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Review

The Effects of Nitrogen Fertilisation on Plant Species Richness in European Permanent Grasslands: A Systematic Review and Meta-Analysis

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Abstract: Nitrogen fertilisation is a common form of agricultural intensification, aimed at increasing biomass, which can affect plant species diversity and ecosystem functioning. Using a systematic review and meta-analysis of nitrogen fertilisation studies in European permanent grasslands, we asked: (i) what relationship form exists between nitrogen application rate and change in plant diversity, compared to zero fertilisation controls; and (ii) how grassland, management and study characteristics affect this relationship. Meta-analysis of 34 control-treatment effects from 14 studies conducted across nine European countries revealed a negative linear relationship between nitrogen fertilisation rate and change in plant species richness, equivalent to approximately 1.5 species/m^2 lost for every 100 Kg ha^{-1} yr^{-1} of nitrogen added. Fertilisation induced reductions in plant species richness were greater when defoliation rates were lower. We found some evidence that grasslands with a higher baseline plant diversity lost more species when fertilised compared to more species poor grasslands, although uncertainty was high. Due to the diverse grassland types included in the analysis, the variability in fertilisation-driven changes in plant diversity was high. We identified several remaining limitations to our understanding, including uncertainty about non-linear effects, which could aid efforts to optimise the trade-off of plant diversity and increasing grassland yields.

Keywords: enrichment; plant diversity; agricultural intensification; management intensity

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1. Introduction

Permanent grasslands, defined by the European Union as "land used to grow grasses or other herbaceous forage that has not been included in the crop rotation for a duration of five years or longer" [1], have been the basis of livestock production and farm nutrient cycling across Europe for centuries [2–4]. Grass production continues to provide one of the cheapest and highest-quality sources of forage for dairy and beef systems [5]. Furthermore, permanent grasslands deliver a wide range of ecosystem services crucial for human well-being [6–9], including protection from soil erosion, water flow management, aesthetic and cultural services, the storage of around a third of Earth's terrestrial carbon, and provision of habitat for biodiversity [10–12]. Despite this multifunctional importance, global reductions in permanent grassland extent and quality have occurred throughout the last century [7,13–15]. Whilst COP26 recognised the importance of forested land for carbon sequestration and biodiversity with a commitment to halt further loss, there is no such aim to prevent loss of permanent grasslands [16], which are often ignored or undervalued in policy agendas for sustainable development [15].

Permanent grasslands cover almost 60 million hectares across the EU and UK [17], constituting a third of the total EU agricultural area [18]. Over past centuries, permanent grasslands have been the mainstay of European ruminant livestock production [2–4], and their contribution to multifunctional landscapes is well documented [9], including their importance as one of the most species-rich habitats in Europe [10,11]. In recent decades, in regions with typically more marginal agricultural conditions, such as mountainous or high latitude areas, management intensity of some permanent grasslands has either greatly declined or been abandoned altogether, leading to conversion to scrub and eventually forest [7,14]. However, more common has been the conversion of permanent grasslands to temporary grasslands or croplands, or intensification of their management [19]. Nitrogen is one of the principal limiting nutrients in grasslands [20–22], and as such, the addition of nitrogen rich fertilisers to facilitate plant growth is a principal form of management intensification on many permanent grasslands in Europe. The addition of nitrogen in grasslands stimulates aboveground biomass production and along with this, competitive exclusion, lowered colonisation and subsequent loss of plant diversity [23–25]. A key mechanism determining plant species loss is increased competition for light in dense, fertilised stands leading to the exclusion of especially small growing species [26]. Previous meta-analyses have demonstrated reductions in plant diversity as a result of nitrogen fertilisation in terrestrial ecosystems [27–30]. For example, in a global meta-analysis, Soons et al. [30] found that the addition of nitrogen decreased herbaceous species richness by c.16%, although results varied geographically and by land-use.

Management intensification generally increases target production services (e.g., crops, forage), but often results in biodiversity declines and reductions in associated ecosystem services [31–35]. While fertilisation is an essential aspect of high agricultural productivity per unit land area, it comes at significant environmental costs such as emissions of greenhouse gases and the risk of nutrient leaching [36]. Therefore, the maximum amount of nitrogen that can be applied to grassland is regulated, with for example 340 and 385 Kg N ha^{-1} yr^{-1} in the UK and the Netherlands, respectively, partly depending on local environmental conditions [37,38]. However, the amount of nitrogen fertiliser actually applied to permanent grassland is often considerably below these maximum values, potentially showing distinct inter-annual variability [39].

The trade-offs associated with nitrogen fertilisation can lead to land-use conflicts between stakeholders who prioritise different groups of ecosystem services [40]. For management policies to reduce these trade-offs, it is crucial that the exact shape of these relationships are understood [41]. For instance, non-linear relationships between management intensification, production and biodiversity may highlight points of ‘minimum-effective dose’ which balance financial return, production yield and biodiversity losses. Such non-linear relationships between nitrogen fertilisation and pasture production have already been documented, e.g., [42], but the relationship with biodiversity loss, particularly on European permanent grasslands, is currently poorly understood.

Generalities are difficult to determine without considering outcomes from many individual studies. Thus, a broad survey of the available relevant literature is required to bridge the gap between experiments and application of management intensification. Here, we aim to explore the relationship between a common form of agricultural intensification (nitrogen fertilisation) and a key measure of grassland biodiversity (plant species richness) using a meta-analysis of control vs. treatment effects from experimental studies conducted on European permanent grasslands. We seek to examine the shape and strength of the relationship between nitrogen fertilisation rates and changes in plant diversity, accounting for a range of other relevant factors. We provide an overview of study characteristics from the wider body of relevant literature, and finally, we suggest how future research can help in strengthening our understanding of the themes discussed here, by identifying remaining gaps in our knowledge.

2. Materials and Methods

2.1. Literature Review and Data Extraction

We obtained 345 scientific research articles which formed a subset of the >70 k articles systematically reviewed by Schils et al. [9]. All studies pertained to research on plant diversity values in European permanent grassland (see Appendix A for details of how these studies were obtained).

From this body of research, we applied strict inclusion criteria when selecting data for our meta-analysis: (i) study includes a control (no nitrogen addition) and a treatment (nitrogen addition); (ii) the study measured changes in plant species richness in both control and treatment conditions; and (iii) the study reported basic statistical data (mean, standard deviation and number of replicates (n)), or these could be calculated from the information provided. This process led to the inclusion of 14 independent studies reporting results from nitrogen fertilisation field experiments conducted in European permanent grasslands, from which we performed a meta-analysis utilising 34 effect sizes contained within these studies.

We used the mean difference in plant species richness between control and each treatment level in each study as the effect size in the analysis, which allowed for ease of interpretation in the results presented. We did not include studies that only provided other metrics of plant diversity in this meta-analysis (e.g., Simpson's or Shannon's Diversity Indices) owing to the low number of studies using these metrics that had extractable data or data that could be readily standardised. Where means and standard deviations were given in graphical form, values were extracted using open source, web-based application Web Plot Digitizer [43]. If the study reported more than one value for the same experiment at multiple time points, we used the data for the final time point.

We focussed on the addition of nitrogen, rather than phosphorus or potassium. Therefore, for a study to be eligible, the level of nitrogen had to be manipulated, although other nutrients may also have been applied or manipulated alongside (e.g., if organic fertilisers were used, which would alter levels of other nutrients beside nitrogen). Accordingly, we tested for any effect of nitrogen source, by comparing results from studies where nitrogen alone was manipulated vs. those that also applied other nutrients whether in known quantities (i.e., nitrogen in synthetic form) or in manure which will also contain other nutrients. Where nitrogen enrichment level was not provided in Kg N ha^{-1} yr^{-1} (e.g., was given in volume of manure applied) we converted these values to Kg N ha^{-1} yr^{-1} using manure specific conversions according to livestock type and manure state as given in Section 2 of the AHDB Nutrient Management Guide [44].

We recorded defoliation rates (i.e., number of cuts or grazing episodes per year) for each contrast of nitrogen fertilisation rate. We excluded contrasts where defoliation levels differed between control and nitrogen addition treatment. Therefore, within each control vs. treatment contrast defoliation rate was constant, although for some studies it differed between contrasts when more than one contrast could be extracted. This approach reduced any confounding effect of defoliation and helped us to isolate the effect of nitrogen fertilisation on plant diversity. We also recorded plant species richness at control levels of nitrogen addition (i.e., zero) for all eligible contrasts, herein referred to as 'baseline species richness'. We further considered plot size (since diversity can be expected to increase with plot size [45]), duration of nitrogen fertilisation before richness values were recorded, and whether fertilisation occurred as 'nitrogen only' or as 'nitrogen + other nutrients' (herein referred to as 'fertilisation type').

For studies not eligible for the meta-analysis, but which met the other inclusion criteria (see Appendix A), we did not seek to quantify the direction or strength of any relationship between nitrogen fertilisation and plant diversity since this could amount to 'vote counting' and risk statistical bias [46]. Instead, we summarised the characteristics of these studies, including when and where these were conducted, and what other forms of permanent grassland management were studied alongside nitrogen fertilisation.

2.2. Meta-Analysis

We analysed eligible data with a three-level mixed-effects meta-analysis model. These models are more equipped to handle clustered effect sizes than more traditional methods used in meta-analyses [47]. Models were fitted using the 'rma.mv' function in the package 'metafor' in R [48] using restricted maximum likelihood (REML) procedures to explore the effect of nitrogen fertilisation rate on differences in mean plant species richness between control and treatment plots. Models were generated using 'study' as a random effect to address potential non-independence without compromising sample size [49]. We compared model heterogeneity both within studies (level 2) and between studies (level 3) using the 'var.comp' function within the 'dmetar' package.

Each effect size (mean difference in plant species richness between control and nitrogen addition treatment) extracted from eligible studies was associated with a nitrogen fertilisation rate, as well as with a number of additional variables, each of which we had a priori justification to include based on prior knowledge of their possible effect on plant richness. Namely, these included: baseline species richness, defoliations per year, plot size, fertilisation type, and years of nitrogen fertilisation. Additionally, we included an interaction term between nitrogen fertilisation rate and defoliation rate, and we included nitrogen fertilisation rate as both a linear and non-linear term given the importance of non-linear effects outlined above. We built a full model with each of these variables included as moderators, after checking for any strong and/or significant correlations between variables.

We aimed to balance adequate data exploration by considering this range of possible influencing variables, with the risk of overfitting models. As such, we utilised an information-theoretic, model selection approach for meta-analysis using the 'glmulti' package [50]. This process involved fitting a 'full' model of all variables, outlined above, before examining fit and plausibility of all possible models and selecting a set of candidate models within two Akaike Information Criteria values of the 'best' model (AICc) [51]. The variables within these models were then tested for effects on change in plant species richness in our selected studies between control and treatment levels. All data were analysed in R (version 1.4.1106). We checked our reporting against 'PRISMA' checklists (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) to ensure compliance for ecological studies [52].

3. Results

Study screening and exclusion resulted in 52 permanent grassland studies that tested the effect of nitrogen fertilisation on plant diversity in European permanent grasslands according to the criteria set for this review. We were then able to retrieve and extract data for our meta-analysis from 14 of these eligible studies [53–66], which were conducted across nine European countries (Czech Republic, Estonia, France, Germany, Ireland, Poland, Romania, Slovenia and United Kingdom), and provided 34 effect sizes. Thus, many of these studies included more than one rate of fertilisation which could be compared to a zero-nitrogen control. Summary information for all 52 studies quantitatively or qualitatively included in this review are given in the Appendix A.

Meta-analysis revealed a mean change in plant species richness between zero-nitrogen controls and all treatment levels of -2.75 species (95% CI: -5.02–-0.47; Figure 1). The mean treatment level for all 34 interventions was 136.2 Kg N ha^{-1} yr^{-1} (range: 12.5–600). There was a high degree of inconsistency in effect, with 22 out of 34 contrasts (65%) showing a negative effect of nitrogen fertilisation on plant species richness, and 12 showing a positive effect. Furthermore, confidence intervals overlapped across studies for most effects (Figure 1).

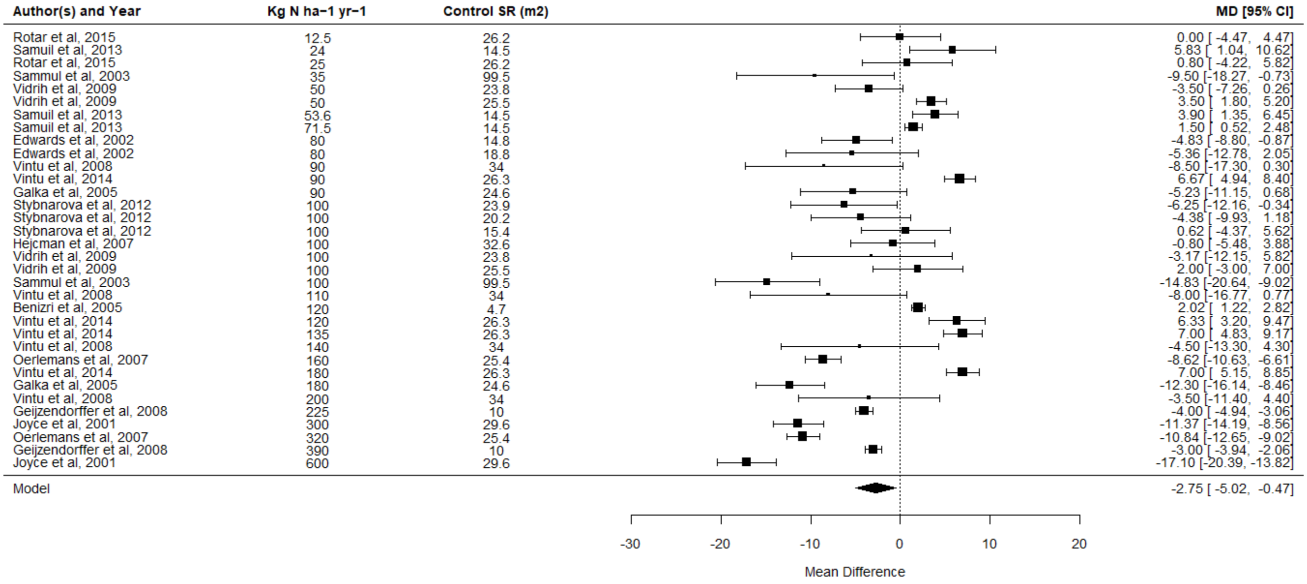

Figure 1. The effects of nitrogen (N) fertilisation on the mean difference (MD) in plant species richness for 34 effect sizes in 14 studies conducted on European permanent grasslands included in a meta-analysis. A mean difference of zero would indicate no change in species richness, whereas negative values indicate the number of species lost and positive values the number of species gained between zero N controls and N addition treatment. Error bars show 95% confidence intervals, and MD and 95% CIs are given in the right column for each effect and for the overall model. Nitrogen application rates and control species richness (SR) values are shown for each effect. Effects are ordered in ascending order of nitrogen treatment rate.

A three-level model was found to provide a significantly better fit compared to a two-level model with level 3 heterogeneity constrained to zero (LRT = 18.15, p = 0.001), justifying the use of the full three-level meta-analysis model. No significant correlations existed between any of the explanatory variables. There was no strong indication of substantial publication bias (Figure 2).

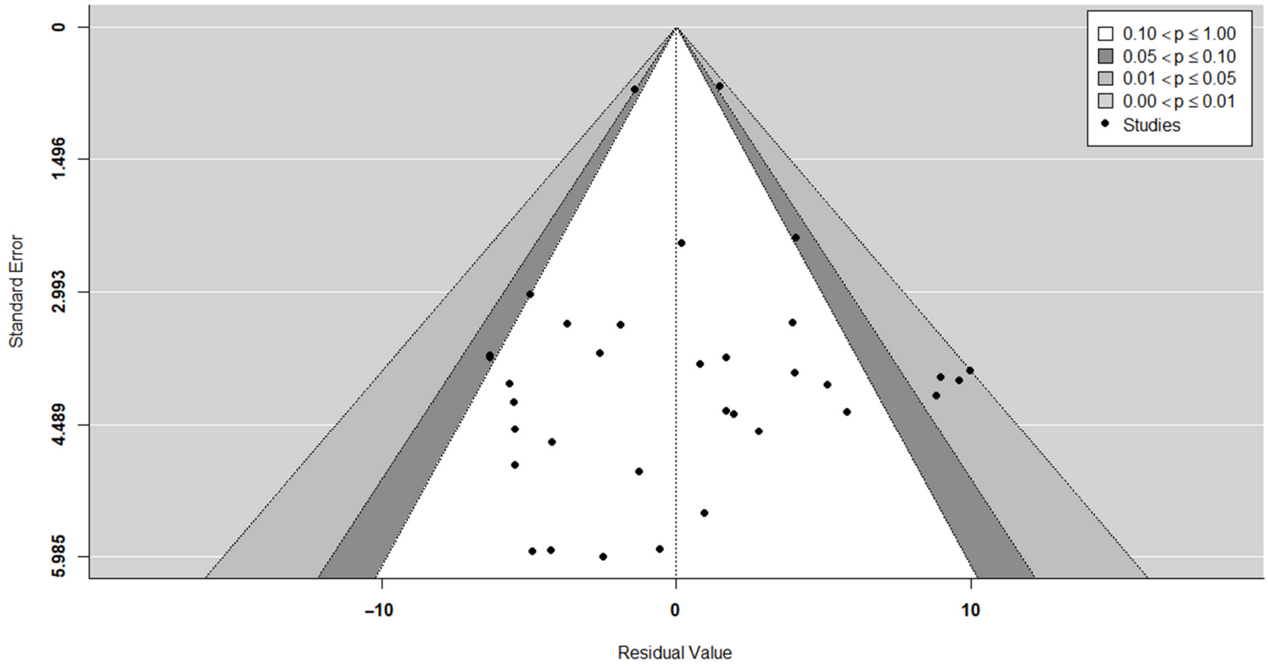

Figure 2. Contour enhanced funnel plot of the residuals of plant species richness response to different levels of nitrogen addition from studies when fitted with a mixed effect linear model.

Model selection resulted in six candidate models within two AICc values of the 'best' model. Four moderators (explanatory variables) featured in these six models: the linear term of nitrogen application rate, the non-linear nitrogen term, number of defoliations per year, and baseline species richness. Our next step was to determine whether the non-linear term improved model fit compared to the linear term when included in a model with the defoliations per year and baseline species richness moderators. Comparison showed that inclusion of a non-linear term did not improve model fit compared to a linear term (LRT = 0.02, $p = 0.88$). Furthermore, the model-averaged importance of the non-linear term was lower than all other moderators in the top set of candidate models, and was the only moderator with a model-averaged importance of <0.5, considered a conservative low value for important variables [50,67]. As such, we consider there to be no sufficient evidence for a non-linear effect of nitrogen fertilisation on plant species richness.

We therefore considered the effect of the linear nitrogen term, defoliations per year and baseline species richness within a reduced model. This model revealed a significant negative linear relationship between nitrogen application rate and plant species richness between control and nitrogen addition treatment (−0.015, s.e. 0.006, $p = 0.01$). This indicates that plant species richness declined on average with increasing nitrogen fertilisation rate in the included studies, representing an average loss of approximately 1.5 species per 100 kg N added per hectare per year (Figure 3).

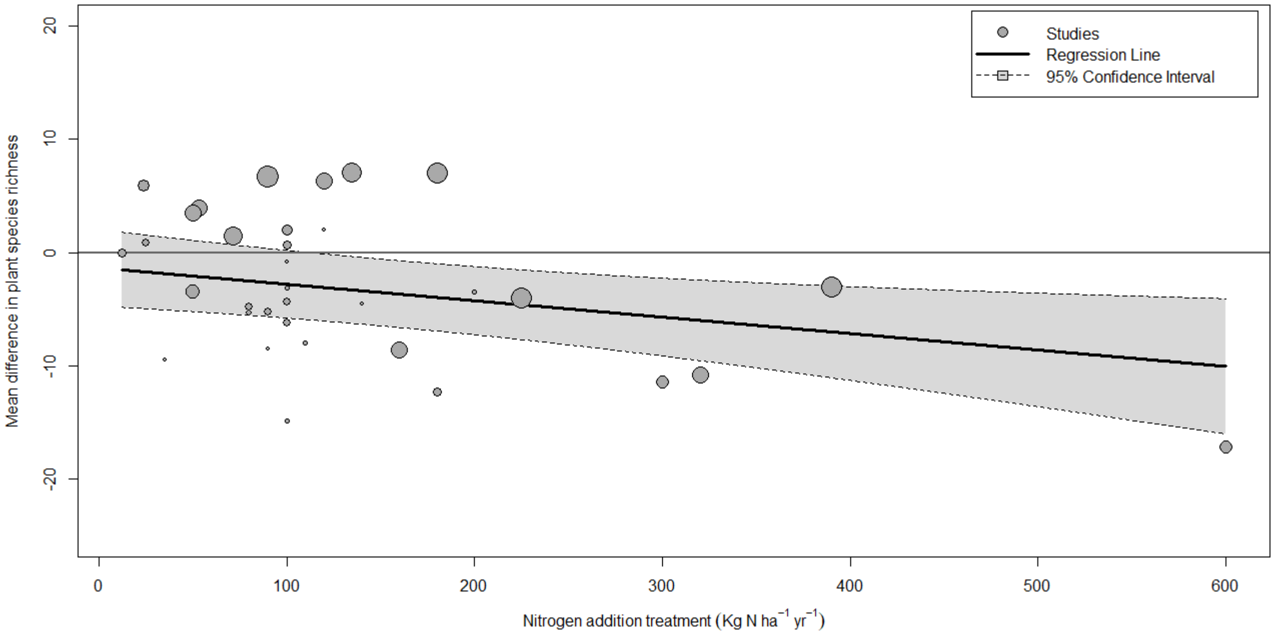

Figure 3. Mean difference in plant species richness in response to 34 different levels of nitrogen addition compared to zero-nitrogen controls reported by 14 studies included in a meta-analysis. Symbol size is a function of the weight in the model, with larger studies having a greater weight on model fit.

Owing to the high uncertainty at nitrogen fertilisation rates above 400 Kg N ha^{-1} yr^{-1} shown in Figure 2, we were interested to see if this negative relationship was driven by the relatively few data points representing studies with very high fertilisation rates. Current UK Nitrate Pollution Prevention Regulations [38] permit a maximum total nitrogen application on any farm holding of 340 Kg N ha^{-1} yr^{-1}, while regulatory values for Nitrogen Action Plans in the Netherlands [37] on frequently cut grassland on clay soils permit up to 385 Kg N ha^{-1} yr^{-1}. As such, we considered values above this to be outside the maximum use on European permanent grassland, and so of little practical use. As such, we repeated this analysis using only data representing fertilisation rates of 385 Kg N ha^{-1} yr^{-1} or below. The negative relationship between nitrogen fertilisation rate and change in plant species richness remained significant (−0.019, s.e. 0.008, $p = 0.03$), demonstrating that the relationship shown in Figure 2 was not driven by the data points above regulatory limits for European grasslands.

Model results also indicated that the number of defoliations per year had a positive effect on the mean change in plant species richness when nitrogen fertilisation was applied (2.49, s.e. = 1.21, $p < 0.05$, Figure 4), indicating that higher defoliation frequencies may have buffered against plant species richness declines that occurred with the addition of nitrogen fertilisation.

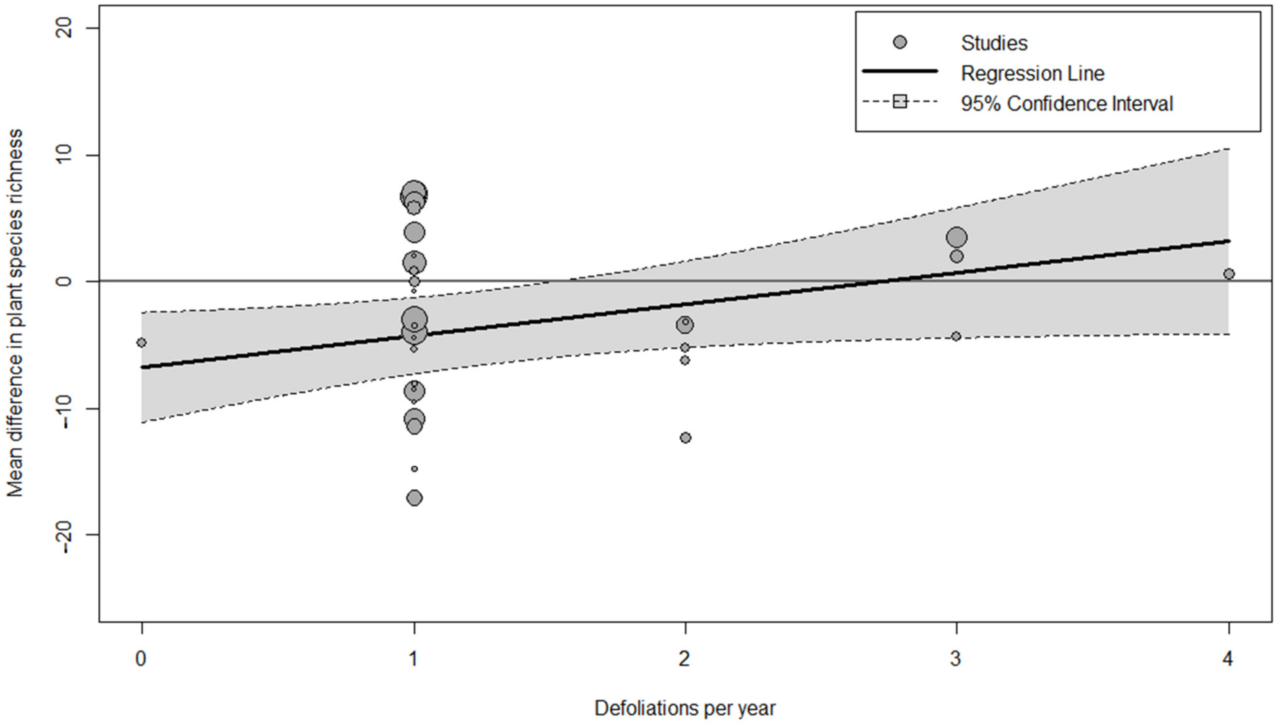

Figure 4. Mean difference in plant species richness with nitrogen addition in relation to the number of defoliation episodes per year from 14 studies included in the meta-analysis. Symbol size is a function of the weight in the model, with larger studies having a greater weight on model fit.

Furthermore, baseline plant species richness was also found to have a significant negative effect on mean change in plant species richness (−0.15, s.e. = 0.07, $p < 0.05$, Figure 5). This indicated that declines in plant species richness with the addition of nitrogen were greater when baseline plant richness was higher.

Owing to the apparent high level of dependency of this result on two effects from one study which had particularly high baseline plant species richness, we repeated our analysis with this study removed. This had no effect on the results from the model selection process, or on the other included moderators, and baseline species richness remained an important moderator in the top set of candidate models. However, baseline species richness was no longer a significant negative predictor of change in species richness when included in the reduced model with these two effect sizes removed. As such, we have high uncertainty about our finding that greater declines in plant species richness occurred in plots with higher baseline species richness.

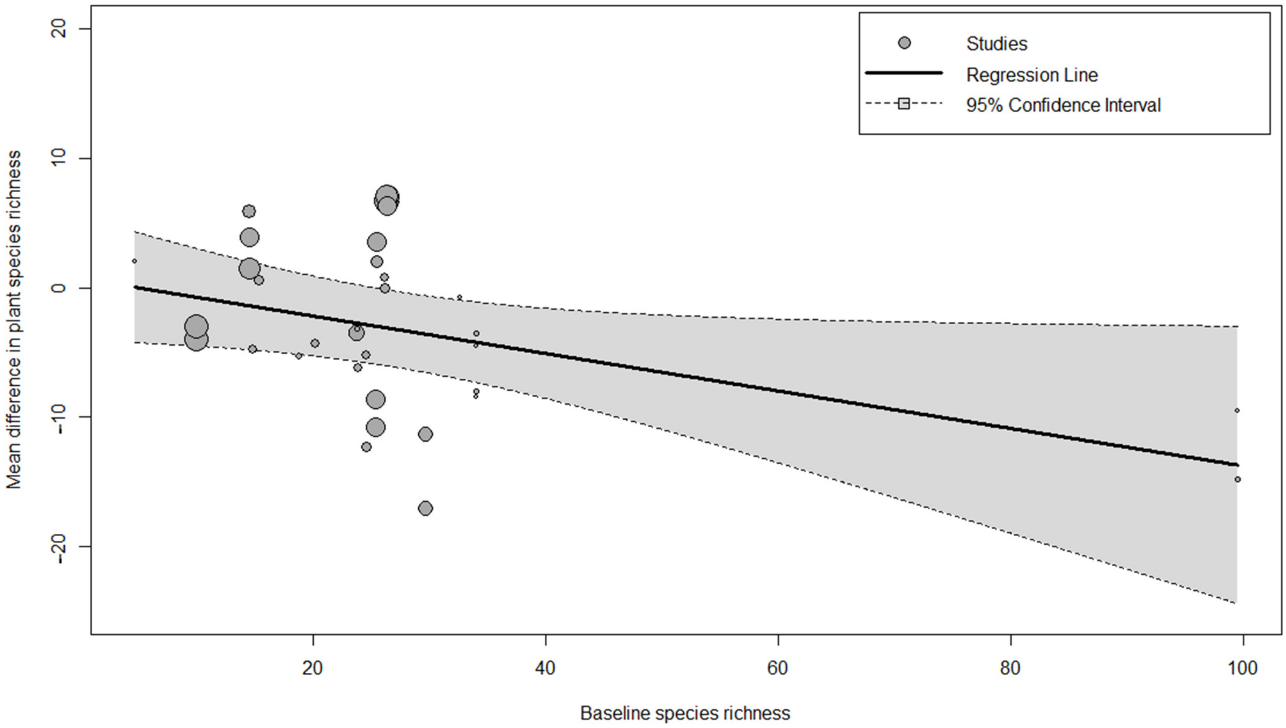

Figure 5. Mean difference in plant species richness with the addition of nitrogen in relation to baseline plant species richness in 14 studies included in the meta-analysis. Symbol size is a function of the weight in the model, with larger studies having a greater weight on model fit. Note: this result was dependent on the inclusion of the two effects from one study on the right-hand side of this plot which had particularly high baseline plant species richness, and there was no significant effect of baseline species richness when this study was removed from the final model.

4. Discussion

Our review and meta-analysis of European permanent grassland studies investigating the effects of nitrogen fertilisation on plant species richness revealed several important insights. First, our analysis of control-treatment effects showed that nitrogen fertilisation had a mean negative effect on plant species richness, equivalent to an average loss of approximately 1.5 species per 100 Kg N added per hectare per year. This negative effect is concordant with previous meta-analyses of nitrogen enrichment in other terrestrial ecosystems [27–30], but is an important new addition to the evidence base of the strength and shape of this effect on European permanent grasslands. It is, however, important to note that our study included a wide range of different grassland types, from semi-natural to improved (originally sown) grasslands, with a wide range of control (i.e., baseline) species richness. This is likely to have a strong effect on the rate of species loss under fertilisation, and means that many important local conditions remain important underlying factors here [68]. For example, a multi-site experiment in mountainous hay meadows found a much stronger decrease of 6 species in response to addition of each 50 kg N ha^{-1} yr^{-1} in previously only marginally fertilised or unfertilised grasslands [69]. However, in already nutrient rich and productive grasslands such as alluvial meadows, the negative impact of N additions on plant diversity is considerably smaller compared to that of introducing fertilisation to nutrient poor, low yielding grasslands [70]. Thus, the inclusion of many studies conducted on grasslands with already low species richness values (e.g., [53]), with limited potential for further species loss under fertilisation, may in part explain the low loss of plant diversity under fertilisation we document here. Due to the diverse grassland types included, our study shows a broad range in individual responses of plant diversity to N addition, from strongly negative to weakly positive. This is in agreement with the finding that only few plant species respond positively to grassland fertilisation (i.e., winners of intensification), while the large majority of typical grassland plant species are losers of intensification, particularly of increasing fertilisation intensity [71]. Finally, changes in plant diversity often need several years to become entirely apparent [72]. Therefore, our study might have underestimated the long-term effect of fertilisation on plant diversity, as we did not control for the duration of the experiments included.

Our analysis also suggested that, in the presence of nitrogen fertilisation, declines in plant species richness were of a lower magnitude as defoliation rate increased. This may suggest that more frequent defoliation reduces the competitive advantage, afforded by the raised nitrogen level, of relatively few species, and thus mitigates against their domination at low defoliation rates [73,74]. However, it may also simply suggest that plant diversity was already reduced by higher defoliation rates [71], thus limiting the potential for further reductions in diversity as a result of the nitrogen fertilisation. We had insufficient information to disentangle these mechanisms or the relative effects of different cutting or grazing management practices, such as grazing density, defoliation timing, or cutting height.

Our analysis also suggested that the negative impact of nitrogen enrichment on changes in plant species richness was greater on plots with a higher baseline species richness (e.g., [62,65]). However, we had very high uncertainty for this result, which was dependent on the inclusion of two effects from one study conducted in particularly species rich plots. More dramatic species losses from plots with higher species richness could be explained by strengthened competition for light or space leading to more rapid out-competition by more competitive species [26], or as random loss, as more species are lost to stochastic events in species rich grasslands [27]. Species loss is likely to be non-random, and instead trait-specific [27,75] and dependent on community composition, as well as other biotic and abiotic conditions such as environmental context and sward age [72].

We found no clear evidence of a non-linear effect of nitrogen fertilisation on plant species richness in European permanent grasslands. There could be a number of reasons for this lack of evidence. Firstly, it may indicate that local conditions are more important at determining changes in plant species richness than the general shape of the relationship with N fertilisation [70]. We considered some of these local factors here, but many other factors are also likely to be important for which we were not able to account. For example, landscape and land use history, soil type, altitude, aspect and the available local plant species pool may influence colonisation by new species and interact with the effects of N fertilisation [15,68,76]. Furthermore, the lack of a non-linear relationship may also be due to a relative lack of studies experimentally testing the effects of nitrogen application on particularly species rich grasslands.

Our analysis had high uncertainty, owing to a lack of relevant studies, for both the effects of very high nitrogen fertilisation rates (above regulatory limits) and for the effects of fertilisation on particularly species rich grasslands. However, we show that the negative relationship between nitrogen fertilisation and change in plant species richness remained when only fertilisation rates below regulatory limits were considered, which improved the relevance of our results to current practice in Europe. The tendency of studies to focus their research efforts on grasslands with low or moderate plant species richness likely coincides with typical species richness values found on agriculturally improved grasslands [77,78]. A notable absence of nitrogen studies conducted on grasslands with high species richness is perhaps not surprising due to the commonly held belief that nitrogen application reduces plant diversity, the ecological and environmental effects of which can be long-lasting [79–81]. However, to increase scientific understanding, it would be beneficial to understand the response of different types of species rich grasslands to varying levels of nitrogen enrichment. Indeed, in the UK for example, many of the most species rich grasslands suffered substantial reductions in plant species richness as agricultural intensification drove increases in yield out of a desire for greater self-sufficiency in food production after the two world wars, yet these changes are poorly monitored and documented [19]. Understanding the strength of any loss at these high richness levels will be crucial to understanding the broader shape of the relationship we document here.

We used strict inclusion criteria for our analysis: studies had to include a zero-nitrogen control and a treatment level; had to be conducted on European permanent grasslands as per the EU definition; had to present results in terms of plant species richness; and had to control for effects of defoliation within control-treatment contrasts. This approach improved the robustness of our review but may have reduced the strength of the evidence overall. In agricultural practice, however, N fertilisation is often associated with more frequent defoliations due to increasing yields [39]. Furthermore, nitrogen addition can have negative effects through both eutrophication and soil acidification, which can significantly and independently contribute to species loss, once more highlighting the relevance of the specific environmental context [82,83].

Overall, we judge that our meta-analysis provides a limited overall strength of evidence for a small magnitude effect of nitrogen fertilisation on reductions in plant species richness (Table A2). This is due to constraints of multi-dimensional data and inconsistency, which may serve to highlight that effects depend on a number of local contextual factors. It may also highlight the variety of mechanisms through which changes in plant diversity can occur as a result of nitrogen fertilisation such as altered plant-soil relationships, increased sensitivity to pests [84–86] or even reduced dispersal capacity [30]. It may also highlight that we currently lack the evidence base, particularly at high species richness as outlined above. Although, it could be argued that the evidence base within typical N application rates is more important, and more relevant to practice is the effect of N application over multiple years [72], for which we also have limited ability to make conclusions about. Further studies, particularly those that address the high uncertainty we demonstrate here, may enable us to gain a better understanding of non-linear relationships, and therefore potentially understanding ‘optimum’ levels for a given context, as well as the trade-offs between increasing yield and other ecosystem services. Currently, we believe this is a warning against extrapolation of our results, and of the results of similar reviews and meta-analyses, to many specific contexts.

Recent events, including climate change driven extreme weather events and the invasion of Ukraine, have increased calls for increased food production in many European countries, and this could impact management practices on European permanent grasslands. History has shown how drives for increased yield and self-sufficiency have had desired effects, but with undesired, and dramatic, impacts on biodiversity and associated ecosystem services [15,87]. We show that plant species richness, and its likely impacts on overall ecosystem functioning [31], needs to be carefully factored into any decisions to intensify management of European permanent grasslands, which may take much more effort to restore once species are lost, than to conserve the richness they currently hold.

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Data Availability Statement: The data for the 14 studies included in the meta-analysis, along with code used for analysing these data, are available in the following GitHub repository: https://github.com/RMFrancksen/nitrogenPGmeta (accessed 1 November 2022).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Appendix A

We utilised a subset of articles obtained by Schils et al. [9] who conducted the literature search, performed initial screening and compiled topic-based libraries for their review of multiple ecosystem service delivery in European permanent grasslands. Schils et al. [9] obtained 92,418 research articles from scientific literature databases CAB Abstracts and SCOPUS, and after removal of duplicates, screened 70,456 of these studies. Literature searches were restricted to articles published between 1980 and 2019, and search terms for all 19 indicators can be found in Schils et al. [9] and supporting information. Search terms relevant for the present study were as follows:

- For grassland: (dryland? or grass* or grazed or grazing or hayfield? or hay field? or haymeadow? or herbaceous or herbage? or meadow? or pasture? or rangeland? or range land? or ryegrass* or silvopast* or steppe or steppes or sward? or woodpast*)
- For Europe: exp europe/or (albania or andorra or austria or belarus or belgium or bosnia or british isles or bulgaria or croatia or cyprus or czech republic or czechia or czechoslovakia or denmark or england or estonia or estonian soviet socialist republic or estonian ssr or europe* or finland or flanders or france or german democratic republic or german federal republic or germany or great britain or greece or hercegovina or herzegovina or hungary or iceland or ireland or irish republic or italy or kosovo or latvia or latvian ssr or liechtenstein or lithuania or lithuanian soviet socialist republic or lithuanian ssr or luxemb?urg or macedonia or moldova or monaco or montenegro or netherlands or norway or poland or portugal or romania or san marino or scotland or serbia or slovakia or slovenia or spain or sweden or switzerland or uk or ukraine or united kingdom or wales or wallonia or walloon or yugoslavia) not (new england or new south wales)
- For plant diversity: ((plant? adj3 composition?) or (plant? adj3 diversit*) or (plant? adj3 richness) or (botanical adj3 composition? or (botanical adj3 diversit*) or botanical richness or (floristic adj3 composition?) or (floristic adj3 diversit*) or (floristic adj3 richness) or (species adj4 composition?) or (species adj3 diversit*) or (species adj3 richness) or (vegetation? adj3 composition?) or (vegetation? adj 3 richness) or biodiversity or agrobiodiversity) not (anti oxidant? or anti-oxidant? or biochemical composition or biofuel or chemical composition or extract* or medical or medicinal or pharmaceut*)

After removal of duplicates, the titles and abstracts of the remaining 70,456 research articles were screened, and the following exclusion criteria applied:

- Outside these European Natura 2000 biogeographic zones of interest: Alpine, Atlantic, Boreal, Continental, Mediterranean or Pannonian. Biogeographical boundaries are a combination of official delineations used in the Habitats Directive (92/43/EEC) and for the EMERALD Network under the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). They are independent of political boundaries of Emerald Network countries or EU Member States (https://www.eea.europa.eu/data-and-maps/data/biogeographical-regions-europe-3. (accessed on 1 February 2021)).
- Outside these countries in Europe: Member states of the EU-28 or Albania, Belarus, Bosnia Herzegovina, Kosovo, Macedonia, Moldova, Montenegro, Norway, Serbia, Switzerland or Ukraine.
- Unit of study was not grassland.
- Not in the English language.
- The outcome was not one of the indicators of interest.
- Papers on urban amenity grasses.
- Reviews.
- Modelling studies.
- Experiments under controlled conditions: laboratories, greenhouses or pots

Following screening and exclusion 3154 articles remained. These 3154 papers were stored in separate ‘libraries’ in ‘Eppi-Reviewer’ software (eppi.ioe.ac.uk) according to which of the 19 ecosystem service indicators they pertained to, based on the original search strings. The subset of papers we use here were taken from the 'plant diversity' sub-library within the 'biodiversity' ecosystem service library of research articles, and consisted of 345 records pertaining to plant diversity in European permanent grasslands (see above for search terms). The plant diversity indicator was defined by Schils et al. (2022) as "The richness of vascular plant species on a specific area, expressed as the number of species or a diversity index (Shannon, Simpson). Thus, only part of the values included here utilised information on the abundance of the individual species of the respective plant community. Plant richness is the results of (visual) assessments of the aboveground vegetation and does not include soil seed banks or other measures of dark diversity". We retained this definition for the current study, although refined our inclusion for the meta-analysis to those papers expressly recording species richness (see text).

Where available, we reviewed full-texts of the 345 articles in two stages. The first stage consisted of screening articles to ensure those retained conformed to all the following inclusion criteria:

- Study took place within Europe
- Study contained permanent grassland, defined as "land used to grow grasses or other herbaceous forage that has not been included in the crop rotation of the holding for a duration of five years or longer" (EU, 2004).
- Study reported a change in plant diversity as a response of nitrogen fertilisation
- Full-text, or required data could be readily obtained
- Study results were not duplicated in other records
- Article was not a review or modelling study
- Written in the English language

Our justification for excluding review and modelling studies was so we could include only the most relevant data applicable to permanent grassland management in Europe. This approach improved the overall robustness of the review but may have reduced the strength of the evidence for contrasts with a relatively few underlying cases.

The second stage was to review the remaining full-text articles and supplementary information in more detail. During this stage we also excluded articles if they did not meet one of the inclusion criteria listed above and were not picked up in the first stage screening. Additionally, we applied the following inclusion criteria to ensure selection of articles of relevance to the current study:

- Articles reported a measure of plant richness, whereby data, trends or qualitative results on these measures could be obtained from text, tables, figures or supplementary information.
- Articles included a measure of nitrogen fertilisation, whether from artificial or organic (e.g., manure) sources, whereby the total amount of nitrogen per unit area per unit time was given or could be calculated. We excluded studies of nitrogen enrichment from atmospheric deposition or from nearby sources (i.e., not applied directly to the plots being measured).

As such, we excluded articles that compared land-uses (i.e., permanent grassland compared to another stated land-use) or management intensification that did not involve changes in nitrogen fertilisation (i.e., manipulated defoliation, or performed grassland renewal/resowing, without also manipulating nitrogen fertilisation rate).

Data were extracted from all eligible studies into an extraction form developed in MS Excel. Data were extracted at two levels: (i) the study/article level, which included bibliographical identification, study type, geography, experimental contrasts, and methods for assessment of the relevant indicators, and (ii) the 'contrast' level, that is, at each level of nitrogen fertilisation given in the study. Each paper contained at least one contrast (i.e., compared at least two levels of nitrogen fertilisation, or gave an overall effect or trend). Data were extracted from the text, tables, figures or supplementary materials.

At the contrast level, data pertaining to the nitrogen fertilisation rate, as well as levels of defoliation at each level and the corresponding plant diversity measure were extracted and recorded. Data at this level were extracted in as much detail as possible using the information provided. The most detailed data included raw data, or means, standard deviations and replicates (n) for effects on the eligible measure at one or more contrast level of nitrogen fertilisation. Studies with these data were deemed suitable for a meta-analysis (see text) since they allowed for quantitative synthesis of effect sizes across multiple studies (Gurevitch and Mengersen, 2010).

First stage screening of the 345 plant diversity articles resulted in the exclusion of 92 records, the majority of which were excluded based on full-text articles or required data being inaccessible (71%, n = 65). A further 15 articles (16%) were excluded because they did not contain permanent grassland per the EU definition (EU, 2004) with the remaining 12 articles excluded because they were duplicates (n = 8), not in English language (n = 2), were review studies (n = 1) or were from sites outside Europe (n = 1).

Assessment of the 253 full-text articles remaining after screening resulted in the exclusion of a further 201 studies (79%). The 201 studies were excluded as either containing no extractable data (n = 116) (e.g., no extractable measure of plant diversity or unspecified nitrogen fertilisation rate) or containing no relevant contrast (n = 85) (e.g., no measure or comparison of different nitrogen fertilisation rates). This process of screening and selection resulted in 52 studies that tested the effect of nitrogen fertilisation on plant diversity in European permanent grasslands (Figure A1).

Year of publication for the 52 included studies ranged from 1981 to 2018. However, publications were not evenly spread over this period, with 96% of studies (n = 50) published after 2000, and 26 studies (50%) published after 2009 (Figure A2).

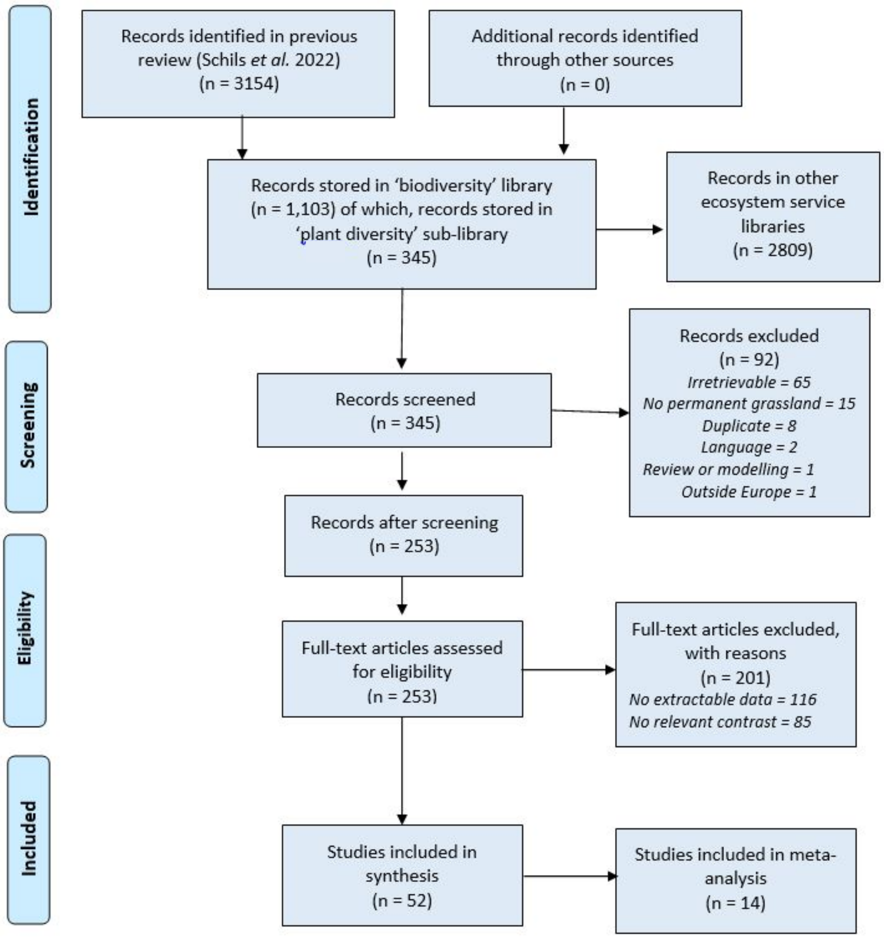

Figure A1. Prisma flow diagram for screening, exclusion and full-text assessment in this study (adapted from [52]).

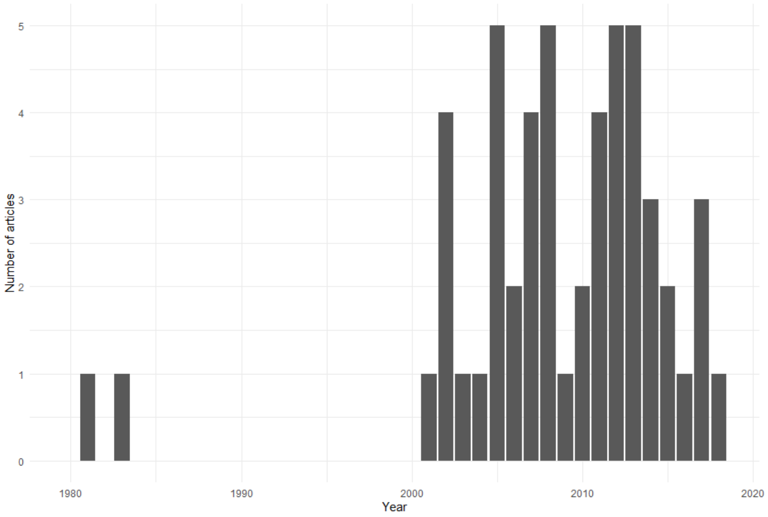

Figure A2. Year of publication of 52 articles that investigated the effect of nitrogen fertilisation on plant diversity on European permanent grasslands.

The 52 studies were conducted in 16 of the EU-27 member states and the United Kingdom, where permanent grassland contributes between 15 and 91% of each country's total Utilised Agricultural Area (UAA) (Eurostat, 2020). Fourteen of these studies (27%) were conducted in Romania, despite just 34% of Romania's UAA consisting of permanent grassland (Eurostat, 2020). The United Kingdom and Germany were the two next greatest contributors to the total number of studies, with five each (Figure A3). There was no relationship between the number of eligible studies conducted and the total percentage UAA of permanent grassland in each country ($r(14) = -0.06$, p = 0.82; Figure A4).

The majority (n = 36, 69%) of the 52 included studies that tested the effects of nitrogen fertilisation on plant diversity did so with no manipulation of defoliation (cutting and/or grazing) either within or between nitrogen fertilisation rate contrasts. Eleven studies (21%) also manipulated cutting rates, three manipulated grazing frequency and/or density (6%), and two (4%) manipulated both cutting and grazing as well as nitrogen fertilisation rates (Table A1).

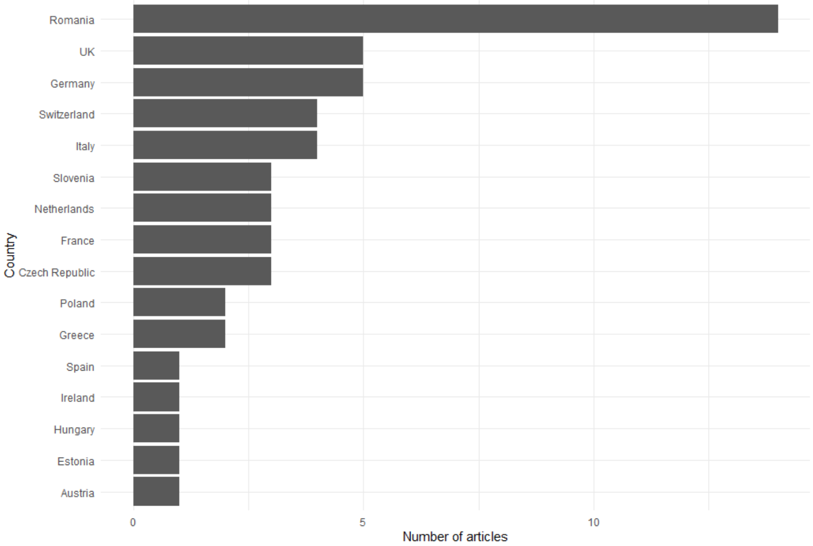

Figure A3. Number of included articles and the country in which they were conducted. Note that whilst 52 articles were included, the total here sums to 53 since one study was conducted in both France and Switzerland and is included in the count of both countries here.

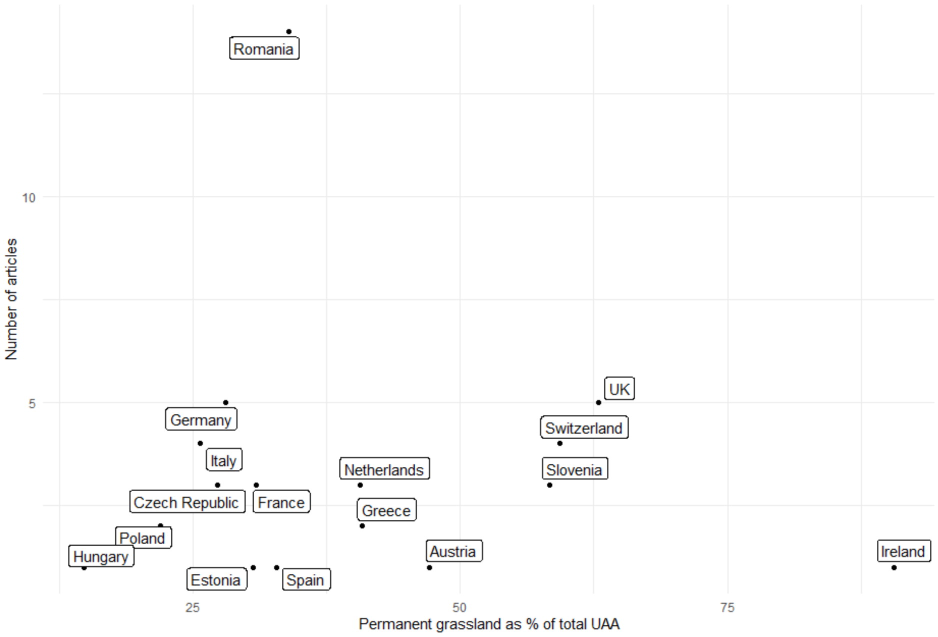

Figure A4. Relationship between number of included articles and the share of UAA consisting of permanent grassland in 16 countries from which the 52 included articles were conducted. There was no significant relationship (see text).

Table A1. Number of included articles that contained contrasts of nitrogen fertilisation and defoliation rates.

Contrast(s)	Articles (n)
Nitrogen fertilisation	36
Nitrogen fertilisation and cutting	11
Nitrogen fertilisation and grazing	3
Nitrogen fertilisation, cutting and grazing	2

Table A2. Summary of co-author ratings of strength of evidence components.

Category	Rating	Rationale
Risk of bias (RoB)	High	We rated the overall RoB as high, mostly driven by a lack of specificity of measurements making changes in plant species richness difficult to directly attribute to the addition of nitrogen fertilisers. High heterogeneity between studies (inconsistency) make generalisations difficult. Limitations in study design and execution compared to, for example, randomised control trials, also increase the overall RoB.
Indirectness	High	Owing to study designs, and our aim to include studies most relevant to 'real-world' agricultural practice, indirectness of measurements is considered to be high.
Inconsistency	High	Figure 1 shows high heterogeneity and inconsistency between studies, making generalisations difficult and highlighting the importance of contextual factors.
Imprecision	High	Our findings suggest that local context, (some of which we included, i.e., defoliation, baseline richness, etc., and other factors which we did not include, i.e., soil type, landscape history) may be more important than the strength or shape of the effect of nitrogen fertilisation, which overall explained a low amount of data variation.

Table A2. *Cont.*

Category	Rating	Rationale
Publication bias	Low	Funnel plot examination showed no great evidence of publication bias, however it is reasonable to expect that some studies finding no significant effect (in either direction) were not published and therefore not included here. Furthermore, our review is biased towards studies published in the English language.
Magnitude of effect	Low	The majority of studies found a low magnitude effect, and over 40% of included effect sizes had confidence intervals that crossed 0 (i.e., no effect)
Overall Strength of Evidence	Low-moderate	An overall negative effect was observed, and we have reasonable confidence in this. This confidence is constrained by the number, size and quality of individual studies, high inconsistency in effects between studies, and lack of information at some scales of fertilisation and species richness. More information has a high potential to change this conclusion.

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