




## Article

# Increasing Wood $\delta^{15}\text{N}$ in Response to Pig Manure Application

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**Abstract:** Intensive livestock management impacts forest and trees in different ways. Pig manure is a major source of nitrogen (N) pollution of surface and ground waters in some European regions such as north-eastern Spain, but it is understudied how manure application impacts agroforestry systems. How pig manure affects tree radial growth and the N cycle was assessed by measuring N concentrations in soil, leaves and wood and  $\delta^{15}\text{N}$  in tree-ring wood in two tree species widely planted for agricultural (*Prunus dulcis*) and reforestation (*Pinus halepensis*) purposes in the study area. Soil physicochemical characteristics and the biomass and structure of major soil microbial groups were also measured. Trees irrigated with pig effluent (manure application) and control trees not subjected to manure application were compared. Soil N, phosphorus (P) and potassium (K) concentrations of fertilized trees increased, but soil microbiota biomass decreased. Similar growth between fertilized and non-fertilized pine trees was found, but lower growth in fertilized almond trees was observed. Leaf N concentrations decreased but  $\delta^{15}\text{N}$  wood increased in trees subjected to pig manure application. Pig manure application alters the N cycling in the soil and within trees.

**Keywords:** animal waste;  $\delta^{15}\text{N}$ ; dendroecology; pig effluent pollution; *Pinus halepensis*; *Prunus dulcis*



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## 1. Introduction

Nitrogen (N) is a key element for the functioning of terrestrial ecosystems which is obtained through biological N fixation [1,2]. However, mankind has altered the global N biogeochemical cycle. Human N fixation, through industrial processes for producing fertilizers and the cultivation of N-fixing crops, was twice the natural N sources in 2010 and, nowadays, anthropogenic N inputs have more than doubled natural inputs [3]. Anthropogenic N deposition may increase forest productivity and the rate of carbon (C) sequestration, but these responses are still debated since they depend on local climate and soil conditions and are also modulated by disturbances related to historical changes in land use [4]. Complex interactions among C and N cycles and climatic conditions may lead to synergistic or antagonistic, and not simply additive, effects on forest ecosystems [5]. For instance, strong N deposition during several years or decades may lead to nutrient imbalances in soils, including phosphorus (P) deficit and nitrate ( $\text{NO}_3$ ) leaching, triggering the loss of soil base cations and reducing tree growth [6]. This saturation in N availability can reverse fertilization effects on forests and lead to indirect adverse impacts including N-enriched streams and groundwater,  $\text{NO}_3$ -pollution of aquifers, eutrophication or a loss in tree vigor and forest productivity [1,7].

The impacts of such increasingly anthropogenically-driven N cycle are spatially and temporally heterogeneous with multiple economic and environmental challenges [3]. For instance, the consumption of N-rich products such as meat is exponentially rising in some countries, whereas the environmental impacts of that intensified livestock production are

experienced by other countries [3]. This is the case of Spain, the largest pig producer in Europe, where the pig population has steadily increased since 2014 at a mean rate of +1 million yr<sup>-1</sup>, accounting for 24% of the European Union pig livestock, being mainly driven by meat export to Asia [8].

In north-eastern regions of Spain (Aragón and Catalonia), pork supply has rapidly increased in the past decade, accounting for half the Spanish pig production [8]. In some of these areas, point sources of ammonia (NH<sub>4</sub>), a N compound abundant in pig effluents, have been identified through remote sensing and linked to intensive pig farming and groundwater nitrate pollution [9,10]. Therefore, these areas, where pig manure is widely applied as a fertilizer in crop fields, are suitable settings to investigate the impacts of such N-fertilization on tree growth and functioning and also on soils.

Since tree nutrient acquisition depends on soil microbial communities, considering the biomass and structure of soil microbiota groups is fundamental to understand nutrient imbalances caused by fertilizer addition. Nitrogen addition can alter the balance between major soil microbial functional groups by favoring decomposers in detriment of symbionts thus affecting the tree–soil continuum. For instance, N addition accelerates the mineralization of humus N, but it is unclear how the increase in N concentration affects soil microbial communities and particularly mycorrhiza [11].

Most tree species produce annual, conspicuous rings in regions with marked seasonality so investigating wood  $\delta^{15}\text{N}$  (<sup>15</sup>N/<sup>14</sup>N) in tree rings allows us to reconstruct changes in N cycling [12,13]. Stable N isotopes and N concentrations in wood are considered a proxy for N supply relative to demand and have revealed contrasting long-term trends in different biomes. Increased N availability is associated to N losses via gaseous or leaching pathways due to biological processes which discriminate against <sup>15</sup>N and favor <sup>14</sup>N, leading to higher  $\delta^{15}\text{N}$  values. For instance, in temperate forests of the USA, strong declines in wood  $\delta^{15}\text{N}$  after 1950 have been attributed to increases in atmospheric CO<sub>2</sub> concentration and increasing N demand [14–16]. Such long-term oligotrophication, which leads to reduced N availability relative to demand, may be caused by a reduction in relative denitrification rates or nitrification rates, or an increase in reliance on mycorrhizal fungi for N acquisition.

However, constant or increasing values of  $\delta^{15}\text{N}$  have been measured in tree-ring wood of tropical forests where trees are more limited by P than by N availability which could constrain increased growth response to higher CO<sub>2</sub> and thus not stimulate N demand [17–19]. Alternatively, increased disturbance rates in tropical forests could enhance NO<sub>3</sub> losses and explain the increase in  $\delta^{15}\text{N}$  wood [17]. The divergent long-term trends of N availability in temperate and tropical forests show how spatially variable is the global N cycle, thus demanding local studies which consider dominant N sources impacting terrestrial ecosystems.

Here, it was assessed how pig manure application affected soil physicochemical features, soil microbiota mass, tree radial growth, N concentration in leaves and wood and tree-ring  $\delta^{15}\text{N}$  in wood. Two functionally different tree species were compared: a conifer (Aleppo pine, *Pinus halepensis* Mill.) and a hardwood (almond tree, *Prunus dulcis* (Mill.) D.A. Webb). These two species naturally grow or are widely planted in north-eastern Spain and are potentially subjected to the impacts of pig manure applied to nearby crop fields.

We expect that the application of pig manure would increase N and P concentrations in soil, albeit NH<sub>4</sub> can be easily leached, and also would increase the N concentrations in leaves and wood. Increased N availability (high N supply relative to demand) should increase the  $\delta^{15}\text{N}$  of the inorganic N pool available to plants, make them less dependent on <sup>15</sup>N-depleted N provided by mycorrhizal fungi and lead to enriched  $\delta^{15}\text{N}$  values in tree-ring wood.

## 2. Materials and Methods

### 2.1. Study Sites and Tree Species

Two sites were chosen situated near crop fields where stands of the two study tree species were subjected to pig manure application since 2016. The estimated amount of pig effluent applied was in the range 20,000–30,000 L ha<sup>-1</sup>. The stands were located near

intensive pig farms situated in El Bayo village, in the Cinco Villas region, an area with intensive agricultural farming situated in Aragón, north-eastern Spain (see more details on their location in Table 1 and Figure S1, Supplementary Material).

**Table 1.** Features of the two study sites. The values of the diameter at breast height (dbh) are means  $\pm$  SD.

Tree Species	Latitude N	Longitude W	Elevation (m)	Dbh (cm)
<i>Pinus halepensis</i>	42.192°	1.253°	398	45.1 $\pm$ 6.8
<i>Prunus dulcis</i>	42.134°	1.141°	320	18.3 $\pm$ 2.1

The study stands are dominated by an evergreen conifer (Aleppo pine) and a deciduous hardwood (almond tree), two tree species widely distributed in the western Mediterranean Basin. Both species produce distinct, annual rings. The pine forms typical conifer wood with clearly defined earlywood and latewood tracheids, whereas the almond tree forms semi-ring porous wood.

In each site, five trees subjected to pig manure application as a fertilizer, and five nearby trees (located about 20–50 m away) which were not subjected to manure application (control trees) but growing under similar climatic and soil conditions, were selected. These trees were dominant and apparently healthy.

In the study area, climate conditions are Mediterranean with dry summers and wet-cool spring and autumn conditions. The mean annual temperature is 14.2 °C and the annual precipitation is 631 mm with October and July being the wettest (75 mm) and driest (31 mm) months, respectively (data from Ejea de los Caballeros meteorological station, 42.127° N, 1.135° W, 349 m a.s.l.). Soils are basic and of a sandy loam texture.

## 2.2. Field Sampling

The diameter at breast height (dbh) of selected trees was measured at 1.3 m using tapes. In each site, soil samples below five trees subjected to manure application and five control trees were taken. Three soil samples below the tree crown and near the trunk (0.5 m) were obtained and pooled in order to obtain a composite sample per tree. Soil samples were taken using a 5 cm diameter soil auger from the uppermost 20–25 cm. Soil samples were stored in plastic bags and transported to the laboratory for their analyses. Soil sampling was conducted in April 2021.

For each site, healthy, 1-year old leaves were collected from the upper third crown. This was done in the same five trees subjected to manure application and in the five control trees. Two branches per tree were sampled and 50 leaves were sampled and then pooled. Leaf sampling was conducted in late July 2021 when leaves are mature and fully expanded.

For the analyses of  $\delta^{15}\text{N}$  wood and tree-ring width, tree cores were extracted at 1.3 m from the same 10 trees of each site and species using a Pressler increment borer. Core sampling was conducted between January and April 2021. In general, two opposite cores were taken per tree separated by ca. 180°.

## 2.3. Soil Analyses

Soil samples for physicochemical analyses were air dried and sieved with a 2 mm mesh size. Soil texture was determined with a laser diffraction method in a particle analyzer (Coulter Mastersizer 2000, Malvern Panalytical, Spectris, London, UK). Soil C and N concentrations were determined with an elemental analyzer (Elementar VarioMAX N/CM, Hanau, Germany). We also measured soil pH. Soil phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) concentrations were measured by inductively coupled plasma optical emission spectrometry (ICP–OES; Thermo Elemental Iris Intrepid II XDL, Thermo Electron, Langenselbold, Germany) after a microwave-assisted digestion with  $\text{HNO}_2:\text{H}_2\text{O}_2$  (4:1, v/v).

To quantify the biomass of the main groups of soil microbiota (eukaryotes, Gram-positive and Gram-negative bacteria, actinobacteria, fungi, arbuscular mycorrhizal fungi

and anaerobic bacteria) phospholipid fatty acids (PLFA) were measured. PLFAs were extracted from 2 g of sieved and lyophilized soil, separated and methylated [20]. The resulting fatty acids methyl esters (FAMES) were separated by gas chromatography using an Agilent 7890A GC System equipped with a HP-ULTRA 2 column (length 25 m, ID 0.20 mm; J&W Scientific Inc., Agilent, Folsom, CA, USA) and a flame ionization detector. The individual FAME peaks were identified and quantified by the PLFAD1 method of Sherlock<sup>®</sup> software version 6.3 from MIDI, Inc. (Newark, NJ, USA). The internal standard 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) was used for quantification of FAMES. Total microbial biomass was estimated by summing the contents of all individual PLFAs and is presented as nanomoles of PLFAs per gram of soil [21–23].

#### 2.4. Tree Growth, Leaf and Wood Chemical Data

Leaves were oven-dried 72 h at 60 °C, milled and homogenized to a fine powder using a ball mill (Retsch ZM1, Haan, Germany). Leaf N concentrations were also analyzed with an elemental analyzer (Element Analyzer VarioMAX N/CM).

The cores were prepared following dendrochronological procedures [24]. They were air-dried and their surface was cut using a sledge microtome to distinguish tree-ring boundaries. The tree-ring widths were measured with a 0.001 mm resolution on images obtained in a scanner (Epson Expression 10000XL, Seiko Epson Co., Suwa, Japan) and using the CDendro and CooRecorder software ver. 9.3.1 (Cybis Elektronik & Data AB, Stockholm, Sweden) [25]. We validated the visual cross dating by calculating moving correlations between the individual series and the mean series of each species using the COFECHA software [26]. Tree-ring width data were converted into basal area increment assuming a concentric stem growth.

Since N may be mobile in sapwood rings [13], we pooled several adjacent rings for analyzing changes in  $\delta^{15}\text{N}$  wood. Specifically, we analyzed the last five formed rings (period 2016–2020). For each tree, the rings formed in this period were separated using scalpels, milled and homogenized using a ball mill (Retsch ZM1, Haan, Germany). Cores were not pre-treated to remove mobile N compounds (e.g., resin) since this pre-treatment does not modify significantly the final  $\delta^{15}\text{N}$  values [13]. All sampled rings were located in the sapwood.

#### 2.5. Statistical Analyses

Values of several variables (soil variables, leaf N concentrations, tree-ring width statistics, wood  $\delta^{15}\text{N}$ ) were compared between control and manure-applied trees using *t* or Dunn tests [27] in the case of variables following or not following the normal distribution, respectively.

To summarize the information of soil microbiota (PFLA), a Non-Metric Multidimensional Scaling (NMDS; [28]) was used. The Euclidean distance was selected to quantify the differences in soil microbial biomass between the two tree groups (control and manure-applied trees) and species (Aleppo pine, almond tree). PLFA data were converted into arcsin square root transformed molar percentages. The first and the second ordination axes of the NDMS (NMDS1 and NMDS2) were plotted to visualize differences between groups.

Additionally, a permutational multivariate analysis of variance (PERMANOVA) was used to test for the differences in soil microbial community structure between groups and species [28]. We calculated the sum of squares (SS) and  $R^2$  to summarize the PERMANOVA results using the following formulas:

$$SS = \frac{1}{N} \sum d_{ij}^2 \quad (1)$$

$$R^2 = 1 - \frac{SS_R}{SS_T} \quad (2)$$

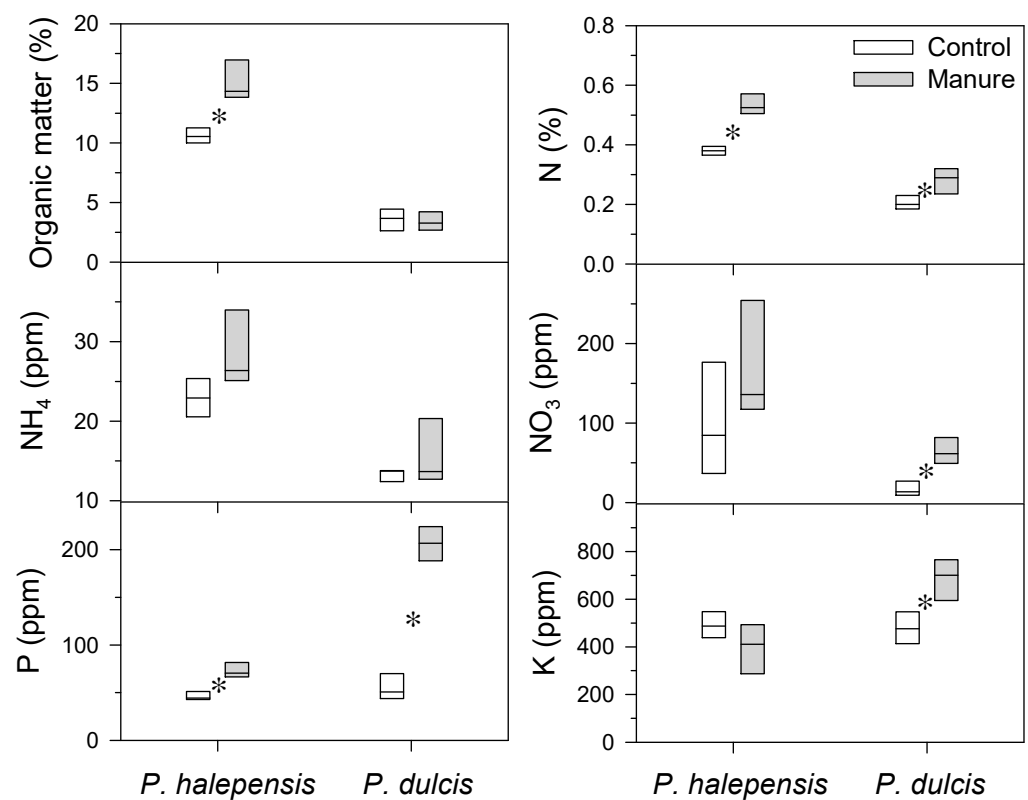
where *d* is the difference (Euclidean distances) between observations *i* and *j*, and  $SS_R$  and  $SS_T$  are the residual and total sums of squares, respectively.

Analyses were separated considering both species together but also were done separately for each species (i.e., testing for groups effect only). NMDS was calculated using the adonis function from the vegan package [29] in the R software ver. 4.1.3 [30].

### 3. Results

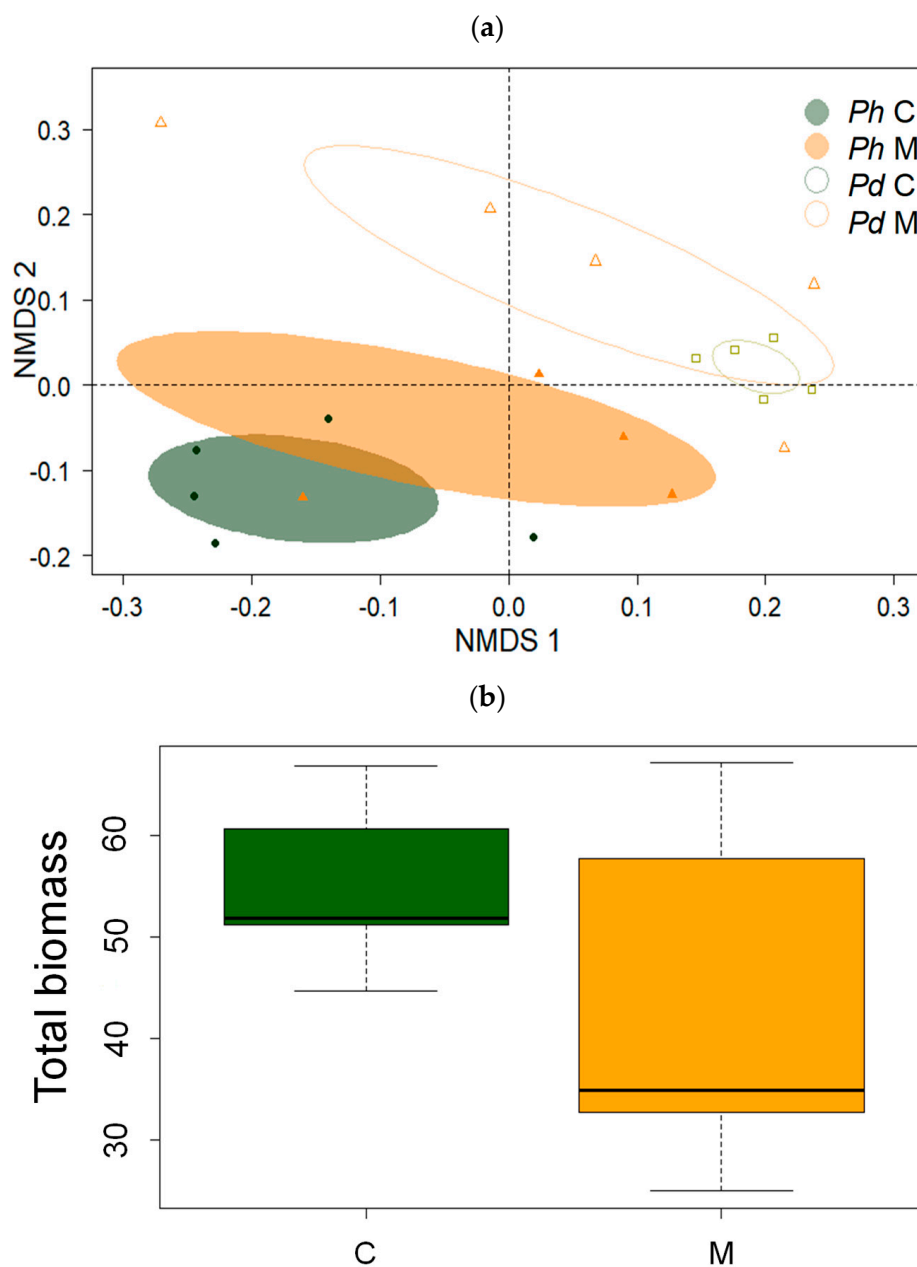
#### 3.1. Soils

In the case of Aleppo pine, soils of manure-applied trees presented significantly higher percentages of organic matter ( $t = 5.10$ ,  $p = 0.005$ ) and N ( $t = 7.98$ ,  $p = 0.002$ ) and higher P concentrations ( $t = 5.21$ ,  $p = 0.006$ ) than control trees (Figure 1). In the case of almond trees, soils of manure-applied trees also presented higher N ( $t = 2.89$ ,  $p = 0.007$ ) and P ( $t = 2.95$ ,  $p = 0.005$ ) concentrations and higher  $\text{NO}_3$  ( $t = 1.72$ ,  $p = 0.017$ ) and K ( $t = 2.76$ ,  $p = 0.009$ ) concentrations (Figure 1). No differences in soil pH were observed as a function of pig manure application, albeit soil pH was significantly lower ( $t = -7.25$ ,  $p < 0.001$ ) in the pine stand (mean  $\pm$  SD =  $7.75 \pm 0.08$ ) than in the almond trees stand ( $8.14 \pm 0.14$ ).



**Figure 1.** Differences between control (empty box-plots) and manure-applied (filled box-plots) trees of the two study species in soil chemical characteristics. Asterisks indicate significant ( $p < 0.05$ ) differences between groups according to  $t$  tests.

The NMDS stress value was 0.015. The NMDS displayed differences between soils of pines and almond trees, but no evident differences between control and manure-applied soils (Figure 2a). The PERMANOVA results (SS = 0.87; df = 16,  $R^2 = 0.57$ ) confirmed this pattern and the existence of significant differences between the two tree species (SS = 0.45;  $F = 0.29$ ,  $R^2 = 0.29$ ,  $p < 0.01$ ) but not between treatments (SS = 0.08;  $F = 1.45$ ,  $R^2 = 0.05$ ). Marginally significant differences were found for the interaction between trees and treatment (SS = 0.13;  $F = 2.41$ ,  $R^2 = 0.09$ ). When comparing almond trees, marginally significant differences in soil microbial composition were found (SS = 0.12; df = 8;  $F = 2.64$ ,  $R^2 = 0.25$ ,  $p = 0.05$ ), while no differences were found between the two pine groups (SS = 0.09; df = 8;  $F = 1.39$ ,  $R^2 = 0.15$ ).



**Figure 2.** Comparisons of soil microbiota features between control (C) and trees subjected to manure application (M) in *P. halepensis* (*Ph*) and *P. dulcis* (*Pd*). (a) Multivariate analysis showing the biplot of scores of a non-metric multi-dimensional scaling (NMDS). The scores correspond to the first (NMDS1) and second (NMDS2) axes and are represented by different symbols (*Ph*C—filled black circles; *Ph*M—filled orange triangles; *Pd*C—empty squares; *Pd*M—empty orange triangles). Filled and empty ellipses group samples corresponding to *P. halepensis* (*Ph*) and *P. dulcis* (*Pd*), respectively. Green and orange colors indicate C and M trees. (b) Soil biomass values (y axes units are nanomoles of PLFAs per gram of soil) in *P. dulcis* showing higher values in control trees (see Table 2).

Significantly higher values of soil microbiota biomass were found in soils of control trees as compared with manure-applied soils in the case of the almond trees (Table 2, Figure 2b). The biomass of fungi, arbuscular mycorrhizal fungi and bacteria was also higher in control almond trees, but the biomass of actinomycetes was higher in soils from manure-applied almond trees. In the case of Aleppo pine no significant differences in soil microbiota between the two types of soils were found.



**Table 2.** Comparison of soil features between control and manure trees of the two study species based on the biomass data of the main microbiota groups. Statistics show the values of Dunn tests and their associated probability level. Significant ( $p < 0.05$ ) differences are indicated with values in bold characters. AM Fungi are arbuscular mycorrhizal fungi.

Variable	<i>P. halepensis</i>		<i>P. dulcis</i>	
	Dunn	<i>p</i>	Dunn	<i>p</i>
Biomass	−0.940	0.347	<b>2.611</b>	<b>0.009</b>
Eukaryotes	−0.731	0.465	−0.522	0.602
Gram − bacteria	1.149	0.251	−1.149	0.251
Gram + bacteria	0.104	0.917	−1.358	0.175
Actinomycetes	1.358	0.175	<b>−2.611</b>	<b>0.009</b>
Total Gram + bacteria	−0.104	0.917	<b>2.193</b>	<b>0.028</b>
Total Bacteria	−1.358	0.175	<b>1.984</b>	<b>0.047</b>
Fungi	−0.940	0.347	<b>2.611</b>	<b>0.009</b>
AM Fungi	−0.104	0.917	<b>1.984</b>	<b>0.047</b>
Total Fungi	−1.149	0.251	0.940	0.347

### 3.2. Leaves

In both tree species, the N concentration of leaves from control trees were significantly higher than those in trees subjected to manure application (*P. halepensis*,  $t = 9.47$ ; *P. dulcis*,  $t = 5.90$ ,  $p = 0.008$  in both cases). In *P. halepensis* leaf N concentrations (mean  $\pm$  SD) were  $1.80 \pm 0.23\%$  vs.  $0.79 \pm 0.06\%$ , whereas in *P. dulcis* they were  $3.40 \pm 0.17\%$  vs.  $2.55 \pm 0.13\%$  in the case of control and manure-applied trees, respectively.

### 3.3. Tree Growth

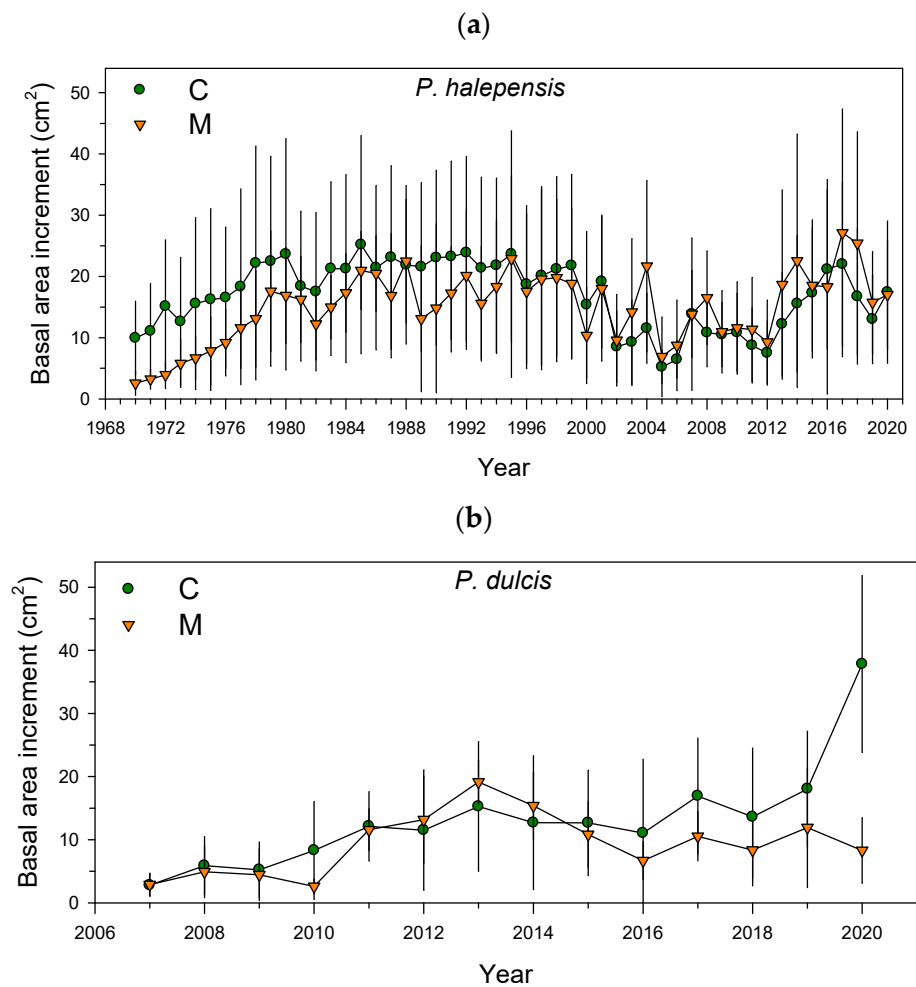
In both species, growth rates (tree-ring widths) and the other tree-ring statistics were similar between control and manure-applied trees (Table 3). However, control individuals presented a significantly higher basal area increment in 2020 ( $t = 38.0$ ,  $p = 0.004$ ) than manure-applied individuals in the case of the almond trees (Figure 3b).

**Table 3.** Tree-ring width data and related variables. Abbreviations: AR1, first-order autocorrelation; MSx, mean sensitivity; Corr, mean correlation with the mean site series. Values are means  $\pm$  SD.

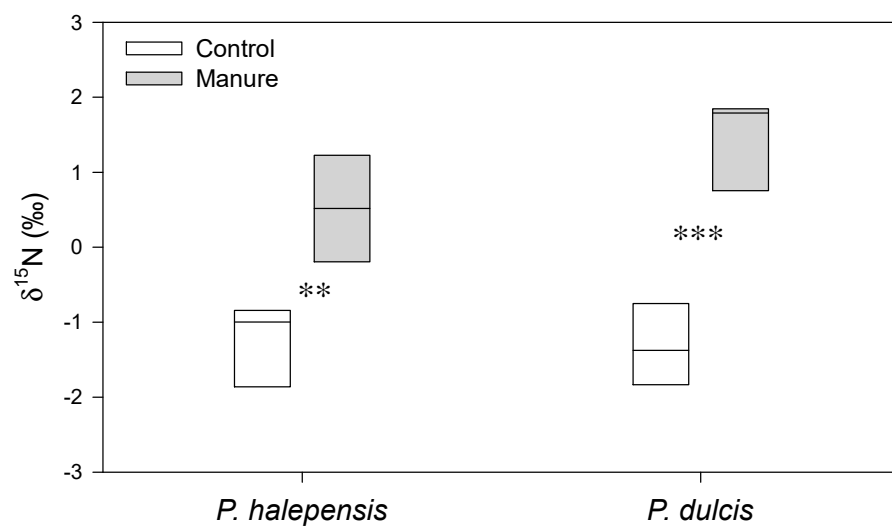
Tree Species	Tree Type	No. Trees (No. Cores)	Period	Tree-Ring Width (mm)	AR1	MSx	Corr
<i>P. halepensis</i>	Control	5 (10)	1970–2020	$3.11 \pm 0.87$	$0.87 \pm 0.08$	$0.28 \pm 0.05$	$0.46 \pm 0.11$
	Manure	5 (10)	1970–2020	$3.11 \pm 0.80$	$0.77 \pm 0.11$	$0.39 \pm 0.09$	$0.59 \pm 0.15$
<i>P. dulcis</i>	Control	5 (10)	2007–2020	$5.92 \pm 1.38$	$0.47 \pm 0.24$	$0.54 \pm 0.17$	$0.60 \pm 0.25$
	Manure	5 (10)	2007–2020	$5.52 \pm 0.67$	$0.44 \pm 0.23$	$0.55 \pm 0.12$	$0.64 \pm 0.19$

### 3.4. Wood N Concentrations and $\delta^{15}\text{N}$ Values

Neither in the Aleppo pines nor in the almond trees were differences in wood N concentration between control and manure-applied trees found, with mean values of 0.07% and 0.14% (SD was 0.01% in both cases), respectively. In both species, the wood of manure-applied trees had higher  $\delta^{15}\text{N}$  values than the wood of control trees (Figure 4).



**Figure 3.** Tree growth (basal area increment) in control (C, green circles) and manure-applied (M, orange triangles) trees of the two study species: (a) *P. halepensis*; (b) *P. dulcis*. Values are means  $\pm$  SD.



**Figure 4.** Values of  $\delta^{15}\text{N}$  in tree-ring wood of control (empty bars) and manure-applied (grey bars) trees of the two study species. The asterisks indicate significant differences between the two groups of trees in each species according to Dunn tests (*P. halepensis*, \*\*  $p < 0.01$ ; *P. dulcis*, \*\*\*  $p < 0.001$ ).



#### 4. Discussion

Pig effluent and manure are important sources of organic matter, N and other nutrients such as P and K [11]. That explains why higher N and P concentrations were found in the soils subjected to pig manure application of both tree species as expected. In some cases, differences were found only in one species with higher concentrations of some components in manure-applied soils (organic matter in Aleppo pine,  $\text{NO}_3$  and K in almond trees). This indicates local differences in soil characteristics and/or potential soil–tree interactions. In addition,  $\text{NO}_3$  and  $\text{NH}_4$  transformation and leaching are fast; thus, their concentrations could be influenced by rainfall seasonality or different irrigation regimes after manure application [6].

Regarding the soil microbiota main groups, we only found a higher biomass in the case of soils sampled below control almond trees which have higher relative abundances of fungi, arbuscular mycorrhizal fungi and bacteria (Gram negative plus Gram positive bacteria, excluding actinobacteria) than manure-applied soils. In contrast, the relative abundance of actinobacteria was higher in the fertilized soils of this species. Soil bacterial groups that are generally considered to be copiotrophic, such as actinobacteria, tend to increase in relative abundance with the addition of labile organic matter or N [31,32]. Conversely, N addition to soils frequently depletes the relative abundance of oligotrophic bacteria, like acidobacteria, planctomycetes and Verrucomicrobia [33–36]. Therefore, as a consequence of a higher N availability, the lower relative abundance of bacteria in soils of manure-applied almond trees might have been due to the decay of oligotrophic bacterial taxa, but a methodological approach with higher taxonomic resolution than PLFAs profiling (e.g., metagenomic 16S amplicon sequencing) would be needed to completely elucidate this issue. In accordance with our results, it has been recently shown that pig manure application tends to increase the abundance of copiotrophic bacterial groups, while decreasing the abundance of oligotrophic bacterial groups [37]. It is also important to note that in the case of almond trees multivariate analyses suggested the existence of differences in the structure of the soil microbial community between the control and the manure applied orchards (Figure 3; PERMANOVA analyses), which agrees with results of other studies on the effects of pig manure on soil microbiota [37–40]. The higher N, P and K concentrations in soils of manure-applied almond trees could explain the abundance of actinomycetes, which are considered biofertilizers associated with improved growth and higher N availability [41,42]. The higher biomass of arbuscular mycorrhizal fungi would improve the capacity to uptake water and nutrients (e.g., P) of almond trees not subjected to manure application, enhancing their long-term growth rate and productivity [43–45].

Our findings indicate that the soil microbiome was probably altered through nutrient addition [46]. It is unclear what the consequences of such alterations are, but they point to a transition from C demanding organisms to N consuming ones as a consequence of fertilizer addition. Further studies should be carried out including more samples but also including formerly agricultural areas recolonized by shrubs and trees. This will provide an interesting picture on how temporal changes in manure addition (i.e., livestock grazing vs. mechanic) affect soil microbiome and plant-soil interactions.

In the case of almond trees, multivariate analyses suggested the existence of differences between control and manure applied microbiota (Figure 3, PERMANOVA analyses). The fact that no differences were observed in the case of pines soils may be explained because they were growing near a steep slope, and their roots may be uptaking deeper N sources rather than N inputs derived from pig manure applied to shallow soils.

We did not find higher radial growth rates in manure-applied trees, but rather the opposite in the case of control almond trees which grew more than manure-applied trees and also presented higher N concentrations in leaves. Manure from pigs has been shown to increase the concentration of N and K in leaves and the productivity in plantations of fast-growing tree species such as willows, poplars and eucalyptus [47,48]. In some cases, pig slurry increased the growth of fertilized plantations but also lead to rising soil levels of  $\text{NO}_3$ , copper and zinc as slurry amounts increased [49].

In the study Mediterranean sites, where tree radial growth is mainly controlled by water availability from winter to spring [50], the application of pig manure did not increase growth and stem wood production. The fertilization effect of N input on tree growth is modulated by climatic conditions and physiological stress caused by water deficit may cancel out the expected tree growth enhancement [51]. Increased N availability can impair tree water balance through the reduction of C allocation to fine roots and mycorrhizal fungi, thus reducing water uptake capacity [52]. In fact, the higher recent growth rates of control almond trees could be caused by long-term soil pollution or redundant N compounds in manure-applied individuals presenting lower wood productivity. Perhaps further irrigation combined with manure applications could enhance the radial growth rate of almond trees.

As expected, wood  $\delta^{15}\text{N}$  concentrations were higher in manure-applied trees indicating increased N supply relative to demand [16], which could be related to the lower biomass of arbuscular mycorrhizal fungi observed in the soils of manure-applied almond trees. In agreement with our findings, wood  $\delta^{15}\text{N}$  also increased in forests subjected to fertilization treatments (N and NPKCa), whereas radial growth did not change significantly [53].

The small sample size of this study prevents us from drawing robust conclusions. To achieve stronger conclusions, it is advisable to expand this research, considering further sites and tree species and sampling a higher number of trees (at least 10 per species) in each treatment type. Nevertheless, the fact that we obtained similar findings (increases in wood  $\delta^{15}\text{N}$ ) with two functionally contrasting types of trees (evergreen conifer vs. deciduous hardwood) provides some support to our results.

## 5. Conclusions

Higher  $\delta^{15}\text{N}$  values were found in the tree-ring wood of manure-applied trees of two tree species (Aleppo pine, almond tree), indicating increased N supply relative to demand, despite this not translating into growth enhancement. By contrast, control almond trees grew more than manure-applied trees. Trees subjected to manure application formed leaves with lower N concentrations despite their soils being more fertile and containing higher N and P concentrations than non-fertilized control soils.

Our results are preliminary and would be more robust if based on more sites, species and trees, but they provide a first step to assess the long-term effects of intensive farming and related manure application on tree growth and productivity.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f14010008/s1>, Figure S1, (a) Location of the study site (El Bayo) in the “Cinco Villas” region, Aragón, north-eastern Spain. (b) View of the sampled stand of almond trees.

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