	Agrii	09/03/2020	20/04/2020	13/05/2020	20/05/2020	25/06/2020
2020	SRUC	23/03/2020	14/04/2020	06/05/2020	27/05/2020	18/06/2020
2021	Agrii	08/03/2021	16/04/2021	10/06/2021	24/06/2021	09/07/2021
2021	SRUC	17/03/2021	13/04/2021	05/05/2021	18/05/2021	01/07/2021
2021	NIAB Caythorpe	05/03/2021	24/03/2021	27/04/2021	12/05/2021	28/06/2021
2021	NIAB Sutton Scotney	05/03/2021	08/04/2021	07/05/2021	19/05/2021	30/06/2021

8.2. Sampling Dates

Year	Site	Sampling Date		
		GS 32 (S1)	**GS 39 (S2)**	**GS 70 (S3)**
2019	Agrii	15/04/2019	19/05/2019	01/07/2019
2019	SRUC	01/05/2019	28/05/2019	01/07/2019
2019	OMEX	06/05/2019	29/05/2019	03/07/2019
2019	NIAB	07/05/2019	29/05/2019	14/07/2019
2020	Agrii	12/05/2020	19/05/2020	22/06/2020
2020	SRUC	18/05/2020	03/06/2020	06/07/2020
2021	Agrii	23/06/2021	08/07/2021	21/07/2021
2021	SRUC	25/05/2021	01/06/2021	19/07/2021
2021	NIAB Caythorpe	10/05/2021	24/05/2021	13/07/2021
2021	NIAB Sutton Scotney	18/05/2021	31/05/2021	14/07/2021