

25 mesocosms, due to concurrent presence of elevated pH values and use of highly oxidized substrates
26 (like acetate).

27 This study suggests that agricultural practices aimed to increase the availability of labile organic
28 matter, such as acetate, are beneficial in buffering reactive N excess in soils and to reduce NO_3^-
29 leaching towards groundwater and surface-water.

30

31 **Keywords**

32 Agricultural soils; denitrification; N_2 :Ar method; reactive N loss; NO_3^- groundwater pollution.

33

34 **1. Introduction**

35 Under the pressure of an increasing world population and food demand, the use of nitrogen (N)
36 synthetic fertilizers, mainly in form of urea is increasing exponentially (Glibert et al., 2006; Liu et al.
37 2015). As a result, an immense mass of N is driven from the atmosphere to agroecosystems, which
38 then partly is lost to surface and ground waters as reactive N (Nr) and partly returns to the atmosphere
39 via volatilization of ammonia (NH_3) and denitrification. The latter process can end up in the forms of
40 both non-reactive dinitrogen (N_2) and the greenhouse gas nitrous oxide (N_2O). Environmental and
41 health consequences of this man-driven global Nr flux are serious, unresolved and not fully
42 understood (Leip et al., 2015; Reis et al., 2016; Zhang et al., 2015). Nitrate (NO_3^-) loss to surface
43 water and the consequential eutrophication is by far the most studied topic in previous decades.
44 Recent studies have highlighted NO_3^- contamination of groundwater and its role as potential
45 temporary or permanent sink for N (Böhlke, 2002; Sacchi et al. 2013; Sebilo et al. 2013). Conversely,
46 much less is known on denitrification in agricultural soils and particularly on the proportions between
47 the gaseous products, i.e. N_2 and N_2O . The latter is one of the most alarming greenhouse gases since
48 it has global warming potential 265-times that of CO_2 (Bouwman et al., 2013; Saggar et al., 2013;
49 Smith, 2017).

50 Denitrification is an essentially anaerobic respiration process, in which N, mainly in form of NO_3^- , is
51 used as an electron acceptor in the oxidation of carbon (C) and reduced to N gases (N_2 and N_2O). It
52 is an important and widespread microbial process which occurs in a variety of waterscapes and
53 landscapes at the global scale (Bothe et al., 2006; Seitzinger et al., 2006). Denitrification in sediments
54 of rivers, canals, lakes, lagoons and coastal waters has been intensively investigated since the '70s
55 (Nixon, 1995), mainly focusing on the regulation of the process by abiotic parameters (Piña-Ochoa
56 and Alvarez-Cobelas, 2006; Birgand et al., 2007; Veraart et al., 2016), and biotic factors, such as
57 benthic macrofauna and rooted macrophytes (Bartoli et al., 2008; Bonaglia et al., 2014; Soana et al.,
58 2017). However, to the best of our knowledge, denitrification process in agroecosystems has not been
59 fully evaluated to date. Soil features (e.g. organic matter), agricultural practices and climatic evolution
60 are all important elements regulating denitrification (Rivett et al., 2008; Aguilera et al., 2013; Barakat
61 et al., 2016). Furthermore, the role of organic C in driving the denitrification and emission of N_2O
62 has been recently reviewed (Charles et al., 2017), demonstrating that, varying organic C amendments
63 can alter N_2O emission factors. Particularly, highest N_2O emissions were attributed to the use of liquid
64 manure whereas opposite effects were observed in compost use. The fate of N excess and relevance
65 of denitrification in agricultural soils is nevertheless open to debate (Burgin et al., 2013; Duncan et
66 al., 2013). This topic is central not only in environmental policy, i.e. the Water Framework Directive
67 or the Clean Water Act but is also vital for the agricultural practice itself (van Grinsven et al., 2015).
68 Besides denitrification, other processes like dissimilatory NO_3^- reduction to NH_4^+ (DNRA) can affect
69 the N cycle in soils (Rütting et al., 2011). Although, NO_3^- reduction by DNRA is favoured over
70 denitrification only in intensively reduced and C-rich environments (Yin et al., 2002; Schmidt et al.
71 2011) with C: NO_3^- ratios usually higher than 12 (Yin et al., 1998). Questions on the availability of
72 the residual N after a cropping cycle, on the fate of N distributed in pre-seeding, on coverage of the
73 most demanding crops, such as maize and wheat, are equally relevant, but nevertheless left
74 unanswered. What is the fate of NO_3^- if heavy rains bring the soil to saturation? How do microbial N
75 transformations change according to soil features? How much of N distributed is lost in the surface

76 and ground waters, how much is lost to the atmosphere, and how much consequently remains
77 available for the crops? The answers to these questions are evidently difficult if not impossible in
78 most cases and generally in agricultural practice the strong unpredictability of N processes is
79 overcome based on farmers' experience. Farmers in western regions generally distribute more N
80 compared to crop needs to avoid the risk of N scarcity. Relatively low price of N fertilizers further
81 facilitates overuse. An attempt to overcome the problem is the use of slow-release N fertilizers or the
82 combined use of urea and nitrification inhibitors (Cameron et al., 2013; McGeough et al., 2016).
83 However, results of the use of these technical alternatives are not always clear and neither are the
84 consequences of introduction of large amounts of nitrification inhibitors or other chemicals in nature
85 (Ruser and Schulz, 2015; Coskun et al., 2017). Regardless of these environmental aspects, the higher
86 price of such fertilizers has prevented their wide use and simple urea has remained by far the most
87 applied fertilizer (Nishina et al. 2017).

88 In the present study, N removal via denitrification was quantified in an intensively cropped lowland
89 of the Po River delta (Northern Italy). These agricultural lands are characterized by fine textured soils,
90 generally impoverished of labile organic matter and fertilized mainly with synthetic urea. In this area,
91 the most important hydrological and microbiological processes affecting Nr fate in both surface and
92 ground waters have been extensively studied (Mastrocicco et al., 2011a; Mastrocicco et al., 2011b;
93 Aschonitis et al., 2012; Aschonitis et al., 2013; Castaldelli et al., 2013; Castaldelli et al., 2015).
94 Denitrification was evaluated in two soil types characterized by different soil textures and content of
95 organic matter by the concomitant measurements of NO_3^- consumption and N_2 production via $\text{N}_2:\text{Ar}$
96 analyses by Membrane Inlet Mass Spectrometry (MIMS), by means of soil core incubations in
97 waterlogged conditions. The selection of $\text{N}_2:\text{Ar}$ technique rather than the classical ^{15}N tracer
98 technique was done since comparable results can be obtained in freshwater core incubations (Smith
99 et al. 2006). The specific aims of the study were: i) to describe the temporal evolution of
100 denitrification in saturated soils fertilized with synthetic fertilizers by monitoring reactants (NO_3^- and
101 acetate) and products (N_2 and dissolved inorganic carbon-DIC) of the reaction; ii) to determine the

102 role of labile organic matter limitation, iii) to analyze possible unwanted accumulation of intermediate
103 by-products like NO_2^- and N_2O .

104

105 **2. Material and Methods**

106 **2.1. Study sites**

107

108 The Po Plain lowlands, in Northern Italy, are intensively cultivated, thanks to the flat topography and
109 to the large availability of surface water for irrigation purposes; maize and wheat are the dominant
110 crops. The Province of Ferrara, located near the Po delta, is the lowest territory, at an altitude ranging
111 from 5 to -3 m above sea level. Following the enactment of the European Directive for water
112 protection (2000/60 CE), the whole territory was declared “vulnerable to nitrates from agricultural
113 sources” and limits were set on N fertilization.

114 Soil cores were collected in two sites, named LOAM (silty-loam) and CLAY (silty-clay), selected on
115 the basis of results previously achieved by field monitoring and experiments on denitrification
116 (Mastrocicco et al., 2011a; Mastrocicco et al., 2011b; Castaldelli et al., 2013). The silty-loam soil is
117 typically Haplic Calcisols, moderately alkaline, with the upper horizons characterized by silty clay
118 loamy texture and lower horizons with calcareous silty loamy textures, while the silty-clay soil is
119 typically deep Vertic Cambisols, moderately alkaline, with silty clay or clay loamy textures. LOAM
120 and CLAY are typical soil types of the lower Po River floodplain, intensively fertilized with synthetic
121 compounds and not amended with livestock manure for decades. For a detailed characterization of
122 the sites, see Castaldelli et al. (2013).

123

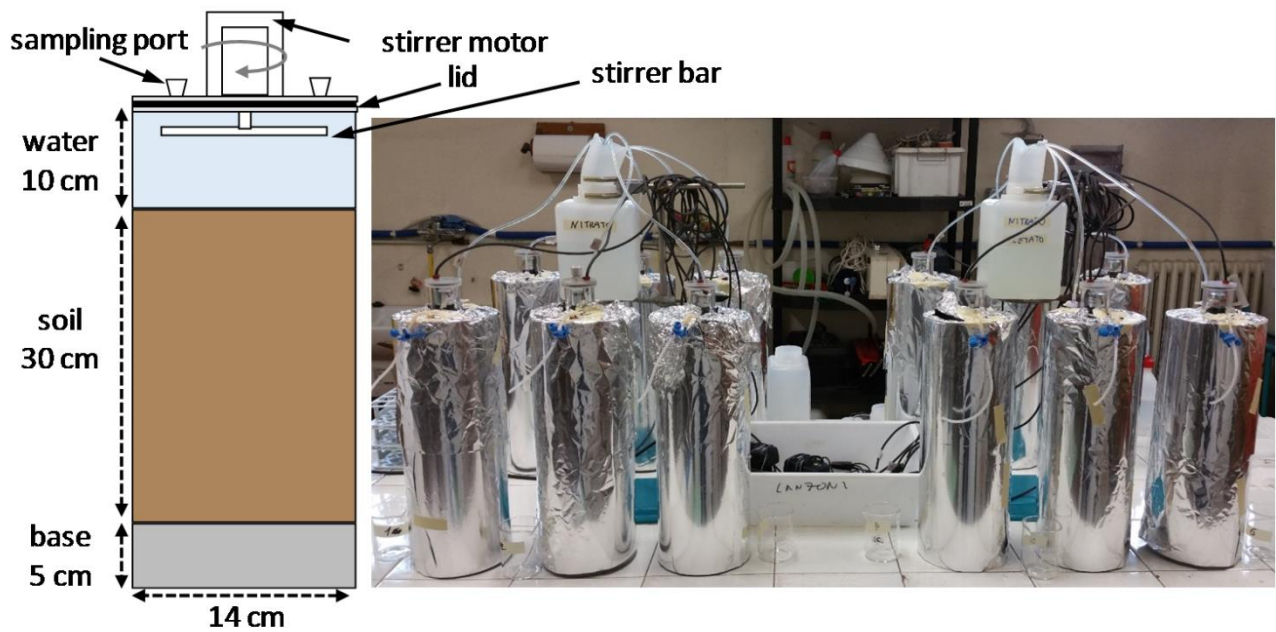
124 **2.2. Soil sampling and incubation**

125

126 Soil sampling was performed on the 18th of April 2016, a period representative of the first N coverage
127 fertilization on wheat for the study area. Soils were manually excavated from the plough layer (from

128 topsoil to 30 cm below ground level) and re-packed in Plexiglass columns (Fig.1) maintaining the
129 same orientation and degree of compaction of the fields. In an hour after sampling, the cores, six for
130 each site, were brought to the laboratory and placed in a thermostatic room. The day after the
131 sampling, the cores were slowly saturated with rain water ($\text{NO}_3^- < 50 \mu\text{mol/L}$) collected from the field
132 sites using a collection tank and amended with NO_3^- to a final concentration of 6.4 mmol/L. According
133 to the local enactment of the European Directive for water protection (2000/60 CE), in areas declared
134 “vulnerable to nitrates from agricultural sources”, N fertilization for wheat is limited to the maximum
135 amount of $170 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, in various forms of synthetic fertilizers, as ammonium nitrate, trivalent
136 fertilizer (nitrogen, phosphorus and potassium, NPK) and synthetic urea. The Nr amount provided in
137 the solutions recapitulated the limits of the EU directive and followed the common fertilization
138 practices. For both soils, an intense rain event of 100 mm after the first Nr distribution in coverage
139 on wheat (equivalent to 90 kg N ha^{-1}) was simulated. Thus, the Nr amount was dissolved in a
140 hypothetical cumulative rainfall of 100 mm, resulting in 6.4 mmol/L of NO_3^- mentioned above. In
141 these soils the conversion of urea and ammonia to NO_3^- occurs in a few days and to avoid any lag
142 phase, all experiments were performed by adding Nr already in form of NO_3^- . Three of the six cores
143 from each site were amended with acetate (LOAM-ace and CLAY-ace) to a final concentration of
144 5.40 mmol/L, according to the stoichiometric relationship describing denitrification using acetate as
145 electron donor (Lew et al., 2012). Low molecular weight organic acids like acetate originate both
146 from root exudates and decomposition of crop residues. For the investigated site, the burial of crop
147 residues into soil at the end of the cropping cycle usually corresponds to the only organic amendment.
148 Three cores from each site were left unamended (LOAM and CLAY) and served as blank. Once
149 saturated with rain water, the cores were left open and unstirred for a few hours to allow for trapped
150 air bubbles to escape and reach anoxia. Then cores were sealed with a screw cap equipped with a
151 screw-tight rubber seal between two PVC disks. When sealed, each core consisted of 30 cm of soil
152 overlaid by 10 cm of water (Fig. 1).

153



154

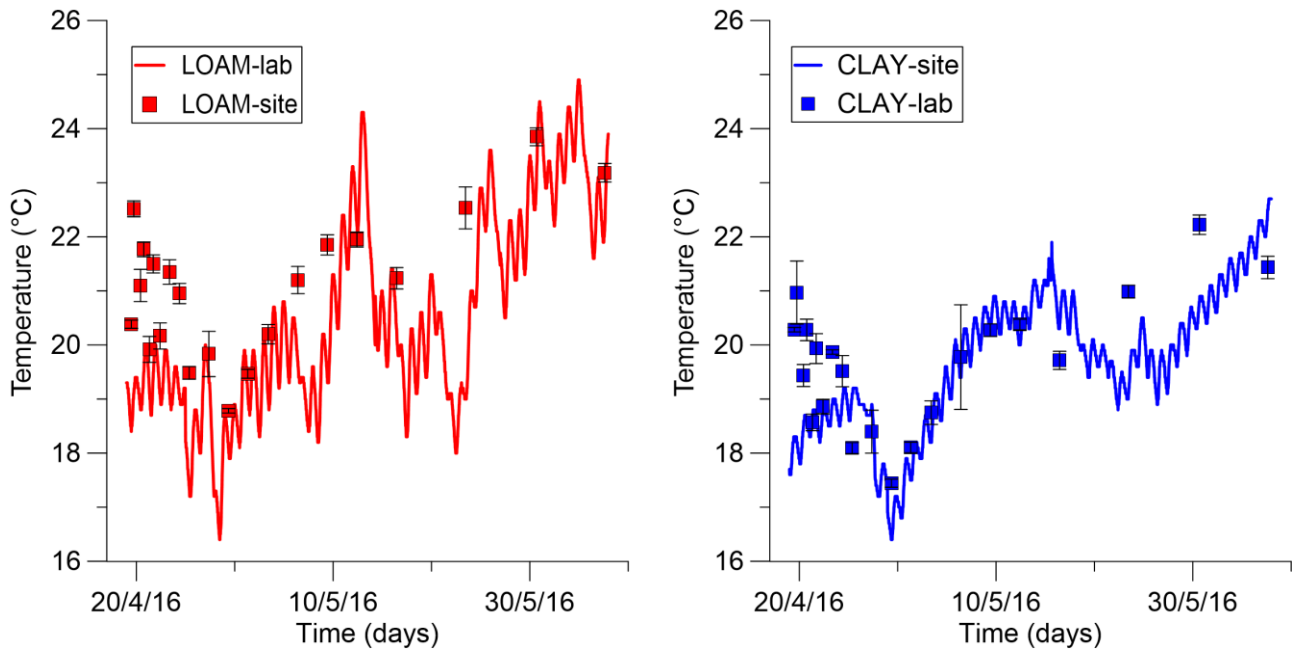
155 Figure 1. Experimental set up, with the mesocosm scheme (on the left) and the picture (on the right)
 156 of the complete laboratory experiment, consisting of twelve soil cores, three for each treatment, i.e.
 157 unamended (LOAM and CLAY) and acetate amended (LOAM-ace and CLAY-ace).

158

159 The superior lid was equipped with a 12 V motor mounted externally and a squared Plexiglass cross,
 160 rotating at 10 rpm, maintained the water column slowly stirred and in equilibrium with soil pore
 161 water, thus avoiding resuspension. The experiment was started when all liners were closed. Pre-
 162 incubation and incubation procedures were performed according to standard protocols (Dalsgaard et
 163 al., 2000). Cores were wrapped in aluminum foils to avoid exposure to light and kept in a thermostatic
 164 room at an average temperature of superficial soil in the field from April to June. The latter was
 165 measured in the field with a thermistor placed from 10 to 20 cm below ground and connected to a
 166 data logger (Decagon Devices, Inc., Pullman WA, USA) in both LOAM and CLAY sites. Here, soil
 167 temperature increased approximately from 18 °C to 24 °C between April and June, and experimental
 168 room temperature was consequently adjusted to mimic this behavior (Fig. 2).

169

170



171

172 Figure 2. Temperature monitored at the LOAM site (red line), at the CLAY site (blue line) and in
 173 laboratory mesocosms (LOAM red squares and CLAY blue squares) with their standard deviations
 174 (error bars)

175

176 Overlying water of each core was sampled at decreasing frequencies as the experiment progressed:
 177 twice a day for the first three days, daily during the following four days, every two days during the
 178 following ten days, and every three days until the end of the incubation, after a total of five weeks.
 179 Water was collected using a 50-mL gas-tight glass syringe through a sealed sampling plastic tube
 180 inserted in the superior lid and equipped with a small one-way valve, which was opened only on
 181 occasion of the sampling. At each sampling time, a withdrawn volume of 40 mL, accounting for <5%
 182 of the core headwater volume, was replaced in real time by NO_3^- amended or NO_3^- and acetate
 183 amended rain water, according to the treatment. Similar sampling port, connected by a plastic tube
 184 and a one-way valve was used to refill and flow of replaced volume in to each core was aided by
 185 gravity. NO_3^- and acetate concentration in the refill was set daily to the value measured in the water
 186 sampled from the single core in the previous day.

187 Water from each core was analyzed for dissolved inorganic N forms (NO_3^- , NO_2^- , NH_4^+), volatile
 188 fatty acids (VFA) as acetate, dissolved gasses (N_2 , N_2O) and dissolved inorganic carbon (DIC). At
 189 each sampling, water temperature was measured with a multiparametric probe and dissolved oxygen
 190 with a microsensor (OX-500, Unisense, Science Park Aarhus, Denmark) in the stirred water column.
 191 Anoxia was reached less than one day after the cores were sealed.
 192 Twelve additional cores (Plexiglas liners, i.d. 4.5 cm, height 20 cm), six from each site, were collected
 193 for soil characterization: grain size analysis, dry bulk density, porosity, soil organic matter (SOM)
 194 and CaCO_3 . Grain size analyses were performed with the sieve-pipette method (Day, 1965). Dry bulk
 195 density and porosity were quantified gravimetrically at 105 °C for 24 hours. SOM was quantified as
 196 loss on ignition in a muffle furnace at 350 °C for 3 hours on dry powdered soil aliquots (Tiessen and
 197 Moir, 1993). Soil CaCO_3 was measured titrimetrically following the method of Bundy and Bremner
 198 (1972). Soil parameters are shown in Table 1.

199
 200 Table 1. Grain size distribution, dry bulk density (ρ), porosity (θ), soil organic matter (SOM), and
 201 CaCO_3 measurements for LOAM and CLAY (average \pm standard deviation, $n=6$).

Parameter	LOAM	CLAY
Grain size (%)		
Sand (63-2000 μm)	20.8 \pm 0.3	4.8 \pm 0.3
Silt (2-63 μm)	59.6 \pm 0.4	35.6 \pm 0.4
Clay (<2 μm)	19.6 \pm 0.6	59.6 \pm 0.6
ρ (kg/m^3)	1.43 \pm 0.1	1.37 \pm 0.1
θ (-)	0.46 \pm 0.1	0.49 \pm 0.1
SOM (%)	3.1 \pm 0.3	3.7 \pm 0.4
CaCO_3 (%)	10.7 \pm 0.4	5.3 \pm 0.3

204 2.3. Analytical methods

205 Samples for dissolved inorganic N (NO_3^- , NO_2^- , NH_4^+) determinations were filtered through
 206 Whatman GF/F glass fiber filters, transferred to polyethylene vials and frozen for later analysis. NO_3^-
 207 and NO_2^- were measured on a Technicon AutoAnalyser II (Armstrong et al., 1967). The NH_4^+

208 concentration was determined on a double beam Jasco V-550 spectrophotometer using salicylate and
209 hypochlorite in the presence of sodium nitroprussiate (Bower and Holm-Halsen, 1980). However,
210 NH_4^+ concentrations were always below detection limits ($<0.5 \mu\text{mol/L}$). Acetate concentration was
211 determined as total fatty acids on a double beam Jasco V-550 spectrophotometer after esterification
212 with ethylene glycol and subsequent reduction with Fe(III) salts.

213 Samples for gas determinations were collected by overflowing at least 3 times 12-mL gas-tight glass
214 vials (Exetainer®, Labco, High Wycombe, UK), and preserved by adding 100 μL of 7M ZnCl_2
215 solution. Water samples were analyzed for concentrations of dissolved $^{28}\text{N}_2$, Ar and CH_4 gases at the
216 laboratory of Aquatic Ecology, University of Ferrara, by MIMS (Bay Instruments, Easton, Maryland;
217 Kana et al., 1994).

218 Previous studies have highlighted an agreeable correlation between N_2 production rates obtained by
219 measurements of $\text{N}_2:\text{Ar}$ (MIMS). In coupled process of nitrification/denitrification, addition of ^{15}N -
220 labeled tracers, followed by isotope ratio mass spectrometry (IRMS) analysis was relatively
221 unimportant (Smith et al. 2006). This most likely recapitulates our experimental conditions since
222 anoxia was reached less than one day after the cores were sealed.

223 For MIMS analysis, the water sample, after equilibration at 20°C , is pumped through a gas-permeable
224 silicone membrane that is under vacuum. The extracted gasses then pass through: 1) a liquid nitrogen
225 cryogenic trap to remove carbon dioxide and water vapor that may interfere with measurement of N_2
226 gas, 2) a copper reduction column operating at 600°C to remove oxygen, and 3) a second liquid
227 nitrogen trap before ionization and detection by a PrismaPlus quadrupole mass spectrometer. Oxygen
228 removal is necessary to avoid production of NO within the MIMS ion source that can potentially
229 affect not only the signal of $^{28}\text{N}_2$ but also that of other gases due to nonlinear ionization efficiency
230 (Eyre et al. 2002; Kana and Weiss 2004).

231 The CH_4 , N_2 and Ar concentrations were quantified by the ion current detected at m/z ratios of 15,
232 28, and 40. For MIMS determinations the analytical precision of gas ratio measurements (coefficient

233 of variation $\sim 0.04\%$) is generally higher than the analytical precision of gas concentration
234 measurements (coefficient of variation $\sim 0.4\%$). Thus, the N_2 concentrations were calculated from the
235 measured $N_2:Ar$ multiplied by the theoretical saturated Ar concentration at the sampling water
236 temperature determined from gas solubility tables (Weiss, 1970), assuming that Ar concentration
237 reflected only physical processes since not affected by biological processes. The approach does not
238 distinguish between denitrification and anammox (Anaerobic Ammonium Oxidation), both N_2
239 producing processes. Data on anammox in soils are scarce but its occurrence is generally irrelevant
240 if compared to denitrification in waterlogged conditions (Bai et al., 2015; Shan et al., 2016).

241 The MIMS response is linear to a wide range of gas concentrations; thus, a single point calibration is
242 usually employed for each mass. The primary standard for MIMS analyses is de-ionized water
243 maintained at a constant temperature ($20^\circ C$) in a circulating bath with headspace at 100% relative
244 humidity and equilibrated to atmospheric gases by low stirring. The ion currents were standardized
245 by applying gas solubility equations of Weiss (1970) and the instrument drift was corrected by
246 measuring thermally equilibrated water every six samples.

247 The best m/z for CH_4 measurements is 15, corresponding to the CH_3 ionization fragment and for
248 which there are no substantial interferences. Moreover, the liquid nitrogen trap does not condense
249 CH_4 but all the other volatile organic compounds that pass through the membrane and may contribute
250 to CH_3 formation. Due to the very low concentration in air, a separate standard procedure for CH_4
251 was used following the headspace equilibration technique. Standards were prepared by injecting
252 known amounts of pure gas ($>99.0\%$, Sigma Aldrich) into the headspace of 12-mL gas-tight glass
253 vials filled with de-ionized water. Prior to MIMS analyses, the vials were shaken vigorously for a
254 minute and placed in a thermostatic bath for solubility equilibration. The CH_4 concentrations never
255 exceeded $8 \mu g/L$, with most values $<3 \mu g/L$, without any temporal increases or differences among
256 treatments (data not shown). Methanogenesis was thus considered a negligible pathway in anaerobic
257 degradation of organic matter. The N_2O analyses were not performed extensively for all the monitored
258 times and cores, but only in a few selected samplings to assess the relevance of N_2O accumulation

259 with respect to N₂. N₂O was measured by gas chromatography (Trace GC, 2000 Series, Thermo
260 Finnigan, San Jose, CA, USA equipped with an ECD detector) on samples collected in the same way
261 of those for N₂:Ar determinations. Samples for dissolved inorganic carbon were transferred into 12-
262 mL gas-tight glass vials (Exetainer®, Labco, High Wycombe, UK) and immediately titrated with 0.1
263 eq/L HCl (Anderson et al. 1986).

264

265 **3. Results and discussion**

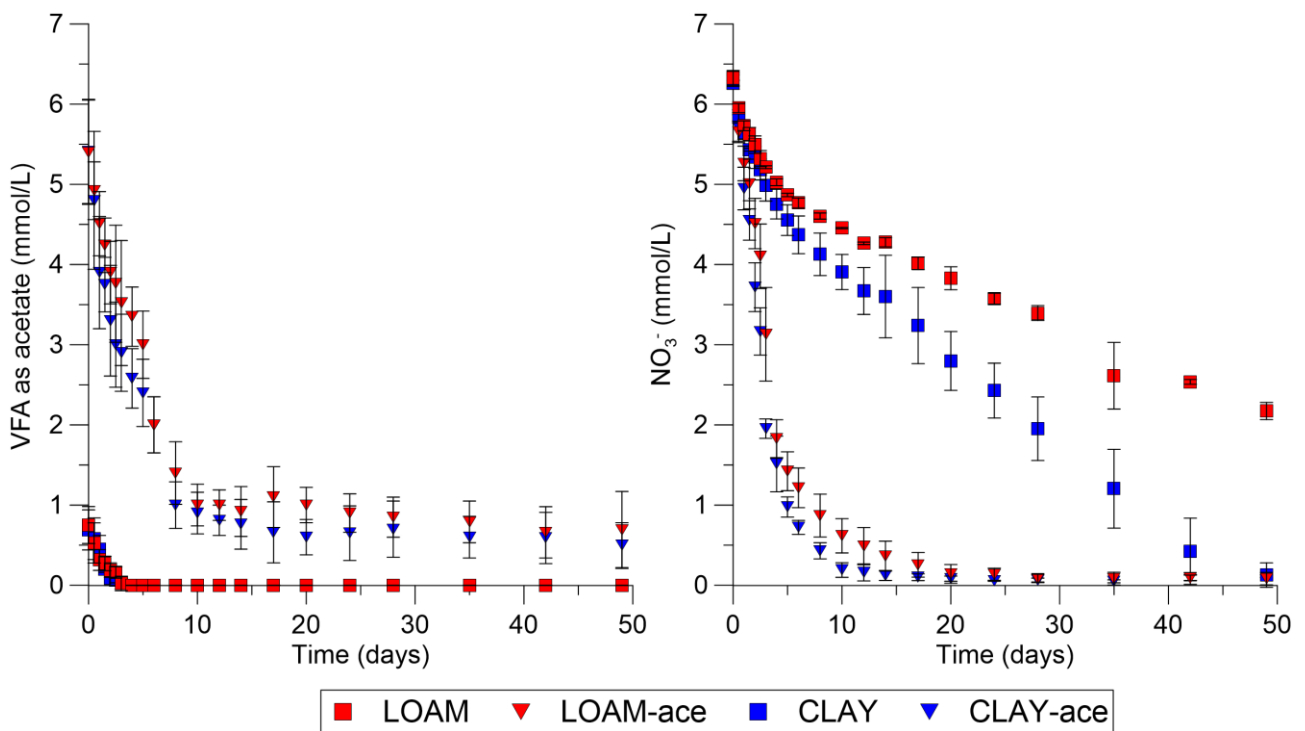
266 **3.1. NO₃⁻ and acetate consumption**

267 The consumption of both NO₃⁻ and VFA during the incubation time are depicted in Figure 3. NO₃⁻
268 concentrations decreased in all the incubated mesocosms. However, in control conditions (LOAM
269 and CLAY) there was a significant delay in NO₃⁻ removal compared to amended conditions (LOAM-
270 ace and CLAY-ace) due to the lack of labile organic substrates. In amended conditions, NO₃⁻
271 concentrations dropped by more than 75% and 90% of initial readings, after 4 and 10 days,
272 respectively. Nevertheless, in CLAY mesocosms, only one third of the initial NO₃⁻ remained at the
273 end of the experiment due to the higher SOM background (Table 1) compared to LOAM mesocosms
274 that instead halved their NO₃⁻ concentrations, denoting intrinsic limited NO₃⁻ reduction capacity of
275 this soil. Since NH₄⁺ concentrations were always below detection limits (<0.5 μmol/L) in all the
276 mesocosms, both DNRA and anammox processes were excluded as main pathways. In addition, the
277 C:NO₃⁻ ratio was approximately 0.8 in acetate amended mesocosms and even lower in unamended
278 ones, a condition that does not facilitate DNRA (Rütting et al., 2011). This is also congruent with a
279 recent study on DNRA in coastal wetland sediments, where other organic C substrates (i.e. oxalate,
280 citrate, glucose) showed a higher stimulating effect on DNRA with respect to acetate (Liu et al.,
281 2016).

282 The VFA were found also in control mesocosms with initial concentrations <0.8 mmol/L, since they
283 are typical by-products of SOM degradation processes in these agricultural soils (Castaldelli et al.,

284 2013). In unamended conditions, the natural VFA background was consumed in the first three days,
 285 while in amended conditions VFA decreased rapidly in the first stage of the experiment, i.e. the first
 286 ten days, followed by stable or slight decrease of concentrations towards the end of the incubation.
 287 Thus, the availability of VFA and in general of labile organic substrates can dramatically increase the
 288 denitrification rate while their scarcity, can promote NO_3^- -leaching from these soils.

289



290

291 Figure 3. VFA concentrations (left plot) and NO_3^- concentrations (right plot) versus time in
 292 unamended (LOAM and CLAY) and acetate amended (LOAM-ace and CLAY-ace) mesocosms
 293 (average \pm standard deviation, $n=3$).

294

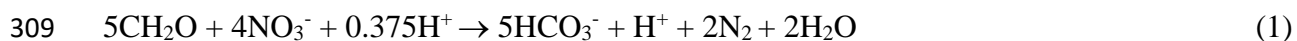
295 3.2. DIC and N_2 production

296 The production of both DIC and N_2 during the incubation time are depicted in Figure 4. DIC and N_2
 297 were produced also in control mesocosms due to the presence of SOM background in both soils
 298 undergoing degradation (Table 1) but exhibiting different patterns with respect to acetate amended
 299 mesocosms. Congruently with the consumption of VFA and NO_3^- reported before, the accumulation

300 of DIC and N₂ in amended conditions (LOAM-ace and CLAY-ace) largely exceeded the unamended
301 conditions (LOAM and CLAY) in the first two weeks of incubation.

302 In acetate-amended conditions, a steep increase of both N₂ and DIC concentrations was detected
303 during the first days of the experiment, followed by almost stable values towards the end. Differently,
304 in LOAM and CLAY mesocosms DIC and N₂ increased slowly but constantly during the course of
305 the incubation. However, as pointed out previously for NO₃⁻ consumption, the smaller DIC and N₂
306 accumulation detected for LOAM confirmed its intrinsically limited NO₃⁻ reduction capacity with
307 respect to CLAY.

308 The general stoichiometry for the reduction of SOM via denitrification:



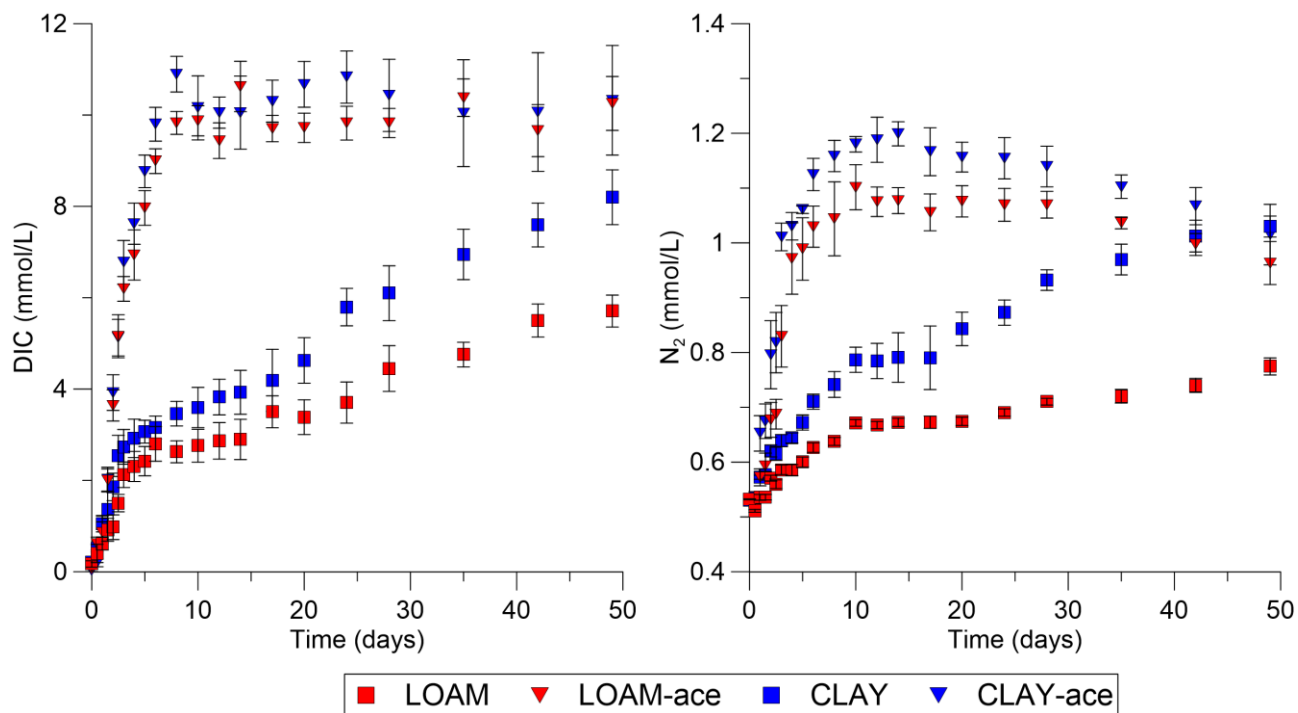
310 denotes that per mole of NO₃⁻ consumed, half a mole of N₂ is produced. Therefore, the expected N₂
311 concentrations increase should be up to 3 mmol/l in all the mesocosms, except in LOAM, where the
312 expected N₂ concentration increase should be around to 2 mmol/l. In our experiments, the dissolved
313 N₂ concentrations reached an upper limit of 1.2 mmol/l and then decreased to values around 1 mmol/l
314 in the acetate amended mesocosms (LOAM-ace and CLAY-ace); while in the unamended ones
315 (LOAM and CLAY) a more gradual N₂ increase was observed (Fig. 4). This apparent inconsistency
316 is easily explained by the N₂ solubility in water that is quite low around 20 °C (Kolev, 2011), thus the
317 formation of gas bubbles (largely observed during our experiments) in the all the acetate amended
318 mesocosms could have caused the observed N₂ decrease in the water phase.

319 As pointed out in the previous sub-section, in CLAY mesocosms DIC and N₂ reached the values
320 observed for the acetate amended mesocosms after 50 days of incubation due to the elevated presence
321 of SOM (Table 1), while in LOAM mesocosms DIC and N₂ increased slowly confirming the
322 intrinsically limited NO₃⁻ reduction capacity of these soils.

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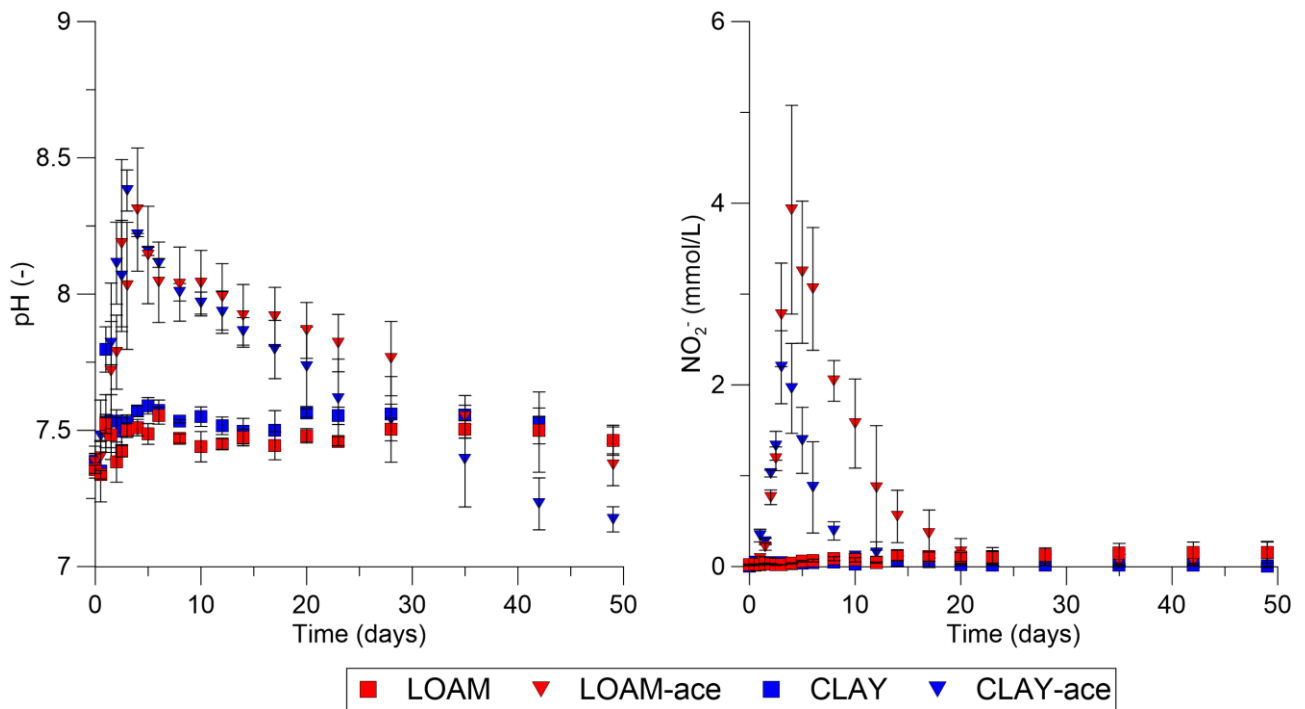
326

327 Figure 4. DIC concentrations (left plot) and N₂ concentrations (right plot) versus time in unamended
 328 (LOAM and CLAY) and acetate amended (LOAM-ace and CLAY-ace) mesocosms (average ±
 329 standard deviation, *n*=3).

330

331 3.3. pH variations and NO₂⁻ accumulation

332 In Figure 5 variations of both pH and NO₂⁻ during the incubation time are depicted. Both pH and NO₂⁻
 333 varied sensibly only in the acetate amended mesocosms, with a peak approximately five days after
 334 the start of the incubation. In carbonate buffered systems as the ones studied here (see Table 1), pH
 335 variations are usually constrained within half an order of magnitude, but the rapid denitrification as a
 336 consequence of acetate addition led to a steep increase in DIC that temporary exceeded the buffering
 337 capacity of the systems. The pH increase could have partially inhibited the complete conversion of
 338 NO₃⁻ into N₂ leading to temporary NO₂⁻ accumulation (Stevens et al. 1998). Besides, since NH₄⁺ was
 339 always below detection limits, the process of dissimilatory NO₃⁻ reduction to NH₄⁺ could not have
 340 been the prevailing mechanism of NO₃⁻ removal. Most probably, the temporary NO₂⁻ increase in these
 341 anaerobic systems was due to the combination of pH increase and the accumulation of extracellular
 342 NO₂⁻ when highly oxidized substrates (like acetate) are employed (Mastrocicco et al. 2011).



343

344 Figure 5. pH (left plot) and NO₂⁻ concentrations (right plot) versus time in unamended (LOAM and
 345 CLAY) and acetate amended (LOAM-ace and CLAY-ace) mesocosms (average ± standard deviation,
 346 *n*=3).

347

348 Despite the number of N₂O samples being too small to compute N₂O fluxes (Table 2), the N₂O
 349 accumulation between two consequent sampling times often represented less than 0.5% of the
 350 corresponding N₂ accumulation. The N₂O production and consumption rates highlight prevailing
 351 consumption of N₂O in organic substrate limited soils and production of N₂O in acetate amended
 352 soils. This observation is consistent with the findings of Charles et al. (2017) that showed increasing
 353 N₂O emission factors with increasing organic C and Nr availability. Besides, other authors have
 354 highlighted the role of C:N ratio on N₂O emission factors (Huang et al. 2004; Saggar et al. 2013;
 355 Smith 2017). Unsurprisingly, the N₂ production rates are directly related to the organic substrates
 356 availability, given that heterotrophic denitrification in the current study, is the principal N₂ production
 357 mechanism. Small sample size of N₂O experiments potentially limits statistical significance,
 358 nevertheless it is interesting to note that N₂O production could be elevated if both reactants (organic
 359 substrates and NO₃⁻) and soils are in saturated conditions. In fact, the environmental factors for

360 production of N₂O are optimal when fertilized fields are flooded (Hansen et al. 2014). This is an
 361 indication that flooding practices in agricultural soils with shallow groundwater table could be major
 362 contributors of N₂O emissions, i.e. paddy soils in waterlogged conditions (Kajiura et al., 2018).
 363 Furthermore, a recent study has shown that drip irrigation can be beneficial to reduce N₂O emissions
 364 in Mediterranean climatic conditions (Cayuela et al., 2017). Given that the climatic conditions and
 365 agricultural practices can alter considerably N₂O emissions, specific field tests should be performed
 366 to quantitatively extend these laboratory results to real agricultural practices.

367

368 Table 2: Dissolved N₂O (mmol/l) and dissolved N₂ (mmol/l) concentrations for selected LOAM and
 369 CLAY experiment time intervals and incubation experiments.

Time (days)	Dissolved N ₂ O (mmol/l)	LOA M	LOAM- ace	CLA Y	CLAY- ace
2		1.22	0.14	0.58	0.22
4		0.97	1.58	0.06	0.71
10		0.09	0.20	0.06	0.13
17		0.12	0.08	0.08	0.13
28		0.22	0.10	0.10	0.06
35		0.21	0.01	0.10	0.02
N ₂ O production and consumption		Net rate (mmol- N/m ² /d)			
		-0.08	+0.14	-0.09	+0.05
Time (days)	Dissolved N ₂ (mmol/l)	LOA M	LOAM- ace	CLA Y	CLAY- ace
2		0.576	0.693	0.632	0.761
4		0.593	0.943	0.647	1.045
10		0.676	1.084	0.791	1.182
17		0.684	1.06	0.805	1.147
28		0.703	1.045	0.931	1.147
35		0.715	1.037	0.969	1.099
N ₂ production and consumption		Net rate (mmol- N/m ² /d)			
		+8.67	+44.8	+17.2	+52.1

370

371 4. Concluding remarks and management implications

372

373 From a methodological point of view, the soil mesocosms, incubated in water saturation conditions
 374 at temperatures resembling field conditions, showed higher capacity of reducing NO₃⁻ to N₂ when

375 supplied with acetate, while in control conditions, the natural NO_3^- attenuation potential was limited
376 by the scarce availability of labile organic substrates. Working in field conditions, it is generally
377 extremely difficult to isolate the effect of a single process, (e.g. water saturation on N loss via
378 denitrification), since other important regulating factors, such as water content, NO_3^- concentration
379 and labile organic matter availability may vary at the same time. Thus, the use of laboratory
380 mesocosms represents a viable alternative to *in situ* studies, where the variability of soil features is
381 controlled or excluded and tested variables may be set to precise values to address specific questions.
382 The outcomes of the present study have two main implications, in terms of both optimizing N
383 fertilization and prevention of NO_3^- pollution of surface and ground waters. The N fertilization
384 followed by the establishment of waterlogged conditions, may result in large N losses via
385 denitrification in as early as a few days, especially in fine texture soils characterized by low
386 permeability. Denitrification in soils occurs when three conditions are fulfilled, i.e. anoxia,
387 availability of NO_3^- and that of a labile carbon source. Thus, high denitrification rates may
388 periodically occur when elevated NO_3^- concentrations in the topsoil are established by fertilizer
389 application and when intense rainfall creates saturation and consequently oxygen deficiency.
390 Although, in soils poor of labile organic substrates, denitrification efficiency could be very low. In
391 temperate regions, poorly drained lowland soils may remain saturated for several days in late
392 winter/early spring. This represents the period of highest probability of Nr losses via denitrification
393 since it overlaps with the fertilizer applications for the most N-demanding crops (e.g. wheat and
394 maize), usually performed in coverage, in the early growing period. Thus, the understanding of this
395 phenomenon could significant impact on both economic interests and direct deleterious effects on the
396 environment. The risk of economic losses is two-fold. First, if Nr distribution is performed according
397 to the precise crop needs, the loss of NO_3^- in case of soil water saturation may lead to nutritional
398 deficiency and yield losses. Second, on the contrary, farmers' empirical knowledge of this unknown
399 NO_3^- loss could lead to an over-protective behaviour resulting in use of higher amounts of Nr than
400 those needed for the crops. In the latter case, the economic burden consists of increased expenditure

401 on excess distributed fertilizer. Moreover, in this second case, from an environmental point of view,
402 the lack of knowledge of this NO_3^- loss mechanism may lead, to a higher risk of losses into the
403 environment. In particular to surface waters via runoff and leaching into tile drains.

404 According to the present results, acetate addition (here used as a proxy of labile organic C substrates)
405 strongly increased NO_3^- reduction via denitrification. This highlights the fact that the actual quantity
406 and bioavailability of organic carbon, is the key regulating factor of soil N buffer capacity against the
407 risk of NO_3^- groundwater pollution (Rivett et al., 2008; Jahangir et al., 2012; Castaldelli et al., 2013).
408 Therefore, a proper N management in agro-ecosystems should be based on land practices that promote
409 accumulation of readily available organic carbon in soils (e.g. no tillage, minimum tillage, compost
410 amendment) (Palm et al., 2014). Most importantly, compost amendment could not only improve the
411 soil quality thereby enhance crop productivity but could also potentially reduce NO_3^- leaching to
412 groundwater.

413

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415

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422

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