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Biogeosciences Discussions

Effects of elevated CO_2 and extreme climatic events on forage quality and *in vitro* rumen fermentation in permanent grassland

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Abstract. This study was aimed at analyzing changes in botanical and chemical composition, and *in vitro* rumen fermentation characteristics of an upland grassland exposed to climate changes in controlled conditions except for light intensity, which was the natural one. Grassland was exposed to future climate scenario coupled with CO_2 treatments (390 and 520 ppm) from the beginning of spring. During summer, an extreme event (two weeks of increased temperature, +6°C, associated with severe drought, ECE) was associated. After the ECE, a recovery treatment was performed. Three cutting dates in April, June and November were considered. Our results indicate that increases in greenness, nitrogen (N) content and changes in water-soluble carbohydrate profile for the cut of November result in higher *in vitro* dry matter degradability (IVDMD) in the rumen. The neutral detergent fiber:nitrogen (NDF:N) ratio appeared to be a main driver of forage quality affected in opposite ways by elevated CO_2 and ECE, with a strong impact on rumen fermentation. A trend towards an interaction between atmospheric CO_2 concentration and ECE was observed in IVDMD, indicating that their effects could partly offset each other. These findings indicate that the different factors of climate change have to be considered together to characterize their effects on forage quality and use by ruminants.

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

1 Introduction

Global livestock production has substantially and rapidly increased in recent decades as a result of world population growth and a shift towards diets with a higher animal protein content in many countries (Tilman and Clark, 2014). The livestock sector is considered to be a significant contributor to global human-induced greenhouse gas (GHG) emissions (Gerber et al., 2013). In particular, energy and protein losses occurring during ruminant digestion in the form of enteric methane (CH_4) and ammonia (NH_3), urea, and nitrous oxide (N_2O), respectively, are important sources of GHG. Using 34 and 298 for a 100-year timescale as global warming potential for CH_4 and N_2O (compared to CO_2), respectively (IPCC, 2013), the livestock supply chains emitted an estimated total of 7.1 gigatonnes CO_2-eq per year, ruminants being by far the largest contributors (Gerber et al., 2013). Recent studies show that atmospheric CH_4 grew very rapidly in the last years (Nisbet et al., 2019) and that the contribution of livestock to these emissions may have been even underestimated (Wolf et al., 2017). Reducing GHG emissions appears to be crucial for Earth system management and there is an important mitigation potential in the ruminant sector (Herrero et al., 2016).

The largest part of ruminant diets consists of plant material under the form of forage. Globally, grasses comprised 48% (2.3 billion tons) of the total biomass used by livestock (4.7 billion tons) in 2000 (Herrero et al., 2013). Forage quality appears to be an important lever of action to decrease GHG emissions as variability in forage nutritive value has been shown to generate variability in the level of CH_4 emissions from ruminant (Thornton and Herrero, 2010). Forage quality is closely linked to its ingestibility and digestibility, which largely depend on the concentrations and nature of the major macronutrients such as structural and non-structural carbohydrates or crude proteins (Fig. 1). Digestibility is the main driver of the net energy of diet, rumen microbial synthesis and production of volatile fatty acids (VFAs), which are the main source of energy for the ruminant (INRA, 2018). On the other hand, digestible organic matter (OM) is the best indicator of CH_4 emissions as it is produced by the fermentation of carbohydrates (Fig. 1, INRA, 2018). In the specific case of permanent grasslands, forage quality is affected by agricultural practices and is mainly driven by the botanical composition consisting of a large diversity of self-seeded grasses and dicotyledons, and a broad variability in phenology and competitiveness (Rossignol et al., 2014; Andueza et al., 2016).

Forage quality is also possibly impacted by elevated temperatures, and by the intensity and frequency of extreme climatic events (ECEs) including droughts and heat waves, which are expected to increase (Planton et al., 2008). For instance, by modeling the variation in the nutritive value of forage species growing across a range of bioclimatic zones, Lee et al. (2017) showed that higher temperatures reduce forage nutritive value, likely due to changes in species identity, physiology and phenology. These authors also indicated that elevated temperatures may concomitantly increase CH_4 production by 0.9 % with a 1 °C temperature rise and 4.5 % with a 5 °C rise. Interactions of ECEs with elevated CO_2 in terms of grassland ecology and forage quality are not well known, especially for permanent grasslands (Dumont et al., 2015). There is a need for knowledge of the drivers of forage quality under such climatic conditions in order to adapt grass-based ruminant systems to the global change context.

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

The aim of this study was to analyze changes in the botanical and chemical composition of plants from an upland grassland when exposed to elevated atmospheric CO_2 level combined or not with an ECE (drought combined with a heat wave) in controlled conditions. The impact of these changes on ruminant digestion was investigated by determination of *in vitro* rumen fermentation characteristics. The hypothesis tested was that several drivers of global change (elevated CO_2 and ECEs) may have different effects on forage characteristics and digestion by ruminants with potential offsets.

2 Materials and methods

2.1 Experimental design

The experiment was previously described by Roy et al. (2016) and Volaire et al. (2020). The present study tested the forage quality response of a plant community consisting of an upland grassland near Saint-Genès-Champanelle (central France) to future climate scenarios projected for the 2050s (Ciais et al., 2005). For the representative year 2045 given by the ARPEGEv4 atmosphere-ocean general circulation model with the A2-CO_2 emission scenario (Nakicenovic et al., 2000), and using a multivariate statistical downscaling methodology (Boé et al., 2006) to generate projections over 8 × 8 km grid, the projected annual means for air temperature and precipitation at Saint-Genès-Champanelle were 10.9 °C and 770 mm, respectively. These values correspond to +2.3 °C and -33 mm, compared to the 1990–2009 air temperature and precipitation means, respectively. Therefore, the baseline climatic conditions of this experiment were drier and warmer compared to the origin site (Saint-Genès-Champanelle). Moreover, the atmospheric CO_2 concentration forecasted for the 2050s (according to the A2 scenario) was 520 ppm compared to 390 ppm measured in 2010.

In June 2009, 48 monoliths of 1 m^2 area formed by undisturbed soil and vegetation from an extensively managed upland semi-natural grassland, were excavated up to 60 cm depth. The origin site (Redon, 45°43' N, 03°01' E, 800 a.s.l.) was located near Saint-Genès-Champanelle. The average botanical composition of the plant communities was initially dominated by C_3 perennial grasses (60%), legumes (35%), and forbs (5%). The soil of this site is a Cambisol, namely 59.5% sand, 19.2% silt, and 21.3% clay with a pH of 5.9. The 48 monoliths after excavation were transferred to the INRAE research station (Clermont Ferrand, 45°46' N, 03°08' E, 350 a.s.l.) where, between natural precipitation and additional irrigation, the soil water content (SWC) was maintained near 80% of the field capacity.

At the beginning of 2010, the monoliths were transported to the Ecotron CNRS near Montpellier (43°40′N, 03°52′E). In each of the 12 Ecotron macrocosms, were allocated four monoliths representative of the botanical composition in the original grassland. They were exposed from April 2010 to early March 2011 to the future climate scenario forecasted for 2045, according to the ARPEGEv4 model, and to current CO_2 concentration. During this time, the Ecotron climate- regulation system monitored the air temperature and humidity hourly means and daily precipitation. From mid-March 2011 to November 2012, six macrocosms randomly selected were exposed to 520 ppm CO_2 and six to 390 ppm CO_2.

For each CO_2 concentration treatment, a first phase of the ECE treatment (reduction of 50% precipitation) was applied on three out of six monoliths from 25 June to 21 July. A second phase of the ECE treatment (no irrigation and an increase in air

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

temperature of 3.4°C considering the 2050s mean) was applied from 22 July to 4 August. This temperature increase corresponded to 7.1 °C above the average in 2000-2009 at the same period, and was above the 14 consecutive hottest day mean of the summer 2003. From 5 to 31 August, irrigation was progressively applied in the treatment with ECE to allow same cumulative precipitation as in the non ECE treated monoliths. From the end of August until the beginning of November, all macrocosms were exposed to the 2045 climate conditions. Each of the four experimental treatments, combining both CO_2 and ECE treatments was replicated three times. For further details on the experimental conditions, see Roy et al. (2016). In each of the 48 monoliths, SWC was continuously measured at 7, 20 and 50 cm soil depth using Time Domain Reflectometry (TDR) probes (IMKO, Ettlingen, Germany) and averaged across soil depths and monoliths in order to get one value per macrocosm. In addition, to match with data on forage quality, SWC was averaged across regrowth periods before the cuts, i.e. from 1 April to 26 April, from 27 April to 9 June, and from 22 September to 3 November.

2.2 Plant materials, cutting and botanical composition

On three dates (26 April, 9 June, 3 November), above-ground biomass was cut on a fixed center square (0.5 × 0.5 m^2) in each monolith using a precision mower (6 cm cutting height). The cut of April was the expression of the winter and spring growth and no treatments were applied except for one month of CO_2 treatment, while the cut of June was the expression of spring and early summer growth and of CO_2 treatment. The cut of November was the expression of summer and fall growth, and included the ECE, the CO_2 treatment and the recovery phase.

The cut material was weighed to determine fresh above-ground biomass and was separated into three subsamples: the first sample was oven-dried at 60°C for 72 h and used for dry matter (DM) determination and chemical analyses; the second was freeze-dried and used for the *in vitro* rumen fermentation assay; and the third was used to sort out green, dead and flower biomass, and to determine the botanical composition of the green material. Species were sorted by hand, oven-dried (60°C, 72 h) and weighed separately, in order to calculate the relative abundance for each species and then to define the functional groups: grasses, legumes and forbs (shown as relative abundance).

2.3 In vitro rumen fermentation assay

For each macrocosm and each cut, a representative sample was reconstituted with cut freeze-dried material from the four monoliths, weighted according to the values of above-ground biomass measured on each monolith (thereby pooling the four monoliths, n = 12). For each cut, the rumen fermentation assay was performed on the 12 samples, three times over a period of two weeks.

All experimental procedures were performed according to the European Union Directive 2010/63/EU, reviewed by the local ethics committee (C2E2A, “Comité d’Ethique pour l’Expérimentation Animale en Auvergne”) and authorized by the French Ministry for Research (no. CE 69-12).

Freeze-dried plant material (600 ± 0.5 mg) was transferred in 120 mL serum bottles, pre-warmed at 39 ± 0.5 °C and flushed with N_2 to eliminate the oxygen. Buffered rumen fluid (40 mL) was added to the serum bottle, which was subsequently sealed

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

hermetically with a butyl rubber stopper and aluminum crimp seals. The buffered rumen fluid was prepared as follows: rumen contents were collected before morning feeding from three cannulated sheep fed daily with 1200 g of a diet composed of 80% of permanent grassland hay and 20% concentrate mix. Rumen contents were mixed in the same proportions in a container and squeezed through two layers of cheesecloth (mesh opening 800 µm) to obtain the fluid used as inoculum for the *in vitro* rumen fermentation assay. Strained rumen fluid was diluted in an anaerobic buffer solution (phosphate:carbonate, 1:2 v/v) as described by Goering and Van Soest (1970) and modified by Niderkorn et al. (2011). The initial pH of the buffered rumen fluid was 7.03 ± 0.02. All bottles were incubated in a shaking water bath at 39 ± 0.5 °C, and blanks without any plant substrate (only buffered rumen fluid) were included. At t = 0, samples of buffered rumen fluid were taken to determine VFAs and NH_3 present in the medium before incubation.

After 24 h of incubation, the volume of gas produced in the headspace of the serum bottles was determined using a pressure transducer (Theodorou et al., 1994) and gas samples were taken for determination of CH_4 and CO_2 concentrations. Then, the whole contents of the bottle were transferred into a pre-weighed 50 mL Falcon tube, and the pH was immediately measured. Tubes were centrifuged at 3,400 × *g* for 10 min at 4 °C, and samples of supernatant were taken for determination of VFA and NH_3 concentrations (Niderkorn et al., 2011). To recover all the non-degraded particles, the bottle was washed twice with distilled water, and the washing water was transferred into the Falcon tube. Tubes were again centrifuged at 3,400 × *g* for 10 min at 4 °C, and after removal of the supernatant, the residue was used for DM determination.

2.4 Analytical procedures

Plant substrates and leftovers after the fermentation (residues) were analyzed for DM by oven-drying at 60 °C for 72 h, and OM by ashing at 550 °C for 6 h in a muffle furnace. The neutral detergent fiber (NDF) content in plants was determined according to the method described by Van Soest et al. (1991), using a Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA). The leaf carbon (C) and nitrogen (N) contents were determined at the isotopic platform of INRAE Nancy using a stable isotope ratio mass-spectrometer (Isoprime 100, IsoPrime, Manchester, UK). Water-soluble carbohydrates (WSC) were extracted from dry powder successively with 80% ethanol and water, according to Benot et al. (2019). Supernatants were pooled and evaporated under vacuum to eliminate ethanol and water, thus concentrating the samples. The residue was dissolved in water and passed through ion exchange resins to remove charged compounds before HPLC analysis. WSC were separated on a cation exchange column (Sugar-PAK I, 300×6.5 mm, Millipore Waters Milford, MA, USA) and detected using a refractometer (see Benot et al., 2019 for more details). Condensed tannin (CT) content was determined using the colorimetric HCl-butanol method (Grabber et al., 2013). Pepsin-cellulase OM digestibility was evaluated according to the method described by Aufrère and Michalet-Doreau (1988). *In vitro* DM degradability (IVDMD) was determined by the difference between the DM of plant material before the fermentation and the DM of fermentation residue after 24 h of fermentation. The concentrations of CH_4 and CO_2 in gas samples were determined by gas chromatography using a MicroGC 3000A (Agilent Technologies, France). Total and individual VFA (acetate, propionate, butyrate, valerate, caproate,

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

isobutyrate, isovalerate) in the supernatant were measured by gas chromatography and NH_3 was determined using the Berthelot reaction (Park et al., 2009).

2.5 Statistical analysis

All variables related to the chemical composition of plant communities and *in vitro* rumen fermentation parameters were analyzed using a mixed model (MIXED procedure, SAS Enterprise Guide 5.1, SAS Institute Inc., Cary, NC, USA). Each macrocosm was considered as an experimental unit. CO_2 concentration, the effect of the ECE and their interaction were used as fixed effects, and the cuts nested within the macrocosm were used as random factors. Data analysis was also carried out at each cut. For the cuts of April and June, CO_2 concentration was used as a fixed effect, and the effect of the ECE treatment and their interaction were added as fixed effects for the cut of November. For these analyses, the macrocosm was used as a random factor. Fractions (relative abundances) were transformed by the Arcsin of the square root before the analysis of variance. Significance was declared at $p \leq 0.05$ and trend at $0.05 < p < 0.10$.

3 Results

The real values of temperature and atmospheric CO_2 concentration throughout the experiment for the different treatments were reported in Roy et al. (2016). When the ECE was imposed, the mean daily air temperature peaked at 25 °C. As expected, the ECE strongly affected SWC during the stress period (Table 1, $p < 0.001$). A significant interaction CO_2 × ECE was detected during the period preceding the cut of November (Table 1, $p = 0.014$), reflecting a higher SWC for the control at 520 ppm of CO_2 compared to the other treatments.

3.1 Above-ground biomass characteristics and chemical composition

There was no significant difference among treatments on above-ground biomass, while more green material ($p = 0.013$) and less dead material ($p = 0.018$) with the ECE than for the control were observed for the cut of November (Tables 2 and S1, and seasonal pattern shown in Fig. 2). Regarding the relative abundance of the functional groups or the species, very few differences were observed between treatments (Tables 2 and S1, Fig. 3 and 4) due to a large variability among macrocosms, except for *Holcus lanatus*, which decreased dramatically after the ECE ($p = 0.001$). There was a cut effect linked to seasonality on above-ground biomass, the fractions of green and dead materials, flowers ($p < 0.001$), and relative abundance of several species, but without a significant cut × CO_2 effect (Table S2).

The N content in the above-ground biomass was significantly lower at 520 ppm CO_2 concentration compared to 390 ppm (Tables 3 and 4). This was shown in the cuts of April (-11%, $p < 0.001$), June (-9%, $p = 0.003$) and November (-21%, $p = 0.007$). Increasing the CO_2 level caused an increase in the OM content in April (+1%, $p = 0.033$) and a decrease in the NDF content in June (-3%, $p = 0.002$), while the C:N and NDF:N ratios increased for the three cuts ($p < 0.05$). After the recovery of the ECE in November, the N (+54%, $p < 0.001$), sucrose and fructose contents strongly increased (+31%, $p = 0.022$; +23%, $p = 0.031$, respectively). The ECE decreased the NDF content (-7%, $p = 0.027$) and the C:N and NDF:N ratios (-34% and -

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

39%, $p < 0.001$, respectively). Pepsin-cellulase OM digestibility was increased (+14%, $p = 0.005$). No effect of the CO_2 × ECE interaction was observed on the chemical composition of the above-ground biomass ($p > 0.05$). There was a cut effect on all the parameters of chemical composition except the C content, with a significant cut × CO_2 effect on the N content, and the ratios C:N and NDF:N ($p < 0.001$) (Table S3).

3.2 *In vitro* rumen fermentation characteristics

When IVDMD was estimated, it was significantly lower (-3%, $p = 0.041$) in plants exposed to 520 ppm CO_2 concentration compared to 390 ppm for the cut of April, while it tended to be lower for the cut of November ($p = 0.075$) (Tables 3 and 4). Increasing the level of CO_2 drastically decreased the NH_3 concentration in the incubation medium for the cuts of April, June and November (-21%, $p = 0.014$; -31%, $p = 0.001$ and -34%, $p = 0.005$, respectively), decreased the proportion of valerate for the cuts of April and June (-7%, $p = 0.016$ and $p = 0.017$, respectively) and increased acidification for the cut of November (+11%, $p = 0.007$). For the cut of April, increasing the level of CO_2 also tended to increase the total VFA concentration and to decrease the proportion of iso-valerate ($p = 0.056$ and $p = 0.062$, respectively), while the proportion of iso-butyrate tended to decrease for the cut of November ($p = 0.063$).

The ECE increased the IVDMD (+10%, $p = 0.001$) and the NH_3 concentration in the incubation medium (+90%, $p < 0.001$), the proportions of propionate, valerate and iso-valerate (+4%, $p = 0.008$; +21%, $p = 0.004$ and +25%, $p = 0.006$, respectively), tended to decrease the proportion of acetate ($p = 0.064$), whereas the acetate:propionate ratio, which is related to the fermentation pathways in the rumen, decreased (-5%, $p = 0.013$). There were also trends for the effects of interaction CO_2 × ECE on the IVDMD and iso-valerate concentration ($p = 0.053$ and $p = 0.067$, respectively).

A cut effect (seasonality) was observed on all the parameters of *in vitro* rumen fermentation parameters except for the concentration of iso-valerate and the CO_2:CH_4 ratio, with a significant cut × CO_2 effect on the concentrations of NH_3, valerate and iso-valerate (Table S3).

4 Discussion

4.1 How elevated CO_2 and ECE modify the above-ground biomass and botanical composition

In this experiment, above-ground biomass was not affected by elevated CO_2 or an ECE, probably because the control treatment was drier and warmer than the actual climatic conditions. The fact that the control was under little stress may have limited biomass and may explain why no difference was observed between treatments with and without an ECE, as expected. Although no overall effect of elevated CO_2 was detected on the plant fractions, we observed a significant increase of green mass for the control in November. This can be related to additional measurements made in the same experiment, indicating that elevated CO_2 increases leaf area index and canopy photosynthesis linked with higher SWC (Roy et al., 2016).

No effect of the ECE on above-ground biomass was observed for the cut of November, though there was more green tissue and less dead tissue compared to control. This could be attributed to a strong increase in the shoot N pool primarily due to an

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

effect of the ECE on the below-ground compartment (Roy et al., 2016). These authors have shown that ECEs strongly increased the root N pool, thereby increasing N availability. At this time, the interaction CO_2 × ECE indicated that SWC before the cut of November at a CO_2 level of 520 ppm was reduced with the ECE and was enhanced in the control compared to the other treatments. This could be explained by the enhanced Leaf Area Index, photosynthesis, greenness and C sequestration leading to higher water extraction from the soil after an ECE, corresponding to higher recovery under elevated CO_2 (Roy et al., 2016). The lack of significant differences in relative abundances of functional groups and species can be attributed to the huge variability in ecosystem responses to elevated CO_2 and ECE. A notable change was still observed through the high vulnerability of *Holcus lanatus* to the ECE that mostly caused the disappearance of this species. The role of soluble carbohydrate metabolism, in particular that of fructans and sucrose, explaining the lack of resilience of *Holcus lanatus* to ECE has been recently highlighted by Volaire et al. (2020).

4.2 How elevated CO2 and ECE modify the chemical composition of above-ground biomass

We observed contrasted effects of elevated CO_2 and ECE on the chemical composition of above-ground biomass. The lower N concentration under elevated CO_2 compared to control was shown for all the cuts and is consistent with what was described in the meta-analyses of Dumont et al. (2015) and Dellar et al. (2018). It should be noted that this was not accompanied by significant changes in legume proportions, which could have been an explanatory factor due to their high N content. The reduction in N content could be rather attributed to combination of increased growth and changes in photosynthetic N use efficiency (Leakey et al., 2009). Although the NDF concentration was only affected for the cut of June, the NDF:N ratio increased with the CO_2 concentration for all the cuts, but without a negative impact on pepsin cellulase OM digestibility. This parameter could have been affected as a high concentration of partially digestible fiber (NDF) and a limiting N concentration can be detrimental for the microbial ecosystem in the rumen (Sinclair et al., 1995). These results are globally well in line with those described in a recent study carried out on mixed grass prairie (Augustine et al., 2018) and a meta-analysis performed on the effects of climate change on European pasture quality (Dellar et al., 2018).

We observed a clear increase in N concentration in above-ground biomass for the cut of November. The dehydration of plant material during ECEs may have resulted in the asynchrony between plants and soil microbe functioning. High plant litter and microbial detritus during the ECE are both sources of energy for microbial recovery during rehydration, which is faster than in plants (Hofer et al., 2017). Mineralization of the OM by microbes produces inorganic N that can be taken up by plants only if they have recovered. In addition, it has been shown that the maintenance of root exudates during drought can be one of the reasons for above-ground recovery, since root functionality ensures increased N availability (Karlowsky et al., 2018). The flush of N can explain the increase of N and sucrose content in the above-ground biomass due to high photosynthesis and transpiration mirrored by lower SWC (Roy et al., 2016). An increase in fructose content, as we observed after the ECE, is usually linked to hydrolysis of fructans (Simpson and Bonnett, 1993), although we did not detect a significant decrease in the fructan content in this experiment. These changes due to the ECE led to a much lower (~ 50%) NDF:N ratio than for the

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

control. A low value for this ratio, in addition to effects on some sugars, is particularly beneficial in terms of forage quality, as confirmed by the greater values obtained for pepsin cellulase OM digestibility due to an increase in easily degradable nutrients providing increased amounts of energy and nitrogen for rumen microbial synthesis (Nocek and Russell, 1988).

4.3 How changes in chemical composition affect rumen fermentation parameters

The changes in chemical composition in above-ground biomass following the ECE strongly affected rumen fermentation parameters. The lower NDF content led naturally to increased IVDMD as some fibers especially lignin are known to be indigestible (Jung and Allen, 1995). This is consistent with the increase of pepsin cellulase digestibility, a parameter which has been shown to be closely correlated with *in vivo* digestibility (Aufrère et al., 1988). Interestingly, in spite of an increase in IVDMD, we did not find any of the usually observed increase in total gas production, including CH_4 (Getachew et al., 2004). This could mean that a potential increase of energy available for the animal was not accompanied by more energy losses and pollutant emissions. This could be due to the changes observed in VFA profiles (acetate, propionate and valerate) indicating changes in fermentation pathways. In particular, we observed a decrease in the acetate/propionate ratio, which is known to be related to hydrogen availability and CH_4 production in the rumen (Russell, 1998). The increase in N concentration after the ECE also led to an increase in ruminal NH_3, which is a main end-product of protein degradability through amino acid deamination. The increase of isovalerate with the ECE also indicates the increased degradation of protein as this branched-chain VFA results from deamination of branched-chain amino acids such as leucine (Menahan and Schultz, 1964). A part of the NH_3 produced is incorporated into the rumen microbial biomass, but the surplus is transformed into urea, which is excreted into the environment, representing N loss and pollutant emission. Indeed, the fraction of urinary N not used by soil microbes and plants is transformed into N_2O, a potent GHG, during the microbial processes of nitrification and denitrification (Firestone et al., 1980).

The changes of chemical composition under elevated CO_2 affected rumen fermentation parameters in a different manner compared to the ECE. Interestingly, we observed contrasted effects according to the date of cut. While elevated CO_2 decreased IVDMD for the cut of April, the inverse effect was observed in November, but only following the ECE, resulting in a trend for interaction CO_2 × ECE. This could indicate that the ECE counteracts the negative effect of elevated CO_2 on IVDMD, likely due to the decrease in NDF concentration mentioned above. However, it should be noted that we never observed any significant change in VFA production, which is a main driver of energetic value for the animal as VFAs provide more than 70% of the ruminant's energy supply (Bergman, 1990). For all the cuts, the decrease of the N content in above-ground biomass under elevated CO_2 led to lower concentrations of NH_3 in the rumen for the reasons given above.

Another relevant result obtained when analyzing the correlation matrix (data not shown) is the positive correlation between the CT content in above-ground biomass and the CO_2:CH_4 ratio in the fermentation gas ($r = 0.51$, $p = 0.002$), but also the negative correlations between the CT content and IVDMD ($r = -0.38$, $p = 0.024$) and total VFA production ($r = -0.40$, $p = 0.015$). The antimethanogenic effect of CT and the reduction of IVDMD are consistent with the literature data (see meta-

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

analysis by Jayanegara et al., 2012). However, in this experiment, although average values of CT content were higher following the ECE compared to control, the difference was not significant.

Fig. 5 provides a schematic and pedagogic overview of the results obtained in this experiment, showing the impact of the ECE and elevated CO_2 on the ruminants' digestive degradation of plant macronutrients (carbohydrates and protein). This suggests that the ECE, by increasing the N content in plants, increases nitrogen metabolism in the rumen, probably with a positive effect on rumen microbial synthesis, which may explain, with the lower NDF content in plants, the increase of IVDMD. In contrast, the elevated concentration of atmospheric CO_2 reduced the N content in plants and ruminal nitrogen metabolism, thereby having a negative impact on IVDMD.

5 Conclusions

Our study shows that the impact of different drivers of global change, namely elevated atmospheric CO_2 concentration and ECEs (drought and heat wave), have contrasted impacts on forage quality. A main result is the increase in greenness, and in N and water-soluble carbohydrate contents in the above-ground biomass produced during the regrowth stage following an ECE, resulting in higher digestibility. Overall, the NDF:N ratio appeared to be a main driver of forage quality, which is highly likely to be affected differently by elevated CO_2 and ECE, with a subsequent strong impact on rumen fermentation. In addition, the interaction CO_2 × ECE indicates that elevated CO_2 could limit the gain of IVDMD due to ECEs.

Author contributions

CPC designed the study, AMB, ALM, MLB, AA, MLD, VN and CPC contributed to acquisition of data, and contributed to the analysis and interpretation of data. VN wrote the manuscript with contributions from all co-authors.

Competing interests

The authors declare that they have no conflict of interest.

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Preprint. Discussion started: 11 January 2021

Biogeosciences Discussions Open Access EGU

Tables

Table 1. Effects of factors (*p*-values) on soil volumetric water content (SWC)

	1-26 April	27 April – 9 June	21 July – 3 August (stress period)			22 September – 3 November		
	CO_2 effect	CO_2 effect	CO_2 effect	ECE effect	CO_2 × ECE effect	CO_2 effect	ECE effect	CO_2 × ECE effect
SWC	0.736	0.430	0.775	**<0.001**	0.312	0.111	0.366	**0.014**
d.f (num/den)	1/10	1/10	1/8	1/8	1/8	1/8	1/8	1/8

d.f (num/den), degrees of freedom (numerator and denominator). ECE, extreme climatic event. In bold: $p < 0.05$; Underlined: $0.05 < p < 0.1$

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Preprint. Discussion started: 11 January 2021

Table 2. Effects of factors (*p*-values) on above-ground mass and fractions of green material, flower and dead material, and relative abundance of functional groups, the ten most abundant species and other species (< 15%) in plant communities subject to two levels of CO_2 concentration (390 and 520 ppm) with or without an extreme climatic event (ECE) at three different cuts

	Cut April	Cut June	Cut November		
	CO_2 effect	CO_2 effect	CO_2 effect	ECE effect	CO_2 × ECE effect
Above-ground mass	0.315	0.301	0.467	0.405	0.818
Fractions					
Green material	0.396	0.332	0.222	**0.013**	0.355
Flower	0.076	0.329	0.406	0.066	0.650
Dead material	0.267	0.718	0.278	**0.018**	0.417
d.f (num/den)	1/10	1/10	1/8	1/8	1/8
Relative abundance of functional groups					
Grasses	0.845	0.822	0.335	0.632	0.129
Legumes	0.451	0.986	0.647	0.339	0.310
Forbs	0.918	0.968	0.091	0.939	0.176
d.f (num/den)	1/10	1/10	1/8	1/8	1/8
Relative abundance of species					
Agrostis spp	0.305	0.989	0.987	0.070	0.126
Arrhenaterum elatius	0.429	0.802	0.811	0.544	0.414
Alopecurus pratensis	0.251	0.212	0.130	0.452	**0.026**
Dactylis glomerata	0.834	0.668	0.696	0.721	0.643
Holcus lanatus	0.693	0.999	0.475	**0.001**	0.379
Lolium perenne	0.497	0.777	0.736	0.732	**0.037**
Poa angustifolia	0.809	0.788	0.415	0.754	0.644
Poa pratensis	0.967	0.952	0.957	0.988	0.846
Trisetum flavescens	0.856	0.475	0.469	0.519	0.192
Ranunculus acris	0.299	**0.017**	0.584	**0.028**	0.166
Other species	0.377	0.443	0.426	0.201	0.324
d.f (num/den)	1/10	1/10	1/8	1/8	1/8

d.f (num/den), degrees of freedom (numerator and denominator). In bold: $p < 0.05$; Underlined: $0.05 < p < 0.1$

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Preprint. Discussion started: 11 January 2021

Biogeosciences Discussions Open Access EGU

Table 3. Chemical composition and *in vitro* rumen fermentation parameters of plant communities subject to two levels of CO_2 concentration (390 and 520 ppm) with or without an extreme climatic event (ECE) at three different cuts

	Cut April		Cut June		Cut November			
					Control		ECE	
CO_2 (ppm)	390	520	390	520	390	520	390	520
Chemical composition								
OM (g.kg^{-1} DM)	918 ± 6	926 ± 5	918 ± 4	921 ± 4	909 ± 10	918 ± 6	918 ± 2	909 ± 13
C (g.kg^{-1} DM)	448 ± 4	447 ± 2	448 ± 2	446 ± 3	444 ± 6	444 ± 3	449 ± 3	442 ± 8
N (g.kg^{-1} DM)	23.1 ± 1.1	20.5 ± 0.8	18.2 ± 0.5	16.5 ± 0.9	17.7 ± 1.5^{bc}	14.3 ± 0.6^{c}	27.6 ± 4.0^{a}	21.6 ± 1.6^{b}
C:N ratio	19.5 ± 0.9	21.8 ± 0.8	24.6 ± 0.7	27.1 ± 1.4	25.2 ± 2.3^{b}	31.0 ± 1.3^{a}	16.4 ± 2.2^{c}	20.5 ± 1.1^{bc}
NDF (g.kg^{-1} DM)	515 ± 23	513 ± 22	586 ± 11	568 ± 11	550 ± 8^{a}	546 ± 37^{ab}	534 ± 6^{ab}	490 ± 26^{b}
NDF:N ratio	22.3 ± 0.9	25.0 ± 1.2	32.2 ± 1.1	34.5 ± 2.1	31.2 ± 2.5^{b}	38.0 ± 1.3^{a}	19.5 ± 2.5^{c}	22.7 ± 0.5^{c}
WSC (g.kg^{-1} DM)	263 ± 36	241 ± 36	187 ± 27	192 ± 33	146 ± 39	194 ± 50	167 ± 36	195 ± 4
Glucose (g.kg^{-1} DM)	19.8 ± 4.2	17.7 ± 2.7	20.2 ± 3.7	16.8 ± 4.2	10.3 ± 2.7	7.8 ± 1.3	10.8 ± 1.7	10.6 ± 1.2
Sucrose (g.kg^{-1} DM)	39.7 ± 6.2	41.4 ± 9.1	29.4 ± 4.9	30.5 ± 6.5	18.7 ± 2.9	20.0 ± 3.4	23.0 ± 5.2	27.6 ± 2.5
Fructose (g.kg^{-1} DM)	21.6 ± 4.2	19.0 ± 3.6	20.5 ± 3.1	17.9 ± 4.5	13.0 ± 2.2	12.7 ± 2.1	15.1 ± 2.3	16.6 ± 1.0
Fructans (g.kg^{-1} DM)	182 ± 29	163 ± 22	117 ± 21	127 ± 22	104 ± 40	153 ± 47	118 ± 27	140 5
CT (g.kg^{-1} DM)	20 ± 7	16 ± 3	30 ± 14	31 ± 15	19 ± 3	17 ± 4	25 ± 13	21 ± 4
Pepsin-cellulase OM digestibility (g.kg^{-1})	775 ± 24	765 ± 28	644 ± 14	649 ± 47	610 ± 37^{b}	645 ± 56^{ab}	696 ± 42^{ab}	737 ± 2^{a}
In vitro rumen fermentation parameters								
Acidification (dpH)	0.91 ± 0.05	0.94 ± 0.05	0.82 ± 0.04	0.84 ± 0.03	0.69 ± 0.04^{ab}	0.77 ± 0.03^{a}	0.66 ± 0.03^{b}	0.73 ± 0.03^{ab}
IVDMD (g.kg^{-1})	602 ± 13	584 ± 14	525 ± 24	516 ± 19	515 ± 17^{b}	512 ± 17^{b}	541 ± 15^{ab}	584 ± 20^{a}
NH_3 (mmol.l^{-1})	9.60 ± 1.18	7.54 ± 1.20	7.10 ± 0.89	4.92 ± 0.67	7.99 ± 1.72^{bc}	4.18 ± 1.38^{c}	13.32 ± 2.23^{a}	9.80 ± 0.85^{ab}
Total VFA (mmol.g^{-1} DM)	8.10 ± 0.17	8.37 ± 0.25	7.58 ± 0.45	7.45 ± 0.27	7.14 ± 0.39	7.37 ± 0.05	7.42 ± 0.33	7.72 ± 0.55
Acetate (%)	63.5 ± 0.9	64.2 ± 1.1	64.5 ± 0.6	65.0 ± 1.3	65.2 ± 0.9	65.8 ± 0.5	64.8 ± 0.3	63.9 ± 1.5
Propionate (%)	23.7 ± 0.5	23.1 ± 0.7	23.0 ± 0.3	22.7 ± 0.5	22.2 ± 0.2^{b}	22.6 ± 0.4^{ab}	23.0 ± 0.06^{ab}	23.4 ± 0.7^{a}
Butyrate (%)	9.19 ± 0.42	9.27 ± 0.41	9.10 ± 0.32	9.12 ± 0.83	8.90 ± 0.35	8.67 ± 0.25	8.21 ± 0.12	8.79 ± 0.67
Valerate (%)	1.19 ± 0.04	1.11 ± 0.05	1.12 ± 0.04	1.04 ± 0.06	1.15 ± 0.12^{ab}	0.99 ± 0.07^{b}	1.31 ± 0.03^{a}	1.27 ± 0.12^{a}
Iso-butyrate (%)	0.93 ± 0.08	0.87 ± 0.10	0.85 ± 0.07	0.83 ± 0.16	0.97 ± 0.18	0.79 ± 0.09	1.07 ± 0.16	0.88 ± 0.02
Iso-valerate (%)	1.41 ± 0.10	1.27 ± 0.12	1.34 ± 0.07	1.25 ± 0.12	1.47 ± 0.22^{ab}	1.12 ± 0.11^{b}	1.61 ± 0.11^{a}	1.64 ± 0.15^{a}
Acetate:propionate ratio	2.69 ± 0.09	2.79 ± 0.12	2.82 ± 0.06	2.89 ± 0.11	2.95 ± 0.06^{a}	2.93 ± 0.07^{ab}	2.84 ± 0.02^{ab}	2.75 ± 0.13^{b}
Total gas (mmol.g^{-1} DM)	7.07 ± 0.23	7.14 ± 0.16	6.43 ± 0.41	6.41 ± 0.21	6.06 ± 0.16	6.13 ± 0.39	6.25 ± 0.28	6.50 ± 0.13
CH_4 (mmol.g^{-1} DM)	1.25 ± 0.03	1.26 ± 0.03	1.10 ± 0.08	1.11 ± 0.06	1.06 ± 0.05	1.08 ± 0.08	1.13 ± 0.05	1.12 ± 0.05
CO_2 (mmol.g^{-1} DM)	5.83 ± 0.22	5.86 ± 0.13	5.28 ± 0.29	5.31 ± 0.16	5.00 ± 0.14	5.06 ± 0.31	5.12 ± 0.22	5.38 ± 0.11
CO_2:CH_4 ratio	4.67 ± 0.16	4.66 ± 0.08	4.80 ± 0.08	4.81 ± 0.19	4.72 ± 0.20	4.71 ± 0.11	4.53 ± 0.04	4.80 ± 0.18

Data shown are means ± standard deviation. ^{a,b,c} For the cut of November, means in a given row with different letters differ ($p < 0.05$).

OM, organic matter; C, carbon; N, nitrogen; NDF, neutral detergent fiber; WSC: water-soluble carbohydrates; CT, condensed tannins; IVDMD, *in vitro* dry matter degradability, DM, dry matter; VFAs, volatile fatty acids

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Biogeosciences Discussions Open Access EGU

Table 4. Effects of factors (*p*-values) on chemical composition and *in vitro* rumen fermentation parameters of plant communities subject to two levels of CO_2 concentration (390 and 520 ppm) with or without an extreme climatic event (ECE) at three different cuts

	Cut April	Cut June	Cut November		
	CO_2 effect	CO_2 effect	CO_2 effect	ECE effect	CO_2 × ECE effect
Chemical composition					
OM	**0.033**	0.158	0.973	0.989	0.123
C	0.547	0.333	0.299	0.603	0.304
N	**<0.001**	**0.003**	**0.007**	**<0.001**	0.346
C:N ratio	**<0.001**	**0.003**	**0.002**	**<0.001**	0.425
NDF	0.883	**0.018**	0.108	**0.027**	0.175
NDF:N ratio	**0.001**	**0.038**	**0.002**	**<0.001**	0.128
WSC	0.337	0.784	0.112	0.616	0.662
Glucose	0.379	0.170	0.227	0.147	0.303
Sucrose	0.712	0.744	0.198	**0.022**	0.452
Fructose	0.306	0.262	0.619	**0.031**	0.473
Fructans	0.261	0.445	0.106	0.986	0.515
Condensed tannins	0.252	0.887	0.495	0.295	0.788
Pepsin-cellulase OM digestibility	0.518	0.808	0.139	**0.005**	0.903
d.f (num/den)	1/10	2/10	1/8	1/8	1/8
In vitro rumen fermentation parameters					
Acidification (dpH)	0.201	0.560	**0.007**	0.112	0.734
IVDMD	**0.041**	0.496	0.075	**0.001**	0.053
NH_3	**0.014**	**0.001**	**0.005**	**<0.001**	0.882
Total VFA	0.056	0.577	0.256	0.186	0.871
Acetate	0.243	0.427	0.779	0.064	0.237
Propionate	0.107	0.210	0.110	**0.008**	0.865
Butyrate	0.749	0.956	0.464	0.251	0.121
Valerate	**0.016**	**0.017**	0.111	**0.004**	0.317
Iso-butyrate	0.308	0.755	0.063	0.289	0.999
Iso-valerate	0.062	0.134	0.111	**0.006**	0.067
Acetate:propionate ratio	0.151	0.223	0.261	**0.013**	0.484
Total gas	0.545	0.916	0.308	0.103	0.554
CH_4	0.567	0.876	0.908	0.122	0.750
CO_2	0.788	0.828	0.225	0.105	0.413
CO_2:CH_4 ratio	0.841	0.960	0.181	0.566	0.137
d.f (num/den)	2/10	2/10	1/8	1/8	1/8

OM, organic matter; C, carbon; N, nitrogen; NDF, neutral detergent fiber; WSC: water-soluble carbohydrates; CT, condensed tannins; IVDMD, *in vitro* dry matter degradability, VFAs, volatile fatty acids; d.f (num/den), degrees of freedom (numerator and denominator). In bold: $p < 0.05$; Underlined: $0.05 < p < 0.1$

https://doi.org/10.5194/bg-2020-464
Preprint. Discussion started: 11 January 2021

Figures

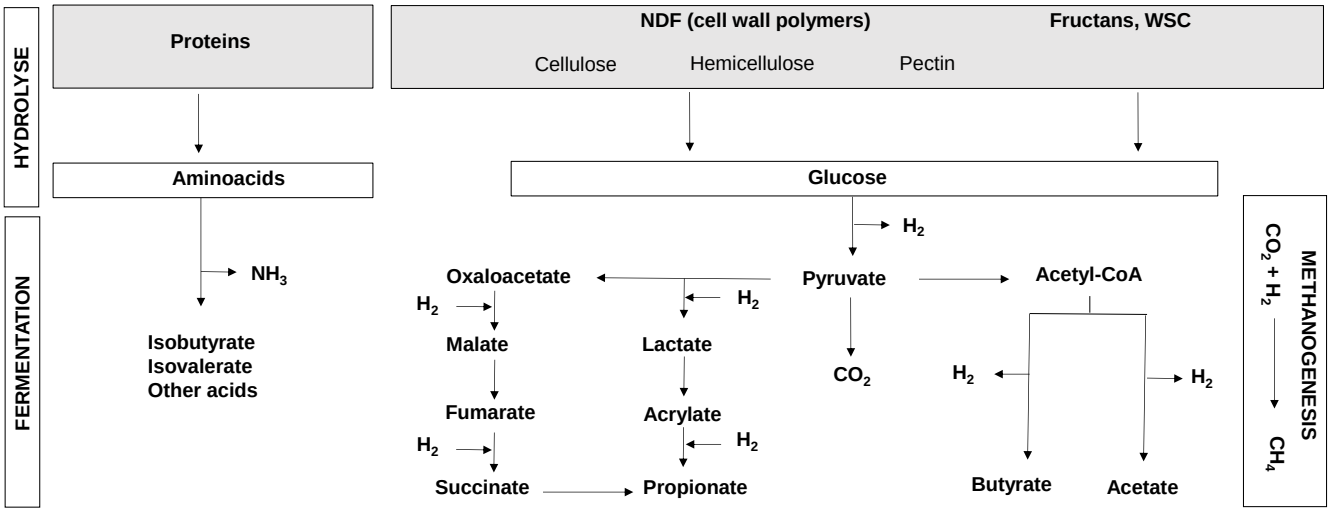

Figure 1. Overview of protein and carbohydrate metabolism in the rumen. WSC: water-soluble carbohydrates; NDF: neutral detergent fiber (cell wall carbohydrates); CH_4: methane; CO_2: carbon dioxide; NH_3: ammonia; H_2: hydrogen.

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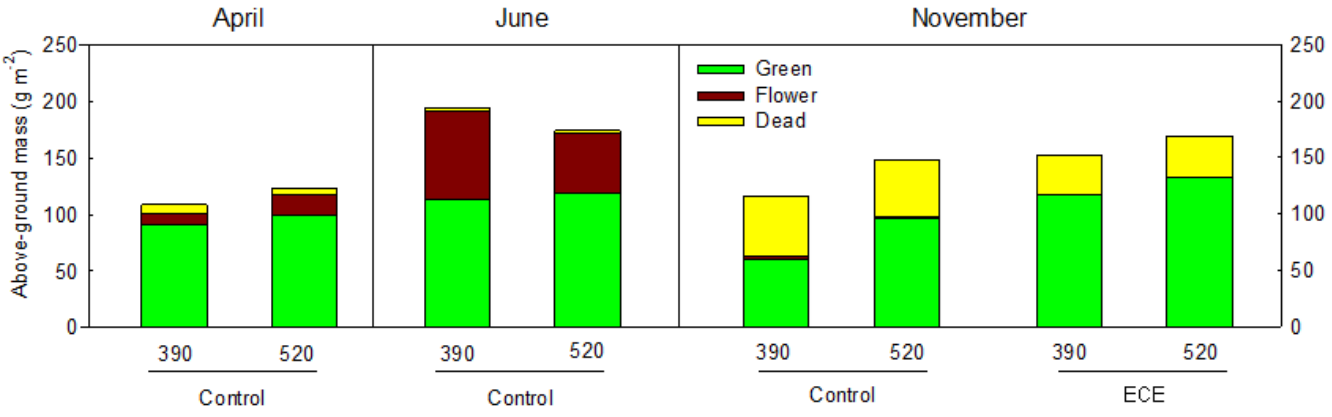

Figure 2. Above-ground biomass of green and dead materials, and flowers measured before (April, June) and after the extreme climatic event (ECE, November), grown under four climatic scenarios: 390 or 520 ppm atmospheric CO_2 concentration combined without (Control) or with the ECE. n = 3.

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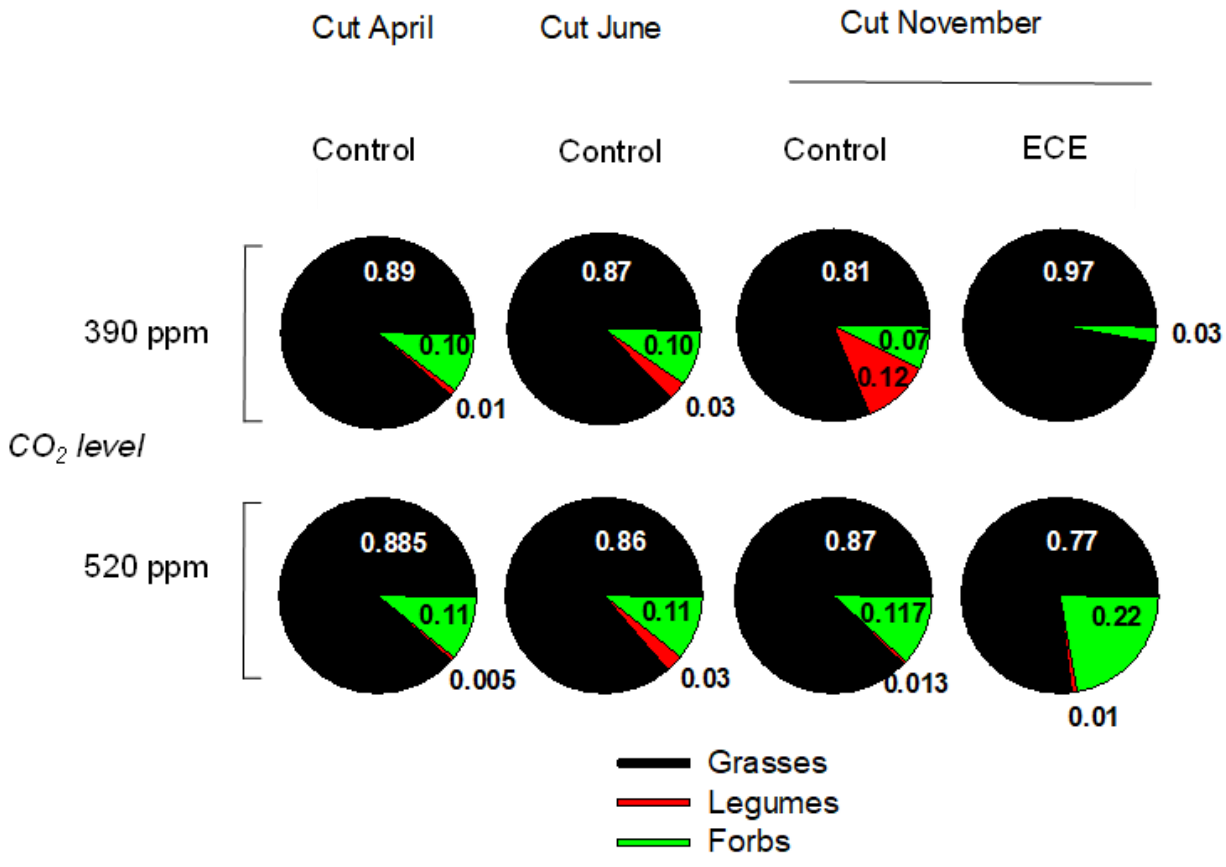

Figure 3. Relative abundance of functional groups (Grasses, Legumes, Forbs) present in the community measured before (April, June) and after the extreme climatic event (ECE, November), grown under four climatic scenarios: 390 or 520 ppm atmospheric CO_2 concentration combined without (Control) or with the ECE. n = 3. Numbers correspond to average values per functional group.

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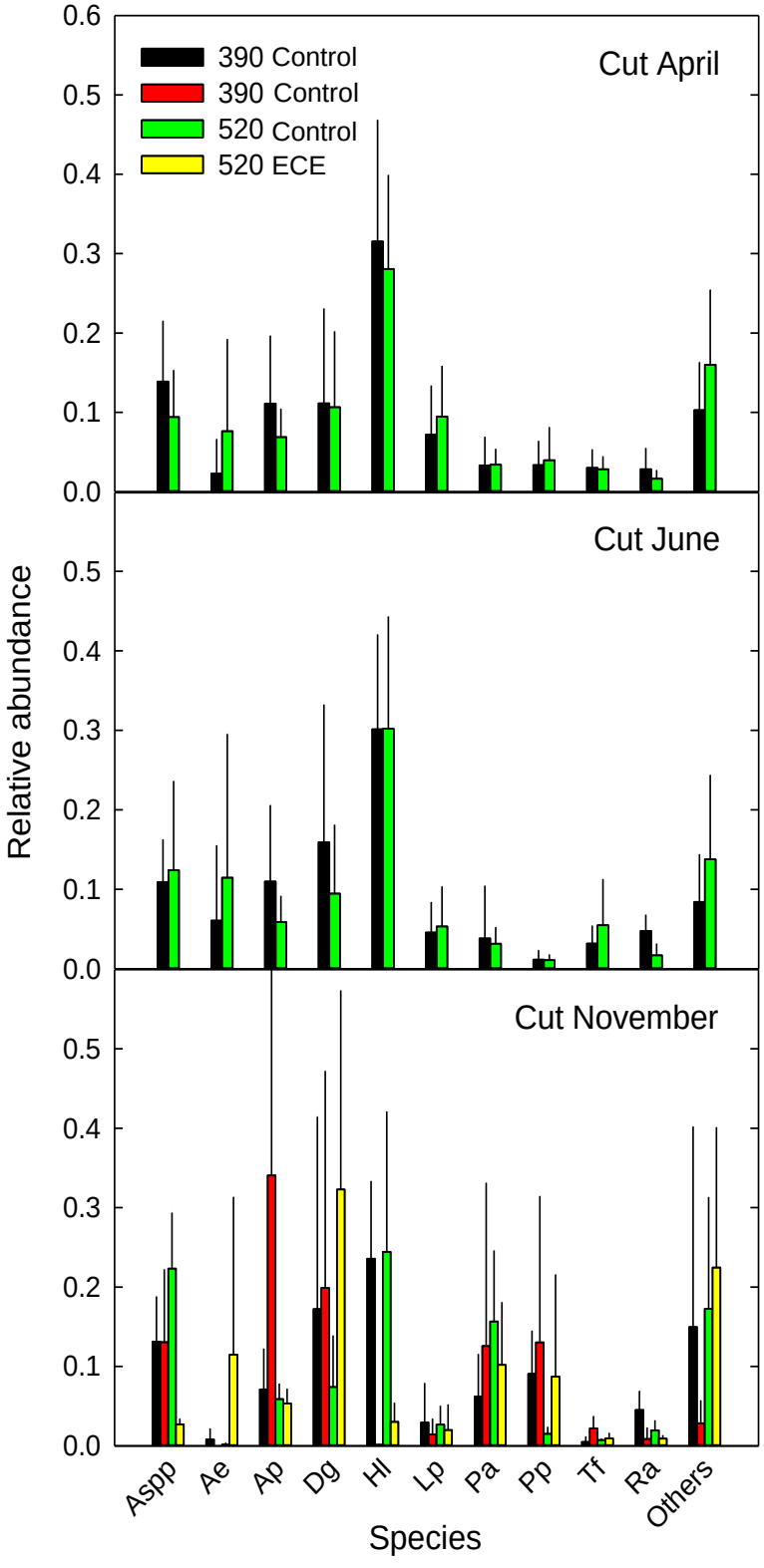

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Figure 4. Relative abundance of the 10 most abundant species present in the community measured before (April, June) and after the extreme climatic event (ECE, November) grown under four climatic scenarios: 390 or 520 ppm atmospheric CO_2 concentration combined without (Control) or with the ECE. Aspp: *Agrostis spp*; Ae: *Arrhenatherum elatius* L.; Ap: *Alopecurus pratensis* L.; Dg: *Dactylis glomerata* L.; Hl: *Holcus lanatus* L.; Lp: *Lolium perenne* L.; Pa: *Poa angustifolia* L.; Pp: *Poa pratensis* L.; Tf :*Trisetum flavescens* L.; Ra: *Rumex acetosa* L.; Others: <15% of species. n = 3.

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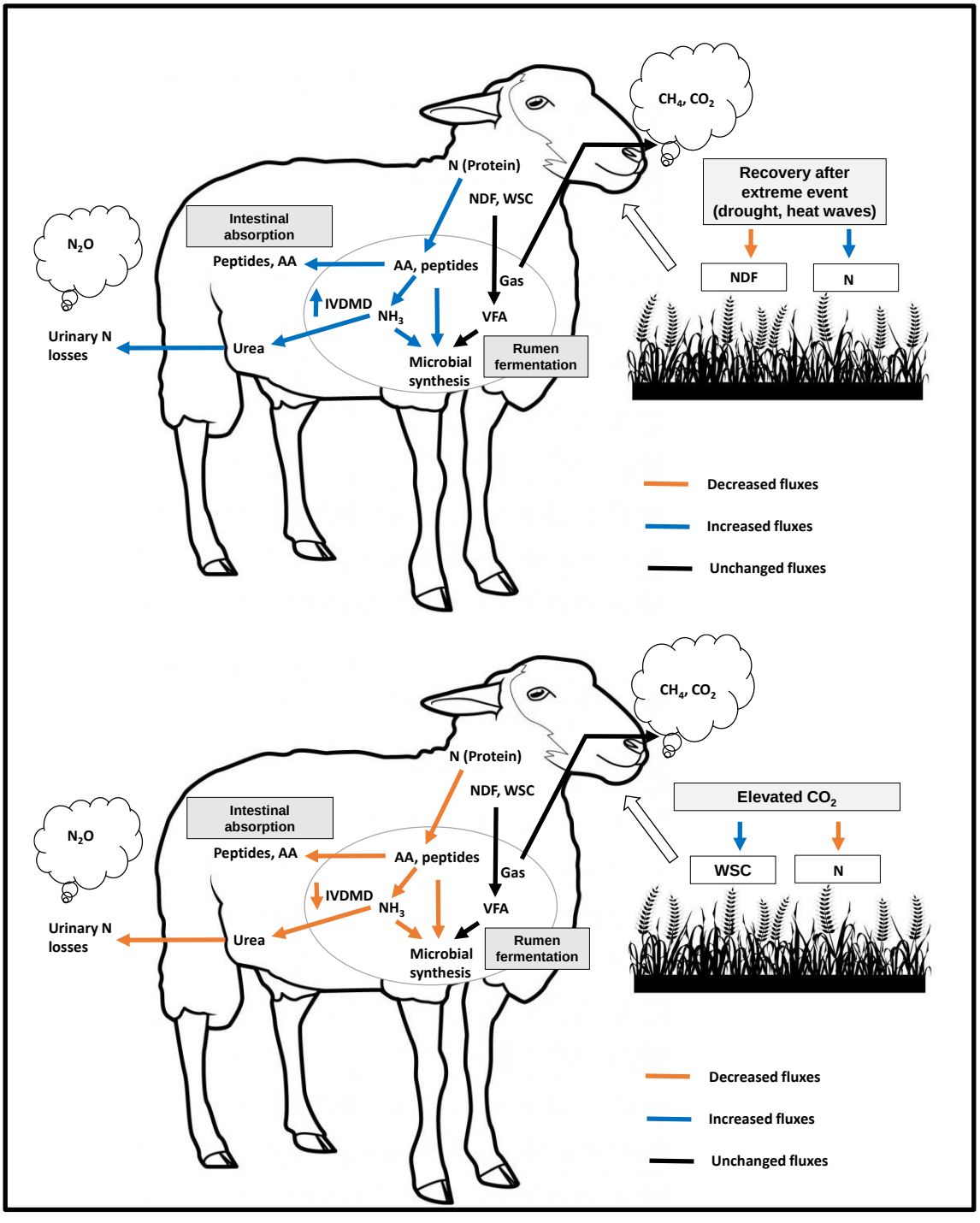

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Figure 5. Schematic overview of treatment effects (recovery after an extreme climatic event, ECE and effect of elevated atmospheric CO_2) on forage quality, digestive use of macronutrients by the ruminant and atmospheric greenhouse gas emission. WSC: water-soluble carbohydrates; N: nitrogen; NDF: neutral detergent fiber (cell wall carbohydrates); AA: amino acids; VFAs: volatile fatty acids; CH_4: methane; CO_2: carbon dioxide; NH_3: ammonia; N_2O: nitrous oxide; IVDMD, *in vitro* dry matter degradability