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

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Originality Significance Statement

We propose the use of *nir* gene-based co-occurrence networks to infer the mechanisms of co-existence of denitrifying bacteria. We take advantage of the fact that *nirS* and *nirK* genes i) perform the same biological function, i.e. nitrite reduction, and are most often exclusive at the individual level, and ii) are carried by soil bacteria that compete for the same resource, but have distinct abiotic requirements and may have differential environmentally-mediated fitness. By monitoring an experimental fire, we find that *nir* gene-based networks are more sensitive to disturbance than other community metrics and help understanding the assembly mechanisms of soil denitrifiers.

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 one 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.

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 (Hu et al 2015, Syakila & Kroeze 2011). 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 assembly of communities that compete for the same resource. However, the reduction of nitrite to nitric oxide in the denitrification pathway is catalyzed 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.

105 2017). For the same reasons, mutual exclusion between *nirS-nirS* or *nirK-nirK* pairs suggests
106 competition by limiting similarity (bottom right panel in Fig. 1), whereas that between *nirS-nirK*
107 would be interpreted as environmental filtering (upper right panel in Fig. 1). While the
108 interpretation of mutual exclusion links mirrors that of co-presence links, it adds evidence on
109 the mechanisms of microbial co-existence since two non-coexisting species do not necessarily
110 exclude each other.
111 [*Figure 1*]
112
113 As a model system, we used a Mediterranean ecosystem in which N is a limiting resource
114 (Hooper and Johnson 1999), thus setting the conditions for competition to occur. We analysed
115 *nirS*- and *nirK*-possessing soil bacteria under natural conditions and after exposure to an
116 experimental fire, which causes a burst in mineral N in the soil (Certini 2005; Goberna *et al*.
117 2012). By changing resource availability, burning alters the activity of N-cycling enzymes
118 (Pérez-Valera et al. 2019) and soil denitrifiers (Andersson *et al*. 2004), and thus likely the
119 strength of competition. Other soil conditions, such as pH or the concentration of trace
120 elements, also change after fire (Certini 2005). We monitored post-fire shifts in soil abiotic
121 factors, 16S rRNA, *nirK* and *nirS* gene copy numbers, and sequenced both *nir* genes to
122 characterise changes in denitrifier community structure, diversity and *nir*-based networks. We
123 hypothesized that environmental filtering is the dominant force determining the prevalence of
124 positive links between bacteria bearing the same *nir* variant (*nirS-nirS* and/or *nirK-nirK*) and
125 negative links between bacteria with different *nir* variants (*nirS-nirK*) (Fig. 1). Our objectives
126 were i) to unravel the processes that structure soil denitrifying communities by separately
127 analysing co-presence and mutual exclusion links following the framework detailed above, and
128 ii) to test how changes in resource availability (e.g. carbon, nitrate, micronutrients) and abiotic
129 conditions (e.g. pH, salinity) in response to the experimental fire alter the balance of forces
130 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 one 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 significantly reduced soil moisture (GH) and pH after one 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 the one-year period of monitoring after the fire (Fig. 2).

[*Figure 2*]

Abundance and community structure of denitrifiers with different nir types

The abundance of 16S rRNA, *nirS* and *nirK* gene copy numbers peaked one week after fire, and prefire levels were restored after one 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 one 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.

[*Figure 3*]

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. Fig. 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.

[Figure 4]

The *nirK*/*nirS* abundance ratio, which originally averaged 14.4 ± 1.6, dropped significantly one day after fire to 10.2 ± 1.1, but displayed prefire levels after one month (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 (Fig. SI2). The relative abundance 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 (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 one week after fire, while for *nirK* it was 4.4 ± 0.1 and did

not differ significantly across sampling times (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 (Table SI1).

[Figure 5]

Detection of co-present and mutually excluding denitrifiers

nir gene-based co-occurrence network analysis detected 2,587 positive (co-presence) links and 1,376 negative (mutual exclusion) links between denitrifiers considering all sampling times (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-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; Table SI2). Positive *nirS* links between OTUs related to different genera were mostly *Magnetospirillum-Cupriavidus* pairs (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 one day and one 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* (Table SI2).

[*Figure 6*]

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* (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 (χ^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%) (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 one 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%).

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. By 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 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

261 environment. Nevertheless, we detected other signs of ecological interactions. A signal for
262 potential positive interactions is the fact that pairs of taxa sharing co-presence links doubled
263 those of mutually excluding taxa. This pattern is commonly found in networks built for
264 different organisms and has been attributed to the easiness to detect physical co-aggregation
265 (Pascual-García *et al*. 2014; Freilich *et al*. 2018; Goberna *et al*. 2019). Organisms that co-
266 aggregate, e.g. in biofilms, might leave more detectable signals because they greatly increase
267 cell density and expand the niche for other species (Freilich *et al*. 2018; Nadell *et al*. 2016;
268 Goberna *et al*. 2019). Under prefire conditions, we detected a prevalence of *nirS*-*nirS* links
269 between bacteria whose *nirS* was most closely related to that in *Magnetospirillum*. These
270 bacteria, which typically thrive in water and sediments, have been reported in biofilms in
271 industrial systems (Osvald *et al*. 2017) and soils (Dearing *et al*. 2001). Furthermore, their
272 magnetotactic behavior allows them to co-aggregate in stable cell bands (Guell *et al*. 1988).
273 Spatial aggregation could also be the basis of the prevalence in co-presence links of organisms
274 related to *Cupriavidus*, which can rapidly form biofilms through the production of an
275 exopolysaccharide matrix (Lerch *et al*. 2017).
276
277 In support for the existence of antagonistic interactions, we observed that mutual
278 exclusion links occurred between bacteria carrying the same *nir* variant (*nirK*-*nirK*) more often
279 than expected at random prior to burning. Such reciprocal exclusion between ecologically
280 similar bacteria is typically attributed to competition based on niche similarities (Webb *et al*.
281 2002; Russel *et al*. 2017). The effects of competition between bacteria with the same *nir*
282 variant can be difficult to detect in the environment, particularly for organisms with high
283 dispersal rates, since dispersal of competitors among habitat patches can blur checkerboard
284 patterns (Dallas *et al*. 2019). However, we have previously shown in simulated communities
285 that co-occurrence networks are able to capture mutual exclusion patterns when competition
286 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 one 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 harboring *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 one 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 (FAO-ISRIC-IUSS, 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 two cm below the surface. Further details can be found in 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 to 3 m apart within a 150 m^2 area. Samples were collected immediately before (prefire), one day, one week, one month, and one 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 analyzed 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 Goberna *et al*. (2012) and published in 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

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

368 The number of copies of the bacterial *16S rRNA*, *nirS* and *nirK* genes were quantified in an
369 iQ5 Multicolor real-time PCR detection system (Bio-Rad Laboratories Inc., CA, USA). Quantitative
370 real-time PCRs (qPCR) were carried out in 20 µL reactions, containing 1 X Dynamo™ Flash
371 SYBR®Green qPCR kit (Finnzymes, Finland), 1 µM of each forward and reverse primer (Table SI3),
372 1 mg mL^{-1} bovine serum albumin, 10 ng of soil DNA and sterile water. Serial dilutions of linearized
373 plasmids (pGEM® T Easy Vector, Promega Corp., WI, USA) containing inserts of the target genes
374 were prepared as in Hallin *et al*. (2009). The standard curves contained a minimum of five
375 standard concentrations and were linear in the range used (R^2=0.99, in all cases). Thermal cycling
376 for the *16S rRNA* gene had a denaturation step at 95°C for 10 minutes, followed by 40 cycles
377 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*
378 and *nirK* included 95°C for 15 minutes, 6 cycles consisting of 95°C for 15 s, decreasing annealing
379 temperatures (ramp -1°C per cycle) for 30 s, 72°C for 30 s and 80°C for 30 s. Forty amplification
380 cycles were performed as above using the touchdown and annealing temperatures
381 corresponding to each primer pair (Table SI3). All qPCRs were terminated with 15 s at 95°C,
382 followed by the construction of a melting curve (60 to 95°C; ramp 0.5°C per 10 s). The PCR
383 efficiencies were 98% for *16S rRNA* genes, 100% for *nirK* and 98.7% for *nirS*.

384 Prior to quantification, the presence of inhibitors in the samples was tested by amplifying
385 positive controls including circular plasmids, non-template controls and samples spiked with
386 circular plasmids. PCR reactions and thermal cycling conditions were performed as for the *16S*
387 *rRNA* gene, but with the plasmid-specific primers T7 (5'-TAATACGACTCACTATAGG-3') and SP6
388 (5'- TATTTAGGTGACACTATAG-3') (Promega, MA, USA) and an annealing temperature of 55°C.

389 Negligible differences in amplification of spiked samples and positive controls indicated the
390 absence of inhibitors.

391

392 *Sequencing of nir genes and sequence processing*

393 The nitrite reductase genes (*nirK* and *nirS*) were amplified for barcoded pyrosequencing with a
394 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) under the following
395 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
396 1 min, after which a final elongation step at 72°C for 5 min was performed. *nirS* genes were
397 amplified with the primer pairs cd3aF/R3cd (Table SI3) and *nirK* with nirK1F (5'-
398 GGMATGGTKCCSTGGCA-3') / nirk5R (5'-GCCTCGATCAGRTTRTGG-3') (Braker *et al*. 1998). Both
399 primer sets have a reasonable coverage and high specificity for *nir* in Proteobacteria (Bonilla-
400 Rosso *et al*. 2016). We specifically focused on denitrifying Proteobacteria, since this phylum is
401 dominant in our study soils and highly responsive to fire (Pérez-Valera *et al*. 2017). Primers
402 included sequencing key and adaptor and with the forward primer preceded with 8 bp
403 barcodes. All amplicons were mixed in equimolar amounts and purified using Agencourt
404 Ampure beads (Agencourt Bioscience Corporation, MA, USA). Sequencing was performed with
405 Roche 454 FLX titanium instruments and reagents by MR DNA (Shallowater, TX, USA).
406 Sequences were quality filtered using QIIME v1 (Caporaso *et al*. 2010). No mismatches were
407 allowed in the barcode sequence and sequences shorter than 150 base pairs, including
408 homopolymer runs longer than 6 base pairs or including ambiguous base calls were removed,
409 as well as sequences with an average Phred quality score lower than 25 analysed by using a
410 sliding window of 50 nucleotides. Sequences were dereplicated and chimeras removed with
411 USEARCH 6.0 in Fungene (Fish *et al*. 2013), after which we obtained 83,483 *nirS* and 129,025
412 *nirK* sequences. Framebot was used to correct frameshift errors and calculate the nearest
413 neighbour to each sequence (Wang *et al*. 2013). Only frameshift-corrected nucleotide
414 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 1/3 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 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 × 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 analyzed 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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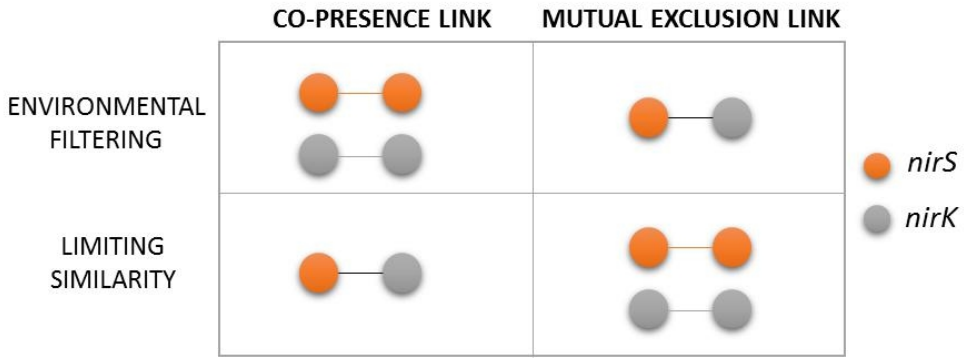

Fig. 1. Expected outcome of community assembly processes in co-occurrence networks of denitrifiers. Spheres with different colours represent microorganisms with different nitrite reductase (nir) variants (orange, nirS; grey, nirK). Solid lines joining two spheres represent significant links between a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

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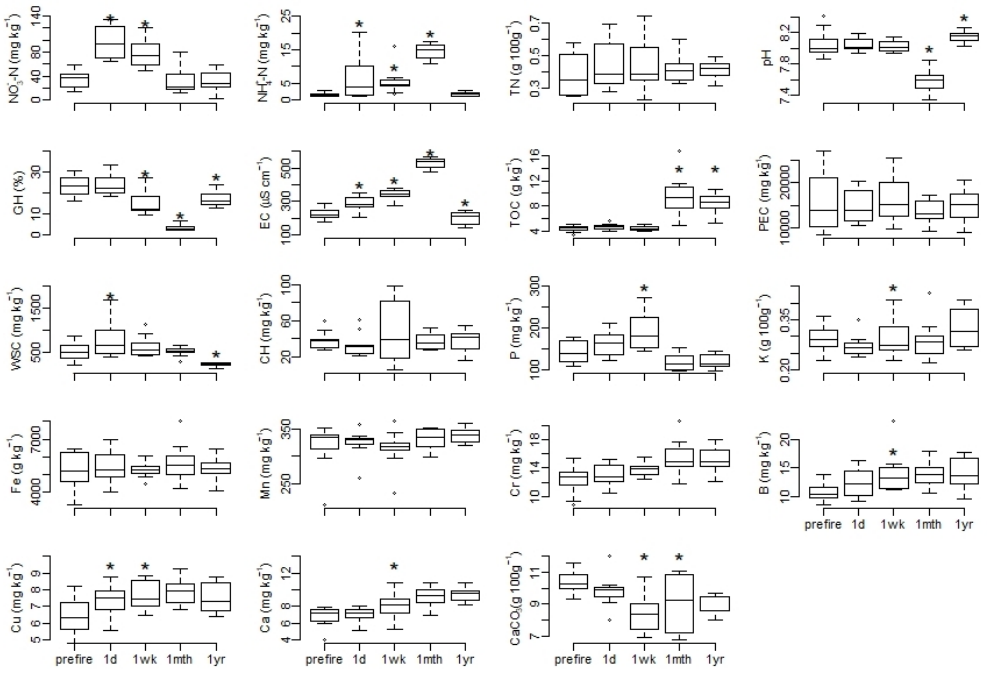

Fig. 2. Soil abiotic factors (expressed in DW) before and during one year after an experimental fire. Asterisks indicate significant differences of each variable and sampling time compared to prefire levels, after accounting for the effects of temperature and rainfall (see text for statistical details). 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). Part of these analyses were published in Pérez-Valera et al. (2017).

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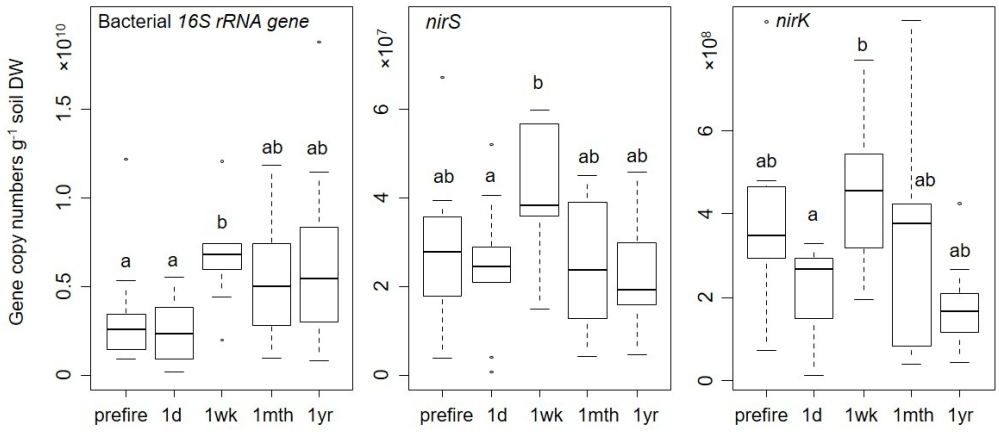

Fig. 3. Number of copies of the 16S rRNA, nirK and nirS genes (expressed per g soil DW) before and at different time points during one year after an experimental fire. Different letters denote statistically significant differences, after accounting for the effects of temperature and rainfall.

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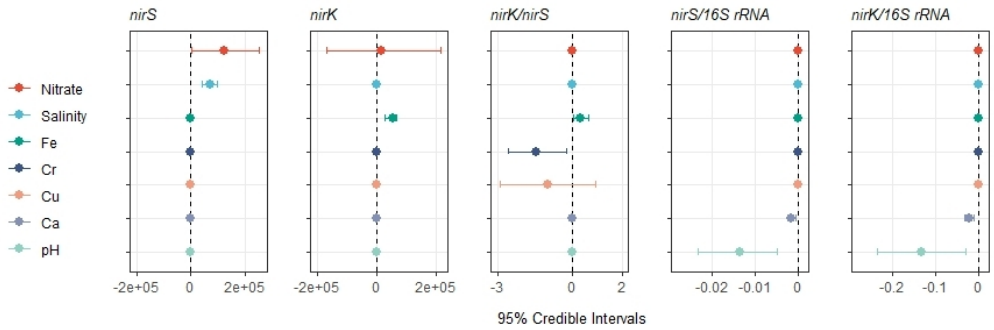

Fig. 4. Bayesian post-mean estimates (and their expected 95% credible intervals) of the best statistical models explaining the effect of soil abiotic factors on the number of nir gene copies and ratios. Factors with intervals not including zero are significant.

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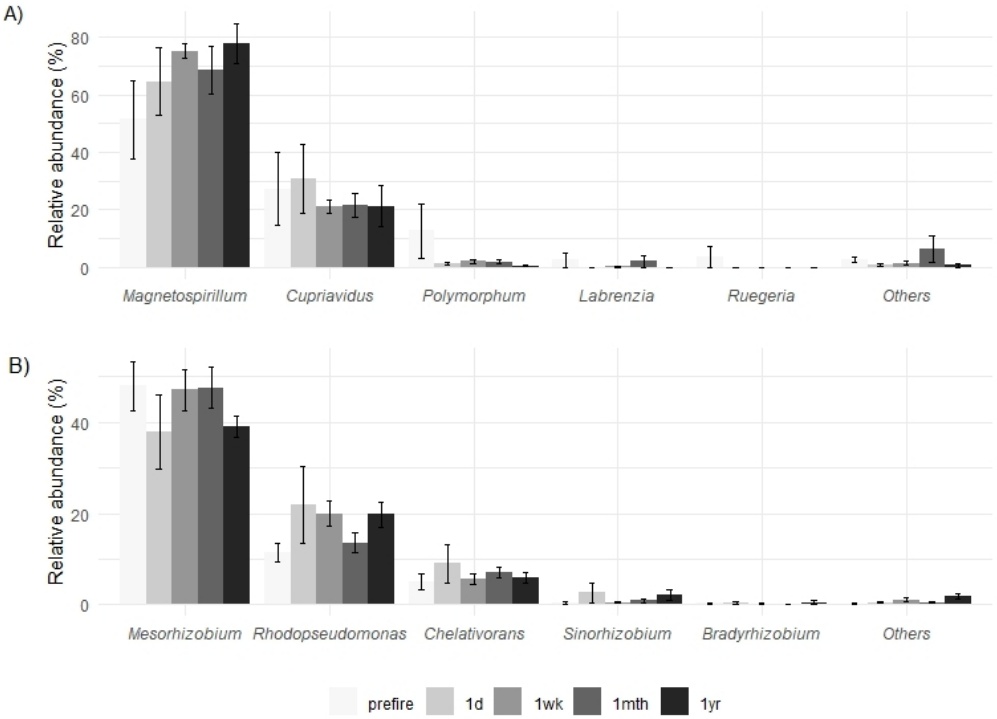

Fig. 5. Taxonomic distribution of A) nirS- and B) nirK-carrying bacteria before and during one year after an experimental fire. Error bars indicate standard errors.

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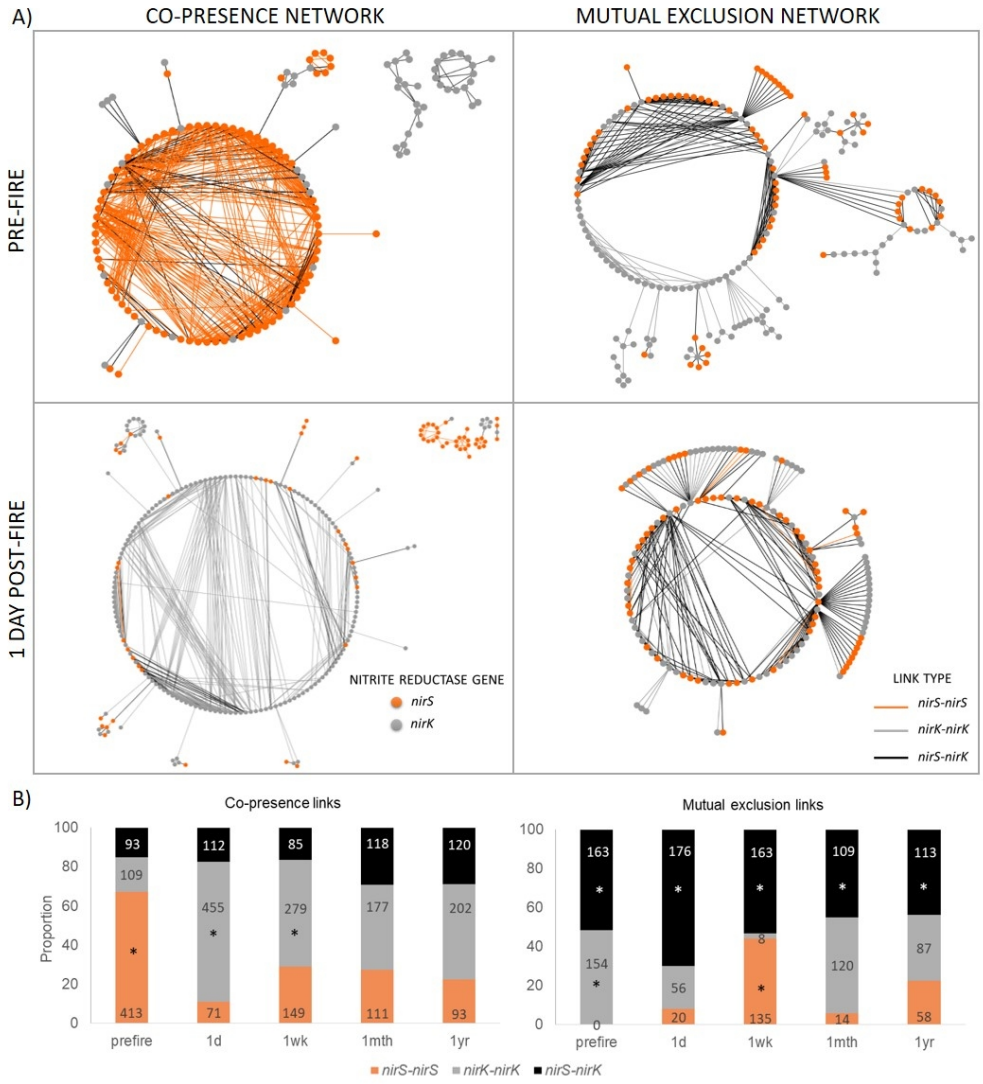

Fig. 6. A) Nitrite reductase (nir) co-occurrence networks. Co-presence (left panel) and mutual exclusion networks (right panel) are shown for pre- and post-fire (1d) conditions for illustrative purposes. Network nodes indicating OTUs are depicted as spheres (orange, nirS; grey, nirK) and links as solid 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 one 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.

178x195mm (150 x 150 DPI)