



# Stand age, degree of encroachment and soil characteristics modulate changes of C and N cycles in dry grassland soils invaded by the N<sub>2</sub>-fixing shrub *Amorpha fruticosa*



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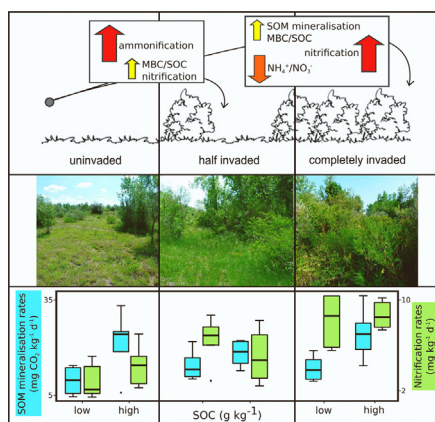
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## HIGHLIGHTS

- Comprehensive study of a vast region undergoing invasion by N<sub>2</sub> fixing shrubs
- Nitrification increased with SOC at 0% and 100% invasion, but not at 50%.
- Direct effects modulated by soil characteristics such as SOC and clay contents.
- Plant growth and density affected BC/SOC, but ratios increased with stand age.
- Remediation potentially effective at intermediate stage, when trends are reverted.

## GRAPHICAL ABSTRACT



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## ABSTRACT

The N<sub>2</sub>-fixing shrub *Amorpha fruticosa* L. is rapidly spreading in the dry riparian natural grasslands of Europe, altering ecosystem functions and depleting plant diversity. Alteration of the N cycle represents the key factor involved in invasions by N<sub>2</sub>-fixing plants with cascading effects on plant species richness. We hypothesized that *A. fruticosa* encroachment strongly impacts not only the N but also the C cycle and that the magnitude of such alterations may be modulated by soil characteristics. To test these hypotheses, we selected four river floodplains in North East of Italy and compared natural uninvaded grasslands with half invaded and completely invaded sites, based on *A. fruticosa* stand characteristic and relevant leaf traits and on soil properties related to soil texture and to C and N cycles. Soil organic matter mineralisation, ammonification and nitrification rates were determined. Soil nitrification increased remarkably with plant invasion while ammonification was significantly higher only in half invaded sites. Soil organic matter mineralisation, microbial biomass C sustained per soil organic C unit and nitrification positively correlated with stand age, regardless to the stage of the encroachment. Mineralisation and nitrification increased with soil organic C and total N in uninvaded and completely invaded sites, but decreased in half invaded sites. At the half invasion stage, trends in nitrification and CO<sub>2</sub> mineralisation were transitionally reverted and remediation may be facilitated by less pronounced changes in soil properties compared to completely invaded sites. Direct effects of plant invasion are modulated by the action of soil characteristics such as soil organic C and clay contents, with soils rich in organic C showing larger nitrification and mineralisation rates.

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## 1. Introduction

Invasion by alien species is a major threat for biodiversity and ecosystem services, including trophic chains and nutrient cycling (Levine et al., 2003; Vilà et al., 2010; Simberloff et al., 2013). The list of possible impacts is extensive and comprises direct and indirect effects (Weidenhamer and Callaway, 2010), acting at different levels (Vilà and Hulme, 2017). By definition, alien plants become invasive when they are able to spread spontaneously into natural environments (Blackburn et al., 2011). Some of them do it so aggressively, that they can be reasonably called “transformer species”, as they drastically modify ecosystem properties (Richardson et al., 2000). In such cases, environmental shifts due to invasion can hardly be reversed (Vitousek et al., 1996) and consequences on native plant communities are difficult to predict, generally inducing a decrease of native species diversity (Simberloff et al., 2013; Boscutti et al., 2018; Vitti et al., 2020). Woody N<sub>2</sub>-fixing species are actually among the top plant invaders worldwide (Raizada et al., 2008). The resprouting response to disturbance is enhanced in woody plants compared to grasses at elevated CO<sub>2</sub> (Manea and Leishman, 2019), therefore the raise in atmospheric CO<sub>2</sub> concentration due to climate change is expected to boost their invasiveness, especially in dry grasslands.

Even though alien trees and shrubs take more time for invasion than herbaceous plants (Richardson et al., 2014), shrub encroachment is nowadays favoured by the degradation of grasslands and has become increasingly common (Li et al., 2016), particularly in arid and semiarid climates (Eldridge et al., 2011). Shrub encroachment modifies litter inputs, microbial and soil enzymatic activities (Ehrenfeld, 2003) and alters soil C pools (Yang et al., 2011) as well as soil physical properties (Raizada et al., 2008). Among invaders, N<sub>2</sub>-fixing plants may cause the most conspicuous changes (Liao et al., 2008), by acting on the N cycle (Vilà et al., 2011). In fact, by altering soil N availability (Liao et al., 2008; Ehrenfeld et al., 2001; Broadbent et al., 2017), N<sub>2</sub>-fixing plants enhance ecosystem productivity (Kleinbauer et al., 2010) and nitrification (Ehrenfeld, 2010; Vilà et al., 2011; Lazzaro et al., 2015). They also affect soil organic matter (SOM) decomposition rates, by altering litter quality (Yelenik et al., 2007; Liao et al., 2008) and may decrease soil pH and pedofauna biodiversity (Lazzaro et al., 2018).

Despite impacts of N<sub>2</sub>-fixing invaders on soil N are well-known (Ehrenfeld, 2003; Hawkes et al., 2005; Liao et al., 2008; Stewart et al., 2019), their consequences on soil microbial biomass and SOM mineralisation are commonly overlooked (Ehrenfeld, 2003; Liao and Boutton, 2008; Yang, 2019). The increased availability of N and change in plant species composition may, in fact, affect microbial use (Mooshammer et al., 2014) and the overall efficiency of the N cycle (Zhang et al., 2018).

Contradictory results emerge from studies on soil organic C (SOC) dynamics during and after plant invasion, showing either accumulation (Hagos and Smit, 2005), negligible changes (Lett et al., 2004) or even significant losses (Jackson et al., 2002; Qiu et al., 2012). These contrasting results could be rationalized by considering neglected site-specific factors (Liao et al., 2008), among which soil characteristics, may play a major role (Broadbent et al., 2017). Most studies were performed in experimental field trials or in small natural areas having the same type of soil, but the extent and direction of changes regarding SOC, which accompany shrub encroachment, also strongly depend on soil properties and in particular on soil texture and soil total N (STN) (Yang et al., 2011; Li et al., 2016).

It is well known that clay content affects SOM mineralisation (Hassink, 1994) and has a significant influence on N mineralisation and nitrification, although the latter is highly dependent on soil moisture regimes (Strong et al., 1999). Moreover, despite the extent and directions of changes depend on plant traits (Yokomizo et al., 2009) or stand age (Liao and Boutton, 2008), these parameters and their combined effects have been scarcely considered. Plant community traits may also contribute: recently, Broadbent et al. (2017) reported that

grassland soils invaded by a N<sub>2</sub>-fixing broom (*Cytisus scoparius*) underwent a decrease in SOM decomposition rates, which was related to plant density, but not to litter quality. Changes in SOM decomposition rates are of great importance because linked not only to C sequestration, but also to availability of mineral N and other important nutrients, such as calcium and phosphorous (Hobbie, 2015). The contrasting results, reported in the literature, may derive from the fact that, up to now, modulation of encroachment effects by soil properties has been mostly neglected.

*Amorpha fruticosa* L. (false indigo, Fabaceae Lindl.) is a shrub native from North America and introduced in Europe at the beginning of the 18th century (Schnitzler et al., 2007). Among alien N<sub>2</sub>-fixing species, *A. fruticosa* is one of the most invasive species (Invasive species compendium - <https://www.cabi.org/isc>) due to its large success of reproduction in pioneer environments (Tickner et al., 2001; Sakai et al., 2001; Schnitzler et al., 2007). Within a few years, its encroachment leads to the development of monospecific and dense populations, causing a collapse of plant diversity (Boscutti et al., 2020) and making difficult the environmental restoration, also due to the allelopathic action of this species that displays the highest juglone index among N<sub>2</sub>-fixing shrubs (Csiszár, 2009). Because of this, *A. fruticosa* has been included in the “Black list” of the most aggressive and hard to eradicate species in North-East of Italy (Buccheri et al., 2019).

In this work, we examined the impact of *A. fruticosa* on the biological properties of different soils located in natural areas within the floodplains of four rivers in North-East Italy. Within each area, we selected sites with different degrees of plant invasion (species cover) and stand age. We hypothesized that: a) *A. fruticosa* invasion strongly impacts not only the N cycle, but also the C cycle of invaded soils and that b) its action may depend on stand age and degree of encroachment and be modulated by soil characteristics. To our knowledge, this is the first time that a comprehensive approach of this kind has been applied to a vast region undergoing spontaneous invasion by N<sub>2</sub> fixing shrubs.

## 2. Materials and methods

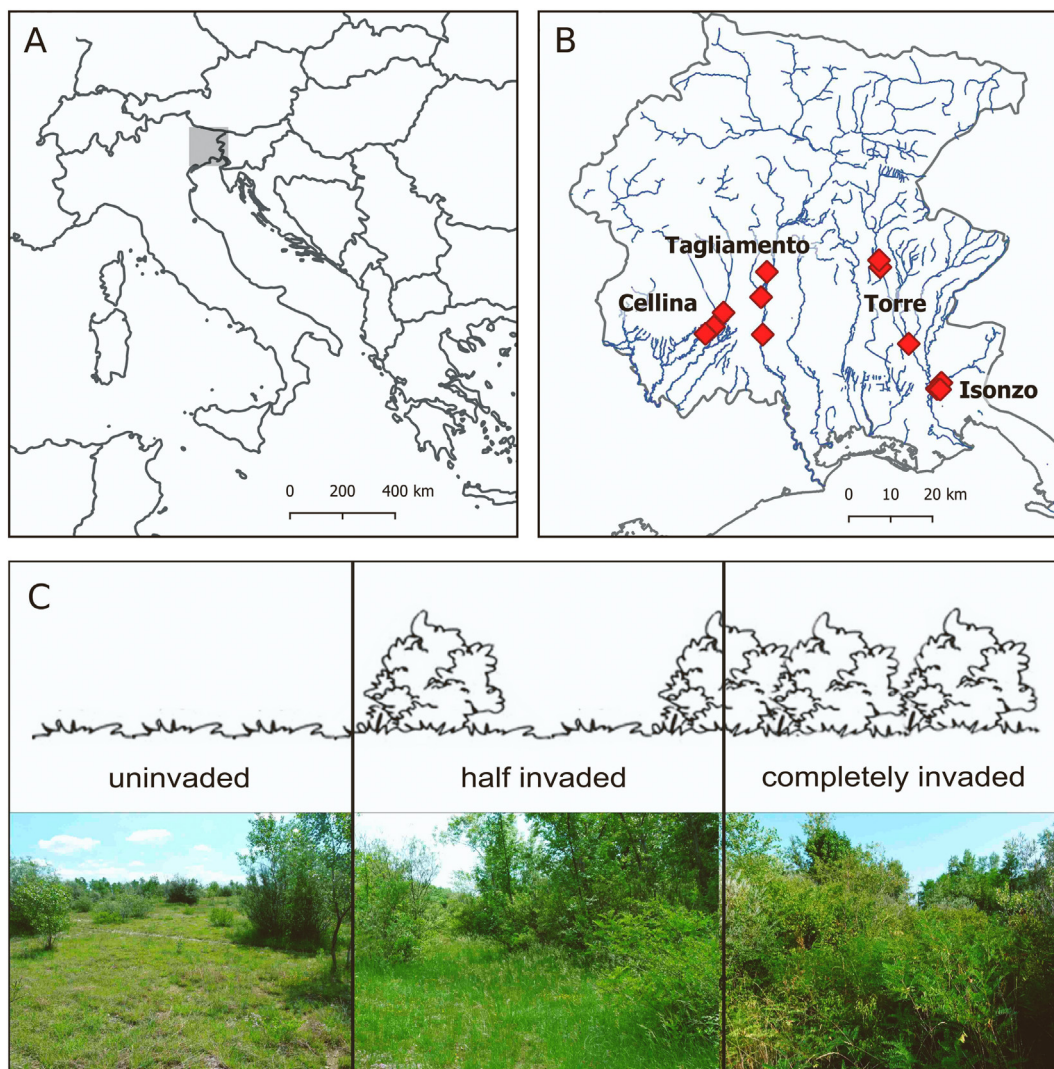
### 2.1. Study area

The present study was carried out in the North-East of Italy within the floodplains of four rivers, namely Cellina, Tagliamento, Torre and Isonzo (centroid of the area 46°01'48" N, 13°30'53" E, Fig. 1A). The average annual temperature is 13 °C and the annual rainfall is between 1100 mm and 1600 mm (Nordio et al., 2003). The pedological substrate is un-coherent calcareous coarse gravel underlain by Holocene alluvial deposits (Fontana et al., 2008). Soils are generally shallow (25 to 50 cm deep), rich in coarse gravel and excessively draining. They are mostly classified as Episkeletic-Calcaric Regosols and Fluvisols (FAO, 1998). One of the most common natural soil cover is represented by xeric grasslands, historically maintained by mowing once a year.

In each of the four floodplains, three sites were randomly selected (Fig. 1B). All sites were at least 0.7 km apart (max distance 58 km). Within each site, three 25 m<sup>2</sup> circular plots were randomly selected in three different areas, each representing a different level of invasion: i) uninvaded dry grassland, ii) half invaded (40–70% of cover of *A. fruticosa*) and iii) completely invaded areas (>70% of cover of *A. fruticosa*, Fig. 1C). In total 36 plots were sampled (4 floodplains × 3 replicate sites × 3 levels of invasion). Three soil samples were collected (0–15 cm) within each plot and pooled to obtain a unique representative sample per plot.

### 2.2. Stand characteristics and leaf traits

*A. fruticosa* plants were counted within each 25 m<sup>2</sup> plot (number of plants) and the basal diameters of five randomly selected stems were measured (mean diameter of stems). The selected shoots were cut at the base and a wood section was collected to measure the mean



**Fig. 1.** Geographical location of this study (A), the selected four riparian floodplains of the rivers Cellina, Tagliamento, Torre and Isonzo (north-east of Italy) (B) and examples of the three separate sampling zones chosen within each site (C), namely natural dry grasslands, half invaded and completely invaded stands by *Amorpha fruticosa*.

width of stem rings (mm) and the mean shoot age (age of plants), obtained by counting tree rings using the Dendrotab 2003 measuring table (© Walesch Electronic GmbH, CH).

*A. fruticosa* leaves were collected from each cut stem, dried at 70 °C for 48 h and then ball-milled and stored in plastic vials. Total C and N were measured to obtain the leaf C/N ratio and leaf isotopic signature ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) was assessed using a CHN Elemental Analyser (Vario Microcube, © Elementar) coupled to a stable isotope ratio mass spectrometer (IRMS; Isoprime 100, © Elementar). C/N ratio and isotopic signature of grassland native species were also collected.

### 2.3. Soil analysis

Soil samples were transported to the lab and stored at 4 °C. A soil aliquot was air-dried and sieved at 2 mm to remove gravel and pieces of roots. Soil organic C (SOC), soil total N (STN),  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were measured on dry ground soil using a CHN Elemental Analyser (Vario Microcube, © Elementar) coupled to an isotope ratio mass spectrometer (IRMS; Isoprime 100, Elementar). Soil texture was measured in a Bouyoucos' cylinder with an ASTM 152H hydrometer.

The remaining fresh soil was stored at 4 °C for the analyses of microbial biomass C and N (MBC and MBN, respectively), SOM mineralisation rate, nitrification and ammonification rates.

Fresh soil aliquots corresponding to 50 g of oven dry soil were pre-incubated in the dark at 25 °C and 40% of water holding capacity (WHC), to allow soil re-equilibration after disturbance. Microbial biomass C (MBC) and N (MBN) were measured after 5-days incubation by the fumigation-extraction method (Vance et al., 1987). Extraction was performed on 20 g of soil (dry weight) with 80 mL of 0.5 M  $\text{K}_2\text{SO}_4$  for 30 min, while a corresponding aliquot (20 g) was fumigated with ethanol-free chloroform for 24 h and then extracted following the same procedure. MBC and MBN were determined using a Total Carbon Analyser (VPCN and TN-1 Shimadzu).

To determine nitrification and ammonification rates of soils, aliquots of fresh soil samples, corresponding to 10 g dry weight, were extracted ( $t_0$ ) with 40 mL of 0.5 M KCl for 1 h, following the procedure of Bremner and Keeney (1966). Afterwards, fresh soils (50 g dry weight) were incubated for 10 days in the dark at 25 °C and extracted ( $t_1$ ) in the same way (using 10 g dry weight soil). Nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) were determined using a Segmented Flow Injection Analyzer (Skalar San<sup>++</sup>). Potential nitrification and net ammonification rates were calculated as the difference between the amounts of nitrate or ammonium extracted from soil at  $t_1$  minus that found at  $t_0$  divided by the incubation time (10 d). For gross ammonification rates, the moles of nitrate developed at the end of the incubation were added to the moles of ammonium to account for the ammonium transformed into nitrate during

the 10-days incubation. The  $\text{NH}_4^+/\text{NO}_3^-$  molar ratio was also calculated. During the 10-days incubation, emitted  $\text{CO}_2$  was trapped in 25 mL of 0.5 M NaOH and determined by titration with 0.2 M HCl, using an automatic DL50 tritator (Mettler Toledo). Containers without soil were also incubated for 10 days. SOM mineralisation rates were calculated from the  $\text{CO}_2$  trapped during incubation minus the blank (no soil) and divided by the incubation time. Metabolic quotients ( $q\text{CO}_2$ , Anderson and Domsch, 1993) were calculated dividing  $\text{CO}_2$  emission rates ( $\text{ng CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) by the MBC ( $\mu\text{g g}^{-1}$ ).

All soil analyses were done in triplicate and hence pooled as mean values for statistical analysis.

#### 2.4. Statistical analysis

All statistical analyses were performed in R statistical software v. 3.6.0 (R Core Team, 2020).

Analysis of Variance (ANOVA,  $P < 0.05$ ) was applied on linear mixed-effects models (LMMs) to detect significant differences on leaf traits and stand characteristics driven by *A. fruticosa* in half or completely invaded sites. The degree of *A. fruticosa* invasion was included as fixed factor in the models (uninvaded, half or completely invaded areas). Floodplains and sites were considered as random factors. LMMs and ANOVA were also applied on soil traits considering all three stages of the encroachment, thus uninvaded, half invaded and completely invaded grasslands. Prior to ANOVA, data normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test) were verified. Tukey test ( $P < 0.05$ ) was applied as post hoc test on ANOVA results.

ANOVA and Tukey post hoc test were also applied in order to investigate the effect of plant invasion on SOM mineralisation and nitrification rates, grouping the sites into low or high SOC content level in each encroachment stage based on the respective SOC median.

Confidence intervals of each variable were calculated using the "emmeans" function within the "emmeans" package (Russell, 2021). In order to compare effects of *A. fruticosa* encroachment, uninvaded and completely invaded sites (half invaded sites excluded) were evaluated using Hedges'  $g$  as a measure of the Standardized Effect Size (Del Re, 2013). Hedges'  $g$  is an unbiased estimate of Cohen's  $d$  not affected by sample size, scale of measure or magnitude. Null values indicate no difference between the original (dry grassland) and the final condition (*A. fruticosa* monospecific stands), whereas positive or negative values imply an average increase or decrease, respectively, following invasion. Effect sizes lower than 0.2 were considered negligible.

LMMs were applied to examine the direct effect of plant traits on soil properties and the indirect effects of soil properties on soil nitrification, ammonification and SOM mineralisation processes (see Table S1 for the complete dataset). The degree of *A. fruticosa* invasion was included as fixed factor in the models (half or completely invaded areas). Floodplains and sites were considered as random factors. Multicollinearity of LMM was analysed using the Variation Inflation Factor ( $\text{VIF} < 2$ ) and the Pearson's correlation test ( $r < 0.75$ ). In addition, a Principal component analysis (PCA) was applied to plant traits and soil properties in order to graphically detect variable collinearity and to identify the main variables driving changes in soil properties due to plant encroachment (Fig. S1). Biplot of PC1 and PC2 was obtained using the "ggfortify" package (Tang et al., 2016). In case of high collinearity, variables were removed from the models (Tables S2, S3).

LMMs were applied using the "nlme" R package (Pinheiro et al., 2017). Model assumptions were verified using diagnostic plots and Shapiro-Wilks normality test ( $P > 0.05$ ) on model residuals. Models did not break any linear model assumption. In order to select the best models describing the effect of plant invasion, a multivariate analysis was applied to LMMs using the MultiModel Inference (MuMIn) approach. The MuMIn allows to rank models based on the AICc criterion (Akaike's Information Criterion corrected for small samples) and to provide one or more plausible models fitting with the dataset

considering more variables at the same time. The "dredge" function of the MuMIn R package (Burnham and Anderson, 2002) was applied for model selection. The best fit refers to the model with the lowest AICc. Results arisen from the best models were tested based on the Analysis of Variance (ANOVA) and plotted using the "visreg" function (Breheny and Burchett, 2013). Slopes of significant interactions between the considered variable and the stage of invasion were further pairwise compared using the "emtrends" function within the "emmeans" package.

### 3. Results

Stand characteristics and leaf traits of *A. fruticosa* of invaded stands are reported in Table 1. As expected, stand characteristics significantly differed between half invaded and completely invaded areas, with older and larger plants becoming more frequent upon complete invasion. In completely invaded areas, *A. fruticosa* populations showed a higher proportion of plants with high number of tree rings and larger mean ring widths. The difference between the average age of plants in half invaded and completely invaded areas was small (1.3 years), but statistically significant. *A. fruticosa* leaf traits related to N (C/N and  $\delta^{15}\text{N}$ ) did not significantly differ between half and completely invaded sites, but  $\delta^{13}\text{C}$  was significantly more negative in the *A. fruticosa* leaves from completely invaded sites. C/N ratio,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of the grassland community (aboveground tissue) did not differ between half and completely invaded sites. Effects of half or complete encroachment on soil properties were compared, among each other, using Hedges'  $g$  as a measure of the Standardized Effect Size. Fig. 2 shows that *A. fruticosa* encroachment determined different effect sizes on soil traits, among which the increase of the nitrification and ammonification rates and  $^{15}\text{N}$  enrichment of STN were the most remarkable ( $g > 1.5$ ). Nitrification rates and  $\delta^{15}\text{N}$  were positive and meaningful in half (Fig. 2A) and completely invaded (Fig. 2B) sites. The principal component analysis (Supporting information Fig. S1, A) confirmed the relevance of plant invasion in driving changes especially on these soil properties.

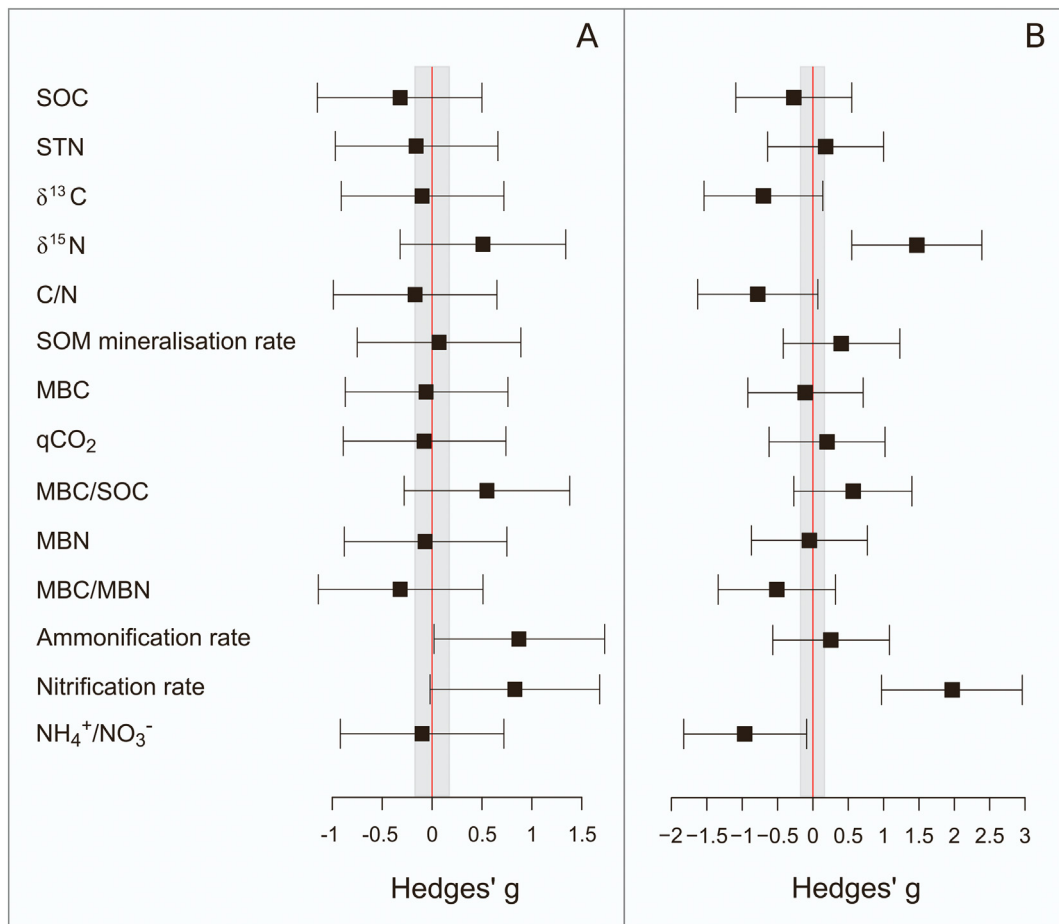
Soil C/N ratios,  $\delta^{13}\text{C}$  of SOC and mineral N composition ( $\text{NH}_4^+/\text{NO}_3^-$ ) were all negatively affected by *A. fruticosa*, and more consistently in completely invaded sites (Fig. 2B) compared to half invaded sites (Fig. 2A). STN seemed not to be affected ( $g < 0.2$ ) whereas soil  $\delta^{15}\text{N}$  increased moderately in half invaded grasslands soils, reaching slightly positive values in completely invaded soils (Table S4).

MBC and metabolic quotient ( $q\text{CO}_2$ ) seemed not to be affected ( $g < 0.2$ ) by plant encroachment as well as the amount of N in the microbial biomass pool (MBN) and MBC/MBN ratios, which did not vary substantially. The latter were, in all soils, typical of a mostly bacterial population of microorganisms (Tables S1, S4). The amount of microbial biomass sustained per unit SOC (MBC/SOC) increased during the invasion and, compared to uninvaded grasslands, MBC/SOC increased by about 40 and 60% respectively upon half and complete invasion (Table S4). Soil properties of uninvaded sites were highly variable in terms of MBC, MBN and SOM mineralisation (Supporting information Fig. S1, B).

**Table 1**

Measured *A. fruticosa* stand characteristics and considered leaf traits, and of the grassland community (aboveground tissues). Values are mean  $\pm$  confidence interval based on 95% confidence level ( $n = 12$ ). \* refer to statistically significant differences obtained with Tukey's post hoc test ( $p < 0.05$ ).

Plant traits	Half invaded	Completely invaded
Number of plants ( $\text{n m}^{-2}$ )*	0.41 $\pm$ 0.13	0.62 $\pm$ 0.15
Mean width of growth rings (mm)*	1.75 $\pm$ 0.17	2.18 $\pm$ 0.11
Mean diameter of stems (mm)*	10.4 $\pm$ 2.0	19.2 $\pm$ 2.7
Age of plants (number of growth rings)*	3.5 $\pm$ 0.5	4.8 $\pm$ 0.5
Leaf C/N	14.4 $\pm$ 1.1	13.8 $\pm$ 0.8
Leaf $\delta^{13}\text{C}$ (‰)*	-27.0 $\pm$ 0.8	-29.3 $\pm$ 1.0
Leaf $\delta^{15}\text{N}$ (‰)	-4.6 $\pm$ 0.6	-5.1 $\pm$ 0.7
C/N of the grassland community	51.2 $\pm$ 8.1	51.9 $\pm$ 8.0
$\delta^{13}\text{C}$ (‰) of the grassland community	-24.1 $\pm$ 2.6	-24.1 $\pm$ 2.6
$\delta^{15}\text{N}$ (‰) of the grassland community	-8.6 $\pm$ 1.2	-8.7 $\pm$ 1.2



**Fig. 2.** Changes of soil properties during *A. fruticosa* encroachment in half (A) or completely invaded sites (B). Effect sizes ( $g$ ) lower than 0.2 are considered not meaningful and displayed within a grey band. Positive or negative mean effect sizes indicate an increase or a decrease of the soil property after the invasion, respectively. Changes are shown using forest plots of mean effect sizes, based on Hedges'  $g$  index. SOC = soil organic C; STN = soil total N; SOM = soil organic matter; MBC = biomass C; qCO<sub>2</sub> = metabolic quotient; MBN = biomass N. Bars represent 95% confidence intervals.

The contribution of *A. fruticosa* inputs to soil was reflected by the isotopic composition of SOC and STN of invaded soils (Fig. 2, Table S4). The  $\delta^{13}\text{C}$  of SOC decreased (depletion slightly increased) during invasion, probably mirroring inputs from shrubs' leaves that are relatively depleted in  $^{13}\text{C}$  (Table 1). The decrease was more consistent upon complete invasion (Fig. 2B).

Soil  $\delta^{15}\text{N}$ , SOM mineralisation and nitrification rates increased after complete invasion (Fig. 2B). SOM mineralisation rates, measured under optimal moisture and temperature conditions and therefore corresponding to maximum potential mineralisation rates, increased (Fig. 2B) and can explain the observed decrease in SOC. Both ammonification and nitrification rates increased with the invasion. The increase in nitrification was, however, stronger and it modified the composition of soil mineral N which showed a larger proportion of nitrate (NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup>), albeit the effect was still not significant due to site variability (Table S4). Ammonification rates increased significantly in half invaded soils ( $g > 0.8$ , Fig. 2A) and decreased upon complete invasion ( $g \sim 0.3$ , Fig. 2B). This denoted an increased microbial activity at the initial stage of the invasion, which can be probably explained by a change in litter composition.

### 3.1. Effects of stand and plant traits on soil biological properties

Rates of SOM mineralisation were faster in old stands (Fig. 3A, best model with lowest AICc).

While SOC was not affected by any stand characteristics (Table S5), the amount of microbial biomass C sustained per unit SOC (MBC/SOC)

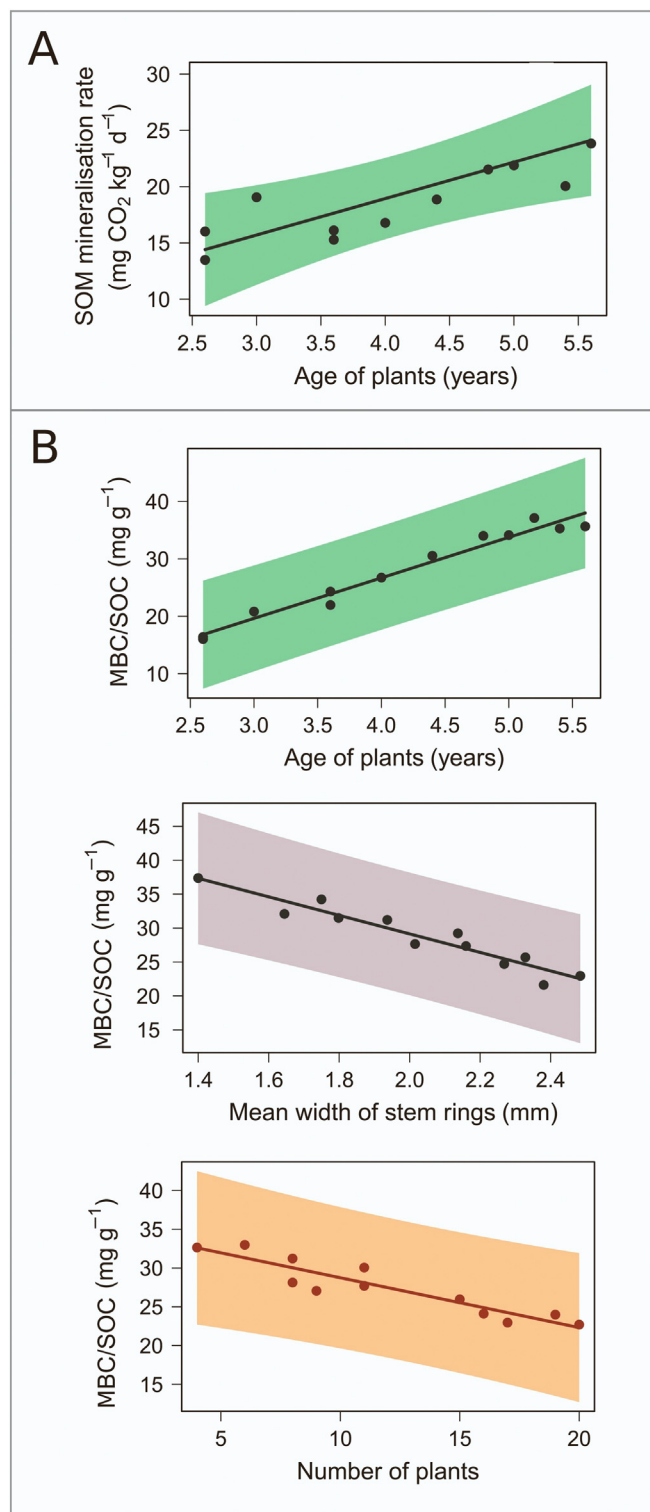
increased with plant age (Fig. 3B). On the other side, MBC/SOC showed a significant inverse relationship with plant number and growth (Fig. 3B). This apparent contradiction points out to the likely concomitant action of effecting factors based on soil characteristics.

Biological soil properties related to the N cycle, such as ammonification and nitrification were highly variable, but more coherent in their response to invasion. Being closely associated to SOC mineralisation, ammonification rates increased with population density (Fig. 4A) and were not affected by plant growth or stand age (Table S5). Nitrification rates were related positively to both plant growth and stand age (Fig. 4B and Table S5).

### 3.2. Modulation by soil characteristics of invasion effects on soil biological processes

The observed trends in SOM mineralisation, ammonification and nitrification rates in invaded areas, were strongly related not only to stand characteristics (Figs. 3 and 4), but also to soil properties. In fact, the best models (lowest  $\Delta$  AIC) obtained from the Multimodel inference analysis showed that, in invaded and uninvaded soils SOM mineralisation rates were strongly positively correlated to SOC and clay content (Fig. S2A, Table S6), while ammonification rates decreased with the increase in clay, but were positively related to SOM mineralisation rates (Fig. S2B, Table S6).

Larger STN values stimulated SOM mineralisation in uninvaded soils, but acted differently at the two stages of *A. fruticosa* invasion. The CO<sub>2</sub> emission from uninvaded grassland soils and completely invaded soils



**Fig. 3.** Effects of stand age of *A. fruticosa* on soil organic matter (SOM) mineralisation (A) and effect of stand age (number of xylem rings), plant size (mean width of xylem rings) and stand density on the amount of biomass C sustained per unit soil organic C (MBC/SOC = microbial biomass C/soil organic C) (B). Effect plots are reported, displaying the results of fitted models. The best models (lowest  $\Delta$  AIC) obtained from the Multi-model inference analysis are plotted ( $R^2 = 0.68$ ,  $R^2 = 0.98$  for SOM mineralisation rate and MBC/SOC, respectively;  $p < 0.05$ ). The dataset includes only sites with half and completely invaded areas (uninvaded sites were excluded). No significant differences were found between half and completely invaded sites.

of increasing STN showed that SOM mineralisation was boosted by availability of STN, however, this trend was not observed in half invaded soils (Fig. 5A) and seemed not dependent on SOC (Fig. 5B).

Nitrification rates were related to SOC at all degrees of invasion (Fig. 6A, Table S6). However, in half invaded sites, soils of low SOC content still displayed nitrification rates that did not differ from those of uninvaded grasslands soils of comparable SOC levels (Fig. 6C). Conversely, in soils of half invaded sites with larger SOC pools, nitrification was comparatively impeded. This represented only a transient stage: after encroachment, the positive relationship was restored ( $P = 0.05$ ). At the same time, independently from the stage of invasion, nitrification rates appeared to be restrained in soils with larger clay contents (Fig. 6A).

Finally, soil  $\text{NO}_3^-$  content increased along with the amount of mineralised SOM, but the effect depended upon the degree of plant invasion (Fig. 6B, Table S6).

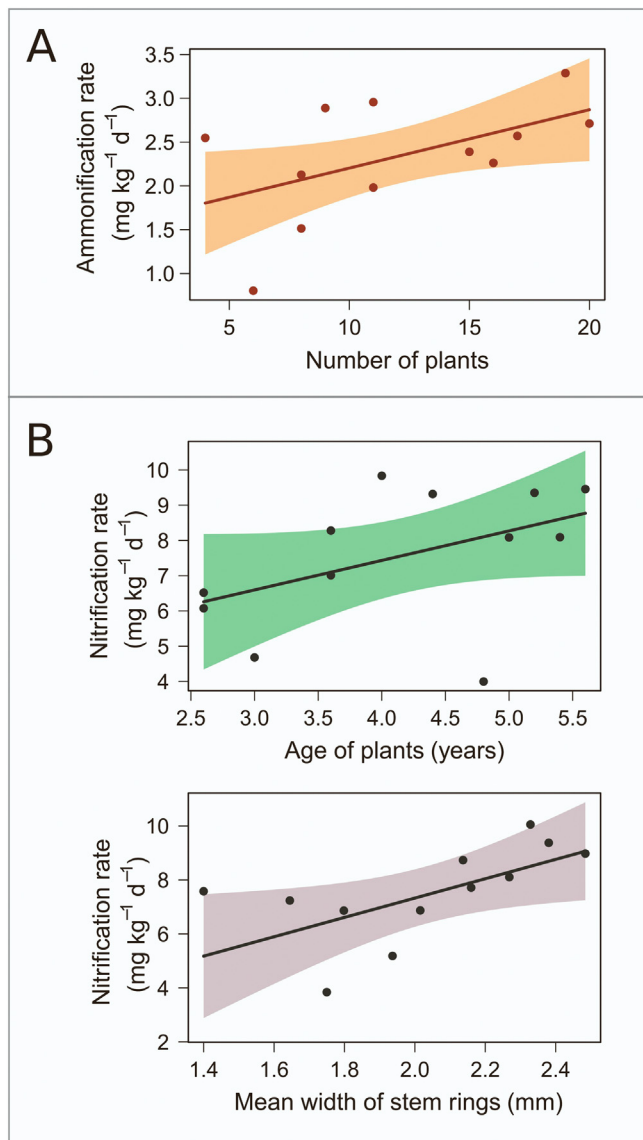
#### 4. Discussion

The progress of *A. fruticosa* invasion in the studied area was an extremely fast process as stands reached a full cover in approximately 4–5 years (Table 1). In addition to the high competitiveness of the species (Takagi and Hioki, 2013), the invasion was probably facilitated by the low biodiversity of these grasslands, which host, on average, about 0.6 species  $\text{m}^{-2}$  (Boscutti et al., 2020). In fact, it has been already reported that plant invasion climaxes faster where the ecological diversity of the habitat is low (Elton, 2000; Naeem et al., 2000). Moreover, *A. fruticosa* inhibits the germination and growth of other species by the release of allelopathic substances from roots (Csizsár, 2009), so that the final part of the invasion may occur even faster.

Even though an increase in STN and mineral N have been often reported in soils invaded by  $\text{N}_2$ -fixing species (Stock et al., 1995; Liao et al., 2008; Raizada et al., 2008; Hellmann et al., 2011), the rapidity of the invasion in the studied areas may justify the fact that we did not observe any significant increase in STN. However, our results show that a positive feedback loop for invaders is probably established before any change in STN becomes detectable. In fact, nitrification was significantly faster in completely invaded sites (about +57%), confirming the reports by Liao et al. (2008), and it increased with stand age, representing the largest impact caused by the species (Hedges'  $g \sim 2$ ). N inputs by *A. fruticosa* were crucial for these changes of the soil N cycle, as shown by the isotopic signature of STN which displayed a  $\delta^{15}\text{N}$  increase that reached, upon complete invasion, values typical of soils under  $\text{N}_2$ -fixing species (Rascher et al., 2012; Gei and Powers, 2013).

More favourable soil conditions for the growth of *A. fruticosa* were therefore established after the intermediate stage of the invasion because of changes in soil mineral N composition (i.e. lower  $\text{NH}_4^+/\text{NO}_3^-$  ratios). Enhanced nitrification and changes in N isotopic signature have been widely documented as constant traits of  $\text{N}_2$ -fixing invaders (Lorenzo et al., 2010; Ehrenfeld, 2010; Lazzaro et al., 2014).

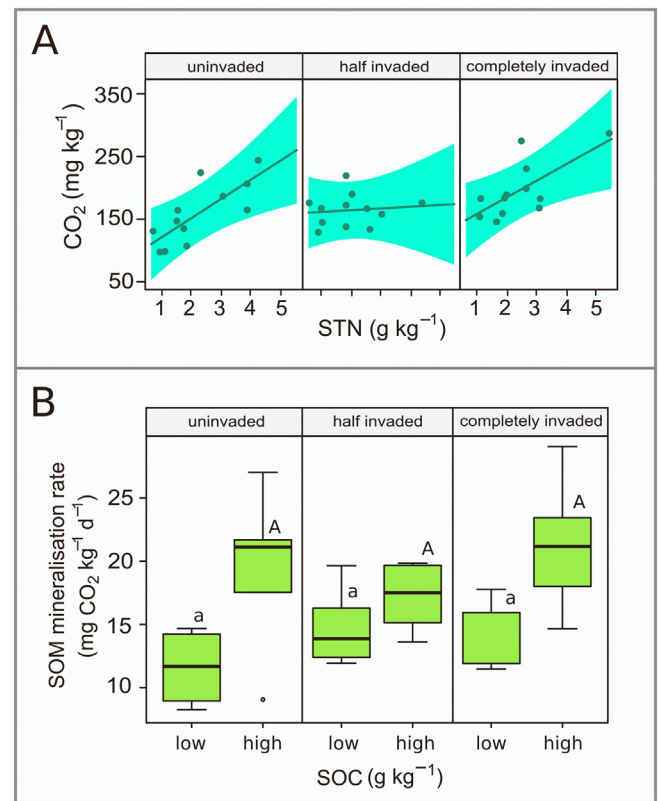
However, complete encroachment is preceded by an intermediate stage when *A. fruticosa* invasion seems to temporarily decouple nitrification from availability of organic C and N (i.e. from SOM mineralisation and ammonification Figs. 5A and 6A). Enhancements in nitrification lower the N-cycling efficiency of terrestrial ecosystems (Subbarao et al., 2015), as they may disrupt species specific N preferences (Zhang et al., 2018). For this reason, many plants living in environments of low N availability are often able to release, from roots and litter, biological nitrification inhibitors (BNI). The synthesis of these substances, which is stimulated by the abundance of  $\text{NH}_4^+$  in the rhizosphere, was first observed in tropical grasslands (Boudsocq et al., 2009), but recent findings suggest this may be a widely adopted strategy (Subbarao et al., 2015). The observed trend may therefore result from the reaction of the autochthonous grasses to changes brought by invaders. After complete encroachment, this action obviously disappears, revealing the full effect of boosted N availability on soil nitrification potential.



**Fig. 4.** Effects of stand density on ammonification (A) and of stand age and plant size (mean width of growth rings) of *A. fruticosa* on nitrification (B). Effect plots are reported, displaying the results of fitted models. The best models (lowest  $\Delta$  AIC) obtained from the Multi-model inference analysis are plotted ( $R^2 = 0.29$  and  $R^2 = 0.50$  for ammonification and nitrification rates, respectively;  $p < 0.05$ ). The dataset includes only sites with half and completely invaded areas (uninvaded sites were excluded). No significant differences were found between half and completely invaded sites.

The intermediate stage could therefore represent a key step of plant invasion where remediation could be more effective. Long term effects of *A. fruticosa* invasion last for decades leading to reduced species richness (Von Holle et al., 2013; Szigetvári, 2002) and the favourable establishment of nitrophilic species (Grove et al., 2015). Most of natural species adapted to poor soils (e.s. sandy soils) could not be able to re-colonize the degraded grasslands, even after several years from *A. fruticosa* eradication (Szigetvári, 2002).

Similar to nitrification, at the intermediate stage of encroachment, also CO<sub>2</sub> emission does not positively correlate with SOC nor STN. This unexpected finding can likely be explained by the fact that changes brought by invaders occur more quickly in soils with smaller SOC pools (soils below the median SOC value registered in the examined range) than in soils with larger SOC pools. Easily degradable litter inputs by *A. fruticosa* undoubtedly represent a substantial contribution to the overall respiration of soils originally low in SOC, but may have a much

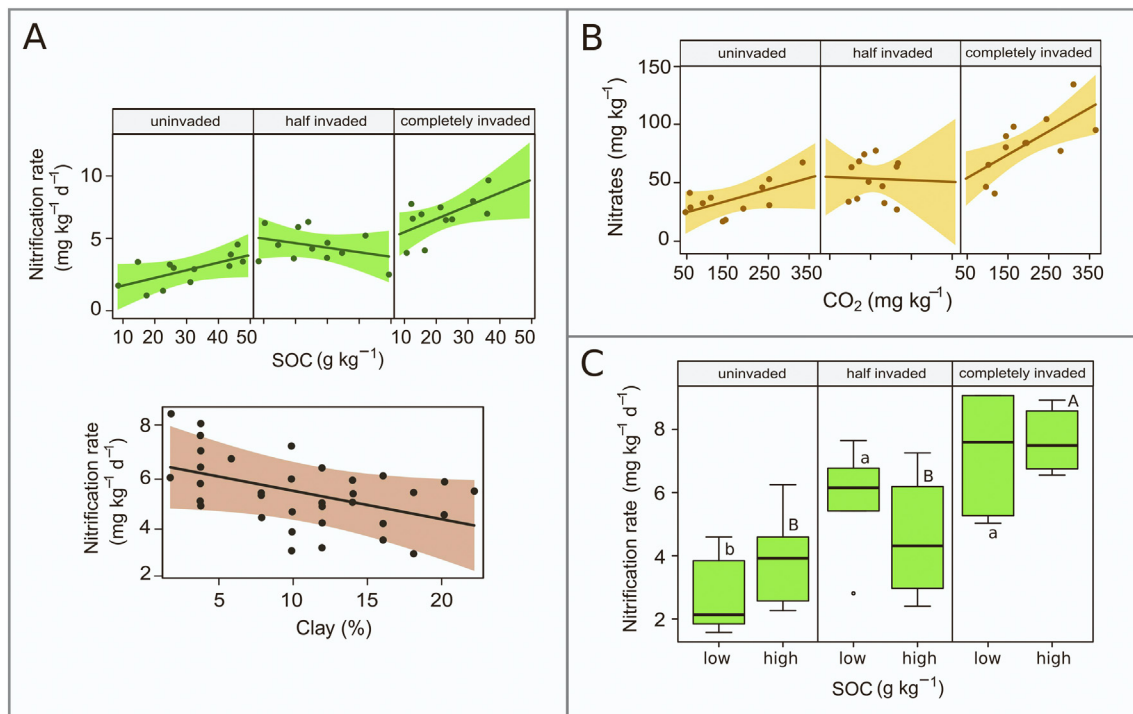


**Fig. 5.** Effects of soil total N (STN) on mineralised CO<sub>2</sub> (A) and changes in SOM mineralisation rates in soils with low or high SOC upon the three degrees of invasion (B). The effect plot is reported, displaying the result of the fitted model (A). The best model (lowest  $\Delta$  AIC) obtained from the Multi-model inference analysis is plotted ( $R^2 = 0.75$ ;  $p < 0.05$ ; A). *A. fruticosa* cover was considered as a discrete factor (uninvaded, half invaded, completely invaded areas) to test possible interactions with independent variables. Low and High SOC were grouped based on SOC median in each degree of invasion (B). Letters refer to one-way ANOVA and Tukey's post hoc test applied on the three degrees of invasion at low (lowercase) or high SOC (uppercase).

lower impact, particularly at the intermediate stage on soils of larger SOC contents.

Besides the aforementioned suppressive action of biological nitrification inhibition, the activity of the soil microbial community was altered by changes in litter composition brought by the invader. The amelioration of the resource quality of inputs, enhanced, in fact, mineralisation and nitrification in SOC poor soils (Fig. 6A, B). Occurrence of a transition stage during the invasion of alien species (negative relationship between nitrification and SOC in half invaded grasslands) was already observed and included in conceptual models describing plant invasion (Dietz and Edwards, 2006). Our results suggest that it could represent a favourable circumstance for effective restoration plans. Long-term invasion will alter N pools but also nutrient distribution along the soil profile (Blank, 2008). Marchante et al. (2009) highlighted that after the removal of a N<sub>2</sub>-fixing invasive species, several years are necessary for the restoration of initial soil conditions and soil biological processes. However, they proved that susceptibility of native plants to recolonization of degraded areas seems to be a function of the time elapsed since the removal of the invader.

Stronger impacts were driven by population density and age compared to plant cover. The increase of nitrification with stand age was already recorded for other woody plant communities (Inagaki et al., 2004; Hawkes et al., 2005) and nitrification is boosted by faster ammonification (Ehrenfeld et al., 2001). In our work, ammonification rates increased with plant density, which was expected, being *A. fruticosa* a N<sub>2</sub>-fixing species (Castro-Díez et al., 2012).



**Fig. 6.** Effects of soil organic C (SOC), clay and mineralised CO<sub>2</sub> on nitrification (A, B) and changes in nitrification rates in soils with low or high SOC and upon the three degrees of invasion (C). Effect plots are reported, displaying the results of fitted models. The best models (lowest  $\Delta$ AIC) obtained from the Multi-model inference analysis are plotted ( $R^2 = 0.75$  and  $R^2 = 0.69$  for nitrification rates and nitrates, respectively;  $p < 0.05$ ; A, B). *A. fruticosa* cover was considered as a discrete factor (uninverted, half inverted, completely inverted sites) to test possible interactions with independent variables. Low and High SOC were grouped based on SOC median in each degree of invasion (C). Letters refer to one-way ANOVA and Tukey's post hoc test applied on the three degrees of invasion at low (lowercase) or high SOC (uppercase).

After complete invasion *A. fruticosa* increased SOM mineralisation rates and showed a significant positive effect size (Hedges'  $g = 0.4$ ) on this soil activity. This is only in part accounted for by the change in litter quality. In fact, in accordance with other studies (Inagaki et al., 2004; Liao and Boutton, 2008), SOM mineralisation rates were significantly boosted by stand age, but not by plant density, contrary to what reported for soils invaded by *Cytisus scoparius* by Broadbent et al. (2017). The impact of *A. fruticosa* on SOM mineralisation appears to be relatively slower, as it does not manifest itself at the early stages of plant encroachment when plant density increases.

Enhanced SOM mineralisation rates are generally explained by the more rapid decay of the litter of invasive plants (Ehrenfeld, 2003). Indeed, in the present work, the better resource quality of litter is reflected by the larger amount of biomass C sustained per unit SOC (MBC/SOC). SOC is usually slightly enriched in <sup>13</sup>C compared to the dominant vegetation (Fry, 2006) and the more intense mineralisation observed is in agreement with the shift in isotopic composition of SOC, which displayed a tendency towards a more intense depletion of <sup>13</sup>C. This trend, however, also reflected the isotopic composition of *A. fruticosa* leaves. The stronger depletion in <sup>13</sup>C of the shrub's leaves in completely invaded sites could depend on a more intense shading of soil resulting in reduced evaporation (Maestre et al., 2003). Stronger depletion in <sup>13</sup>C could, on the other hand, derive from a larger contribution of lignin by shrubs. However, considering the rapidity of the encroachment (only about one year on average between half and complete invasion) and the comparatively slow rates of decomposition and incorporation of lignin into SOM, this is unlikely to have occurred so far.

The increase of SOM mineralisation at optimum temperature and moisture conditions is normally considered to be the direct consequence of the increase in microbial biomass, which normally accompanies the accumulation of C (Alvarez and Alvarez, 2000). In our study, MBC/SOC ratios positively correlated to stand age, similarly to trends reported in literature for shrub plantations (Jia et al., 2010), but showed a negative correlation with plant size and plant density. An

increasing number of taller plants, such as occurs at half invasion, might temporarily inhibit microbial activity (Castro-Díez et al., 2012) until the establishment of older stands. Even more likely, the effect is caused, at this intermediate stage, by restriction of the diversity of microbial community resources. In fact, microorganisms are progressively forced to use only one kind of litter, in contrast to what happens in natural plant communities of larger species richness (Kowalchuk et al., 2002).

In soils under similar climatic conditions and net primary production, SOC content is expected to be related to soil texture, with clayey soils containing more organic matter (Hassink, 1994; Kaiser et al., 1992). SOM mineralisation rates are often hampered in soils of finer texture as a consequence of the physical protection exerted by clay particles. In the soils examined, on the contrary, SOM mineralisation was positively related to the clay content. This possibly derives from the coarse textures (sandy to loam) of these excessively draining soils, in which small increments in clay may enhance water retention and allow for longer periods of microbial activity. This hypothesis is also in agreement with the trend observed for the isotopic signature of SOC.

Clay, on the other hand, negatively influenced ammonification and nitrification in these soils. The depressing effect of clay on ammonification has been ascribed to physical stabilization of SOM and sorption of organic N forms. That on nitrification to a regulation of ammonium concentration in the soil solution caused by attraction from negatively charged clay surfaces (Sahrawat, 2008), so that less ammonium is free to diffuse to nitrifiers. Clay content is therefore another soil factor that may concur in the modulation of the effects of invasion by N<sub>2</sub>-fixing shrubs on soil C and N cycles.

## 5. Conclusions

Dry grasslands are inhabited by plants with low nutrient demands and are therefore associated with soils of low fertility. Exotic N<sub>2</sub>-fixing plants take advantage of their extra N supplies to easily colonize these



grasslands and alter soil N and C cycles, quickly establishing favourable conditions for further invasion. Encroachment by *A. fruticosa* was extremely fast and increased significantly soil ammonification and nitrification, but also SOM mineralisation rates. All these changes are in agreement with a faster cycling of nutrients and in particular with a faster but less efficient N cycling in completely invaded soils. The microbial biomass C sustained by unit SOC also increased, reflecting the improved resource quality of litter. Nevertheless, this was not the only affecting factor, as it displayed strong negative relationships with plant size and stand density.

This work highlights the existence of an intermediate stage during invasion, at which nitrification and ammonification trends are transitionally reverted. This is probably the stage when remediation may still be possible. Afterwards, even after removal of *A. fruticosa*, faster nutrient recycling driven by SOM mineralisation coupled with faster nitrification would favour the establishment of other more demanding nitrophilic species, permanently altering vegetation.

Direct effects of plant invasion are modulated by the action of soil characteristics such as SOC and clay contents and these factors must also be considered when planning remediation actions, as they act on the longstanding changes that persist after eradication of N<sub>2</sub>-fixing invaders.

Based on our results, remediation should be managed considering not only plant traits but also stand age and the degree of encroachment that drive most of the changes on soil mineralisation and nitrification.

#### CRediT authorship contribution statement

**E. Pellegrini:** Investigation, Formal analysis, Data curation, Writing – original draft. **F. Boscutti:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing – review & editing. **G. Alberti:** Methodology, Investigation, Resources, Writing – review & editing. **V. Casolo:** Resources, Writing – review & editing. **M. Contin:** Resources, Writing – review & editing. **M. De Nobili:** Resources, Supervision, Data curation, Writing – original draft.

#### 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.

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#### Appendix A. Supplementary data

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