Agriculture, Ecosystems and Environment 368 (2024) 109006

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Effect of organic fertilisation on soil phosphatase activity, phosphorus availability and forage yield in mountain permanent meadows

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A R T I C L E I N F O

Keywords:
Soil phosphorus
Nutrient input
Slurry
Farmyard manure
Manure effluent
Phosphomonoesterases
Plant diversity

A B S T R A C T

Grassland management aims to ensure sufficient yield, forage quality and biodiversity. Robust knowledge supports sustainable management practices. In South Tyrol (NE Italy), we studied the effect of organic fertilisation on soil acid and alkaline phosphatase activity (ACP, ALP, or both APase), phosphorus (P) availability and forage yield in mountain permanent meadows. Three factors were included in the experimental design, which was arranged as a split-plot design: the initial vegetation class (C1 = moderately species-poor or C2 = moderately species-rich), being the main plot, randomised within three study areas, as well as the manure type (slurry, farmyard manure, and a combination of farmyard manure and manure effluent) and the nitrogen (N) fertilisation input (0, 55.5 and 111 kg N ha^{-1} yr^{-1}), both randomised within the vegetation class. Soil samples were collected from the top 10 cm before the last cut in summer 2022. Results showed that the combined use of farmyard manure and manure effluent decreased ACP activity with increasing N input, whilst ALP activity remained unaffected. These novel findings show that organic N input does not imply an increase in APase activity. Moreover, the C2 meadow class showed higher ACP activity than C1, possibly due to higher species diversity, a lower mowing frequency and the legacy effect of more extensive management prior to the start of the trial. Both ACP and ALP activity responded to pH (negatively for ACP activity and positively for ALP activity) and both were negatively affected by soil moisture, highlighting their sensitivity to changes in the soil conditions. ALP activity was positively influenced by total organic carbon (TOC) and by the Shannon diversity index of the plant communities, possibly due to its link with the soil microbial community. Soil available P increased with pH, TOC, soil moisture, K_2O content, and N organic input from farmyard manure, which provided the highest P input. The forage yield of the last growth cycle was positively affected by organic N input but negatively affected by TOC, whilst the activity of both APase had no effect on it. The annual yield increased with the N input and was higher in the C1 meadow class than in the C2. On the whole, the results suggest that organic fertilisation, rather than APase activity, was the main driver of forage yield.

1. Introduction

Phosphorus (P) is a crucial element for all living organisms and a major limiting nutrient for plant growth in many agroecosystems (Zhu et al., 2018). In these systems, the activity of soil P enzymes is essential for mobilising P by catalysing the hydrolysis of P esters into phosphate ions (i.e., $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}), thereby making them soluble and accessible for plant uptake (Schmidt and Laskowski, 1961). Acid phosphatase (ACP, EC 3.1.3.2) and alkaline phosphatase (ALP, EC 3.1.3.1) are the predominant forms among the monoester phosphatases (APase) (Alef and Nannipieri, 1995). ACP is secreted by plants and fungi, while ALP is secreted by microorganisms and mesofauna

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https://doi.org/10.1016/j.agee.2024.109006
Received 31 January 2024; Received in revised form 12 March 2024; Accepted 27 March 2024
Available online 8 April 2024

(Carricondo-Martínez et al., 2022; Juma and Tabatabai, 1988). The available scientific literature has extensively shown that enzymes play a significant role in the decomposition of organic matter, facilitating nutrient recycling (Burns, 1978).

Grassland covers a significant portion of Europe's agricultural area, contributing to over one-third of the land (Eurostat, 2022). It provides a vital role in livestock farming systems, providing forage for herbivores (Jäger et al., 2020). Grassland also delivers essential ecosystem services including erosion control, water management and water purification (Hernández-Becerra et al., 2016). The vegetation of grassland can include grasses, graminoids, legumes and forbs, and woody species may also be present (Velthof et al., 2014). The vegetation cover and absorption capacity of grasslands significantly reduce nitrogen (N) leaching, thanks to their effective absorption rates throughout the growing season (Scherer-Lorenzen et al., 2003). Additionally, grasslands contribute significantly to enriching soils in organic matter, leading to a global decrease in carbon (C) dioxide emissions (Dignac et al., 2017). Permanent meadows are a form of utilising grassland, harvested mostly by mowing over the last five years and not having been completely renewed for ten years or longer. They are one of the most common forms of agriculturally managed grassland in Europe (Euromontana, 2021). N and P addition increases soil P availability, promoting plant nutrient uptake of nutrients, potentially increasing leaf N and P contents (Shi et al., 2020) and resulting in lower C inputs into soils through roots (Zi et al., 2022). Low APase activity values indicate that biological communities have adapted to acquiring most of their P from inorganic sources, where inorganic P content is much higher than organic P, or the remaining organic P is relatively resistant to mineralisation (Zhang et al., 2021). In general, ACP and ALP activity are thought to increase following N and P fertilisation, often associated with increased plant production (Touhami et al., 2021). However, a comprehensive investigation of patterns of APase activity in permanent meadows and their relationship with soil, biodiversity, yield and the agricultural management (especially concerning organic fertilisation) is still lacking.

Over the whole range of fertilisation intensity there is a clear trade-off in managed grasslands between yield and plant diversity (Fraser et al., 2015; Humbert et al., 2016; Müller et al., 2016). Agricultural practices involving the use of N mineral fertilisers can stimulate the activity of hydrolase enzymes such as ACP and enhance microbial biomass content in grasslands (Jian et al., 2016). In P-deficient soils, some plants hydrolyse soil organic P compounds into inorganic phosphate to transfer phosphate from senesced tissue to young tissue, reducing their reliance on soil P and P fertilisation (Yang and Yang, 2021). Organic manure fertilisation aids this plant nutrient resorption process and contributes substantial amounts of organic matter to the soil, which positively affects APase activity (Zhang et al., 2015) and other nutrient cycles. P supplementation enhances nutrient cycling in P-limited and N:P imbalanced grasslands, potentially improving nutrient use efficiency in these systems (Gong et al., 2022). In conditions of poor N and P availability, microorganisms may increase enzyme production to mobilise resources from complex substrates (Garcia et al., 1997; Lucas-Borja et al., 2011). However, the relationship between changes in productivity and the response of APase is still not fully understood. Additionally, climate indices like mean annual temperature or mean annual precipitation have been found in two meta-analyses to have a linear, positive relationship with APase activity (Meng et al., 2020; Sun et al., 2020). To comprehensively understand the factors affecting ACP and ALP activity in organically fertilised permanent meadows, we investigated the effect of different kinds of manures along a gradient of total N up to 111 kg^{-1} ha^{-1} yr^{-1} consistently applied over five growing seasons in mountain meadows located in the Alps (South Tyrol, NE Italy), corresponding to two different classes of vegetation, a moderately species-poor and a moderately species-rich botanical composition, along with other site factors related to soil and vegetation. Furthermore, we investigated the effect of the above-mentioned factors plus ACP and ALP activity on P availability, and finally the combined effect of all of them on forage yield.

We hypothesised that (I) the short-term application of organic N would positively affect the activity of both ACP and ALP, (II) P availability would increase with APase activity, (III) soil physiochemical parameters, along with vegetation-related parameters and meteorological indices, would affect the activity of both APase and P availability and (IV) forage yield would be positively affected by increased APase activity and P availability.

2. Materials and methods

2.1. Study areas and experimental design

In 2017, a field experiment, laid out as a split-plot design, was established at three study areas within South Tyrol (NE Italy): Rüdeferia/Rudiferia, Radsberg/Monterota, and Montal/Mantena, encompassing altitudes between 1112 and 1714 m above sea level (m a.s.l.), from the montane to the subalpine zone (Table 1). Within each study area, two experimental sites were established, each corresponding to different starting botanical compositions, herein referred to as meadow classes, being the main plot; C1: moderately species-poor and C2: moderately species-rich, according to Tomasi et al. (2016), also taking into consideration minor adaptations decided by the working group "Managementleitlinien zur Ausbringung von Wirtschaftsdünger in Natura 2000 Gebieten" of the Autonomous Province of Bozen-Bolzano (South Tyrol, Italy). C2 fulfils the requirements to be classified as habitat type 6510 lowland hay meadow or 6520 mountain hay meadow (European Commission, 2007), whilst C1 does not. At the start of the trial, the botanical composition of C1 meadows resembled nutrient-rich

Table 1
Location, mean topographic features, soil pH and soil texture (mean of sand, silt and clay percentage) ± SD of the six experimental sites. The coordinates refer to the centroid of the respective experimental field; altitude, aspect and inclination are mean values of the respective experimental site.

Feature	Meadow class	Study site		
		Montal/ Mantena	Radsberg/ Monterota	Rüdeferia/ Rudiferia
Coordinates (N, E)	C1	46° 42' 33.8" N 11° 55' 5.0" E	46° 44' 46.7" N 12° 13' 19.9" E	46° 35' 06.1" N 11° 55' 36.8" E
	C2	46° 42' 33.4" N 11° 55' 3.1" E	46° 45' 07.0" N 12° 12' 27.7" E	46° 35' 08.6" N 11° 55' 35.3" E
Altitude (m a.s.l.)	C1	1120 (montane)	1542 (higher-montane)	1678 (subalpine)
	C2	1112 (montane)	1714 (subalpine)	1698 (subalpine)
Aspect	C1	WNW	SSO	S
	C2	W	S	SSW
Inclination (°)	C1	5	18	12
	C2	18	19	23
pH	C1	7.36 ± 0.063	7.03 ± 0.259	7.63 ± 0.028
	C2	7.41 ± 0.098	6.09 ± 0.171	7.79 ± 0.046
Soil texture	C1	Loam	Clay	Loam
	C2	Loam	Clay loam	Loam
Sand (%)	C1	47.3 ± 3.87	44.8 ± 2.17	33.0 ± 3.43
	C2	50.6 ± 3.94	49.7 ± 3.04	37.2 ± 2.11
Silt (%)	C1	30.9 ± 2.47	44.1 ± 2.03	24.1 ± 1.54
	C2	30.4 ± 1.51	36.6 ± 1.81	23.1 ± 1.05
Clay (%)	C1	21.8 ± 1.86	11.1 ± 1.05	42.9 ± 2.26
	C2	19.0 ± 3.24	13.8 ± 1.86	39.7 ± 2.45

forms of the alliance *Centaureo transalpinae-Trisetetum flavescentis* (Scotton et al., 2012) or *Poo-Trisetetum* (Tasser et al., 2010), whilst that of C2 meadows resembled nutrient-poor forms of the alliance *Centaureo transalpinae-Trisetetum flavescentis* (Scotton et al., 2012) or *Trisetetum flavescentis* (Tasser et al., 2010; Zwack, 2019).

Altitude, aspect and inclination were not entangled with the meadow class (Table 1). Regarding the texture, it is worth noting that the soils in Radsberg/Monterota had a higher proportion of clay (clay to clay loam), while the other sites had a similar loamy texture.

Within each experimental site, nine plots with a size of 5 ×5 m (Fig. S1) were established, with two fertilisation-related factors and all the possible combinations of their levels randomly allocated to the plots. These plots were separated by a 0.5 m wide buffer zone.

The first factor was the manure type including three levels: (1) S = slurry: a mixture of faeces and urine, which may also contain water (rainwater, cleansing water), fragments of bedding material, waste feed and silage effluents; (2) F = farmyard manure: a mixture of straw or other forms of bedding, which are used to absorb faeces and urine of animals housed in tie stalls, with only a part of the urine being absorbed and bound by the bedding material; (3) L = combined use of farmyard manure (as previously described) and manure effluent, with manure effluent providing 30% of the total N. Manure effluent (German: Jauche) primarily consists of urine of animals housed in tie stalls that has not been bound by the bedding material. It may also contain, to a minor extent, effluents from stored farmyard manure or small amounts of faeces and bedding material and it is usually collected in specifically designed pits.

All manures used in the field experiment were obtained by cattle farms located in the surroundings of the experimental sites.

The second factor was N input, corresponding to 0, 55.5 and 111 kg ha^{-1} yr^{-1} of total N provided by the manures.

2.2. Determination of nutrient content in manures and manure application

Before each fertilisation event, the total N content in the manure charge to be used was measured in the laboratory by means of an Elemental Analyzer (Truspec N, LECO, Miami, USA) according to DIN-EN-ISO16634–1:2009 and the exact amount of manure to be applied in each plot was determined. The potassium (K) and P content was determined by means of ICP-OES 720 (Agilent, Santa Clara, USA) and according to Naumann et al. (1997). Total organic C was determined by the method 10.2 of VDLUFA (1995), after incineration at 550°C for four hours.

The ratio between total N, other nutrients, and C depends on the manure type (Peratoner et al., 2022) and, to a lesser extent, on the single manure batch (Table S1). Therefore, a variable input of the other nutrients and of total organic C is unavoidable. For example, P input increased from S to F (from 8.7 to 13.3 kg ha^{-1} yr^{-1} at intermediate nutrient input), K input more than doubled from F to L (from 45.5 to 98.1 kg ha^{-1} yr^{-1} at intermediate nutrient input) and total organic C increased by about 50% from S to F (from 1013.7 to 1546.7 kg ha^{-1} yr^{-1} at intermediate nutrient input) (Table 2).

The application of manure followed a specific pattern in accordance with good agricultural practice: farmyard manure was applied in autumn at the end of the growing season, while liquid manures (slurry and manure effluent) were applied at the beginning of the growing season (see Table S2 for details). At the higher nutrient input level, the total N input of the liquid manures was applied in two equal doses at the beginning of the growing season and just after the first cut. All manures were manually applied at plot level. Farmyard manure was evenly spread using pitchforks, while liquid manures were distributed using watering cans.

The mowing schedule of the experimental plots closely followed the mowing dates chosen by the owner of the meadows in which the experimental sites were located. Whenever the last grass regrowth of the grassland surrounding the experimental site was grazed, we performed an additional cut in the experimental plots at the time the grazing event in the surrounding grassland was concluded. In 2022, C1 meadows were mown three times per year, while C2 meadows were mown twice. Over the preceding years (2018–2021), the average cut frequency was 2.8 cuts yr^{-1} for C1 and 2.0 cuts yr^{-1} for C2.

Table 2
Annual mean fertilisation input ± SD of total nitrogen (N), phosphorus (P), potassium (K) and total organic carbon (C) over the whole investigation period (2017–2022) depending on manure type and nutrient input level.

Manure type	Total N (kg ha^{-1} yr^{-1})	P (kg ha^{-1} yr^{-1})	K (kg ha^{-1} yr^{-1})	Total organic C (kg ha^{-1} yr^{-1})
S	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	55.5 ± 0.00	8.7 ± 2.20	78.2 ± 12.50	1013.7 ± 345.12
	111.0 ± 0.00	17.5 ± 4.63	151.5 ± 19.78	2007.4 ± 647.93
F	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	55.5 ± 0.00	13.3 ± 3.31	45.5 ± 20.62	1546.7 ± 335.26
	111.0 ± 0.00	26.6 ± 6.62	91.1 ± 41.24	3094.3 ± 670.79
L	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	55.5 ± 0.00	10.4 ± 2.53	98.1 ± 28.95	1237.7 ± 226.57
	111.0 ± 0.00	20.9 ± 4.93	194.1 ± 59.60	2464.8 ± 468.78

S: slurry, F: farmyard manure, L: combined use of farmyard manure and manure effluent.

2.3. Soil sampling and preparation

In spring 2022, prior to the start of the growing season, composite soil samples for determining soil texture and total organic C (TOC) content were gathered from each plot using a grassland soil core sampler (2 cm diameter). Twenty subsamples were collected per plot from the topsoil layer (0–10 cm).

ACP and ALP activity, and thus available P, are known to be affected by the nutrient input as well as by the time elapsed since the application of manures (Gong et al., 2022). As the fertilisation timetable differed depending on manure type and nutrient input level (Table S2), the timing of the sampling event to quantify APase activity and available P was chosen to maximise the time since the last fertilisation event and to avoid the disturbance caused by cutting. Hence, soil samples were collected in each plot in late summer 2022, just before the last cut at each experimental site. This corresponded to the third cut at C1 sites and to the second cut at the C2 sites: August 23rd (C2) and September 17th (C1) at Montal/Mantena; August 24th (C1) and September 18th (C2) at Radsberg/Monterota; September 12th for both meadow classes at Rüdeferia/Rudiferia. This ensured that a minimum of 54 days had passed since the last fertilisation event (Table S3). Within each plot, four samples were taken within a 0.5 ×0.5 m metal frame placed randomly along a plot diagonal. Each sample comprised four to six subsamples, depending on the stoniness and root density in the soil, ensuring the minimum amount of soil required for analyses. Subsamples were blended, transferred to sealed plastic bags, and kept in a cooling box at approximately 4°C for up to four hours. Subsequently, they were stored in a refrigerator at a temperature of 4°C ± 2 °C in dark, anaerobic conditions for a maximum of three months until analyses completion.

2.4. Physicochemical soil analyses

Half of the fresh soil sample from each sampling event in summer was air-dried (7 days) and sieved to 2 mm to remove stones and plant parts debris and used to analyse the physicochemical parameters. Soil pH was determined in H_2O (1:2.5 soil:water ratio) using a digital pH meter (Robotics analyzer SP2000, Skalar, Breda, NL). TOC was assessed by an Elemental Analyzer (Primacs SNC 100, Skalar, Breda, NL), according to ISO10694:1995, the P and K soil content, expressed as phosphorus pentoxide (P_2O_5) and potassium oxide (K_2O) content, by means of ICP-OES 720 (Agilent, Santa Clara, USA) according to ÖNORM L1087:2019 A.5. Soil moisture was measured as weight loss of 30 g of fresh soil after drying at 105°C for 24 hours and expressed as a percentage of dry soil on a weight basis. Soil texture was gravimetrically determined according to ÖNORM L1061–2:2019.

Soil solution P (hence available P) was extracted in sodium bicarbonate (Olsen et al., 1954) and quantified by using the ammonium molybdate tetrahydrate-malachite green reaction as modified by Ohno and Zibilske (1991). This method was selected because of its greater sensitivity in soil-water extracts with low P concentrations in a small sample volume. Four grams of dry soil were mixed with sodium bicarbonate solution 0.5 M, and the suspension was shaken for 30 minutes, and then centrifuged for 5 minutes at 8000 rpm. The liquid extract was then filtered with qualitative filter paper (MN 619 eh, Macherey-Nagel GmbH & Co.KG, Düren, Germany). Then two reagents were added to the filtrate: ammonium molybdate 4-hydrate BioChemica 14.2 nM (AppliChem GmbH, Darmstadt, Germany) prepared in sulphuric acid 3.15 M, and a mixture composed of polyvinyl alcohol (Mowiol® 6–98, Sigma-Aldrich, Darmstadt, Germany) and malachite green oxalate (TCI Europe N.V, Zwijndrecht, Belgium). The green colour obtained was measured after 30 minutes using a SHIMADZU UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto-Shi, Japan) at 630 nm wavelength. The actual P availability concentration in soil extract was extrapolated from a simultaneously assessed calibration curve that enabled the absorbance to be related to that of an increasing concentration of standard phosphate solution submitted to the same analytical steps of the method (Ohno and Zibilske, 1991). The concentration of P extract or available P in soils was expressed in mg P kg^{-1} soil.

2.5. Soil phosphatase enzyme assay

ACP and ALP activity were measured following the method proposed by Tabatabai and Bremner (1969) and the following improvements (Eivazi and Tabatabai, 1977; Tabatabai, 1994). The other half of the fresh soil samples collected in late summer (see Section 2.3) was sieved to 5 mm. One gram of soil was then mixed with 0.2 ml of toluene, 4 ml of modified universal buffer (MUB) (pH 6.5 for assay of ACP and pH 11 for assay of ALP), and 1 ml of 4-nitrophenyl phosphate (sodium salt hydrate) (Cayman chemical, MI, USA). The mixture was placed in a sealed centrifugation tube and incubated at 37°C for one hour. After the incubation, 1 ml of 0.5 M calcium chloride ($CaCl_2$) and 4 ml of 0.5 M sodium hydroxide (NaOH) were added and thoroughly mixed. The soil suspension was then filtered with qualitative filter paper (MN 619 eh, Macherey-Nagel GmbH & Co.KG, Düren, Germany) and the yellow colour of para-nitrophenol (pNP) released by the enzyme was measured by a SHIMADZU UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto-Shi, Japan) using the colorimetric absorbance at 405 nm wavelength. The actual pNP concentrations in soil extracts were extrapolated from a simultaneously assessed calibration curve relating the absorbance to increasing pNP concentration solutions (Supelco®, Merck Life Science, Milano, Italy) (Tabatabai and Bremner, 1969). The actual pNP concentrations in samples were then translated to the enzymatic activity rates by expressing them in mg of pNP kg^{-1} dry soil h^{-1}. Analytical methods only generate estimates of the potential APase activity due to the possibility that the results might not accurately portray the true activity of APase on site (Duly and Nannipieri, 1998; Wang et al., 2013). In this case, the pH of the buffer used for ALP activity strongly differed from that of the meadow soil samples (ranging between 5.7 and 7.9) and also the pH buffer used for ACP activity was not exactly the same as those in all soil samples.

2.6. Vegetation-related parameters

In 2022, a detailed assessment of the botanical composition was performed shortly before the end of the first growth cycle. In one third of the plots, the plant cover of each species across all vegetation layers was measured by means of point quadrat analysis (Levy and Madden, 1933) with 80 observation points per plot (20 points spaced 10 cm apart along each plot side), starting 1.5 m from the plot side and keeping a distance of 25 cm from the plot margin in order to avoid margin effects (Fig. S1). At each observation point, all contacts of plant parts with the tip of iron rods vertically led towards the soil with the aid of a wooden frame (Peratoner, 2003) were recorded. Species occurring in the plot, but not having been hit by the iron rods, were assigned a conventional value of 0.5% (Peratoner and Pötsch, 2019). The cover of single species in the remaining plots was visually estimated using the results of the measurements as a reference. The Shannon-Index, combining species richness and their evenness (Shannon, 1948), was calculated based on this data related to the first growth cycle alone. For the last growth cycle, evenness was calculated based on the yield proportions of the functional groups (grasses, including also graminoids, legumes and forbs), which were visually estimated in each plot just before the last cut. Community-weighted mean of Ellenberg indicator values (Ellenberg and Leuscher, 2010) for nutrient availability (Ellenberg N) were computed based on the cover of each species.

In each plot, just before each cut, forage yield on dry matter basis (DM) was quantified by subsequently placing a 0.5 $\times$0.5 m metal frame along one of the plot diagonals, and by harvesting the biomass within the frame at a stubble height of 5 cm by means of electric scissors (Gardena ClassicCut, Husqvarna Italia, Erba, Italy). The samples were oven-dried at 60°C until weight constancy, and average values at plot level were computed.

2.7. Meteorological indices

Meteorological data were obtained by automated weather stations in Bruneck/Brunico at 828 m a.s.l., St. Martin in Thurn/S. Martino in Badia at 1150 m a.s.l., and Toblach/Dobbiaco at 1219 m a.s.l., located near Montal/Mantena, Rüdeferia/Rudiferia and Radsberg/Monterota, respectively (Meteo Browser Eurac Research, 2022). Daily maximum temperature and minimum temperature correspond to the average at each experimental site over the time between the date of farmyard manure application in autumn 2021 and the sampling date of the last growth cycle. An adjustment of each temperature was applied (-0.59 ºC for every 100 m a.s.l. increase in altitude). Precipitation was summed at each experimental site over the time comprising between the date of farmyard manure application in autumn 2021 and the sampling date of the last growth cycle.

2.8. Statistical analysis

Four different dependent variables (ACP activity, ALP activity, available P and forage yield) were studied in separate analyses. Concerning ACP and ALP activity, along with the factors of the experimental design (meadow class, manure type, N input), further soil, vegetation, and meteorological variables were tested for inclusion as explanatory variables into the statistical model: i) soil-related variables: TOC before the start of the growing season, pH, phosphorus pentoxide (P_2O_5) and K_2O content, sand proportion, clay proportion, soil moisture content and available P, ii) fertilisation-related variables: C, P and K input during the growing season 2021–2022, iii) vegetation-related parameters: Shannon-Index at the end of the first growth cycle, evenness based on the yield proportion of the functional groups (grasses, legumes and forbs) in the last growth cycle and yield proportion of grasses, forbs and legumes (both from the last growth cycle and the whole growing season), and iv) meteorological indices: mean maximum and minimum temperature, and precipitation sum. In the analysis concerning available

P, ACP and ALP activity were included together with all the already mentioned set of independent variables possibly affecting available P in the soil. Finally, ACP and ALP activity and available P in soil were included in the cited set of independent variables potentially affecting forage yield. Additional data analyses according to the experimental design only and following the methods described above were performed at plot level for additional dependent variables needed for the discussion of the results: the species number and the weighted community mean of Ellenberg N at the time of the first cut as well as the total N content in soil at the end of the last growth cycle.

All the statistical analyses were performed by means of linear mixed models. As a first step, a baseline model was investigated, accounting for the split plot design (study site as fixed term and the interaction between study site and meadow class as random term), for the main terms of meadow class, manure type and N input (all treated as categorial factors) as well as their interactions. Repeated measurements within the plots were accounted for by considering the plot as a random term. This term was omitted for the analyses of the variables describing the yield, for which it was impossible to establish a univocal relationship between pseudoreplicates of soil parameters and yield measurements. Therefore, measurements of all variables were averaged at plot level. In a second step, the non-significant interactions between N input and the other factors were dropped, and then all further analyses were performed by treating N input as a covariate and by performing a stepwise forward model development to explore the improvement of model fit by adding further available explanatory variables (all metric). The Satterthwaite approximation for degrees of freedom in combination with type III sum of squares and treatment contrasts were used. The choice of including new explanatory variables was made based on the Akaike Information Criterion (AIC, the lower the better) (Akaike, 1998) and the p-value of the terms (a p-value of 0.1 was regarded as a threshold for keeping nonsignificant terms in case of AIC improvement). Interactions of designed factors that became non-significant during model development and exceeded a p-value of 0.1 were dropped at the end of model development. During model development Maximum Likelihood (ML) was used in order to compare models differing in fixed effects via AIC, whilst Restricted Maximum Likelihood (REML) was used to get the final results. Terms turning non-significant in this last step were retained in the model. Diagnostic plots were used to visually check the assumptions concerning the normal distribution of residuals and homoscedasticity (Kozak and Piepho, 2018). In case they were not fulfilled, the analysis was performed with natural log-transformed data after checking that the transformation allowed fulfilling the assumptions. Results were back transformed and are always mentioned or shown on the original scale. Collinearity due to the inclusion in the model of not designed factors was checked by means of Variance Inflation Factor (VIF) using 10 as a threshold. In the event of collinearity among the designated explanatory variables, priority was assigned to the latter ones, followed by the covariate that resulted in a better model fit. This prevented C input, P input and K input from being included in the models. Metric variables were centred around their mean for the models having yield as a dependent variable, in order to achieve convergence. Post-hoc comparisons of estimated marginal means were performed by the Sidak test. When p-values were less than 0.05 they were considered statistically significant, and the respective explanatory variables were interpreted in the results and discussion sections. Back-transformed predicted values, estimated marginals means and 95%-confidence intervals, given in square brackets, are reported in the text.

All analyses were conducted in R version 4.3.2 (R Core Team, 2023) and RStudio (version 2022.07.2+576). The function *lmer* from the package *lmerTest* was used to conduct the linear mixed model analyses. The package *effects* was used to visualize the results. Post-hoc comparisons and calculation of estimated marginal means were performed by using the *emmeans* package.

3. Results

3.1. Overview of factors affecting the dependent variables

The statistical analyses revealed that different groups of independent variables exerted a significant influence on the investigated dependent variables (Table 3).

Of the soil-related variables, soil moisture and pH consistently played a relevant role in determining ACP activity, ALP activity (Section 3.2) and available P (Section 3.3). Whilst both ACP activity and available P were affected by the interaction between N input and manure type, no effect of the fertilisation was detected on ALP activity. Interestingly, available P was the only dependent variable affected by K soil content and ALP activity. The meadow class affected ACP activity alone, whilst ALP activity was the only soil-related dependent variable being affected by TOC and a diversity-related parameter, namely the Shannon Index (Section 3.2). None of the investigated meteorological variables were retained for inclusion into the models during their development. Concerning the yield-related parameters (Section 3.4), N input emerged as the primary influential factor, followed by meadow class, which also interacted with manure type in the case of the yield of the last growth cycle. Additionally, TOC also affected the yield of the last growth cycle. Neither the ACP or ALP activity nor the available P had any detectable influence on the yield-related dependent variables.

Regarding the yield-related parameters (Section 3.4), N input emerged as the foremost influential factor, with meadow class following closely behind. Meadow class also displayed an interaction with manure type in the model constructed for the yield of the last growth cycle. The latter was also affected by TOC. Neither the ACP or ALP activity nor the available P were found to affect the yield.

3.2. Effects of management and physiochemical parameters on ACP and ALP activity

Several soil physiochemical parameters were found to affect ACP and ALP activity (Table 3). Concerning the designed factors, ACP activity was affected by all of them, whereas none of them had an effect on ALP activity. The meadow class strongly affected the activity of ACP ($F=53.4$, $p<0.001$), which increased by 151 [115–198] mg pNP kg^{-1} h^{-1} from C1 (91 [70–119] mg pNP kg^{-1} h^{-1}) to C2 (242 [185−317] mg pNP kg^{-1} h^{-1}).

The influence of N input on ACP activity varied depending on the type of manure used ($F=6.0$, $p=0.006$). Whilst no effect of increasing N input was found for slurry, a slightly negative effect was found for farmyard manure and the combination of farmyard manure and manure effluent along the N input gradient from 136 [111–165] to 145 [119–176] mg pNP kg^{-1} h^{-1} when using slurry, from 171 [140–208] to 154 [127–188] mg pNP kg^{-1} h^{-1} when using farmyard manure and from 176 [144–214] to 118 [97–144] mg pNP kg^{-1} h^{-1} when using a combination of farmyard manure and manure effluent (Fig. 1a).

When investigating additional physiochemical parameters, ACP activity was observed to decrease with rising pH levels (from 229 [171−307] at pH 5.5–123 [103–146] mg pNP kg^{-1} h^{-1} at pH 8.0, $F=10.2$, $p=0.002$) (Fig. S2a), with a more pronounced decline linked to increasing soil moisture content (from 282.2 [226−353] at approximately 10% soil moisture content to 96 [80–114] mg pNP kg^{-1} h^{-1} at 70% soil moisture content, $F=40.6$, $p<0.001$) (Fig. S2b). Both relationships exhibited a convex pattern, displaying a steeper decrease at lower values of pH and soil moisture values.

ALP activity was also affected by the observed pH gradient ($F=7.4$, $p=0.008$). In contrast to ACP activity, ALP activity exhibited an opposite trend, increasing from 166 [121−227] to 277 [213−360] mg pNP kg^{-1} h^{-1} with a slight convex course (Fig. S3a). Like ACP, ALP activity was strongly negatively affected by soil moisture ($F=71$, $p<0.001$), displaying a considerable decrease of 448 [338−593] mg pNP kg^{-1} h^{-1} between 10% and 70% soil moisture with a marked convex course

Table 3
Factors affecting ACP activity, ALP activity, available P, and forage yield (last growth cycle = forage yield of the last growth cycle; whole year = annual cumulative forage yield over all growth cycles).

Source		ACP#		ALP#		Available P#		Forage yield (last growth cycle)#		Forage yield (whole year)#	
	df	F	p	F	p	F	p	F	p	F	p
Site	2	2.5	0.249	2.1	0.324	10.6	**0.047**	8.4	**0.001**	4.0	**0.025**
Meadow class (MC)	1	53.4	**<0.001**	2.5	0.248	0.4	0.581	10.7	**0.002**	61.0	**<0.001**
Manure type (MT)	2	2.5	0.094	0.5	0.602	3.2	**0.048**	3.0	0.060	3.2	**0.048**
N input (N)	1	3.2	0.082	0.2	0.684	5.9	**0.018**	12.2	**0.001**	64.2	**<0.001**
MC x MT	2	3.0	0.060	-	-	-	-	3.4	**0.043**	2.5	0.094
MT x N	2	6.0	**0.006**	-	-	9.2	**<0.001**	-	-	-	-
MC x MT x N	3	2.2	0.099	-	-	-	-	-	-	-	-
pH	1	10.2	**0.002**	7.4	**0.008**	6.3	**0.014**	-	-	-	-
Soil moisture	1	40.6	**<0.001**	71.0	**<0.001**	11.7	**0.001**	-	-	-	-
TOC	1	-	-	4.1	**0.048**	3.7	0.060	12.1	**0.001**	-	-
Shannon-Index	1	-	-	7.8	**0.008**	-	-	-	-	-	-
K_2O soil content	1	-	-	-	-	23.3	**<0.001**	-	-	-	-
ALP	1	n.t.		n.t.		4.1	**0.045**	-	-	-	-
ACP	1	n.t.		n.t.		-	-	3.2	0.081	-	-

df = degrees of freedom; F = Fisher's F, p = probability; p-values < 0.05 are highlighted in bold. # Analysis with natural logarithm-transformed data. – = not taken up in the model according to the stepwise forward model selection. n.t. = not tested for inclusion.

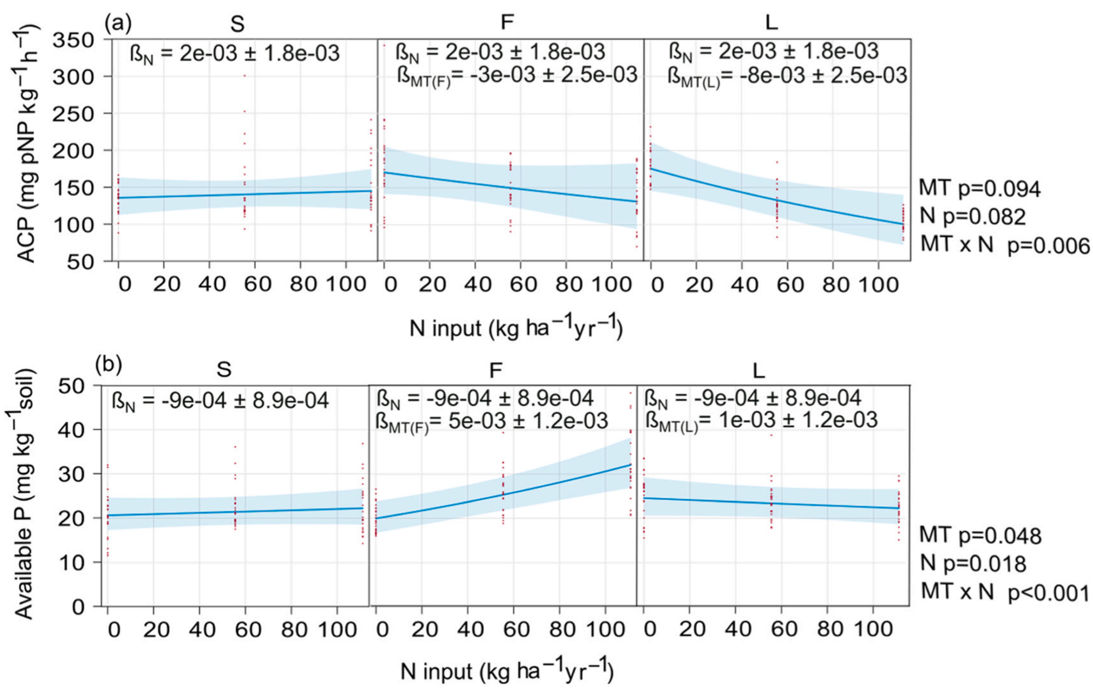

Fig. 1. Effect of N input and manure type on (a) ACP activity and (b) available P at the end of the last growth cycle. S: slurry, F: farmyard manure, L: combined use of farmyard manure and manure effluent. Predicted values and 95%-confidence interval are shown against partial residuals. Analysis with natural logarithm-transformed values; back-transformed values are shown (n=72). MT= Manure type, N = N input; estimated slope parameters (β) ± SE of the reference level ($β_N$) and the difference for levels F ($β_{MT(F)}$) and L ($β_{MT(L)}$) are reported on the transformed scale.

(Fig. S3b). Unlike ACP activity, two additional physiochemical parameters positively influenced ALP activity, which increased from 193 [147–254] to 293 [221–389] mg pNP kg^{-1} h^{-1} as TOC increased from 6% to 20% (F=4.1, p=0.048) and from 196 [152–253] to 267 [202–353] mg pNP kg^{-1} h^{-1} as the botanical diversity at the end of the first growth cycle, expressed as the Shannon index, increased from 2.5 to 3.3 (F=7.8, p=0.008) (Fig. 2a and Fig. 2b respectively).

3.3. *Effects of management and physiochemical parameters on available P*

When considering the designed factors, meadow class had no effect on available P (F=0.4, p=0.581), which was instead affected by the interaction between N input and manure type (F=9.2, p<0.001) (Fig. 1b). N input from slurry or the combination of farmyard and manure effluent did not significantly impact available P, whereas an increase was observed with increasing N input from farmyard manure (from 20.0 [16.0–25.0] to 32.1 [25.6–40.2] mg P kg^{-1} soil along the N input gradient).

Available P exhibited a positive curvilinear relationship with soil physicochemical parameters such as K_2O (F=23.3, p<0.001), soil moisture content (F=11.7, p=0.001) and pH (F=6.3, p=0.014). Increasing K_2O content in soil (ranging between 9 and 118 mg 100 g^{-1} soil) led instead to a substantial increase in available P from 19.4 [15.2–24.8] to 45.7 [33.4–62.5] mg P kg^{-1} soil (Fig. 3a). Moreover, with a pH increase from 5.5 to 8.0, available P increased from 14.6 [9.8–21.9] to 28.7 [22.4–36.8] mg P kg^{-1} soil (Fig. S4a). Concerning the APase, only ALP activity positively affected available P (F=4.1, p=0.045)

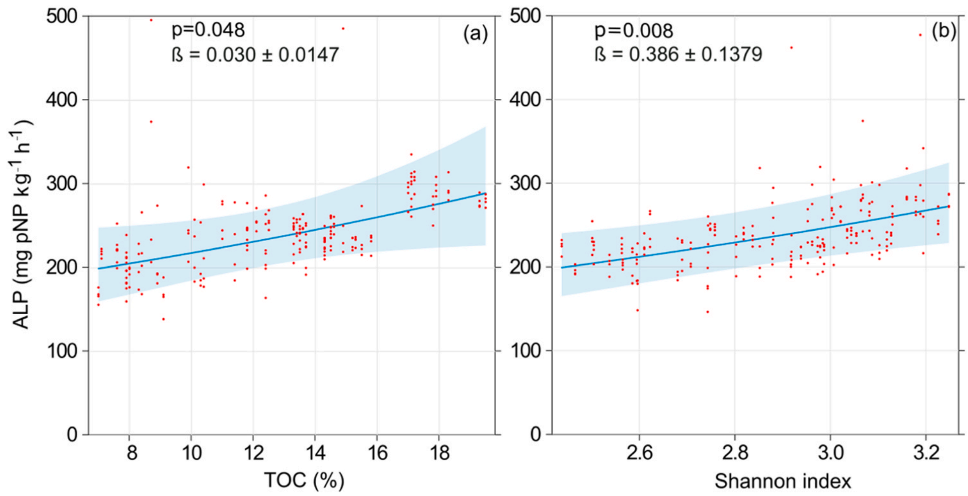

Fig. 2. Effect of (a) total organic carbon (TOC), and (b) Shannon index on the ALP activity at the end of the last growth cycle. Predicted values and 95%-confidence interval are shown against partial residuals. Analysis carried out with natural logarithm-transformed values; back-transformed values are shown (n=216). Estimated slope parameters (β) ± SE are reported on the transformed scale.

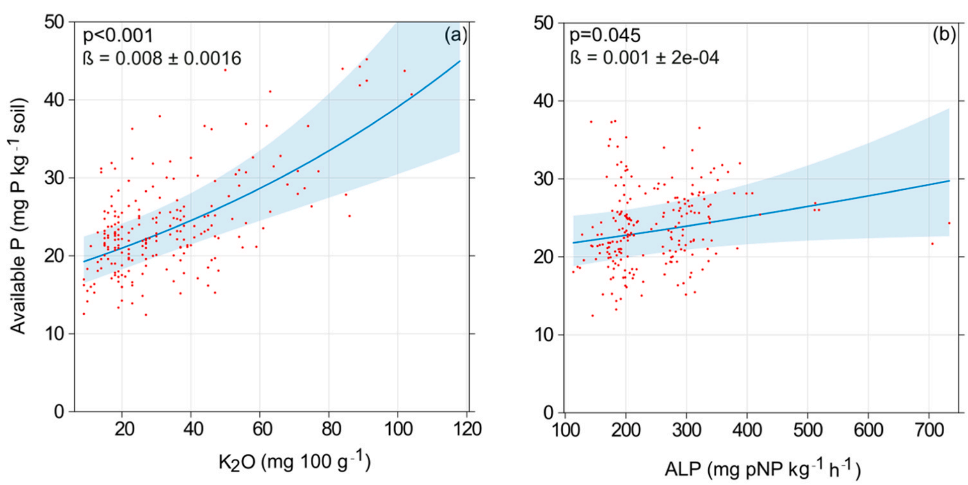

Fig. 3. Effect of (a) K_2O content in soil and (b) ALP activity on the available P at the end of the last growth cycle. Predicted values and 95%-confidence interval are shown against partial residuals. Analysis with natural logarithm-transformed values; back-transformed values are shown (n=216). Estimated slope parameters (β) ± SE are reported on the transformed scale.

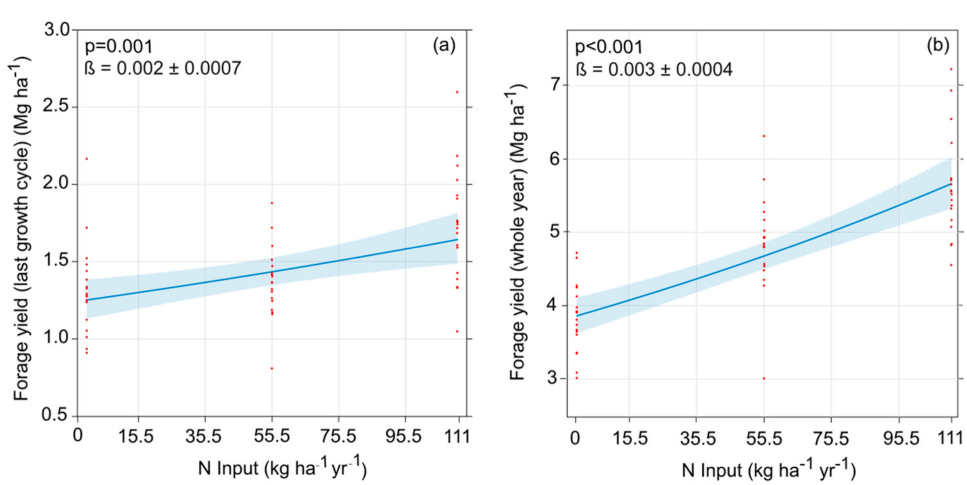

Fig. 4. Effect of N input (a) on the forage yield of the last regrowth at the end of the last growth cycle and (b) on the annual cumulative forage yield over all growth cycles. Predicted values and 95%-confidence interval are shown against partial residuals. Analysis with natural logarithm-transformed values; back-transformed values are shown (n=54). Estimated slope parameters (β) ± SE are reported on the transformed scale.

(Fig. 3b). However, despite ALP activity increasing from 100 to 700 mg pNP kg^{-1} h^{-1}, the increase in available P was only 7.6 [5.3–10.8] mg P kg^{-1} soil (from 21.7 [17.0–27.6] to 29.3 [22.3–38.4] mg P kg^{-1} soil). Similarly, a moderately steep increase was observed depending on soil moisture from 13.5 [9.6–19.2] to 33.9 [25.9–44.3] mg P kg^{-1} soil along a moisture gradient ranging from 10% to 70% (Fig. S4b).

3.4. Effects of management and physiochemical parameters on forage yield

The forage yield of the last growth cycle exhibited a positive relationship with N input (F=12.1, p=0.001) and a negative relationship with TOC (F=12.1, p=0.001). As N input increased, forage yield increased from 1.24 [1.11–1.38] to 1.66 [1.49–1.85] Mg ha^{-1} (Fig. 4a). Conversely, yield strongly decreased from 2.49 [1.80–3.44] to 0.83 [0.60–1.14] Mg ha^{-1} as the TOC increased from 6.75% to 19% (Fig. S5). We did not find any effect of other physiochemical parameters on forage yield.

Furthermore, the forage yield of the last growth cycle was affected by the interaction of manure type and meadow class (F=3.4, p=0.043) (Table 4). Whilst no effect of the manure type was found within C1, fertilisation with farmyard manure resulted in higher yields than those resulting from fertilisation with slurry. Only when the combination of farmyard manure and manure effluent was used for fertilisation, higher yield was observed in C1 than in C2.

The cumulative annual forage yield was influenced by the meadow class (F=61.0, p<0.001) and was 1.44 [1.35–1.52] Mg ha^{-1} higher for C1 (5.45 [5.15–5.76] Mg ha^{-1}) than for C2 (4.01 [3.80–4.24] Mg ha^{-1}). Additionally, it was positively affected by N input (p<0.001) and it increased from 3.80 [3.56–4.06] to 5.75 [5.39–6.14] Mg ha^{-1} along the investigated N input gradient (Fig. 4b). The influence of manure type was less pronounced: the combined use of farmyard manure and manure effluent led to the highest mean annual forage yield (4.96 [4.63–5.31] Mg ha^{-1}), the lowest one was observed with slurry (4.39 [4.10–4.70] Mg ha^{-1}), whilst that of farmyard manure achieved an intermediate value (4.69 [4.38–5.02] Mg ha^{-1}) (F=3.2, p=0.048).

4. Discussion

4.1. Effects of management and physiochemical parameters on ACP and ALP activity

Several findings of this study align with evidence from existing literature concerning ACP and ALP activity. Firstly, ACP activity exhibited a negative correlation with soil pH, while ALP activity showed a positive correlation. This observation is consistent with various meta-analyses conducted in different agroecosystems (Janes-Bassett et al., 2022; Sun et al., 2020; Pokharel et al., 2020). Soil pH influences P availability, impacting plant access to essential forms of P. The study emphasizes the critical role of soil pH within the common range of meadows, where enzyme activity is vital for making P accessible to plants and microorganisms.

Secondly, plant diversity positively influenced both ACP and ALP activity. Increased soil microbial diversity, which is known to be linked to higher plant diversity (Liu et al., 2020), resulted in elevated enzyme activity. Specifically, ACP activity was higher in meadow class C2 than C1, attributed to the higher species diversity in C2. Indeed, despite five years of differentiated N input, the initial status in terms of meadow class hasn't changed in most of the plots (in 51 out of 54 plots, corresponding to 94.4%) and there is still a significant effect of the meadow class (F=26.2, p=0.036), leading to a higher species number in C2 (45.4 [37.9–52.9]) compared to C1 (32.7 [25.2–40.2]), (Table S4). This suggests that species diversity results in a variety of coexisting N and P acquisition strategies (Lambers et al., 2008), enhancing ACP activity. Similarly, ALP activity is positively correlated with the Shannon index based on the botanical composition of the vegetation at the time of the first cut, indicating the importance of vegetation diversity in influencing the activity of both APase. The importance of species diversity for ACP and ALP activity can also be attributed to a higher likelihood of having community-level combinations of conservative and acquisitive traits that regulate tissue P demand and the need for acquiring P through APase activity function (White and Hammond, 2008). Indeed, a positive correlation between the diversity of the soil microbial community and ACP activity has been found in arable crops elsewhere (Diallo-Diagne et al., 2016; Sun et al., 2018). Additionally, Liu et al. (2021) found out in a newly established ryegrass (*Lolium perenne* L.) sward that the treatment showing the highest ALP activity was that also showing the highest value of diversity-related parameters such as Shannon index and Pielou's evenness for the fungal soil community.

As a third finding, we confirm the positive association between ALP activity and soil organic C that has been well demonstrated in previous studies (Shi et al., 2020; Pokharel et al., 2020).

Finally, the statistical analysis detected an inverse relationship between soil moisture and the activity of both APase. A negative correlation between the activity of APase and soil moisture has been reported in pastures with P input only (Speir and Cowling, 1991) or input of P and N (Touhami et al., 2022a; Touhami et al., 2022b) in addition to the nutrient input provided by grazing animals.

Nevertheless, our study also yielded an unexpected outcome, as no effect of the N input was found on ALP activity, whilst a clear interaction between manure type and N input was detected for ACP activity. ACP activity decreased with increasing N input only in the treatment combining farmyard manure and manure effluent, whilst it remained unaffected by the N input when using the other manures. Our results seem to contradict the outcome of several meta-analyses based on natural soils, including grasslands, demonstrating that N mineral fertilisation (Jian et al., 2016; Margalef et al., 2021; Marklein and Houlton, 2012) and organic fertilisation (Miao et al., 2019) increase ACP and ALP activity. In any case, the activity of ACP increases if P plant demand increases (Nannipieri et al., 1978), provided that the P availability in the soil is insufficient for both plant and microorganism growth. Considering that the available P is directly related to the type of manure and N input gradient (see Section 4.2), the obtained results concerning ACP activity are surprising and at the same time novel suggesting that an increase in N input does not consistently result in increased ACP activity when utilizing organic manures. Moreover, ACP activity can even decrease when using increasing addition of manure effluent in combination with farmyard manure. Although our design does not allow us to provide an explanation for it, the examination of the composition of the utilized manures (Table S1) and of the respective nutrient inputs (Table S2) highlights that the combined use of farmyard manure and manure effluent differs by the other treatments mainly by the very low N:K and very high K:P ratio. Moreover, as a difference to the fertilisation with farmyard manure only, its application was closer in time to the sampling date at the highest fertilisation rate. This suggests a complex mechanism governing ACP's activity response, which is not readily

Table 4
Effect of manure type (S: slurry, F: farmyard manure, L: combined use of farmyard manure and manure effluent) and meadow class (C1: moderately species-poor meadows, C2: moderately species-rich meadows) on the DM forage yield of the last growth cycle (Mg ha^{-1}). Multiple comparisons by Sidak. Analysis with natural logarithm-transformed data. Back-transformed estimated marginal means and 95%-confidence intervals (in square brackets) are shown. Means between manure type within the same meadow class sharing no uppercase letters and means between meadow classes within the same manure type sharing no lower caser letters significantly differ from each other (n=9).

Meadow class	Manure type		
	S	F	L
C1	1.61 [1.33–1.95]^{Aa}	1.66 [1.38–2.00]^{Aa}	2.04 [1.68–2.46]^{Aa}
C2	1.03 [0.84–1.25]^{Ba}	1.37 [1.13–1.65]^{Aa}	1.14 [0.95–1.36]^{ABb}

explicable for our study. Further data would be required to interpret the acquired results, including additional sampling data during growth and available soil macronutrient ratios.

Consequently, our first hypothesis was proved false, as short-term organic N fertilisation did not positively affect the activity of either enzyme. However, our third hypothesis holds true, since there was an effect of pH and soil moisture on ACP and ALP activity. The response of both ACP and ALP activity to pH was as expected, whereas their response to soil moisture was found to be negative. Furthermore, organic matter had a positive and significant influence on ALP activity and plant diversity positively affected the activity of both APase.

4.2. Effects of management and physiochemical parameters on P availability

The results indicate an interaction between manure type and N input, as well as a positive relationship between soil moisture, K_2O, pH and ALP activity, influencing P availability, and these align with the existing literature.

Depending on their P content and on the applied amount, manures contribute organic P to P cycling, increasing P content (Edmeades, 2003) and P availability (Kidd et al., 2017) in soils. Indeed, in our study, available P was shown to increase by increasing nutrient input only when fertilisation was implemented with farmyard manure, the manure type having the highest P and C content (Table S1) and providing the highest input of both elements (Table 2). A long-term study investigating the effect of organic and inorganic fertilisation in meadows has shown a positive correlation between ALP activity and organic extractable P (i.e., check the method explained by Bowman and Cole, 1978) in plots receiving mineral P and farmyard manure (Colvan et al., 2001). We found a positive correlation between ALP activity and available P, which is likely to be related to increased microbial biomass, as ALP activity is released by microorganisms. Indeed, long-term application of farmyard manure has been found to result in both higher microbial biomass and ALP activity in comparison to mineral fertilisation (Langer and Klimanek, 2006). On the other hand, having observed a neutral or negative response of ACP activity to the addition of slurry, farmyard manure or the combination of farmyard manure and manure effluent respectively, it is logical that no significant correlation in our statistical analysis has been found between ACP activity and available P. It is well known that physicochemical parameters that strongly influence APase activity also impact P availability. In some cases, the presence of potential P-mobilizing microbes in grasslands with high P availability may be influenced by environmental factors like soil pH and moisture (Graça et al., 2021). Therefore, low soil moisture content leads to lower P availability (Meisser et al., 2019). In our study, both pH and soil moisture positively affect available P, and these correlations also explain the link between P and uptake of other nutrients, such as N, since N-cycling microbial communities are influenced by soil P content under P application (O'Neill et al., 2022). Moreover, under conditions of high N content in the soil, the enhanced ability of organic C degradation leads to further degradation of complex organic matter, releasing more organic phosphoric acid and ultimately improving the utilization rate of organic P by soil microorganisms (Chen et al., 2021).

In our study, we observed a positive relationship between soil K content and P availability. When abiotic conditions improve and plants and microbes increase their productivity, various mechanisms for mobilizing nutrients become activated. These mechanisms include enzyme activity, exudation, release of acidic compounds, and increased soil volume occupancy (Sardans et al., 2011; Sardans et al., 2023). As a result, both K and P are mobilized into soluble and available forms (Sardans et al., 2023). The enhancement of physiological activities and interactions among plants and microbes leads to their greater mobilization and availability in the soil. For instance, root proton release facilitates the mobilization of P and K in soil (Jungk and Claassen, 1986). Over evolutionary time, numerous synergistic processes likely developed between these two essential nutrients for plants and microbes. Although the molecular mechanisms underlying K and P interaction require further investigation, existing literature suggests for example the existence of cross-talk between signalling pathways involved in plant responses to K and P (Wang et al., 2002).

These results are partially consistent with our second hypothesis, as it appears that microbial communities, which release ALP, play a positive role concerning P availability, similar to other parameters such as manure type, N input, K_2O, pH and soil moisture.

4.3. Effects of management and physiochemical parameters on forage yield

The primary aim in forage production is to attain a satisfactory yield and quality of forage, with P being a key macroelement that could potentially limit the grass growth. However, to our best knowledge, the only study simultaneously examining the relationship between forage yield, APase activity and available P is based on pastures (Speir and Cowling, 1991), with no study addressing this topic in permanent meadows. We investigated the effect of organic fertilisation as well as of soil physiochemical parameters on the forage yield of the last growth cycle and in a second step, we focused on the effect of these factors on the forage yield of the whole year (henceforth, annual yield). Concerning the forage yield of the last regrowth, we found a positive effect of N input, a negative effect of TOC, as well as an interaction of manure type with meadow class. Regarding the annual yield, the results showed a positive relationship with N input, an effect of the meadow class and one of the manure type.

The consistent increase of forage yield with increasing N input along the whole gradient is a typical agronomic response, suggesting that even at the highest fertilisation rate the yield potential was not fully exploited yet. Moreover, the results did not indicate any effect of P availability on the forage yield of both the last growth cycle and the annual yield, suggesting that P was not limiting. As for the annual yield, the higher annual forage yield observed for C1 compared with C2 is likely to be due to a combination of factors entangled within the factor meadow class. The results suggest that after five years of differentiated fertilisation treatment there is still a residual effect of the meadow class on nutrient availability beside that of the fertilisation itself. This can be interpreted as a legacy effect of the management of the experimental fields prior to the start of the trial. So, C1 exhibited a constant soil N content at the end of the last growth cycle, whilst that of C2 was observed to rise with increasing N input (Table S5 and Fig. S6). However, no significant differences were found between C2 and C1 at the two extremes of the N input gradient (6.8 [4.7–9.0] vs. 8.4 [6.3–10.6] in case of 0 kg N ha^{-1} yr^{1-} and 7.3 [5.0–9.5] vs. 8.4 [6.1–10.6] at an input of 111 kg N ha^{-1} yr^{-1}), suggesting that the this is not the main cause for the observed yield differences. The second factor involved is the higher proportion of competitive species in C1 than in C2 as shown by the higher values of the community-weighted Ellenberg N, which increased with increasing N input in both C1 and C2, but exhibited greater differences at the lower end of the N-input gradient (5.1 [4.9–5.4] for C1 and 3.5 [3.2–3.8] for C2) than at the upper end of it (5.7 [5.4–6.0] for C1 and 4.5 [4.2–4.8] for C2) (Table S6, Fig. S7). A further role of the mowing frequency cannot be excluded, although there is evidence that, if not supported by increased nutrient availability, it does not necessarily positively affect forage yield (Pavlů et al. 2011).

Furthermore, the forage yield of the last growth cycle was negatively influenced by TOC. Organic matter affects soil structure, water-holding capacity, and its accumulation can fix minerals whereas its decomposition will increase mineral availability (Hoogerkamp, 1973). The observed inverse correlation may be explained by the diversity and composition of the microbial community altering organic matter components and stocks, and ultimately impacting forage production (Li et al., 2021). Under specific weather conditions, this excess organic matter could temporarily sequester nutrients during microbial decomposition, diminishing their availability to plants. The higher yield in C1 compared to C2, specifically where the combination of farmyard manure and manure effluent was used, is probably due to the greater presence of competitive species in C1. These species tend to respond more strongly to N availability and in manure effluent N is almost entirely in ammonium form which is readily available (Table S1). The relationship between crop yield and APase activity has primarily been studied in arable crops, showing a positive correlation between ACP activity and yield (Antolín et al., 2005; Gao et al., 2016; Moharana and Biswas, 2022; Wei et al., 2021). However, we found no effect of ACP activity on the forage yield of the last growth cycle and on the annual yield, which requires further investigation. The results obtained are inconsistent with our third hypothesis, as neither ACP nor ALP activity have an impact on the forage yield of the investigated permanent meadows. This suggests that organic fertilisation under the given conditions is the main driver of forage yield.

4.4. *Limitations of the study*

Whilst most of the investigated independent and dependent variables are a summary of the whole growing season or at least of single growth cycles, or are expected not to greatly change during the growing season, APase values refer to the end of the growing season only. On one hand, this was necessary to minimise the effect of the time elapsed from the last fertilisation event, which was treatment-dependent. On the other hand, this is not sufficient to fully characterise the APase activity in terms of activity peak or changes over time. For this reason, it cannot be excluded that APase played a more significant role in specific parts of the growing season. Future studies may benefit from repeated sampling throughout the entire growing season to elucidate the role of APase at other specific stages of the growing season, thereby providing a more comprehensive understanding of their dynamics and contributions to plant development.

5. Conclusions

This study demonstrates that the activity of both APase, which are directly related to the P cycle, are influenced by soil physicochemical factors, such as pH and soil moisture, but also by factors related to biodiversity; ACP, primarily released by plants, exhibits higher activity in moderately species-rich meadows, whilst ALP activity is positively influenced by biological factors like TOC and the Shannon diversity index of the aboveground vegetation.

The combined use of farmyard manure and manure effluent, along with an increasing rate of nutrient input, causes a negative response in ACP activity, whilst there is no response when using farmyard manure alone or slurry.

Regarding available P in soil, multiple factors play a significant role, including soil physicochemical factors and concentrations of other macronutrients like N and K. The type of organic manure also affects the P availability emphasising the importance of being aware of the ratios between the macronutrients in different manures. Concerning the APase, their impact is of limited relevance at the sampling time.

In the investigation of the annual yield, the study did not detect significant effects of APase activity and available P, indicating that nutrient input from organic fertilisation plays a predominant role here, overshadowing the positive impact of APase activity and P availability. However, as in the long term a negative relationship between management intensity and biodiversity is expected, our findings suggest that APase activity contributes more strongly to the P cycle under extensive management.

CRediT authorship contribution statement

Patrícia Campdelacreu Rocabruna: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xavier Domene:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Aldo Matteazzi:** Writing – review & editing, Resources, Investigation. **Ulrich Figl:** Writing – review & editing, Investigation. **Alois Fundneider:** Investigation, Writing – review & editing. **Marcos Fernández-Martínez:** Writing – review & editing, Formal analysis. **Elena Venir:** Writing – review & editing, Resources. **Peter Robatscher:** Writing – review & editing, Resources. **Catherine Preece:** Writing – review & editing. **Josep Peñuelas:** Writing – review & editing. **Giovanni Peratoner:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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

Data will be made available on request.

Acknowledgements

We thank Hans-Peter Piepho for advice concerning the experimental design, Federico Fava and the other colleagues from Laimburg Research Centre for support in organising the lab work, Matias Pons from CREAF who contributed to lab work prior to analyses, Bernat Perramon and Joan Casas who provided useful information and training on biodiversity in Pyrenees grasslands. Additionally, Àngela Bosch, Rosa Ma. Poch and Jimena Ortiz for help in understanding the ACP and ALP activity assay. The field experiment in which this investigation took place was partially funded by the Action Plan 2016–2022 for Research and Training in the Fields of Mountain Agriculture and Food Science of the Autonomous Province of Bozen/Bolzano. Marcos Fernández-Martinez was supported by the European Research Council project ERC-StG-2022–101076740 STOIKOS, and a Ramón y Cajal fellowship (RYC2021–031511-I) funded by the Spanish Ministry of Science and Innovation, the NextGenerationEU program of the European Union, the Spanish plan of recovery, transformation and resilience, and the Spanish Research Agency. Laimburg Research Centre is funded by the Autonomous Province of Bolzano.

Supporting information

Supplementary materials associated with this article can be found in the online version.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2024.109006.

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