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nir gene-based co-occurrence patterns reveal assembly mechanisms of soil denitrifiers in response to fire

Marta Goberna,[1*] **Santiago Donat**,[2] **Eduardo Pérez-Valera**,[2,3] **Sara Hallin**[4] **and Miguel Verdú**[2]

[1]*Department of Environment and Agronomy, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain.*

[2]*Department of Ecology, Centro de Investigaciones sobre Desertificación (CIDE - CSIC), Valencia, Spain.*

[3]*Biology Centre of the Czech Academy of Sciences, Institute of Soil Biology, České Budějovice, Czech Republic.*

[4]*Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden.*

Summary

Denitrification causes nitrogen losses from terrestrial ecosystems. The magnitude of nitrogen loss depends on the prevalence of denitrifiers, which show ecological differences if they harbour *nirS* or *nirK* genes encoding nitrite reductases with the same biological function. Thus, it is relevant to understand the mechanisms of co-existence of denitrifiers, including their response to environmental filters and competition due to niche similarities. We propose a framework to analyse the co-existence of denitrifiers across multiple assemblages by using *nir* gene-based co-occurrence networks. We applied it in Mediterranean soils before and during 1 year after an experimental fire. Burning did not modify *nir* community structure, but significantly impacted co-occurrence patterns. Bacteria with the same *nir* co-occurred in space, and those with different *nir* excluded each other, reflecting niche requirements: *nirS* abundance responded to nitrate and salinity, whereas *nirK* to iron content. Prior to fire, mutual exclusion between bacteria with the same *nir* suggested competition due to niche similarities. Burning provoked an immediate rise in mineral nitrogen and erased the signals of competition, which emerged again within days as *nir* abundances peaked. *nir* co-occurrence patterns can help infer the assembly mechanisms of denitrifying communities, which control nitrogen losses in the face of ecological disturbance.

Received 15 July, 2020; revised 23 October, 2020; accepted 26 October, 2020. **For correspondence. E-mail marta.goberna@inia.es; Tel. (+34) 913476752; Fax (+34) 913572293.

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Introduction

Denitrification, the reduction of nitrate to gaseous N compounds, is an essential microbial process that accounts for the majority of nitrogen (N) losses from terrestrial ecosystems to the atmosphere (Canfield *et al*., 2010). Further, denitrification is the main source of nitrous oxide, a potent greenhouse gas, emitted from soil (Syakila and Kroeze, 2011; Hu *et al*., 2015). Dentrification is found among a diverse range of microorganisms with different genetic make-up (Jones *et al*., 2008; Graf *et al*., 2014). Owing to their impact on essential ecosystem processes like primary production and decomposition by reducing the amount of N available as well as on climate change, it is paramount to discern the assembly mechanisms of denitrifying communities.

Competition by limiting similarity, i.e. the classical Darwinian competition, can be thought of as the main mechanism controlling the assembly of communities that compete for the same resource. However, the reduction of nitrite to nitric oxide in the denitrification pathway is catalysed by two types of nitrite reductases, one with iron as cofactor and encoded by the *nirS* gene, and the other using copper and encoded by *nirK*. Both enzymes are thought to be mutually exclusive, as 99% of all known *nir*-possessing denitrifiers either have *nirS* or *nirK* genes in its genome (Graf *et al*., 2014). Denitrifiers with different *nir* types show ecological differences in at least two features that are relevant for community assembly. First, bacteria carrying *nirS* or *nirK* respond to different abiotic factors (Smith and Ogram, 2008; Enwall *et al*., 2010; Jones and Hallin, 2010; Bru *et al*., 2011). Second, enzymatic studies suggest that NirS has higher affinity for nitrite than NirK (Rinaldo and Cutruzzolà, 2007; Rinaldo *et al*., 2017). The biosynthesis of NirK is less costly (Van Lis *et al*., 2011) and it depends on either only the *nirK* gene or in some cases also an accessory gene (Zumft, 1997). This suggests that organisms with either *nir* type may have different fitness advantages depending

on the environmental conditions, although expression patterns are inconclusive (Wittorf *et al*., 2018). Altogether, these observations indicate that several ecological assembly processes, i.e. abiotic filtering, competition based on limiting similarity or on relative fitness differences, might simultaneously structure denitrifier communities (Chesson, 2000; Jones and Hallin, 2010; HilleRisLambers *et al*., 2012). We propose that the concomitant effects of assembly mechanisms can be inferred based on the analysis of functional co-occurrence networks (Zhou *et al*., 2010; Jones *et al*., 2014).

Co-occurrence networks allow detection of microorganisms that co-occur more or less frequently than expected by chance across multiple soil assemblages. Positive and negative associations between pairs of community members can be quantified based on correlation analyses and significance tested against a null model (Faust and Raes, 2012). Patterns of co-presence (positive associations) and mutual exclusion (negative associations) can be interpreted in terms of either niche preferences or ecological interactions (Faust and Raes, 2012; Barberán *et al*., 2012; Pascual-García *et al*., 2014; Jones and Hallin, 2019). We have previously shown that the phylogenetic analysis of bacteria that co-occur or mutually exclude each other based on 16S rRNA gene networks allows discerning among assembly processes that shape soil bacterial communities (Pérez-Valera *et al*., 2017; Goberna *et al*., 2019). In *nir* gene-based co-occurrence networks (hereinafter '*nir*-based networks'), the co-presence of microorganisms bearing the same *nir* variant (i.e. *nirS-nirS* or *nirK-nirK* links) can be interpreted as the result of environmental filtering (upper left panel in Fig. 1) since this process favours the co-existence of organisms with similar environmental preferences. Environmental filters, according to Mayfield and Levine 2010, can be the result of (i) abiotic factors that benefit the organisms most tolerant to the prevailing conditions, and/or (ii) environmentally mediated relative fitness differences that promote organisms with superior competitive abilities. With microbes carrying different *nir* variants (i.e. *nirS-nirK* links), the co-presence can result from competition based on niche similarities (bottom left panel in Fig. 1), since it precludes the co-existence of organisms that are ecologically similar (HilleRisLambers *et al*., 2012; Russel *et al*., 2017). For the same reasons, mutual exclusion between *nirS-nirS* or *nirK-nirK* pairs suggests competition by limiting similarity (bottom right panel in Fig. 1), whereas that between *nirS-nirK* would be interpreted as environmental filtering (upper right panel in Fig. 1). While the interpretation of mutual exclusion links mirrors that of co-presence links, it adds evidence on the mechanisms of microbial co-existence since two non-coexisting species do not necessarily exclude each other.

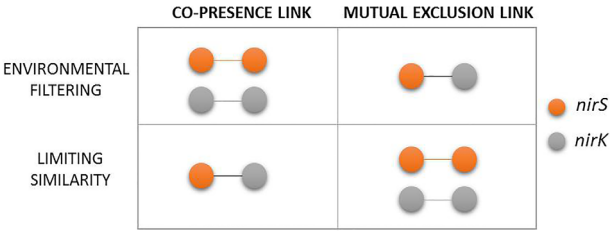

Fig 1. Expected outcome of community assembly processes in co-occurrence networks of denitrifiers. Spheres with different colours represent microorganisms with different nitrite reductase (nir) variants (orange, nirS; grey, nirK). Solid lines joining two spheres represent significant links between a pair of microorganisms. [Color figure can be viewed at wileyonlinelibrary.com]

As a model system, we used a Mediterranean ecosystem in which N is a limiting resource (Hooper and Johnson, 1999), thus setting the conditions for competition to occur. We analysed *nirS*- and *nirK*-possessing soil bacteria under natural conditions and after exposure to an experimental fire, which causes a burst in mineral N in the soil (Certini, 2005; Goberna *et al*., 2012). By changing resource availability, burning alters the activity of N-cycling enzymes (Pérez-Valera *et al*., 2019) and soil denitrifiers (Andersson *et al*., 2004) and thus likely the strength of competition. Other soil conditions, such as pH or the concentration of trace elements, also change after fire (Certini, 2005). We monitored post-fire shifts in soil abiotic factors, 16S rRNA, *nirK* and *nirS* gene copy numbers, and sequenced both *nir* genes to characterize changes in denitrifier community structure, diversity and *nir*-based networks. We hypothesized that environmental filtering is the dominant force determining the prevalence of positive links between bacteria bearing the same *nir* variant (*nirS-nirS* and/or *nirK-nirK*) and negative links between bacteria with different *nir* variants (*nirS-nirK*) (Fig. 1). Our objectives were (i) to unravel the processes that structure soil denitrifying communities by separately analysing co-presence and mutual exclusion links following the framework detailed above and (ii) to test how changes in resource availability (e.g. carbon, nitrate, micronutrients) and abiotic conditions (e.g. pH, salinity) in response to the experimental fire alter the balance of forces shaping the co-existence between soil denitrifiers.

Results

Soil properties

Fire imposed significant shifts in several soil abiotic factors relevant to denitrifiers (Fig. 2; Supporting Information Fig. SI1). Nutrient contents peaked during the first week after fire and reestablished at prefire levels after 1 month (Fig. 2). In particular, we detected sharp increases in nitrate and ammonium contents, together with other macro- (phosphorous and potassium) and micronutrients, such as copper, boron and calcium (Fig. 2). The experimental fire

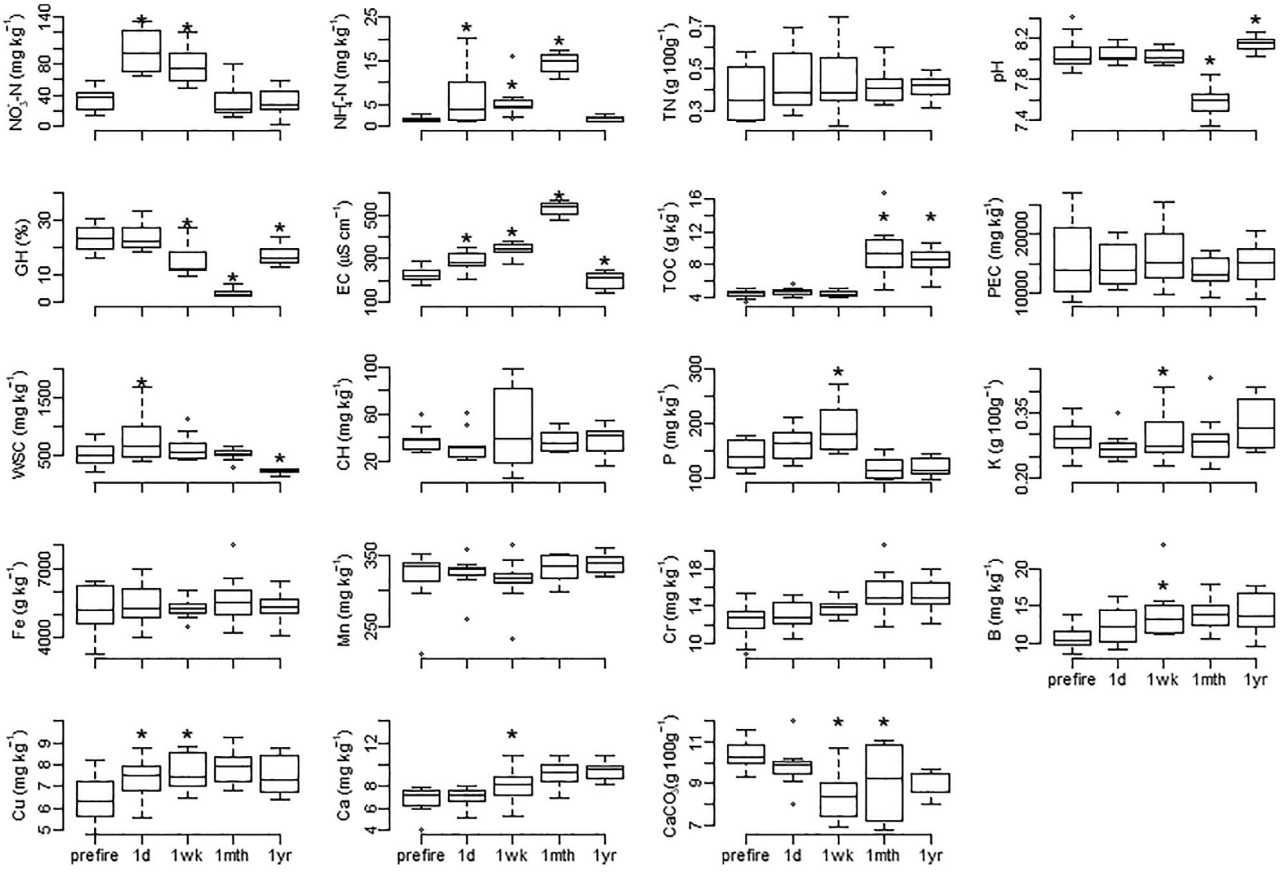

Fig 2. Soil abiotic factors (expressed in DW) before and during 1 year after an experimental fire. Asterisks indicate significant differences of each variable and sampling time compared to prefire levels, after accounting for the effects of temperature and rainfall (see text for statistical details). CH (Water Soluble Carbohydrates), EC (Salinity), GH (Gravimetric Humidity), PEC (Pyrophosphate Extractable Carbon), TN (Total Nitrogen), TOC (Total Organic Carbon), WSC (Water Soluble C). Part of these analyses were published in the study by Pérez-Valera *et al*. (2017).

significantly reduced soil moisture (GH) and pH after 1 month, while increasing total organic carbon (TOC) and electrical conductivity (EC, which is a measure of the amount of salts in the soil solution and hereafter referred to as salinity). Prefire levels of GH, pH and TOC were not restored during 1-year period of monitoring after the fire (Fig. 2).

Abundance and community structure of denitrifiers with different nir types

The abundance of 16S rRNA, *nirS* and *nirK* gene copy numbers peaked 1 week after fire, and prefire levels were restored after 1 month (Fig. 3). Specifically, 16S rRNA gene copies increased from $0.35 \times 10^{10} \pm 1.0 \times 10^{9}$ (mean ± SE) gene copy numbers per gram soil [dry weight (DW)] prior to fire to $1.2 \times 10^{10} \pm 5.4 \times 10^{9}$ gene copies g^{-1} DW 1 week after fire. During the same period, *nirS* increased from $2.9 \times 10^{7} \pm 5.5 \times 10^{6}$ to $5.0 \times 10^{7} \pm 7.3 \times 10^{6}$ gene copies g^{-1} DW and *nirK* from $3.7 \times 10^{8} \pm 6.6 \times 10^{7}$ to $4.7 \times 10^{8} \pm 5.1 \times 10^{7}$ gene copies g^{-1} DW.

The abundance of *nirS* and *nirK* genes responded to different soil abiotic factors, according to generalized linear mixed models in which the sampling time was included as a random factor. Figure 4 shows the post-mean estimates and expected 95% credible intervals of the fixed factors for the best-fit models (see description of model selection in Experimental Procedures). Results indicated that *nirS* gene copies were significantly explained by the variation in nitrate content and salinity (i.e. their 95% credible intervals did not cross zero), whereas *nirK* abundance significantly responded to iron content.

The *nirK*/*nirS* abundance ratio, which originally averaged 14.4 ± 1.6, dropped significantly 1 day after fire to 10.2 ± 1.1, but displayed prefire levels after 1 month (Supporting Information Fig. SI2). The *nirK*/*nirS* ratio responded positively to iron and negatively to chromium (Fig. 4). The number of *nirS* and *nirK* gene copies constituted 0.97 ± 0.14% and 13.2 ± 1.8% (mean ± SD), respectively of the bacterial 16S rRNA gene copy numbers under prefire conditions (Supporting Information Fig. SI2). The relative abundance

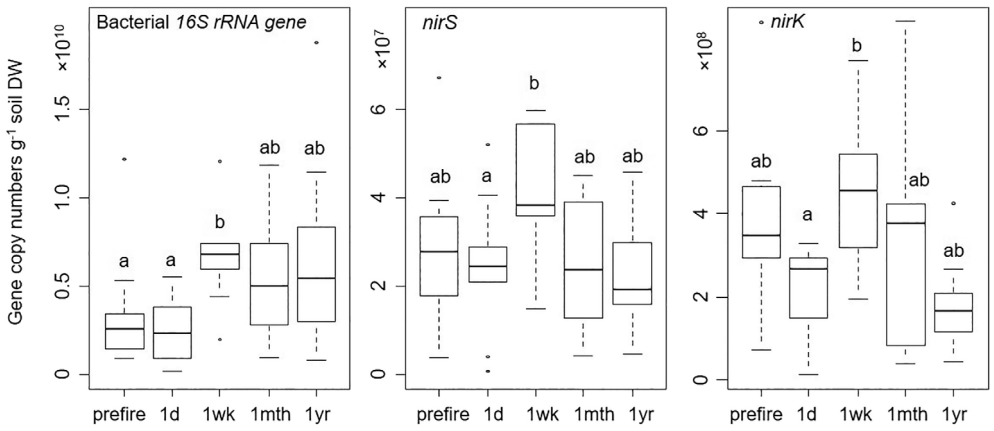

Fig 3. Number of copies of the 16S rRNA, nirK and nirS genes (expressed per g soil DW) before and at different time points during 1 year after an experimental fire. Different letters denote statistically significant differences, after accounting for the effects of temperature and rainfall.

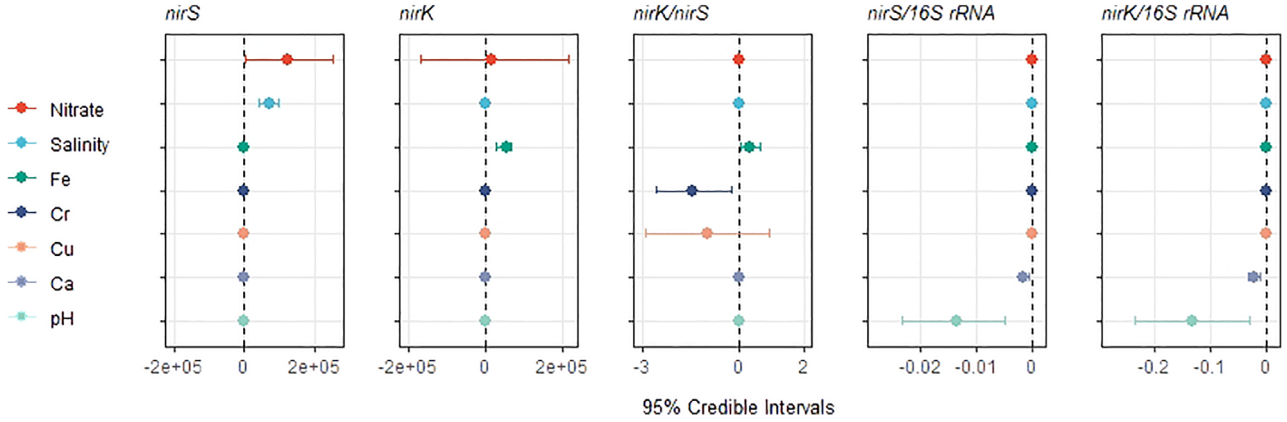

Fig 4. Bayesian post-mean estimates (and their expected 95% credible intervals) of the best statistical models explaining the effect of soil abiotic factors on the number of nir gene copies and ratios. Factors with intervals not including zero are significant. [Color figure can be viewed at wileyonlinelibrary.com]

of both nitrite reductase genes increased significantly at lower levels of soil pH and calcium contents (Fig. 4). However, they did not vary significantly over the study period (Supporting Information Fig. SI2).

The *nirS* sequences in prefire communities were assigned with higher probability to *nirS* in *Magnetospirillum* (51 ± 14%), *Cupriavidus* (27 ± 13%) and *Polymorphum* (13 ± 9%) (Fig. 5). The remaining 9% corresponded to bacteria assigned to *nirS* in six other genera, namely, *Labrenzia*, *Ruegeria*, *Pseudogulbenkiania*, *Acidovorax*, *Pseudomonas* and *Rhodobacter*. The prefire community was dominated by bacteria whose *nirK* was most closely related to that in *Mesorhizobium* (48 ± 5%) and *Rhodopseudomonas* (11 ± 2%), and organisms assigned to 13 other genera, including *Chelativorans*, *Sinorhizobium* and *Bradyrhizobium* (Fig. 5). Fire did not induce statistically significant fluctuations in the relative abundance of the dominant genera (Fig. 5). Likewise, we did not detect any significant changes in the community structure of either *nir* type of denitrifier due to fire (all PERMANOVAs, $F \leq 1.6$, $P > 0.07$, $R^2 \leq 0.14$). Alphadiversity (Shannon's index) for *nirS* averaged 3.3 ± 0.5 under pre-fire conditions and increased temporarily to 4.3 ± 0.1 1 week after fire, while for *nirK* it was 4.4 ± 0.1 and did not differ significantly across sampling times (Supporting Information Fig. SI3). For both *nir* genes, betadiversity mainly originated from a high taxon turnover across plots (≥99.94% of betadiversity values), while nestedness was negligible throughout the study (Supporting Information Table SI1).

Detection of co-present and mutually excluding denitrifiers

nir gene-based co-occurrence network analysis detected 2587 positive (co-presence) links and 1376 negative (mutual exclusion) links between denitrifiers considering all sampling times (Supporting Information Table SI2). Thus, the majority (65 ± 4%) of links detected were positive regardless of fire. Among these co-presence links, 70–80% occurred within the same *nir* variant across all sampling times (Fig. 6A and B, left panel). Under prefire-

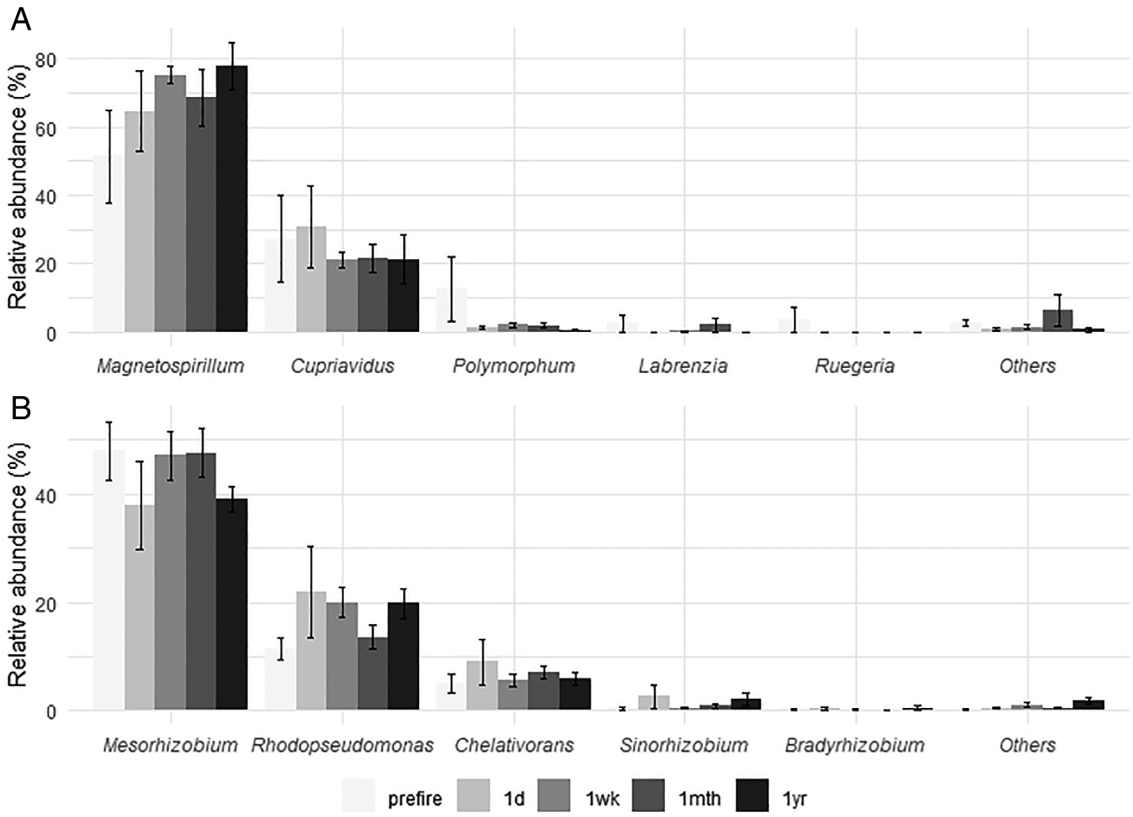

Fig 5. Taxonomic distribution of (A) nirS- and (B) nirK-carrying bacteria before and during 1 year after an experimental fire. Error bars indicate standard errors.

conditions, positive *nirS-nirS* links prevailed (Fig. 6A and B, left panel), mainly between OTUs most closely related to the same genus (*Magnetospirillum* in almost half of all positive links; Supporting Information Table SI2). Positive *nirS* links between OTUs related to different genera were mostly *Magnetospirillum-Cupriavidus* pairs (Supporting Information Table SI2). Fire reverted this pattern, and co-presence *nirK-nirK* links dominated over *nirS-nirS* immediately after fire (Fig. 6A and B, left panel). Positive *nirK-nirK* links were detected to be more frequent than expected by chance 1 day and 1 week after fire between OTUs assigned to the same genus in 40%–47% of all cases (mainly *Mesorhizobium*), and pairs involving *nirK* in *Mesorhizobium* and *Rhodopseudomonas* (Supporting Information Table SI2).

Mutual exclusion links between the two *nir* variants (i.e. negative *nirS-nirK* links) were more frequent than expected by chance irrespective of fire (Fig. 6A and B, right panel). Most of these negative links occurred between OTUs most closely related to *Magnetospirillum* and either *Mesorhizobium* or *Rhodopseudomonas* (Supporting Information Table SI2). Negative *nirS-nirK* links were not explained by the dominance of any of the two forms, as bacteria carrying *nirS* excluded those with *nirK* in 397 ± 122 cases and the opposite occurred in 408 ± 118 cases, considering all sampling times ($\chi^2 > 0.01$, df = 1, $P > 0.2$). We also detected mutual exclusion links that occurred between the same *nir* variant more often than expected at random. Under prefire conditions, we found a dominance of negative *nirK-nirK* links (Fig. 6A and B, right panel) that occurred between OTUs related to *nirK* in the same genus (*Mesorhizobium*, 15% of all negative links) and between different genera (34%) (Supporting Information Table SI2). Such mutual exclusion between *nirK* forms was lost immediately after fire and replaced by negative *nirS-nirS* links, which were more frequent than expected at random. This was detected exclusively 1 week after fire (Fig. 6B, right panel) and coincided with the increase in *nirS* gene copy numbers (Fig. 3). Negative *nirS-nirS* links mainly involved OTUs related to the genus *Magnetospirillum* (26%; Table SI2).

Discussion

nir-gene based co-occurrence patterns revealed that denitrifying bacteria bearing the same *nir* variant in their respective genomes appeared aggregated over multiple soil assemblages, suggesting ecological similarities. In

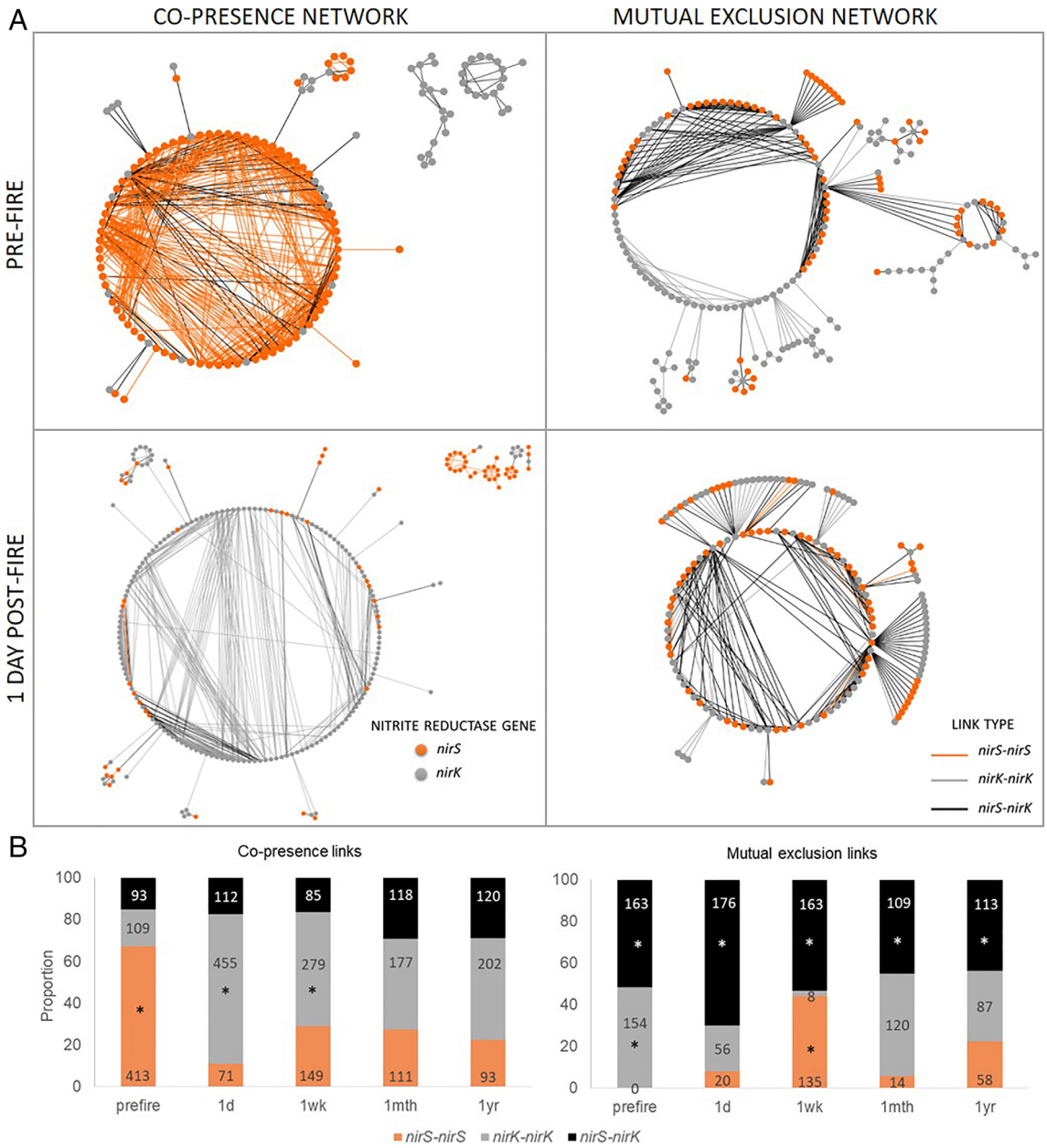

Fig 6. (A) Nitrite reductase (nir) co-occurrence networks. Co-presence (left panel) and mutual exclusion networks (right panel) are shown for pre- and post-fire (1 day) conditions for illustrative purposes. Network nodes indicating OTUs are depicted as spheres (orange, nirS; grey, nirK) and links as solid lines connecting nodes (orange, nirS-nirS; grey, nirK-nirK; black, nirS-nirK links). All networks are given in Supporting Information Fig. SI4. B. Proportion of co-presence (left panel) and mutual exclusion links (right panel) detected for the pairs nirS-nirS, nirK-nirK and nirS-nirK before and at different time points during 1 year after an experimental fire. The number of links is shown for each category. Asterisks indicate that observed frequencies of each link type are larger than expected by chance. [Color figure can be viewed at wileyonlinelibrary.com]

contrast, bacteria with different *nir* variants tended to mutually exclude each other. Consistent with our hypothesis, these results suggest environmental filtering as a major structuring force of soil denitrifier communities. Environmental filters were most likely abiotic, rather than biotic, irrespective of fire, as discussed below.

The abundance of denitrifiers with different *nir* types responded to distinct soil abiotic factors. While *nirS* type denitrifiers were explained by soil salinity and nitrate content, *nirK* types were exclusively responsive to iron content agreement with earlier work (Enwall *et al*., 2010; Jones and Hallin, 2010; Bru *et al*., 2011; Yuan *et al*., 2012). These studies, which were carried out at broader

geographic scales, highlighted other abiotic factors as relevant in explaining the variation of *nirS* and *nirK* genes, including pH, Cu, Ca, Mn, Cr or B content (Enwall *et al*., 2010; Jones and Hallin, 2010; Bru *et al*., 2011). In our 150 m^2 study area, Cr negatively impacted the *nirK*/*nirS* ratio. In agreement, Hu *et al*. (2019) showed that the relative expression of *nirK* dominated under no-Cr conditions, while that of *nirS* increased after the addition of Cr(VI). The differential response to abiotic factors by *nirK* and *nirS* denitrifiers suggests that *nir* gene-based co-occurrence patterns responded to a great extent to abiotic filtering. That is to say, shared abiotic preferences led to the co-presence of ecologically similar organisms in similar habitats, and unshared preferences of dissimilar organisms were the basis of their mutual exclusion (Webb *et al*., 2002). In addition, betadiversity of *nirK* and *nirS* communities was mainly explained by a high spatial turnover of taxa across plots, rather than a high nestedness which would indicate an orderly species loss in poorer compared to richer communities (Baselga, 2010). This observation, although not being a direct evidence, might indicate environmental filtering due to local abiotic conditions (Soininen *et al*., 2018). Our results add up to previous studies suggesting that abiotic filtering is the prevailing assembly mechanism of bacterial communities (Pascual-García *et al*., 2014; Goberna *et al*., 2019).

We did not find any evidence for biotic filtering, which refers to competition caused by differences in competitive ability (Mayfield and Levine, 2010). Biotic filtering is thought to be the basis of the widespread co-existence in soils of closely related heterotrophic bacteria, particularly under carbon enriched conditions (Goldfarb *et al*., 2011; Goberna *et al*., 2014, 2016). In the case of denitrifiers, biotic filtering could have arisen due to the lower cost associated with the synthesis of NirK (Van Lis *et al*., 2011). However, we could not attribute the mutual exclusion patterns between *nirS*- and *nirK*-carrying bacteria to the dominance of any of the two types of nitrite reductases across plots. Thus, the spatial distribution of every *nirS*-*nirK* pair that was significantly segregated over multiple plots did not respond to the overrepresentation of either partner. These patterns do not support that differences in competitive abilities between types of denitrifiers underlie their mutual exclusion in the environment. Nevertheless, we detected other signs of ecological interactions. A signal for potential positive interactions is the fact that pairs of taxa sharing co-presence links doubled those of mutually excluding taxa. This pattern is commonly found in networks built for different organisms and has been attributed to the easiness to detect physical co-aggregation (Pascual-García *et al*., 2014; Freilich *et al*., 2018; Goberna *et al*., 2019). Organisms that co-aggregate, e.g. in biofilms, might leave more detectable signals because they greatly increase cell density and expand the niche for other species (Nadell *et al*., 2016; Freilich *et al*., 2018; Goberna *et al*., 2019). Under prefire conditions, we detected a prevalence of *nirS*-*nirS* links between bacteria whose *nirS* was most closely related to that in *Magnetospirillum*. These bacteria, which typically thrive in water and sediments, have been reported in biofilms in industrial systems (Osvald *et al*., 2017) and soils (Dearing *et al*., 2001). Furthermore, their magnetotactic behaviour allows them to co-aggregate in stable cell bands (Guell *et al*., 1988). Spatial aggregation could also be the basis of the prevalence in co-presence links of organisms related to *Cupriavidus*, which can rapidly form biofilms through the production of an exopolysaccharide matrix (Lerch *et al*., 2017).

In support for the existence of antagonistic interactions, we observed that mutual exclusion links occurred between bacteria carrying the same *nir* variant (*nirK*-*nirK*) more often than expected at random prior to burning. Such reciprocal exclusion between ecologically similar bacteria is typically attributed to competition based on niche similarities (Webb *et al*., 2002; Russel *et al*., 2017). The effects of competition between bacteria with the same *nir* variant can be difficult to detect in the environment, particularly for organisms with high dispersal rates, since dispersal of competitors among habitat patches can blur checkerboard patterns (Dallas *et al*., 2019). However, we have previously shown in simulated communities that co-occurrence networks are able to capture mutual exclusion patterns when competition operates based on niche similarities (Pérez-Valera *et al*., 2017). Our negative *nirK*-*nirK* links involved organisms whose nitrite reductase was most closely related to that in several genera of the order Rhizobiales, mainly *Mesorhizobium*. These results support previous 16S rRNA gene-based network analysis at the same site, which revealed that mutual exclusion links involved phylogenetically closely related alpha-Proteobacteria (Pérez-Valera *et al*., 2017). Since we sampled the soil matrix in a shrubland that was highly dominated by Lamiaceae prior to disturbance, the rhizobia we detected are most likely free-living alpha-Proteobacteria that combine denitrification and nitrogen fixation abilities (e.g. Delgado *et al*., 2007). The fact that the abundance of *nirK* did not respond to its main resource (mineral N) or cofactor (copper) but to iron, suggests that the basis for competition between these *nirK* bearing rhizobia could be the synthesis of the Fe-dependent nitrogenases (Raymond *et al*., 2004).

Fire abruptly increased the levels of mineral N, as well as those of other relevant macro- and micronutrients, due to the mineralization of organic substances (Certini, 2005). With a slight delay in time, total organic carbon increased, and thus pH dropped, in soil due to the supply of burned plant material that did not combust completely in our experimental fire. Concurrent with the pulse of mineral N, we detected a peak in the abundance of genes coding for both

nitrite reductases that fainted away in the first week after burning. Apart from this short-term increase in *nir* copy numbers, burning did not alter the relative abundance of the dominant denitrifying taxa, their community structure, or the partition of the betadiversity into a large spatial turnover and a negligible nestedness component. In agreement, others have reported that burning does not significantly modify the abundance of molecular markers of denitrifiers or the denitrification activity in the mid-term (Castaldi and Aragosa, 2002; Liu *et al*., 2013). Nevertheless, burning significantly shifted the patterns of spatial association of denitrifying taxa, suggesting changes in their community assembly mechanisms. Most remarkably, the disturbance reverted the co-presence and mutual exclusion detected between bacteria bearing the same *nir* variant. As soon as 1 day after fire, negative *nirK-nirK* links were lost suggesting an immediate relaxation of competition between denitrifiers carrying *nirK*. Although increased nitrate levels can inhibit Cu-dependent nitrite reductases (Tocheva *et al*. 2008), our data do not suggest that, as the number of *nirK* copies were promoted by fire. Alternatively, nitrogenase activity in the rhizobia harbouring *nirK* could have been inhibited by ammonia, a process that can take place within minutes and is magnified as pH drops (Klugkist and Haaker, 1984; Hartmann *et al*., 1986). In contrast to the decreasing negative *nirK-nirK* links, fire led to increased *nirS-nirS* exclusion that peaked 1 week after fire coinciding with the significant increase in *nirS* copy numbers. Such exclusion links mostly involved organisms most closely related to *Magnetospirillum*, which can have three *nirS* copies in the genome (Jones *et al*. 2008). The ecological significance is not clear, but experiments with *Thauera*, another genus of Proteobacteria carrying more than one *nirS* copy, have shown that strains with two copies of *nirS* express one of them constitutively and the other one in response to increased nitrate (Etchebehere and Tiedje, 2005). This feature provides them with increased competitive ability compared to denitrifiers with a single *nirS* copy.

In conclusion, *nir* gene-based co-occurrence patterns were more sensitive to disturbance than metrics quantifying community structure or the organization of diversity across space. Even if a different fire regime would change the specific results reported here, the present study illustrates how *nir* gene-based co-occurrence networks can help infer the multiplicity of ecological processes that govern the assembly of soil denitrifier communities. Our results suggest that abiotic filtering was a prevailing structuring force and revealed signals of competition between ecologically similar bacteria, a process that is difficult to trace in complex microbial communities in nature. This indicates that functional network analysis that considers potential biotic interactions as well as niche requirements can assist in the understanding of ecological assembly of microorganisms responsible for critical soil functions.

Experimental procedures

Experimental fire and soil sampling

An experimental fire was provoked on April 2009 in a 500 m^2 area in the province of Valencia (E Spain; UTM: 30N 676565.50, 4332416.06 m; 950 m a.s.l.; 20% NE facing slope; 446 mm mean annual rainfall; 13.7°C mean annual temperature). The soil is a Humic Leptosol (IUSS Working Group WRB, 2006) and the area was covered by a dense shrubland dominated by *Rosmarinus officinalis* that was completely burned out by the experimental fire. Temperature reached 611 ± 94°C (average ± SE; $n = 3$) at 50 cm over the soil surface, 338 ± 83°C ($n = 10$) on the soil surface and 106 ± 35°C ($n = 8$) within the upper 2 cm below the surface. Further details can be found in the study by Goberna et al. (2012). After removing the ash layer, surface soil samples (0–2 cm; 300 g) were randomly taken from 1 × 1 m plots ($n = 10$) located 1–3 m apart within a 150 m^2 area. Samples were collected immediately before (prefire), 1 day, 1 week, 1 month, and 1 year after fire. Sampling prefire soils as the unburned control considerably reduces the effects of environmental and spatial heterogeneity that results from sampling an adjacent unburned area typically done when wildfires are investigated (e.g. Pérez-Valera *et al*., 2018). Seasonal variation in microbial parameters with respect to the control was accounted for in the statistical models (see details below).

Soil samples were transported to the laboratory on ice, immediately sieved (<2 mm) and soil physical and chemical factors were analysed using standard procedures. Soil pH, electrical conductivity (EC), gravimetric humidity, carbonate content ($CaCO_3$), total organic C (TOC), water soluble C (WSC), water soluble carbohydrates (CH), pyrophosphate extractable C (PEC), Total N (TN), nitrate N (NO_3^-), ammonium N (NH_4^+), P and K were determined as in the study by Goberna et al. (2012) and published in the study by Pérez-Valera et al. (2017). In addition, total Cu, Fe, Mn, Cr, B and Ca were determined by digestion with HNO_3 and H_2O_2 using an Ultraclave microwave digestion system (Milestone SRL, Milan, Italy) followed by analysis by ICP (ICAP 6500 ICP Spectrometer, Thermo Fischer Scientific, MA, USA).

DNA extraction and quantitative PCR

DNA was extracted within 24 h after sampling from ca. A 0.25 g soil using the PowerSoil DNA isolation kit (MO BIO Laboratories, CA, USA). Extracted DNA was checked for quality in 1% agarose gels, quantified with the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, CA, USA) and the extracts were stored at −20°C.

The number of copies of the bacterial *16S rRNA*, *nirS* and *nirK* genes were quantified in an iQ5 Multicolor

real-time PCR detection system (Bio-Rad Laboratories Inc., CA, USA). Quantitative real-time PCRs (qPCR) were carried out in 20 μl reactions, containing 1 X Dynamo^{Tm} Flash SYBR®Green qPCR kit (Finnzymes, Finland), 1 μM of each forward and reverse primer (Supporting Information Table SI3), 1 mg ml^{-1} bovine serum albumin, 10 ng of soil DNA and sterile water. Serial dilutions of linearized plasmids (pGEM® T Easy Vector, Promega Corp., WI, USA) containing inserts of the target genes were prepared as in the study by Hallin *et al*. (2009). The standard curves contained a minimum of five standard concentrations and were linear in the range used (R^2 = 0.99, in all cases). Thermal cycling for the *16S rRNA* gene had a denaturation step at 95°C for 10 min, followed by 40 cycles consisting of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, and 80°C for 30 s. Thermal cycling for *nirS* and *nirK* included 95°C for 15 min, 6 cycles consisting of 95°C for 15 s, decreasing annealing temperatures (ramp −1 °C cycle^{-1}) for 30 s, 72°C for 30 s, and 80°C for 30 s. Forty amplification cycles were performed as above using the touchdown and annealing temperatures corresponding to each primer pair (Supporting Information Table SI3). All qPCRs were terminated with 15 s at 95°C, followed by the construction of a melting curve (60–95°C; ramp 0.5°C per 10 s). The PCR efficiencies were 98% for *16S rRNA* genes, 100% for *nirK* and 98.7% for *nirS*.

Prior to quantification, the presence of inhibitors in the samples was tested by amplifying positive controls including circular plasmids, non-template controls and samples spiked with circular plasmids. PCR reactions and thermal cycling conditions were performed as for the *16S rRNA* gene, but with the plasmid-specific primers T7 (5'-TAATACGACTCACTATAGG-3′) and SP6 (5′- TATTT AGGTGACACTATAG-3′) (Promega, MA, USA) and an annealing temperature of 55°C. Negligible differences in amplification of spiked samples and positive controls indicated the absence of inhibitors.

Sequencing of nir genes and sequence processing

The nitrite reductase genes (*nirK* and *nirS*) were amplified for barcoded pyrosequencing with a 30 cycles PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) under the following conditions: 94°C for 3 min, and 28 cycles consisting of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. *nirS* genes were amplified with the primer pairs cd3aF/R3cd (Table SI3) and *nirK* with nirK1F (5′- GGMATGGTKCCSTGGCA-3')/nirk5R (5'-GCCTCGATCAGRTTRTGG-3′) (Braker *et al*., 1998). Both primer sets have a reasonable coverage and high specificity for *nir* in Proteobacteria (Bonilla-Rosso *et al*., 2016). We specifically focused on denitrifying Proteobacteria, since this phylum is dominant in our study soils and highly responsive to fire (Pérez-Valera *et al*., 2017). Primers included sequencing key and adaptor and with the forward primer preceded with 8 bp barcodes. All amplicons were mixed in equimolar amounts and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Sequencing was performed with Roche 454 FLX titanium instruments and reagents by MR DNA (Shallowater, TX, USA).

Sequences were quality filtered using QIIME v1 (Caporaso *et al*., 2010). No mismatches were allowed in the barcode sequence and sequences shorter than 150 base pairs, including homopolymer runs longer than 6 base pairs or including ambiguous base calls were removed, as well as sequences with an average Phred quality score lower than 25 analysed by using a sliding window of 50 nucleotides. Sequences were dereplicated and chimeras removed with USEARCH 6.0 in Fungene (Fish *et al*., 2013), after which we obtained 83 483 *nirS* and 129 025 *nirK* sequences. Framebot was used to correct frameshift errors and calculate the nearest neighbour to each sequence (Wang *et al*. 2013). Only frameshift-corrected nucleotide sequences were kept for downstream analyses. The taxonomic assignments of the nearest neighbour to each frameshift-corrected sequence was obtained in GeneBank. Operational taxonomic units (OTUs) were constructed from a total of 18 647 *nirS* and 57 856 *nirK* sequences with the complete linkage clustering method using mcCLUST in Fungene (Fish *et al*., 2013) at a distance cutoff of 0.05 in the amino acid sequence. In total, we obtained 500 *nirS* and 1046 *nirK* OTUs. Sequence data have been submitted to the European Nucleotide Archive under accession number PRJEB39377 (http://www.ebi.ac.uk/ena/data/view/PRJEB39377).

Network analysis

Co-occurrence network analysis was used to detect OTUs co-occurring more (co-presence links) or less (mutual exclusion links) frequently than expected at random using CoNet 1.0b6 (Faust and Raes, 2012; Faust *et al*., 2012) and the script available at http://psbweb05.psb.ugent.be/conet/cmdline.php. Five networks, one per sampling time, were constructed based on the relative abundances of *nirS* and *nirK* OTUs using seven replicated plots which had a sufficient number of sequences for both marker genes. Recall that these are 1 × 1 m plots distributed in a small area of 150 m^2, which is highly homogeneous in terms of abiotic conditions. Such low environmental heterogeneity most likely underlies our ability to detect biologically meaningful patterns using co-occurrence network analysis despite the limited number of replicated plots (e.g. Pérez-Valera *et al*., 2017). Furthermore, in order to reduce the chance of detecting

spurious associations, we used stringent methodological settings as follows.

Prior to network construction, *nirS* and *nirK* relative abundance matrices were grouped into a single matrix in such a way that links could be computed between OTUs of the same *nir* variant (i.e. pairs of *nirK-nirK* or *nirS-nirS*) and between OTUs of different *nir* variants (i.e. *nirS-nirK*). Low-abundant OTUs (i.e. present in less than one-third of the samples) were removed to reduce artefactual associations (Faust *et al*., 2012). Co-presence and exclusion links were identified with an ensemble-based approach, including two measures of correlation (Pearson and Spearman) and dissimilarity (Bray Curtis and Kullback–Leibler), to increase the robustness of the analysis (Faust and Raes, 2016). The interaction sign was used to distinguish between co-presence and exclusion links, which were considered as undirected due to the nature of the correlation/dissimilarity measures used. Networks were computed with the 1000 initial top- and bottom-scoring links for each measure. Statistical significance was tested by obtaining the link- and measure-specific p-value as the mean of the permutation distribution under the bootstrap distribution, using 1000 iterations for each distribution. Probability values of different correlation/dissimilarity measures supporting the same link were merged using Brown's method and corrected for multiple testing using Benjamini–Hochberg's procedure. Finally, to reduce the detection of false positives only those links supported by at least two measures of correlation/dissimilarity and having an adjusted merged *P*-value below 0.05 were included.

Statistical analysis

We evaluated post-fire changes in soil abiotic factors, *16S rRNA*, *nirS* and *nirK* gene copy numbers, as well as the relative abundance of bacterial taxa having *nirS* or *nirK* through generalized linear models (GLM) in R 4.0.0 (R Core Team, 2020). To account for the variation in all factors due to shifts in climatic conditions, we performed two consecutive GLMs in all cases. In the first model, we used each soil parameter as a dependent variable, and mean air temperature and precipitation as independent factors (climatic data are given in the study by Pérez-Valera *et al*., 2017). In the second model, we used the residuals of the first model as the dependent variable and the sampling time as a categorical independent factor.

To test which soil abiotic factors determine the abundance of *nirS* and *nirK* genes in our study soils, we performed generalized linear mixed models (GLMMs) with the MCMCglmm package for R (Hadfield, 2010). We included gene copy numbers as the dependent variable and a collection of soil abiotic factors as independent factors, including the sampling time as a categorical random factor. We performed model selection based on Deviance Information Criteria of GLMMs including decreasing numbers of soil abiotic predictors.

We tested the existence of changes in the structure of *nirS*- and *nirK*- bacterial communities by using permutational multivariate analysis of variance (PERMANOVA) based on Bray Curtis dissimilarity matrices with the *adonis* function in the vegan package for R (Oksanen *et al*., 2017). PERMANOVAs were carried out using pairwise orthogonal contrasts comparing the OTU $\times$ plot relative abundance matrix of each post-fire sampling time against that under prefire conditions. We calculated alphadiversity values (Shannon's index) using the *diversity* function in the *vegan* package for R. We computed the turnover and nestedness components of betadiversity to analyse whether there is spatial species replacement across plots (high turnover) or poorer communities contain a subset of the species in richer communities (high nestedness) (Baselga, 2010). We used the *beta.multi.abund* function in the *betapart* package for R based on Bray-Curtis multiple-site dissimilarity (Baselga *et al*., 2018).

To detect which *nir* type significantly co-occur or mutually exclude each other, we analysed the departure from randomness of observed frequencies in the co-presence and mutual exclusion links obtained from co-occurrence networks between OTUs belonging to the same *nir* variant (i.e. pairs *nirS-nirS*, *nirK-nirK*) and to different *nir* variants (i.e. pairs *nirS-nirK*). To do so, we performed log linear analyses for each sampling time with the *loglm* function in the MASS package for R (Venables and Ripley 2002).

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References

Andersson, M., Michelsen, A., Jensen, M., and Kjøller, A. (2004) Tropical savannah woodland: effects of experimental fire on soil microorganisms and soil emissions of carbon dioxide. *Soil Biol Biochem* **36**: 849–858.

Barberán, A., Bates, S.T., Casamayor, E.O., and Fierer, N. (2012) Using network analysis to explore co-occurrence patterns in soil microbial communities. *ISME J* **6**: 343–351.

Baselga, A. (2010) Partitioning the turnover and nestedness components of beta diversity. *Glob Ecol Biogeogr* **19**: 134–143.

Baselga, A., Orme, D., Villeger, S., De Bortoli, J., and Leprieur, F. (2018) Betapart: partitioning beta diversity into

turnover and nestedness components. R package v 1.5.0 https://cran.r-project.org/web/packages/betapart/index.html

Bonilla-Rosso, G., Wittorf, L., Jones, C.M., and Hallin, S. (2016) Design and evaluation of primers targeting genes encoding NO-forming nitrite reductases: implications for ecological inference of denitrifying communities. *Sci Rep* **6**: 39208. https://doi.org/10.1038/srep39208.

Braker, G., Fesefeldt, A., and Witzel, K.P. (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl Environ Microbiol* **64**: 3769–3775.

Bru, D., Ramette, A., Saby, N.P.A., Dequiedt, S., Ranjard, L., Jolivet, C., *et al*. (2011) Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. *ISME J* **5**: 532–542.

Canfield, D.E., Glazer, A.N., and Falkowski, P.G. (2010) The evolution and future of Earth's nitrogen cycle. *Science* **330**: 192–196.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al*. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Meth* **7**: 335–336.

Castaldi, S., and Aragosa, D. (2002) Factors influencing nitrification and denitrification variability in a natural and fire-disturbed Mediterranean shrubland. *Biol Fertil Soils* **36**: 418–425.

Certini, G. (2005) Effects of fire on properties of forest soils: a review. *Oecologia* **143**: 1–10.

Chesson, P. (2000) Mechanisms of maintenance of species diversity. *Annu Rev Ecol Syst* **31**: 343–366.

Dallas, T., Melbourne, B.A., and Hastings, A. (2019) When can competition and dispersal lead to checkerboard distributions? *J Animal Ecol* **88**: 269–276.

Dearing, J.A., Hannam, J.A., Anderson, A.S., and Wellington, E.M.H. (2001) Magnetic, geochemical and DNA properties of highly magnetic soils in England. *Geophys J Int* **144**: 183–186.

Delgado, M.J., Casella, S., and Bedmar, E.J. (2007) Denitrification in rhizobia-legume symbiosis. In *Biology of the Nitrogen Cycle*, Bothe, H., Ferguson, S.J., and Newton, W.E. (eds). Amsterdam, The Netherlands: Elsevier, pp. 83–91.

Enwall, K., Throbäck, I.N., Stenberg, M., Söderström, M., and Hallin, S. (2010) Soil resources influence spatial patterns of denitrifying communities at scales compatible with land management. *Appl Environ Microbiol* **76**: 2243–2250.

Etchebehere, C., and Tiedje, J. (2005) Presence of two different active *nirS* nitrite reductase genes in a denitrifying *Thauera* sp. from a high-nitrate-removal-rate reactor. *Appl Environ Microbiol* **71**: 5642–5645.

Faust, K., and Raes, J. (2012) Microbial interactions: from networks to models. *Nature Rev Microbiol* **10**: 538–550.

Faust, K., and Raes, J. (2016) CoNet app: inference of biological association networks using Cytoscape [version 2; referees: 2 approved]. *F1000Res* **5**: 1519. https://doi.org/10.12688/f1000research.9050.2.

Faust, K., Sathirapongsasuti, J.F., Izard, J., Segata, N., Gevers, D., Raes, J., and Huttenhower, C. (2012) Microbial co-occurrence relationships in the human microbiome. *PLoS Comput Biol* **8**: e1002606.

Fish, J.A., Chai, B., Wang, Q., Sun, Y., Brown, C.T., Tiedje, J.M., and Cole, J.R. (2013) FunGene: the Functional Gene Pipeline and Repository. *Front Microbiol* **4**: 291. https://doi.org/10.3389/fmicb.2013.00291.

Freilich, M.A., Wieters, E., Broitman, B.R., Marquet, P.A., and Navarrete, S.A. (2018) Species co-occurrence networks: can they reveal trophic and non-trophic interactions in ecological communities? *Ecology* **99**: 690–699.

Goberna, M., García, C., Insam, H., Hernández, M.T., and Verdú, M. (2012) Burning fire-prone Mediterranean shrublands: immediate changes in soil microbial community structure and ecosystem functions. *Microb Ecol* **64**: 242–255.

Goberna, M., García, C., and Verdú, M. (2014) A role for biotic filtering in driving phylogenetic clustering in soil bacterial communities. *Glob Ecol Biogeogr* **23**: 1346–1355.

Goberna, M., Navarro-Cano, J.A., and Verdú, M. (2016) Opposing phylogenetic diversity gradients of plant and soil bacterial communities. *Proc Roy Soc B* **283**: 20153003.

Goberna, M., Montesinos, A., Valiente-Banuet, A., Colin, Y., Gómez, A., Donat, S., *et al*. (2019) Incorporating phylogenetic metrics to microbial co-occurrence networks based on amplicon sequences to discern community assembly processes. *Molec Ecol Res* **19**: 1552–1564.

Goldfarb, K.C., Karaoz, U., Hanson, C.A., Santee, C.A., Bradford, M.A., Treseder, K.K., *et al*. (2011) Differential growth responses of soil bacterial taxa to carbon substrates of varying chemical recalcitrance. *Front Microbiol* **2**: 94.

Guell, D.C., Brenner, H., Frankel, R.B., and Hartman, H. (1988) Hydrodynamic forces and band formation in swimming magnetotactic bacteria. *J Theor Biol* **134**: 525–542.

Graf, D.R.H., Jones, C.M., and Hallin, S. (2014) Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N_2O emissions. *PLoS ONE* **9**: e114118.

Hadfield, J.D. (2010) MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. *J Stat Softw* **33**: 1–22.

Hallin, S., Jones, C.M., Schloter, M., and Philippot, L. (2009) Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *ISME J* **3**: 597–605.

Hartmann, A., Fu, H., and Burris, R.J. (1986) Regulation of nitrogenase acitivity by ammonium chloride in *Azospirillum* spp. *J Bacteriol* **165**: 864–870.

HilleRisLambers, J., Adler, P.B., Harpole, W.S., Levine, J.M., and Mayfield, M.M. (2012) Rethinking community assembly through the lens of coexistence theory. *Annu Rev Ecol Evol Syst* **43**: 227–248.

Hooper, D.U., and Johnson, L. (1999) Nitrogen limitation in dryland ecosystems: responses to geographical and temporal variation in precipitation. *Biogeochem* **46**: 247–293.

Hu, H.-W., Chen, D., and He, J.-Z. (2015) Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. *FEMS Microbiol Rev* **39**: 729–749.

Hu, Y., Chen, N., Li, M., Feng, C., and Liu, T. (2019) Insights into simultaneous microbial chromium and nitrate

reduction: inhibitory effects and molecular mechanisms. *J Chem Technol Biotechnol* **94**: 2589–2596.
IUSS Working Group WRB. (2006) World reference base for soil resources 2006. World Soil Resources Reports No. 103. FAO, Rome.
Jones, C.M., and Hallin, S. (2010) Ecological and evolutionary factors underlying global and local assembly of denitrifier communities. *ISME J* **4**: 633–641.
Jones, C.M., and Hallin, S. (2019) Geospatial variation in co-occurrence networks of nitrifying microbial guilds. *Mol Ecol* **28**: 293–306.
Jones, C.M., Stres, B., Rosenquist, M., and Hallin, S. (2008) Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. *Mol Biol Evol* **25**: 1955–1966.
Jones, C.M., Spor, A., Brennan, F.P., Breuil, M.-C., Bru, D., Lemanceau, P., *et al*. (2014) Recently identified microbial guild mediates soil N_2O sink capacity. *Nature Clim Change* **4**: 801–805.
Klugkist, J., and Haaker, H. (1984) Inhibition of nitrogenase activity by ammonium chloride in *Azotobacter vinelandii*. *J Bacteriol* **157**: 148–151.
Lerch, T.Z., Chenu, C., Dignac, M.F., Barriuso, E., and Mariotti, A. (2017) Biofilm vs. planktonic lifestyle: consequences for pesticide 2,4-D metabolism by *Cupriavidus necator* JMP134. *Front Microbiol* **8**: 904. https://doi.org/10.3389/fmicb.2017.00904.
Liu, X., Chen, C.R., Wang, W.J., Hughes, J.M., Lewis, T., Hou, E.Q., and Shen, J. (2013) Soil environmental factors rather than denitrification gene abundance control N_2O fluxes in a wet sclerophyll forest with different burning frequency. *Soil Biol Biochem* **57**: 292–300.
Mayfield, M.M., and Levine, J.M. (2010) Opposing effects of competitive exclusion on the phylogenetic structure of the communities. *Ecol Lett* **13**: 1085–1093.
Nadell, C.D., Drescher, K., and Foster, K.R. (2016) Spatial structure, cooperation and competition in biofilms. *Nature Rev Microbiol* **14**: 589–600.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., *et al*. (2017). vegan: Community Ecology Package. R package version 2.4-4. https://CRAN.R-project.org/package=vegan
Osvald, M., Maróti, G., Pap, B., and Szanyi, J. (2017) Biofilm forming bacteria during thermal water reinjection. *Geofluids* **2017**: 9231056.
Pascual-García, A., Tamames, J., and Bastolla, U. (2014) Bacteria dialog with Santa Rosalia: are aggregations of cosmopolitan bacteria mainly explained by habitat filtering or by ecological interactions? *BMC Microbiol* **14**: 284.
Pérez-Valera, E., Goberna, M., Faust, K., Raes, J., García, C., and Verdú, M. (2017) Fire modifies the phylogenetic structure of soil bacterial co-occurrence networks. *Environ Microbiol* **19**: 317–327.
Pérez-Valera, E., Verdú, M., Navarro-Cano, J.A., and Goberna, M. (2018) Resilience to fire of phylogenetic diversity across biological domains. *Mol Ecol* **27**: 2896–2908.
Pérez-Valera, E., Goberna, M., and Verdú, M. (2019) Fire modulates ecosystem functioning through the phylogenetic structure of soil bacterial communities. *Soil Biol Biochem* **129**: 80–89.
R Core Team. (2020) *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing URL http://www.R-project.org/.
Raymond, J., Siefert, J.L., Staples, C.R., and Blankenship, R.E. (2004) The natural history of nitrogen fixation. *Mol Biol Evol* **21**: 541–554.
Rinaldo, S., and Cutruzzolà, F. (2007) Nitrite reductases in denitrification. In *Biology of the Nitrogen Cycle*, Bothe, H., Ferguson, S.J., and Newton, W.E. (eds). Amsterdam, The Netherlands: Elsevier, pp. 37–55.
Rinaldo, S., Giardina, G., and Cutruzzolà, F. (2017) Nitrite reductase – cytochrome cd1. In *Metalloenzymes in Denitrification: Applications and Environmental impacts*, (RSC Metallobiology Series No. 9), Moura, I., Moura, J.J.G., Pauleta, S.R., and Maia, L.B. (eds). London, UK: The Royal Society of Chemistry, pp. 59–90.
Russel, J., Røder, H.L., Madsen, J.S., Burmølle, M., and Sørensen, S.J. (2017) Antagonism correlates with metabolic similarity in diverse bacteria. *Proc Natl Acad Sci U S A* **114**: 10684–10688.
Syakila, A., and Kroeze, C. (2011) The global nitrous oxide budget revisited. *Greenh Gas Meas Manag* **1**: 17–26.
Smith, J., and Ogram, A. (2008) Genetic and functional variation in denitrifier populations along a short-term restoration chronosequences. *Appl Environ Microbiol* **74**: 5615–5620.
Soininen, J., Heino, J., and Wang, J. (2018) A meta-analysis of nestedness and turnover components of beta diversity across organisms and ecosystems. *Glob Ecol Biogeogr* **27**: 96–109.
Tocheva, E.I., Eltis, L.D., and Murphy, M.E.P. (2008) Conserved active site residues limit inhibition of a copper-containing nitrite reductase by small molecules. *Biochemistry* **47**: 4452–4460.
Van Lis, R., Ducluzeau, A.L., Nitschke, W., and Schoepp-Cothenet, B. (2011) The nitrogen cycle in the Archaean: an intricate interplay of enzymatic and abiotic reactions. In *Nitrogen Cycling in Bacteria: Molecular Analysis*, Moir, J. W.B. (ed). Norwich: Caister Academic Press.
Venables, W.N., and Ripley, B.D. (2002) *Modern Applied Statistics with S*, 4th ed. New York, USA: Springer.
Wang, Q., Quensen, J.F.I.I.I., Fish, J.A., Lee, T.-K., Sun, Y., Tiedje, J.M., and Cole, J.R. (2013) Ecological patterns of *nifH* genes in four terrestrial climatic zones explored with targeted metagenomics using FrameBot, a new informatics tool. *mBio* **4**: e00592–e00513.
Webb, C.O., Ackerley, D.D., McPeek, M.A., and Donoghue, M.J. (2002) Phylogenies and community ecology. *Annu Rev Ecol Syst* **33**: 475–505.
Wittorf, L., Jones, C.M., Bonilla-Rosso, G., and Hallin, S. (2018) Expression of nirK and nirS genes in two strains of *Pseudomonas stutzeri* harbouring both types of NO-forming nitrite reductases. *Res Microbiol* **169**: 343–347.
Yuan, Q., Liu, P., and Lu, Y. (2012) Differential responses of nirK- and nirS-carrying bacteria to denitrifying conditions in the anoxic rice field soil. *Environ Microbiol Rep* **4**: 113e22.
Zhou, J., Deng, Y., Luo, F., He, Z., Tu, Q., and Zhi, X. (2010) Functional molecular ecological networks. *MBio* **1**: e00169–e00110.

Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**: 533–616.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. SI1. Biplot depicting the principal component analysis of main soil abiotic factors in pre-fire and post-fire plots. Total variance explained by each principal component (PC) is given in parenthesis. PC analysis was performed with the *prcomp* function in R using the *scale* argument. Abbreviations: CH (Water Soluble Carbohydrates), EC (Salinity), GH (Gravimetric Humidity), PEC (Pyrophosphate Extractable Carbon), TN (Total Nitrogen), TOC (Total Organic Carbon), WSC (Water Soluble C).

Fig. SI2. Ratios of the number of gene copies before and during one year after an experimental fire. Different letters denote significant differences across sampling times, after accounting for the effects of temperature and rainfall (statistical details in main text).

Fig. SI3. Shannon's diversity index for *nirS* and *nirK* before and during one year after an experimental fire.

Fig. SI4. Nitrite reductase (*nir*) co-occurrence networks. Co-presence and mutual exclusion networks are shown for pre- and post-fire conditions. Network nodes indicating OTUs are depicted as spheres (orange, *nirS*; grey, *nirK*) and links as solid lines connecting nodes (orange, *nirS-nirS*; grey, *nirK-nirK*; black, *nirS-nirK* links).

Table SI1. Betadiversity partition into turnover and nestedness components for *nirK* and *nirS* community matrices. Turnover and nestedness are expressed as the values of the balanced variation (turnover) and abundance-gradient (nestedness) components of Bray-Curtis multiple-site dissimilarities (Baselga *et al*. 2018. Betapart: Partitioning beta diversity into turnover and nestedness components. R package v 1.5.0. Retrieved from https://cran.r-project.org/web/packages/betapart/index.html).

Table SI2. Number of co-presence (A) and mutual exclusion links (B) detected for all sampling times between *nir* genes in OTUs most closely related to bacteria belonging to the same genus or different genera.

Table SI3. Primers used for quantitative PCR.