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Abstract

Application of manure (urine and/or feces) to agricultural soils enhances emissions of gases such as nitrous oxide (N_2O) and carbon dioxide (CO_2). Some minor N compounds such as hippuric acid and benzoic acid present in urine can be controlled through diet manipulation to mitigate these emissions. The aim of this study was to evaluate how the inclusion of fibrous by-products in the diet of pigs affects hippuric and benzoic acid concentrations in the excreted urine/slurry, and their possible effect on N_2O emissions following application of these manures to soil. Slurries were obtained from growing-finishing pigs fed five contrasting diets: a conventional diet (pig slurry control, PSC); and orange pulp and carob meal at a dietary fiber level of 75 or 150 g kg-1 (OP-75; OP-150; CM-75; CM-150) and were then applied to mesocosms containing young ryegrass plants. A control treatment without slurry was also included. The N_2O and CO_2 emissions were measured using static chambers following slurry application, alongside measurements of soil ammonium (NH_4^+), nitrate (NO_3^-), and dissolved organic carbon (DOC). Soils amended with slurries obtained from fibre by-products, OP and CM, decreased N_2O emissions by 65 and 47%, respectively, compared with slurries obtained through a conventional pig diet.

Benzoic acid was negatively correlated with N_2O emission for slurries from OP diets, which had over double the hippuric acid content, and more than 1.8 times the benzoic acid content than the CM. However, this effect only occurred during the first week due to rapid degradation of this compound within soil. The possible toxic effect of benzoic acid did not appear to affect soil respiration, since a positive correlation was found. Results of a benzoic acid balance (considering both intake through feed and release through urine) indicated that the source of both acids were phenolic compounds (polyphenolic or lignin) present in the fibrous fraction. These results show that N_2O emissions are more affected than CO_2 by to compounds within urine/faeces that can be manipulated indirectly through the diet.

Keywords: nitrous oxide emissions, carbon dioxide emissions, diet manipulation, diet fibre content

1. Introduction

Manures from intensive pig production systems are generally inefficiently recycled and can potentially lead to atmospheric pollution, from the entire manure management chain, including housing (IPCC, 2007). Nitrous oxide (N_2O) is one of the main pollutants emitted after application of pig slurries to agricultural soils (Aguilera et al., 2013). This gas is a by-product of soil biochemical processes, mainly nitrification and denitrification (Firestone and Davidson, 1989). These processes are directly controlled by soil moisture, with low values of water-filled pore space (WFPS) favoring nitrification (WFPS<60%) and high values (WFPS>60%) being suitable for denitrification (Sanchez-Martin et al., 2010a). Emissions of N_2O contribute considerably to the radiative forcing of the atmosphere, having a global warming potential 298 times higher than that of CO_2 expressed on a weight basis (i.e. per kg) over a 100 year timescale (IPCC, 2007).

Ammonium and other labile N compounds of slurry (e.g. urea, creatinine) can influence the total N_2O emissions when applied to agricultural soils (Whitehead et al., 1989). When slurries are applied to aerated soils, NH_4^+ is rapidly nitrified, producing large fluxes of N_2O (Sanchez-Martin et al., 2010b). Additionally, the nitrate (NO_3^-) obtained from nitrification in soils, together with degradable organic C compounds added with manures, often accelerates denitrification, especially under conditions of high soil WFPS (> 60%) (Cardenas et al., 2007).

Diet manipulation has been identified as a promising technique to modify urine and feacal composition (Hansen et al., 2014) and consequently this is proposed as an acceptable strategy to reduce environmental N pollution. Available information indicates that reducing crude protein (CP) in the diet of growing-finishing pigs can lead to an 8% reduction in N excretion for each percentage decrease in CP of the feed, without reduction of animal performance (Galassi et al., 2010). Moreover, the addition of fibrous feedstuffs to the diet has been related to a reduction of urea-N excretion (Jarret et al., 2011). At present, information regarding the influence of pig diet on slurry composition and its effect on N_2O emission is scarce. Velthof et al. (2004) found that pig diet modified pig manure composition, but its effect on N_2O was dependent on soil properties, especially soil organic matter content.

In laboratory experiments, it has been shown that minor constituents of ruminant urine, such as hippuric acid (HA) and benzoic acid (BA), can contribute to decreased N_2O emissions (Kool et al., 2006a-b). van Groenigen et al. (2006) observed an effect of these aromatic compounds on denitrification, whereas, Bertram et al. (2009) found nitrification was also partially inhibited by these organic compounds. These findings suggest an increase of hippuric and benzoic acid within ruminant urine could potentially be a N_2O mitigation strategy, however, contradictory results have been obtained in the few field experiments conducted to date, with no inhibitory effects detected (Clough et al., 2009; Krol et al., 2015). Further research is required to determine whether manipulation of pig diet to enhance the production of these minor urinary compounds can reduce subsequent N_2O emissions.

Within this context, two combined experiments were conducted, investigating the manipulation of pig diet, to assess the consequent effects on slurry composition, and the application of these slurries as N fertilizer to grassland mesocosms. The aims of the study were to: 1) evaluate the effect of the inclusion of fibrous by-products in pig's diet on hippuric and benzoic acid concentrations in the excreted urine/slurry, and 2) determine the possible effect of both acids on N_2O emissions following application of the slurries to grassland soil. We predict that diets resulting in slurries with higher organic acids (hippuric and benzoic) concentration would result in lower soil N_2O emissions following their application.

2. Material and Methods

2.1 Selection of pig slurries.

Thirty growing-finishing pigs, progeny of Danish Duroc × (Landrace × Large White), were fed five different diets, under controlled conditions, in an experimental farm in Castellón (Spain). The experimental conditions to obtain urine and faeces from each animal, and the collection period, are explained in detail in Beccaccia et al. (2015). The experimental feeds included a conventional diet, formulated to contain the most common ingredients used in commercial diets for growing-finishing pigs (wheat, barley and soybean meal), and either orange pulp (OP) or carob meal (CM), at two dietary concentrations (75 or 150 g kg^{-1}), in replacement of barley grain as a fibrous by-product. The diets were as follows: 1) pig slurry control (PSC), 2) 75 g kg^{-1} of carob meal (CM-75), 3) 150 g kg^{-1} of carob meal (CM-150), 4) 75 g kg^{-1} orange pulp (OP-75), and 5) 150 g kg^{-1} of orange pulp (OP-150). These diets were designed to modify slurry composition by changing dietary fibre sources, but without an influence on pig performance or health (Beccaccia et al., 2015). In order to maintain constant neutral detergent fibre (NDF) dietary level, net energy, protein and essential amino acid levels between diets, lard, soybean meal and synthetic amino acids were added to the feeds including fibrous by-products. Essential nutrients were formulated according to the recommendations of FEDNA (2006).

Urine and faeces were collected and stored (covered) separately at -20ºC in closed plastic bottles. Following the usual pig slurry´s management, urine and faeces from each animal were mixed in the same proportions as they were excreted. Subsequently, slurries of four different animals fed the same diet were immediately mixed, in order to obtain the manure used in the mesocosms experiment. In order to consider the possible side effects of freeze-thaw on the constituents, pH, NH_4^+ and total N (Nt) were determined before application of the reconstituted slurry to soil. Standard electrodes were used to determine pH and titration of the liquid fraction and the Kjeldahl method to NH_4^+ and Nt, respectively. Total N content was used to calculate the amount of slurry to apply in the mesocosms experiment, in order to achieve equal N application rates.

Purine derivatives (allantoin, creatinine, uric acid, hippuric acid and benzoic acid) were analyzed directly in urine samples from the different pigs (n = 46) used for each diet via high performance liquid chromatography (HPLC) on a Varian Pro Star 310 HPLC system (Varian Inc., Palo Alto, CA), using a Phenomenex Luna® 5 µm SCX 100Å column (250 × 4.6 mm) a variable wavelength detection set at 218 nm and a flow rate of 0.7 ml min^{-1}. The method consisted of two mobile phases: mobile phase A (KH_2PO_4; 17 g L^{-1}; adjusted to pH=4) and mobile phase B (40% methanol: 60% mobile phase A). The samples were centrifuged, and prepared for analysis in HPLC vials (1:10, urine: mobile phase A) and mixed with a vortex mixer before analysis.

Additionally, benzoic acid and total polyphenolic compounds were also determined in OP. Benzoic acid was extracted and determined by micellar electrophoresis as indicated in Ding et al. (2015), and polyphenolic compounds were determined using the Folin–Ciocalteu method (Obanda and Owuor, 1997).

2.2 Mesocosms experiment

In an experimental greenhouse of the Technical University of Madrid farm, 36 PVC cylindrical containers (26 cm diameter, 15 cm height) were used for the mesocosms experiment. Each container was filled with 6 kg of dry soil (7cm height). The soil was previously collected randomly from a 700 m^2 area, at the experimental field station ‘El Encín’ (40° 32′N, longitude 3° 17′W) from 0 - 25 cm soil depth. In the laboratory, soil was air-dried at 20 °C, sieved through a 2 mm mesh and repeatedly mixed to ensure homogeneity. Some physico-chemical properties of the top 0–25 cm of the soil layer, measured by standard methods of soil analysis (Burt, 2004) were: total organic C, 8.2 ± 0.4 g kg^{-1}; pH_{H2O}, 7.8 and $CaCO_3$, 13.1± 0.3 g kg^{-1}. According to Soil Survey Staff, 1992, the soil used was a *Calcic Haploxerept* with a clay loam texture (clay, 28%; silt, 17%; sand, 55%). At the beginning of the experiment, the soil mineral N content was 1.1 and 10.2 mg N kg $soil^{-1}$ for NH_4^+ and NO_3^-, respectively. The temperature was maintained between 10 and 15ºC. Ryegrass was sown and the soil moisture content was brought to 60% water-holding capacity (WHC) during 15 days before applying treatments. The containers were maintained at this WHC level throughout the experiment, by replacing the weight loss with distilled water, on a daily basis.

Perennial ryegrass (*Lolium perenne L.)* seed was sown at a density of 6 g of seed per container. Two weeks after sowing, the seedlings were clipped to 3 cm above the soil surface, following which the pig slurries were applied (i.e., day 0) to the soil surface. The slurry was applied at an equal N application rate of 100 kg N ha^{-1} (0.531 g N $container^{-1}$). Due to the different N content of the slurries, different total amounts of slurry were applied to the containers, as follows: 55.2, 61.2, 67.9, 66.8 and 54.7 g of slurry per pot for PSC, OP-75, OP-150, CM-75 and CM-150, respectively. Additionally, water was added to the slurry application, in order to apply an equal total volume of 75 mL $container^{-1}$ across all treatments. The amount of NH_4^+ added to different treatments were: 0.293, 0.274, 0.235, 0.250 and 0.238 g N $container^{-1}$ for the same treatments, respectively. The control treatment received the same water and seed density as the rest of the treatments but it did not receive any N application.

The experiment was arranged in a factorial randomized complete block design, with six containers for each treatment. Half of the pots were used for GHG emission measurements (non-destructive; n = 3), and the other half were used in order to sample the soil (destructive; n = 3).

2.3 Soil sampling and analysis

In the pots designed for soil sampling, two soil cores per container were taken for each sample date, using a 1 cm diameter soil auger (10 cm long). The hole produced by the auger was filled with sand in order to maintain the stability of the soil structure. Soil samples were analyzed for dissolved organic C (DOC), extractable mineral N (NH_4^+ and NO_3^-) and soil moisture. Soil DOC was determined by extracting 8 g soil with 50 mL of deionized water. Afterward, DOC was analyzed with a total organic carbon analyser (multi N/C 3100 Analityk Jena, Jena, Germany). From another 8 g of homogeneously mixed soil, NH_4^+-N and NO_3^--N were extracted with 50 mL of KCl (1 M) over a 1 hour period, filtered and measured by automated colorimetric determination using a flow injection analyzer (FIAS 400 Perkin Elmer, USA) provided with a UV-V spectrophotometer detector.

Water-filled pore space (WFPS) was estimated by dividing the volumetric water content by total soil porosity. Total soil porosity was calculated by measuring the bulk density of the soil according to the relationship: soil porosity = 1 − (soil bulk density/2.65), assuming a particle density of 2.65 g cm^{-3} (Danielson and Sutherland, 1986). The bulk density, which was calculated from the volume of soil in the cores, was 1.29 ± 0.1 Mg m^{-3}. Soil samples were taken three days during the first two weeks after fertilizers applications. After the first week, samples were taken twice or once a week.

2.4 GHG sampling and analysis

Emissions of GHG were measured using a closed dark static chamber approach, following the same sampling schedule as described in section 2.3. Each container was used as a chamber, closing it with a perfectly fitting lid for 40 minutes, resulting in a headspace of approx8 L. The closure period was selected after testing, before the experiment, the linearity of the increasing gas concentrations of N_2O and CO_2 inside the chamber (Ábalos et al., 2014). Gas samples were taken at 0, 20 and 40 min through a three way valve, which was installed in the lid. Gas samples were taken using a 100 ml syringe and stored in 20 ml chromatography vials prior to analysis.

Concentrations of N_2O, and CO_2 were quantified by gas chromatography, using a HP-6890 gas chromatograph (GC; Agilent Technologies, Barcelona, Spain) equipped with a Turbomatrix autoanalyzer (Perkin Elmer, Madrid, Spain). Gas samples were injected through HP Plot-Q capillary columns to a ^{63}Ni electron-capture detector (ECD) to analyze N_2O concentrations and to a flame-ionization detector (FID) fitted with a methanizer for CO_2 concentrations. Helium was used as carrier gas and the oven was operated at a constant temperature of 35 °C. Greenhouse gas flux rates were calculated from the change in gas concentration in the headspace air during the sampling period, where total N_2O-N and CO_2-C fluxes per container were estimated by successive linear interpolations of the flux measurements.

2.5 Biomass sampling and analysis

Grass was cut (to a height of 3 cm) two weeks after the beginning of the experiment and at the end of the experimental period. The biomass obtained from these two cuts were weighed in order to obtain the yield, and total foliar C and N content were determined with an elemental analyzer (TruMac CN Leco, USA).

2.6 Calculations and statistical analysis

For the effect of diet on minor chemical constituents of pig urine, individual animals (n=6 per treatment) were the experimental unit for all the diet treatments studied. Data were analyzed as a completely randomized design with type of diet as main factor, by using PROC GLM of SAS (2008). The effects of type of diet on urine composition were analyzed as a factorial arrangement by using orthogonal contrasts with source and level of inclusion of fibrous by products as main factors. Contrasts of each of the experimental treatments against the control diet were done by using a Dunnet test. For the effect of different slurries on soil N_2O and CO_2 emission, each container (*n*=3 per treatment) was the experimental unit. Differences between treatments at each sampling event and between the mean and cumulative emissions were evaluated using analysis of variance (ANOVA, $P < 0.05$). The least significant difference (LSD) test was used for multiple comparisons between means. Prior to the statistical tests the data were analyzed to determine whether the assumptions of normality (Kolmogorov–Smirnov test) and equality of variance (Levene's test) were satisfied. Where needed to fulfill these assumptions, the data were log-transformed before analysis. Cumulative N_2O and CO_2 fluxes were estimated by successive linear interpolation between weekly sampling dates to study the possible effect of benzoic acid on the emissions due to the rapid degradation of this compound. Correlations between total N_2O and slurry chemical constituents, such as hippuric and benzoic acid or NH_4^+ content, were also performed at these periods during the experiment. Other correlation between N_2O and CO_2 fluxes with soil parameters such as NH_4^+–N, NO_3^-–N, DOC were performed, with a 95% significant level.

3. Results

3.1 Composition and effect of diet on some urine chemical constituents

The chemical composition of the experimental diets can be seen in Table 1, and are further described in detail in Beccaccia et al. (2015). Feeds containing CM generally had a higher concentration of acid detergent lignin (ADL) compared to PSC, OP-75 and

OP-150, and feeds containing OP had a greater soluble fibre content compared to PSC, CM-75 and CM-150.

The composition of the slurries from the contrasting dietary treatments can be seen in Table 2. The PSC diet resulted in the slurry with the highest pH and NH_4^+ content, whereas, the OP-150 diet resulted in a slurry with the lowest NH_4^+ content (3.5 mg N L^{-1}).

Some minor N-containing urine compounds, such as creatinine or allantoin, were not affected by the source and level of dietary fibre by-product, however, the amount of hippuric and benzoic acid was related to the type of fibre included in diets (Table 3). In fact, the OP urines (OP-75 and OP-150) contained the highest amount (P <0.001) of hippuric acid and tended (P = 0.072) to contain a high content of benzoic acid than those obtained from CM diets. Urine from OP diets had more than double the hippuric acid, and more than 1.8 times the benzoic acid content than with CM-75 and CM-150. Hippuric acid concentration increased with the amount of OP (75 g kg^{-1} or 150 g kg^{-1}), but the same trend was not observed for benzoic acid. The concentration of uric acid ranged from 40 to 69 mg L^{-1} for CM-150 and PSC respectively.

The OP contained 30 mg benzoic acid kg^{-1} and 20 g galic acid kg^{-1} (total polyphenol). Taken into account the percentage of this ingredient in feeds, the dietary treatment OP-75 contained 2.25 mg benzoic acid kg^{-1} and 1.5 g galic acid kg^{-1}, and the dietary treatment OP-150 contained 4.5 mg benzoic acid kg^{-1} and 3.0 g galic acid kg^{-1}.

3.2 Nitrous oxide emissions

Nitrous oxide emissions began to increase in the slurry treatments 3 days following application (Fig. 1). At 7 to 17 days following treatment application, emissions were higher than 1 mg N_2O-N m^{-2} d^{-1} for all slurry treatments (Fig. 1). Although during some of the sampling days there were no significant differences between treatments, a clear and significant (P < 0.05) effect was observed during the highest period of N_2O flux. The highest fluxes were always measured from the PSC treatment, with a maximum peak on day 15 (4.3 mg N_2O-N m^{-2} d^{-1}). In contrast, application of slurries obtained from pigs fed with OP diets (OP-75 and OP-150) resulted in lower fluxes than CM diets (CM-75 and CM-150) (Fig. 1). Significant differences (P < 0.05) between the two levels of fibre (75 or 150 g kg^{-1}) in each pig's diet were only found on day 15.

At the end of the experimental period, the N_2O emissions from the soils amended with slurries obtained from fibrous by-products diets, OP and CM, were 65 and 47% (respectively) lower than from soil amended with the slurry obtained through a conventional pig diet. Considering cumulative N_2O emission by periods of a week, significant differences at P < 0.05 between OP and CM were found for the first two weeks (Fig. 2a). However, for the 3rd and 4th weeks, both treatments produced similar cumulative N_2O fluxes.

A strong positive correlation was found between daily N_2O fluxes and soil mineral N content, where r = 0.52, P < 0.01, n = 39 for NO_3^- and r = 0.33, P = 0.04, n = 36 for NH_4^+. Daily mean flux of N_2O was also positive correlated with DOC (P < 0.01, r=0.63, n=54). During the first week, mean N_2O cumulative fluxes were negatively correlated (P < 0.05) with the amount of benzoic acid applied in slurries (r = -0.92, n = 5).

4.3 Carbon dioxide emissions

Carbon dioxide emissions were measured under dark conditions to provide an indication of plant and soil processes (plant respiration + plant root respiration + soil microbial respiration).

Different pig's diets did not influence daily CO_2 emissions, which only showed significant differences (*P* < 0.05) between the control and the slurry treatments on days 9, 11, and 15 (Fig. 3). Overall, CO_2 fluxes from the different treatments showed a similar emissions pattern to that found for N_2O, with the highest fluxes (>1000 mg CO_2-C m^{-2} d^{-1}) were found between 7 and 20 days following slurry application.

At the end of the experimental period, cumulative CO_2 fluxes from all slurry treatments were not significantly different (*P* > 0.05) from the control. During the first week, however, significantly (*P* < 0.05) higher CO_2 losses were found from OP-75, OP-150 and CM-150 treatments than from CM-75 or PSC (Fig. 2b). In the second week, treatments produced by fibrous by-products shown similar emissions than from conventional diet, PSC.

Mean daily CO_2 fluxes during the first week were positive correlated with benzoic acid (r = 0.95; P < 0.05; n = 5) and hippuric acid (r = 0.93; P < 0.01; n=5) added with slurry. The cumulative CO_2 fluxes during the first week were also positively correlated with mean soil DOC (r = 0.88; P < 0.05; n = 5).

4.5 Soil mineral N and DOC

Soil NH_4^+-N concentration increased significantly with the addition of slurry (Fig. 4a). Generally, treatments from diets rich in fibrous by-products decreased the mineral N within in the slurry, and consequently that extracted from the soil, but neither OP nor CM showed lower soil NH_4^+ concentrations than PSC (Fig. 4a). Soil NH_4^+ content was higher than 20 mg N kg^{-1} during the first two weeks of the meso-cosm incubation, after which it returned to that of the control soil. The mean NH_4^+ concentration in soil was positively correlated with hippuric acid ($r = 0.90$, $P < 0.05$, $n = 5$), but not with benzoic acid. The maximum soil NO_3^- content (106.3 mg N kg^{-1}) appeared 20 days after slurry application (Fig. 4b), due to nitrification of soil NH_4^+. During the last two weeks of the experiment, the amount of soil NO_3^- was also higher than 20 mg N kg^{-1} but no significant ($P < 0.05$) differences between dietary's treatments were found.

Soil DOC concentration ranged from 73 to 194 mg C kg^{-1} (Fig. 5a). The maximum concentration (358 mg C kg^{-1}) was found in OP-150 at the beginning of the experiment. Although there were no significant differences between treatments in the daily measurements, the trend was that the OP treatments (OP-75 and OP-150), showed the highest soil DOC content during the first 15 days (Fig. 5a). This is consistent with the average of the soil DOC during the experiment, where the highest concentration were from treatments rich in soluble fibre, 122 y 149 mg C kg^{-1} for OP-75 and OP-150, respectively (Fig. 5b).

4.6 Harvest yield

In general, there was no significant effect of diet on the grass yield (Table 4). The grass biomass obtained in two cuts was higher in conventional than in the fibrous by-products-diets. The control showed a 27% less biomass compared with the diets treatments which indicated that the addition of a labile N source through pig slurry application had a clear effect on the yield, although independent of the type of diet.

The total N analysed in the grass biomass was significantly lower ($P < 0.05$) in the control than in the rest of the slurries treatments (Table 4). The total N obtained in the grass from different pig's diet treatments ranged from 2.7 to 3.2 %, but there were no significant differences ($P > 0.05$) between them.

5. Discussion

5.1 Slurry composition and gas emissions

Manipulation of pig diet has modified the composition of urine and faeces (Philippe et al., 2011), which can directly affect the emissions of N_2O from agricultural soils after manure is applied to soil as fertilizer. In this experiment, we demonstrated that slurries from pig fed with diets based on OP and CM produced lower emission of N_2O than slurry produced from a conventional diet. Although the amount of total N added with slurries was the same in all containers, the emission factor over the experimental period (37 days) ranged from 0.32% CM-75 to 0.19% for OP-150, compared with 0.56% for the conventional slurry.

Slurry contains multiple compounds that could individually affect production or consumption of N_2O in soil after its application. One of the most important components is NH_4^+ within the slurry, as it is the substrate for the process of nitrification, which is one of most important process involved in N_2O emission in Mediterranean cropping systems (Sahrawat and Keeny, 1986). The positive correlation found between the NH_4^+ content in the upper part of soil and N_2O fluxes are in consistent with that. However the NH_4^+ added with slurry was not enough to explain the important differences in fluxes, because the OP-150 and OP-75, which received 20% and 7% less NH_4^+ than PSC, respectively, resulted in <50% of total N_2O in comparison to that of PSC. Probably others minor slurry compounds could have an important effect on N_2O emission. In fact, an interesting finding of this experiment was that only benzoic acid added with slurries explained differences in N_2O fluxes. So, a negative correlation between benzoic acid added with pig slurry and total N_2O emission was detected during the first 15 days after slurry application. Some laboratory studies reported that N_2O losses can be mitigated by >50% by increasing hippuric acid concentration in cattle slurries (van Groenigen et al., 2006), but this effect has only been observed when in the presence of benzoic acid (Kool et al, 2006b), since this compound is a recognized antimicrobial agent (Marwan and Nagel, 1986) and it is the sub-product of the hippuric acid degradation. Our results partly corroborate the results from these studies, as the treatments which had the highest amounts of both acids (OP-75 and OP-150) produced the lowest N_2O emissions. However, the higher concentration of hippuric acid of OP-150 in comparison to that of OP-75 produced a similar total N_2O emission in both treatments. Neither, concentration of hippuric acid in slurries explained differences in fluxes between CM and PSC treatments, because N_2O emission were lower in the CM treatments (CM-75 and CM-150) than in PSC, despite the higher urine hippuric acid concentration from the PSC diet.

There are contradictory results in the literature regarding the effect of hippuric and benzoic acid on N_2O emissions, as studies carried out under field conditions did not find significant differences in N_2O emission from cattle urine with different concentrations of both acids (Clough et al., 2009; Krol et al., 2015). The first study argued that the low WFPS (< 35%) and the high pH (> 6.4) of the soil during the experimental period were not appropriate conditions to promote the inhibitory effect of hippuric and benzoic acid on N_2O emissions. In the case of Krol et al. (2015), the authors suggest that both organic acids loose their power to mitigate N_2O emissions as a consequence of other effects such as leaching, and plant root activity, which are not realistically represented in laboratory incubations that do not contain plants, or have sufficient soil depth. Therefore, this study is the first to show a mitigation effect, likely to be associated with benzoic acid concentration within slurry, where both soil microorganism and plants can compete for the available N. However, the possible inhibitory effect on processes producing N_2O emission was only maintained for a short period of time in this soil, as deduced from the negative correlation during the first two weeks between cumulative N_2O emission and benzoic acid. According to Clough et al. (2009), high soil pH could promote a rapid degradation of benzoic acid into its conjugated base (benzoate) diminishing its inhibitory effect. In our experimental conditions, with high pH both in slurries and soil, the conditions for degradation were very favourable. Benzoic acid could be metabolized in agricultural soils by some microorganisms (*Pseudomonas* and *Burkholderia* species), as demonstrated Pumphrey and Madsen (2008), reducing its concentration after addition. In this study, slurry was applied on the soil surface following the most common practices in the field. Therefore, C and N compounds added with slurry were concentrated on the upper part of the soil where the WFPS ranged from 60-70% on most of the sampling dates. This situation may have favoured N_2O production via denitrification, although, the rapid decrease in soil NH_4^+ concentration during the first two weeks of the study indicates that nitrification was also taking place (Bateman and Baggs, 2005). The inhibitory effect of benzoic acid seems to affect the denitrification process (van Groenigen et al. 2006, Bertram et al. 2009), although nitrification could also be affected (Bertram et al. 2009). Taking into account that both nitrification and denitrification processes contributed to the overall N_2O emissions from the mesocosms. We speculate that in this small soil volume, the amount of benzoic acid (and hippuric acid) added with treatments, such as OP-75 and OP-150, could have been enough to partially inhibit N_2O production. However, this needs to be checked with additional experiments.

An interesting result was the significant correlation between added hippuric acid with slurries and mean NH_4^+ concentration in soil, which could indicate that nitrification rate was retarded by hippuric acid, and therefore the amount of N_2O emission from this process. Also Bertram et al. (2009) suggested that hippuric acid reduced the activity of nitrifiers, however additional experiments are needed to confirm this. Additionally, the higher DOC concentrations and the higher CO_2 soil respiration observed for OP treatments after slurry application could have contributed to increased electron demand for denitrifiers, favouring the consumption of N_2O and consequently the reduction of N_2O/N_2 ratio. This effect, which has been observed when a source of labile C was added to soil (Cardenas et al., 2007), also contributes to a reduction in total N_2O emissions.

Hippuric acid as well as benzoic acid could have been used by soil microorganisms, enhancing soil respiration rate as indicated by the positive correlation found between cumulative CO_2 and added hippuric acid during the first two weeks after slurry application. Based on this finding, it is possible to indicate that both acids did not have a general inhibitory effect of microbial activity, at least at these concentrations, although as discussed before, benzoic acid added with slurry or derived from hippuric acid degradation in soil could have affected denitrification and nitrification activity.

5.2 Diets and N_2O emission in soil

Our results demonstrated that by manipulating pig diet, it is possible to modify urine and faeces composition and subsequently reduce N_2O emissions following soil application of the slurry. As slurries with the highest amount of benzoic acid produced the lowest N_2O emission, strategies based on increasing concentration of this compound in slurry (as well hippuric acid) could be considered as a potential option to mitigate N_2O emissions from slurry applications.

Organic acids and their salts, such as benzoic acid, are used in monogastric animal nutrition as alternatives to antibiotic growth promoters (Hansen et al., 2007). benzoic acid is absorbed in the small intestine, and metabolized in liver producing hippuric acid, which is subsequently excreted in the urine (Bridges et al., 1970). Murphy et al. (2011) observed a linear increase of N retained/intake when pig diet was supplemented with benzoic acid in the range 0 to 30 g kg^{-1}. This was attributed to a reduction in the total aerobic bacteria in the ileum, thus increasing digestibility. Also the lowering of pH in the gastrointestinal microbiota improves N absorption (Sauer et al., 2009). This effect could have contributed to lower NH_4^+ in the OP-150 treatment.

Another possible cause of the reduction of the amount of NH_4^+ in slurry, and its subsequent effect on fluxes, was through the effect of increasing soluble fibre in the diets (Beccaccia et al., 2015). These authors found that increasing soluble fibre, through incorporating OP within feeds, reduced the total N excreted via urine and the urine:faeces N ratio was reduced. This effect was caused as consequence of a consumption of N by microorganisms, which transformed soluble N, such as urea of urine, into organic N. This last fraction (organic N) which is mainly included in faeces, is normally mineralized more slowly in soil than soluble N, reducing the risk of volatilization in the following days after application. In fact, Beccaccia et al. (2015) found an important reduction of NH_3 emissions from diets with high percentage on fibre and lignin such as OP.

Analyzing the component of diets, and considering a mean of 2.4 L urine pig^{-1} day^{-1} excreted (in this experiment), benzoic or hippuric acid excreted in urine for OP-150 diet was 0.35 g BA and 3.96 g HA pig^{-1} day^{-1}, respectively. As the mean consumption of feed per pig per day was 2.5 kg in this experiment, only 11.25 mg benzoic acid was included in the feed for the OP-150 treatment. Therefore, other compounds (e.g. polyphenolic compounds) are necessary as a source of excreted benzoic acid. So, polyphenolic compounds included in OP, and ingested by the pig was 5.5 g galic acid pig^{-1} day^{-1} (OP-150), which was enough to generate the additional benzoic acid excreted in the urine.

However, more studies are needed to understand how degradation of polyphenolic compounds in the intestine generates benzoic acid in pig excreta.

To date, there is scarce literature (Petersen et al., 2013; Eriksen et al., 2014) regarding the use of slurries/urines with manipulated hippuric or benzoic acid content, and most of these have been conducted by increasing their concentration through direct addition of these compounds into the slurry (Fangueiro et al., 2015). However, this practice could be difficult to achieve due to the low solubility of both compounds (Krol et al., 2015). The manipulation of diet, as demonstrated in this experiment, provides an available strategy for increasing these compounds within the excreta (Dijkstra et al., 2013).

Meat producing countries, such as Spain, which is the 4th largest producer of pork in the world, need to develop strategies for sustainable pig meat production in order to decrease the release of N pollutants to the environment. Modifying pig diet using sub-products rich in fibre, such as OP or CM, is a potentially economically viable strategy to reduce N_2O since fibre rich feed ingredients are often cheaper than usual ones and can reduce the competition with food (cereals) for human nutrition. However, these products are many times locally produced and seasonally available and the

N_2O emissions, only represents 1-5% of the total N applied from the slurries which will not result in increased the nitrogen used efficiency (NUE). If this effect were combined with a reduction in nitrate leaching, then it could become important as an increase the N use efficiency of the meat sector butore studies should be necessary to achieve these challenges.

6. Conclusion

Changes in dietary fibre composition, as a consequence of including fibrous by-products, had an important effect on the concentration of urine and faeces compounds. Bezoic and hippuric acid concentrations in urine were related to the type of fibrous by-product in the diet, being higher for OP than for CM or barley grain. Results of a benzoic acid balance considering both intake through feed and release through urine indicated that the source of this acid and its precursor (i.e. hippuric acid) should be phenolic compounds (other than benzoic acid), probably associated with the polyphenolic or lignin content in the fibrous fraction.

The composition of slurry also had an important effect on N_2O. Emission of this gas was correlated with the benzoic acid added with urine, but not directly with hippuric acid concentrations. Under denitrification favoring condition (WFPS close to 70%), the inhibitory effect was only observed for 15 days following slurry application, probably because of the degradation of this compound in soil within that period.

In contrast, microorganisms increased soil CO_2 emissions in these first two weeks from OP or CM treatments. This could indicate that there were not toxic effects of benzoic acid at this relatively low concentration on soil respiration.

Further knowledge is required on which compounds within urine and faeces have a natural inhibitory effect on denitrification or nitrification. Improving knowledge within this area will contribute to the range of approaches that can be used to mitigate greenhouse gas emission from livestock systems. These results show the potential of alternative feeding strategies for the reduction of environmental problems associated with agriculture, including the external dependency of raw material imports for feeding animals in Spain

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Table 1. Chemical composition of experimental diets (g kg^{-1}, as fed basis) (Beccaccia et al., 2015). Control pig slurry (PSC), 75 g kg^{-1} orange pulp (OP-75), 150 g kg^{-1} orange pulp (OP-150), 75 g kg^{-1} carob meal (CM-75) and 150 g kg^{-1} carob meal (CM-150).

	PSC	**OP-75**	**OP-150**	**CM-75**	**CM-150**
Dry matter	912	902	903	895	899
Crude protein	158	156	154	153	157
NDICP[a]	21.4	26.7	21.2	23.8	20.8
Total dietary fibre	194	214	234	200	212
NDF[b]	154	165	158	164	161
ADF[c]	45.6	52.3	56.3	61.0	75.4
ADL[d]	8.0	9.0	10.7	18.9	33.9
Soluble fibre	61.2	75.9	97.3	60.1	71.4

[a]Neutral detergent insoluble crude protein.

[b] Neutral detergent fibre

[c] Acid detergent fibre without residual ash;

[d] Acid detergent lignin

Table 2. Chemical characteristics of pig slurries (faeces+urine) from different dietary treatments[a]. Control pig slurry (PSC), 75 g kg^{-1} orange pulp (OP-75), 150 g kg^{-1} orange pulp (OP-150), 75 g kg^{-1} carob meal (CM-75) and 150 g kg^{-1} carob meal (CM-150).

	PSC	**OP-75**	**OP-150**	**CM-75**	**CM-150**
			Slurry[a]		
Total N (g kg^{-1})	9.64	8.68	7.82	7.95	9.7
NH_4^+ (g kg^{-1})	5.32	4.48	3.46	3.75	4.35
pH	8.89	7.93	8.08	8.38	8.20

[a] Slurry samples were obtained mixing individual excretas (faeces+urine) of 6 pigs by dietary treatment.

Table 3. Effect of source (S) and level of inclusion (L) of fibrous by products on the concentration of minor components in pig urines. Control pig slurry (PSC), 75 g kg^{-1} orange pulp (OP-75), 150 g kg^{-1} orange pulp (OP-150), 75 g kg^{-1} carob meal (CM-75) and 150 g kg^{-1} carob meal (CM-150).

	PSC	OP-75	OP-150	CM-75	CM-150	SEM[1]	S	L	SxL
Allantoin (mg L^{-1} urine)	679	575	653	552	505	141	0.551	0.912	0.661
Creatinine (mg L^{-1} urine)	1888	1821	1744	1636	1690	342	0.730	0.973	0.851
Uric acid (mg L^{-1} urine)	69.2	52.1	64.8	54.4	40.5	439	0.422	0.967	0.331
Hippuric acid[2] (mg L^{-1} urine)	848	1231	1651	552	699	189	<0.001	0.146	0.479
Benzoic acid (mg L^{-1} urine)	61.4	147	149	81.1	73.8	37.3	0.072	0.939	0.906

[1]Standard error of means (n=6)

[2]Contrast PSC vs OP-150 (*P<0.05*)

Table 4. Harvest yield and total N in the ryegrass. Control pig slurry (PSC), 75 g kg^{-1} orange pulp (OP-75), 150 g kg^{-1} orange pulp (OP-150), 75 g kg^{-1} carob meal (CM-75) and 150 g kg^{-1} carob meal (CM-150).

	Harvest Yield (g DM m^{-2})	N (%)
Control	14.33 ± 0.9	1.71 ± 0.0
PSC	19.37 ± 1.0	3.16 ± 0.2
CM-75	19.77 ± 0.5	2.95 ± 0.1
CM-150	19.57 ± 0.4	2.86 ± 0.2
OP-75	20.45 ± 0.5	2.92 ± 0.1
OP-150	20.54 ± 0.9	2.78 ± 0.1

Values are the mean of three replicates ± standard deviation.

Figures

Fig. 1 Daily soil N_2O emissions (mg N_2O-N m^{-2} d^{-1}) from different slurry treatments following application (DAA). Control pig slurry (PSC), 75 g kg^{-1} orange pulp (OP-75), 150 g kg^{-1} orange pulp (OP-150), 75 g kg^{-1} carob meal (CM-75) and 150 g kg^{-1} carob meal (CM-150). Vertical bars indicate standard errors for each sampling date.

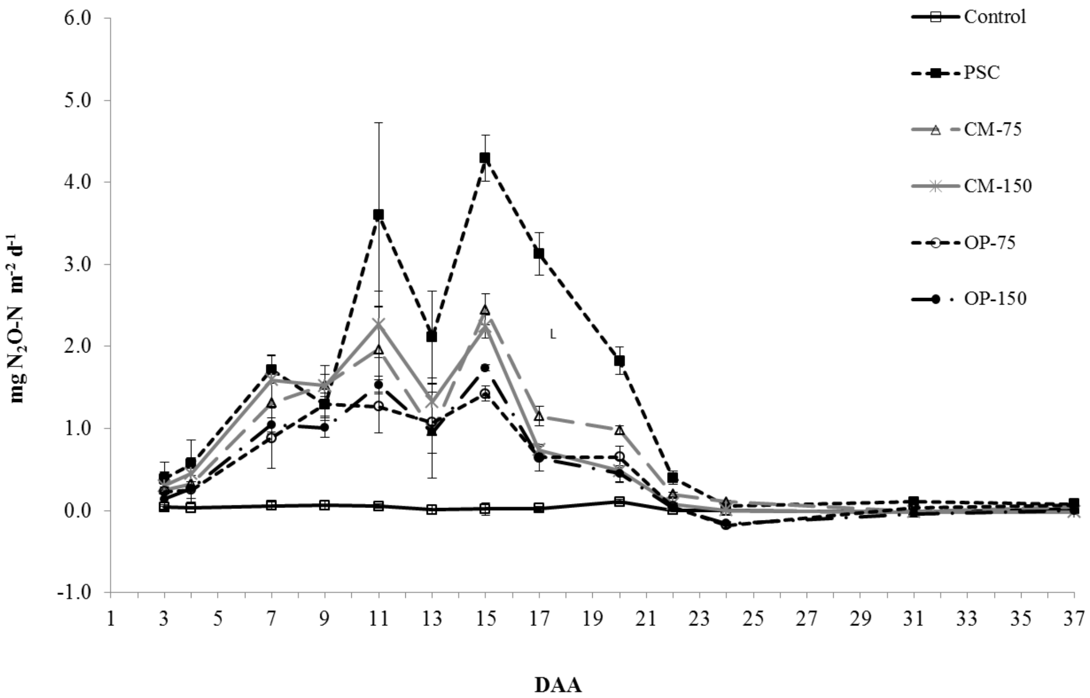

Fig. 2 Cumulative (a) N_2O and (b) CO_2 emissions per week from soil amended with different pig slurries. Control pig slurry (PSC), 75 g kg^{-1} orange pulp (OP-75), 150 g kg^{-1} orange pulp (OP-150), 75 g kg^{-1} carob meal (CM-75) and 150 g kg^{-1} carob meal (CM-150). Vertical bars indicate standard errors for each sampling date. Values are the mean of three replicates ± standard deviation. Different letters indicate significant differences at ($P < 0.05$) between treatments in the same week.

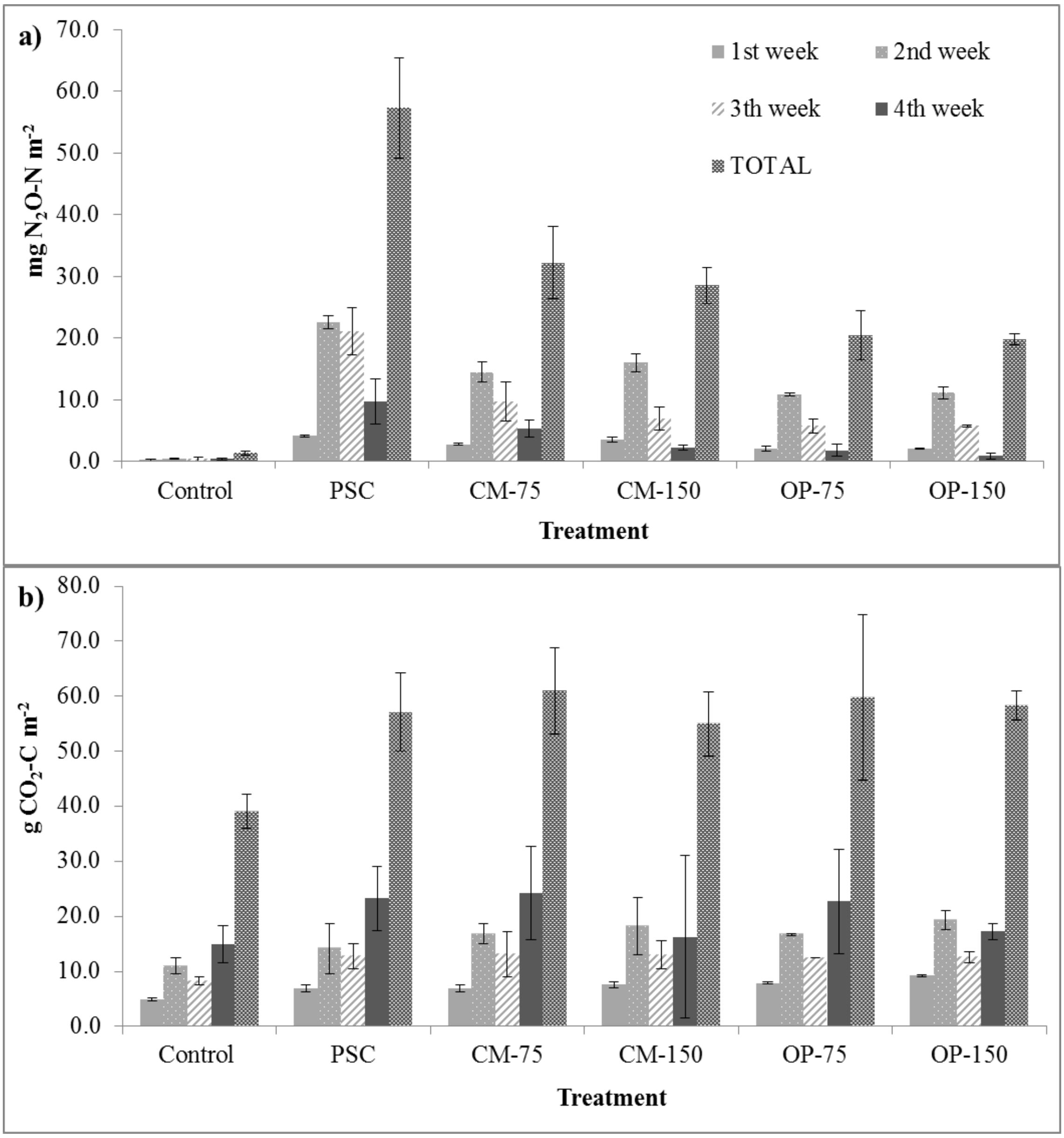

Fig. 3 Daily soil CO_2 emissions (mg CO_2-C m^{-2} d^{-1}) from different slurry treatments following application (DAA). Control pig slurry (PSC), 75 g kg^{-1} orange pulp (OP-75), 150 g kg^{-1} orange pulp (OP-150), 75 g kg^{-1} carob meal (CM-75) and 150 g kg^{-1} carob meal (CM-150). Vertical bars indicate standard errors for each sampling date.

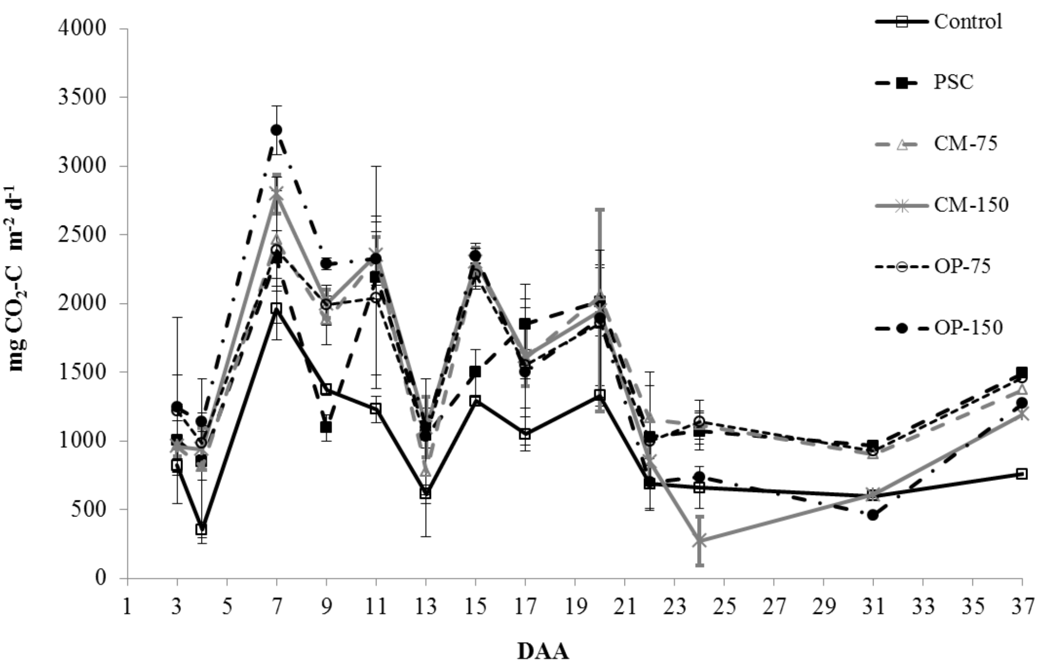

Fig. 4 (a) Soil NH_4^+-N (mg NH_4^+-N kg^{-1}) and (b) NO_3^--N (mg NO_3^--N kg^{-1}) for different slurry treatments following application (DAA). Vertical bars indicate standard errors for each sampling date.

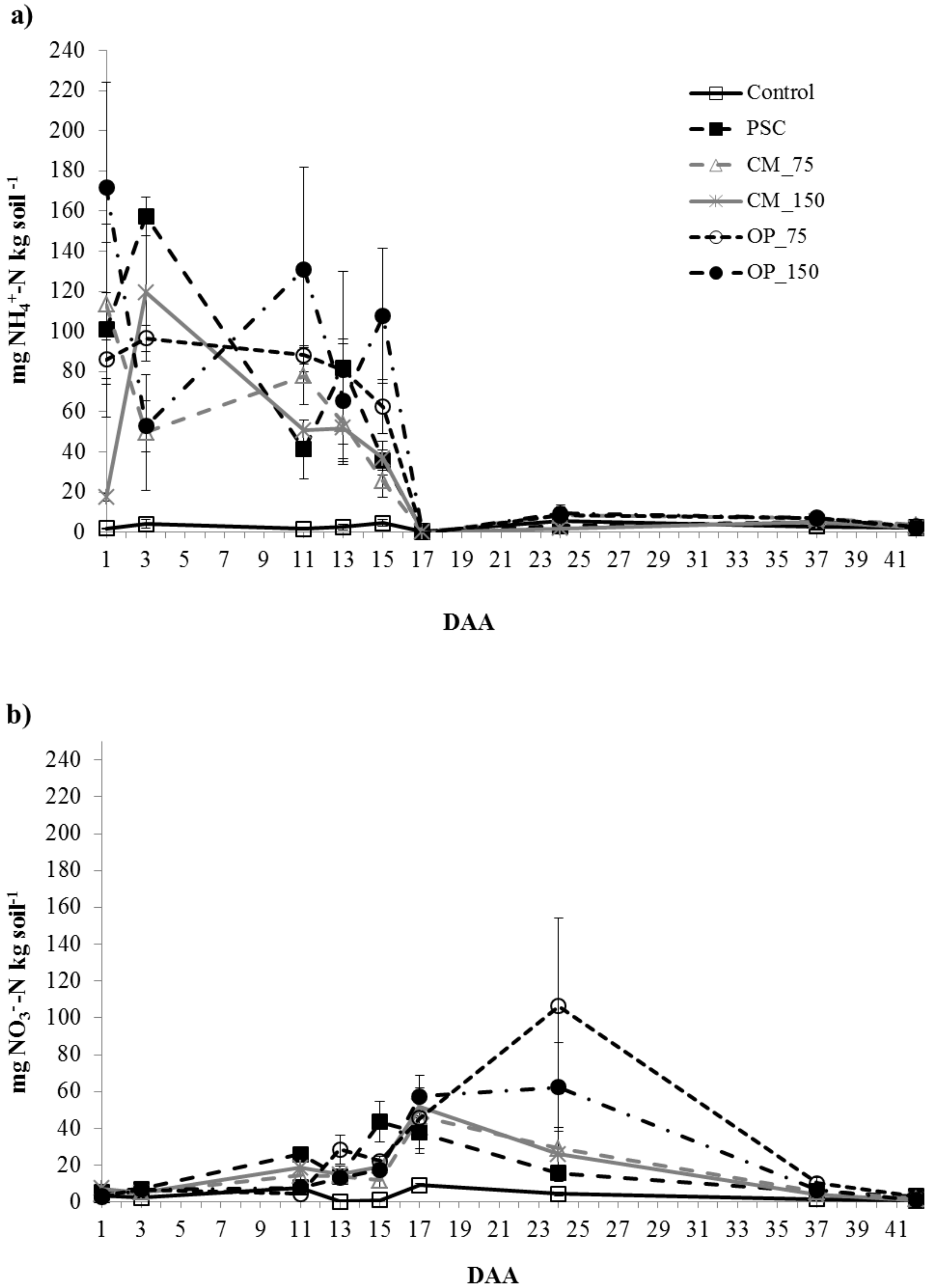

Fig. 5 (a) Soil C (mg C kg^{-1}) for different slurry treatments following application (DAA) during the experimental period and (b) mean soil C (mg C kg^{-1}) during the experimental period for each treatment. Vertical bars indicate standard errors.