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Grazing Intensity Accelerates Surface Soil C and N Cycling in Alpine Pastures as Revealed by Soil Genes and $\delta^{15}N$ Ratio

Salvatore Raniolo [1,*], Luca Da Ros [2], Laura Maretto [1], Damiano Gianelle [3], Federica Camin [4], Luana Bontempo [3], Piergiorgio Stevanato [1], Enrico Sturaro [1], Andrea Squartini [1] and Mirco Rodeghiero [3]

[1] Department of Agronomy Food Natural Resources Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020 Legnaro, PD, Italy; laura.maretto@unidp.it (L.M.); stevanato@unipd.it (P.S.); enrico.sturaro@unipd.it (E.S.); squart@unipd.it (A.S.)
[2] Competence Centre for Mountain Innovation Ecosystems, Free University of Bozen-Bolzano, Piazza Universitá/Universitätsplatz 1, 39100 Bolzano/Bozen, Italy; luca.daros@unibz.it
[3] Research and Innovation Centre, Fondazione Edmund Mach, 38098 San Michele all'Adige, TN, Italy; damiano.gianelle@fmach.it (D.G.); luana.bontempo@fmach.it (L.B.); mirco.rodeghiero@fmach.it (M.R.)
[4] Agriculture Food Environment Centre (C3A), University of Trento, 38098 San Michele all'Adige, TN, Italy; federica.camin@unitn.it
* Correspondence: salvatore.raniolo@unipd.it

Abstract: European grasslands are vital carbon (C) sinks, contributing to climate change mitigation. Grazing intensity significantly influences soil C and nitrogen (N) cycles through effects on soil conditions and microbial communities. While heavy grazing is linked to soil C loss and altered N processes, existing studies show conflicting outcomes. This study examines the impact of cattle grazing on soil C and N cycles in a historical alpine pasture in the eastern Italian Alps (1868 m a.s.l.). The following three grazing intensities were analyzed: heavy (8.19 LU ha^{-1}), moderate (0.59 LU ha^{-1}), and light (0.06 LU ha^{-1}). Soil was sampled from two depth layers (0–5 cm, 5–10 cm) and analyzed for bulk density, C and N content, C/N ratio, exchangeable N, $\delta^{15}N$, and microbial genes targeting general abundance (16S), N fixation (*nifH*), nitrification (*amoA*), and denitrification (*nirK*, *nosZ*) using real-time PCR. The results revealed decreased C and N concentrations with increasing grazing intensity, exclusively in the 0–5 cm soil layer. Higher $\delta^{15}N$ and enhanced nitrification and denitrification suggest a more open N cycle under heavy grazing. These findings highlight the potential of microbial gene markers and $\delta^{15}N$ isotopic ratios to monitor N cycle dynamics in alpine pastures, informing sustainable grazing management.

Keywords: grazing intensity; soil N; soil C stock; N cycle; soil genes

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1. Introduction

Grasslands represent one of the most extensive ecosystems globally, occupying ~25% of the Earth's ice-free land surface [1]. They are used predominantly for forage and livestock production and are considered to contribute significantly to C sequestration [2] and increased biodiversity [3], particularly in grazing systems. Grazing is estimated to occupy as much as one-third of the earth's surface area [4] and has the potential to alter C and N cycles [5]. In particular, grazing animals represent a local direct and indirect affecting factor for the microbial community of grassland soils, as they reintroduce nutrients and live microbes in the soil through their dung [6,7]. This additional input of substances and cells can influence the turnover rates and the absorption of soil nutrients, ultimately affecting plant community and productivity [8–12].

At the same time, such land use can also pose challenges to the ecosystem, since heavy herbivores gathering at the delicate interface layer that surface soil represents is a potential stress factor, particularly in man-managed pasture systems where overgrazing can more frequently occur. The passage of animals, through trampling, can in fact also directly alter the soil physical characteristics, compacting the soil and reducing the amount and availability of oxygen, therefore, favoring anaerobic microbial processes, such as denitrification [13]. Animals feeding on plants can alter the vegetation composition (i.e., long-term changes in species richness, abundance, and cover), with indirect implications for the rhizosphere and the litter, and eventually also modifying the microbial communities [8,14,15]. Research on the microbial processes controlling C and N dynamics, including SOC decomposition, organic N mineralization, ammonia volatilization, nitrification, and denitrification, is needed to understand mechanisms governing C and N cycles [16].

Studies on the effects of grazing on grassland soils show contradictory responses of belowground C and N pools, which could be due to differences in grazing intensity, duration, or climatic conditions [17]. The C/N ratio in soil ecology is a major determinant of both elements' fate and, at the same time, an indicator of the stage reached in the transition from the assimilation to the mineralization steps operated by the microbial communities. In a meta-analysis on the effects of grazing, Zhou et al. [5] reported a general reduction in C and N pools, which could possibly be due to the decreased aboveground plant production and the reduced C allocation to roots. In particular, when analyzing in detail the effect of grazing intensity, they found that light grazing enhanced soil C and N pools, whereas moderate and heavy grazing caused the opposite effect. From the point of view of the carbon cycle, European grasslands were estimated to be below saturation level, therefore, indicating a large capacity for SOC sequestration in their topsoil [18].

Within this scenario, specific approaches delving into the ecological stoichiometry of habitats can be of particular help in casting light on both past and ongoing physiological phenomena, which is the case of isotope-based analyses. The stable N isotopic ratio ($\delta^{15}N$) provides an integrated indicator of the relative rates of soil N inputs and losses [19]; moreover, $\delta^{15}N$ can be used as an index of the ecosystem's nitrogen saturation, with higher soil $\delta^{15}N$ indicating the long history of high N availability, more openness, and a more saturated state [7,20]. However, the effect of grazing on soil $\delta^{15}N$ seems not to be straightforward. Some studies have found that grazing either increased [21,22] or reduced [23,24] soil $\delta^{15}N$, while others showed no change [25].

From the biological side of events, besides plants, a crucial category of soil keepers and mediators of organic matter cycling, ultimately restituting nutrients to the plants, is the soil microbiota. The microbial communities sustain multiple ecosystem processes through enzyme-catalyzed reactions depending on specific genes [26–28]. From this point of view, the microbial genetic content of the soil drives ecosystem metabolic niches and potential functions [27,28], such as nutrient cycles and greenhouse gas (GHGs) modulation [29]. Thus, specific microbial genes can be used as indicators for the potential of ecosystem processes where they are involved [30,31]. In particular, the N cycle incorporates processes composed of multiple reactions, which are catalyzed by single genes. For example, denitrification involves sequential reactions with *nirK* genes encoding nitrite reductase and *nosZ* encoding nitrous oxide reductase (N2OR) for N_2O reduction to N_2 [32]. The ratio between *nosZ* and *nirK* can provide information about the relative abundance of different denitrifiers' guilds, indicating the potential to produce or reduce N_2O as function of the prevalent component [33,34]. Furthermore, nitrification involves sequential reactions, where the first and limiting step is the oxidation of ammonia through ammonia monooxygenase, encoded by the *amoA* gene [35–37], whereas nitrogen fixation is mediated by the *nifH* gene in the soil [38]. The abundance of *nifH*, *nirK*, *nosZ*, and *amoA* genes indicates nitrogen fixation,

denitrification, and nitrification potentials, and it can be quantified through real-time PCR (qPCR) [28,37,39–43]. This technique was successfully applied in different research contexts, such as wastewater treatment [44], croplands [45,46], forests [30], and grasslands [44,47].

In order to understand the impact of grazing on the dynamics of soil C and N, and provide and support the trend in soil $\delta^{15}N$, we compared three different grazing intensities sampled two years apart in an alpine pasture of the eastern Italian Alps by combining classical soil survey methods with molecular analysis of the soil microbial community. Our objective was to investigate the chemical and physical characteristics of soils in the three grazed areas, as well as gene abundances, in order to understand how grazing can affect soil C and N cycles. As $\delta^{15}N$ is expected to be a proxy reporting a history of higher N availability, we considered that the C/N ratio of the depositions and that resulting in the soil after the coupled rounds of microbial nitrification and denitrification processes would be suitable indicators of the balance and that the isotopic ratio would more precisely clarify the undergone dynamics. In this respect, we hypothesized that a higher C and N input through urine and feces in the more heavily grazed areas could accelerate the respective biogeochemical cycles, eventually leading to an increase in soil $\delta^{15}N$ abundance, irrespective of the total mineral nitrogen content.

2. Materials and Methods

2.1. Study Area

This study was conducted in the alpine pasture of the summer farm "Juribello," 1868 m (ASL) (46°18′28″ N 11°44′38″ E), in the Natural Park "Parco Naturale Paneveggio Pale di San Martino," belonging to the Autonomous Province of Trento (eastern Italian Alps). The site is characterized by an alpine climate [48] with rainy and fresh summers (period June–September, 2000–2021: cumulated precipitation, 147.5 mm ± 48.05 (s.d.) mm; mean temperature, 10.9 ± 3.9 (s.d.) °C); and cold and long winters (period October–April, 2000 to 2021: cumulated precipitation, 132.8 mm ± 295.3 (SD) mm; mean temperature, −1.1 ± 5.5 (SD) °C). The pasture covers approximately 180 ha at an altitude of 1950 ± 100 (s.d.) m a.s.l., with Cambisols as the predominant soil type, according to the World Reference Base for Soil Resources. Three areas were identified according to the different grazing intensities (heavy, moderate, light, hereafter H, M, and L, respectively), which were derived from the distribution of positions of GPS collars applied to the herd from July to September of years 2019 and 2020 (Table 1). In both years, GPS collars were attached to two groups of dairy cows to increase the number of monitored animals. In particular, during 2019, 6 GPS collars were applied to a total of 12 dairy cows, while, in 2020, 5 collars were applied to a total of 10 dairy cows. In both years, the GPS collars were programmed to record a position every 2 min. The outlier positions were filtered beforehand to estimate their distribution at pasture as a function of their movement metrics, according to Raniolo et al. [49]. The present situation of the pasture is the result of a long-term management strategy (>100 years), which has persisted for the last century [50].

Table 1. Grazing intensity determined from the count of GPS positions in the three areas of the farm (LU = Livestock Units).

Area	Grazing Intensity (GPS Positions/625 m^2) *	Stocking Rate (LU/ha) **
1	250 (Heavy—H)	8.19
2	70 (Moderate—M)	0.59
3	30 (Light—L)	0.06

* The GPS positions were counted within a square buffer of 25 m sides (625 m^2) for each sampling location. ** The stocking rate was determined by comparing the total number of GPS positions to the expected number of GPS positions per unit area (ha) and the total surface area. The expected number of GPS positions was estimated by multiplying the surface of a single area by the ratio of the total number of GPS positions to the surface area of all pastures.

2.2. Soil Sampling and Analysis

Within each management area, three circular plots of similar topographic characteristics, including elevation, slope (from 6° to 10.5°), and south or south-east aspect, were randomly selected for soil sampling (Figure 1). The topographic characteristics of each plot were derived from the DTM (Digital Terrain Model), with a resolution of 0.5 m. In each plot, 4 soil samples were collected at the cardinal and ordinal points of a circle with a 5 m radius, plus 1 at the center, for a total of 5 sampling points, according to the sampling scheme adopted by Mueller and Koegel-Knabner [51].

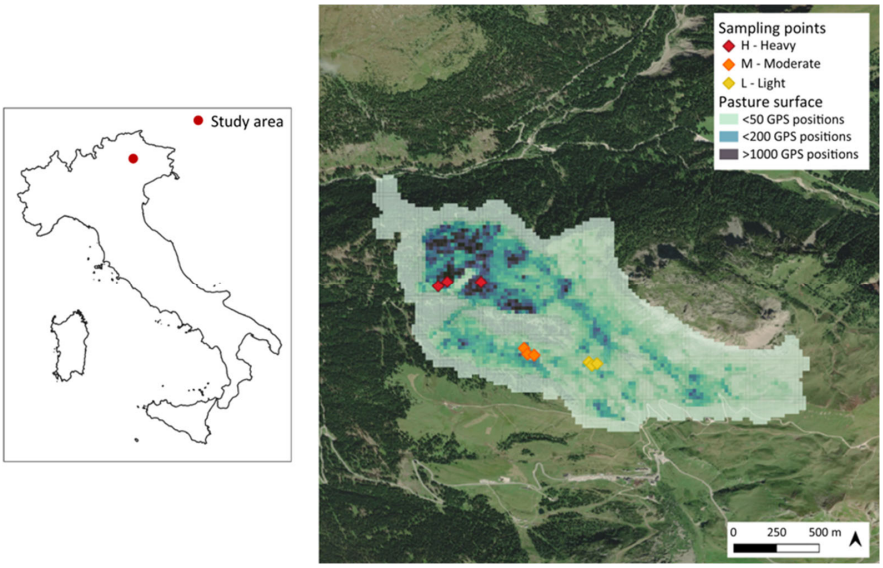

Figure 1. Location of the study area in the north-eastern Italian Alps (**left panel**) and a map of pasture as a function of grazing intensity (**right panel**). Different shades of blue indicate the level of grazing intensity according to the position density of the animals in a square area of 625 m^2, determined with GPS collars (from July to September in the years 2019 and 2020). The soil sampling plots (diamonds) are also reported for the three grazing intensities (H—heavy, M—moderate, L—light).

In each sampling point, the grass was cut on a 20 cm × 20 cm area, and a 30 cm soil core was extracted using a soil corer with an internal diameter of 4.8 cm (Eijkelkamp, Giesbeek, The Netherlands) filled with a clear PVC soil liner to preserve the integrity of the cores. Each core was processed in the field and divided into four depth segments (0–5 cm, 5–10 cm, 10–20 cm, and 20–30 cm) and packed into plastic bags. When it was not possible to reach the maximum sampling depth, due to the presence of bedrock or boulders, the reached depth was recorded, and the remaining part of the core was considered as being occupied by rocks. Two soil sampling campaigns were performed, as follows: In August 2018 ("intensive sampling"), a complete soil core down to a depth of 30 cm was collected in each sampling point as described above; moreover, the chemical analyses were performed separately on all of the collected samples (5 sampling points × 4 depths = 20 samples per plot, i.e., 60 samples per treatment). In contrast, the subsequent campaign in September 2020 ("light sampling") was specifically designed to focus on the superficial soil layers, the first 10 cm. This decision was adopted after the analysis of the 2018 data, which suggested that significant effects were primarily confined to the upper soil strata. The core was, therefore, split into two depths, as follows: superficial (S = 0–5 cm) and deep (D = 5–10 cm). Soil C, N, and $\delta^{15}N$ were determined in both years, soil bulk density was computed only on samples of the year 2018, whereas exchangeable N, pH, texture, and molecular analyses were performed only in the year 2020 on bulked samples (hereafter "composite samples") obtained by pooling the 5 samples collected at the same depth in each plot (1 pooled sample × 2 depths = 2 samples per plot; i.e., 6 samples per treatment).

The soil samples were brought to the laboratory, air-dried for two weeks, and manually sieved at 2 mm to separate the fine soil fraction from stones and roots. Roots were sorted by diameter during sieving and then dried at 105 °C for 48 h for the determination of dry mass. Root volume was estimated from linear equations developed by the soil laboratory (M. Rodeghiero, personal communication) relating root fresh volume (according to diameter class) to dry weight. The soil fraction < 2 mm was oven-dried at 40 °C for 48 h and weighed. A subsample of soil fraction < 2 mm was further oven-dried at 105 °C for the determination of dry mass. The bulk density (Bd) of the soil fraction < 2 mm was determined by dividing its dry weight by its volume (surface area of the corer × core length). The stone content was determined from the mineral soil cores and expressed as a percentage of total soil volume. Stone volumes were derived from their weight, considering an average stone density of 2.65 g cm^{-3} [52]. The soil C and N stocks on an area basis (i.e., C or N density; SC or SN kg m^{-2}) for each sampling layer were determined according to the following equation [53]:

$$SC = C \times Bd \times V \times HF$$

where C is soil organic C (or N) concentration (%); Bd is the fine soil bulk density (kg m^{-3}); V is the reference soil volume (thus, a soil layer of 5 or 10 cm thickness and 1 m^2 surface); and HF is calculated as (1 − (stone volume + root volume)/V) being a dimensionless factor, which represents the fine soil fraction content in the soil volume V.

Before chemical analysis, the organic and mineral soil samples were ground to a fine powder using a ball mill (RETSCH MM200, Haan, Germany). The samples were analyzed for C and N contents by dry combustion with a PerkinElmer PE2400 CHNS/O elemental analyzer (Norwalk, CT, USA). Prior to dry combustion, all mineral soil samples were treated with HCl (10%) for the removal of carbonates [54]. The soil pH (in water, ratio 1:2.5; MIPAF, 1999) and texture of the superficial part of the soil (to 10 cm depth) were measured on composite samples (as stated above). Soil texture was determined using the hydrometer method [55]. Exchangeable N (NO_3^- and NH_4^+) was measured by photometric detection after extraction with K_2SO_4, 0.5 M (ratio 1:4) with an automatic analyzer (Easychem200, Systea spa, Anagni, Italy; [56]). Composite soil samples were weighed and analyzed for C and N content and $\delta^{15}N$ with a continuous flow isotopic ratio mass spectrometer (DELTA V, Thermo Scientific, Bremen, Germany) interfaced with an Elemental Analyzer (Flash EA™1112, Thermo Scientific). The N isotope values are reported in delta notation ($\delta^{15}N$) and were calculated against the international standard air N_2 molar fraction, according to the following equation:

$$\delta^{15}N = (RA - RSTD)/RSTD$$

where RA is the isotope ratio measured for the sample and RSTD is the international standard isotope ratio. Delta values are multiplied by 1000 and commonly expressed in parts per thousand (‰) [57]. The isotopic values for $\delta^{15}N$ were calculated through the development of a linear equation against working in-house standards, which were themselves calibrated against the international reference materials potassium nitrate IAEA-NO-3 (IAEA-International Atomic Energy Agency, Vienna, Austria) and l-glutamic acid USGS 40 (U.S. Geological Survey, Reston, VA, USA). The maximum standard deviations of repeatability accepted for each sample analyzed in duplicate was 0.3‰.

2.3. Molecular Analysis

The total soil DNA was extracted using the DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) from 0.25 g of dried and sieved soil, quantified through the Qubit Flex fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and stored at −20° until analysis. The extracted DNA was used to investigate the potential of specific reactions of N cycle

through qPCR to quantify 6 target genes, as follows: *nosZ*, *nirK*, *nifH*, archaeal *amoA* (AOA), eubacterial *amoA* (AOB), and total 16S rRNA gene (primers used are shown in Table S3). The qPCR was performed using a 5 µL reaction volume, composed of 1 µL of target DNA and 4 µL of reaction, which contained 0.15 µL of each forward (F) and reverse (R) primer (Table S3), 1.2 µL PCR-grade water, and 2.5 µL PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA). The PowerUp SYBR Green Master Mix included the Taq polymerase. The qPCR thermal conditions consisted of a pre-denaturing stage at 95 °C for 10 min, followed by 40 cycles with a denaturation step at 95 °C for 15 s, an annealing step at 60 °C for 60 s, and extension at 72 °C for 60 s. A negative control of PCR-grade water was run for each amplification in three technical replicates. For all of the targeted genes, the cycle threshold (Ct) value obtained from the qPCR was converted to gene copy estimates by applying the equation of Dong et al. [26].

2.4. Statistical Analysis

Statistical analyses were performed with the software R 4.2.0 [58] by means of the following libraries: "vegan 2.6" [59], "lmPerm 2.1" [60], "agricolae 1.3" [61], "mgcv 1.9" [62], "arm 1.14" [63], and "rcompanion 2.4.35" [64].

The following dependent variables were considered to check for differences among grazing intensities: soil bulk density, δ^{15}N, C, N, C/N ratio, exchangeable N, and gene copy estimates (log-transformed), also considered as the ratio between *nosZ* and *nirK* as proxy for N_2O emission potential [65,66]. Considering the low occurrence of AOA and AOB, these two genes were combined into the new variable, *amoA*, which represents the total ammonia oxidation potential. Before statistical analysis, the normality of all distributions of dependent variables was tested with the Shapiro–Wilk test using the "shapiro.test" function from the default function of R [58]. Considering the small number of samples and the frequently non-normal distributions [67], all dependent variables were analyzed using ANOVA based on the permutation test with the function "aovp" of the "lmPerm" library. The ANOVA model for all dependent variables included the grazing intensity (H, M, L), soil depth (S, D), and their 2-way interaction. Specifically, the ANOVA of bulk density was performed only for 2018, while the rest of the pedological variables were analyzed for the two years separately. The "aovp" function considers the last of each factor as a reference for comparison (e.g., when comparing the three grazing intensities, 'L' grazing will be considered as a reference). To explore all possible differences among the factors, a post hoc test based on permutation was performed using the "pairwisePermutationTest" function from the "rcompanion" library. Afterwards, a correlation analysis between genes and soil conditions was performed. The correlations were examined with the Kendall coefficient for non-parametric distributions [68] after the standardization as z-scores using the function "cor" from the default function of R [58].

The statistical analysis included a specific focus on the state of nitrifier genes (AOA and AOB) considering the high percentage of "undetermined" eubacterial *amoA*, which was classified as absence or 0. Specifically, the occurrence of eubacterial *amoA* and the ratio between this gene and the total ammonia oxidation potential (*amoA*) were analyzed to detect possible effects of grazing intensity, soil depth, and their 2-way interaction. The occurrence of the eubacterial *amoA* gene was analyzed with a generalized linear model (glm) based on binomial error distribution and a "probit" link function. To handle the quasi-complete separation of grazing intensity, the binomial model was conducted on a Bayesian framework with weak prior assumptions applying the "bayesglm" functions of the "arm" library. The ratio between AOB and the total ammonia oxidation potential (*amoA*) was analyzed with a general additive model based on beta error distribution using the "gam" function of the "mcgv 1.9" library.

3. Results

3.1. Bulk Density, C and N Concentration, N Pool, and $\delta^{15}N$ Content

The nine plots selected for the experiment had an average slope of 8.3° ± 1.3° (mean ± s.d.), and no significant differences in slope were found between management areas (Kruskal–Wallis ANOVA by ranks, H = 3.8; p = 0.15; n = 9). Therefore, we can exclude the effect of nutrient accumulation in flatter areas. Statistically significant differences in soil bulk density were instead evident among grazing intensities and depths (Figure 2A,B), but not when considering the interaction between grazing intensity and soil depth (Table S1). In particular, the heavy grazing plots had a higher soil bulk density (0.75 ± 0.19 g cm^{-3} for the superficial layer and 1.11 ± 0.19 g cm^{-3} for the deep layer) compared to the intermediate (0.38 ± 0.11 g cm^{-3} for the superficial layer and 0.92 ± 0.10 g cm^{-3} for the deep layer) and light grazing treatments (0.45 ± 0.06 g cm^{-3} for the superficial layer and 0.89 ± 0.09 g cm^{-3} for the deep layer).

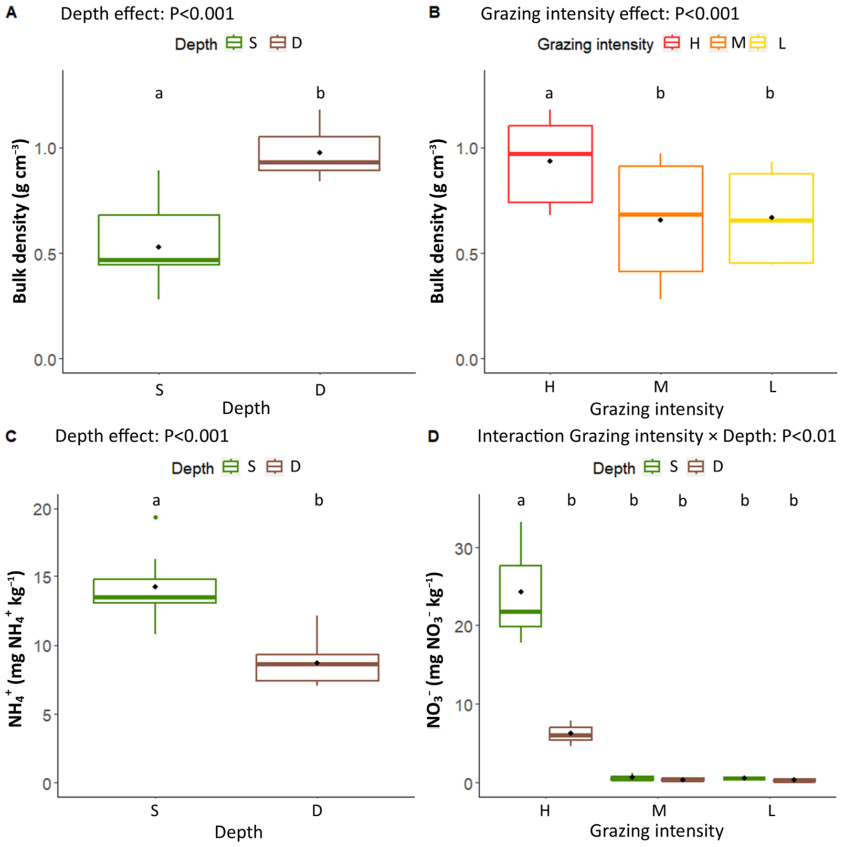

Figure 2. Soil bulk density mean values (g cm^{-3}) in relation to soil depth (panel (**A**); S = superficial layer; D = deep layer) and grazing intensity (panel (**B**); H = heavy; M = moderate; L = light) in 2018. Soil mean content of NH_4^+ (mg NH_4^+ kg^{-1}) as a function of soil depth (panel (**C**)) and soil mean content of NO_3^- (mg NO_3^- kg^{-1}) in a function of the 2-way interaction of grazing intensity and soil depth (panel (**D**)) in 2018. Means not sharing any letter are significantly different by ANOVA based on the permutation test ("aovp" R function) at the specified level of significance. For details, see Table S1.

The concentration of NH_4^+ was not significantly affected by grazing treatments, but it was significantly affected by the depth of the layers, with a higher concentration in the S layer (14.2 ± 2.4 mg kg dw^{-1}) than that found in the D layer (8.7 ± 1.6 mg kg dw^{-1}; Figure 2C). The average NO_3^- content of all samples of the H treatment was 24.3 ± 8.0 mg kg dw^{-1}, compared to the 6.2 ± 1.6 mg kg dw^{-1} of the D layer and to the 0.6 ± 0.1 mg kg dw^{-1} of the S layer of the L treatment (Figure 2D).

The soil carbon (C) and nitrogen (N) contents were measured both as concentrations (in %; i.e., mass of C or N present in 100 g of dry soil) and as densities (kg m^{-2} or g m^{-2}). In

both 2018 and 2020, the D soil layers maintained consistent and not statistically different C% values, while the S soil layers were significantly influenced by grazing intensity (Figure 3A and Table S2). Specifically, in 2018 ("intensive sampling"), the C% in the S layers was significantly lower in the H grazing plots, compared to the M and L grazing plots. In 2020, the C% still remained lower in the H plots and statistically different from the L plots but was not different from the M plots.

The N% patterns mirrored those of C%, with similar and not statistically different values in the D layers and variable values in the S layers (Figure 3B and Table S2). In 2018, the N% peaked in the M grazing plots and was statistically different from that of the H and L grazing treatments, while, in 2020, although lower in the H treatments, the N% was not statistically different from that of M and L. The N density was higher in the H treatment and statistically different from that of M and L (Figure S1). When considering carbon density, no statistically significant differences emerged among the different grazing intensities for the superficial layer nor for the deep layer (Figures S1B and S3A).

Grazing intensity, depth, and their interaction affected the soil C/N ratio ($p < 0.001$; Figures 3C and S2 and Table S2). Both in 2018 and in 2020, the C/N ratio of the S layer in the H treatment resulted to be significantly lower than that of the other two grazing intensities; however, no significant differences in C/N ratio among the grazing intensities emerged for the D layer in 2018, while, in 2020, it significantly increased in the L grazing plots (Figure 3C).

Both in 2018 and in 2020, the $\delta^{15}N$ content was significantly affected by the interaction between grazing intensity and soil depth ($p < 0.01$; Figure 3D and Table S2). The D layers were enriched in $\delta^{15}N$ compared to the S layers (Figure 3D); moreover, in 2018, the S layer of the H grazing treatment was significantly enriched in $\delta^{15}N$ compared to the L grazing treatment but was not statistically different from M (Figure 3D). In 2020, no statistically significant differences emerged in $\delta^{15}N$ among the grazing treatments, whereas depth had a significant effect on $\delta^{15}N$ but just on the M and L treatments.

3.2. Nitrogen Genes Content Analysis

The analysis of Kendall correlations among genes and soil variables revealed various patterns. The 16S rRNA gene did not present any strong and significant correlation with pedological variables (Figure 4). The *nifH* gene showed a significant and negative correlation with bulk density ($r = -0.42$; $p < 0.05$). The *nosZ* gene showed a significant positive correlation with nitrate ($r = 0.33$; $p < 0.05$; Figure 4), while it had a negative relation with the C/N ratio ($r = -0.58$; $p < 0.05$). The *nirK* gene presented a positive and significant correlation with the pH ($r = 0.32$; $p < 0.001$) and with the N content ($r = 0.43$; $p < 0.05$), while it was significantly and negatively related to the clay amount ($r = -0.38$; $p > 0.05$) and soil thickness ($r = -0.12$; $p < 0.01$; Figure 4). The *nirK* gene also had a strong relation with the amount of nitrate ($r = 0.65$; $p < 0.001$). The ratio between *nosZ* and *nirK* did not present significant correlations with the pedological features, but it was negatively related with C% ($r = -0.12$), N% ($r = -0.13$) and nitrate ($r = -0.28$). The *amoA* gene was significantly and negatively correlated with the C/N ratio ($r = -0.58$; $p < 0.001$), while it presented positive and significant correlations with pH ($r = 0.58$; $p < 0.001$; Figure 4).

The ANOVA based on the permutation test highlighted a significant effect of the grazing intensity for the abundance of *nosZ*, *nirK*, and *amoA* genes. The *nosZ* gene was significantly more abundant in the H grazing treatment ($p < 0.05$) compared to the L grazing intensity, as well as the archaeal *amoA* gene ($p < 0.01$; Figure 5; Table S4). The *nirK* gene was significantly more abundant in the H treatment than it was in both the M and L grazing intensities ($p < 0.01$; Figure 5; Table S4). The ratio between *nosZ* and *nirK* was significantly affected by the grazing intensity ($p < 0.01$; Figure 5; Table S4), with a significant increase for the moderate levels with respect to the heavy and light ones

(Figure 5E). The 16S and the *nifH* genes did not present significant differences among the treatments (Figures 5A,B and S2A; Table S4). The topsoil depth factor was not significant for all genes, both for absolute and relative abundances (Tables S4). The interaction between grazing intensity and topsoil depth was significant for only the *amoA* gene abundance (depth: $p < 0.05$; grazing × depth: $p < 0.05$; Figure S4; Table S4). The ratio between AOB and *amoA* genes significantly increased with the grazing intensity, but it was not affected by topsoil depth or the two-way interactions (grazing intensity: $p < 0.001$; Figure 6A; Table S5), while the presence of AOB did not present significant factors, but a positive trend related to the grazing intensity (Figure 6B; Table S5).

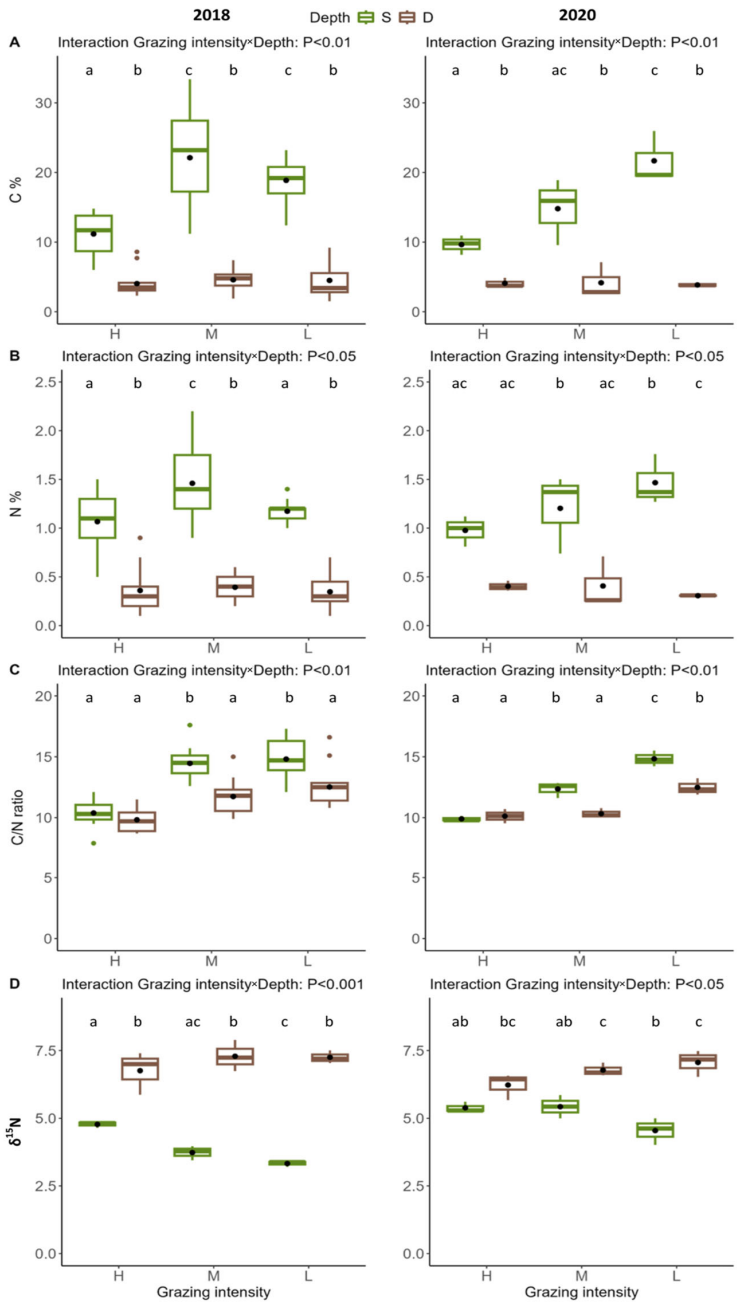

Figure 3. Mean values of total soil N (%) (panel (**A**)), C (%) (panel (**B**)), C/N ratio (panel (**C**)), and $\delta^{15}N$ (panel (**D**)) as a function of the 2-way interactions between grazing intensity and soil depth (grazing intensity: H = high; M = moderate; L = low; soil depth: S = superficial layer; D = deep layer) in 2018 and 2020. Means not sharing any letter are significantly different by ANOVA based on the permutation test ("aovp" R function) at the specified level of significance. For details, see Table S2.

Figure 4. Correlation plot with Kendall rank's coefficient between copies of selected genes (16S, *nifH*, *nosZ*, *nirK*, ratio *nosZ*/*nirK*, and *amoA*) and pedological variables. The copies of genes were expressed on a logarithmic scale. The level of statistical significance is marked with asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

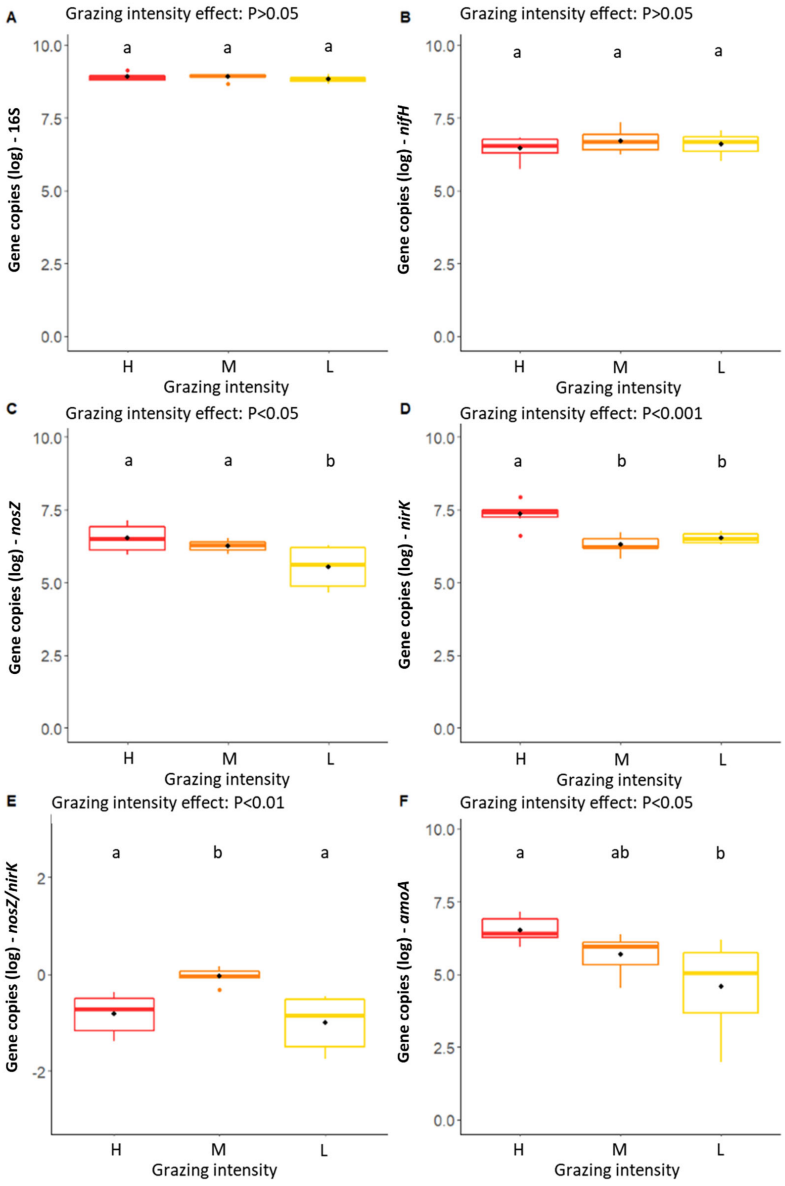

Figure 5. Boxplots showing log mean abundance of the number of copies per gram of soil dry weight for the following genes: 16S (panel (**A**)), *nifH* (panel (**B**)), *nirK* (panel (**C**)), *nosZ* (panel (**D**)), *nosZ*/*nirK*

(panel (**E**)), and *amoA* (sum of AOA and AOB, panel (**F**)) in relation to grazing intensity (H = heavy grazing; M = moderate grazing; L = light grazing). Means not sharing any letter are significantly different by the ANOVA based on the permutation test ("aovp" R function) at the specified level of significance. For details, see Table S4.

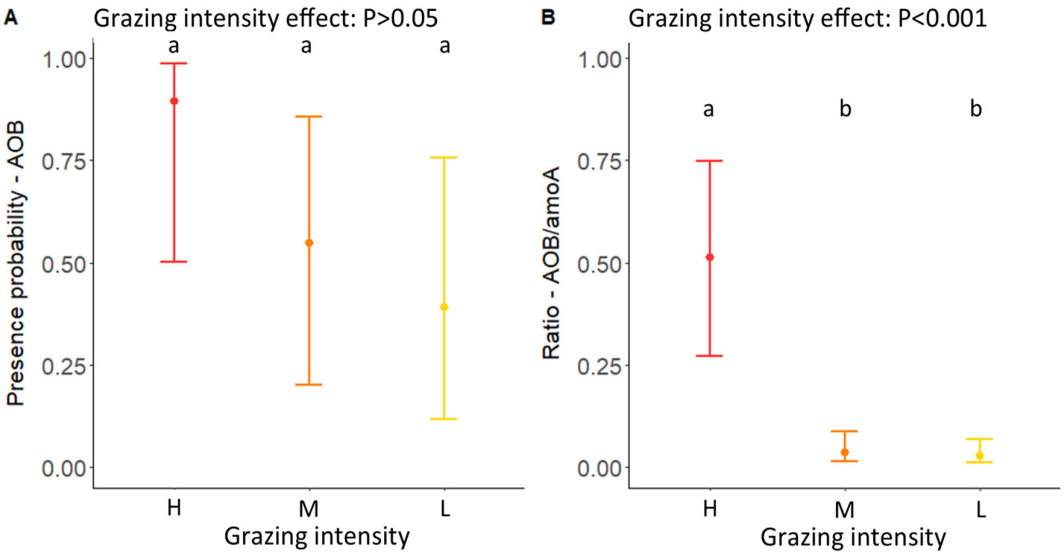

Figure 6. Effects of grazing intensity (H = heavy grazing; M = moderate grazing; L = light grazing) on AOB presence (panel (**A**)) and ratio AOB/*amoA* (panel (**B**)). Means sharing the same letter are not significantly different for $p < 0.05$ by the general linear model based on binomial distribution (panel (**A**)) and general additive model based on beta distribution (panel (**B**)) at the specified level of significance. For details, see Table S5.

4. Discussion

4.1. General Aspects and Baseline Knowledge

This research revealed different patterns of pedological conditions and microbial genes as a function of grazing intensity and soil depth. Pedological parameters differed mainly by soil depth and relatively by grazing intensity, whereas the microbial genes were primarily affected by grazing intensity.

Where animals deposit waste, the concentrated addition of mineral nutrients and organic matter has the potential to alter the N-isotopic composition of both soils and plants [7]. As reported in the literature [69,70], animal trampling in high-intensity grazing plots affects soil properties both physically and chemically. The more evident effect is the compaction of the soil in the superficial layers (i.e., higher bulk density) and a higher pH. Higher pH values in areas of higher grazing pressure [5,69,71] could be related to urine deposition [72] or changes in plant community composition [73].

4.2. Macro-Nutrient Chemistry

Numerous studies have investigated the effects of grazing intensity on soil C and N dynamics, but the results were often contradictory because the responses of belowground C and N processes to grazing may be associated with differences in grazing intensities, grazing duration, type of livestock involved, or climatic conditions [5,74,75]. In this study, we found a significant decrease in C% limitedly to the very superficial (0–5 cm) soil layer in the H grazed plots compared to those of M and L. The soil carbon content per unit surface was, instead, not affected as a possible consequence of the higher variability in soil physical characteristics (soil bulk density, stones, and root contents) affecting the calculation of carbon density. According to some reports, this decrease could be due to

a reduction in soil mycorrhizal hyphal density, which would have implications for the nutrient uptake capacity of fungi and plants, carbon translocation into the bulk soil, or to a decrease in soil microbial biomass [70,75]. Other possible negative effects of heavy grazing were identified in a reduction in plant diversity, cover, and productivity, which may reduce the input of C to soil through root exudates favoring C loss by erosion and microbial turnover [76,77]. Many authors reported a decrease in soil C stock due to high grazing intensity [1,70,75]; however, similarly to Zhou et al. [5], we found a higher reduction in C in the very superficial part of the soil in H plots and no significant effect in the deeper layers. The most linear interpretation in our view is the fact that overgrazed vegetation, being limited in photosynthesis capability, would suffer a shortage of possible root growth and of root exudation to soil microbes, causing a depletion of the soil organic carbon, both by an immediate cut in supplies and by long-term return due to the stalled ecosystem servicing from the undernourished microbiota.

The total soil N content exhibited two distinct patterns between 2018 and 2020, particularly in the superficial layer, which seemed most responsive to external disturbances such as animal presence and grazing. The differences may be due to the heterogeneous use of pasture by cattle and variations in the animals' diets over the years, which can lead to fluctuating N input and redistribution in the pasture. High grazing intensity can also introduce higher N input from animals. In theory, this should lead to an increase in soil N, however, paradoxically, there are consequent actions that could also yield the opposite outcome, as the N of animal origin is accompanied by copious organic C deposition of their excreta. This can be a direct support to the heterotrophic denitrifying guilds that can enroute the reduced nitrogen compounds to the volatile form, which abandons the soil. In addition, the decrease in soil N may result also from increased necessities of plant uptake to compensate for the injuries driven by higher rates of animal feeding. Trimmed plants tend to invest more in root development, as long as the damage is not unbearable and biosynthesis of root is possible [78], increasing, wherever possible, the nutrient uptake from the soil to support regrowth after grazing. Additionally, heavier grazing pressure can intensify competition among plants for nutrients, reducing their overall soil content [79,80]. Conversely, light grazing intensity can lead to lower N content, as observed in 2018, due to reduced animal N input. Alternatively, it can result in higher N content, as seen in 2020, due to N accumulation and reduced plant uptake, as the vegetation was less disturbed. Lighter grazing intensity could restrict nutrient reintroduction, leading to a dilution of N and C content [81], thereby influencing nutrient use by vegetation and microorganisms. This dilution can also explain the consistent significant negative impact of grazing intensity on the C/N ratio. The heightened presence of animals and the subsequent increased reintroduction of nutrients through dung may account for the lower C/N ratio values in the heavy grazing intensity plots. The distinction among topsoil layers vanished in the H grazing plots throughout both years. This convergence suggests that high grazing intensity tends to homogenize the topsoil, likely due to the substantial input from animals.

4.3. N Isotopic Analyses

The analysis of $\delta^{15}N$ over the two years revealed consistently higher values in the deep layers (accumulation of the heaviest N isotope) independently from the grazing intensity, however, this is quite common in natural not-tilled soils [7]. The reason for this trend has been summarized by Szpak [7] in the following three possible mechanisms: (i) plant litter accumulating on top of the soil is depleted in ^{15}N compared to the soil itself; (ii) the migration of ^{15}N enriched organic matter down the soil profile; and (iii) accumulation at a depth of ^{15}N enriched material derived from mycorrhizal fungi [82]. What is instead more interesting, although less pronounced, are the grazing-related differences in $\delta^{15}N$,

as follows: a visible gradient trend of decrease along with the reduced grazing pressure was evident in both years, and, in 2018, was also statistically significant between the H and L plots. The N cycle appeared consistently more open in the superficial layer, potentially involving higher rates of processes such as plant nutrient uptake, ammonification, nitrification, and denitrification [7,83]. All of these processes, with the exception of ammonification, are associated with ^{15}N enrichment in the residual substrates, and, therefore, by increasing both N inputs and outputs, herbivores tend to cause higher $\delta^{15}N$ values in soils and plants [7].

However, the absence of strong and significant correlations between $\delta^{15}N$ and the genes' abundances involved in these processes may indicate a more flexible utilization of all available nitrogen isotopes by microorganisms compared to plants [84,85]. This high flexibility of microorganisms may minimize competition among themselves and with plants, thus maintaining the independence of nitrogen fixation, nitrification, and denitrification potentials from the nitrogen isotopic contents without necessarily altering $\delta^{15}N$ values.

4.4. Bacterial Gene Abundance

In terms of nitrogen fixation potential, the *nifH* gene, along with the 16S gene, was not significantly affected by either grazing intensity or soil depth, although, in theory, the soil bulk density was the only pedological variable capable of significantly influencing the *nifH* abundance, since a lower bulk density would present more aerated conditions, which in turn would not be conducive to a reductive metabolism as the conversion of gaseous nitrogen to ammonia. A further possible explanation for the observed pattern of the *nifH* gene could be its enzyme substrate, which is atmospheric N. Atmospheric N presence is independent and unaffected by livestock, and it may be more concentrated in the surface layer of the soil where gas exchange with the atmosphere takes place [86,87]. Moreover, the lower bulk density of the surface layer allows for a greater available volume for the gas. Our results partially contrast with those of a previous study, where *nifH* showed a stronger and significant decrease with grazing presence and soil depth [85,86]. Thus, the relatively constant abundance of *nifH* and its weak-moderate correlations with pedological conditions suggest a possible continuum of N fixation operated by free microorganisms in alpine pastures, marginally influenced by animal presence and soil depth.

Concerning the nitrification process, the highest abundance of *amoA* genes was found in the heavy grazing intensity and in the deeper layer of soil (Figures 5 and S1). Our results are partially in contrast with those of Song et al. [86], who found a significant increase in *amoA* in relation to soil depth but a significant decrease with the presence of grazing. The peak of *amoA* may be driven by increased availability of ammonium-releasing substrate due to the greater amount of animal waste in heavily grazed areas. The *amoA* was significantly and negatively influenced by the C/N ratio, which decreased, supposedly due to the significant increase in N density and the available organic C, capable of accelerating the mineralization of organic matter. Specifically, the organic matter decrease can competitively favor the growth of the autotrophic nitrifiers, leading to an increase in the *amoA* gene pool, whose advantage can in part be due also to a reduction in plant secondary compounds able to limit the microbial communities [88,89]. The negative relationship between *amoA* and C/N might also be influenced by the different vegetation types in the study areas. Light grazing intensity areas presented *Rhododendron-vaccinion* (*Rhododendro ferruginei-vaccinion myrtilli* Schnyd, 1930) in their vegetation, while heavily grazed areas featured a dominant presence of *Poion alpinae* Gam ex-Oberd, 1950. Different vegetation types can introduce different types of organic C into the soil [90], with varying effects on the microbial communities [91]. Thus, for example, *Rododendron ferrugineum* L., as a shrub, might add more organic C to the soil; however, this organic carbon might not be directly

usable or available to nitrifiers, leading to a decrease in this microbial guild. Regarding the depth effect, an accumulation of ammonium, which serves as the substrate for ammonia monooxygenase, at deeper layers by limited leaching and cationic absorption, is among the possible factors, as well as the fact that, being nitrifier autotrophs, their presence in layers with less organic matter, as the deeper ones, is not competed against by heterotrophic bacteria. Our results showed no direct and positive relationship between the amount of ammonium and *amoA*, according to the absence of significant difference in ammonium amount among areas and depth. This is not actually a contradiction, since the multiplication of the nitrifiers is indeed consuming ammonium to ultimately produce nitrates. Archaea, which constitute a significant portion of the nitrifiers in the microbial community, exhibited an increase in gene abundance in the deeper soil layers, particularly in the moderate and light grazing areas. This observation aligns with the relationship observed between *amoA* and the C/N ratio. In sites of accumulation of organic matter, nitrifying bacteria would not be competitive over heterotrophs. As a result, these nitrifiers may display their higher shares in the soil layers where conditions would be less favorable for groups exploiting different metabolisms. This trend can be the result of the highest nutrient enrichment associated with heavy grazing intensity, which, therefore, can override the differences between the layers. In addition to the general trends of *amoA*, the AOA gene and the AOB gene presented diversified patterns as functions of the grazing intensity. The AOA were always present at all levels of grazing intensity and prevailed on the AOB, which increased its presence and its contribution to the nitrifier guild at the heaviest grazing intensity (Figure 6). The difference between archaeal and bacterial nitrifiers supports the hypothesis of ecological niche partitioning [92], which appears to be directly influenced by grazing intensity. In general, the AOA community seems to prefer areas with lower pH and less substrate and nutrients than the AOB community [42]. The partition of the niche between nitrifiers was also highlighted by the positive and significant correlation between the AOB community and NO_3^-, making the relevant contribution of bacteria to complete nitrification in habitats with high nutrient availability, as well as in areas with heavy grazing intensity.

Regarding denitrification, the significant increase in the abundance of both *nosZ* and *nirK* from light to heavy grazing intensity is compliant with the same pathway and the commonly required reductive conditions, but they also displayed some differences (Figures 5 and S2). Both *nosZ* and *nirK* reactions require a low concentration of oxygen, which can be brought about by animal trampling due to soil compaction, as we found in this and similar studies [92,93]. Both nitrite (NO_2^-) and nitrous oxide (N_2O) are likely produced in soils with high concentrations of ammonium or urea that are transformed to nitrate and then consumed as an electron acceptor under low oxygen conditions, such as those occurring in trampled soils [13]. Nitrite is a highly mobile soluble anion, while nitrous oxide is a gaseous molecule; therefore, the product of the *nosZ* gene could be more active in compacted soil than that of the *nirK* gene, due to the more obstructed leaking rate of nitrous oxide from the soil to the atmosphere. The *nirK* outperforms the *nosZ* under heavy and light grazing intensity conditions due to a possible greater affinity for nutrients, such as NO_3^-, and pH, according to the correlations that we found. Therefore, at high concentrations of NO_3^- and high pH, *nirK* results are more abundant than those of *nosZ*, possibly leading to partial denitrification. In fact, the ratio between *nosZ* and *nirK* reached 0 (value when the logarithmic abundances of the two genes are equal) at a moderate grazing intensity, revealing a prevalence of partial denitrification under both light and heavy grazing intensity conditions. Therefore, it is also possible that denitrifiers, as seen for the two types of nitrifiers, present niche differentiation, in their case with a possible direct implication in terms of N_2O emissions. An increase in

grazing intensity with associated trampling activity appears to be a factor of increase in local soil disturbance, which creates more favorable conditions for the development of specific bacterial guilds both for nitrification and denitrification processes. Interestingly, we did not find a significant effect of depth or any correlation with soil bulk density, even though in the deeper layer the more anoxic environment should, in principle, be favorable to denitrification processes [94,95]. These absences are in contrast with previous studies [86] where the *nirK* increased its abundance with the soil depth.

5. Conclusions

The key findings of this work can be outlined as follows: (1) decreased C and N concentrations upon increasing grazing intensity were found exclusively in the 0–5 cm soil layer; and (2) under heavy grazing, a higher $\delta^{15}N$ concentration and enhanced nitrification and denitrification, which are consistent with a more open N cycle, were found. The main element of novelty, compared to existing reports, is represented by the use of the coupled analyses of gene quantitation by real-time PCR, and, in parallel, $\delta^{15}N$ isotopic ratio. Those yielded consistent results, qualifying both approaches as agreeing reporters testifying an acceleration of the surface soil C% and N% cycling in alpine pastures in direct correlation to cattle grazing intensity.

Such observations embody some consequent theoretical and practical implications. Namely, they highlight the potential of coupling analyses of microbial gene markers with those of $\delta^{15}N$ isotopic ratios to monitor N cycle dynamics in alpine pastures. The applied usefulness of these is the possibility to recommend, as good practices for sustainable agro-ecological land management, specific livestock unit stocking rates, as well as rotational temporal shifts on plots and paddocks. Examining the potential limits of the approach, we could mention the fact that the DNA-abundance-based analyses are estimates of the potential for the corresponding genes' expression, but not direct proof of their actual engagement in the encoded physiological pathways and subsequent ecosystemic phenotypes. For such a reason, an ideal development of future studies would be an RNA-based analysis comparing the specific transcripts' abundance overlap with that of their corresponding encoding genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su17052165/s1, Figure S1. Mean values of C% (panel A), C (kg/m^2—panel B), N% (panel C), and N (kg/m^2—panel D) as function of grazing intensity (H = high; M = moderate; L = low) in 2018. In the analysis the average values of the S and D layers were considered. Means sharing the same letter are not significantly different for $p < 0.05$ by the ANOVA based on the permutation test ("aovp" R function) at the specified level of significance; Figure S2. Mean values of N (kg/m^2) in function of Depth (panel A–S = superficial layer; D = deep layer) and of C (kg/m^2) in function of Depth (panel B) in 2018; Figure S3. Mean values of total soil C (kg/m^2—panel A) and N (kg/m^2—panel B), as function of 2-way interactions (grazing intensity × soil depth; H = high; M = moderate; L = low; S=superficial layer; D = deep layer) in both 2018 and 2020. Means not sharing any letter are significantly different by the ANOVA based on the permutation test ("aovp" R function) at the specified level of significance; Figure S4. Effects of 2-way interactions between grazing intensity and soil depth on absolute amoA abundance log transformed. Means not sharing any letter are significantly different by the ANOVA based on the permutation test ("aovp" R function) at the specified level of significance; Table S1. Results of ANOVA based on permutations test and mean (±standard deviation) for the Bulk density (BD), NH_4^+, and NO_3^- as function of grazing intensity (panel A–H = heavy; M = moderate; L = light–reference level) and soil depth (panel B–S = superficial layer; D = deep layer–reference level) and their 2-way interaction in 2018; Table S2. Results of ANOVA based on permutations test and mean (±standard deviation) for the N%, $\delta^{15}N$ and C/N as function of grazing intensity (panel A–H = heavy; M = moderate; L = light–reference level) and soil depth (panel B–S = superficial layer; D = deep layer–reference level)

and their 2-way interaction in both 2018 and 2020; Table S3. List of primers used for the Realtime PCR; Table S4. Results of ANOVA based on permutation test and mean (±standard deviation) for the gene abundances, in logarithmic scale, as function of grazing intensity (panel A–H = heavy–reference level; M = moderate; L = light) and soil depth (panel B–S = superficial layer; D = deep layer–reference level) and their 2-way interaction in 2020; Table S5. Results of logistic regression for AOB presence and beta regression and least-square means with confidence intervals for AOB ratio (AOB/amoA; amoA: AOA+AOB) as function of grazing intensity (panel A–H = heavy; M = moderate; L = light–reference level) and soil depth (panel B–S = superficial layer–reference level; D = deep layer) and their 2-way interaction in 2020. References [38,39,96–98] are included in the Supplementary Materials.

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