



## *Alnus viridis*: an encroaching species with valuable nutritional value reducing livestock greenhouse gas emissions

Mia Svensk<sup>a,b,\*</sup>, Pierre Mariotte<sup>a</sup>, Melissa Terranova<sup>c</sup>, Marco Pittarello<sup>d</sup>, Ginevra Nota<sup>e</sup>, David Frund<sup>a</sup>, Sébastien Dubois<sup>f</sup>, Elisa Manzacchi<sup>g</sup>, Francesca Napoleone<sup>h</sup>, Susanne Meese<sup>i</sup>, Giampiero Lombardi<sup>e</sup>, Eric Allan<sup>b</sup>, Massimiliano Probo<sup>a</sup>

<sup>a</sup> Grazing Systems, Agroscope, Posieux, Switzerland

<sup>b</sup> Department of Ecology and Evolution, University of Bern, Bern, Switzerland

<sup>c</sup> ETH Zurich, AgroVet-Strickhof, Lindau, Switzerland

<sup>d</sup> Department of Veterinary Sciences (DSV), University of Turin, Turin, Italy

<sup>e</sup> Department of Agricultural, Forest and Food Sciences (DISAFA), University of Turin, Turin, Italy

<sup>f</sup> Feed Chemistry, Agroscope, Posieux, Switzerland

<sup>g</sup> Ruminant Nutrition and Emissions, Agroscope, Posieux, Switzerland

<sup>h</sup> Department of Environmental Biology, Sapienza University of Rome, Rome, Italy

<sup>i</sup> Division Animal husbandry and Dairy Production, Strickhof, Lindau, Switzerland

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### ABSTRACT

*Alnus viridis* is a shrub species that has been increasing in many European mountains over the past decades, leading to a loss of agricultural areas and several negative environmental impacts. Recently, targeted livestock management systems have been investigated to reduce its encroachment. This study aims to provide an exhaustive assessment of *A. viridis* leaf composition and its temporal variation across the grazing season. *A. viridis* leaves were collected throughout the summers of 2020 and 2021 in four encroached sites across the Swiss and Italian Alps, characterized by different pedo-climatic conditions. Based on the data collected by meteorological stations, the growing degree days (GDD) were calculated for each site and year. The leaf functional traits (i.e., Leaf Dry Matter Content – LDMC – and Specific Leaf Area – SLA), chemical composition (macro- and micro-elements and fibre content), phenolic content, in vitro organic matter digestibility (IVOMD), and related gas production (CO<sub>2</sub> and CH<sub>4</sub>) were assessed. The LDMC significantly increased throughout the season, whereas the SLA decreased. All macro- and micro-elements significantly varied during the season, with leaf nitrogen (N) and phosphorus (P) decreasing. In contrast, leaf fibre contents significantly increased as the season advanced. There was a significant increase in condensed tannin content during the summer season. Finally, adding *A. viridis* leaves (20% of diet on a dry matter basis) to cattle diets significantly reduced IVOMD, methane produced per digested organic matter (CH<sub>4</sub>/DOM) and CO<sub>2</sub>/DOM, compared to a 100% hay diet. Moreover, CH<sub>4</sub>/DOM and CH<sub>4</sub>/CO<sub>2</sub> ratio increased during the summer season. These results highlight the potential of *A. viridis* leaves as a valuable forage resource, especially at the beginning of the summer. Such information could be used to optimize grazing of robust livestock in *A. viridis*-encroached alpine pastures in order to reduce its invasion and minimize greenhouse gases production at the same time.

### 1. Introduction

Due to agricultural land abandonment in the most marginal areas, tree and shrub-encroachment has strongly increased in European mountains in the last century (MacDonald et al., 2000; Orlandi et al., 2016). This trend has caused the loss of grassland areas in alpine regions,

with a reduction in landscape diversity and aesthetic value (Strebel and Bühler, 2015; Schirpke et al., 2016). *Alnus viridis* (Chaix) DC is one of the most rapidly spreading shrub species in several European mountain chains (Boscutti et al., 2014; Caviezel et al., 2017; Skoczowski et al., 2021), thanks to high reproduction and growth (Wiedmer and Senn-Irlet, 2006). Its expansion can create unfavourable

\* Corresponding author at: Grazing Systems, Agroscope, Posieux, Switzerland.

E-mail address: [mia.svensk2@gmail.com](mailto:mia.svensk2@gmail.com) (M. Svensk).

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agro-environmental conditions and the reduction of key ecosystem services. Indeed, in addition to the reduction of forage yield due to grassland loss, it also prevents forest succession and does not provide protection from natural hazards such as avalanches (Caviezel et al., 2014, Faccioni et al., 2019). Moreover, its ability to fix nitrogen (N) due to the symbiosis with the N<sub>2</sub>-fixing actinomycete *Frankia alni* can lead to N saturation in soils (Bühlmann et al., 2016). This increases risks of nitrate leaching and emissions of greenhouse gasses, such as nitrous oxide, and reduces animal and plant biodiversity (Bü2020). While *A. viridis* is mostly found on northern and west facing slopes, it is predicted to expand to a wider range of environmental conditions due to its observed occurrence in new habitats (Skoczowski et al., 2021).

Since it is costly and difficult to control its spread through manual cuts, recent studies have investigated the potential of robust livestock to forage on *A. viridis* and reduce its encroachment. For example, Pauler et al. (2022) showed that Dexter cattle, Pfauen goats and Engadine sheep can cause significant damage to *A. viridis* shrubs. Indeed, cattle were able to break branches and trample on young shrubs, while goats and sheep foraged on and debarked the plants, with Engadine sheep being the most effective livestock in clearing encroached areas. Other robust breeds, such as Highland cattle, were also evaluated and shown to be efficient in opening dense *A. viridis* stands (Nota et al., 2023, Svensk et al., 2022). While it was observed that some species and breeds are thus able to feed on *A. viridis* shrubs, it is essential to assess the full potential of this plant species as a forage resource for livestock. Tree and shrub species can have high potential for ruminant nutrition (Luske and van Eekeren, 2017), as they maintain higher nutrient content during the late summer season compared to herbaceous species (Papachristou and Papanastasis, 1994, Ravetto Enri et al., 2020). Therefore, shrubs such as *A. viridis* could become an interesting forage supplement and help compensate for animal feeding during dry summers and periods with forage shortage. Bühlmann et al. (2016) measured the N concentration of *A. viridis* leaves at different elevations (1650 m and 1950 m) and found slightly higher N concentration at higher elevations. Another study assessed the biomass production of *A. viridis* (Wiedmer and Senn-Irlet, 2006), and Stević et al. (2010) measured the total tannin content of *A. viridis* leaves (4.4 ± 0.4%), which is an important factor in leaf digestibility assessment. However, to the best of our knowledge, no study has ever conducted an exhaustive analysis of *A. viridis* leaf functional traits, chemical composition, and nutritive value across the whole summer season. This aspect is crucial to fully understand the forage potential of *A. viridis* and to identify the ideal period for it to be grazed. Several studies have highlighted the importance of assessing leaf characteristics at different development stages to better estimate the nutritive value for livestock. For example, Pauler et al. (2020) pointed out the significant impact of leaf functional traits, such as specific leaf area (SLA) and leaf dry matter content (LDMC), on cattle foraging behaviour, where larger leaves (i.e., with higher SLA) were preferred for grazing, while leaves with higher LDMC were avoided. Other studies found that these plant traits impacted digestibility, as smaller and denser leaves may have reduced ruminal degradation (Pontes et al., 2007). Pauler et al. (2020) also found that leaves with higher N and phosphorus (P) contents were more often selected by cattle, and other research highlighted a significant impact of primary and secondary leaf compounds on forage selection, digestibility, and animal health (Collins et al., 2017; Leng, 1997; Mahieu et al., 2021; Ravetto Enri et al., 2020). The impact of phenolic compounds on forage quality is also well known, as high tannin and non-tannin phenolic concentrations tend to reduce palatability and digestibility (Kumar and Vaithiyathan, 1990; Sunnerheim et al., 1988). Finally, leaf neutral detergent fibre (NDF) content is an essential factor influencing digestibility in ruminants as well, and has previously been found to increase throughout the growing season for woody fodder species (Papachristou and Papanastasis, 1994). Moreover, since livestock production is one of the most important contributors to direct greenhouse gas emissions, especially methane (CH<sub>4</sub>) production from ruminants (Slade et al., 2016), it is relevant to investigate the potential

mitigating effect of *A. viridis* on CH<sub>4</sub> emissions. Previous studies investigated the impact of the inclusion of woody plants on forage intake, nutrient digestibility, and gas production (Terranova et al., 2018, 2019), but the impact of *A. viridis* leaves in ruminant diet has not been assessed yet, hampering the implementation of livestock management systems to control *A. viridis* expansion.

To fill these knowledge gaps, this study aimed to assess the temporal variation in *A. viridis* leaf functional traits, chemical composition and in vitro digestibility and the impact on gas production when they are added to cattle diet in different sites across the Swiss and Italian Alps characterized by varying pedo-climatic conditions. Specifically, the objectives of this study were to assess: (1) the functional traits (LDMC and SLA), (2) the chemical composition (macro/micro- elements and fibre fractions), (3) the phenol composition, (4) the in vitro organic matter digestibility (IVOMD) and gas production of *A. viridis* leaves, as well as (5) the relationships among all measured leaf characteristics. We expected that *A. viridis* leaf functional traits, chemical composition, IVOMD, and gas production varied across sites and throughout the season, with a loss of leaf forage quality as the season advanced. We also expected the IVOMD and related gas production of a ruminant diet partially composed by *A. viridis* leaves to be lower than that of a control diet purely composed of hay.

## 2. Methodology

### 2.1. Study sites

The study was carried out in 2020 and 2021 in four sites in the Italian and Swiss Alps, characterised by different pedo-climatic conditions (Fig. 1, Table 1). The first site was located in Val Vogna, in Northern Italy (province of Vercelli), the second and third sites in Western Switzerland, namely in Bovonne (canton of Vaud) and Champlong (canton of Valais), and the fourth one in Bergün (Alp Weissenstein), in Eastern Switzerland (canton of Grisons). In all the sites, areas highly encroached by *A. viridis* were present at comparable elevations (1800–2000 m). At each site, a meteorological station (DWS Decagon weather station from Decagon devices Inc and HOBO Pro v2 U23–00x from Onset Corp., Pocasset, MA) was placed throughout the two years at two meters from ground level to record air temperature every hour.

### 2.2. *Alnus viridis* leaf sampling

During the summers of 2020 and 2021, 1.75 kg of *A. viridis* fresh leaves were collected three times (i.e. in June, July and August) at each site to assess the seasonal changes in leaf functional traits, chemical

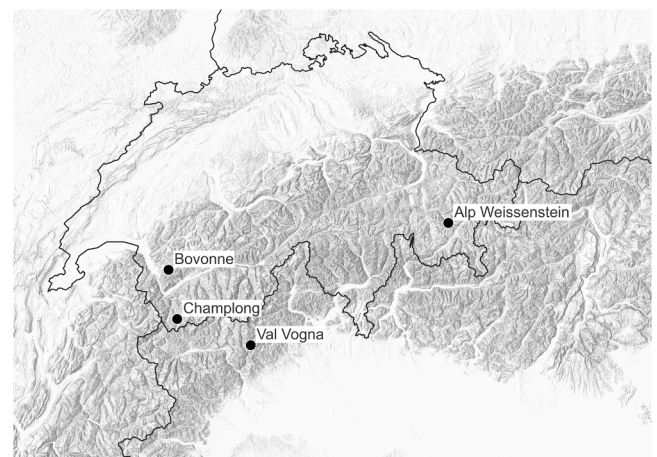


Fig. 1. Location of the four sites of the study in the Swiss and Italian Alps. Basemap: ESRI Terrain.

**Table 1**

Characteristics of the four experimental sites. Soil data refer to the laboratory analyses of the first 10 cm of soil below four different *A. viridis* encroached areas at each site.

	Val Vogna	Bovonne	Champlong	Alp Weissenstein
Coordinates	N45°46'18.815'' E7°54'9.198''	N46°16'20.109'' E7°6'47.327''	N45°56'2.306'' E7°12'14.333''	N46°34'50.711'' E9°47'58.761''
Elevation (m)	1897 ± 67	1820 ± 66	2012 ± 36	2033 ± 26
Aspect (°N)	102.15	105.38	111.43	190.00
Mean annual precipitation (mm)	881 <sup>a</sup>	1674 <sup>b</sup>	1023 <sup>b</sup>	1200 <sup>b</sup>
Mean annual temperature (°C)	7.46 <sup>c</sup>	4.51 <sup>b</sup>	4.21 <sup>b</sup>	1.79 <sup>b</sup>
Soil total organic carbon (TOC mg kg <sup>-1</sup> )	548.53 ± 37.48	282.04 ± 42.45	483.89 ± 70.93	370 ± 33.37
Soil total nitrogen (TN mg kg <sup>-1</sup> )	56.33 ± 4.61	19.48 ± 1.21	49.02 ± 9.41	29.22 ± 3.04
Soil dissolved inorganic nitrogen (DIN mg kg <sup>-1</sup> )	16.74 ± 5.1	12.36 ± 1.1	19.35 ± 4.49	10.44 ± 1.86
Soil available phosphorus (mg kg <sup>-1</sup> )	11.27 ± 5.32	0.56 ± 0.13	9.36 ± 4.47	5.23 ± 1.77
pH (H <sub>2</sub> O)	3.84 ± 0.03	6.27 ± 0.28	5.44 ± 0.18	4.71 ± 0.22

<sup>a</sup> Mean annual precipitation (mm) for the period from 1977 to 2007.

<sup>b</sup> Mean annual precipitation (mm) or mean annual temperature (°C) for the period from 1991 to 2021.

<sup>c</sup> Mean annual temperature (°C) for the period from 1977 to 2007, calculated on a 10 km<sup>2</sup> grid.

composition, phenolic contents, and IVOMD. For each sample, *A. viridis* leaves and petioles were hand-harvested from different points in the canopy of five different shrubs, at a maximum height of 1.80 m to simulate the potential browsing of cattle (Svensk et al., 2022). New shrubs were selected for each harvest at all four sites to analyze seasonal changes without repeated harvesting, which would imply feeding pressure. Moreover, sampled *A. viridis* shrubs were chosen outside of grazed areas and they were comparable in terms of height (three to five meters high).

### 2.3. Leaf functional traits

The LDMC was measured according to the protocol of Cornelissen et al. (2003). For each of the five shrubs and for each sampling time, 10 leaves were randomly selected and then weighed to record their fresh weight (FW). Afterwards the samples were dried at 60°C for 72 hours and their dry weight (DW) was recorded. The LDMC was then calculated as followed:

$$\text{LDMC (mg g}^{-1}\text{)} = \text{DW(mg)} / \text{FW(g)}$$

The SLA was measured according to Cornelissen et al. (2003) using the leaves collected for LDMC described above. Before drying the leaves, we measured the surface of all 10 leaves of each sample by photocopying them and calculating their area with an image analysis software (ImageJ, Schneider et al., 2012). This allowed us to calculate SLA as followed:

$$\text{SLA (cm}^2\text{ g}^{-1}\text{)} = \text{leaf surface (cm}^2\text{)} / \text{DW (g)}$$

### 2.4. Leaf chemical composition

A total of 350 g of fresh leaves were taken from each collection batch and dried at 60°C for 72 h. After being ground to pass a 1-mm screen (Brabender rotary mill; Brabender GmbH & Co. KG, Duisburg, Germany), leaf samples were analysed to determine residual dry matter (DM) content by heating at 105°C for 3 h, followed by incineration at 550°C until a stable mass was reached, to determine the ash content according to ISO 5984:2002 (prepASH, Precisa Gravimetrics AG, Dietikon, Switzerland). Mineral content (i.e. calcium, phosphorus, potassium, magnesium, copper, iron, manganese and zinc) was analysed according to the EN 15510:2008 by ICP-OES (ICP-OES 5800, Agilent Technologies, Switzerland) after microwave digestion. The samples were dissolved in a glass tube (5 ml HNO<sub>3</sub> 65% + 3 ml H<sub>2</sub>O ASTM Class I) using a microwave digester (UltraClave MLS, Leutkirch, Germany) at

235°C for 60 min (1000 W). If necessary, samples were diluted with HNO<sub>3</sub> 2% prior to analysis. The N concentration was determined by the Dumas method (ISO 16634-1:2008) and crude protein (CP) was calculated as N × 6.25.

Fibre fractions were analysed according to Van Soest et al. (1991). The neutral detergent fibre (aNDF<sub>OM</sub>) and acid detergent fibre (ADF<sub>OM</sub>) contents (ISO 16472:2006 for NDF and ISO 13906:2008 for ADF) were determined gravimetrically (ISO 6865:2000) after acid and alkaline digestions using a fibre analyser (Fibretherm Gerhardt FT-12, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) and were expressed without residual ash after incineration at 600°C for 3 h. The aNDF<sub>OM</sub> was evaluated with heat stable α-amylase and sodium sulfite. Acid detergent lignin (ADL) was analysed according to ISO 13906:2008. Hemicellulose and cellulose concentrations were estimated as aNDF<sub>OM</sub> minus ADF<sub>OM</sub> and ADF<sub>OM</sub> minus ADL, respectively.

### 2.5. Leaf phenolic compounds

Concentrations of total extractable phenols (TEP) and non-tannin phenols (NTP) were determined using a modified Folin-Ciocalteu method according to Makkar (2003). For a detailed description of the method see Terranova et al. (2018). The determination of the condensed tannins (CT) was performed with the butanol-HCl-iron method (Makkar, 2003) and the contents were given as leucocyanidin equivalents. Total tannins (TT) and hydrolysable tannins (HT) were calculated as TEP minus NTP and TT minus CT, respectively.

### 2.6. Leaf in vitro organic matter digestibility and gas production measurements

The assessment of IVOMD and gas production was made using the in vitro incubation with the Hohenheim gas test (HGT) method, which was performed as outlined by Menke and Steingass (1988). For each sampling date, the remaining leaves from the five shrubs were pooled in one sample, ending up in 12 samples per year. *A. viridis* leaves were freeze dried and ground with a centrifugal mill (Model ZM1, Retsch GmbH, Haan, Germany) to pass through a 1 mm sieve. The incubation of *A. viridis* leaves was performed by combining the leaves with hay (ryegrass dominated sward, CP = 11.6%; NDF = 48.4 g.kg<sup>-1</sup> DM), in a ratio of 1:4 on a DM basis. This proportion was chosen to simulate the diet of Highland cattle in *A. viridis*-encroached pastures, as described in Svensk et al. (2023). A diet of hay (100%) served as the control. For each year, rumen fluid was collected from three ruminally-cannulated multiparous late lactating Original Braunvieh cows, three times before morning feeding, across three weeks in both 2020 and 2021. It was then transported in a pre-heated thermos flask to the laboratory. Within one hour after collection, rumen fluid was strained through four layers of

gauze and added to a buffer solution in a 1:2 ratio according to the protocol of Menke and Steingass (1988). For incubation, modified 100-ml glass syringes with two outlets, one for fluid and one for gas sampling, were used as described in Soliva and Hess (2007). The incubation lasted for 24 h at 39 °C in an incubator with an integrated rotor. After 24 h, the fermentation gas volume was recorded from the calibrated scale printed onto the syringes, and the fermentation was terminated by removing the incubation fluid from the syringes while the gas phase remained inside. Fermentation gas samples of 150 µl were taken from the incubation syringes and injected using a gas-tight Hamilton syringe (Hamilton AG, Bonaduz, Switzerland) into a gas chromatograph (6890 N, Agilent Technologies, Wilmington, DE, USA) equipped with a thermal conductivity detector. Concentrations of CH<sub>4</sub> and CO<sub>2</sub> were analysed with this detector, and expressed per digested organic matter (DOM). Each pooled sample and the control were incubated in duplicates per run resulting in a total number of n = 6 per each year. Two syringes filled with only rumen fluid buffer mixture were incubated as blank.

### 3. Statistical analyses

All statistical analyses were performed using R version 4.2.2 (R Core Team, 2022). The cumulative growing degree-days (GDD) of each site was calculated from the meteorological stations as described in Grigorieva et al. (2010):

$$GDD = \sum_{i=1}^n \left[ \left( \frac{T_{\max} + T_{\min}}{2} \right) - T_{\text{base}} \right]$$

where  $i = n$  are the days with a temperature above 0°C,  $T_{\max}$  and  $T_{\min}$  are the daily maximum and minimum temperature respectively, and  $T_{\text{base}}$  is a threshold temperature defined here at 5°C following the description for pasture grasses from Grigorieva et al. (2010). The GDD was then used as a proxy to represent the seasonal temperature changes, as it allows comparison between sample dates of both years and is commonly used in agro-ecosystems as an indicator of season advancement (Grigorieva et al., 2010; McMaster and Wilhelm, 1997). We tested the effect of GDD, site and their interaction on plant functional traits, leaf chemical composition, and phenolic contents using a Linear Mixed-effect Model (lme, package “nlme”), with GDD, site and their interaction as a fixed effects and “year” as a random factor. The same model was used to test the effect of GDD on IVOMD and fermentation gas with the “cow” used for the experiment as an additional random factor. When residuals were not normally distributed, a log transformation was applied to the data. Some outliers were also deleted (0.83% of the data was deleted for Fe and Zn and 3.33% for Cu). The marginal and conditional R-squared values were obtained using the “performance” package (function “model\_performance”) to quantify the variance explained by fixed and fixed plus random factors, respectively. From the difference between conditional and marginal R-squared, it was possible to obtain the variance explained by the random factors. For IVOMD and gas production measurements, a comparison between the *A. viridis* treatment (20% DM *A. viridis* leaves, 80% DM hay) and the control (only hay) was made using a Student’s test (t-test) for independent samples. Finally, a principal component analysis (PCA, FactoMineR package) was performed to assess the relationships among all variables. For this analysis, the leaves of the five shrubs of each sampling date were pooled in one sample, ending up in 12 samples per year. A 95% normal confidence ellipses enclosing the samples collected from the different sites were displayed around each site on the PCA biplot (fviz\_pca\_ind, factoextra package).

### 4. Results

A table summarizing model results for all the variables is provided as Supplementary Table S1.

#### 4.1. Leaf functional traits

The GDD were not significantly different between 2020 and 2021 ( $P = 0.41$ ) and ranged from 151.31 to 721.68 in 2020 and from 221.02 to 799.49 in 2021. The LDMC of *A. viridis* leaves significantly increased with GDD for all sites, from 258.70 to 483.41 mg g<sup>-1</sup> (Fig. 2A,  $P < 0.001$ ,  $R^2$  marginal = 0.62). In contrast, the SLA of *A. viridis* leaves significantly decreased with GDD (Fig. 2B,  $P < 0.001$ ,  $R^2$  marginal = 0.21) from 348.72 to 104.16 cm<sup>2</sup> g<sup>-1</sup>.

#### 4.2. Leaf chemical composition

All macro- and micro-elements of *A. viridis* leaves were significantly affected by seasonal temperature changes, except Fe ( $P = 0.052$ ). Indeed, N, P, K, Cu and Zn all significantly decreased with increasing GDD (Fig. 3A, B, D, F, I,  $P < 0.001$ ). In contrast, Ca, Mn, Fe and Mg significantly increased with GDD (Fig. 3C, E, G, H). Depending on the element considered, some significant interactions GDD\*site were detected (Supplementary Table S1). However, the direction of the significant relationships between GDD and each element was always consistent across sites (Fig. 3). Therefore, the significant interactions GDD\*site detected were mainly due to different site-specific amplitudes of these relationships or to the not significant relationship for specific elements within specific sites. The N and P contents ranged from 22.62 g kg<sup>-1</sup> to 46.98 DM and from 0.92 to 5.84 g kg<sup>-1</sup> DM, respectively. Therefore, leaf CP content had minimum and maximum values of 141.37 g kg<sup>-1</sup> DM and 293.63 g kg<sup>-1</sup> DM, respectively. Residual ash ranged from 32.07 g kg<sup>-1</sup> DM to 62.79 g kg<sup>-1</sup> DM, and increased significantly with GDD ( $P < 0.001$ ).

The fibre content of *A. viridis* leaves, i.e.- aNDF<sub>OM</sub>, ADF<sub>OM</sub>, and ADL concentrations significantly increased with increasing GDD (Fig. 4,  $P < 0.01$ ), ranging from 202.77 to 454.59 g kg<sup>-1</sup> DM, from 116.14 to 353.46 g kg<sup>-1</sup> DM and from 41.61 to 257.28 g kg<sup>-1</sup> DM, respectively. Significant interactions GDD\*site were detected and they were mainly related to different site-specific amplitudes of the relationships between fibre fractions and GDD (Supplementary Table S1 and Fig. 4). However, the direction of the significant relationships between GDD and fibre fractions was always consistent across sites. *A. viridis* leaves had a hemicellulose content of 124.8 g kg<sup>-1</sup> DM, and a cellulose content of 97.4 g kg<sup>-1</sup> DM on average over all sites and samplings. Nutrient and fibre concentrations in *A. viridis* leaves for each site and period are summarized in Supplementary Table S2.

#### 4.3. Leaf phenolic compounds

In contrast to macro- and micro-nutrients and fibre fractions, total phenolic compounds and phenolic fractions did not vary over the season, except for CT, which slightly increased with GDD at all sites (Fig. 5C), and ranged from 0.34% DM to 4.09% DM. Significant interactions GDD\*site were found for TEP (Supplementary Table S1 and Fig. 5A) TT (Fig. 5E) and HT (Fig. 5D), which ranged from 7.26% DM to 19.08% DM, 4.32% DM to 15.03% DM, and from 2.36% DM to 13.90% DM, respectively. Indeed, TEP, TT and HT concentrations were significantly related with GDD only at two sites out of four. Finally, the NTP concentrations in the leaves ranged from 1.82% DM to 4.25% DM, with no significant effects of GDD, site and their interaction., (Fig. 5B).

#### 4.4. Leaf in vitro organic matter digestibility and gas production

Including 20% of *A. viridis* leaves in the hay diet for in vitro incubation, reduced the IVOMD by 5.80% on average (Fig. 6A), with an average digestibility of 63.30 ± 0.19% compared to 69.10 ± 0.46% for the control. The IVOMD of the *A. viridis*-composed diet also significantly decreased over the season (Fig. 6A), from a maximum value of 66.80% to a minimum value of 60.11%.

The amount of CH<sub>4</sub> produced per unit of digestible OM (CH<sub>4</sub>/DOM)

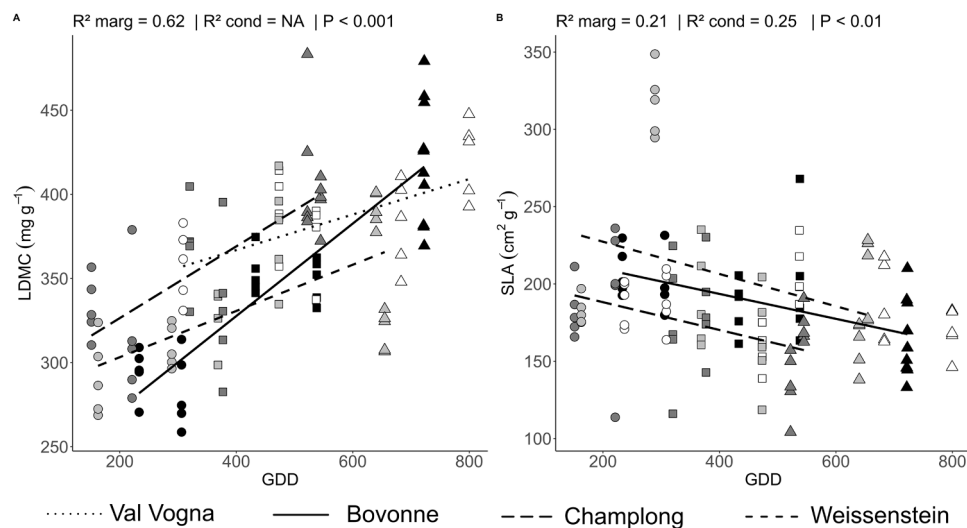


Fig. 2. Variation of (A) Leaf Dry Matter Content (LDMC) and (B) Specific Leaf Area (SLA) in relation to growing degree days (GDD) in Val Vogna, Bovonne, Champlong and Weissenstein. Sampling periods are represented with different shapes as circles (June), squares (July) and triangles (August).

was reduced on average by 17.73% when adding *A. viridis* leaves (Fig. 6B, Student's t-test  $P < 0.01$ ), with a mean value of  $43.83 \pm 0.48 \text{ ml g}^{-1}$ , compared to  $53.27 \pm 0.46 \text{ ml g}^{-1}$  of the control. It significantly increased with GDD (Fig. 6B), ranging from  $34.54 \text{ ml g}^{-1}$  to  $49.86 \text{ ml g}^{-1}$ .

The amount of  $\text{CO}_2$  produced per unit of digestible OM ( $\text{CO}_2/\text{DOM}$ ) followed an opposite trend, with a significant reduction with the seasonal increase in GDD (Fig. 6C). It ranged from  $305.03$  to  $259.84 \text{ ml g}^{-1}$ , and was also significantly reduced (by 5.84%) compared to the control (Fig. 6C, Student's t-test  $P < 0.01$ ), with average values of  $278.74 \pm 0.22 \text{ ml g}^{-1}$  and  $297.03 \pm 2.09 \text{ ml g}^{-1}$ , respectively.

Therefore, the  $\text{CH}_4/\text{CO}_2$  ratio obtained from the *A. viridis* diet was also significantly reduced compared to the control (Fig. 6D, Student's t-test  $P < 0.01$ ), and increased significantly with GDD, ranging from  $121.28$  to  $181.62 \text{ ml l}^{-1}$  (Fig. 6D), with a significant interaction  $\text{GDD} \times \text{site}$  (Supplementary Table S1).

#### 4.5. Principal component analysis

The results of the PCA are presented in Fig. 7. The first PCA axis (PC1, accounting for 33.7% of the variance, Fig. 7A) reflected the seasonal changes in *A. viridis* leaves, with increasing GDD, NDF, LDMC, and Ca, and decreasing P, HT, Zn, Cu and N. The second axis (PC2, accounting for 16.5% of the variance) reflected a gradient of increasing CT, ADL, TEP and TT, and decreasing  $\text{CH}_4/\text{DOM}$ , IVOMD,  $\text{CH}_4/\text{CO}_2$  and Fe. The PCA grouped the different phenolic compounds together, as well as the fibre fractions. The 95% normal confidence ellipses enclosing the samples collected from the different sites largely overlapped, thus suggesting an absence of a site effect (Fig. 7B).

## 5. Discussion

Significant seasonal variations were found in all *A. viridis* leaf nutrient and fibre components, as almost all measured variables were significantly affected by GDD. In contrast, the phenolic components were much stable, with only the concentration of CT displaying a slight increase over time. The PCA results provided a summary of seasonal changes of leaves, as the first axis represented the season progress.

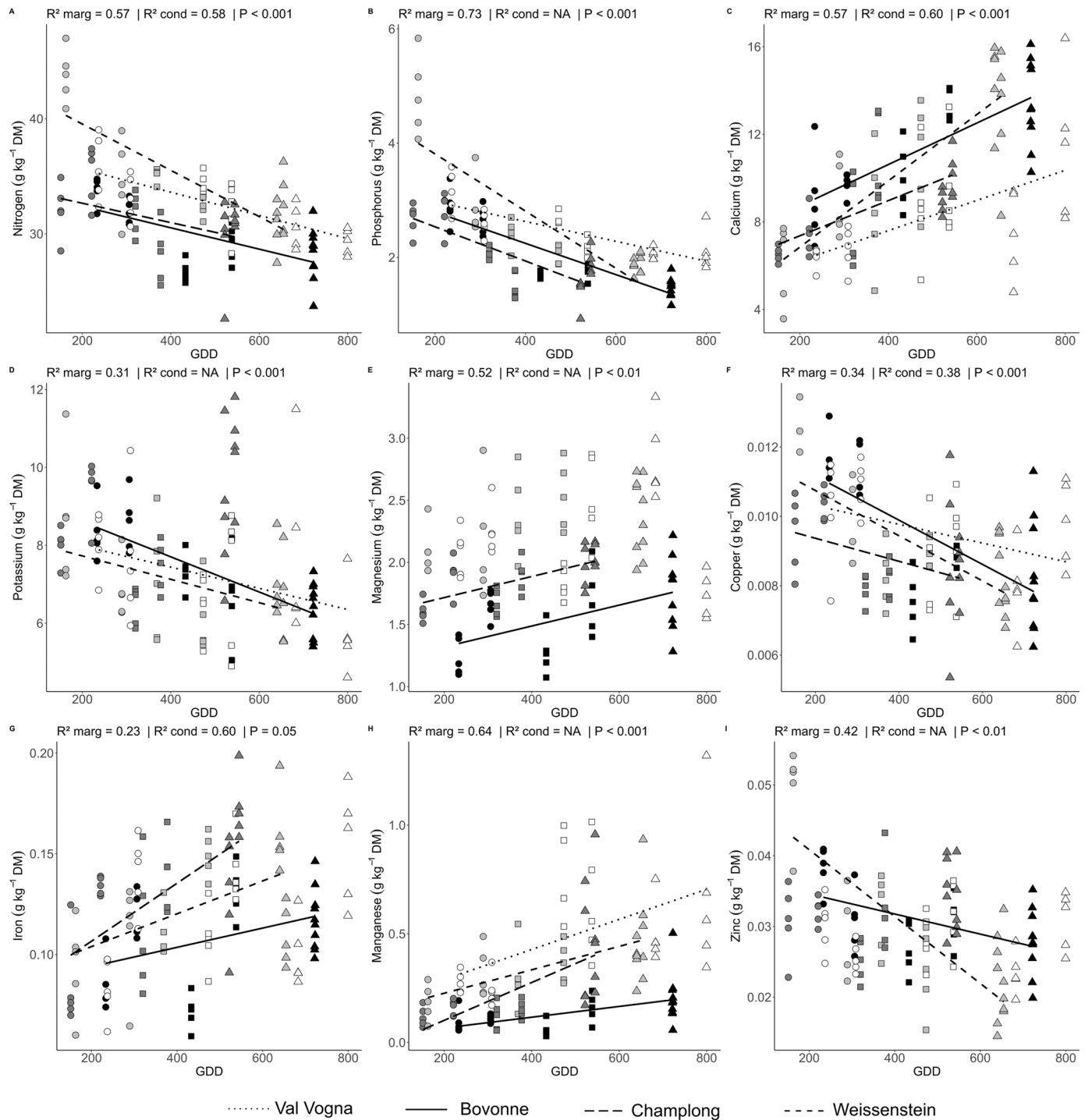
### 5.1. Leaf functional traits

Throughout the season, we found similar SLA and LDMC values for *A. viridis* compared to previous studies (Skoczowski et al., 2021) and

compared to other alder species (Pierce et al., 1994). As we expected, *A. viridis* leaf functional traits varied during the season, and leaf SLA and LDMC changed in opposite directions, leading to denser leaves with higher DM content as the season advanced. This trend was supported by the PCA that showed these two functional traits following opposite patterns, with LDMC positively correlated to GDD. A study on *A. glutinosa* made by De Kort et al. in, (2014) showed similar trends, as the alder had smaller leaves with increasing temperature. Skoczowski et al. (2021) also found variation in *A. viridis* leaf morphology according to elevation, linked to the different climatic conditions and stressful environments. However, even if in our study some differences among sites were detected for LDMC, the relationship between GDD and LDMC was consistent among all the studied sites. Previous studies on other woody shrubs and trees have also provided similar results (Qin et al., 2018, Wilson et al., 1999). This pattern is explained by tree strategies to acquire or retain resources depending on the environment. The SLA and LDMC are directly linked with leaf biomass and nutrient assimilation, and lower SLA and higher LDMC values at the end of the season occur because the plant focuses on conservation of acquired resources (Ravetto Enri et al., 2020, Qin et al., 2018). Consequently, as for other forage plants, *A. viridis* leaves may be less selected by cows at the end of the summer season (Pauler et al., 2020).

### 5.2. Leaf chemical composition

The chemical composition of *A. viridis* leaves varied significantly as the season advanced. Indeed, we found similar decreasing patterns for many important leaf nutrients (N, P, K, Cu, and Zn) during the summer season at all sites, except for site-specific constant concentrations of K and Zn at the Champlong site, and unaltered concentrations of Zn at the Vogna site. This general decrease is in line with the findings on *A. glutinosa* by Rodríguez-Barrueco et al. (1984), who showed that leaf N, P, and K decreased during the vegetative season as well. Other studies on alder species (Dawson and Funk, 1981; Luske and van Eekeren, 2017) or on other woody plants (Gowda et al., 2019; James and Smith, 1978; Niinemets and Tamm, 2005) detected similar trends, with the lowest values for these leaf nutrients in autumn. Our results from the PCA also highlighted tight correlations between some of these elements (i.e., positive correlations between N-P and N-K), as also shown by Rodríguez-Barrueco et al. (1984) for *A. glutinosa* leaves. The relationship between leaf N, P and K is well documented, and plays an essential role in the eco-physiological processes of the plant, contributing to photosynthesis, growth and reproduction (Tian et al., 2019). Moreover, in the

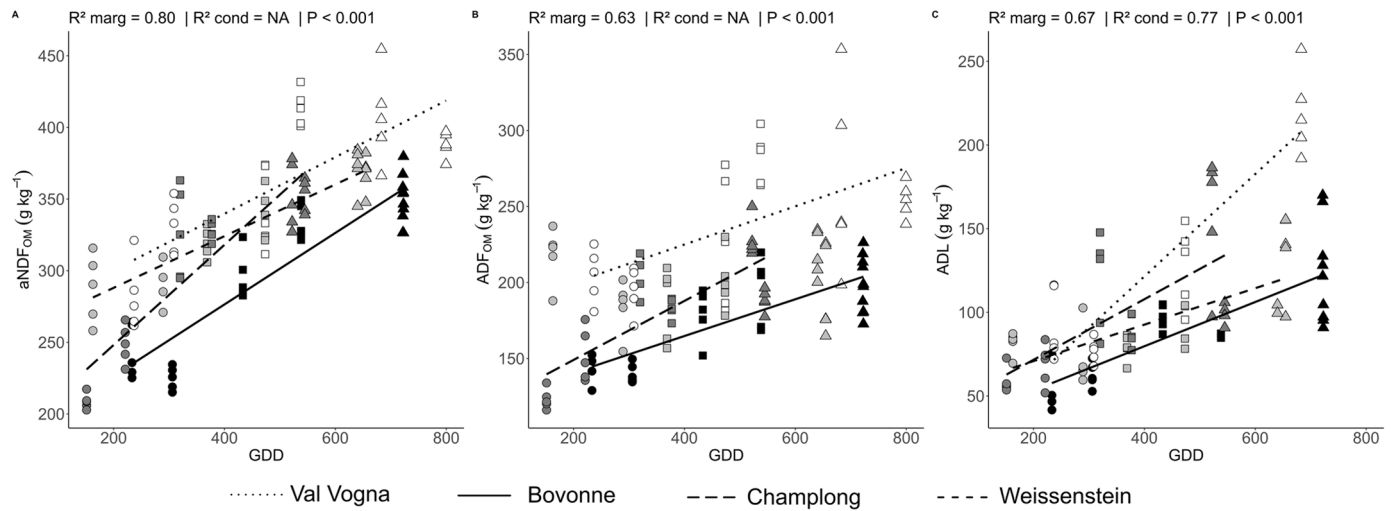


**Fig. 3.** Variation of (A) nitrogen, (B) phosphorus, (C) calcium, (D) potassium, (E) magnesium, (F) copper, (G) iron, (H) manganese and (I) zinc, in relation to growing degree days (GDD) in Val Vogna, Bovonne, Champlong and Weissenstein. Sampling periods are represented with different shapes as circles (June), squares (July) and triangles (August).

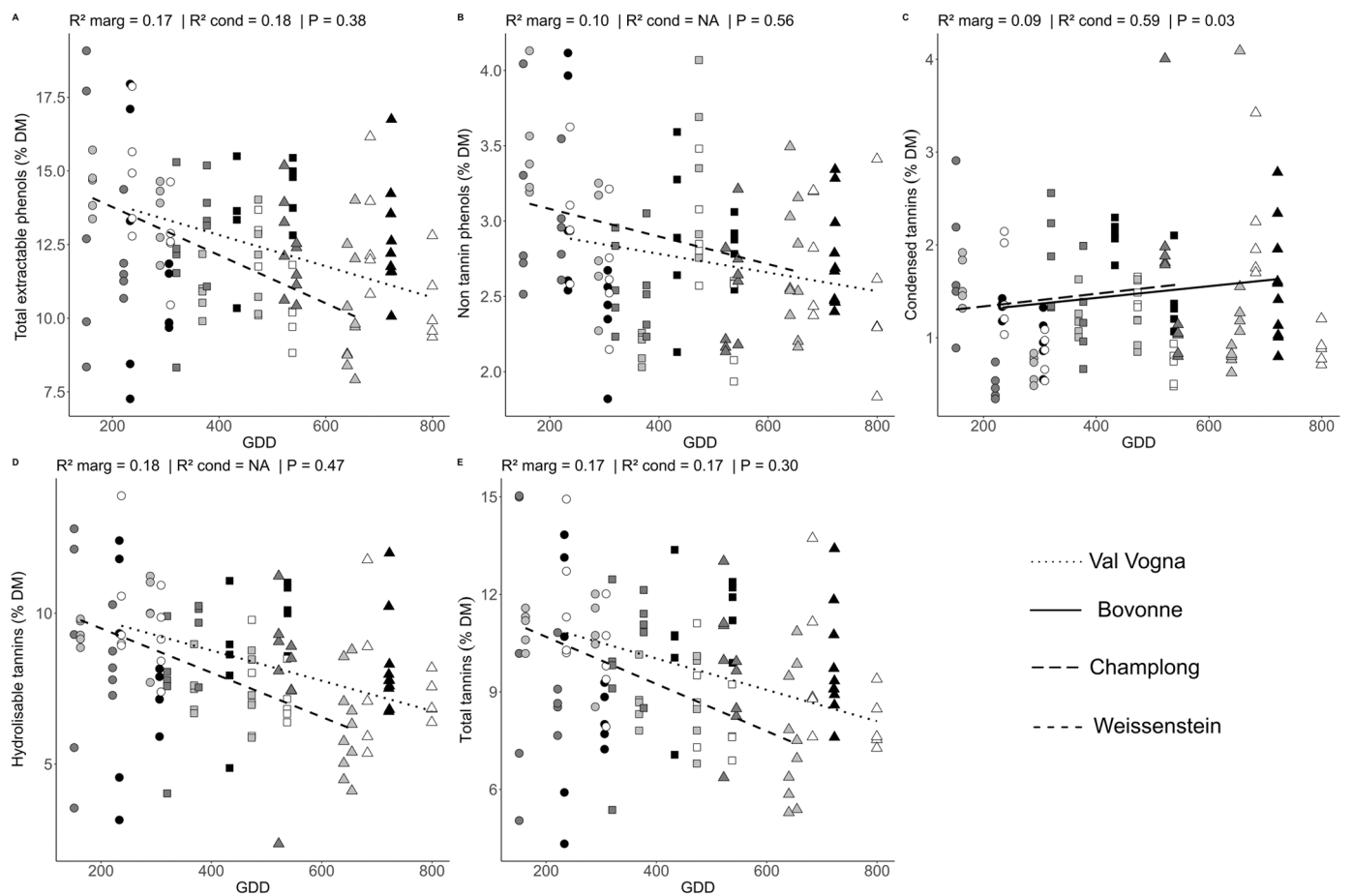
case of N-fixing shrubs such as *A. viridis*, the N fixation leads to a higher P demand compared to non-N-fixing plants, thus creating a positive relationship between N and P (Dawson, 2008). On the other hand, other *A. viridis* leaf nutrients displayed an increase with leaf senescence, such as leaf Ca and Mn, which were also shown to increase in *A. glutinosa* leaves (Rodríguez-Barrueco et al., 1984; Luske and van Eekeren, 2017). However, the increase in *A. viridis* leaf Mg measured in Champlong and Bovonne was not found in *A. glutinosa* but was found in other woody plants (James and Smith, 1978). The increase in *A. viridis* leaf Ca

contrasts with herbaceous species, where Ca is generally reported to be stable during the growing season by Schlegel et al. (2016). This increase, as well as the increase in leaf Mn, could be explained by their low mobility in the phloem and the lack of remobilization of these elements from leaves with senescence (Maillard et al., 2015; White, 2012).

Despite this significant variation throughout the season, *A. viridis* N leaf concentrations (22.62–46.98 g kg<sup>-1</sup> DM) were similar to previous measurements made on this species, e.g. Bühlmann et al. (2016) measured in late July and early August  $28.2 \pm 0.2$  g kg<sup>-1</sup> DM and  $29.4$



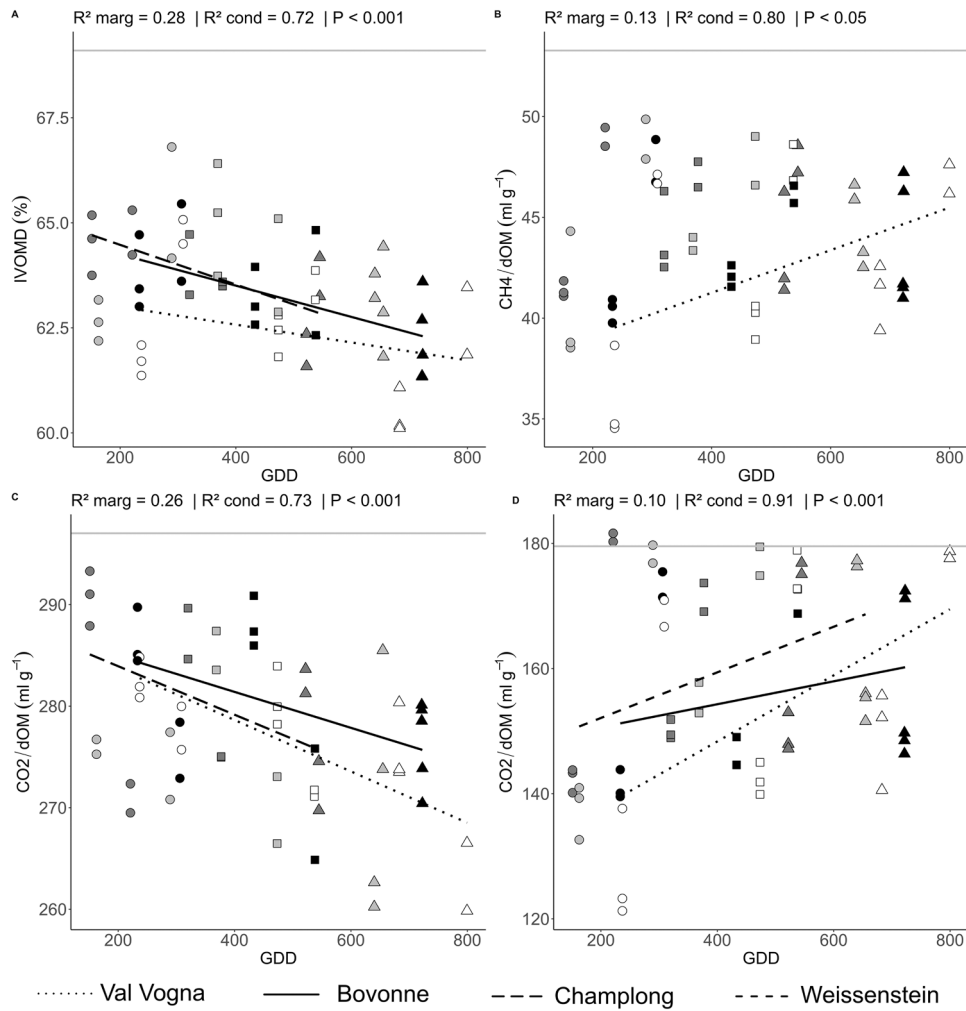
**Fig. 4.** Variation of aNDF<sub>OM</sub> (A), ADF<sub>OM</sub> (B) and ADL (C) in relation to growing degree days (GDD) in Val Vogna, Bovonne, Champlong and Weissenstein. Sampling periods are represented with different shapes as circles (June), squares (July) and triangles (August).



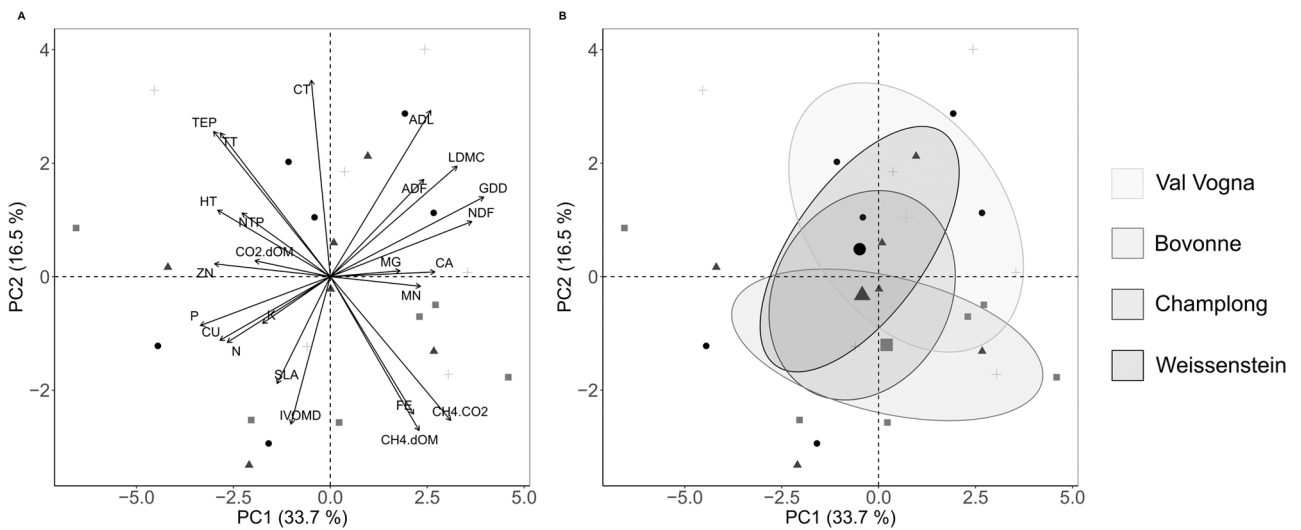
**Fig. 5.** Variation of (A) total extractable phenols, (B) non-tannin phenolics, (C) condensed tannins, (D) hydrolysable tannins and (E) total tannins in relation to growing degree days (GDD) in Val Vogna, Bovonne, Champlong and Weissenstein. Sampling periods are represented with different shapes as circles (June), squares (July) and triangles (August).

$\pm 0.4 \text{ g kg}^{-1} \text{ DM}$  at low (1650 m) and high (1950 m) elevations, respectively. These results are relatively high in comparison to other woody species, as Tian et al. (2018) found a global mean of  $21.13 \text{ g kg}^{-1} \text{ DM}$  when analysing a compiled dataset of several deciduous woody species across continents. This high N concentration in *A. viridis* leaves

was expected due to its symbiosis with *F. alni*, which leads to an accumulation of N in the roots and therefore higher N uptake by the plant (Dawson, 2008; Luske and van Eekeren, 2017). In addition, as they are not N limited, *Alnus* species do not need to resorb leaf N before winter, leading to potentially higher leaf N concentrations at the end of the



**Fig. 6.** Variation of (A) in vitro organic matter digestibility (IVOMD), (B) methane per digested organic matter (CH<sub>4</sub>/dOM), (C) carbon dioxide per digested organic matter (CO<sub>2</sub>/dOM) and (D) the ratio between methane and carbon dioxide (CH<sub>4</sub>/CO<sub>2</sub>) in relation to growing degree days (GDD) in Val Vogna, Bovonne, Champlong and Weissenstein. Sampling periods are represented with different shapes as circles (June), squares (July) and triangles (August). The solid grey line represents the control for comparison, i.e. the diet composed by 100% hay.



**Fig. 7.** Principal component analysis (PCA) for *A. viridis* leaf functional traits, macro- and micro-elements, fibre fractions, phenolic compounds, gases per digested organic matter, in vitro organic matter digestibility, and the GDD. The samples are represented in black dots (June), dark grey triangles (July) or light grey squares (August).



season (Dawson et al., 1981; Rodríguez-Barrueco et al., 1984; Han et al., 2013; Maillard et al., 2015). Consequently, as previously found for other alder species (Mahieu et al., 2021; Luske and van Eekeren, 2017), *A. viridis* leaf CP concentrations, which always exceeded 140 g kg<sup>-1</sup> DM, were higher than those usually found in temperate green fodder or in permanent grassland at the beginning of the season (Agabriel, 2010; Schlegel, 2021). Indeed, if compared to typical leguminous forage species found in nutrient-rich grasslands, such as *Trifolium pratense* L. or *Trifolium repens* L., the CP content is similar and sometimes higher for *A. viridis* leaves, with values ranging from 141.37 to 293.63 g kg<sup>-1</sup> DM compared to 161–261 g kg<sup>-1</sup> DM for the two *Trifolium* species (Daccord and Arrigo, 2001). Additionally, *A. viridis* leaf N content also mostly exceeded some nutrient optimum requirements for sheep and cattle, as described by Hejzman et al. (2016), above all at the beginning of summer. Since leaf CP concentrations lower than 7% have been shown to decrease forage intake by ruminants (Minson and Milford, 1967), *A. viridis* could remain a good source of CP for ruminants throughout the season, despite its seasonal decrease (Gonzalez-Hernandez et al., 2000).

Similarly, high concentrations were found for other nutrients measured in *A. viridis* leaves. For instance, leaf Ca, Mg and P concentrations were similar or higher than that found in typical leguminous forage species. Indeed, *A. viridis* leaf P content ranged between 0.92 and 5.84 g kg<sup>-1</sup> DM compared to 4.0–4.1 g kg<sup>-1</sup> DM usually found in *T. pratense* and *T. repens* (Daccord and Arrigo, 2001). *A. viridis* leaf P was similar to that of *A. glutinosa* measured by Luske and van Eekeren (2017), and to other deciduous woody plants (Tian et al., 2018). On the other hand, *A. viridis* leaf K was lower than that of leguminous species but within the range defined by Hejzman et al. (2016). A few out-of-range values for P were found in the samples from Weissenstein at the beginning of the 2020 season, for which the highest N, Cu and Zn concentrations were also recorded. Because macro- and micro-elements are essential for animal health, in terms of growth and reproduction (Mahieu et al., 2021), *A. viridis* leaves could represent a significant resource for ruminants.

The range of fibre and lignin concentrations of *A. viridis* leaves were very similar to those found for other alder species in France by Mahieu et al. (2021), and were higher than contents found for herbaceous vegetation used as a comparison in the same study. As the season advanced, leaves became more fibrous, as all fibre fractions showed an increase from June to August. This is in line with previous studies on other alder species such as *A. rubra* (Gonzalez-Hernandez et al., 2000) and other deciduous shrubs and trees (Papachristou and Papanastasis, 1994; Happe et al., 1990), which showed that NDF, ADF, and lignin leaf content increased from spring to autumn, leading to a lower forage quality at the end of the summer season. Indeed, forage with high fibre contents leads to lower nutrient digestibility and assimilation with plant senescence, as lignin has been described as one of the most important factors limiting the degradation of cell walls in the rumen (Mahieu et al., 2021; Van Soest et al., 1991; Cherney et al., 1993). Moreover, high fibre content tends to reduce voluntary intake from livestock (Allison et al., 1985), which would lead to less selection of the shrub at the end of the summer.

These results show that *A. viridis* leaves decrease in overall nutritive value across the grazing season, which is similar to previous studies on fodder shrubs and trees. Indeed, in 2022, Navale et al. studied the variation in the leaf composition of several fodder trees throughout the season and found that most of the nutritive and mineral content decreased as leaves matured.

### 5.3. Leaf phenolic compounds

Leaf total tannin concentrations from our study were higher than what has been previously found for *A. viridis* (as well as for *A. incana*), with values up to 3.5 times higher than the values measured by Stević et al. (2010). Because their results focused on lower elevations, we could hypothesize that *A. viridis* could display higher total phenol

concentrations at higher elevations, which has been described for other plant species due to their tendency to synthesise phenols to protect against UV radiations that increase with altitude (Alonso-Amelot, 2007; Bernal et al., 2013; Zargoosh et al., 2019). Except for leaf CT, the phenolic compound contents of *A. viridis* leaves did not significantly change as the season advanced. In contrast, previous studies on alder species and on other woody plants have shown a general phenol decrease during the season, with a higher phenolic allocation to young leaves, while the variation in leaf CT seems to be highly species-dependent (Gonzalez-Hernandez et al., 2000; Gowda et al., 2019). While leaf phenol content has been primarily linked to leaf ontogeny and air temperature, the high phenol content of *A. viridis* leaves in June could also be linked to the seasonal variations of UV radiations that tend to peak in June in Europe (Bernal et al., 2013). However, in our study TEP, NTP, TT and HT concentrations decreased along the summer season only at Vogna and Weissenstein sites. The variation in phenols in *A. viridis* leaves during the season may have a significant impact on livestock feeding behaviour, as they are responsible for astringency and affect digestibility. For instance, if consumed in large quantities, HT can be toxic, while CT can lead to lower voluntary feed intake and negatively impact ruminant digestion rate (Waghorn, 2008; Piñeiro-Vazquez et al., 2015; Gowda et al., 2019). Indeed, CT create binding complexes with protein that protect them from ruminal degradation and intestinal absorption, leading to a reduced apparent total tract N digestibility (Waghorn, 2008; Woodfield et al., 2019). This is supported by the results of our PCA, which showed a negative relationship between CT and IVOMD. On the other hand, this phenomenon may lead to a higher proportion of N excreted through dung instead of urine, which is less subjected to ammonia volatilization and nitrate leaching (Woodmansee et al., 1981; Tamminga, 2006; Angelidis et al., 2019). Woodfield et al. (2019) recommended a CT concentration to range between 2% and 4% DM. Including phenols in that proportion in the diet of grazing ruminants might increase energy and N utilisation (Gowda et al., 2019).

### 5.4. Leaf in vitro OM digestibility and gas production

The mean IVOMD of *A. viridis* leaves was very similar to the OM digestibility found for *A. glutinosa* leaves by Luske and van Eekeren (2017). As expected, including 20% *A. viridis* leaves in a hay-based diet led to a lower IVOMD when compared to the hay alone. This is most probably due to the higher concentrations of phenols in *A. viridis* leaves compared to hay, which can highly affect digestibility. On the other hand, the IVOMD of the mixed diet, including *A. viridis* leaves, seemed to be at its maximum at the beginning of the season, which is in line with our results on CT. Moreover, this is consistent with previous studies on fodder tree species (Papachristou and Papanastasis, 1994) and with our results on functional traits and nutrient variation during the season. Indeed, SLA and LDMC have a positive and negative effect on digestibility respectively (Pontes et al., 2007), while leaf N content may have a positive effect on digestibility (Bumb et al., 2018), and the highest values of N in *A. viridis* leaves at the beginning of the season might thus be one factor for the highest measured digestibility. In addition, the lower concentrations of fibre fractions found at the beginning of the summer might also explain the highest IVOMD in the early season.

Interestingly, a diet including 20% of *A. viridis* leaves in the DM significantly decreased in vitro total gas production when compared to the control diet consisting of hay only. This is in line with the findings of Terranova et al. (2018), where most of the woody plant leaves tested led to a decrease in CH<sub>4</sub> production in comparison to the control diet. However, CH<sub>4</sub>/dOM production from *A. viridis* leaves diet significantly increased as the season advanced, highlighting that leaves have a better CH<sub>4</sub>/dOM mitigation effect in the early season. These CH<sub>4</sub>/dOM emission values are partly in line with our results on leaf CT concentrations, which were significantly influenced by GDD but slightly increased along

the season only at two of the four study sites. Indeed, CT were repeatedly reported to reduce methanogenesis in ruminants (Patra and Saxena, 2010; Jayanegara et al., 2011; Piñeiro-Vázquez et al., 2015). Therefore, the general CH<sub>4</sub>/dOM increase during the season may be related to the strong increase of ADL common to all sites, and partly to the decrease of TEP observed at two of the four study sites, which is confirmed by the PCA results displaying ADL and phenolic fractions in opposite positions with respect to CH<sub>4</sub>/dOM. Similarly to CH<sub>4</sub> production, CO<sub>2</sub> production from *A. viridis* leaves diet significantly decreased in comparison to the control, showing that the fermentation was generally diminished with increasing GDD. However, contrary to CH<sub>4</sub>, the CO<sub>2</sub>/dOM ratio decreased with increasing GDD, which is the consequence of the reduced IVOMD that is in line with the greatly increasing ADL concentrations in leaves across the season. Consequently, the CH<sub>4</sub>/CO<sub>2</sub> ratio increased with increasing GDD.

Therefore, *A. viridis* leaves tend to have a better mitigation effect on gas emissions at the beginning of the season. According to Tavendale et al. (2005) and Terranova et al. (2018), a CT concentration below 8% in the DM as found in our study could reduce CH<sub>4</sub>/dOM production without restraining fermentation rate. Because CH<sub>4</sub> is the most important greenhouse gas emitted in animal production (Slade et al., 2016), making robust livestock graze in *A. viridis*-encroached pastures at the beginning of the season could thus help mitigate greenhouse gas emissions in these mountain livestock production systems, while keeping an appropriate ruminal fermentation and therefore a high animal performance. Finally, future trials will need to investigate *A. viridis* response to robust livestock grazing, in terms of leaf biomass resprout and chemical composition in relation to different defoliation periods, frequencies and intensities, in order to find the most suitable grazing regimes to control its encroachment.

## 6. Conclusion

This study described the composition of *A. viridis* leaves and their OM digestibility and related gas production throughout two summer seasons at four different subalpine sites. The high amounts of CP and macro- and micro-elements found in *A. viridis* leaves highlighted its value as fodder for robust livestock, offering a complementary forage resource to grass that could be well integrated in an adapted management of encroached mountain pastures. High seasonal variation in leaf chemical composition was found, and because fibre, lignin, CP and other nutrients can impact voluntary intake in foraging behaviour, these results could help defining targeted management strategies for alpine pastures to optimize robust livestock grazing in *A. viridis* encroached areas while increasing beef cattle productivity and reducing the CH<sub>4</sub> emissions. In particular, the beginning of the summer (June and July) seems to be the best period to graze *A. viridis* leaves, as in this period of the year they have the highest nutritional value and potential to reduce greenhouse gas emissions.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

I have shared the link to my data at the comment section step.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2024.108884.

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