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1 **The nitrification inhibitor nitrapyrin has non-target effects on the soil microbial**
2 **community structure, composition, and functions**

3

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22 **Key words**: nitrification inhibitor, nitrogen cycle, microbial community structure, ammonia

23 oxidation, volatiles, functional genes

24 **Abstract**

25 Nitrapyrin is a nitrification inhibitor used to retain ammonia-N in soil to improve crop yields and
26 quality. Nitrapyrin targets specifically the ammonia oxidizers, but it is not known if it has non-
27 target effects on the soil microbial communities. Here, we tested the hypothesis that nitrapyrin
28 also leads to large shifts in soil microbial community structure, composition, diversity and
29 functions. To test this hypothesis, we set-up a field experiment where wheat (*Triticum aestivum*
30 cv. AC Walton) was fertilized with ammonium nitrate (NH_4NO_3) and supplemented or not with
31 nitrapyrin. Rhizosphere and bulk soils were sampled twice, the 16S rRNA gene and ITS region
32 were amplified and sequenced to follow the changes in archaeal, bacterial and fungal community
33 structure, composition and diversity. To assess microbial functions, several genes involved in the
34 nitrogen cycle were quantified by real-time qPCR, and volatile organic compounds (VOCs) were
35 trapped in the rhizosphere at the moment of sampling. Sampling dates and soil compartments
36 had overwhelming effects on the microbial communities. However, nitrapyrin still significantly
37 affected the relative abundance of Thaumarchaeota, Proteobacteria, Nitrospirae and
38 Basidiomycota, and several genera. Nitrapyrin also significantly affected bacterial and fungal
39 community structure, and the abundance of all the N-cycle gene tested, but always in interaction
40 with sampling date. In contrast, nitrapyrin had no significant effect on the emission of VOCs,
41 where only sampling dates significantly influenced the profiles observed. Our results show that
42 nitrapyrin influences non-target soil- and plant-associated microbial communities. In the longer
43 term, these shifts might counteract the positive effect of nitrapyrin on crop nutrition and
44 greenhouse gas emissions.

45 **Introduction**

46 Nitrogen (N) is the most limiting nutrient for plant growth, especially in agroecosystems. One of
47 the key advances of the green revolution was the intensive use of inorganic fertilizers to increase
48 crop yields. However, N fertilization has become at the center of the environmental and
49 sustainability issues faced by agriculture. The adverse effects of the inefficient use of N
50 fertilizers range from N losses through ammonia volatilization, nitrate leaching and nitrous oxide
51 (N_2O) emissions, leading to atmospheric and groundwater pollution (Gu *et al.*, 2009). A lot of
52 these adverse effects are linked to soil microbial processes such as nitrification.

53 Nitrification is the oxidation of ammonia to nitrate carried out by chemoautotrophs that use the
54 energy thereby generated to fix atmospheric CO_2. The transformation of ammonia to nitrate by
55 nitrifiers is environmentally and agriculturally unfavorable. Nitrate, because of its negative
56 charge, is more mobile in soil and more prone to leaching than ammonia. At the same time,
57 nitrate is the substrate for denitrification, which, if incomplete, can lead to N_2O emissions.
58 Nitrate, although more mobile, is energetically less favorable for plant growth, as it needs to be
59 actively taken up and transformed back to ammonia for protein synthesis (Moreau *et al.*, 2019).
60 To counteract these adverse effects of nitrification, nitrification inhibitors (NI) have been used in
61 recent decades in combination with ammonia-based fertilizers. Nitrapyrin (2-chloro-6-
62 (trichloromethyl)-pyridine) is one of the most commonly applied commercial NIs and was shown
63 to increase soil N retention (Subbarao *et al.*, 2006) and yields of a variety of crops (Wolt, 2004),
64 while decreasing N_2O emissions (Omonode *et al.*, 2013). On average, following nitrapyrin
65 application crop yields increase by 7% and soil N retention by 28%, while N leaching decreases
66 by 16% and greenhouse gas emissions by 51% (Wolt, 2004). In a global meta-analysis of field

67 trials, nitrapyrin increased average grain production by 2% and 9% for wheat and maize,
68 respectively (Qiao *et al.*, 2015).

69 Nitrapyrin delays nitrification by temporarily deactivating the ammonia monooxygenase
70 (AMO), the enzyme responsible for ammonia oxidation, the first and rate-limiting step of
71 nitrification (Powell & Prosser, 1986; Vannelli & Hooper, 1992). The α subunit of AMO is
72 encoded by the *amoA* gene, which is homologous in archaeal (AOA) and bacterial (AOB)
73 ammonia-oxidizers (Prosser & Nicol, 2012), suggesting that both AOA and AOB could be
74 inhibited by nitrapyrin. However, AOA and AOB were reported to be affected or not by
75 nitrapyrin (Fisk *et al.*, 2015; Lehtovirta-Morley *et al.*, 2013; Shen *et al.* 2013), indicating that
76 other factors such as soil physico-chemical properties, environmental conditions or soil biotic
77 properties might influence the efficiency of nitrapyrin. Yet, it remains unresolved whether
78 nitrapyrin has non-target effects on the soil and plant-associated microbial communities, either
79 directly or through its effect on ammonia-oxidizers. We hypothesized that nitrapyrin affects the
80 overall soil and rhizosphere microbial community structure, composition, diversity, and
81 functions. We set-up a field experiment where we exposed wheat-associated microbial
82 communities to nitrapyrin and observed the effects on 1) the microbial community structure,
83 composition and diversity, 2) the abundance of genes encoding for enzymes involved in
84 ammonia oxidation, N-fixation and denitrification, and 3) the microbial metabolic activities.

85 **Materials and methods**

86 *Field experiment and sampling*

87 The field trial was set up in June 2019 at the Armand-Frappier Santé Biotechnologie Research
88 Center (Laval, Québec, Canada, 45.54159° N, 73.71734° W) (Figure S1). Before the setup of the
89 field trial, the sod was removed and the soil was kept free of vegetation throughout the
90 experiment by weekly weeding. No other crops were planted in this area for over 20 years. Soil
91 samples taken in 2016 from our experimental field had a pH of 7.3 and contained 3,000 mg/kg of
92 total N (Kjeldahl extraction), 1,300 mg/kg of total phosphorus (P) and 1,110 mg/kg of total
93 potassium (K) (Giard-Laliberté et al. 2019). The climatic data were retrieved from the weather
94 station of Montreal International Airport (QC), Canada (Station CWTQ, 45.4678, -73.7417;
95 http://www.agrometeo.org/indices/mcd/cwtq), located 8.4 km away from our experimental field.
96 The mean daily precipitation and temperature from June to September 2019 was 4.0 mm and
97 21.2 °C, respectively.

98 A total of twelve plots (1 m × 1 m) were established based on a randomized block design with
99 six replicated plots per treatment. For each plot, approximately 20 g of wheat seeds (*Triticum*
100 *aestivum* cv. AC Walton) were sown in 4 rows separated by 20 cm (Figure S1). A solution of
101 ammonium nitrate (NH_4NO_3) and the nitrification inhibitor nitrapyrin (2-chloro-6-
102 (trichloromethyl)-pyridine (NI) (Dow AgroScience, AB, Canada) were applied twice during the
103 growing season by spraying uniformly on the soil surface. On June 11th, 2019, 10 g N (28.57g
104 ammonia nitrate) was applied to each plot and 0.05 g NI to each NI-treated plot. On July 10th,
105 2019, 10 g N was applied to each plot and 0.1 g NI to each NI-treated plot. NI application rates
106 and method were determined from the manufacturer's instructions eNtrench can be used at rates
107 up to 5.4 L ha^{-1} for spring application in wheat. According to these rates and the concentration of

108 nitrapyrin in eNtrench, we diluted the appropriate amount of nitrapyrin for each 1-m^2 plot in 50
109 ml of water.

110 Sampling of bulk soil and rhizosphere soil, as well as entrapment of VOCs using PDMS tubes
111 (see below for more details) in the rhizosphere was conducted twice. The first sampling was
112 carried out at the stage of grain filling (July 23rd, 2019). The second was carried out at the time
113 of harvest (September 5th, 2019). There was a total of 48 samples for soil DNA extraction,
114 consisting of 2 treatments (NI and control), 2 compartments (bulk and rhizosphere soil) and 2
115 sampling dates from 6 replicated blocks. For VOC analyses, only the rhizosphere was sampled,
116 resulting in 24 samples. For bulk soil, five soil cores (2cm diameter × 5cm depth) of the upper 5
117 cm of soil were collected from four corners and the center of each plot and pooled together to
118 create one composite sample per plot. Rhizosphere soil was collected from the root of one plant
119 located in the center of each plot. The plant was vigorously shaken by hand to remove non-
120 adhering soil around the roots and the remaining rhizosphere soil was then collected in 2-ml
121 polyethylene tube. Soils were sieved (2.0 mm mesh size) and placed into a sterile plastic bag and
122 immediately stored at -80ºC prior to DNA extraction. VOCs were collected with Rotilabo®-
123 silicone tubes (PDMS tubes, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), which were
124 placed in the rhizosphere of one uprooted plant per plot (Figure S1). The PDMS tubes were pre-
125 treated as described previously (Kallenbach *et al.*, 2015). After 20 minutes, PDMS tubes were
126 removed and kept at -20 °C until analysis.

127

128 *DNA extraction and PCR amplicon sequencing*

129 Soil DNA was extracted from 0.3 g of well-mixed soil for each sample using the DNeasy
130 PowerLyzer PowerSoil Kit (Qiagen, Toronto, Canada), following the manufacturer's

131 instructions. Extracted genomic DNA was quantified using a PicoGreen™ assay (Thermo
132 Scientific, OR, U.S.A.) with a Tecan Infinite M1000 PRO (Thermo Scientific). DNA was stored
133 at -20°C until used for PCR.

134 PCR amplicon libraries were prepared for the archaeal and bacterial 16S rRNA gene using
135 primers 515F and 806R targeting the V4 region (Caporaso *et al.*, 2012) and for the fungal ITS1
136 region using primers ITS1F and 58A2R (Martin & Rygiewicz, 2005). The first step of PCR
137 contained the template specific primers with a short adaptor sequence, and the second step of
138 PCR was conducted with primers containing the Illumina barcodes. Two PCR amplification
139 steps were performed in a T100™ Thermal Cycler (Bio-Rad, U.S.A.). Reagents and
140 amplification conditions for each of the PCR are shown in Table S1. Amplicons were verified on
141 1% agarose gels and purified using AMPPure XP beads (Beckman Coulter, Indianapolis, U.S.A.)
142 following the manufacturer's instructions. Sequencing was conducted on an Illumina MiSeq
143 sequencer (2 x 250 pair-end) at the McGill University and Genome Québec Innovation Center
144 (Montréal, Canada). A total of 7,416,206 16S rRNA gene reads and 6,649,643 ITS region reads
145 were produced. The raw datasets and associated metadata are available through NCBI BioProject
146 accession PRJNA634744.

147

148 *Quantitative real-time PCR (qPCR)*

149 qPCR was performed using the iTaq universal SYBRGreen® kit (Bio-Rad Laboratories Inc,
150 Hercules, CA) following the manufacturer's protocol on an Agilent Mx3005P qPCR Systems
151 (Agilent Technologies, CA, U.S.A.). Primers crenamoA23f and crenamoA616r (Tourna *et al.*,
152 2008), amoA1f and amoA2r (Levy-Booth *et al.*, 2014), Cd3af and R3cd (Throbäck *et al.*, 2004),
153 nirK876f and nirK1040r (Kandeler *et al.*, 2006) and nifHPo1f and nifHPo1r (Poly *et al.*, 2001)

154 were used for quantification of the archaea ammonia monooxygenase A subunit gene (*amoA*-
155 AOA hereafter), bacterial ammonia monooxygenase A subunit gene (*amoA*-AOB hereafter),
156 *nirS*, *nirK* and *nifH* genes, respectively. Standards were prepared prior to qPCR as described
157 previously (Yergeau *et al.*, 2020). Briefly, the genes of interest were amplified from DNA
158 extracted from an agricultural soil, and the resulting amplicons were purified and concentrated
159 using a Wizard SV gel purification kit (Promega Corporation, U.S.A.), and cloned into a P-Gem
160 T plasmid (Promega Corporation, U.S.A.). The plasmids were linearized with the restriction
161 enzyme SacII, quantified using a PicoGreen assay and then serially diluted (10^8–10^1 copies μl^{-1})
162 for creating a standard curve. Reagents and reaction conditions for qPCR are shown in Table S2.
163

164 *VOC analysis*

165 VOCs were desorbed from the PDMS tubes by using an automated thermodesorption unit
166 (model UnityTD-100, Markes International Ltd., UK) at 250°C for 12 min (flow 50 ml/min).
167 The desorbed VOCs were subsequently collected on a cold trap at -10°C and introduced into a
168 GC-QTOF (model Agilent 7890B GC and the Agilent 7200AB QTOF, USA) by heating the cold
169 trap for 10 min to 280°C. A split ratio was set to 1:10. The column used was a 30 × 0.25 mm ID
170 DB-5MS with as film thickness of 0.25 µm (Agilent 122-5532, USA). The temperature program
171 was as follows: 2 min at 39°C, 3.5°C/min to 95°C, 4°C/min to 165°C and finally 15°C/min to
172 280°C that was hold for 15 min. VOCs were detected by the MS operating at 70 eV in EI mode.
173 Mass spectra were acquired in full scan mode (30-400 AMU, 4 spectras/sec). Collected GC/MS
174 data was converted to mzData files using the Chemstation B.06.00 (Agilent Technologies, Santa
175 Clara, USA) and further processed (peak picking, baseline correction and peak alignment) in an
176 untargeted manner with MZmine 2 (Pluskal *et al.*, 2010). Detected compounds were identified

177 using NIST-MS Search by comparing the spectra, accurate mass, linear retention indices and
178 spectra match factor with NIST 2014 V2.20 (National Institute of Standards and Technology,
179 USA, http://www.nist.gov), Wiley 9th edition, and in-house spectral libraries. Putative
180 compounds were identified using AMDIS 2.72 (National Institute of Standards and Technology,
181 Gaithersburg, USA) and linear retention indices of VOCs were calculated according to the
182 method described by Strehmel *et al.*, (2008). PDMS contaminants were removed from the final
183 list.
184

185 *Bioinformatic analyses*

186 Primers were trimmed with up to one mismatch allowed and starting position ≤ 1. Forward and
187 reverse reads of same sequence were merged with at least 30 bp overlap and <0.25 mismatches
188 by using FLASH v1.2.5 (Magoč & Salzberg, 2011). The sequences were then quality trimmed
189 using Btrim (Kong, 2011) with a QC threshold > 30 over a 5bp window size. Merged sequences
190 with an ambiguous base, or < 240 bp for 16S and < 200 bp for ITS were discarded. Chimera
191 sequences were detected and removed with the UCHIME algorithm (Edgar *et al.*, 2011).
192 Sequences were clustered into operational taxonomic units (OTUs) with a 97% identity cut-
193 off using UPARSE (Edgar, 2013) and their taxonomic affiliation was assigned using RDP 16S
194 rRNA reference database (Wang *et al.*, 2007) and UNITE ITS reference database (Nilsson *et al.*,
195 2019). To correct for sampling effort (number of reads retrived per sample), the samples were
196 rarefied at 11,554 sequences for 16S and 2,066 sequences for ITS for subsequent statistical
197 analysis of microbial communities. The above mentioned steps were performed using an in-
198 house pipeline that was built on the Galaxy platform at the Research Center for Eco-

199 Environmental Sciences, Chinese Academy of Sciences (http://mem.rcees.ac.cn:8080/) (Feng *et*
200 *al.*, 2017).
201
202 *Statistical analysis sequencing data*
203 All statistical analysis and figure generation were performed in RStudio v1.2.5042 running R
204 v3.6.3. All plots were generated using the ggplot2 package v 3.3.0 (Ginestet, 2011). 16S rRNA
205 gene and ITS region data were analysed using the phyloseq package v 1.28.0 (McMurdie &
206 Holmes, 2013), the microbiome package v 1.6.0 (Lahti *et al.*, 2017) and the microbiomeutilities
207 package v 0.99.00 (Sudarshan & Lahti, 2018). Shannon diversity index, Inverse Simpson index
208 matrices were calculated with the *estimate_richness* function of the phyloseq package. Faith's
209 Phylogenetic Diversity (PD) (Faith, 1992) was estimated using the *pd* function of the picante
210 package v 1.8.1 (Kembel *et al.*, 2010). Three-way repeated measures analysis of variance
211 (ANOVA) was performed for alpha diversity indices across treatment, date and compartment
212 using the *aov* function of the stats package v 3.6.3 (R Core Team, 2020). After removal of
213 singletons and unclassified phyla, top 10 phyla and genera were extracted using the *taxa_sums*
214 function of the phyloseq package. Three-way repeated measures ANOVA was performed for the
215 top 10 phyla and genera across treatment, date and compartment using the *aov* function of the
216 stats package. Principal Coordinates Analysis (PCoA) with Bray-Curtis dissimilarity was used to
217 visualize similarity between samples based on bacterial and fungal composition using the
218 *ordinate* function of the phyloseq package. The effects of the treatment, date and compartment
219 on the bacterial and fungal community structure were tested using permutational multivariate
220 analysis of variance (PERMANOVA) with the *adonis* function of the vegan package v 2.5-6
221 (Dixon, 2003) based on Bray–Curtis dissimilarity indices.

222 For qPCR, values (log copies per g soil) were first tested for homogeneity of variance
223 using the *leveneTest* function of the car package v 3.0-7 (Fox *et al.*, 2014), followed by Shapiro-
224 Wilk's normality test using the *shapiro.test* function of the rstatix package v 0.4.0 (Kassambara,
225 2020). The non-parametric *krusk.test* function of the of the stats package was used to compare
226 samples, followed by post-hoc analysis using the *dunnTest* function of the FSA package v 0.8.30
227 (Ogle & Wheeler, 2020).

228 For VOCs, aligned m/z features were filtered and normalised as time-series data using the
229 metaboAnalystR package v 2.0.1 (Chong & Xia, 2018) as follows: features containing a 0.5%
230 cut-off of missing values were removed, missing values with a minimum positive value (here:
231 2463.451875). Further feature filtering was performed based on Interquantile Range. Finally,
232 data were normalised by median, log transformed and auto scaled. The normalised data were
233 then used to perform ANOVA ($P < 0.01$) and Principal Component Analysis (PCA) using the
234 metaboAnalystR package. The effects of treatment and date as well as their interaction on the
235 overall VOC composition were tested using PERMANOVA with the *adonis* function of the
236 vegan package based on Euclidean distances. A heatmap of significant features based on
237 ANOVA was created using the *ggheatmap* function of the heatmaply package 1.1.0 (Galili *et al.*,
238 2018) by hierarchical clustering based on Euclidean distances. Values of individual compounds
239 were subsetted and plotted as log median values.

240 **Results**

241 *Diversity, composition and structure of soil microbial communities*

242 The sequences obtained from the 16S rRNA gene and the ITS region sequencing were grouped
243 into 26,979 and 4,482 OTUs with a 97% sequence similarity threshold, respectively. Bacterial
244 and archaeal, as well as fungal alpha diversity estimated by the Shannon, inverse Simpson and
245 phylogenetic diversity (PD) indices did not differ significantly between the NI treated samples
246 and the controls ($P > 0.05$) (Table 1). For bacterial and archaeal communities, sampling date
247 significantly affected the Inverse Simpson index ($P < 0.01$) with an increased diversity at the
248 second sampling date (Table 1, Figure 1). Compartment significantly affected the Faith's PD (P
249 < 0.01) (Table 1), with a lower diversity in bulk soil as compared to the rhizosphere for the first
250 sampling date for both the NI and control treatments (Figure 1). For fungal communities,
251 compartment significantly affected the Shannon diversity index ($P < 0.01$) and the Inverse
252 Simpson index ($P < 0.001$) (Table 1) with a lower diversity in rhizosphere as compared to the
253 bulk soil (Figure 1). Sampling date also significantly affected the Shannon ($P < 0.001$), the
254 inverse Simpson ($P < 0.001$) and Faith's PD ($P < 0.001$) indices for fungal communities (Table
255 1), with a decreased diversity at the second sampling date (Figure 1).

256 Nitrapyrin had a significant effect on the relative abundance of the archaeal phylum
257 Thaumarchaeota with an increased relative abundance in the NI treatment (Table 2, Figures 2
258 and 3). In contrast, compartment and sampling date significantly affected the relative abundance
259 of most bacterial, archaeal and fungal phyla (Table 2 and Figure 3). The interaction between the
260 NI treatment and compartment was significant for Thaumarchaeota, Proteobacteria and
261 Basidiomycota, whereas the interaction between nitrapyrin and sampling date was only
262 significant for Nitrospirae. The interaction between compartment and sampling date was

263 significant for Thaumarchaeota, Actinobacteria, Firmicutes, Mortierellomycota and Ascomycota.
264 The three-way interaction was not significant for any of the major phyla tested.
265 At the genus level, we focused our analyses on the 10 relatively most abundant genera for the
266 archaea and bacteria, and the fungi (listed in Table 3). The NI treatment was only significant for
267 the genus *Nitrososphaera* (Table 3, Figure 4). In contrast, the compartment was significant for 5
268 out of 10 bacterial and archaeal genera and for one fungal genus (Table 3), whereas the sampling
269 date was significant for 6 out of 10 bacterial and archeal genera and 7 out of 10 fungal genera
270 (Table 3). The interaction between nitrapyrin addition and compartment was significant for
271 *Nitrososphaera*, *Gaiella*, *Rhodoplanes*, *Gliomastix* and *Ganoderma*, whereas the interaction
272 between nitrapyrin and sampling date was not significant for any of the most abundant genera
273 (Table 3). The interaction between compartment and date was significant for *Nitrososphaera*,
274 *Solirubrobacter*, *Hyphomicrobium, Rhodoplanes*, *Mortierella* and *Thelonectria* (Table 3).
275 Furthermore, the three-way interaction was significant for *Gaiella*, *Solirubrobacter*,
276 *Hyphomicrobium* and *Gliomastix* (Table 3).
277 The overall structures of bacterial and archaeal, and fungal communities are shown separately
278 for the two sampling dates in the principal coordinates analysis (PCoA) ordinations in Figure 5.
279 For both 16S and ITS ordinations, the first axis clearly separated the rhizosphere and bulk soil
280 samples, but only for the first sampling date (Figure 5). No effect of nitrapyrin could be observed
281 in the ordination plots. The permanova analyses confirmed this visual interpretation for both
282 archaeal, bacterial and fungal communities, with significant effects for compartment and
283 sampling date, but not for nitrapyrin addition (Table 4). Additionally, for both communities, the
284 interaction effects between nitrapyrin and compartment and between sampling date and

285 compartment were significant, whereas the three-way interaction was also significant for bacteria
286 and archaea (Table 4).
287
288 *Abundance of functional genes of the N cycle*
289 The abundances of the AOA-*amoA*, AOB-*amoA*, *nirK*, *nifH* and *nirS* genes as well as the AOA
290 to AOB ratio were not affected by the NI treatment but were strongly affected by sampling date
291 (Table 5). However, the interaction between the NI treatment and sampling date, as well as the
292 three-way interaction were highly significant for all the genes and for the AOA to AOB ratio
293 (Table 5). Specifically, the abundance of *amoA*-AOA, *nirK* and *nifH* significantly increased over
294 time across all treatments, whereas the abundance of *nirS* increased over time only in NI
295 treatments in rhizosphere soil (Figure 6). In contrast, the abundance of *amoA*-AOB significantly
296 decreased over time across all treatments (Figure 6). AOA to AOB ratios increased over time in
297 both compartments, showing a higher ratio in bulk soil as compared to the rhizosphere (Figure
298 6).
299
300 *Volatile organic compound (VOC) composition*
301 The overall composition of rhizosphere VOCs across treatment and date can be visualized
302 through the first two components of the principal component analysis (PCA) (Figure 7). In
303 permanova tests, VOC profiles were not significantly affected by the NI treatment but differed
304 significantly among sampling dates (Table 6, Figure 7). No individual compounds were found to
305 be significantly different between the control and the NI treatment. However, thirteen
306 compounds showing significant ($P < 0.001$) differences between sampling dates were identified
307 (Figure 8, Table S3). These compounds, ordered by decreasing significance values in Table S3,

308 were tentatively annotated as members of the chemical classes of terpene, ester, alcohol and
309 alkene. The monoterpene alpha-pinene showed the most significant respond to sampling date,
310 with a large increase over time in both the NI and control treatments (Figure 8B).

311 **Discussion**

312 The inhibitory effect of nitrapyrin on soil microbial communities is not totally understood. It
313 remains unresolved whether the application of nitrification inhibitors (NIs) leads to direct or
314 indirect shifts in the overall soil microbial community structure, composition and functioning.
315 The effects of adding various NIs together with N-containing fertilizers have been shown to
316 range from no changes in the community structure of ammonia-oxidizers and in the abundances
317 of total bacteria (Shi *et al.*, 2016; Gu *et al.*, 2019) to only minimal and transient changes on the
318 overall microbial community structure, depending on the compartment (Morales *et al.*, 2015). In
319 contrast to these findings, we found here widespread effects of nitrapyrin on soil and wheat
320 rhizosphere microbial communities across different sampling dates.

321 The nitrapyrin treatment affected the relative abundance of many bacterial, archaeal and
322 fungal taxa. For example, Proteobacteria were affected by the nitrapyrin treatment depending on
323 the compartment, with a decrease in relative abundance in the bulk soil of the nitrapyrin plots.
324 The nitrite-oxidizing bacterial phylum Nitrospirae, that often occurs in close association with
325 AOB or AOA (Daims & Wagner, 2018), was affected by the nitrapyrin treatment depending on
326 the sampling date. Basidiomycota was the only fungal phylum that was affected by the nitrapyrin
327 treatment (in interaction with compartment), and it contains many denitrifiers that play key roles
328 in the N cycle (Hayatsu *et al.*, 2008). Similarly, genes related to denitrification were affected by
329 nitrapyrin in interaction with sampling date, most probably through changes in the availability of
330 nitrate. It is difficult to ascertain if these shifts were caused directly by nitrapyrin (e.g. off-target
331 toxic effects) or indirectly through the reduced activities of ammonia-oxidizers. Our results
332 suggest that, in agreement with our hypothesis, nitrification inhibitors have off-target but
333 unpredictable effects on soil microbial communities and associated nitrogen-cycle functions.

334 Nitrapyrin also had significant effects on its expected targets, the ammonia-oxidizers.
335 Nitrapyrin is generally thought to be a strong inhibitor of AOBs, with limited efficiency for AOA
336 (Coskun *et al.*, 2017a,b). We were thus expecting a decrease in AOB for the nitrapyrin plots, and
337 potentially an increase in AOA because of the lowered competition from AOB. For AOA, we did
338 find an increase in both the qPCR and 16S rRNA gene data. The dominant genera found in our
339 samples, the AOA *Nitrososphaera*, and the associated phylum (Thaumarcheota) had significantly
340 higher relative abundance following the addition of nitrapyrin. This finding is consistent with a
341 laboratory experiment in which the inhibitory effect of nitrapyrin was stronger on a
342 representative AOA strain (*Ca. Nitrososphaera*) than a AOB strain (*Nitrosospira multiformis*)
343 (Shen *et al*., 2013). Here, the increase observed in the abundance of the AOA-*amoA* gene was
344 more important at the first sampling date. Surprisingly, similar trends were observed for the
345 abundance of AOB-*amoA* gene in the rhizosphere of wheat, suggesting a weak efficiency of the
346 nitrapyrin on its target group. These strong temporal patterns suggest that environmental factors
347 are likely to play a dominant role in structuring ammonia-oxidizers. It was previously shown that
348 ammonia-oxidizers are affected greatly by soil pH (Nicol *et al*., 2008; Liu *et al*., 2015) and
349 temperature (Tao et al., 2021). Irrespective of these temporal patterns, the effects of nitrapyrin on
350 the abundance of ammonia-oxidizers might not necessarily have consequences on nitrification
351 rates. Previous studies on the effect of nitrapyrin on the AOA to AOB ratio and its effect on
352 nitrification rates have reported inconclusive results, (Fisk *et al.*, 2015; Lehtovirta-Morley *et al.*,
353 2018; Gu *et al.*, 2019).
354 Nitrapyrin did not significantly influence the volatile metabolic profile in the rhizosphere of
355 wheat, suggesting that the changes observed in the microbial community composition, structure
356 and functions at the DNA level did not result in major shifts in the metabolic activities of the

357 rhizosphere microbes and plant roots. This could be related to the high functional redundancy
358 typically found in agricultural soils that is responsible for maintaining ecosystem functioning
359 under fluctuating environmental conditions (Mendes *et al.*, 2015). Interestingly, alpha-pinene
360 was identified as the most significantly different compound between the two sampling dates,
361 with a sharp increase at the second sampling date. Alpha-pinene, amongst other monoterpenes
362 has been previously shown to inhibit nitrification by targeting the ammonia monooxygenase
363 enzyme (Bremner & McCarty, 1988; White, 1994), which could explain part of the large
364 decrease in AOB-*amoA* abundance at the second sampling date. These findings contrast a
365 previous study that linked bacterial VOC emissions to an increasing AOB-*amoA* gene
366 abundance, but without identifying the responsible terpenes (Yuan *et al.*, 2017). It remains
367 unclear what ecological role alpha-pinene plays in the interaction between the plant, soil
368 microbes and nitrifiers and what consequences it might have on the N-cycle, but it could have
369 substantial ecological implications.

370 In summary, we showed that, in agreement with our hypothesis, nitrapyrin application results
371 in non-target changes in microbial communities and in the abundance of N-cycle functional
372 genes. These effects were however not mirrored in the metabolic activities of the community, as
373 estimated by VOC concentrations in the rhizosphere. The effects of nitrapyrin were constrained
374 by sampling date and soil compartment, suggesting that the non-target effects of nitrapyrin vary
375 seasonally and across soil compartments. Our results are important as non-target effects of
376 nitrification inhibitors could disturb other soil processes and plant-microbe interactions, negating
377 their potentially positive environmental and agricultural impacts.

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383 **Author contributions**

384 XW, EY designed the study, XW and RS performed the experiment and RS analyzed the data.
385 RS wrote the manuscript with the revisions and comments from EY, XW and PG. All coauthors
386 participated in discussions at the working group meetings and granted valuable advices of the
387 manuscript.

388 **Conflict of Interest**

389 The authors declare no conflict of interest.

390 **Figure legends**
391
392 **Figure 1**: **Alpha diversity of bacterial, archaeal (16S) and fungal (ITS) communities**.
393 Diversity indices were calculated using Shannon diversity index, Inverse Simpson index and
394 Faith's Phylogenetic Diversity (PD) across NI treatments and sampling dates. The boxplot shows
395 quartile values per compartment (bulk soil and rhizosphere) and are colored by treatment and
396 facetted by sampling date (2019-07-23, 2019-09-05).
397
398 **Figure 2**: **Bacterial (16S, A) and fungal (ITS, B) community composition at the phylum**
399 **level**. The phyla showed had a mean relative abundance above 1% based on Illumina amplicon
400 sequencing of the 16S rRNA gene and the ITS region for bacteria and archaea, and fungi,
401 respectively. Treatment: NI: Fertilizer with nitrapyrin, Control: Fertilizer without nitrapyrin.
402
403 **Figure 3**: **Variation in the relative abundance of the dominant bacterial, archaeal (16S) and**
404 **fungal (ITS) phyla across treatments**. Treatment: NI: Fertilizer with nitrapyrin, Control:
405 Fertilizer without nitrapyrin, compartment (bulk soil and rhizosphere) and sampling dates (2019-
406 07-23, 2019-09-05).
407
408 **Figure 4**: **Variation in the relative abundance of the top 10 most abundant bacterial,**
409 **archaeal (16S) and fungal (ITS) genera across treatments**. Treatment: NI: Fertilizer with
410 nitrapyrin, Control: Fertilizer without nitrapyrin, compartment (bulk soil and rhizosphere) and
411 sampling dates (2019-07-23, 2019-09-05).
412

413 **Figure 5**: **Principal Coordinates Analysis (PCoA) of the bacterial, archaeal (16S) and
414 fungal (ITS) community composition based on Bray-Curtis dissimilarity**. The first and
415 second axes of the 16S ordination explained 34.6% and 6% of the variance for sampling date
416 2019-07-23, and 38.1% and 13.9% of the variance for sampling date 2019-09-05. For ITS, the
417 first and second axes of explained 23.1% and 15.1% of the variance for sampling date 2019-07-
418 23, and 29.3% and 22.8% of the variance for sampling date 2019-09-05. Treatment: NI:
419 Fertilizer with nitrapyrin, Control: Fertilizer without nitrapyrin, compartment (bulk soil and
420 rhizosphere).
421
422 **Figure 6**: **Real-time PCR quantification of the abundance of the archaeal (A) and bacterial
423 (B) ammonia monooxygenase A subunit (*amoA*-AOA and *amoA*-AOB, respectively) genes,
424 the AOA:AOB ratio (C), and the *nirK* (D), *nirS* (E) and *nifH* (F) genes.** The boxplot shows
425 quartile values colored by treatments. Treatment: NI: Fertilizer with nitrapyrin, Control:
426 Fertilizer without nitrapyrin, compartment (bulk soil and rhizosphere) and sampling dates (2019-
427 07-23, 2019-09-05).
428
429 **Figure 7**: **(A) Principal Component Analysis (PCA) of the VOCs emitted in the
430 rhizosphereof wheat plants**. The first and second axes of the ordination explained 37.1% and
431 13.3% of the variance. Treatment: NI: Fertilizer with nitrapyrin, Control: Fertilizer without
432 nitrapyrin, and sampling dates (2019-07-23, 2019-09-05).
433
434 **Figure 8: Heatmap of the VOCs emitted in the rhizosphere of wheat with Euclidian
435 distance-clustering of samples (A)**. 'Unknown' indicates that no match was found in the NIST

436 library 2014 V2.20, or based on mass spectra, retention time and retention index (Table S3).
437 Treatment: NI: Fertilizer with nitrapyrin, Control: Fertilizer without nitrapyrin, and sampling
438 dates (2019-07-23, 2019-09-05). **Alpha-pinene concentration across treatment and sampling**
439 **dates (B)**. Treatment: NI: Fertilizer with nitrapyrin, Control: Fertilizer without nitrapyrin, and
440 sampling dates (2019-07-23, 2019-09-05). Values are depicted as log median normalised values.

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