



# Potential of high-throughput eDNA sequencing of soil fungi and bacteria for monitoring ecological restoration in ultramafic substrates: The case study of the New Caledonian biodiversity hotspot

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## ABSTRACT

Due to their central role in ecosystems functioning and their ability to rapidly respond to environmental changes, soil microorganisms could potentially be used for monitoring ecosystems recovery in the context of degraded land restoration. However, these belowground organisms have been, to date, largely neglected. Here, we investigated fungal and bacterial community diversity, composition, and structure from ultramafic soils in New Caledonia, an archipelago in the southwest Pacific recognized as a priority for conservation and restoration. The emerging approach of high-throughput amplicon sequencing of environmental DNA (eDNA) – metabarcoding of eDNA – was used to compare soil microbial communities from four different native vegetation types, representing different stages of a chronosequence and defined as reference ecosystems, to five distinct post-mining sites revegetated several years ago. Our results clearly revealed changes in soil microbial phyla and functional groups along the reference chronosequence and variable responses at the different revegetated sites, with two of the five sites showing a good trajectory of recovery. We thus propose three ratios as metrics for monitoring the restoration trajectory of soil microorganisms: the Ascomycota:Basidiomycota and Saprotrophic:Ectomycorrhizal ratios for fungi, and the Cyanobacteria:Chloroflexi ratios for bacteria. Our study, combined with recent works undertaken in other geographical areas, underpins the great promise that could represent soil microbial eDNA metabarcoding for monitoring restoration progress and success. With the emergence of these new cost-effective and scalable sequencing technologies, soil microbes could, in the near future, be included in guidelines for restoration operations in complement to more conventional approaches.

## 1. Introduction

Restoring degraded lands has become an urgent priority worldwide in order to mitigate the decline of biodiversity and ecosystem functions (IPBES, 2018; UN Environment Programme, 2020). Through human intervention, ecological restoration aims to initiate or accelerate the recovery of degraded ecosystems (IPBES, 2018; McDonald et al., 2016). It thus relies on identifying effective tools for monitoring the recovery

trajectory of systems of interest (Harris, 2003).

Terrestrial ecosystems are composed of both aboveground and belowground components. However, to date, the belowground part has been largely disregarded. Yet, a single gram of soil can harbor hundreds of meters of fungal hyphae, billions of bacteria, and thousands of species (Godat et al., 2010). Moreover, there is an increasing awareness of the crucial roles of soil microorganisms in ecosystem functioning. These neglected organisms are involved in major processes, such as

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biogeochemical cycles (especially in carbon and nitrogen cycles), soil aggregation, and plant community dynamics (e.g., Bever et al., 2010; Demenois et al., 2020; Hartnett and Wilson, 2002; Madsen, 2011). Furthermore, they may promote plant establishment in unvegetated areas by modifying soil conditions (Nemergut et al., 2007; Schmidt et al., 2008). Soil microorganisms can thus be a large and key component of biodiversity in ecosystems where plants are absent or poorly represented (Nara et al., 2003; Nemergut et al., 2007).

Several studies have examined changes in soil microbial diversity, composition and structure in natural succession processes (e.g., Cutler et al., 2014; Jangid et al., 2013; Knelman et al., 2012; Tarlera et al., 2008). For instance, Cutler et al. (2014) showed changes in soil fungal richness and composition in a primary succession on an 850-year chronosequence of lava flows. Similarly, shifts in soil bacterial communities were observed in ecosystems that were established from 60 to 120,000 years after the retreat of a glacier (Jangid et al., 2013). At shorter time-scales (a 20 years chronosequence), an increase of bacterial diversity and of certain bacterial groups has also been observed following glacial retreat (Nemergut et al., 2007). Differing environmental conditions along an ecological succession can thus result in different microbial communities, and soil microbiota are potentially able to respond rapidly to these environmental changes (Gourmelon et al., 2016; Nemergut et al., 2007).

Given these elements, a growing number of studies investigate soil microbial communities, particularly bacteria and fungi, to compare restored sites to natural reference sites and thereby assess restoration progress (e.g., Araújo et al., 2014; Banning et al., 2011; Chen et al., 2020; Gellie et al., 2017; Harris, 2009; Harris, 2003; Yan et al., 2018). Very recently, in this restoration context, high-throughput amplicon sequencing of environmental DNA (eDNA) – metabarcoding of eDNA – has been used for characterizing soil communities (Chen et al., 2020; Gastauer et al., 2019; Gellie et al., 2017; Nurulita et al., 2016; Sun et al., 2017; van der Heyde et al., 2020; Yan et al., 2020; Yan et al., 2018). Metabarcoding of eDNA is an approach that uses specific primers to amplify, from environmental samples (e.g., soil or water), an informative DNA region of a group of organisms of interest (e.g., bacteria or fungi) (Ji et al., 2013). This approach presents several advantages over traditional field-based visual surveys, such as botanical inventories. Field taxonomic inventories rely on expert observers, are time-consuming, may overlook species, may vary between observers, and are not standardized among projects (Thomsen and Willerslev, 2015; Vittoz and Guisan, 2007). Comparatively, high-throughput sequencing of eDNA is cost-effective, easy to standardize, quicker to produce, verifiable by a third party, and does not specifically require taxonomic expertise beforehand (Bouchez et al., 2016; Ji et al., 2013; Thomsen and Willerslev, 2015).

Overall, investigation of soil microbial communities for restoration purposes using these emerging sequencing technologies have failed to detect clear trends in terms of species richness, or other diversity indices (Gellie et al., 2017; Sun et al., 2017; Yan et al., 2018; Yan et al., 2018). However, several studies have detected a gradual and directional shift of soil microbial communities in terms of structure and composition towards native reference ecosystems with restoration age. For example, in sites historically submitted to long-term grazing in southern Australia, bacterial and fungal communities were more similar to natural reference ecosystems a decade after native plant revegetation (Gellie et al., 2017; Yan et al., 2018). Similarly, in northern America, a transition of soil microbiota was detected, with soil bacterial and fungal communities becoming more similar to the unmined reference sites with increasing age of reforested stands (Sun et al., 2017). Interestingly, these authors also showed some differences in trends between these two types of microorganisms. More recently, in post-mining revegetated landscapes, inconsistencies in bacterial and fungal community changes have been highlighted by comparing patterns at three distinct locations in western Australia (van der Heyde et al., 2020). The authors concluded that the trajectory of soil microbial communities was dependent on location and type of microorganism. Overall, these contrasting results, plus the

novelty and the paucity of studies, clearly highlight the need of additional research investigating the efficiency of eDNA metabarcoding of soil microorganisms for monitoring restoration progress, and success, in distinct ecosystems in different geographical regions.

New Caledonia, a subtropical archipelago located in the southwest Pacific renowned for its exceptional biological diversity (Carriconde et al., 2019; Chazeau, 1993; Morat et al., 2012), is recognized as a biodiversity hotspot (Myers et al., 2000) and as one of the highest priority areas for restoration (Strassburg et al., 2020). With one third of its territory covered by ultramafic substrates and 20–30% of the global nickel ore reserve (L'Huillier and Jaffré, 2010), mining has drastically increased across the main island since the 19th century (DIMENC, 2008; L'Huillier and Jaffré, 2010). Over this past century and a half, anthropogenic pressure on New Caledonian terrestrial environments has subsequently increased considerably due to the transition to open-cast mining in the 1920s and post-second world war mechanization (Losfeld et al., 2015). This tendency has been recently accentuated by the establishment of a new hydrometallurgical plant, extracting both nickel and cobalt from the Goro deposit in the south of the main island. Facing this landscape degradation, local awareness of protecting natural ecosystems and restoring degraded lands has emerged, leading to changes in mining practices and the development of revegetation actions, with the first experimental field test performed in 1971 (DIMENC, 2008; L'Huillier et al., 2010). Over the past fifty years, land management expectations have been upgraded from solely soil stabilization, without understanding the underlying mechanisms, to a more holistic consideration, taking into account the native diversity, the landscape, the ecosystem structure, the soil microorganisms, and various ecological services (L'Huillier et al., 2010).

Information on fungal and bacterial biodiversity in New Caledonia is relatively sparse, with investigation of microbial communities being mostly recent (e.g., Carriconde et al., 2019; Demenois et al., 2020; Gourmelon et al., 2016; Houles et al., 2018; Navarro et al., 1999). A high microbial diversity has been detected in ultramafic soil (Bordez et al., 2016; Carriconde et al., 2019; Gourmelon et al., 2016; Houles et al., 2018); Carriconde et al. (2019) even hypothesized the existence of a very high species endemism rate (95%) within the symbiotic group of ectomycorrhizal fungi. Interestingly, variation in relative abundances of Ascomycota and Basidiomycota phyla in fungi using a next generation sequencing approach was observed along the same chronosequence at two distant ultramafic outcrops (Gourmelon et al., 2016). In particular, a large representation of Ascomycota was detected in “maquis” vegetation (shrub-like vegetation), a secondary vegetation resulting, here, from degradation of the initial rainforests (Isnard et al., 2016). In contrast, Basidiomycota was most abundant in the undegraded forests. The authors concluded that a larger representation of Ascomycota could therefore be an indicator of ecosystem degradation. Such results hold great promise for assessing ultramafic soil microbial trajectory after revegetation.

In this present work, high-throughput amplicon sequencing of soil eDNA was used to assess fungal and bacterial diversity, composition, and structure in ultramafic soils of the Goro plateau (located in the south of New Caledonia, near the hydrometallurgical plant) (Fig. 1). Four common types of maquis vegetation in this area were used as references. These maquis formations, growing on iron crust soils, represent different stages of a chronosequence (McCoy et al., 1999), ranging from a sparse vegetation (open low maquis) to a closed formation enriched in forest species (preforest maquis) (Fig. 2; see Materials and Methods). The microbial communities of five distinct revegetated sites on the Goro plateau were compared to these references. We thus addressed the following questions: 1) do soil fungal and bacterial communities show a gradual shift, in terms of diversity, composition and structure along the local chronosequence of reference sites? 2) which native maquis formations do the soil microbial communities of the revegetated sites most closely resemble? 3) might soil microbial richness, phyla composition (e.g., Ascomycota versus Basidiomycota), and fungal functional groups (i.e.,

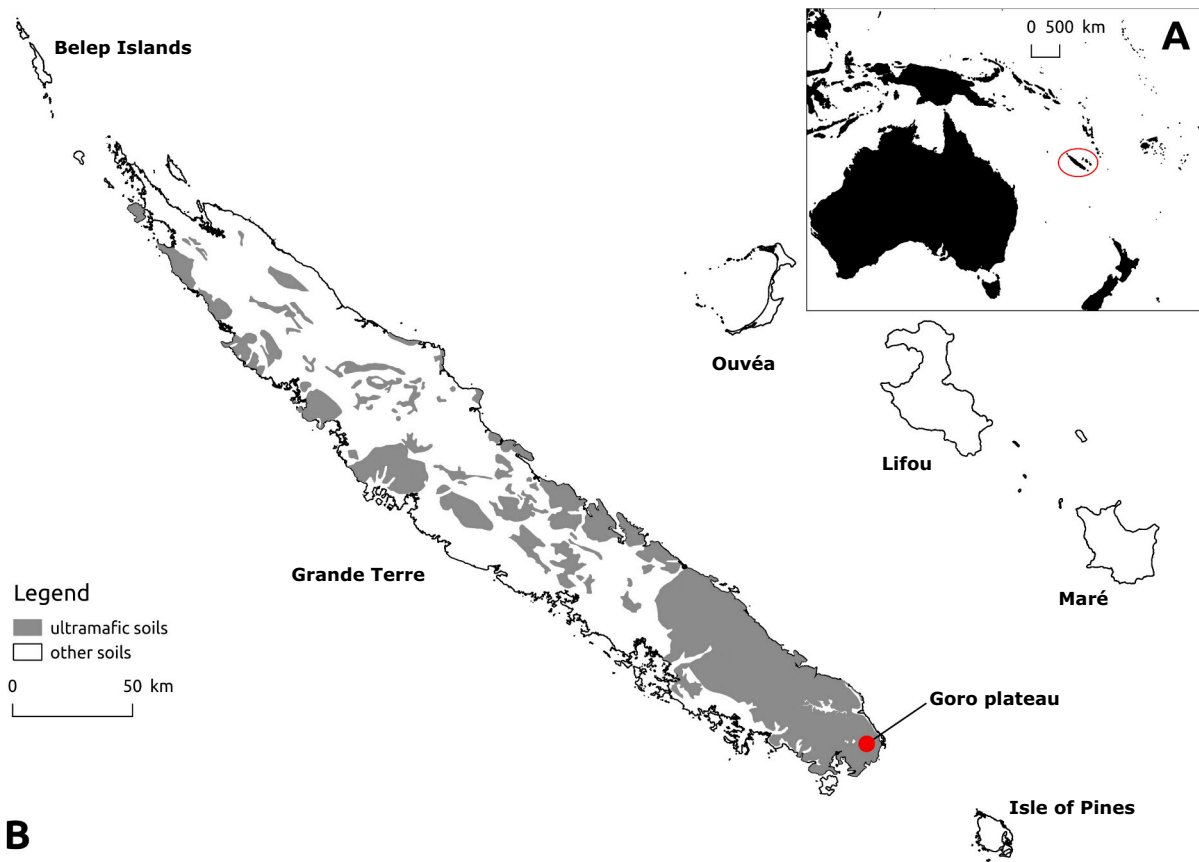


Fig. 1. (A) Location of New Caledonia in the southwestern Pacific, and (B) of Goro plateau in the south of the main island (ultramafic substrates are shown in grey).

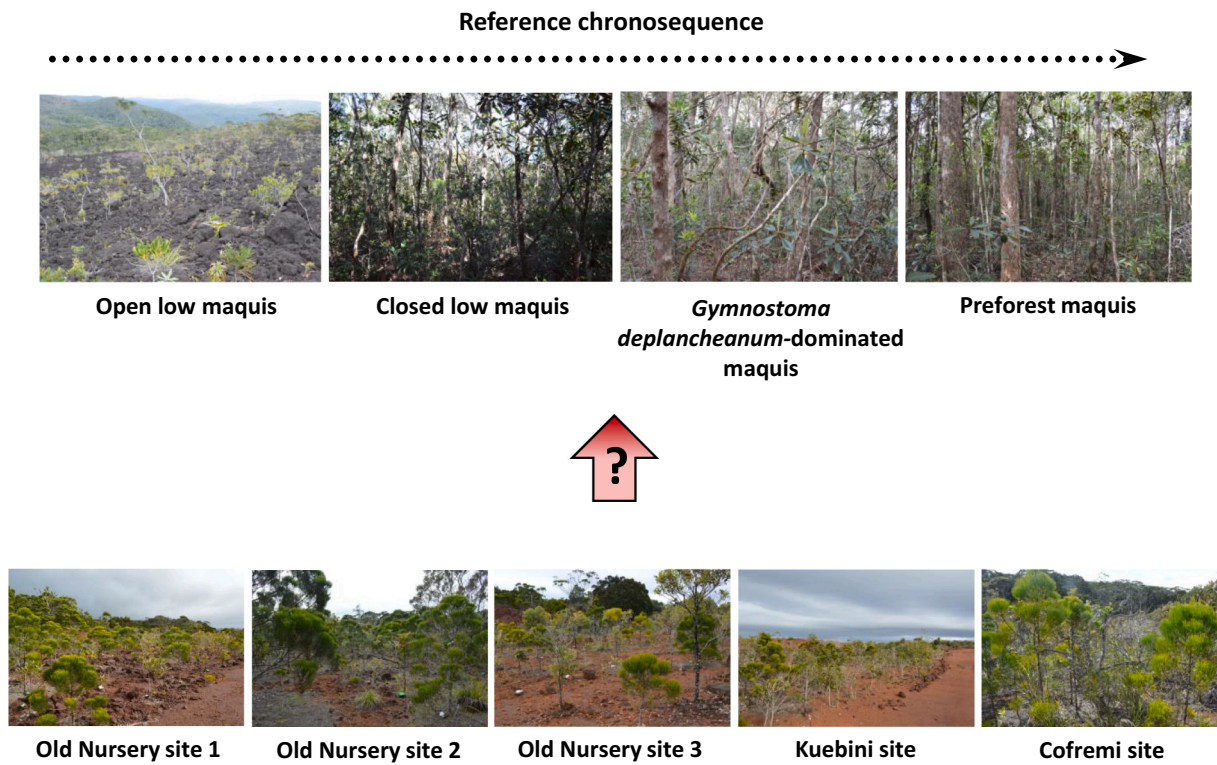


Fig. 2. Comparison of soil microbial communities (fungi and bacteria) of the five studied revegetated sites to the reference chronosequence in southern New Caledonia (Goro plateau).

fungus Saprotophs versus Symbiotrophs), therefore be useful as monitoring tools for assessing revegetation progress and success? And finally, 4) is there any relationship between microbial changes and soil chemical properties?

## 2. Material and methods

### 2.1. Experimental design

#### 2.1.1. Study sites

New Caledonia is an archipelago located in the southwestern Pacific, just above the Capricorn tropic, about 1500 km east of Australia and 2000 km north of New Zealand (Fig. 1). With a total area of 19100 km<sup>2</sup>, it extends from north to south for approximately 450 km and from east to the west for 40 to 70 km. The archipelago consists of a main island, commonly called “Grande Terre”, and extends to the Belep Islands to the north, the Isle of Pines to the south, and the three Loyalty Islands to the east (Ouvéa, Lifou and Maré) (Fig. 1). The archipelago has a subtropical climate with a hot and humid season from December to March, a cooler season from June to August, and dry season from September to November. The mean annual minimum and maximum precipitations are 800 mm and 2500 mm; and mean annual temperatures range from 20.7 to 26.6 °C (METEO-FRANCE).

The present study was undertaken at an ultramafic area located in the Massif du Grand Sud in New Caledonia, called Goro plateau (22°16'14.0"S 166°57'44.0"E) (Fig. 1). Situated approximately 300 m above sea level, the mean annual precipitation is 2760 mm and the minimal and maximal mean annual temperatures are 13.8 and 28.9 °C (METEO-FRANCE).

The soil sampling regime at Goro plateau was designed to compare revegetated sites to native adjacent ecosystems (Fig. S1). The study site consists of a 2 × 2 km area where twenty-four 20 × 20 m plots were placed on a grid of 100 × 100 m by semi-random selection (Fig. S1). These plots were positioned in four locally well-represented maquis vegetations (McCoy et al., 1999) that constitute a reference chronosequence (Fig. 2). A chronosequence was defined by Sun et al. (2017) as a “space-for-time substitutions using multiple sites with similar starting conditions but of different ages”. In each of the vegetation types, six plots were established. These selected plant formations corresponded to: (i) “open low maquis”; (ii) “closed low maquis”; (iii) “tall closed maquis dominated by *Gymnostoma deplancheanum*”, also called the “*G. deplancheanum*-dominated maquis”; and finally (iv) “tall closed maquis rich in forest species”, also called “preforest maquis” (Fig. 2).

Five revegetated sites, presenting various soil conditions, initially planted species, surfaces, ages, and natural environment vicinities were assessed (Fig. 2; Tables S1 and S2). The first three sites were located in an area called the “Old Nursery”, and subsequently named “Old Nursery sites 1 to 3”. They were all revegetated in 1997. At the Old Nursery site 1, three native plant species were directly planted on the iron crust soil (Tables S1 and S2). For the Old Nursery sites 2 and 3, four plants were planted after spreading red laterite and yellow laterite, respectively, on soil after removing the naturally occurring iron crust (Tables S1 and S2). The fourth revegetated site was located in an area called “Kuebini”, an abandoned iron crust quarry with exposed underlying red laterite (Fig. 2; Tables S1 and S2); revegetation was performed in 2002 using 34 native plants. The fifth and last site was situated in an area named “Cofremi”, on which four plants were planted on yellow laterite in 1988 (to our knowledge, no information is available on the original conditions) (Fig. 2; Tables S1 and S2). In total, seven plots of 20 × 20 m were set up in these revegetated sites (two plots at Old Nursery site 2 and at Cofremi, and one on each of the other sites).

To complement information on the studied plots, percentages of vegetation cover were estimated from aerial pictures for the five revegetated sites and selected stands of the reference ecosystems (see Table S1 and Fig. S2). Boundaries of revegetated sites were delimited, exported, and duplicated. On the duplicated images, a selection by

colour was applied, and, finally, a ratio of the number of pixels was made between both pictures using the newly created *Forest\_cover()* function of the *ImaginR* package v. 2.0.1 (<https://github.com/PLStenger/ImaginR>) (Stenger et al., 2019).

#### 2.1.2. Soil sampling

In each of the 20 × 20 m plots, four 2 × 2 m subplots were established (Figs. S1 & S3). Within each of these subplots, five soil subsamples at 0–10 cm depth were taken and mixed to form a representative composite sample (Fig. S3). A total of 124 soil samples (31 plots × 4 subplots) were thus collected from the 29th of February until the 4th of March 2016. All samples were immediately placed at 4 °C and stored at –20 °C in the 2–4 following hours. Soil samples were both used for both chemical analyses and assessing microbial communities.

### 2.2. Soil chemical properties

Soil chemical analyses were conducted by the LAMA laboratory (Nouméa, New Caledonia) according to standardized methods. Assimilable nitrogen (N-NO<sub>3</sub> and N-NH<sub>4</sub>) was extracted using 1 M KCl on fresh soils and analysed by flow injection analysis (AutoAnalyzer 3 HR, SEAL®) coupled with spectrophotometric detection. The other analyses were made on air-dried soils. The pH was measured on 2-mm soil fraction in water and KCl medium with a 1:5 v:v ratio (ISO 10390). Total carbon (C) and nitrogen (N), exchangeable cations and cation exchange capacity, as well as assimilable phosphorus (Pass) were measured on 2 mm soil fraction crushed and sieved at 100 µm. C and N content were assessed by dry combustion using an elemental analyzer-IRMS (Integra 2, Sercon) (ISO 10694). Exchangeable cations (Ca, Mg, Na, and K) were extracted using ammonium acetate (NF X 31–108) and cation exchange capacity (CE) was assessed on based on these extracts using Metson's method at pH 7 (NF X 31–130). Assimilable phosphorus (Pass) was determined according to the Olsen-Dabin method (Dabin, 1967).

### 2.3. Molecular methods

#### 2.3.1. Environmental DNA extraction

Soil samples were lyophilised with FreeZone Freeze Dry System (Labconco Corporation, Kansas City, MO, USA) at –51 °C and 0.015 bar for 24 h prior to eDNA extractions. One gram of dry soil was extracted per sample (four extractions of 0.25 g each) using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. DNA concentration and DNA quality were checked with a NanoDrop™ (Thermo Fisher Scientific, Waltham, MA, USA) and on a 1% Agarose gel.

#### 2.3.2. Libraries generation and sequencing

The Regional Genotyping Platform (GPTR Génotypage, <https://www.gptr-lr-genotypage.com/>) of the UMR AGAP (Cirad - Inra - Montpellier SupAgro) performed a two-step PCR strategy combined with the dual-index paired-end sequencing approach described in Kozich et al. (2013). Two independent PCR assays were performed for each eDNA sample as technical replicates and negative controls libraries were prepared in parallel. Beforehand, eDNA was purified with Agen-court AMPure beads (Beckman Coulter™ Life Sciences, Indianapolis, IN, USA) and diluted by half to obtain a better elimination of PCR inhibitors. The ITS2 region of the 18S nuclear ribosomal RNA gene for the fungal community was amplified using the primers 18S-Fwd-ITS7 5'-GTGARTCATCGAATCTTTG-3' (Ihrmark et al., 2012) and 18S-Rev-ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). The V4 region of the bacterial 16S RNA gene for the bacterial community was amplified using the primers 16S-Fwd-515 5'-GTGCCAGCMGCCGCGGTAA-3' and 16S-Rev-806 5'-GGACTACHVGGGTWTCTAAT-3' (Caporaso et al., 2011). Partial overhang Illumina sequencing primers in 50-end for each primer was added. During the first PCR (PCR1), DNA amplification was conducted in replicate, in 4.6 µl of Type-it Microsatellite PCR Kit

mastermix (Kit Qiagen®, Germany), 0.2 µl of each primer pair (10 µM), and 5 µl of template DNA for a final volume of 10 µl. Amplification was run on a 384-well plate (Mastercycler® EP384, Eppendorf® Thermal Cycler, Germany) under the following conditions: 95 °C for 5 min, 10 cycles of 30 s at 95 °C, 62 °C for 1 min 30 s and 72 °C for 30 s, 25 cycles of 30 s at 95 °C, 57 °C for 1 min 30 s and 72 °C for 1 min 30 s, followed by 30 min at 60 °C. PCR products were verified on an Agilent TapeStation with a D5000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). The second PCR (PCR2) consisted of a limited cycle amplification step to add the I5 and I7 indexes (different 8-base index sequences described in Kozich et al., 2013). The PCR2 was carried out in a 20 µl reaction volume using 2 µl pooled PCR1-dilution 1/10, 4 µl of indexing primers (10 µM), 10 µl of Taq MasterMix2 (New England Biolabs, ON, Canada) and 4 µl of H<sub>2</sub>O (PCR conditions: 95 °C for 30 s, 5 cycles of 10 s at 95 °C, 60 °C for 30 s and 72 °C for 30 s, 5 min at 72 °C). An equimolar pool of all PCR products by fragment was created and purified with a Promega Wizard® PCR Preps DNA Purification System DNA kit (Promega, Madison, WI, USA). The pool of the amplicon ITS2 and V4 libraries was quantified using the DNA Standards for Library Quantification kit (Clontech Takara, USA). The libraries were sequenced on a MiSeq System (Illumina, San Diego, CA, USA) with a V2 reagent kit. 12 pM and 10% PhiX were loaded on a MiSeq flow cell according to the manufacturer's instructions with 500 cycle cartridges (2 × 250 bp). Approximately 37 million paired reads of 250 bp length were obtained for both ITS2 and V4 independent sequencing runs. The Illumina MiSeq sequences are available under the NCBI BioProject PRJNA743931 and submission numbers SUB9939738, SUB9957890, SUB9966585, SUB9965709 and SUB9966666.

## 2.4. Bioinformatics

### 2.4.1. Working environment

For this work, we used the Canonical Ubuntu 16.04.4 LTS with MATE Desktop Environment 1.12.1, an open-source operating system using the Linux kernel and based on Debian ([ubuntu.com](http://ubuntu.com)). Conda v. 4.5.5, an open-source package management system ([conda.io](http://conda.io)), was also used. The workflow engine Snakemake v. 3.5.5 was used to automate the analyses (Köster and Rahmann, 2012). As suggested by Garris et al. (2016), a tailored bioinformatic workflow has thus been developed for the project (Fig. S4). This pipeline, freely available at [https://gitlab.com/IAC\\_SolVeg/CNRT\\_BIOINDIC](https://gitlab.com/IAC_SolVeg/CNRT_BIOINDIC) under the GNU General Public License v3 (GPLv3) license rights, allows the automatization, tracing, and reproduction of the analyses.

### 2.4.2. Pre-processing

Raw Illumina sequences from the ITS2 and V4 datasets were processed by first pooling each technical replicate. Illumina sequencing adapters in 3' ends were removed from sequences using cutadapt v. 1.18 (Martin, 2011). Low-quality sequences with a PHRED score of less than Q30 were trimmed, and sequences that were less than 150 nt in length after trimming were removed using Sickle v. 1.33 (Joshi and Fass, 2011). Finally, the paired forward and reverse sequences were joined, and single sequences were removed using fastq-join v. 1.3.1 (Aronesty, 2011). Quality control of the sequences was performed using FastQC v. 0.11.8 (Andrews, 2010), and reports were generated using MultiQC v. 1.6 (Ewels et al., 2016).

### 2.4.3. Qiime 2 framework

Microbiome analysis was performed using the QIIME 2 framework v. 2018.6.0 (Bolyen et al., 2018). Dereplicated and trimmed sequences were imported into the framework as single-end (joined) sequences and denoised using the DADA2 plugin, based on the DADA2 v. 1.8 R library (Callahan et al., 2016), which removed singletons, chimaeras, and sequencing errors and processed the sequences into a table of exact amplicon sequence variants (ASVs) (Callahan et al., 2017). Negative control library sequences, as putative contaminant sequences, were

removed from each sample sequence (Galan et al., 2016). ASVs that were present in only a single sample were filtered, based on the idea that these may not represent real biological diversity but rather PCR or sequencing errors. Finally, all samples were rarefied to the sample with the lowest number of reads, in order to keep at least three subplots by plot as biological replicates (subsequently, the lowest number of reads were of 3215 sequences for the ITS2 dataset (fungi) and 4171 sequences for the V4 dataset (bacteria)).

A multiple sequence alignment was produced using MAFFT v. 7.310 (Katoh, 2002; Katoh and Standley, 2013), and a rooted phylogenetic tree relating the ASV sequences to one another was constructed using Fast-Tree methods v. 2.1.10 (Price et al., 2010; Price et al., 2009). The observed number of ASVs (equivalent to species richness) (DeSantis et al., 2006), Chao1 (the expected richness) (Chao, 1984), Simpson evenness (diversity that accounts for the number of organisms and the number of species) (Simpson, 1949), Pielou evenness (measure of the relative evenness of species richness) (Pielou, 1966), Shannon entropy (richness and diversity that accounts for both abundance and evenness of taxa) (Shannon and Weaver, 1949), and Faith PD (measure of biodiversity that incorporates phylogenetic differences between species using the sum of length of branches) (Faith, 1992), were calculated with the q2-diversity plugin. Bray-Curtis dissimilarity (Sørensen, 1948) and Jaccard similarity index (Jaccard, 1908) matrices were also calculated with the q2-diversity plugin.

Naive Bayes feature classifiers were trained using the q2-feature-classifier plugin to assign taxonomy to the sequences (Bokulich et al., 2018). For the fungal classifier training, two databases were combined and used for reference sequences and taxonomy. The first database was the UNITE-V7.2-2017.10.10-dynamic QIIME pre-formatted database with dynamic homology clustering, including 8756 reference sequences and 21,793 representative sequences (RepS) (Köljalg et al., 2013), and the second was an in-house database, including 311 New-Caledonian ectomycorrhizal reference sequences and taxonomy from previous work (Carriconde et al., 2019). As recommended by the QIIME 2 development team, the fungal classifier was trained on the full reference sequences. For bacterial classifier training, the SILVA-V132-2018.04.10-99 QIIME pre-formatted database with 99% homology clustering, including 412,168 sequences (Quast et al., 2012), was used as the reference.

## 2.5. Statistical analyses

### 2.5.1. Soil microbial diversity, composition and structure

Statistical analyses were mostly performed using the R software environment (R Core Team, 2014). Differences in richness (i.e., number of ASVs) and diversity indices (see above) between the distinct conditions (i.e., the reference chronosequence and revegetated sites) were performed using Kruskal-Wallis one-way ANOVA analyses on rank followed by pairwise Dunn post-hoc test (`dunn.test` v. 1.3.5 and `multcompView` v. 0.1-5 R packages). Hill numbers and extrapolation curves (Chao et al., 2014) were also calculated using `iNEXT` v. 2.0.15 R package (Hsieh et al., 2016).

For fungal functional assignments, the FUNGuild database and the `Guilds_v1.0.py` python script were used (Nguyen et al., 2016). "Possible", "Probable" and "Highly Probable" confidence assignments were kept. Some ASVs were assigned to two or more guilds; only assignments to a unique guild were considered. A focus on ectomycorrhizal fungal communities was undertaken, consequently the related data were extracted from our global fungal dataset. To show co-occurrence of ectomycorrhizal fungal species among the different conditions (i.e., between revegetated sites and the ecosystems of reference), an UpSet plot was constructed with `UpSetR` R package v. 1.4.0 (Lex et al., 2014).

Regarding soil microbial community structure analyses, distance matrices (based on the Bray-Curtis measurement) were visualized using principal coordinate analysis (PCA) and non-metric multidimensional

scaling (NMDS) with FactoMineR R package v. 2.4 (Lê et al., 2008) and vegan v. 2.4.6 (Oksanen, 2015). Differences between microbial communities were tested using PERMANOVA, with 9999 permutations. In addition, bipartite networks were generated using Gephi 0.9.2, an open-source network exploration and manipulation software, using the ForceAtlas2 spatialization algorithm (Bastian et al., 2009; Jacomy et al., 2014). ASV abundance matrices were used with subplots and ASVs as nodes and ASVs abundances as weighted edges. Thereafter, partition of networks into communities was performed using the Blondel et al. (2008) algorithm and Lambiotte et al. (2009) resolution.

Normalized Community Structure Integrity Index (CSIInorm) and the Higher Abundance Index (HAI) were also calculated using the Jauvatre CSII R package (Jauvatre et al., 2013). These indices have been initially developed on plant communities to assess restoration success (Jauvatre et al., 2013). Here, the CSIInorm index was used to measure the proportion of the abundance of microbial species which is higher in the reference than in the restored system. Values range from 0 to 1; a value of 1 indicates that all species have the same abundances in both communities, whereas values lower than 1 indicate that some species from the reference are less abundant or absent from the restored ecosystem. Regarding the HAI index, *in contrario*, it indicates the proportion of the abundance of the species higher in the restored than in reference. This index also varies from 0 to 1. Thus, a value of 1 reveals that all species in the restored ecosystem display higher abundances.

### 2.5.2. Relationships between soil chemical properties and microbial communities

Distribution of soil chemical values were visualized through boxplot representations, using ggplot2 R package v. 3.3.3 (Wickham, 2011). To test differences among all conditions, a one-way ANOVA followed by a Tukey HSD test was realized using multcomp R package v. 1.4–16 (Hothorn et al., 2008). Thereafter, based on the Bray-Curtis dissimilarity index, distance based-Redundancy Analysis (db-RDA) was used to examine the relationships between soil properties and soil fungal and bacterial communities (Legendre and Anderson, 1999) using R packages pmultcomp v. 1.4–16, factoextra v. 1.0.7 (Kassambara and Mundt, 2020) and vegan v. 2.5–7. Finally, structure of soil microbial communities according to soil chemical properties was tested using PERMANOVA analyses, with 999 permutations. Significance of each variable was further tested.

## 3. Results

In total, 372940 quality-filtered fungal sequences (ITS2) from 116 soil samples and 488007 quality-filtered bacterial sequences (V4) from 117 soil samples were generated and further analysed. From these, 3221 fungal ASVs and 1572 bacterial ASVs were delineated.

### 3.1. Microbial diversity

For fungal communities within the reference chronosequence, species richness (*i.e.*, the number of observed ASVs), Chao index (*i.e.*, the expected number of ASVs), Shannon index, and Faith (PD) index were significantly lower in the open low maquis in comparison to the other native vegetation types (Table 1). Species accumulation curves corroborated this discrepancy (Fig. S5). Looking at revegetated sites, the calculated diversity indices were not significantly different to those of the open low maquis, except for the Old Nursery site 2 that showed observed and expected species richness similar to the closed low maquis and the preforest maquis (Table 1).

For bacteria, higher significant values in species richness, Chao index and Shannon index were observed in the open low maquis than in the *G. deplancheanum*-dominated maquis and preforest maquis (Table 1). This tendency was, in some extent, supported by the species accumulation curves (Fig. S5). Comparison of revegetated sites to the ecosystems of reference showed, in contrast, no clear pattern (Table 1). Finally,

overall, despite some differences in bacterial and fungal diversity between the reference open and the closed vegetations, revegetated sites harbored unclear patterns.

### 3.2. Microbial phyla composition

Approximately 50% of soil fungal ASVs were identified at the phylum level, and most of these ASVs belonged to two phyla: the Ascomycota and Basidiomycota (both representing 97.6% of the reads). A shift in the relative abundances of these taxonomic groups was observed between the open low maquis and the three others reference ecosystems (Fig. 3). Indeed, Ascomycota was found to dominate in the open maquis, whereas Basidiomycota dominated in the closed plant formations. Consequently, calculation of the Ascomycota:Basidiomycota (A:B) ratio gave higher values in the open low maquis (A:B > 1) than in the three other closed vegetations (A:B ratios <1) (Fig. 3). In the Old Nursery revegetated sites 1 and 3, as for the open low maquis, Ascomycota was found to be the most abundant phyla (A:B ratios >1) (Fig. 3). As in closed native vegetations, Basidiomycota dominated in the Old Nursery site 2 (A:B ratio < 1). For the two last revegetated sites, Kuebini and Cofremi, both phyla were encountered in equivalent proportions (A:B ratios ~ 1).

Regarding bacteria, 94.3% of ASVs were identified at the phylum level, and nine phyla dominated the communities (representing 98.7% of the reads): the Acidobacteria, Actinobacteria, Chloroflexi, Cyanobacteria, GAL15, Planctomycetes, Proteobacteria, Verrucomicrobia and WPS-2. The relative abundance of bacterial phyla varied substantially between the reference ecosystems. These differences were mainly due to changes in Proteobacteria, Cyanobacteria, and Chloroflexi relative abundances. Indeed, a higher representation of Proteobacteria and Cyanobacteria was observed in closed plant formations, while a higher relative abundance of Chloroflexi was detected in open vegetation (Fig. 1; Table S3). In most revegetated sites, both Chloroflexi and Proteobacteria were well-represented, and Cyanobacteria was essentially present at the Old Nursery site 2 and Cofremi site (Fig. 1; Table S3). To represent these discrepancies in bacterial phyla composition, a Cyanobacteria:Chloroflexi ratio was calculated, similar to the Ascomycota:Basidiomycota ratio for fungi (Fig. 3).

### 3.3. Fungal functional groups

Out of the 3221 initially delineated fungal ASVs using the FUNGuild tool, 883 ASVs (27%) were assigned to functional groups, and 596 ASVs (18.5%) were kept for further analyses (see Material and Methods). In total, 12 guilds were detected: Animal Pathogen, Arbuscular Mycorrhizal, Ectomycorrhizal, Endophyte, Ericoid Mycorrhizal, Fungal Parasite, Lichenized, Orchid Mycorrhizal, Plant Pathogen, Soil Saprotroph, Undefined Saprotroph and Wood Saprotroph (Fig. 4). Two clear patterns emerged from relative abundances of these different guilds. Saprotrophic fungi (soil saprotroph, undefined saprotroph, and woods saprotroph combined) were the most abundant functional group in the open low maquis and in three revegetated sites as well, namely the Old Nursery sites 1 and 3, and the Kuebini site (Fig. 4). In contrast, an over-representation of ectomycorrhizal fungi characterized the closed vegetations of the chronosequence of reference, and the Old Nursery site 2, and the Cofremi site (Fig. 4). Calculating of an Saprotrophic:Ectomycorrhizal fungi ratio gave values superior to 1 in the first case and inferior to 1 in the second (Fig. 4). When looking at the species richness, results for ectomycorrhizal fungi corroborated these observations to some extent (Table S4).

Focusing more deeply on ectomycorrhizal fungi, the Upset plot revealed that the Cofremi site and the Old Nursery site 2 were, by far, the two revegetated sites that showed the highest number of co-occurring species (Fig. S6). The most shared ectomycorrhizal species was encountered in six distinct conditions (Fig. S6) and was assigned to the *Pisolithus microcarpus* (Cooke & Masee) G. Cunn. species (Fig. S7).

**Table 1**

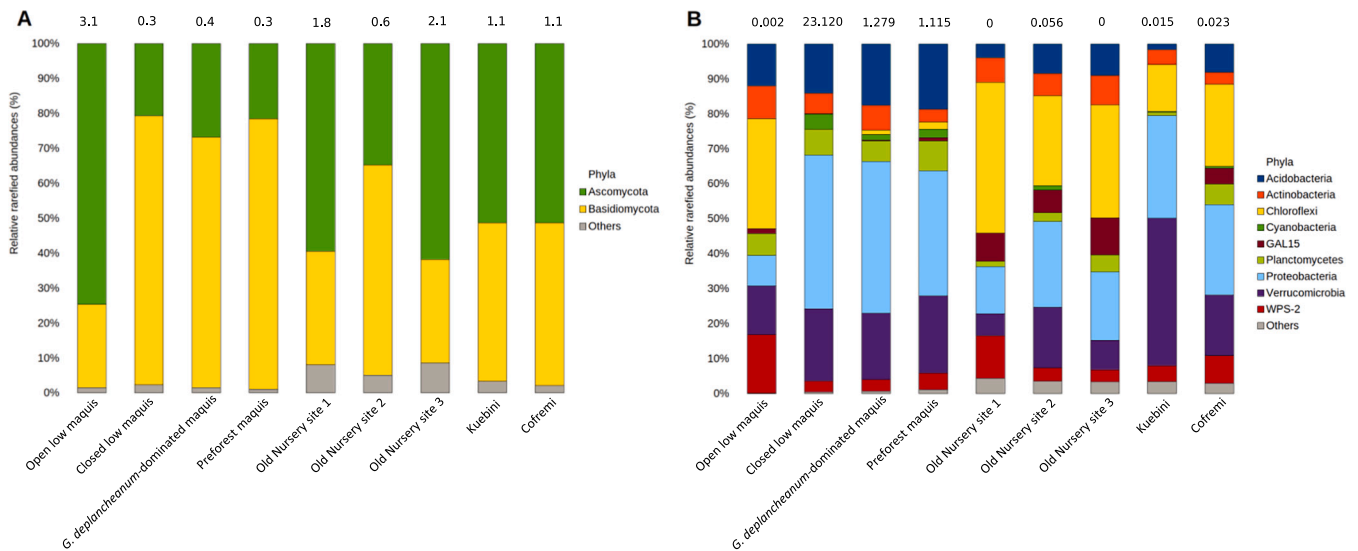
Soil fungal and bacterial diversity indices calculated for the reference chronosequence and the revegetated sites. Letters indicate differences among all pairwise comparisons using the Dunn *post hoc* test. The results of Kruskal-Wallis one-way ANOVAs are also presented.

Soil microorganism group	Formation	Number of subplots	Observed ASVs	Chao 1	Simpson (1-D)	Shannon (H)	Faith (PD)	Pielou evenness (J)	
Fungi	<i>Reference chronosequence</i>								
		Open low maquis	21	97 ± 5 a	104 ± 6 a	0.928 ± 0.008 a	4.97 ± 0.13 a	25.6 ± 1.1 a	0.757 ± 0.014 a
		Closed low maquis	22	133 ± 7 bc	141 ± 8 bc	0.947 ± 0.009 a	5.61 ± 0.16 bc	32.1 ± 1.2 bc	0.799 ± 0.018 ab
		<i>G. deplancheanum</i> -dominated maquis	24	161 ± 6 b	173 ± 7 b	0.954 ± 0.007 a	5.84 ± 0.13 b	37.2 ± 1.3 b	0.798 ± 0.014 ab
		Preforest maquis	22	131 ± 11 c	139 ± 11 c	0.935 ± 0.018 a	5.56 ± 0.22 bc	32.1 ± 2.1 bc	0.802 ± 0.024 b
	<i>Revegetated sites</i>								
		Old Nursery site 1	4	74 ± 14 a	76 ± 15 a	0.907 ± 0.016 a	4.45 ± 0.19 a	21.4 ± 3.3 a	0.730 ± 0.037 ab
		Old Nursery site 2	8	106 ± 11 ac	114 ± 13 ac	0.921 ± 0.012 a	4.84 ± 0.15 a	28.8 ± 2.2 ac	0.729 ± 0.025 ab
		Old Nursery site 3	3	79 ± 8 a	80 ± 9 a	0.920 ± 0.009 a	4.73 ± 0.15 ac	23.8 ± 1.5 ac	0.752 ± 0.024 ab
		Kuebini site	4	79 ± 13 a	83 ± 14 a	0.928 ± 0.018 a	4.80 ± 0.18 ac	24.5 ± 3.0 ac	0.773 ± 0.038 ab
		Cofremi site	8	93 ± 6 a	99 ± 7 a	0.943 ± 0.006 a	5.07 ± 0.15 ac	25.4 ± 1.2 a	0.777 ± 0.014 ab
		<i>KRUSKAL-WALLIS H test</i>	116	7.4e-09***	7.1e-09***	0.002**	1.6e-05***	1.1e-07***	0.007**
	Bacteria	<i>Reference chronosequence</i>							
		Open low maquis	22	48 ± 4 a	48 ± 4 a	0.952 ± 0.007 ab	4.95 ± 0.15 a	5.5 ± 0.3 a	0.903 ± 0.007 a
		Closed low maquis	22	37 ± 2 ab	37 ± 2 ab	0.952 ± 0.003 abc	4.73 ± 0.09 ab	4.9 ± 0.2 abc	0.919 ± 0.003 ab
		<i>G. deplancheanum</i> -dominated maquis	23	30 ± 2 b	30 ± 2 b	0.931 ± 0.007 b	4.30 ± 0.13 b	4.4 ± 0.2 bc	0.897 ± 0.007 a
		Preforest maquis	23	33 ± 3 b	33 ± 3 b	0.935 ± 0.007 ab	4.43 ± 0.14 b	4.7 ± 0.2 abc	0.904 ± 0.006 a
<i>Revegetated sites</i>									
		Old Nursery site 1	4	36 ± 4 ab	36 ± 4 ab	0.955 ± 0.003 abc	4.90 ± 0.22 ab	5.2 ± 0.4 abc	0.914 ± 0.006 ab
		Old Nursery site 2	7	39 ± 6 ab	39 ± 6 ab	0.948 ± 0.008 abc	4.53 ± 0.21 ab	5.7 ± 0.6 abc	0.909 ± 0.007 a
		Old Nursery site 3	4	46 ± 3 ab	46 ± 3 ab	0.965 ± 0.001 ac	4.53 ± 0.07 ab	5.5 ± 0.2 abc	0.924 ± 0.004 a
		Kuebini site	4	24 ± 3 b	24 ± 3 b	0.929 ± 0.012 ab	4.10 ± 0.24 b	3.5 ± 0.2 c	0.901 ± 0.011 a
		Cofremi site	8	58 ± 7 a	58 ± 7 a	0.970 ± 0.004 b	5.42 ± 0.19 a	6.1 ± 0.5 a	0.939 ± 0.004 b
		<i>KRUSKAL-WALLIS H test</i>	117	1.3e-04***	1.2e-04***	1.2e-04***	9.7e-05***	0.002**	0.002**

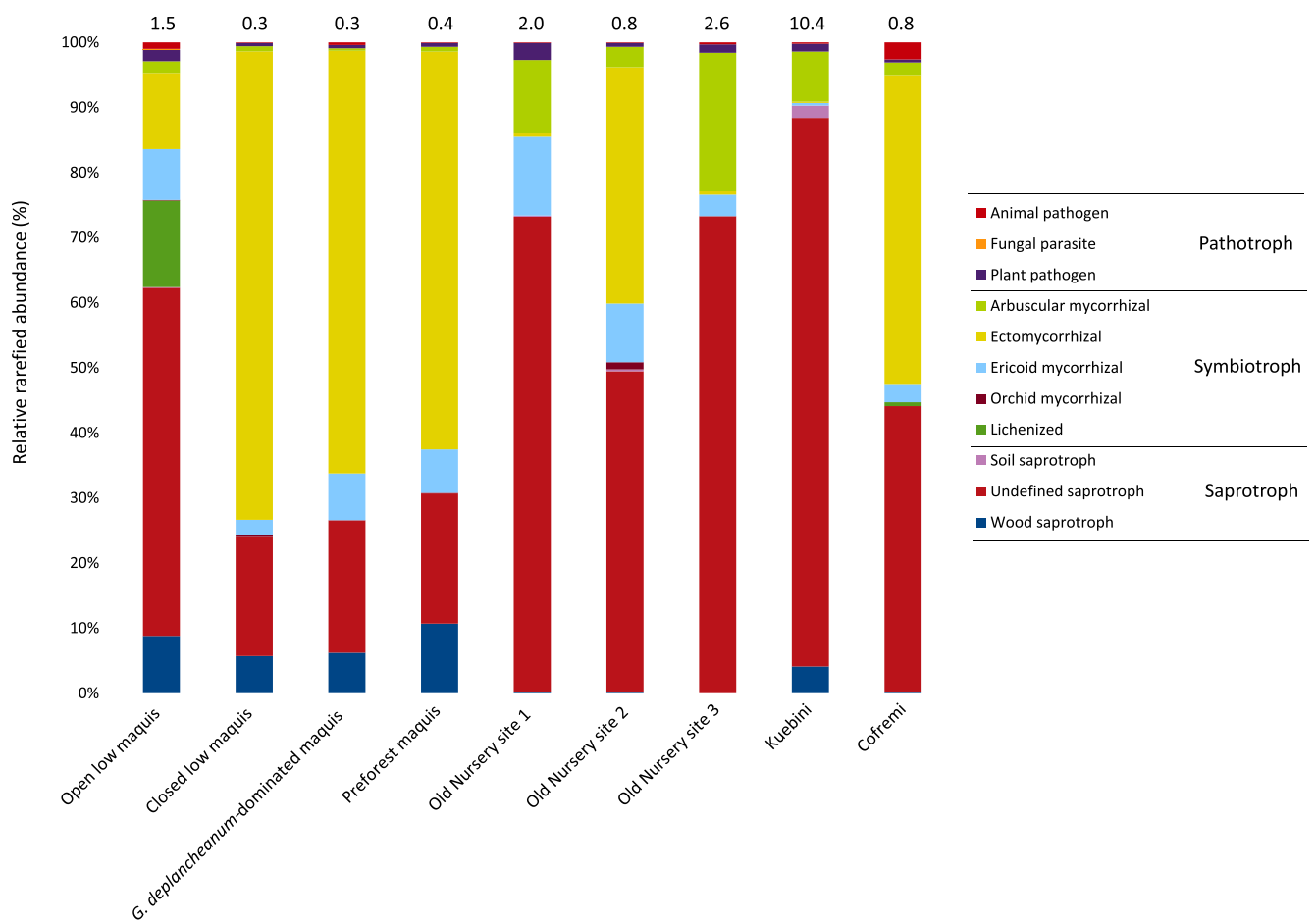
The number of 2 × 2 m subplots kept after filtering (*i.e.*, ASVs shared by at least two subplots) are shown. The standard deviation and the standard error are indicated for the observed number of ASVs (*i.e.*, the species richness) and the other indices (*e.g.*, Chao 1, Simpson, Shannon), respectively.

\*\*  $P < 0.01$ .

\*\*\*  $P < 0.001$ .



**Fig. 3.** Relative rarefied abundances of soil (A) fungal and (B) bacterial phyla across the reference chronosequence (open low maquis, closed low maquis, *Gymnostoma deplancheanum*-dominated maquis, and preforest maquis) and within the five revegetated sites studied (Old Nursery sites 1, 2 and 3, Kuebini site and Cofremi site). Ascomycota:Basidiomycota ratios for fungi (A) and Cyanobacteria:Chloroflexi ratios for bacteria (B) are indicated above bar plots.



**Fig. 4.** Fungal guild relative rarefied abundances at the four reference ecosystems (open low maquis, closed low maquis, *Gymnostoma deplancheanum*-dominated maquis, and preforest maquis) and at the five revegetated sites studied (Old Nursery sites 1, 2 and 3, Kuebini site and Cofremi site). Guilds are grouped by trophic modes (Pathotroph, Symbiotroph and Saprotroph).



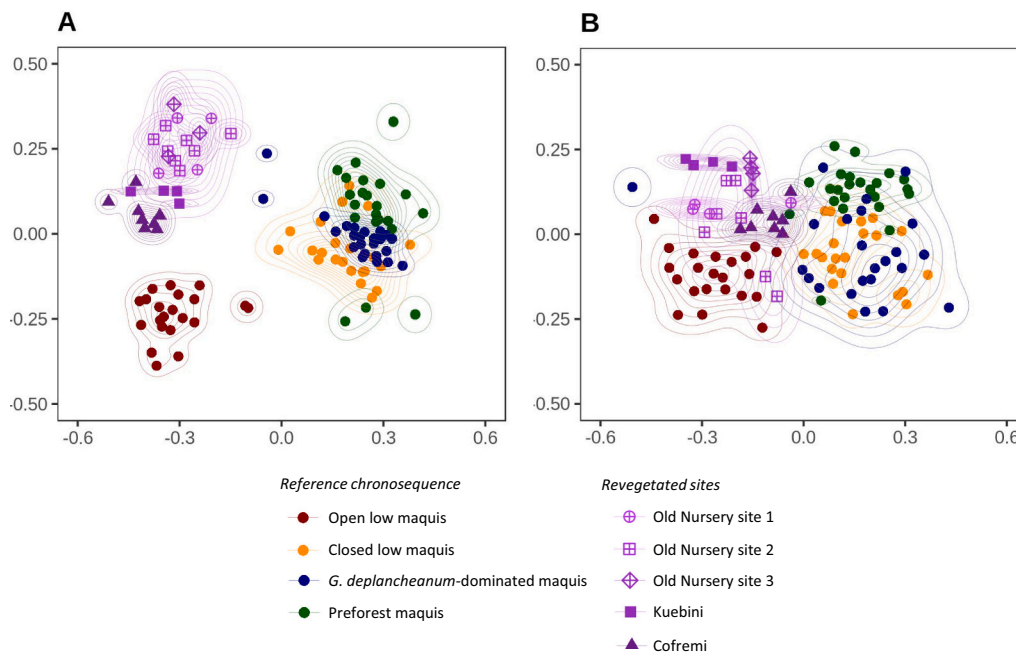


Fig. 5. NMDS ordination based on Bray-Curtis dissimilarity index for the (A) fungal and (B) bacterial soil communities. Stresses are 0.197 and 0.165, respectively.

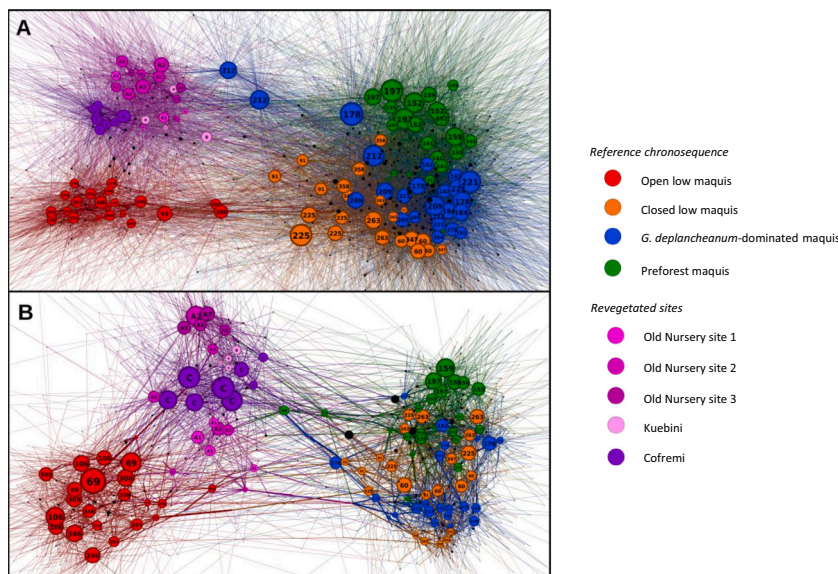


Fig. 6. Bipartite networks performed on soil fungal (A) and bacterial (B) communities. Colored nodes correspond to the subplots positioned either in the ecosystems of reference or the revegetated sites. Black nodes represent the ASVs. Numbers showed in nodes correspond to subplot names (the same name can thus appear at most four times (four 2 × 2 m subplots per 20 × 20 m plot)). The size of the nodes is proportional to the number of edges and, consequently for the subplots, to the observed species richness. For the ASV nodes, they are proportional to the number of subplots the nodes are connected to.

### 3.4. Microbial community structure

NMDS ordinations, based on the Bray-Curtis dissimilarity index, showed that for both fungi and bacteria, soil communities were distinct among them (Fig. 5). The distinction was particularly marked between the open low maquis and the rest of the native vegetations, as well as for the revegetated sites that were separated from all the reference ecosystems (Fig. 5). A certain overlap has to be noted between the three closed plant formations, i.e., between the closed low maquis, the *G. deplancheanum* maquis, and the preforest maquis. To support NMDS results, PERMANOVA analyses were carried out. A significant partitioning was observed for fungi using the Bray-Curtis dissimilarity index (51.4%,  $R^2 = 0.078$ ,  $p$ -value = 0.018) and the Jaccard dissimilarity index (51.3%,  $R^2 = 0.075$ ,  $p$ -value = 0.013). For bacteria, the result was non-significant with the Bray-Curtis dissimilarity index (50.8%,  $R^2 =$

0.073,  $p$ -value = 0.065), and significant using the Jaccard dissimilarity index (50.8%,  $R^2 = 0.072$ ,  $p$ -value = 0.047). Hence, overall, PERMANOVA analyses seem to confirm the NMDS representations.

Visualization of bipartite networks (Fig. 6) and the partition of networks into communities (which can be considered as a non *a priori* approach) (Table 2) also validated the microbial community structure observed in soil. In more details, the extraction of the community structure from the networks (Table 2) detected ten and eight communities for fungi and bacteria, respectively. Regarding fungi, the first community encompassed almost all open low maquis samples (20 out of the 21 retained), whereas from the second to the seventh community, the repartition of subplots showed a gradation along the reference chronosequence from the closed low maquis to the preforest maquis (Table 2). Concerning revegetated sites, they were mostly distinct from the chronosequence and grouped into three different communities

**Table 2**

Community partitioning based on bipartite networks for both soil fungi and bacteria. The number of subplots belonging to a given community, noted as 1 to 10, are given for each reference native vegetation and revegetated site.

Soil microorganism group	Formation	Number of subplots	Bipartite network communities													
			1	2	3	4	5	6	7	8	9	10				
Fungi	<i>Reference chronosequence</i>															
	Open low maquis	21	20	1												
	Closed low maquis	22		3	4	6	6	3								
	<i>G. deplancheanum</i> -dominated maquis	24			3	7	11	1					1	1		
	Preforest maquis	22			4	3	2	8		5						
	<i>Revegetated sites</i>															
	Old Nursery site 1	4											1	3		
	Old Nursery site 2	8											5	3		
	Old Nursery site 3	3												3		
	Kuebini site	4											4			
	Cofremi site	8													8	
Bacteria	<i>Reference chronosequence</i>															
	Open low maquis	22	22													
	Closed low maquis	22	1	2	3	6	5	5								
	<i>G. deplancheanum</i> -dominated maquis	23		1	3	1	11	7								
	Preforest maquis	23			2	1	2	16	1	1						
	<i>Revegetated sites</i>															
	Old Nursery site 1	4												4		
	Old Nursery site 2	7	1				1			3	2					
	Old Nursery site 3	4								4						
	Kuebini site	4								4						
	Cofremi site	8								3	5					

The total number of 2 × 2 m subplots kept after filtering (i.e., ASVs shared by at least two subplots) are indicated.

(Table 2). A relatively similar pattern was observed for bacteria, with all open low maquis plots belonging to one community, followed by a gradation along the chronosequence, and a clustering of revegetated sites.

In addition, two indices, the CSII<sub>norm</sub> and HAI indices, were used to measure differences in soil microbial species composition from the reference ecosystems. Although relatively low, the highest CSII<sub>norm</sub> values were observed when the comparison was made between the Old Nursery site 2 and Cofremi site to the open low maquis (0.160 to 0.246) (Table S5). This indicates that at these two revegetated sites about 16% to 25% of microbial species composition and abundance of the reference open maquis were recovered in the soil. The HAI index was high for all comparisons (> 0.95) (Table S5), indicating that most soil microbial species in revegetated sites are more abundant than in the reference ecosystems.

### 3.5. Soil chemical properties and relationships to microbial communities

Notable changes in soil properties were observed between the open low maquis and the rest of the reference ecosystems (Table S6 and Fig. S8). For instance, carbon, nitrogen, and phosphorus were drastically lower in the open maquis. Such limitations in soil nutrients were also observed at all revegetated sites (Table S6 and Fig. S8). Nevertheless, despite non-significant differences between them, a slight enhancement in organic carbon, total nitrogen and ammonium seems to take place at the Old Nursery site 2, Cofremi, and Kubeni (Table S6). Significant differences in pH were also encountered; the open native vegetation and the revegetated sites harboring the highest values (Table S6 and Fig. S8). The relationships between microbial communities and soil chemical parameters were supported by the db-RDA representations (Fig. S9) and PERMANOVA tests (for fungi: 90.6% of the variance explained,  $p$ -value = 0.019; for bacteria: 59.6% of variance explained,  $p$ -value > 0.001). Further analyses showed that the pH and total nitrogen were the significant explanatory variables explaining the variations in fungal and bacterial communities (Table S7). For bacteria, ammonium was also detected as a significant contributing variable. Despite it slightly non-significance, soil organic carbon may also be involved in structuring soil fungal communities (Table S7).

## 4. Discussion

Comparison of soil microbial communities between the reference ecosystems and revegetated sites at Goro plateau led to several key findings. In particular, clear changes were apparent in soil phyla composition for both fungal and bacterial communities, as well as shifts in soil fungal functional groups. Ratios of taxonomic and functional groups are subsequently proposed for monitoring soil development following revegetation. Notably, no clear trend was detected for microbial diversity indices (e.g., observed and expected richness), as encountered in other studies (e.g., Gellie et al., 2017; van der Heyde et al., 2020; Yan et al., 2018). This may suggest that microbial diversity, in contrast to microbial composition, is not efficient for detecting soil environmental changes in the context of ecological restoration.

### 4.1. Changes in soil fungal and bacterial phyla composition

In our study, Ascomycota and Basidiomycota were the two most abundant fungal phyla and both showed a drastic change in their relative abundances along the reference chronosequence. Two distinct patterns can be distinguished. The first consists of the dominance of the Ascomycota over the Basidiomycota and was observed in the earliest stage of the native vegetation succession, i.e., in the open low maquis. In a contrasting pattern, there was a higher representation of Basidiomycota in the following succession stages, i.e., in the closed low maquis, the *G. deplancheanum*-dominated maquis, and finally the preforest maquis, all three grouped as closed vegetation in terms of plant cover (Fig. 2 and Table S1). To report these variations in relative abundance, an Ascomycota:Basidiomycota (A:B) ratio was calculated; values superior to 1 correspond to dominance by Ascomycota and less than 1 to dominance by Basidiomycota. Our findings are consistent with the results obtained by Gourmelon et al. (2016) for another native plant succession developing on a different ultramafic soil type in the archipelago. In their study, overall, A:B ratios were found to exceed 1 in the first stages of the succession and drop below 1 in the last stages. The authors concluded that a larger representation of Ascomycota may be an indicator of ecosystem degradation (Gourmelon et al., 2016).

In our study, the five revegetated sites showed variable responses compared with the reference ecosystems. As in the closed vegetations of

the reference ecosystems, Basidiomycota dominated at the Old Nursery site 2. In contrast, similarly to the open maquis, Ascomycota was present in higher proportions in the Old Nursery sites 1 and 3 (A:B ratio > 1). At Kuebeni and Cofremi, both phyla were equally represented (A:B ~1). Recently, in southern Australia, in a study system where native plant species were planted after long-term grazing, Ascomycota were shown to decrease with revegetation age (Yan et al., 2018). In a western Australia landscape restored after mining, the same trend was observed at only one of the three sites (van der Heyde et al., 2020). The relative abundance of Ascomycota and Basidiomycota may thus be an indicator of successional trajectory, and therefore may permit evaluation of revegetation progress and success, but this may also be location dependent.

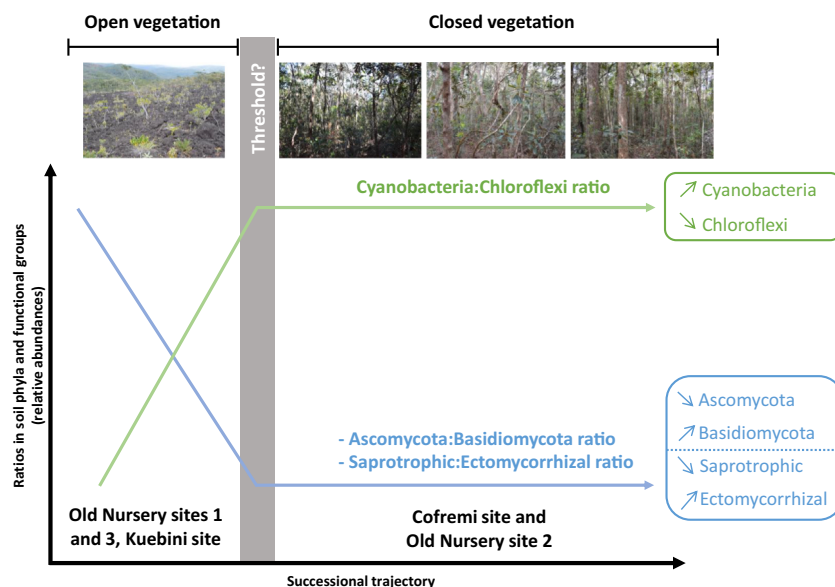
As for fungal composition, clear differences in bacterial phyla composition were observed along the reference chronosequence. These discrepancies were mainly due to the higher abundance of Chloroflexi within the open vegetation and of Proteobacteria within the closed vegetations. In addition, Cyanobacteria were mostly encountered in closed vegetations and nearly absent from the open vegetation. The large representation of Chloroflexi was characteristic of most revegetated sites, and Cyanobacteria were essentially detected only at two sites: the Old Nursery site 2 and Cofremi. Such abundances of Chloroflexi have been observed after the active revegetation of pasture lands in southern Australia (Yan et al., 2020). Interestingly, based on a meta-analysis of more than 100 articles, Trivedi et al. (2016) found that Chloroflexi was one of the dominant phyla in human-impacted lands, whereas Proteobacteria and Cyanobacteria were in higher abundances in natural systems. The authors suggested that, potentially due to their metabolic flexibility, Chloroflexi may have a competitive advantage over Cyanobacteria in nutrient-limiting environments. Supporting this idea, members of Chloroflexi phylum belonging to the Ktedonobacteriales Class dominated in cave sediments in Venezuela, a typical nutrient-poor environment (Barton et al., 2014). In our study, this Class was dominant in terms of relative abundance (61.8% of the reads) and species richness (242 ASVs detected out of the 313 delineated in the phylum). As such, the large proportion of Chloroflexi at the first stage of the reference succession and in all revegetated sites may be explained by the oligotrophic nature of the soil. Indeed, low concentrations of soil organic carbon, nitrogen, and also phosphorus characterized these plant formations. It has been suggested that the relationship between Chloroflexi and Cyanobacteria could be used as an early warning tool of soil degradation (Trivedi et al., 2016). In the present work, a Cyanobacteria:

Chloroflexi ratio has been proposed. Comparing revegetated sites a higher ratio value was obtained, in a decreasing order, at the Old Nursery site 2, Cofremi, and Kuebeni (due to the absence of Cyanobacteria, values were nulls at the Old Nursery sites 1 and 3). Despite non-significant differences in soil chemical properties among these managed areas, enrichment in soil nutrients (in carbon and nitrogen) seems, nonetheless, to occur at these three locations and could explain these differences in ratios. Supporting this trend, Proteobacteria, which is commonly associated with soil rich in organic carbon and globally categorized as copiotrophic (e.g., Fierer et al. (2007), displayed higher relative abundances and species richness at these sites (in decreasing order: at Cofremi, Old Nursery site 2, and Kuebeni).

As for Ascomycota and Basidiomycota, our findings may indicate that relative abundances of Chloroflexi and Cyanobacteria could constitute relevant biological indicators for monitoring the soil evolution after revegetation in our study system (Fig. 7). Taking both microbial communities into consideration, at this step of the investigation, we could argue that Cofremi, the Old Nursery site 2, and potentially Kuebeni might be on a good recovery trajectory.

#### 4.2. Changes in fungal functional groups: Saprotrophic versus ectomycorrhizal fungi

In addition to phyla composition, assignment to functional guilds could give further information on revegetation progress. Indeed, through a field survey of macrofungi (i.e., fungi producing visible reproductive structure), Avis et al. (2017) found that functional guild ratios, more precisely the ratio of saprophyte to ectomycorrhizal fungal richness, may allow restoration success evaluation. In our study, two unambiguous patterns arose from relative read counts. Saprotrophic fungi largely dominated in the open low vegetation of the reference chronosequence and the following revegetated sites: Kuebeni, and Old Nursery sites 1 and 3 (Saprotrophic:Ectomycorrhizal fungi ratio > 1). Conversely, ectomycorrhizal fungi were found in larger proportions in the closed maquis formations of the reference chronosequence and within the Old Nursery site 2 and Cofremi site (Saprotrophic:Ectomycorrhizal fungi ratio < 1). Although less obvious, ectomycorrhizal fungal richness gave equivalent results. Convergence to closed vegetation may suggest better success after active revegetation at these two sites. Furthermore, the different responses observed in revegetated sites that were originally subjected to different management practices (Table S1) seem to support the use of functional groups for monitoring ecological



**Fig. 7.** Synthesis of soil changes in fungal and bacterial phyla composition and fungal functional groups along the successional trajectory at Goro plateau in New Caledonia. The green arrow represents the evolution of the Cyanobacteria:Chloroflexi ratio in bacteria that increase across the ecosystem succession. The decrease in Ascomycota:Basidiomycota and Saprotrophic:Ectomycorrhizal ratios in fungi corresponds to the blue arrow. Increase and decrease for each of the microbial groups are also shown. The drastic shift occurring along the reference chronosequence between the open vegetation and the closed vegetation, and indicated as a potential threshold, is represented by a grey bar. The five investigated revegetated sites (i.e., Old Nursery sites 1, 2 and 3, Cofremi site, and Kuebeni site) are replaced according to their soil trajectory of recovery. Changes in Proteobacteria are not represented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

restoration programs. Nevertheless, using the same approach, Yan et al. (2018) did not find clear changes in fungal trophic modes (saprotrophs versus symbiotrophs) several years after planting native trees and shrubs. In another study, topsoils transfer (i.e., stripping and spreading of the soil surface) gave contrasting results, with differences in species richness of fungal functional groups observed only at one of the three studied locations (van der Heyde et al., 2020).

Differences in proportions of saprotrophic and symbiotic fungi found in our study raise questions about the relative functional roles of these two major soil groups in revegetated areas and surrounding natural ecosystems in ultramafic landscapes, particularly in terms of nutrient cycling and plant community dynamics. In ectomycorrhizal-dominated forests, ectomycorrhizal fungi have been suggested to inhibit saprophytic organisms, leading to reduced rates of litter decomposition; this phenomenon is known as the Gadgil effect (Gadgil and Gadgil, 1975; Gadgil and Gadgil, 1971). One of the potential mechanisms involved would be the ability of ectomycorrhizal fungi to decompose organic matter for nitrogen mobilization (Lindahl and Tunlid, 2015; Shah et al., 2016). Both groups may thus compete for nitrogen acquisition.

The ectomycorrhizal symbiosis might also be involved in plant community dynamics. Indeed, the presence of plants involved in this association could facilitate the establishment of heterospecific seedlings through their fungal partners and, consequently, promote plant succession. Indeed, ectomycorrhizal fungi can act as inoculum sources for the newly colonizing seedlings, and can also establish a mycelia network allowing connections and translocations of elements between plants (e.g., van der Heijden et al., 2008). Greenhouse and field experiments at Koniambo Massif, an ultramafic Massif in northern New Caledonia, showed the potential for the legume, *Acacia spirorbis* (Labill.), to behave as an ectomycorrhizal plant nurse (Houles, 2017). Therefore, better success at Cofremi might be explained by facilitation from planting this species at this site. Other ectomycorrhizal plant species could also be involved in the success at the Cofremi site, as well as at Old Nursery site 2. However, despite the abundance and diversity of ectomycorrhizal fungi at Goro plateau, only 14 species are known to be involved in this mutualistic association in the archipelago (Amir and Ducouso, 2010; Carriconde et al., 2019). Of these 14 species, in addition to *A. spirorbis*, only one *Tristaniopsis* species was recorded in very low abundance from our revegetated plots (*T. guillainii* Vieill. ex Brongn. & Gris), and three species were observed in the surrounding native vegetation (*T. guillainii*, *T. calobuxus* (Brongn. & Gris) and *T. macphersonii* (J.W.Dawson)) (Table S2; Y. Ititiaty, pers. comm.). We consequently do not know to what extent ectomycorrhizal plants may influence vegetation structure and dynamics in this area, as suggested in other vegetation types in New Caledonia (Carriconde et al., 2019; Demenois et al., 2017a). Further determination of plant mycorrhizal status is required. Moreover, our results suggest the possible value of using *A. spirorbis* in revegetation, a shrub and tree recommended in the past but put aside for its dominance and invasive characteristics in degraded lands, as well as the observed absence of natural regeneration in its understorey (L'Huillier et al., 2010; Meyer et al., 2006). However, its use might be valuable in very low density and in ecological conditions that do not favor persistence and expansion (e.g., in relation to altitude and type of soil). Additional research on the community ecology of *A. spirorbis*-dominated stands will enable a better understanding of functional processes within this plant formation.

Finally, in regard to ectomycorrhizal fungi, one particular species has been recovered from four revegetated sites out of the five (i.e., from the Old Nursery sites 1, 2 and 3, and Cofremi) and two of the native vegetations (the low open maquis and the *G. deplancheanum*-dominated maquis) (Fig. S7). This species is *Pisolithus microcarpus*, a taxon whose fruit bodies (i.e., the reproductive structures) are commonly found on ultramafic soils in open or degraded areas in the southern massif in New Caledonia (F. Carriconde, pers. obs.) (Fig. S10), and originally described from Australian specimens (Cunningham, 1944 in Anderson et al., 1998). In a greenhouse experiment, Demenois et al. (2017b) showed the

substantial benefits of this native ectomycorrhizal fungus for inoculating plant roots to aid revegetation. After less than one year, inoculation of Myrtaceous endemic plant species increased plant growth, modified root structure and enhanced soil aggregate stability, i.e., reduced the susceptibility of the soil to erosion. Remarkably, the current study, through the detection of *P. microcarpus* in the soil, confirms the significance of using this ectomycorrhizal fungus as an inoculum source. This supports the idea that future revegetation programs could incorporate soil eDNA metabarcoding for selecting ectomycorrhizal strains, or other strains from other microbial groups (e.g., endomycorrhizal fungi, or nitrogen fixing bacteria), for plant inoculation purposes.

#### 4.3. Differences in soil microbial community structure at the species level

In accordance to what is expected in an ecological succession, microbial community structure analyses (NMDS ordinations, bipartite networks, and community assignments) showed a gradual progression from the open low maquis to the preforest maquis. Clearly, revegetated sites were all dissimilar to these reference ecosystems. Hence, our results emphasize that revegetated sites harbor distinct molecular species (here, ASVs), and/or differences in abundances. These dissimilarities were supported by the calculation of the CSII<sub>norm</sub> and HAI indexes, comparing the proportion of the species abundance between revegetated and reference systems. Despite differences in soil species composition, equivalent functional roles seem to be recovered from these species. Indeed, taking the Old Nursery site 2 and Cofremi site identified as the most successful sites (see discussion above), fungal species differed from those of the closed vegetations, whereas the same patterns were observed in functional groups (e.g., dominance of ectomycorrhizal fungi). These observations could lead us to the following question: is the aim to recover the original species diversity or the functional profile in soil, or both? The answer depends on the targets set by the stakeholders, which themselves depend on the current knowledge of soil biodiversity.

We note that our study system allowed us to determine the status of soil communities at a given time, e.g., 27 and 17 years after revegetation at Cofremi and the Old Nursery, respectively, but not how soil microbial species have gradually shifted with the revegetation age. To the best of our knowledge, despite the relevant number of programs set up on ultramafic soils in New Caledonia (Amir et al., 2018), monitoring of revegetated sites at different times over a long period (at least a few years), or when using other practices, such as topsoil transfer, has never been performed. Detailed and careful monitoring is, therefore, an important priority for future work in this biodiversity hotspot in particular, and for the understanding of ecological restoration processes in general.

## 5. Conclusion

Investigating soil microbial communities via high-throughput amplicon sequencing of eDNA has undoubtedly revealed changes in phyla and functional groups along the reference chronosequence, as well as variable responses at the different revegetated sites studied. Two patterns emerged from our analyses (Fig. 7). The first, encountered in the open low maquis and in three revegetated sites, i.e., at Kuebini and the Old Nursery sites 1 and 3, is essentially characterized by higher proportions of Ascomycota and saprotrophic fungi, and a lower abundance of Cyanobacteria. The second, encountered in the closed native vegetations and two revegetated sites, i.e., Cofremi and the Old Nursery site 2, is mainly characterized by the dominance of Basidiomycota and ectomycorrhizal fungi, and higher relative abundances of Cyanobacteria (Fig. 7). Based on these results, we could argue that the latter two revegetated sites are on a good trajectory of recovery. We proposed three distinct ratios as metrics for monitoring the restoration trajectory of soil microorganisms (Fig. 7). A validation of these microbial metrics would be necessary by testing them in different restoration conditions in the archipelago (for instance, when topsoil transfer is undertaken).

Nevertheless, we suggest that our findings, combined with recent studies undertaken in southern and western Australia in different environmental situations (e.g., in terms of climate, type of soil, and plant community) (Gellie et al., 2017; van der Heyde et al., 2020; Yan et al., 2020; Yan et al., 2018), underpin the great promise of soil microbial eDNA metabarcoding for monitoring restoration progress and success. In the near future, with the emergence of these new cost-effective and scalable sequencing technologies, soil microbes could potentially be included in guidelines for restoration operations in comparison and complement to more conventional approaches (e.g., botanical inventories). In addition, we showed that metabarcoding of soil eDNA could contribute to identifying suitable symbionts for plant inoculation purposes, and could consequently guide future revegetation protocols. Finally, for a more global and comprehensive understanding of soil functioning in a restoration context, other soil organisms, including soil engineers such as earthworms or termites (Fig. S11), could also be targeted (Basset et al., 2020; Bienert et al., 2012). Ultimately, the combination of an eDNA metabarcoding approach (encompassing a large array of soil organisms) with a soil metatranscriptomics approach (Carvalho et al., 2012), could provide relevant information on the mechanisms taking place at the ecosystems level (e.g., in terms of the food web), and consequently help monitor and optimize restoration operations.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecoleng.2021.106416>.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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