High soil phosphorus levels overrule the potential benefits of organic farming on arbuscular mycorrhizal diversity in northern vineyards

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Abstract

Organic farming is a key approach to reconcile food production, biodiversity conservation and environmental sustainability. Due to reduced inputs of agrochemicals, the success of organic farming is heavily dependent on the ecosystem services provided by the soil microbial community, and in particular by arbuscular mycorrhizal fungi (AMF). Numerous studies have already shown that also grapevines (*Vitis vinifera*) depend on AMF for normal growth and development. To what extent organic agriculture benefits the AMF communities on vines at regional scales, however, is still poorly understood. Here, we first quantified the relative importance of organic management, soil chemical characteristics, and geography on vineyard AMF diversity and community composition. Second, we tested whether soil nutrients fundamentally change the host-AMF community dynamics through changing universality of dissimilarity overlap curves. To identify AMF communities, we used high-throughput pyrosequencing on 170 root samples from grapevines originating from 18 conventionally and 16 organically managed Belgian and Dutch vineyards. We found no differences in AMF diversity between conventionally and organically managed vineyards. Soil phosphorus content and soil acidity, however, was strongly negatively associated with AMF diversity. Together with management type (organic vs. conventional), these two soil variables did also explain most of the variation in AMF community composition. The observed accumulation of soil copper, used to control fungal diseases, especially in organically managed vineyards, did not affect AMF communities. We observed, however, that copper concentration in the soil increased with vineyard age, indicating copper accumulation in the soil over time. AMF communities showed a regularity in interactions among taxa and their host. Under high soil P availability, however, interactions became more irregular. The potential benefits of organic vineyard management in terms of a high diversity of AMF are highly compromised by elevated soil phosphorus levels which may jeopardize the role of these symbionts in improving plant health and soil fertility.

Decreasing nutrient inputs, even organic, is a key step in developing diverse AMF communities in vineyards.

Keywords: AMF; biodiversity; bordeaux mixture; copper; eutrophication; vitis vinifera;

1 Introduction

The use of high-yielding crop varieties, chemical fertilizers and pesticides in combination with mechanization have dramatically increased worldwide agricultural production since the 1950s. At the same time, the application of agrochemicals has resulted in the eutrophication and contamination of soil and water, and has severely simplified agricultural ecosystems in terms of their species richness (Tilman *et al.*, 2001; Geiger *et al.*, 2010). As there is compelling evidence that biodiversity benefits the provision of a range of ecosystem services (Cardinale *et al.*, 2012), this simplification can be expected to jeopardize the ecosystem services delivered by agricultural ecosystems. It is in this context that organic farming has been proposed as a key approach to reconcile food production, biodiversity conservation and environmental sustainability. As organic farming practices exclude the use of chemical fertilizers and pesticides, it heavily relies on natural biological processes for both the nutrient supply and the protection of the crops grown (Tittonell, 2014). Therefore, the soil microbial community is vital for the success of organic farming and for the functioning of agroecosystems in general (Bowles *et al.*, 2016). Particularly arbuscular mycorrhizal fungi (AMF) are important components of the soil microbial community in agricultural ecosystems as they contribute to plant health and soil fertility (Rillig *et al.*, 2016). As compared to other crop species, the AMF communities that associate with grapevine (*Vitis vinifera*) may be of even greater importance because they may contribute to the microbial terroir of vines, providing distinct characteristics to the grapes and the wine produced (Trouvelot *et al.*, 2015).

AMF are key components in agricultural ecosystems and form a symbiosis with the majority of the land plants. In return for plant photosynthates, AMF provide a range of benefits to the host through their extraradical hyphal network, which acts as a living interface between the roots and the soil. Numerous studies have already shown that also grapevines depend on AMF for normal growth and development (reviewed in Schreiner, 2005). AMF mainly increase phosphorus (P)

and nitrogen (N) uptake by grapevines, but increased uptake of other nutrients, such as zinc, copper, potassium and calcium have been reported as well (Schreiner, 2005). AMF can also enhance grapevine tolerance to abiotic stress conditions, such as drought (Valentine *et al.*, 2006), salinity (Belew *et al.*, 2010) or heavy metals (Karagiannidis and Nikolaou, 2000). These effects are thought to partly stem from systemic plant responses that are associated with marked changes in secondary metabolite composition of tissues (Doehlemann *et al.*, 2014). Furthermore, AMF can protect grapevine from soil-borne pathogens (Hao *et al.*, 2012) and stabilize the soil through entangling soil particles with their hyphae (Rillig and Mummey, 2006). Given both their potential importance for developing a microbial terroir and the reported beneficial effects of AMF on grapevine, it is crucial to understand how organic vineyard management practices and local soil characteristics can influence AMF communities in the roots of grapevine, across larger geographical scales.

Organic agriculture has been shown to increase the diversity of AMF in many crop species (e.g. Verbruggen *et al.*, 2010). How organic agriculture affects AMF diversity in grapevine, however, is hardly known (only from small scaled studies using microscopic analysis or genetic fingerprinting techniques, e.g. Balestrini *et al.*, 2010; Likar *et al.*, 2013). Furthermore, high fertilizer inputs have widely been recognized to negatively affect AMF abundance in a large variety of crop and plant species (Jansa *et al.*, 2009). Also in grapevine, it has been shown that high soil P levels reduce root colonization of specific AMF taxa (Karagiannidis and Nikolaou, 1999), whereas N fertilization suppressed colonization and sporulation of specific AMF taxa (Karagiannidis *et al.*, 2007). How entire AMF communities in grapevine change with increasing soil P or N levels, and to what extent the AMF community shows signs of an altered dynamic in response to high nutrient levels, is still poorly known. Since the end of the nineteenth century, copper sulfate (Bordeaux mixture) has been used in vineyards to control vine fungal diseases, such as Downy mildew (*Plasmopara viticola*). Copper based fungicides are currently also the only

allowed way to control plant pathogenic fungi in organically managed vineyards. The practice has resulted in a widespread accumulation of copper in the soil. Whereas normal background concentrations of copper range from 5-30 mg kg^{-1}, copper concentrations ranging from 100 up to 1500 mg kg^{-1} have been measured in European vineyards with a long history of copper-based fungicide use (Flores-Vélez *et al.*, 1996). High soil copper concentrations have been shown to negatively affect a wide range of soil biota in agricultural ecosystems (e.g. Van Zwieten *et al.*, 2004), but to what extent copper affects AMF communities is still unknown.

Recent advances in microbiome bioinformatics have greatly increased the toolbox at our disposal to test for patterns in metagenomic data that can inform us on underlying community ecological processes. One such tool is the recently formulated and successfully applied dissimilarity overlap curve (DOC) (Bashan *et al.*, 2016), which analyzes the relationship between overlap in community composition and the dissimilarity in relative abundances of all taxa. A negative slope in the high-overlap region of the DOC indicates a regularity in interactions among taxa and their host, *i.e.* 'universality'. In contrast, the absence of this relationship indicates that interactions are irregular, or 'individual' based. Applying DOC analysis to AMF communities interacting with grapevines informs us on the universality of hosts interacting with their AMF symbionts, which has been found to commonly occur in other host-microbe interactions such as those between humans and gut and mouth microbiomes that display pronounced universal dynamics (Bashan *et al.*, 2016). This further allows us to assess whether the host-AMF community interaction is resistant to the disturbance imposed by high nutrient levels in terms of its stability across individual grapevines.

Here, we applied high-throughput pyrosequencing on 170 root samples from grapevines originating from 18 conventionally and 16 organically managed vineyards in northern Belgium and the southern part of The Netherlands and aimed to (i) evaluate the benefits of organic farming on the AMF communities present; (ii) quantify the relative importance of soil chemical

variables, including nutrients and copper, and management type (conventional *vs.* organic)) on AMF diversity and community composition; and (iii) test whether soil nutrients fundamentally change the host-AMF interaction through changing universality of dissimilarity overlap curves.

2 Materials and methods

2.1 Study sites and sampling

The study was conducted in Flanders, the northern part of Belgium, and the most southern part of the Netherlands. Annual average precipitation is 785 mm and average annual temperature is 9.8°C. A total of 34 vineyards were examined within this study (average distance between vineyards was 87.9 km, minimal 1 km, maximal 223 km) (Supporting information Fig. S1 and Table S1). Three vineyards were located in the Netherlands, just across the Flemish border (Vineyard 30, 31 and 32) (Supporting information Fig. S1). A stratified random sampling design, stratified by the type of management, was used. We sampled 18 conventionally and 16 organically managed vineyards (Supporting information Fig. S1). In the organic vineyards, no chemical fertilizers, or pesticides were used since transformation to organic management. However, organic fertilizers and small amounts of copper-based fungicides to control Downy mildew (*Plasmopara viticola*) were allowed. Planting density or plant age did not differ between both types of vineyards. All grapevines were grafted on SO4 rootstocks, a frequently used rootstock for commercial grapevine production. In October 2015, roots from five randomly chosen grapevines per vineyard were excavated. Root samples were collected at three random locations around each grapevine and were pooled afterwards to obtain one pooled root sample per grapevine. Especially fine roots were collected, as these are known to contain AMF. A soil sample for chemical analysis was also collected near each sampled individual. Root samples were stored at 4 °C until further analysis. Soil samples were stored at 4 °C for maximum one week to prevent nitrogen loss. Preservation at 4 °C slows down microbial mediated denitrification to the

143 extent that virtually no nitrogen is lost in the sample in the time span of one week. In total, 170
144 root and 170 soil samples across the 34 vineyards were obtained.

145 **2.2 Soil chemical analysis**

146 Soil pH was quantified using a pH probe in a 1:10 soil/water mixture. As a measure of the plant-
147 available N content of the soil, ammonium and nitrate availability were quantified by shaking 10
148 g of soil in 200 mL of 1 M potassium chloride solution for one hour. Extracts were analyzed
149 colorimetrically using a segmented flow auto analyzer (Skalar, Breda, the Netherlands). As a
150 measure of the plant-available P content of the soil, Olsen P values were quantified by shaking
151 2 g dry soil for 30 minutes with 0.5 M sodium bicarbonate at pH 8.5 and subsequent colorimetric
152 analysis of the extracts using the molybdenum blue method (Robertson *et al.*, 1999). Organic
153 carbon content was quantified by shaking 10 g of soil in an excess volume of 0.27 M potassium
154 dichromate and 18 M sulfuric acid at a temperature of 135 °C. Extracts were analyzed
155 colorimetrically. Copper concentration in the soil was measured by digesting 50 mg of dried and
156 sieved soil with 7.5 ml concentrated hydrochloric acid and 2.5 ml concentrated nitric acid. The
157 digested solution was diluted to 10 ml and measured with ICP-OES.

158 **2.3 DNA extraction, PCR amplification and pyrosequencing**

159 Root samples (which were approximately 15 cm long) were cut in 1 cm pieces and rinsed twice
160 with sterile distilled water. For each sample, 0.1 g root material was used to extract DNA, using
161 the UltraClean Plant DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA)
162 according to the manufacturer's instructions. Subsequently, the obtained DNA was diluted 10
163 times prior to PCR amplification. PCR amplification was performed using primer pair
164 AMV4.5NF-AMDGR (Sato *et al.*, 2005), as this primer pair is highly AMF specific and is able
165 to consistently describe AMF communities using 454 pyrosequencing based on the most
166 variable part of the small subunit (SSU) rRNA gene region (Van Geel *et al.*, 2014). 'Fusion'
167 primers, required for the 454 process, were designed according to the guidelines for 454 GS-

168 FLX Titanium Lib-L sequencing containing the Roche 454 pyrosequencing adapters and a
169 sample-specific MID barcode in between the adapter and the forward primer. In total, 57 MID
170 barcodes (recommended by Roche, Mannheim, Germany) were used for sample-specific
171 amplicon tracking of all 170 root samples. PCR reactions were performed on a Bio-Rad T100
172 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15
173 mM of each dNTP, 0.5 µM of each primer, 1x Titanium *Taq* PCR buffer, 1U Titanium *Taq*
174 DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. Before
175 amplification, DNA samples were denatured at 94°C for 2 min. Next, 35 cycles were run,
176 consisting of 45 s at 94°C, 45 s at 65°C and 45 s at 72°C, followed by a final elongation of 10
177 min at 72°C. After resolving the amplicons by agarose gel electrophoresis, amplicons within the
178 appropriate size range were cut from the gel and purified using the Qiaquick gel extraction kit
179 (Qiagen, Hamburg, Germany). Purified dsDNA amplicons were quantified using the Quant-iT
180 PicoGreen® dsDNA Assay Kit and the Qubit fluorometer (both from Invitrogen, Ghent,
181 Belgium), and pooled in equimolar quantities over three amplicon libraries, each representing
182 57 samples tagged with a unique MID barcode. The quality of the amplicon libraries was
183 assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The
184 amplicon libraries were each loaded on a $1/4^{th}$ of a 454 Pico Titer Plate and pyrosequencing was
185 performed using the Roche GS-FLX instrument and Titanium chemistry according to the
186 manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

187 **2.4 Bioinformatics**

188 Sequences obtained from the 454 pyrosequencing run were clustered into operational
189 taxonomic units (OTUs) using the UPARSE algorithm, following the recommended pipeline
190 (Edgar, 2013). First, quality filtering of the reads was performed with the 'fastq_filter' command,
191 allowing a maximum expected error of 0.5 for the individual sequences. In order to optimize
192 the number and length of retained sequences, truncation length was set to 225 bp. Next, the

sequences were dereplicated and sorted by abundance. Subsequently, singletons, i.e. sequences only occurring once in the entire dataset, were removed prior to clustering as this has been shown to improve the accuracy of diversity estimates (Brown *et al.*, 2015). Then, sequences were clustered into OTUs defined at 97% sequence similarity, which is commonly used to define SSU-based OTUs in AMF, with the 'cluster_otus' command. In this step, chimeric OTUs predicted by the *de novo* method built from more abundant reads were discarded as well. However, as advised by Edgar (2013) all obtained OTUs were double-checked for chimeric sequences against the MaarjAM database (Öpik *et al.*, 2010) using the 'uchime_ref' command. OTUs were assigned to a taxonomic identity by querying the representative sequence (as determined by the 'cluster_otus' command) against GenBank using the BLAST algorithm (Altschul *et al.*, 1990). Taxonomic assignments were considered reliable when a ≥200 BLAST score value was found (Lumini *et al.*, 2010). OTUs not belonging to the Glomeromycota or having a BLAST score lower than 200 were discarded. To accurately identify the obtained AMF OTUs, the representative sequence for each OTU was also queried against the MaarjAM database (Öpik *et al.*, 2010; accessed April 13, 2016), a database that aims to provide a quality-controlled repository for published sequence data from Glomeromycota.

2.5 Data analysis and statistics

To assess the adequacy of the sampling effort, rarefaction curves were made in MOTHUR (Schloss *et al.*, 2009) for all 34 vineyards, and for all conventional and organic vineyards separately, using a re-sampling without replacement approach. AMF richness was determined as the number of AMF OTUs present in a sample. AMF diversity was approximated by the Shannon diversity index (H) and was calculated using the 'summary.single' command in MOTHUR. Shannon diversity was exponentially transformed (Exp(H)) (Jost, 2006). Subsequently, a set of spatial predictors were calculated from the geographical coordinates of the vineyards by principle coordinates of neighbor matrices (PCNM), using the 'pcnm' function

of the R-package Vegan (Borcard and Legendre, 2002). Next, we explored whether conventionally and organically managed vineyards differed in soil chemical composition using linear mixed models in SPSS 22.0 (SPSS Inc., Chicago, IL), with the soil variables as the dependent variables, and management as the fixed factor. Because five samples were taken within a vineyard, we included 'vineyard' as a random factor to account for pseudoreplication. Next, we used linear mixed models with a forward selection procedure to test for relationships between AMF richness and diversity, soil chemical variables, management and the spatial PCNM variables. To account for sequencing depth and pseudoreplication, 'sequencing depth' (covariate) and 'vineyard' (random factor) were also included in the model.

To test for relationships between AMF community composition (i.e. presence/absence of certain OTUs in the AMF community), soil chemical variables, management type and geography, we performed a non-metrical multidimensional scaling (NMDS) on the sample * OTU matrix, using Bray-Curtis distances based on presence/absence data (R- package Vegan, Oksanen *et al.*, 2016). Subsequently, soil chemical variables, management type and PCNM variables were fitted onto the ordination and tested for significance based on a permutation test with 1000 iterations, using the function 'envfit' (Vegan package).

We took two further approaches to evaluate AMF community patterns in response to local environments. First, we tested whether AMF OTUs detected in OTU-poor vineyard are a subset of the OTUs found in OTU-rich vineyards through estimating the degree of nestedness. This was done using BINMATNEST (Rodriguez-Girones and Santamaria, 2006) which calculates the matrix temperature, a measure of nestedness varying between 0° (perfectly nested) and 100° (perfectly non-nested). The significance of nestedness was tested using default input parameters and null model 3. Almeida-Neto *et al.* (2008) demonstrated that matrix temperature may be sensitive to both matrix size and shape, and designed a new metric for nestedness analysis to overcome these flaws. This metric is based on overlap and decreasing fill (NODF) and was

243 calculated using the software package ANINHADO (Guimarães and Guimarães, 2006). To test
244 the significance of nestedness, two different randomization models were used. In the first model
245 (ER) presences are randomly assigned to any cell within the matrix. In the second model (CE)
246 the probability of each cell being occupied depends on the number of presences in the row and
247 column (Almeida-Neto *et al.*, 2008). The CE model allows us to test for statistical significance,
248 given that some vineyards have higher diversity and some taxa are more common than others.
249 In order to assess the relation between the nestedness of the AMF communities, management
250 and soil chemical variables, a Spearman rank correlation coefficient was calculated between the
251 position of the vineyards in the maximally stacked matrix and the soil chemical variables. A
252 Mann-Whitney U test was performed to test for a significant difference in position of the
253 vineyards in the maximally stacked matrix between both management types. Finally, to test
254 whether variation in sequencing depth affected the degree of nestedness (Ulrich and Almeida-
255 Neto, 2012), we rarefied all vineyards to the lowest sequencing depth and recalculated the
256 NODF metric.

257 Second, we applied dissimilarity-overlap curve (DOC) analysis, a novel method recently
258 developed by Bashan *et al.* (2016), to test for universal patterns in AMF community-host
259 interactions. Based on our results that soil P had a major effect on AMF community
260 composition, we first divided all our 170 samples in two groups: the high-P samples (P-levels >
261 median P) and the low-P samples (P-levels < median P) (median P = 44.01 mg/kg). For both
262 sample groups, we calculated the overlap and dissimilarity of all the sample pairs and plotted
263 each pair in the dissimilarity-overlap plane. Next, we performed non-parametric regression and
264 bootstrap sampling to calculate the dissimilarity-overlap curve (DOC) and its confidence
265 interval. To test whether the DOC displays a negative slope in the high-overlap region, one-
266 tailed P values are calculated as the fraction of 200 bootstrap realizations with a non-negative

slope, and adjusted for multiple comparisons with the Benjamini-Hochberg procedure (for more details see Bashan *et al.*, 2016).

3 Results

3.1 Pyrosequencing

For all 170 samples together, pyrosequencing resulted in a total of 450 334 filtered reads, with a minimal length of 225 bp and containing the correct barcode and primer sequence. Further taxonomic assignment revealed the presence of 129 782 (28.8 %) Glomeromycota reads, ranging from 8 to 3969, and an average of 763 AMF reads per sample.

3.2 AMF diversity

In total, 123 AMF OTUs were detected. The majority of OTUs belonged to the Glomeraceae (72.4 %, 89 OTUs, 119 472 sequences) and Claroideoglomeraceae (15.4 %, 19 OTUs, 9 443 sequences), whereas only a few OTUs belonged to the Gigasporaceae (5.7 %, 7 OTU, 406 sequences), Diversisporaceae (2.4 %, 3 OTU, 143 sequences), Acaulosporaceae (1.6 %, 2 OTU, 20 sequences), Paraglomeraceae (1.6 %, 2 OTU, 291 sequences) and Archaeosporaceae (0.8 %, 1 OTU, 7 sequences) (Supporting information Table S2 and S3). The rarefaction curves tended to saturate for almost all vineyards (Supporting information Fig. S2), and cumulative AMF richness ranged from 16 to 62 OTUs per vineyard (Supporting information Table S1). In total, 119 OTUs were observed in the organic vineyards compared to 112 OTUs in the conventional vineyards (Supporting information Fig. S3).

The relative size (highest value divided through the lowest value) of the sampled soil gradient was 1.43 for pH (logarithmic scale), 22.08 for Soil N, 41.53 for Olsen P, 34.12 for organic carbon content, and 29.75 for soil copper. The mixed model to test whether the soil chemical variables differed between management types revealed no significant differences (Table 1). The mixed model with forward selection revealed Olsen P and pH as the only variables significantly related

291 to AMF richness and Exp(H) (Table 2) (Fig. 1 and 2). Soil copper, management type and PCNM
292 variables, were not selected in both models (Fig. 3). No effect of time since conversion to
293 organic management on AMF diversity was found.

294 **3.3 AMF community composition**

295 The NMDS permutation test revealed organic vineyards to harbor significantly different AMF
296 communities as compared to conventional vineyards (Table 3, Fig. 4). From the soil chemical
297 variables, only Olsen P and pH contributed significantly to AMF community composition
298 (Table 3). No significant relationships could be found between AMF community composition
299 and nitrogen, organic carbon or copper concentrations in the soil (Table 3). PCNM2 was the
300 only spatial variable that was significantly related to AMF community composition (Table 3,
301 Supporting information Fig. S4).

302 **3.4 Nestedness**

303 The distribution of AMF OTUs showed a nested pattern, as indicated by a matrix temperature
304 of 36.8, which was significantly lower than expected by chance ($P < 0.001$). In agreement, the
305 matrix NODF(Er) was 37.85 ($P < 0.001$) and NODF(Ce) was 44.91 ($P < 0.001$), indicating that
306 the matrix was significantly more nested than expected by chance. The row and column
307 permutated presence/absence vineyard-OTU matrix closest to perfect nestedness is shown in
308 Fig. 5. A Mann-Whitney U test revealed no significant difference in position in the stacked
309 minimum temperature matrix between conventional and organic vineyards ($P = 0.88$). In
310 contrast, matrix position significantly correlated with Olsen P (Spearman's rank, $r = 0.372$, $P =$
311 0.030) and not with pH, nitrogen, organic carbon and copper in the soil. Therefore, vineyards
312 with higher P availability harbored increasingly nested AMF communities. Finally, to test
313 whether variation in sequencing depth affected the degree of nestedness, we rarefied all
314 vineyards to the lowest sequencing depth, i.e. 1471 sequences per vineyard. Subsequently, the

matrix NODF(Er) was 34.8 ($P < 0.001$) and NODF(Ce) was 39.95 ($P < 0.001$), indicating sequence depth had little or no effect on the degree of nestedness.

3.5 DOC analysis

DOC analysis for both the high-P samples and the low-P samples yielded different results (Fig. 6). The DOC of the high-P samples is nearly flat in the high-overlap region and shows broad confidence intervals ($P = 0.383$). In contrast, the DOC of the low-P samples displays a pronounced negative slope in the high-overlap region ($P < 0.001$), consistent with 'universal' dynamics. In general, the DOC of the low-P samples shows higher dissimilarity levels compared to the DOC of the high-P samples.

4 Discussion

This is the first study characterizing AMF communities in organically and conventionally managed vineyards across a regional scale using a next-generation sequencing approach. The few studies that have investigated management effects on AMF communities in vineyards were either performed on a very small scale or used fingerprinting methods, which may lack sufficient resolution to thoroughly characterize AMF communities (Balestrini *et al.*, 2010; Lumini *et al.*, 2010; Likar *et al.*, 2013). Although several studies have shown that organic farming can increase AMF diversity in agricultural settings (e.g. Verbruggen *et al.*, 2010; Van Geel *et al.*, 2015), we found no differences in AMF diversity between organically and conventionally managed vineyards. Instead, plant-available P content of the soil and pH were the only variables significantly related to AMF diversity. Soil P content and pH, however, were similar in both organically and conventionally managed vineyards. Although no chemical fertilizers are allowed in organically managed orchards, still high levels of available P occurred in these vineyards. The two vineyards with the highest available P content in the soil (vineyard 26 and 34, Supporting information Table S1) were both managed organically. Therefore, organic management is no

guarantee for high AMF diversity, as organic fertilization can still lead to high plant available P levels in the soil. This can overrule any beneficial effects of organic management, and consequently still result in a low AMF diversity. This negative relationship between AMF diversity and available P content in the soil was also found in apple orchards in Belgium (Van Geel *et al.*, 2015) and maize fields in northern China (Xiang *et al.*, 2014). Karagiannidis and Nikolaou (1999) also showed that high phosphorus inputs reduced AMF root colonization in vineyards. High P in the soil can increase the competition among AMF taxa or suppress certain AMF taxa. Phosphorus enrichment through fertilization will reduce plant allocation to roots and consequently the mycorrhizal symbiosis. A reduced plant allocation to AMF will increase competition for plant photosynthates between AMF, thereby leading to reduced AMF diversity (Johnson *et al.*, 2013).

The DOC analysis of the low-P samples suggests that grapevine interactions with AMF microsymbionts exhibits a universal dynamic. A given set of AMF taxa colonizing roots will thus lead to a regular distribution of their relative abundances. Such regularity could occur through fixed life-history strategies of AMF, fixed interactions among AMF, and/or a stable colonization regime imposed by hosts, each determining AMF relative abundance in roots in a predictable manner. In contrast, the DOC of the high-P samples, which were impoverished in AMF diversity, was undistinguishable from a flat line, suggesting that the interactions among AMF and their host that led to a predictable pattern in the low-P samples no longer hold. Potential causes for this include loss or gain of particular keystone AMF species with strong interactions or a reduction in the strength with which plants favour or disfavour particular AMF taxa given their presence with increasing P.

Additionally, a positive correlation between pH and AMF diversity was found. In general, there is a broad agreement that soil acidity can strongly affect soil microbial communities. Jansa *et al.* (2014) also showed that soil acidity was one of the most important drivers of AMF communities

364 in Swiss agricultural soils. Moreover, soil acidity strongly affected AMF communities in the
365 roots of Arabica coffee (De Beenhouwer *et al.*, 2015). Therefore, our results agree with previous
366 studies. It is possible that N enrichment through fertilization lowered pH, as N enrichment can
367 acidify the soil (Vitousek *et al.*, 1997). Although it has been shown that N fertilization can affect
368 AMF colonization (Nikolaou *et al.*, 2002; Karagiannidis *et al.*, 2007), we observed no effect of
369 soil N on AMF communities. Nitrogen mobilizes easily in the soil, especially under humid
370 conditions. Therefore, effects of N on AMF communities may be difficult to measure.

371 In agreement with our diversity analysis, the NMDS ordination revealed that the available P
372 content in the soil and pH explained most variation in AMF community composition,
373 suggesting that there is a regularity in which taxa are lost with increasing soil P levels. This was
374 confirmed by the nestedness analysis. The second spatial predictor (PCNM2) also significantly
375 contributed to AMF community composition. PCNM2 separates the vineyard according to their
376 longitude and may correlate with unmeasured environmental variables such as soil texture.
377 Although organic farming did not affect AMF diversity in vineyards, AMF communities
378 significantly differed between conventionally and organically managed vineyards. However,
379 management type could explain only very little variation in AMF community composition (R^2
380 = 0.021).

381 We found no effects of copper concentration in the soil on AMF diversity and community
382 composition. This can be explained by the relatively low copper concentrations measured in the
383 vineyard soils, i.e. the majority of the vineyards (75%) had lower copper concentrations than
384 the background level (30 mg/kg). Also no differences in copper concentration were found
385 between conventionally and organically managed vineyards. However, we observed that copper
386 concentration in the soil increases with vineyard age (Fig. 7). Older vineyards (> 15 years)
387 showed copper concentrations above the background level (30 mg/kg), indicating copper is

388 accumulating in the soil over time. If this trend continues, the copper concentration of a
389 vineyard may reach 100 mg/kg after 73 years of viticulture.

390 The AMF communities originating from 34 vineyards across Flanders and the South of The
391 Netherlands were organized in a nested pattern. Therefore, poor AMF communities are a subset
392 of the richer AMF communities, indicating a gradual loss of specialist taxa and the occurrence
393 of general taxa. In a total of 170 samples, OTU_2 (identified as VTX00113) occurred in 167
394 samples. Therefore, this AMF taxon can be considered a generalist. VTX00113 was not only
395 the most frequent taxon in our dataset, but it is also the most abundant taxon in the MaarjAM
396 database. In some entries VTX00113 is identified as *Rhizophagus intraradices*, one of the most
397 widespread mycorrhizal fungi. It has been observed in a wide range of natural and
398 anthropogenic ecosystems, from forests and grasslands to orchards and arable fields. VTX0013
399 also occurs in high-input agricultural ecosystems, suggesting it tolerates high nutrient levels in
400 the soils (Hijri *et al.*, 2006). Indeed, Sylvia and Schenk (1983) showed that P enrichment did not
401 affect sporulation of *R. intraradices*. Still, at high plant-available P levels, *R. intraradices* has been
402 shown to reduce the growth of citrus trees (Peng *et al.*, 1993), suggesting that the absence of its
403 downregulation may be detached from the provision of plant nutritional benefits. Furthermore,
404 we found that the degree of nestedness was positively correlated to the plant-available P in the
405 soil. Therefore, higher plant-available P levels in the soil were related to a gradual loss of
406 specialist taxa. Consequently, vineyards with high plant-available P (Olsen P > 70 mg P/kg soil)
407 were dominated by generalists. Conversely, vineyards with low plant-available P (Olsen P < 70
408 mg P/kg soil) harbored more specialist species.

409 ## 5 Conclusion

410 Grapevine depends on AMF for normal growth and development and its AMF communities
411 can be expected to contribute to the microbial terroir of vines (Trouvelot *et al.*, 2015). Although

it has been shown that organic farming may increase AMF diversity in some crops, we found no positive effects of organic farming on the AMF diversity of vines. Instead, plant-available phosphorus and soil pH levels strongly affected AMF diversity, AMF community composition and nestedness in vineyards. Furthermore, high soil phosphorus levels led to a more irregular plant-AMF community dynamic, suggesting that the interactions that led to a predictable pattern under low soil P availability no longer hold. Especially high soil phosphorus levels seem to overrule any potential benefit of organic farming on AMF diversity. Through impoverishing and homogenizing AMF communities, high soil phosphorus levels may jeopardize the potential role of these symbionts in plant nutrition, pathogen protection, stress tolerance and soil structure provisioning. In the specific case of grapevine, homogenized AMF communities may also jeopardize the development of a specific microbial terroir. Decreasing soil nutrient additions, even organic ones, and increasing soil pH are the first steps in improving AMF diversity in vineyards, and likely the ecosystem services they deliver.

Acknowledgements

We are grateful to all vineyard managers that participated in this study to allow sampling in their vineyards. We wish to thank Kasper Van Acker and Gerrit Peeters for assistance in the laboratory. EV acknowledges funding from ERC Synergy Grant 610028 entitled "Imbalance-P".

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5.2 Author Contributions

MVG, BL and OH designed the study. MVG performed the field sampling, analyzed the data and wrote the first manuscript version. MDB assisted in lab analysis, data analysis and provided

575 useful comments on the manuscript. EV assisted in data analysis and provided useful
576 suggestions on the manuscript. MDB, EV, BL and OH edited the manuscript. GVR provided
577 contact information of wine growers and commented on the final version of the manuscript.
578 All authors contributed critically to the drafts and gave final approval for publication.

579 **Tables**

580 **Table 1** Results of the mixed model analysis to test for differences in soil chemical variables between
581 management types. To account for pseudoreplication, 'vineyard' was included as a random factor. Soil
582 N, Olsen P and Cu are expressed in mg/kg soil.

	Conventional Mean (S.E.)	Organic Mean (S.E.)	*F*	*P*
pH	7.28 (0.064)	7.31 (0.068)	0.046	0.832
Soil N	14.67 (1.61)	16.82 (1.71)	0.834	0.368
Olsen P	49.17 (6.66)	49.33 (7.07)	<0.001	0.988
Organic carbon	0.042 (0.0053)	0.057 (0.0056)	3.738	0.063
Cu	22.59 (2.96)	20.40 (3.13)	0.256	0.616

583

584

585 **Table 2** Results of the mixed models to test for relationships between AMF diversity measures, soil
586 chemical variables and management. To account for pseudoreplication, 'vineyard' was included as a
587 random factor. To prevent bias due to different sequencing depth, 'sequencing depth' was included as a
588 covariate in both models. Soil N, organic carbon, management and the spatial PCNM variables were
589 excluded by forward selection model procedures.

	Richness			Exp(H)		
	Coefficient	*F*	*P*	Coefficient	*F*	*P*
Intercept	-5.205	0.24	0.633	-2.508	0.41	0.521
Sequencing depth	0.002	6.53	0.012	-0.001	9.03	0.003
Olsen P	-0.081	10.86	0.002	-0.0309	13.53	0.001
pH	3.474	5.59	0.019	1.356	6.60	0.011

590

591 **Table 3** Results of the permutation tests of the two dimensional NMDS ordination testing for significant
592 relationships between AMF community composition, soil chemical variables, management and spatial
593 PCNM variables. The results are based on 1000 permutations.

	R^2	*P*
Management	0.021	0.022
pH	0.053	0.017
Soil N	0.005	0.689
Olsen P	0.155	<0.001
Organic carbon	0.004	0.711
Cu	0.010	0.416
PCNM1	0.008	0.465
PCNM2	0.056	0.006
PCNM3	0.001	0.884
PCNM4	0.016	0.265
PCNM5	0.001	0.894
PCNM6	0.002	0.825
PCNM7	0.001	0.898
PCNM8	0.013	0.339

594

5.3 Figure legends

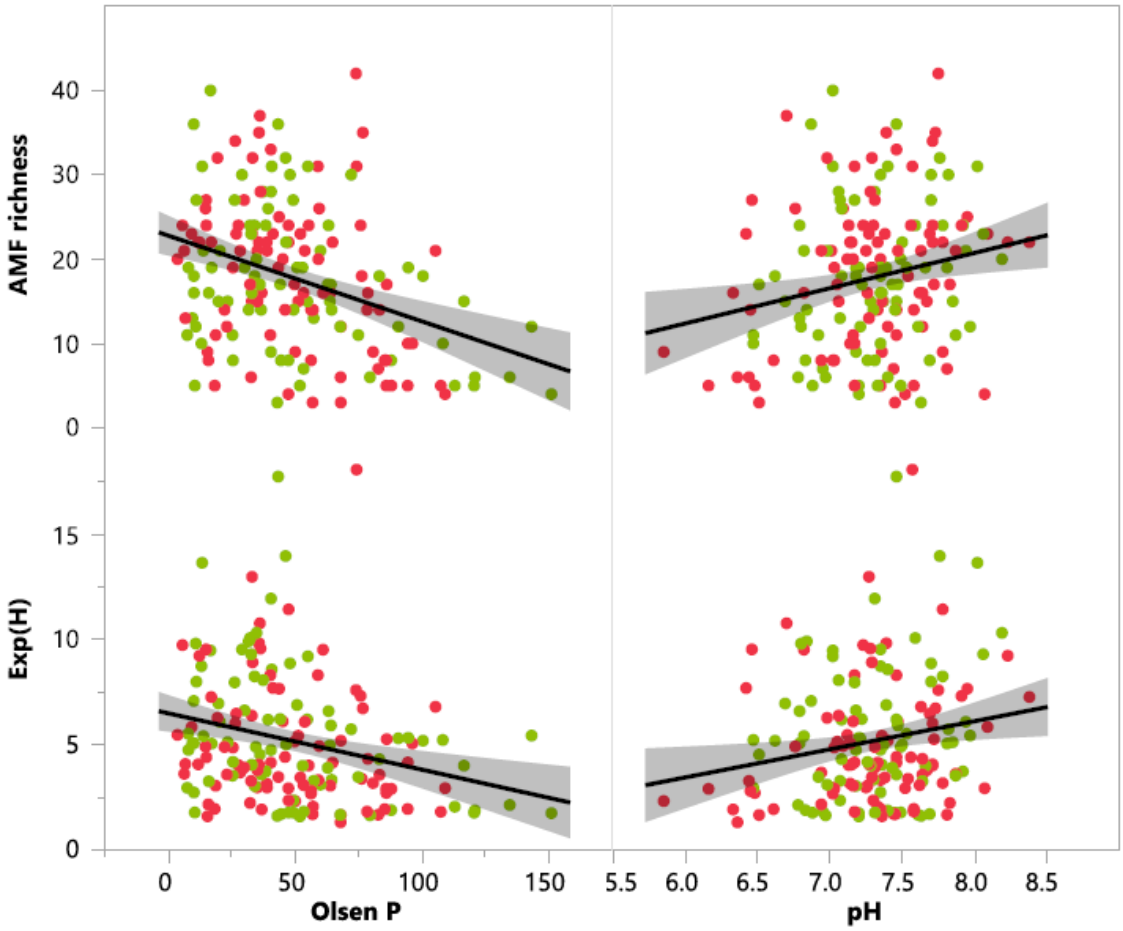


Figure 1 Relationship between AMF diversity measures, soil Olsen P and pH. Lines represent marginal models as calculated from linear mixed models containing all predictor variables (Table 2).

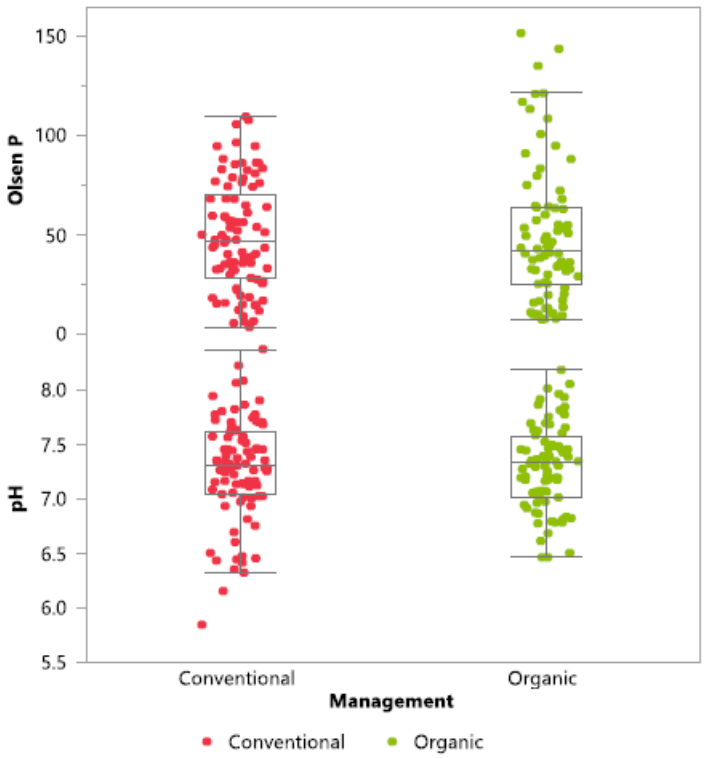
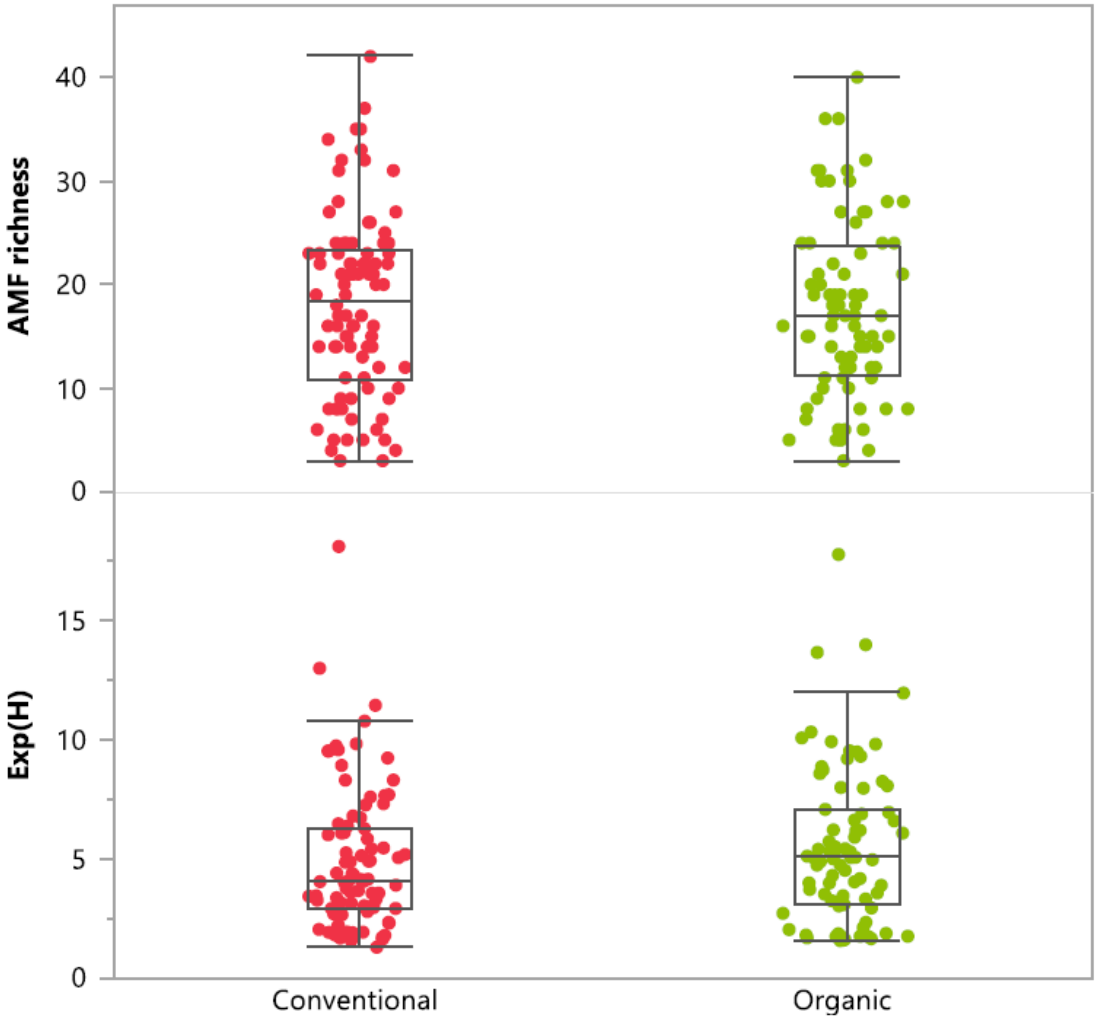


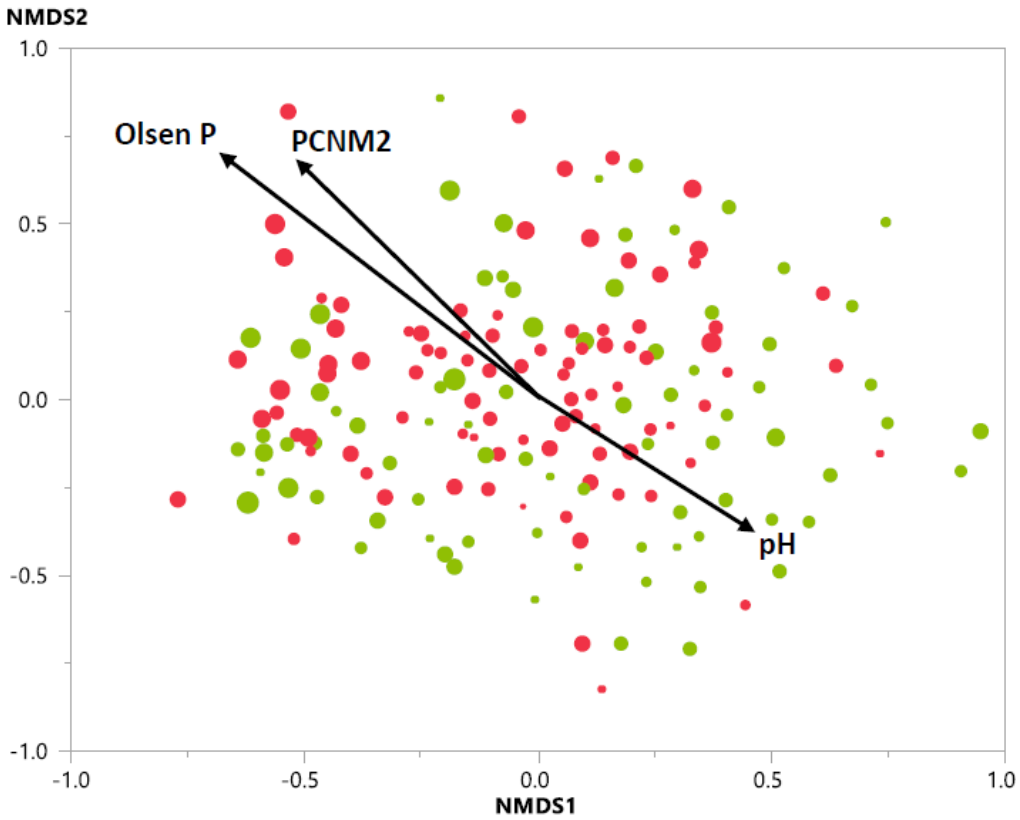
Figure 2 The soil chemical variables selected in the forward selection procedure of the mixed model to test for relationships between soil variables and AMF diversity measures, i.e. Olsen P and pH, did not differ between management types. Box plots show 25, 50 and 75 percentiles, and outliers.



603

604 **Figure 3** No differences in AMF diversity measures were found between conventionally and organically
605 managed vineyards. Box plots show 25, 50 and 75 percentiles, and outliers.

606



607

608 **Figure 4** NMDS ordination plot of AMF communities from 34 vineyards (5 samples per vineyard).
609 AMF communities between conventional (red) and organic (green) vineyard were significantly different
610 (Table 4). Significant relationships between ordination scores, soil chemical and PCNM variables are
611 shown with an arrow, representing the direction of the increasing gradient. Point size represents Olsen
612 P values. Stress value: 19.3.

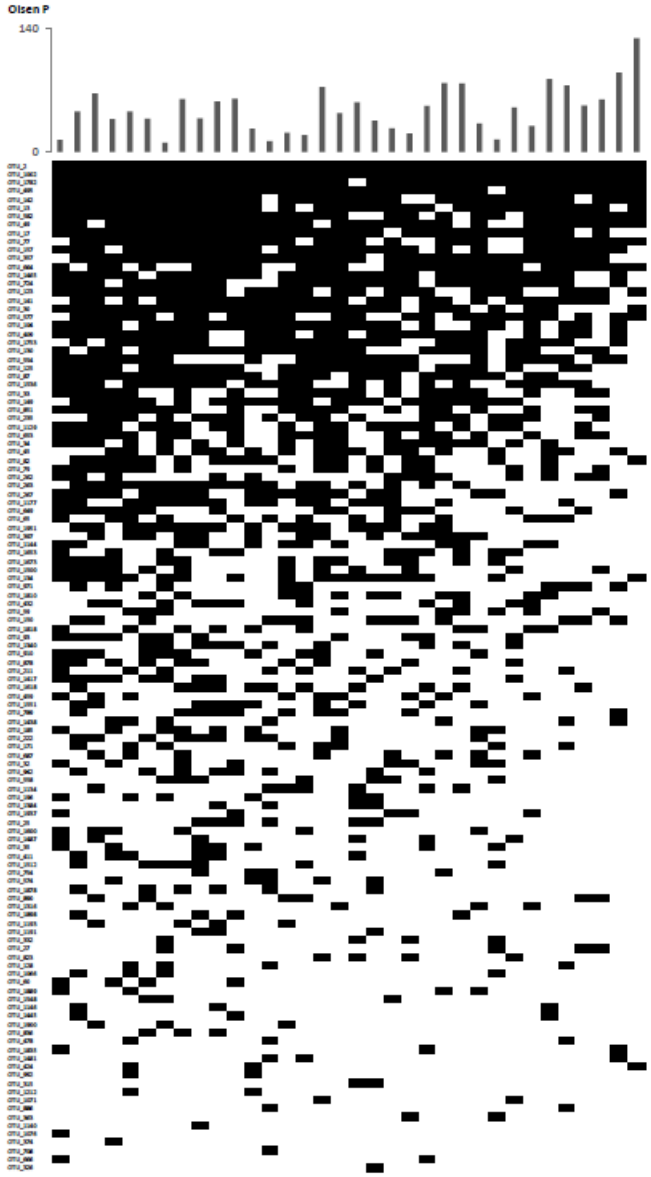
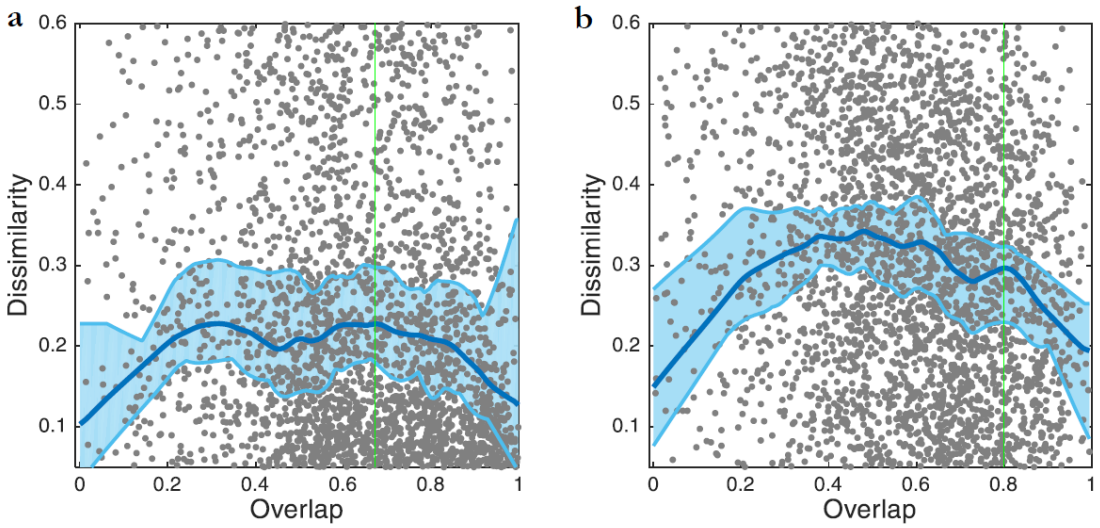
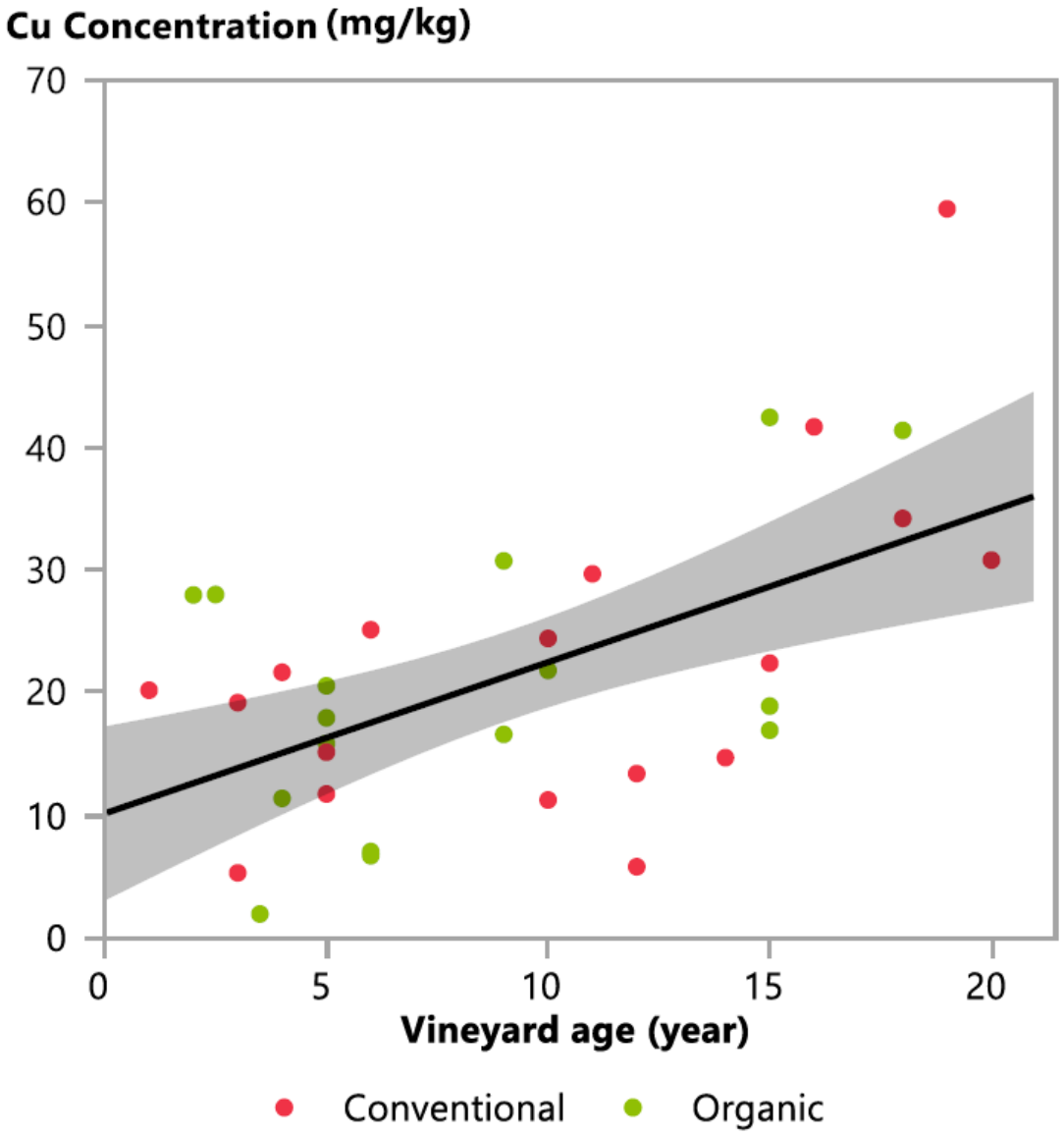


Figure 5 Nestedness of AMF communities across 34 vineyards as shown by the row and column permutated presence/absence matrix that is closest to perfect nestedness. Columns represent vineyards (sorted according to their degree of nestedness) and rows are OTUs. The average P availability (Olsen P) per vineyard is indicated on top to show that vineyards are ranked according to P availability.



618

619 **Figure 6** DOC analysis on both high-P (a) and low-P (b) sample groups. The DOC of the high-P
620 samples was undistinguishable from a flat line in the high-overlap region and shows broad confidence
621 intervals ($P = 0.383$), while the DOC of the low-P samples displays a pronounced negative slope in the
622 high-overlap region ($P < 0.001$). The vertical green line represents the change point from where the P
623 value of the slope of the DOC is calculated.



624

625 **Figure 7** The relation between copper concentration in the soil and vineyard age ($P < 0.001$). Older
626 vineyards (> 15 years) show copper concentration above the background level (30 mg/kg).

Supporting information

Figure S1 Map of Flanders (Belgium) showing the distribution of the 34 sampled vineyards with organic (green) and conventional (red) management.

Figure S2 Rarefaction curves of AMF richness for all 34 vineyards. For the sake of graphical representation, the curves are shown in three separate graphs, (a), (b) and (c). Vineyards are shown in different colors.

Figure S3 Rarefaction curves of AMF richness per management type.

Figure S4 The relationship between geographical location (latitude and longitude) and the spatial predictor PCNM2 that significantly contributed to AMF community composition. PCNM2 separates the sampled vineyards according to their longitude and increases with higher longitudes.

Table S1 Coordinates, soil properties, management and AMF diversity measures of all 34 vineyards sampled.

Table S2 List of the 123 operational taxonomic units (OTUs) identified at a 3% sequence dissimilarity cut-off. The taxonomic affiliations were obtained by BLAST analysis against the MaarjAM database.

Table S3 The sample*OTU matrix and all accompanying environmental data.