Dear Dr Sébastien Barot

Thank you for your helpful comments and those from the reviewers on our manuscript.

All reviews were very constructive and we think they really help to improve the manuscript quality.
We provide below in red font, a detailed point-by-point response to the reviewers' comments, with explanations of the changes we made and their locations in the text.

All comments have been addressed.

We hope that this new version will be suitable for publication.

Yours sincerely,

Nicolas Legay, on behalf of all co-authors**.**

Review of Vincent Maire 19th of October 2018:
The manuscript, entitled "Studies of NH_4^+ and NO_3^- uptake ability of subalpine plants and resource-use strategy identified by their functional traits" by Fabrice Grassein and coauthors proposes in a research paper to study **the intraspecific variability of leaf and root functional traits related to plant nitrogen nutrition along an intensity management gradient and throughout two seasons for three grass species.** The objective of the paper is to study **how root functional traits are related with the leaf economic spectrum** and **to test if the root nutrition strategy for nitrogen (maximal uptake of N, preference for NO_3^- or NH_4^+) is related with the leaf N utilisation strategy** as defined according to the leaf economic spectrum. This manuscript is written with a good English, and results are of interest for the readership of PCI Ecology. The study will be particularly relevant in plant functional ecology and grassland ecology disciplines.
I found that the study suffers from **two main major issues** that prevent to give my support for acceptance. Let me explain: All the introduction, theoretical and statistical approaches as well as objectives and discussion of the study are presented to focus on differences between species.

However, the study design and sampled data (including the number of species which is three) are mainly based to test the differences within species.

There is then a discrepancy between what the authors want to test and their message and what the data permit to do. For instance, the leaf economic spectrum or the NO_3^-/ NH_4^+ trade-off have been observed at the interspecific level. However, you cannot expect to address interspecific differences with three species. In addition, it is not really clear if interspecific patterns occur at the intraspecific level too. It seems that correlations between traits involved in the leaf economic spectrum are weaker or even broken at intraspecific levels (Messier et al 2010 ELE; Derroire et al 2018 Scientific Reports; Fajardo and Siefert 2018 Ecology; Osnas et al 2018 PNAS). At least, one would expect to show how the trait variations of this study fit with the worldwide or grassland LES or with the NO3-/NH4+ trade-off as the data are easily available in the literature or through TRY database. Then, I strongly suggest to the authors to better streamline the conceptual framework on which their interesting results will be interpreted. I do not think that LES or NO3-/NH4+ are the good ones. I suggest to better focus on the intraspecific variation across the season and along the management gradient.

RESPONSE: Thanks for these remarks. In the discussion, we clarified the interpretation of our results taking into account our experimental design and statistical analyses. Also, we answered in more details below to the questions and comments of the two reviewers.

The second important issue concerns how authors define the nitrogen uptake term, which is key in their study but has not been clearly defined. All over the article, we have the feeling that nitrogen uptake is the total nitrogen that plant acquire along the season. However, this is the root instantaneous maximal uptake rate of either nitrate or ammonium and the leaf N content that have been measured and used in results. By the way, the **root instantaneous maximal uptake rate of total mineral nitrogen does not appear in the results**. Altogether, this leads then to many confusions when authors discuss their results (for instance L336-337). It will be very important to clearly define what plant N uptake is to further understand the results and their interpretation.

RESPONSE: Indeed N uptake here is NH_4^+ and NO_3^- uptake as mentioned in the title, in the key words section, and in the introduction. Anyway, we agree that this could be even clearer for the reader. We therefore modified the abstract and the introduction accordingly by replacing N by NH_4^+ and NO_3^- when necessary.

Other important issues but less problematic to me are listed below:

Abstract

The abstract is too general and do not give enough result to understand what have been done. How many species, many grasslands, and seasons did you investigate for instance?

RESPONSE: Thanks for this remark, we added the requested information.

L29 What is the plant resource economic spectrum RES? It is defined neither in the abstract nor in the introduction.

RESPONSE: We agree and decided to replace it by “the whole-plant economic spectrum” (L33).

Introduction

L11-12 The leaf economic spectrum seems to not have been fully understood. First, some important traits of the LES were not measured for this study but are central to the LES: leaf photosynthetic capacity and leaf lifespan for instance.

RESPONSE: We are not sure to understand the point here. First the line numbers do not match with those of the introduction. Second, though we agree that we did not measure all traits of the LES and that some of them would have been of interest, we did not mention that they were actually measured in our study.

Second, high nitrogen use efficiency belong more to the conservative strategy rather than the exploitative one.

RESPONSE: Although we agree with this point it is important to note that the spectrum is continuous, rather than divided into distinct categories either by growth form or by habitat. Furthermore, we did not look at NUE per se

Third, photosynthetic capacity and photosynthetic N use efficiency are two different variables with different meaning but are presented as the same variable (L49).

RESPONSE: Thanks for this remark; we corrected this mistake (L50).

L50: Reich et al 1998 is lacking in the reference list.

RESPONSE: Actually we were referring to Reich 2014, thanks for pointing this out (L50-51).

L6-74 One would consider that some N transporters are constitutive while others are adaptive. As such, it is important to study interspecific differences for N, Nitrate and Ammonium at a given site, which is not the case in the study. Otherwise, it is difficult to interpret differences as resulting from species differences. Please mention this point.

RESPONSE: L73, the sentence "N uptake ability also differs between species…"was replaced by "NH_4^+ and NO_3^- uptake ability also differs between species…". Anyway, we agree with this comment which has been included in the text (L75-79).

L83-L92 This paragraph needs to be rewritten. First, it is not clear why a methodological point is introduced in the introduction. It seems to me that it is not important to present this here. Second, this paragraph is very unclear suffering from a bad logical flaw and very long sentences.

RESPONSE: We agree and moved parts of these sentences to the M &M section (L176-180). We also corrected the flaw of the paragraph.

L94 What do you mean by 'contrasted LES'. There is only one universal leaf economic spectrum. Do you mean contrasted leaf economic strategies?

RESPONSE: Thanks for this comment, we corrected accordingly (L90-91).

L101 It seems that the objective 2 has never been statistically tested. I do not see any result where the total nitrogen uptake is analysed.

RESPONSE: We agree that the hypothesis needed clarification and we specified that the N uptake is ammonium and nitrate (L98). This hypothesis was statistically tested and results were presented in the figure 3 & 4.

L103. The number 3 of the third hypothesis seems to be wrongly located in the sentence. I would place it before 'As functional traits…'

RESPONSE: Done (L99)

Method

L109: Please quote table 1.

RESPONSE: Done (L107)

L161 and L192 - CaSO4: It seems problematic to me to use the same solution in order to preserve the integrity of roots before uptake measurement as well as to stop the uptake of nitrate and ammonium at the end of the root incubation. Please clarify this.

RESPONSE: The first solution of $CaSO_4$ used was maintained at 4°C to remove unlabeled nitrate and unlabeled ammonium from the surface of the root and to maintain the functional integrity of the roots. The second $CaSO_4$ solution was maintained at 4°C to remove the labeled nitrogen (^{15}N) at the root surface but also to stop any metabolic process (including any efflux of ^{15}N).

L159-164: The paragraph was rewritten to clarify the protocol used in the present study.

Data analysis is another major issue of the article! First, it is totally obscure how many data are used for each analysis. For instance, one would expect to see 15 points (3 species* 5 individuals) or 24 points (4 habitats * 2 seasons * 3 species) on figure 2, while only 12 are presented without any explanation.

RESPONSE: The sampling design of plant species was already detailed in the text (L130-132 and 133-143) of the submitted version. We have not sampled all species in all habitats but only in the habitat where they are dominants and one habitat where they were all presents. Consequently, we have sampled *Dactylis glomerata* in Terraced Mown & Fertilized (TMF) and in Unterraced Unmown (UU) grasslands, *Bromus erectus* in Terraced Unmown (TU) and in Unterraced Unmown (UU) grasslands, and *Festuca paniculata* in Unterraced Mown (UM) and Unterraced Unmown (UU) grasslands. In total, we have 12 points (3 species*2 seasons*2 habitats per species). We added a sentence in this section to summarize our point number (L143).

Second, the statistical models used to test differences are not appropriate with the hierarchical (site, season, species, individuals) and unbalanced design of the data (not the same number of species for the different sites).

RESPONSE: We were aware about this unbalanced design and we took it into account in our statistical analyses. The statistical analyses in the table 2 used two-way ANOVA only to compare the effect of season and land-use within each species. In each case, we have the same number of sites and seasons for each species.
Concerning the figure 3 where we tested the effect of land-use and sampling time within each species on maximal uptake rate, we also took into account the unbalanced design. Indeed, we only tested for each species at a given site the effect of sampling time using one-way ANOVA whereas the effect of land-use was tested only within species using Student test.
Concerning, the figure 4, the mean we used to represent our results could have led to a misinterpretation of our statistical analyses. In fact, we tested the effect of land-use and sampling time within each species using two-ways ANOVA and, as sampling time had no effect, we only illustrated the land-use effect within a species using a star in the figure. Then, we tested only in UU grasslands, the species effect. The differences between species were illustrated with lower case letters but we agree that the letter above the horizontal bars could have led to confusion. We modified the figure in the revised version and clarified the caption of the figure 4.
For the table 4, we tested the effect of land-use on soil properties using one-way ANOVA. For this, we have the same number of soil replicates and we think that the method used was appropriate.
We also detailed in the method section, the different statistical analyses used in each case (L210-225).

L211: Km, which seems to be N affinity, has not been defined. Please remove it as you do not use it in the study. Finally, it is not clear how each statistical tests address each of the three hypotheses of the introduction. Please give more details.
RESPONSE: Thanks for this remark; we removed Km from the paper (L215). To help the reader to understand which statistical test addressed each of the three hypotheses, we added some information and rephrased a sentence (L216-218).

Result
L234: Looking at table 3, LNC seems also a good predictor of nitrogen uptake.
RESPONSE: We agree, yet, our sentence was also correct as we say that no other trait (beside RDMC) is a <u>better</u> predictor than the functional axis 1.

Figure 2: Please use subscript and superscript in the y axis legend. Please use only two digits for root square
RESPONSE: Done

Discussion

L270-273 You use three negatives in the same sentence, resulting a very unclear sentence. Please remove at least two negatives.
RESPONSE: Thanks for this comment, we modified the sentence accordingly (279-281).

L274 It is unclear why 'similar' refers to. Please clarify it.
RESPONSE: Agree, we removed "similar" (L281).

L283-285 The statistics used here are not appropriate to test the causality and it is then not appropriate to say that leaf traits determine root traits. In addition, other studies show that root N maximal uptake rate is strongly correlated with leaf nitrogen content (Osone et al 2005 ABO; Soussana et al 2005 NPH; Osone et al 2008 NPH; Maire et al 2009 FEC), which is in contradiction with the assertion proposed in this sentence.
RESPONSE: Agree, our statistical tests are only showing relationships and not causality (L290-292). We also modified our sentences according to the reviewer suggestion (L292-295).

L305-306 It seems very contradictory with the sentence 283-285. Please clarify this.
RESPONSE: Thanks for pointing this out, we removed the end of the sentence (L312-314).

L322 TDN has been defined far away from this place. Please write total dissolved nitrogen
RESPONSE: Done (L328).
L336-338 'the effects of soil NH4+ : NO3- ratio concentration on plant N uptake' are not strictly presented in your results. Please do it if you want to assert some purpose.
RESPONSE: We agree that we did not strictly test the relationship between soil parameters and plant N uptake rates (as acknowledged L350-351). We argue that we can discuss this because our results (based on NH_4:NO_3 uptake ratio) at least partially support that plant N preference depends on soil availability of the various N forms.

L391-395 Only one year has been investigated. It is then not possible to conclude on a seasonal effect. Multiple years of investigation would be necessary for that.
RESPONSE: Thanks for this remark. We agree that our study is only one-year long, and that a seasonal effect is therefore difficult to catch. Yet, here we compared two very distinct and typical alpine vegetation seasons (peak of biomass and senescence), which have been previously investigated and presented in other papers, some of which being cited here. Nevertheless, we added a sentence to take this remark into account (L400-401).

I read your paper with great interest and I belief it is very relevant to PCI Ecology readership, providing the consideration of the issues presented here.
Hope will the comments be useful.

Reviewed by anonymous reviewer, 2018-08-14 09:41

The ms compared for three grasses in four different grasslands (but only one grassland contained all three species!) patterns of ammonium and nitrate uptake capacity in relation to several aboveground and belowground functional traits. When reading the ms I noted that very little literature post-2010 was cited. I found this curious, as the field of root traits (and their possible coordination with leaf traits) is a flourishing research field. However, the explanation is simple: the ms is just around four years old; and rather than let it rest in peace the authors decided to submit it.

RESPONSE: Indeed this paper has been submitted in a different journal in 2014 and, after the rejection, the lead author of the previous version decided not to resubmit the paper. However, most of us thought that the results deserved publication and that PCI Ecology was appropriate to give a visibility of this paper. We are sorry concerning the literature, and we are aware that only 1/3 of the cited paper was post-2010; we have partly corrected this by updating some references even if older papers do not mean bad science.

However, they cited a paper by Grassein et al. in press (l. 467; note that in the text, but not in the references there are papers by Grassein et al. 2010, 2015 & in press). But the paper in press (in Ann. Bot.) was published in January 2015 (with on-line availability at the end of 2014). As I do not think it is the duty of reviewers to come up with suggestions of important literature, when the authors could easily have done so themselves, I will not go in details where the paper could be improved.

RESPONSE: Thanks for these comments. Our manuscript has its own long history and we apologize for these discrepancies that are now corrected. We did our best to better refer to an up-to-date literature (there is 18 references on 54 which were published post-2010. Moreover, at less 12 references concerned plant physiology papers on nitrogen uptake in controlled conditions. This subject was mainly studied in 90's that explains why we have numerous old papers.

The paper is also sloppy in other respects. The description on functional traits is partly repetitive (SRL is mentioned twice),

RESPONSE : corrected (L169)

while the paper makes inconsistent claims how long excised roots were stored before measurements (l. 161 mentions less than 2 hours, l. 177 less than sixty minutes).

RESPONSE : For consistency, we corrected the time during which roots stayed in the solution before measurements (L162) and entirely described the method used (L157-164). We are sorry for this mistake but these two sentences are not contradictory since root samples must be processed within three hours after excision and we did it within sixty minutes.

Again, these mistakes should have been seen by at least one of the six authors!

RESPONSE: Apologies again.

I will only give a few examples where I think that more careful ecological considerations would have been important.

The authors decided to measure Vmax rather than Km (although the text in l. 211 suggests that the latter parameter was also measured but not reported). However, they did not provide a rationale for it. As they studied the high-affinity uptake systems they looked at uptake at low concentrations. Under such conditions plants may be selected to optimise Km to ensure a sufficient influx of nutrients under conditions of low availability. While Vmax is potentially important (especially in cases of clear nutrient pulses, e.g. immediately after snow melt or after sufficient moisture at the end of the summer), I think the authors should have presented both parameters and should give a rationale why maximum uptake capacity rather than Km would fit better with a plant economics spectrum.

RESPONSE: We have decided not to show Km data since we considered that they were of no interest for this paper. Indeed, this variable is useful only when ammonium and nitrate soil concentration are expressed in function of the soil solution. Here, we only measured soil ammonium and nitrate concentrations per gram of soil after K_2SO_4 soil extraction, so we have suppressed the mention of Km and only presented Vmax data.

The authors also report to have measured δ15N at natural abundance (l. 169) but do not report them. While there has been substantial debate to what extent these data relate to differential uptake from separate sources (peoples have overconfidently used such data to link these results to mycorrhizal preferences for various N sources) and / or to isotopic discrimination during uptake, I think it would have been helpful if the authors has measured the 15N signature of both ammonium and nitrate in their soils. Such data could also increase the ecological significance.

RESPONSE: We have indeed measured the δ15N at natural abundance but only for root and leaf tissues. This allowed us calculating isotopic enrichment of plant tissues and consequently plant N uptake rates. However, we did not measure the δ15N at natural abundance of both ammonium and nitrate in our soils; this is the reason why these data were not reported in the paper.

The authors decided not to supply both nutrients at the same time, but I did not read a rationale why this would have been important. Considering that N-uptake has a major effect on the proton balance in the rhizosphere, may plants would likely take up both with a mixed supply to minimise pH-changes in the rhizosphere. In terms of functionality, such a mixed uptake system may be very relevant. A view that looks at these as independent (and synergistic –l. 298l; how was this tested?), misses functionality of uptake systems.

RESPONSE: This is a good point. Yet, in our study we were specifically interested in discriminating the plant NO_3 vs. NH_4 uptake individually to avoid possible interaction (see L188-190 of the first version). Also, we are interested by plant uptake preferences and unless both N forms were labeled differently (not both with ^{15}N), it is not possible to

differentiate which form is taken up preferentially by the plant when both labeled N forms are mixed as suggested.

The design is pretty minimalistic (three grass species; one grassland in which all three species occurred; and three grasslands where only one species was found). And disentangling species effects from site effects is only possible with n = 2.

RESPONSE: Indeed, this is typically an exploratory study managed by Ph.D. students and postdocs with no extra funding, which explains this minimalistic design. Yet, we explained the sampling strategy in the manuscript (L116-122 of the first version and 136-143 of the revised version) and we believe it still carries some interesting results for the community.

In order to increase significance of their data, the others sampled more plants per site. At a distance of a few meters, one could differ in the opinion whether these constitute 'true' replicates or pseudoreplicates (which increase the degrees of freedom and hence the significance of statistical tests; only Fig. 2 seems to aggregate the individual root samples into one average value with 12 data points).

RESPONSE: We explained that we sampled five individuals that were at least 2 m apart and considered as genetically distinct (L136-138, first version and 134-136 of the revised version). Consequently, we do not think that these individuals are to be considered as pseudo-replicates.

The other sampled during two seasons and described the roots sampled (l. 160) as young fine roots. There is little detail and one wonders whether root age and root nutritional status (rather than seasonality) contributed to seasonal differences. (There could also have been differences in mycorrhizal colonisation in both seasons, and even though these three species show only limited growth responses to mycorrhiza, it does not follow that differences in uptake patterns are related to difference in mycorrhizal fungal mycelium surrounding the roots).

RESPONSE: We sampled roots at two distinct times during the vegetation season. Although we only used young fine and living roots due to the standardized protocol, we think it implicitly means that both sets of roots are integrating different ages and nutritional status.