Reactive nitrogen losses via denitrification assessed in saturated agricultural soils

Giuseppe Castaldelli¹, Nicolò Colombani¹, Elisa Soana¹, Fabio Vincenzi¹, Elisa Anna Fano¹, Micol Mastrocicco²#

¹SVeB - Department of Life Sciences and Biotechnology, University of Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy
²DiSTABiF - Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Campania University “Luigi Vanvitelli”, Via Vivaldi 43, 81100 Caserta, Italy
#Corresponding author:

Abstract

The aim of the present study was to quantify nitrate (NO₃⁻) removal via denitrification in an intensively cropped lowland of the Po River delta (Northern Italy). These agricultural lands are characterized by fine textured soils, generally poor of labile organic matter and amended mainly with synthetic fertilizers. Laboratory core incubations in water saturation conditions were performed on two contrasting soil types distinguished by different soil textures (silty-loam and silty-clay) either amended with NO₃⁻ alone or a combination of NO₃⁻ and acetate. Denitrification was evaluated by concomitant measurements of NO₃⁻ consumption and N₂ production via N₂:Ar analyses by Membrane Inlet Mass Spectrometry (MIMS).

The water-logged soils showed higher capacity to reduce NO₃⁻ to N₂ when supplied with acetate as an organic substrate, while, without acetate amendment, NO₃⁻ removal was limited due to lack of labile organic substrates. Transient nitrite (NO₂⁻) accumulation was documented in acetate amended
mesocosms, due to concurrent presence of elevated pH values and use of highly oxidized substrates (like acetate).

This study suggests that agricultural practices aimed to increase the availability of labile organic matter, such as acetate, are beneficial in buffering reactive N excess in soils and to reduce NO$_3^-$ leaching towards groundwater and surface-water.

**Keywords**

Agricultural soils; denitrification; N$_2$:Ar method; reactive N loss; NO$_3^-$ groundwater pollution.

1. **Introduction**

Under the pressure of an increasing world population and food demand, the use of nitrogen (N) synthetic fertilizers, mainly in form of urea is increasing exponentially (Glibert et al., 2006; Liu et al. 2015). As a result, an immense mass of N is driven from the atmosphere to agroecosystems, which then partly is lost to surface and ground waters as reactive N (Nr) and partly returns to the atmosphere via volatilization of ammonia (NH$_3$) and denitrification. The latter process can end up in the forms of both non-reactive dinitrogen (N$_2$) and the greenhouse gas nitrous oxide (N$_2$O). Environmental and health consequences of this man-driven global Nr flux are serious, unresolved and not fully understood (Leip et al., 2015; Reis et al., 2016; Zhang et al., 2015). Nitrate (NO$_3^-$) loss to surface water and the consequential eutrophication is by far the most studied topic in previous decades. Recent studies have highlighted NO$_3^-$ contamination of groundwater and its role as potential temporary or permanent sink for N (Böhlke, 2002; Sacchi et al. 2013; Sebilo et al. 2013). Conversely, much less is known on denitrification in agricultural soils and particularly on the proportions between the gaseous products, i.e. N$_2$ and N$_2$O. The latter is one of the most alarming greenhouse gases since it has global warming potential 265-times that of CO$_2$ (Bouwman et al., 2013; Saggar et al., 2013; Smith, 2017).
Denitrification is an essentially anaerobic respiration process, in which N, mainly in form of NO$_3^-$, is used as an electron acceptor in the oxidation of carbon (C) and reduced to N gases (N$_2$ and N$_2$O). It is an important and widespread microbial process which occurs in a variety of waterscapes and landscapes at the global scale (Bothe et al., 2006; Seitzinger et al., 2006). Denitrification in sediments of rivers, canals, lakes, lagoons and coastal waters has been intensively investigated since the ‘70s (Nixon, 1995), mainly focusing on the regulation of the process by abiotic parameters (Piña-Ochoa and Alvarez-Cobelas, 2006; Birgand et al., 2007; Veraart et al., 2016), and biotic factors, such as benthic macrofauna and rooted macrophytes (Bartoli et al., 2008; Bonaglia et al., 2014; Soana et al., 2017). However, to the best of our knowledge, denitrification process in agroecosystems has not been fully evaluated to date. Soil features (e.g. organic matter), agricultural practices and climatic evolution are all important elements regulating denitrification (Rivett et al., 2008; Aguilera et al., 2013; Barakat et al., 2016). Furthermore, the role of organic C in driving the denitrification and emission of N$_2$O has been recently reviewed (Charles et al., 2017), demonstrating that, varying organic C amendments can alter N$_2$O emission factors. Particularly, highest N$_2$O emissions were attributed to the use of liquid manure whereas opposite effects were observed in compost use. The fate of N excess and relevance of denitrification in agricultural soils is nevertheless open to debate (Burgin et al., 2013; Duncan et al., 2013). This topic is central not only in environmental policy, i.e. the Water Framework Directive or the Clean Water Act but is also vital for the agricultural practice itself (van Grinsven et al., 2015). Besides denitrification, other processes like dissimilatory NO$_3^-$ reduction to NH$_4^+$ (DNRA) can affect the N cycle in soils (Rütting et al., 2011). Although, NO$_3^-$ reduction by DNRA is favoured over denitrification only in intensively reduced and C-rich environments (Yin et al., 2002; Schmidt et al., 2011) with C:NO$_3^-$ ratios usually higher than 12 (Yin et al., 1998). Questions on the availability of the residual N after a cropping cycle, on the fate of N distributed in pre-seeding, on coverage of the most demanding crops, such as maize and wheat, are equally relevant, but nevertheless left unanswered. What is the fate of NO$_3^-$ if heavy rains bring the soil to saturation? How do microbial N transformations change according to soil features? How much of N distributed is lost in the surface
and ground waters, how much is lost to the atmosphere, and how much consequently remains available for the crops? The answers to these questions are evidently difficult if not impossible in most cases and generally in agricultural practice the strong unpredictability of N processes is overcome based on farmers’ experience. Farmers in western regions generally distribute more N compared to crop needs to avoid the risk of N scarcity. Relatively low price of N fertilizers further facilitates overuse. An attempt to overcome the problem is the use of slow-release N fertilizers or the combined use of urea and nitrification inhibitors (Cameron et al., 2013; McGeough et al., 2016). However, results of the use of these technical alternatives are not always clear and neither are the consequences of introduction of large amounts of nitrification inhibitors or other chemicals in nature (Ruser and Schulz, 2015; Coskun et al., 2017). Regardless of these environmental aspects, the higher price of such fertilizers has prevented their wide use and simple urea has remained by far the most applied fertilizer (Nishina et al. 2017).

In the present study, N removal via denitrification was quantified in an intensively cropped lowland of the Po River delta (Northern Italy). These agricultural lands are characterized by fine textured soils, generally impoverished of labile organic matter and fertilized mainly with synthetic urea. In this area, the most important hydrological and microbiological processes affecting N fate in both surface and ground waters have been extensively studied (Mastrocicco et al., 2011a; Mastrocicco et al., 2011b; Aschonitis et al., 2012; Aschonitis et al., 2013; Castaldelli et al., 2013; Castaldelli et al., 2015). Denitrification was evaluated in two soil types characterized by different soil textures and content of organic matter by the concomitant measurements of NO$_3^-$ consumption and N$_2$ production via N$_2$:Ar analyses by Membrane Inlet Mass Spectrometry (MIMS), by means of soil core incubations in waterlogged conditions. The selection of N$_2$:Ar technique rather than the classical $^{15}$N tracer technique was done since comparable results can be obtained in freshwater core incubations (Smith et al. 2006). The specific aims of the study were: i) to describe the temporal evolution of denitrification in saturated soils fertilized with synthetic fertilizers by monitoring reactants (NO$_3^-$ and acetate) and products (N$_2$ and dissolved inorganic carbon-DIC ) of the reaction; ii) to determine the
role of labile organic matter limitation, iii) to analyze possible unwanted accumulation of intermediate by-products like NO$_2^-$ and N$_2$O.

2. Material and Methods

2.1. Study sites

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

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

2.2. Soil sampling and incubation

Soil sampling was performed on the 18$^{th}$ of April 2016, a period representative of the first N coverage fertilization on wheat for the study area. Soils were manually excavated from the plough layer (from
topsoil to 30 cm below ground level) and re-packed in Plexiglass columns (Fig.1) maintaining the same orientation and degree of compaction of the fields. In an hour after sampling, the cores, six for each site, were brought to the laboratory and placed in a thermostatic room. The day after the sampling, the cores were slowly saturated with rain water (NO$_3^-$<50 µmol/L) collected from the field sites using a collection tank and amended with NO$_3^-$ to a final concentration of 6.4 mmol/L. According to the local enactment of the European Directive for water protection (2000/60 CE), in areas declared “vulnerable to nitrates from agricultural sources”, N fertilization for wheat is limited to the maximum amount of 170 kg N ha$^{-1}$ yr$^{-1}$, in various forms of synthetic fertilizers, as ammonium nitrate, trivalent fertilizer (nitrogen, phosphorus and potassium, NPK) and synthetic urea. The Nr amount provided in the solutions recapitulated the limits of the EU directive and followed the common fertilization practices. For both soils, an intense rain event of 100 mm after the first Nr distribution in coverage on wheat (equivalent to 90 kg N ha$^{-1}$) was simulated. Thus, the Nr amount was dissolved in a hypothetical cumulative rainfall of 100 mm, resulting in 6.4 mmol/L of NO$_3^-$ mentioned above. In these soils the conversion of urea and ammonia to NO$_3^-$ occurs in a few days and to avoid any lag phase, all experiments were performed by adding Nr already in form of NO$_3^-$ . Three of the six cores from each site were amended with acetate (LOAM-ace and CLAY-ace) to a final concentration of 5.40 mmol/L, according to the stoichiometric relationship describing denitrification using acetate as electron donor (Lew et al., 2012). Low molecular weight organic acids like acetate originate both from root exudates and decomposition of crop residues. For the investigated site, the burial of crop residues into soil at the end of the cropping cycle usually corresponds to the only organic amendment. Three cores from each site were left unamended (LOAM and CLAY) and served as blank. Once saturated with rain water, the cores were left open and unstirred for a few hours to allow for trapped air bubbles to escape and reach anoxia. Then cores were sealed with a screw cap equipped with a screw-tight rubber seal between two PVC disks. When sealed, each core consisted of 30 cm of soil overlaid by 10 cm of water (Fig. 1).
Figure 1. Experimental set up, with the mesocosm scheme (on the left) and the picture (on the right) of the complete laboratory experiment, consisting of twelve soil cores, three for each treatment, i.e. unamended (LOAM and CLAY) and acetate amended (LOAM-ace and CLAY-ace).

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

Overlying water of each core was sampled at decreasing frequencies as the experiment progressed: twice a day for the first three days, daily during the following four days, every two days during the following ten days, and every three days until the end of the incubation, after a total of five weeks. Water was collected using a 50-mL gas-tight glass syringe through a sealed sampling plastic tube inserted in the superior lid and equipped with a small one-way valve, which was opened only on occasion of the sampling. At each sampling time, a withdrawn volume of 40 mL, accounting for <5% of the core headwater volume, was replaced in real time by NO$_3^-$ amended or NO$_3^-$ and acetate amended rain water, according to the treatment. Similar sampling port, connected by a plastic tube and a one-way valve was used to refill and flow of replaced volume in to each core was aided by gravity. NO$_3^-$ and acetate concentration in the refill was set daily to the value measured in the water sampled from the single core in the previous day.
Water from each core was analyzed for dissolved inorganic N forms (NO$_3^-$, NO$_2^-$, NH$_4^+$), volatile fatty acids (VFA) as acetate, dissolved gasses (N$_2$, N$_2$O) and dissolved inorganic carbon (DIC). At each sampling, water temperature was measured with a multiparametric probe and dissolved oxygen with a microsensor (OX-500, Unisense, Science Park Aarhus, Denmark) in the stirred water column. Anoxia was reached less than one day after the cores were sealed.

Twelve additional cores (Plexiglas liners, i.d. 4.5 cm, height 20 cm), six from each site, were collected for soil characterization: grain size analysis, dry bulk density, porosity, soil organic matter (SOM) and CaCO$_3$. Grain size analyses were performed with the sieve-pipette method (Day, 1965). Dry bulk density and porosity were quantified gravimetrically at 105 °C for 24 hours. SOM was quantified as loss on ignition in a muffle furnace at 350 °C for 3 hours on dry powdered soil aliquots (Tiessen and Moir, 1993). Soil CaCO$_3$ was measured titrimetrically following the method of Bundy and Bremner (1972). Soil parameters are shown in Table 1.

Table 1. Grain size distribution, dry bulk density ($\rho$), porosity ($\theta$), soil organic matter (SOM), and CaCO$_3$ measurements for LOAM and CLAY (average ± standard deviation, $n=6$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LOAM</th>
<th>CLAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain size (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand (63-2000 μm)</td>
<td>20.8±0.3</td>
<td>4.8±0.3</td>
</tr>
<tr>
<td>Silt (2-63 μm)</td>
<td>59.6±0.4</td>
<td>35.6±0.4</td>
</tr>
<tr>
<td>Clay (&lt;2 μm)</td>
<td>19.6±0.6</td>
<td>59.6±0.6</td>
</tr>
<tr>
<td>$\rho$ (kg/m$^3$)</td>
<td>1.43±0.1</td>
<td>1.37±0.1</td>
</tr>
<tr>
<td>$\theta$ (-)</td>
<td>0.46±0.1</td>
<td>0.49±0.1</td>
</tr>
<tr>
<td>SOM (%)</td>
<td>3.1±0.3</td>
<td>3.7±0.4</td>
</tr>
<tr>
<td>CaCO$_3$ (%)</td>
<td>10.7±0.4</td>
<td>5.3±0.3</td>
</tr>
</tbody>
</table>

2.3. Analytical methods

Samples for dissolved inorganic N (NO$_3^-$, NO$_2^-$, NH$_4^+$) determinations were filtered through Whatman GF/F glass fiber filters, transferred to polyethylene vials and frozen for later analysis. NO$_3^-$ and NO$_2^-$ were measured on a Technicon AutoAnalyser II (Armstrong et al., 1967). The NH$_4^+$
concentration was determined on a double beam Jasco V-550 spectrophotometer using salicylate and hypochlorite in the presence of sodium nitroprussiate (Bower and Holm-Hansen, 1980). However, NH$_4^+$ concentrations were always below detection limits (<0.5 µmol/L). Acetate concentration was determined as total fatty acids on a double beam Jasco V-550 spectrophotometer after esterification with ethylene glycol and subsequent reduction with Fe(III) salts.

Samples for gas determinations were collected by overflowing at least 3 times 12-mL gas-tight glass vials (Exetainer®, Labco, High Wycombe, UK), and preserved by adding 100 µL of 7M ZnCl$_2$ solution. Water samples were analyzed for concentrations of dissolved $^{28}$N$_2$, Ar and CH$_4$ gases at the laboratory of Aquatic Ecology, University of Ferrara, by MIMS (Bay Instruments, Easton, Maryland; Kana et al., 1994).

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

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

The CH$_4$, N$_2$ and Ar concentrations were quantified by the ion current detected at m/z ratios of 15, 28, and 40. For MIMS determinations the analytical precision of gas ratio measurements (coefficient
of variation ~0.04%) is generally higher than the analytical precision of gas concentration measurements (coefficient of variation ~0.4%). Thus, the N₂ concentrations were calculated from the measured N₂:Ar multiplied by the theoretical saturated Ar concentration at the sampling water temperature determined from gas solubility tables (Weiss, 1970), assuming that Ar concentration reflected only physical processes since not affected by biological processes. The approach does not distinguish between denitrification and anammox (Anaerobic Ammonium Oxidation), both N₂ producing processes. Data on anammox in soils are scarce but its occurrence is generally irrelevant if compared to denitrification in waterlogged conditions (Bai et al., 2015; Shan et al., 2016).

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

The best m/z for CH₄ measurements is 15, corresponding to the CH₃ ionization fragment and for which there are no substantial interferences. Moreover, the liquid nitrogen trap does not condense CH₄ but all the other volatile organic compounds that pass through the membrane and may contribute to CH₃ formation. Due to the very low concentration in air, a separate standard procedure for CH₄ was used following the headspace equilibration technique. Standards were prepared by injecting known amounts of pure gas (>99.0%, Sigma Aldrich) into the headspace of 12-mL gas-tight glass vials filled with de-ionized water. Prior to MIMS analyses, the vials were shaken vigorously for a minute and placed in a thermostatic bath for solubility equilibration. The CH₄ concentrations never exceeded 8 µg/L, with most values <3 µg/L, without any temporal increases or differences among treatments (data not shown). Methanogenesis was thus considered a negligible pathway in anaerobic degradation of organic matter. The N₂O analyses were not performed extensively for all the monitored times and cores, but only in a few selected samplings to assess the relevance of N₂O accumulation
with respect to N$_2$. N$_2$O was measured by gas chromatography (Trace GC, 2000 Series, Thermo Finnigan, San Jose, CA, USA equipped with an ECD detector) on samples collected in the same way of those for N$_2$:Ar determinations. Samples for dissolved inorganic carbon were transferred into 12-mL gas-tight glass vials (Exetainer®, Labco, High Wycombe, UK) and immediately titrated with 0.1 eq/L HCl (Anderson et al. 1986).

3. Results and discussion

3.1. NO$_3^-$ and acetate consumption

The consumption of both NO$_3^-$ and VFA during the incubation time are depicted in Figure 3. NO$_3^-$ concentrations decreased in all the incubated mesocosms. However, in control conditions (LOAM and CLAY) there was a significant delay in NO$_3^-$ removal compared to amended conditions (LOAM-ace and CLAY-ace) due to the lack of labile organic substrates. In amended conditions, NO$_3^-$ concentrations dropped by more than 75% and 90% of initial readings, after 4 and 10 days, respectively. Nevertheless, in CLAY mesocosms, only one third of the initial NO$_3^-$ remained at the end of the experiment due to the higher SOM background (Table 1) compared to LOAM mesocosms that instead halved their NO$_3^-$ concentrations, denoting intrinsic limited NO$_3^-$ reduction capacity of this soil. Since NH$_4^+$ concentrations were always below detection limits (<0.5 µmol/L) in all the mesocosms, both DNRA and anammox processes were excluded as main pathways. In addition, the C:NO$_3^-$ ratio was approximately 0.8 in acetate amended mesocosms and even lower in unamended ones, a condition that does not facilitate DNRA (Rütting et al., 2011). This is also congruent with a recent study on DNRA in coastal wetland sediments, where other organic C substrates (i.e. oxalate, citrate, glucose) showed a higher stimulating effect on DNRA with respect to acetate (Liu et al., 2016).

The VFA were found also in control mesocosms with initial concentrations <0.8 mmol/L, since they are typical by-products of SOM degradation processes in these agricultural soils (Castaldelli et al.,
In unamended conditions, the natural VFA background was consumed in the first three days, while in amended conditions VFA decreased rapidly in the first stage of the experiment, i.e. the first ten days, followed by stable or slight decrease of concentrations towards the end of the incubation. Thus, the availability of VFA and in general of labile organic substrates can dramatically increase the denitrification rate while their scarcity, can promote NO$_3^-$ leaching from these soils.

Figure 3. VFA concentrations (left plot) and NO$_3^-$ concentrations (right plot) versus time in unamended (LOAM and CLAY) and acetate amended (LOAM-ace and CLAY-ace) mesocosms (average ± standard deviation, $n=3$).

3.2. DIC and N$_2$ production

The production of both DIC and N$_2$ during the incubation time are depicted in Figure 4. DIC and N$_2$ were produced also in control mesocosms due to the presence of SOM background in both soils undergoing degradation (Table 1) but exhibiting different patterns with respect to acetate amended mesocosms. Congruently with the consumption of VFA and NO$_3^-$ reported before, the accumulation
of DIC and N\textsubscript{2} in amended conditions (LOAM-ace and CLAY-ace) largely exceeded the unamended conditions (LOAM and CLAY) in the first two weeks of incubation.

In acetate-amended conditions, a steep increase of both N\textsubscript{2} and DIC concentrations was detected during the first days of the experiment, followed by almost stable values towards the end. Differently, in LOAM and CLAY mesocosms DIC and N\textsubscript{2} increased slowly but constantly during the course of the incubation. However, as pointed out previously for NO\textsubscript{3} consumption, the smaller DIC and N\textsubscript{2} accumulation detected for LOAM confirmed its intrinsically limited NO\textsubscript{3} reduction capacity with respect to CLAY.

The general stoichiometry for the reduction of SOM via denitrification:

\[
5\text{CH}_2\text{O} + 4\text{NO}_3^- + 0.375\text{H}^+ \rightarrow 5\text{HCO}_3^- + \text{H}^+ + 2\text{N}_2 + 2\text{H}_2\text{O}
\]  

(1)

denotes that per mole of NO\textsubscript{3} consumed, half a mole of N\textsubscript{2} is produced. Therefore, the expected N\textsubscript{2} concentrations increase should be up to 3 mmol/l in all the mesocosms, except in LOAM, where the expected N\textsubscript{2} concentration increase should be around to 2 mmol/l. In our experiments, the dissolved N\textsubscript{2} concentrations reached an upper limit of 1.2 mmol/l and then decreased to values around 1 mmol/l in the acetate amended mesocosms (LOAM-ace and CLAY-ace); while in the unamended ones (LOAM and CLAY) a more gradual N\textsubscript{2} increase was observed (Fig. 4). This apparent inconsistency is easily explained by the N\textsubscript{2} solubility in water that is quite low around 20 °C (Kolev, 2011), thus the formation of gas bubbles (largely observed during our experiments) in the all the acetate amended mesocosms could have caused the observed N\textsubscript{2} decrease in the water phase.

As pointed out in the previous sub-section, in CLAY mesocosms DIC and N\textsubscript{2} reached the values observed for the acetate amended mesocosms after 50 days of incubation due to the elevated presence of SOM (Table 1), while in LOAM mesocosms DIC and N\textsubscript{2} increased slowly confirming the intrinsically limited NO\textsubscript{3} reduction capacity of these soils.
Figure 4. DIC concentrations (left plot) and N₂ concentrations (right plot) versus time in unamended (LOAM and CLAY) and acetate amended (LOAM-ace and CLAY-ace) mesocosms (average ± standard deviation, n=3).

3.3. pH variations and NO₂⁻ accumulation

In Figure 5 variations of both pH and NO₂⁻ during the incubation time are depicted. Both pH and NO₂⁻ varied sensibly only in the acetate amended mesocosms, with a peak approximately five days after the start of the incubation. In carbonate buffered systems as the ones studied here (see Table 1), pH variations are usually constrained within half an order of magnitude, but the rapid denitrification as a consequence of acetate addition led to a steep increase in DIC that temporary exceeded the buffering capacity of the systems. The pH increase could have partially inhibited the complete conversion of NO₃⁻ into N₂ leading to temporary NO₂⁻ accumulation (Stevens et al. 1998). Besides, since NH₄⁺ was always below detection limits, the process of dissimilatory NO₃⁻ reduction to NH₄⁺ could not have been the prevailing mechanism of NO₃⁻ removal. Most probably, the temporary NO₂⁻ increase in these anaerobic systems was due to the combination of pH increase and the accumulation of extracellular NO₂⁻ when highly oxidized substrates (like acetate) are employed (Mastrocicco et al. 2011).
Figure 5. pH (left plot) and NO$_2^-$ concentrations (right plot) versus time in unamended (LOAM and CLAY) and acetate amended (LOAM-ace and CLAY-ace) mesocosms (average ± standard deviation, $n=3$).

Despite the number of N$_2$O samples being too small to compute N$_2$O fluxes (Table 2), the N$_2$O accumulation between two consequent sampling times often represented less than 0.5% of the corresponding N$_2$ accumulation. The N$_2$O production and consumption rates highlight prevailing consumption of N$_2$O in organic substrate limited soils and production of N$_2$O in acetate amended soils. This observation is consistent with the findings of Charles et al. (2017) that showed increasing N$_2$O emission factors with increasing organic C and Nr availability. Besides, other authors have highlighted the role of C:N ratio on N$_2$O emission factors (Huang et al. 2004; Saggar et al. 2013; Smith 2017). Unsurprisingly, the N$_2$ production rates are directly related to the organic substrates availability, given that heterotrophic denitrification in the current study, is the principal N$_2$ production mechanism. Small sample size of N$_2$O experiments potentially limits statistical significance, nevertheless it is interesting to note that N$_2$O production could be elevated if both reactants (organic substrates and NO$_3^-$) and soils are in saturated conditions.
production of N$_2$O are optimal when fertilized fields are flooded (Hansen et al. 2014). This is an indication that flooding practices in agricultural soils with shallow groundwater table could be major contributors of N$_2$O emissions, i.e. paddy soils in waterlogged conditions (Kajiura et al., 2018).

Furthermore, a recent study has shown that drip irrigation can be beneficial to reduce N$_2$O emissions in Mediterranean climatic conditions (Cayuela et al., 2017). Given that the climatic conditions and agricultural practices can alter considerably N$_2$O emissions, specific field tests should be performed to quantitatively extend these laboratory results to real agricultural practices.

Table 2: Dissolved N$_2$O (mmol/l) and dissolved N$_2$ (mmol/l) concentrations for selected LOAM and CLAY experiment time intervals and incubation experiments.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Dissolved N$_2$O (mmol/l)</th>
<th>LOAM</th>
<th>LOAM-ace</th>
<th>CLAY</th>
<th>CLAY-ace</th>
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<tbody>
<tr>
<td>2</td>
<td>1.22</td>
<td>0.14</td>
<td>0.58</td>
<td>0.22</td>
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</tr>
<tr>
<td>4</td>
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<tr>
<td>28</td>
<td>0.22</td>
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N$_2$O production and consumption Net rate (mmol-N/m$^2$/d) -0.08 +0.14 -0.09 +0.05

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<th>LOAM</th>
<th>LOAM-ace</th>
<th>CLAY</th>
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N$_2$ production and consumption Net rate (mmol-N/m$^2$/d) +8.67 +44.8 +17.2 +52.1

4. Concluding remarks and management implications

From a methodological point of view, the soil mesocosms, incubated in water saturation conditions at temperatures resembling field conditions, showed higher capacity of reducing NO$_3^-$ to N$_2$ when...
supplied with acetate, while in control conditions, the natural NO$_3^-$ attenuation potential was limited by the scarce availability of labile organic substrates. Working in field conditions, it is generally extremely difficult to isolate the effect of a single process, (e.g. water saturation on N loss via denitrification), since other important regulating factors, such as water content, NO$_3^-$ concentration and labile organic matter availability may vary at the same time. Thus, the use of laboratory mesocosms represents a viable alternative to in situ studies, where the variability of soil features is controlled or excluded and tested variables may be set to precise values to address specific questions. The outcomes of the present study have two main implications, in terms of both optimizing N fertilization and prevention of NO$_3^-$ pollution of surface and ground waters. The N fertilization followed by the establishment of waterlogged conditions, may result in large N losses via denitrification in as early as a few days, especially in fine texture soils characterized by low permeability. Denitrification in soils occurs when three conditions are fulfilled, i.e. anoxia, availability of NO$_3^-$ and that of a labile carbon source. Thus, high denitrification rates may periodically occur when elevated NO$_3^-$ concentrations in the topsoil are established by fertilizer application and when intense rainfall creates saturation and consequently oxygen deficiency. Although, in soils poor of labile organic substrates, denitrification efficiency could be very low. In temperate regions, poorly drained lowland soils may remain saturated for several days in late winter/early spring. This represents the period of highest probability of Nr losses via denitrification since it overlaps with the fertilizer applications for the most N-demanding crops (e.g. wheat and maize), usually performed in coverage, in the early growing period. Thus, the understanding of this phenomenon could significant impact on both economic interests and direct deleterious effects on the environment. The risk of economic losses is two-fold. First, if Nr distribution is performed according to the precise crop needs, the loss of NO$_3^-$ in case of soil water saturation may lead to nutritional deficiency and yield losses. Second, on the contrary, farmers’ empirical knowledge of this unknown NO$_3^-$ loss could lead to an over-protective behaviour resulting in use of higher amounts of Nr than those needed for the crops. In the latter case, the economic burden consists of increased expenditure
on excess distributed fertilizer. Moreover, in this second case, from an environmental point of view, the lack of knowledge of this NO₃⁻ loss mechanism may lead to a higher risk of losses into the environment. In particular, to surface waters via runoff and leaching into tile drains.

According to the present results, acetate addition (here used as a proxy of labile organic C substrates) strongly increased NO₃⁻ reduction via denitrification. This highlights the fact that the actual quantity and bioavailability of organic carbon, is the key regulating factor of soil N buffer capacity against the risk of NO₃⁻ groundwater pollution (Rivett et al., 2008; Jahangir et al., 2012; Castaldelli et al., 2013). Therefore, a proper N management in agro-ecosystems should be based on land practices that promote accumulation of readily available organic carbon in soils (e.g. no tillage, minimum tillage, compost amendment) (Palm et al., 2014). Most importantly, compost amendment could not only improve the soil quality thereby enhance crop productivity but could also potentially reduce NO₃⁻ leaching to groundwater.

Acknowledgments

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References


