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Metabolic fingerprinting for bio-indication of nitrogen responses in *Calluna vulgaris* heath communities

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Increased atmospheric deposition of nitrogen (N) over the last 50 years is known to have led to deleterious effects on the health of *Calluna vulgaris* heathland, with increased proliferation of grasses and loss of species diversity. However, currently it is difficult to attribute damage specifically to N deposition rather than other drivers of change such as inappropriate management. Metabolic fingerprinting using FT-IR offers a rapid, cost-effective and "holistic" means for quantifying foliar biochemistry responses specifically to N deposition. To test the potential of this approach we used a long term lowland heath N addition study in Cheshire, England. FT-IR spectra of treated *C. vulgaris* shoot material showed that responses were detectable above 20 kg N ha^{-1} $year^{-1}$. Differentiation was also evident in *C. vulgaris* metabolic fingerprints due to additional watering. We have shown that FT-IR is able to identify biochemical variations in *C. vulgaris* related to increases in received N and water. This technique therefore provides a sensitive measure of biochemical change in response to N addition, and allows development towards predictive modelling of N deposition at the landscape level.

KEY WORDS: nitrogen; *Calluna vulgaris*; bio-indication; metabolic fingerprinting; FT-IR.

1. Introduction

Over the last few decades there has been great global ecological concern over the high levels of nitrogen (N) deposition, and expected future increases thereof (Tilman *et al*., 2001), arising from anthropogenic emissions (Vitousek, 1994; Vitousek *et al*., 1997). The eutrophic effect of N deposition is known to have detrimental impacts on a range of terrestrial ecosystems (Fowler *et al*., 1989; Pietila *et al*., 1991; Soares and Pearson, 1997; Bobbink, 1998; Stevens *et al*., 2004), including semi-natural systems such as *Calluna vulgaris* (L.) Hull heaths (Caporn *et al*., 1994; Pitcairn *et al*., 1998; Carroll *et al*., 1999). Heathland communities have been seen to decline across Europe and the UK at a rate that cannot be explained by changes in land use alone (Heil and Diemont, 1983; Pitcairn and Fowler, 1995). Observed deleterious effects due to N include changes in competitive balance (e.g. increases in grasses) and increased vulnerability of *C. vulgaris* to environmental stresses such as frost, winter damage (Power *et al*., 1998; Carroll *et al*., 1999), herbivory (Wilson, 2003) and drought (Cawley, 2000). In many cases these effects show a strong interaction with plant tissue N content. Changes in management may offer a means by which to mitigate N influenced heathland loss (Power *et al*., 1998; Barker *et al*., 2004), but such a strategy would require identifying ecosystems at risk prior to damage. Thus, significant investigation into using *C. vulgaris* shoot biochemistry responses for the measurement of N deposition has been provoked (Pitcairn and Fowler, 1995; Hicks *et al*., 2000; Gidman *et al*., 2004). Previous explorations into developing bio-indicators for N deposition using foliar biochemistry responses have concentrated on using "classic" techniques, such as foliar amino acid (Huhn and Schulz, 1996) and, in *C. vulgaris*, total tissue N (Pitcairn and Fowler, 1995; Hicks *et al*., 2000) responses. However, these approaches are constrained by time, expense (Huhn and Schulz, 1996; Pitcairn *et al*., 2003) and are relatively insensitive and therefore ineffective for measuring low N deposition. This is particularly important as the critical load threshold for *C. vulgaris* heathland is in the region of 10–20 kg N ha^{-1} $year^{-1}$ (Bobbink *et al*., 1996). The critical loads approach (Nilsson and Grennfelt, 1998) estimates levels of exposure to a pollutant above which ecosystem health suffers

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(Nilsson and Grennfelt, 1998; Løkke *et al*., 2000). In addition, complications in measuring N deposition creates difficulties in determining whether a particular ecosystem is receiving N above a threshold level (Skeffington, 1999).

A more recent approach for attacking these problems has been through the application of metabolic fingerprinting, being essentially a study of global shoot chemistry (Fiehn, 2001; Gidman *et al*., 2003), to determine N deposition in *C. vulgaris* (Gidman *et al*., 2004). Here detectable differences in the metabolome of *C. vulgaris* were observed to occur due to simulated N depositions of between 8 and 16 kg N ha^{-1} $year^{-1}$ in open top chambers (OTC) (Gidman *et al*., 2004). This approach was also seen to combine the advantages of rapidity, cost effectiveness and in combination with suitable chemometrics (Goodacre *et al*., 2004) also allows an interrogation of the entire foliar metabolic pool.

Among the various techniques available for metabolic fingerprinting FT-IR can be considered the most rapid (Ellis *et al*., 2003) and this has already proved tractable in the studies of plant metabolic responses to salt stress in tomato (Johnson *et al*., 2003), plant–plant interference in *A. thaliana* (Gidman *et al*., 2003) and open top chamber studies on *C. vulgaris* responses to increasing N (Gidman *et al*., 2004). In an effort to expand on previous work performed on *C. vulgaris* we examined the prospect of detecting metabolic fingerprint shifts in a heathland situation as affected by controlled inorganic N applications.

2. Materials and methods

The study site we used was an example of lowland heath (50 m a.s.l.) situated at Budworth Common near Crewe, Cheshire, UK. This heath, while dominated by *C. vulgaris*, also contains the grass *Deschampsia flexuosa* (L.) Trin. (Cawley, 2000; Wilson, 2003) and is classifiable by the National Vegetation Classification (NVC) as a H9: *Calluna vulgaris-Deschampsia flexuosa* heath. The experimental regime sampled from at this site consisted of applying increasing levels of NH_4NO_3 treatments (a "true" control not receiving any inputs, 0, 20, 60 and 120 kg N ha^{-1} $year^{-1}$) to 2×1 m plots. Hence each nitrogen treatment was replicated four times in plots positioned within a randomized block design (figure 1). Additionally, from 1997 onwards each plot was split into 1×1 m watered and droughted plots, making the overall experimental design a split-plot one. However, only those sections of the plots receiving water at the time of sampling were selected for. All N treatments had been applied since 1996.

Material was harvested from the study site during late autumn/early winter 2001 (23rd November 2001) at ~15:00 (GMT, ±1 h). Five samples of current years shoot growth (~2 g per sample) were randomly selected from each plot ($n = 20$ overall per treatment). These were immediately wrapped in tinfoil following excision from the plant and later transported back to the laboratory on ice. We opted against snap-freezing samples in liquid N_2 at the experimental site due to field safety implications. Thus an assumption was made that transport on ice halted senescence activity sufficiently so as to not affect any subsequent metabolic fingerprints derived from the collected samples. Preliminary experiments were conducted to this effect confirming that this approach was acceptable (data not shown for brevity). Upon arrival at the laboratory samples were taken from ice and immediately oven-dried at 65 °C for 48 h. Dried samples were then ground by pestle and mortar to a fine powder.

FT-IR analysis required preparation of 10 μL sample slurry aliquots (100 mg d.wt. mL^{-1} Milliq H_2O) that were then applied to the wells of an FT-IR sample carrier plate. Loaded sample carrier plates were oven-dried at 50 °C prior to insertion into a robotic stage accessory (Bouffard *et al*., 1994). Automated spectral collection by an IFS-28 FT-IR instrument (Bruker Ltd.) was then controlled using an IBM-PC operating under a standard protocol as outlined elsewhere (Goodacre *et al*., 1998; Timmins *et al*., 1998). Spectra collected were typically complex and uninterpretable by eye (figure 2) and so the multivariate method of principal components – discriminant function analysis (PC-DFA) was used to analyse the data.

Analysis involved randomly splitting the collected spectra into training and validation sets on a respective 70:30 basis. From each treatment one block worth of data, containing five samples' spectra, was randomly selected for the validation set. PC-DFA models (Radovic *et al*., 2001) were then trained within Matlab 6.1 (Mathworks Ltd.) in order to investigate potential clustering (Gidman *et al*., 2003, 2004) and therefore disparity between treatments. All the models calculated were trained to recognise the level of NH_4NO_3 treatment to which a particular sample's spectra had originated from.

3. Results and discussion

A PC-DFA model that used 30 PCs (comprising 98.93% of the total variation) was built that described a pattern running across DF1 relating to increasing N treatment (figure 3). The true control spectra also appeared to differentiate from spectra originating from N treatments, and thus additional watering, along DF2 (figure 3). In order to determine which clusters were significantly different from each other (at $p < 0.05$) the scores for each DF were analysed by one-way ANOVA with a concurrent Tukey's multiple range test (Sokal and Rohlf, 1969). This showed discrimination across DF1 due to N treatment, with the 60 and 120 kg

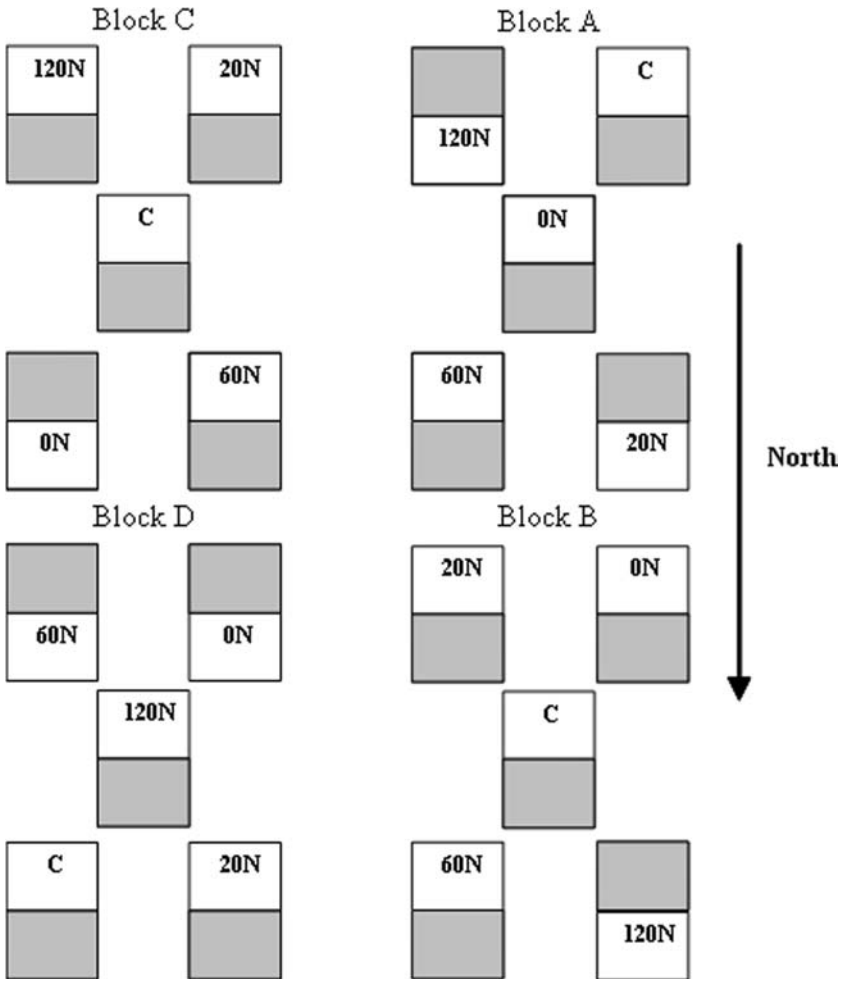

Figure 1. Schematic diagram illustrating experimental regime of the field site at Little Budworth, Cheshire, UK. Five overall NH_4NO_3 treatments (''true'' control [C], 0, 20, 60 and 120 kg N ha^{-1} $year^{-1}$) replicated four times in a randomised block design. Rectangles represent 1×2 m plots and shaded portions of plots indicate drought treatments.

N ha^{-1} $year^{-1}$ treatments differing from each other and those treatments that received less N (table 1). However, the 120 kg N ha^{-1} yr^{-1} training cluster was significantly different from the same treatment's validation cluster (table 1), which suggests validation of this treatment was not successful even though both the relevant clusters are quite distinct from the all the others. DF2 showed a significant difference between the true control, or that treatment not receiving water inputs, and all other treatments, being those receiving N and / or water inputs. Although, as seen in the 120 kg N ha^{-1} $year^{-1}$ treatment, true control training and validation clusters were different from each other (table 1).

A loading plot for DF1 was also examined in an attempt to elucidate which spectral regions could be responsible for the observed trend (figure 4). Whilst this loading plot is rather complex, it was apparent that as NH_4NO_3 treatment increased, several spectral regions were positively responsible for the observed trend, meaning functional groups vibrating in these regions generally positively correlated with increasing N treatment. These included amide I (~1682 cm^{-1}) and amide II vibrations (~1543 cm^{-1}), and a couple of polysaccharide vibrations (~1160 and ~1057 cm^{-1}) (Schmitt and Flemming, 1998; Ellis *et al.*, 2003) (figure 4). Such positive correlations between N deposition and shoot amides in *C. vulgaris* may shed more light on previously observed positive correlations between N deposition and total shoot nitrogen (Pitcairn and Fowler, 1995; Hicks *et al.*, 2000). There were also spectral regions that appeared negatively responsible for the observed trend relating to increasing NH_4NO_3, meaning functional groups vibrating in these regions generally negatively correlated with increasing N treatment. These were a C=O vibration of fatty acid esters (~1732 cm^{-1}), another amide II vibration (~1574 cm^{-1}) and a vibration residing in the fingerprint region (~780 cm^{-1}) (Schmitt and Flemming, 1998; Ellis *et al.*, 2003) (figure 4).

The results display an N deposition response in *C. vulgaris*' shoot biochemistry, which is detectable using

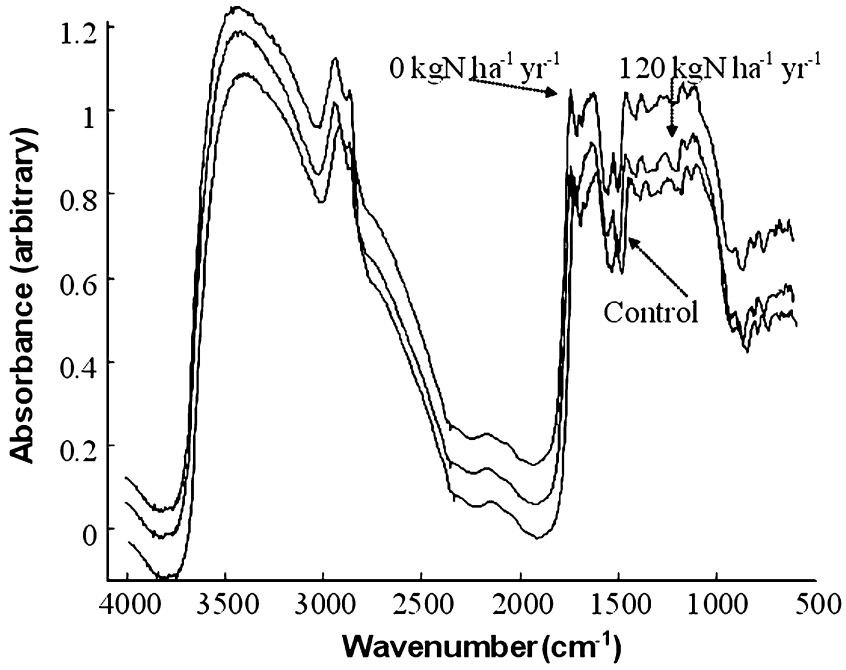

Figure 2. Typical FT-IR spectra as collected from oven-dried and homogenised *C. vulgaris* shoot tissue. Examples of spectra taken from samples at three different treatments given ("true" control, 0 and 120 kg N ha^{-1} $year^{-1}$).

the metabolic fingerprinting technique of FT-IR in samples collected from the field. Whilst this study has only concentrated on a single experimental site, the presented results provide considerable evidence for the further development of this technique. Such metabolic shifts, if evident across a range of heathland communities, may provide an early indicator of change attributable to N deposition. Indeed, in the early years of this field site treatment stimulation of shoot growth and flowering was seen as well as a positive correlation between shoot N concentrations and N treatment (Cawley, 2000; Wilson, 2003). However, it is worth

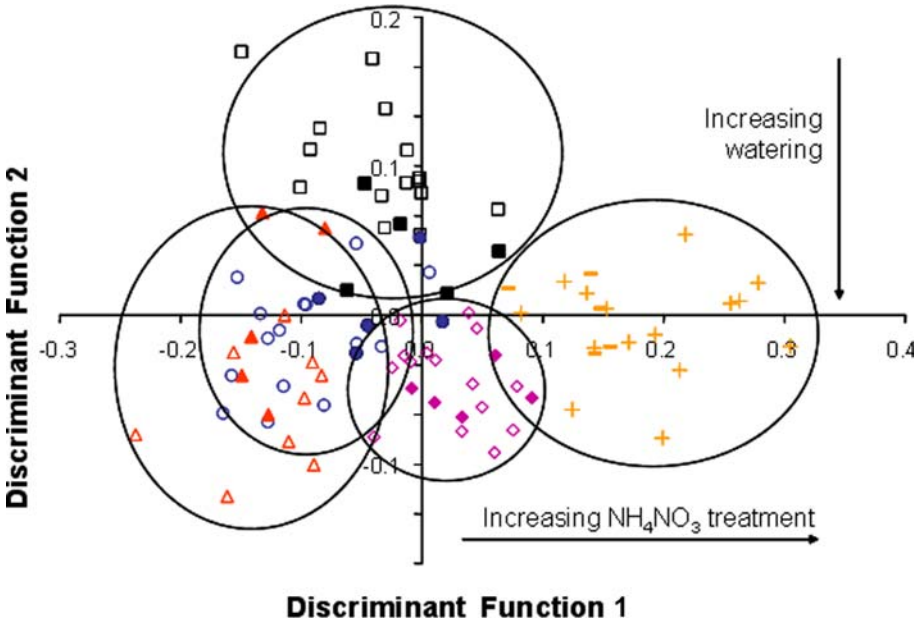

Figure 3. PC-DFA cross-validated ordination plot of *C. vulgaris* spectra using 30 principal components (98.93% of total variation). Clustering shown for samples taken from plants subjected to simulated wet NH_4NO_3 deposition at five different levels (□ = "true" control, △ = 0, ○ = 20, ◇ = 60 and + = 120 kg N,ha^{-1} $year^{-1}$). Projected PC-DF scores for test spectra denoted by solid points (except for 120 kg N ha^{-1} $year^{-1}$, denoted by −). DFA *a priori* class structure based on grouping all spectra per treatment (one *a priori* class per level of NH_4NO_3 treatment) and each point is the mean of three machine replicate sample spectra's PC-DF scores. Circles are provided around treatment clusters for additional clarification, and arrows are given to highlight direction of trends relating to increasing levels of N and additional watering.

Table 1
Mean values (±SE) for training and validation PC-DFA clusters (30 PCs explaining 98.93% of total data variation) of *C. vulgaris* shoot FT-IR spectra subjected to varying levels of N treatment (see figure 3 for original model ordination plot). Results of Tukey's multiple range test as calculated from prior one-way ANOVA, performed on both DF1 and DF2 separately, also given as bold lowercase characters next to each treatment training/validation cluster mean. ANOVA analysis not presented for brevity.

Treatment (kg N ha^{-1} $year^{-1}$)	Discriminant function 1		Discriminant function 2	
	Training clusters	Validation clusters	Training clusters	Validation clusters
True control	0.0356 ± 0.009 **b**	0.0086 ± 0.016 **bc**	0.0614 ± 0.004 **e**	0.0359 ± 0.008 **a**
0	0.1267 ± 0.012 **a**	0.1262 ± 0.010 **a**	0.0093 ± 0.005 **bc**	0.0257 ± 0.005 **b**
20	−0.1006 ± 0.008 **a**	0.0340 ± 0.011 **b**	0.0048 ± 0.004 **bcd**	0.0267 ± 0.006 **d**
60	0.0221 ± 0.007 **c**	0.0374 ± 0.013 **c**	0.0007 ± 0.005 **bcd**	0.0032 ± 0.005 **cd**
120	0.1901 ± 0.010 **e**	0.1314 ± 0.011 **d**	0.0310 ± 0.003 **b**	0.0004 ± 0.006 **bcd**

noting that during a heather beetle outbreak in 1998 and 1999 the high N plots were preferentially selected for (Cawley, 2000; Wilson, 2003), which has also been observed on a lowland heath at Thursley Common in Surrey (Power *et al.*, 1998). It is possible therefore that the changes in FT-IR spectra could partly represent an indirect response to the effect of this environmental stress, and caution is obviously needed in interpretation.

The presented model also suggests there is a marked difference between the "true" control *C. vulgaris* plots and those plants that receive additional water inputs. Responses to this particular source of variation were not considered when initially analysing the *C. vulgaris* shoot metabolome data. Such an outcome of the chemometrics displays the "holistic" and hypothesis generating (Kell and Oliver, 2004) ability of metabolic fingerprinting and the potential for identifying other sources of stress such as drought. However, examination of the PC-DFA axis do suggest that the trend relating to N treatment is the most important effect in the model (figure 3).

4. Concluding remarks

We have shown the potential of metabolic fingerprinting *C. vulgaris* for use as a bioindicator of N deposition. The ability to discriminate between differing levels of N addition with this relatively rapid and sensitive technique could prove to be an invaluable tool in detecting early plant responses to N when used alongside more conventional methods such as total tissue N. Development of this method for detecting N deposition and other sources of environmental variation is currently underway. Further work will concentrate on expanding the findings here across different *C. vulgaris*

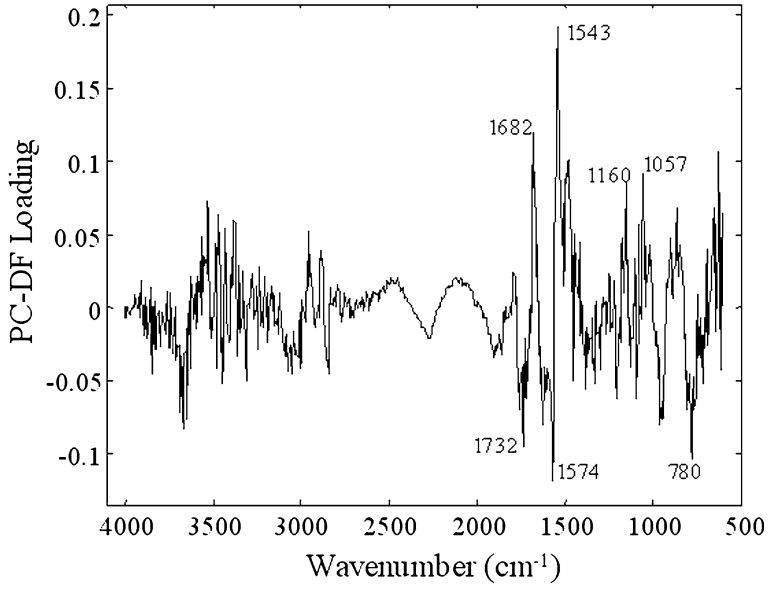

Figure 4. PC-DFA loading plot for DF1 from a model trained to discriminate between oven-dried *C. vulgaris* sample spectra obtained from Little Budworth field site that originate from differing NH_4NO_3 treatments ("true" control, 0, 20, 60 and 120 kg N ha^{-1} $year^{-1}$) where DF1 describes a positive trend relatable to these treatments (see figure 3). Loadings for all wavenumbers (cm^{-1}) given, and notably high loadings selected by eye labelled for clarity.

communities, with differing genotypes and environmental backgrounds, to allow predictive modelling at the landscape level.

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