Blending based optimisation and pretreatment strategies to enhance anaerobic digestion of poultry manure

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ABSTRACT

Anaerobic digestion of poultry manure is limited by the excessive levels of nitrogen and the high concentration of dry matter. These limitations are usually overcome either by applying procedures to remove nitrogen or by employing pretreatments that allows to solubilise organic matter. In this work, the treatment of poultry manure was enhanced by co-digestion with pig manure through the methodological determination of optimal mixtures combined together with a thermochemical pretreatment coupled to ammonia stripping . The optimum poultry-pig mixture, resulting in a 24%:76% (volume basis) poultry-pig manure, was determined by applying a methodology based on linear programming which calculates the proportions of the blend which returns the maximum methane production while keeping a stable process.. Pretreatment batch experiments, consisting of increasing both temperature and pH simultaneously with ammonia stripping process was optimised for a temperature of 90ºC and a pH of 10 resulting ina nitrogen removal efficiency of 72% and a 1.2-fold higher methane production in comparison to the unpretreated mixture. Continuous anaerobic co-digestion of pretreated optimum mixture enhanced the COD removal efficiency by 37% when compared with the treatment of unpretreated feedstock (37% vs 27%, respectively).. This study indicates that combining blending optimisation of substrates, thermochemical pretreatments and ammonia stripping procedures prior to anaerobic co-digestion becomes a good strategy to overtake the limitations offered by solid- and nitrogen-rich substrates, such as poultry manure.

KEYWORDS

Ammonia inhibition; co-digestion; lignocellulosic material; livestock waste; methanisation.

1. **INTRODUCTION**

In European Union 113 millions of tons of poultry manure are yearly generated (Foged et al., 2012). This residue was traditionally applied on land as agricultural amendment due to the valuable nutrient content (Thangarajan et al., 2013). Nevertheless, the direct application of manure on land may provoke severe effects to the environment such as greenhouse gas emissions, odour related issues, eutrophication or releasing of pathogens in the groundwater, among others (ten Hoeve et al., 2014). This barrier is successfully overtaken by applying efficient treatments to manure such as anaerobic digestion (AD) (Nasir et al., 2012; Rodriguez-Verde et al., 2014a; Sakar et al., 2009). Organic wastes with high and readily biodegradable organic matter content are preferablytreated by AD because higher biogas production are expected, thus improving both economic and environmental profiles (Rodriguez-Verde et al., 2014a). Poultry manure (PoM) presents high levels of organic matter (> 300 g O_2/kg); however, the total solid content (>25%) and the nitrogen concentration, up to 30 g N-TKN/kg, most as urea (Kelleher et al., 2002; Tiquia and Tam, 2000) are the two main factors hampering the anaerobic digestion of PoM. The high dry matter content limits the processing in conventional digestion systems (Chamy et al., 2012) which is further aggravated due to the reduction of the methanisation potential caused by the high lignocellulosic fraction coming from the bedding material (sawdust and straw) (Ahring et al., 2015; Güngör-Demirci and Demirer, 2004; Sun et al., 2016). Moreover, the high protein content may conduct to the formation of free ammonia during AD, which was already demonstrated to be an inhibitor for the process (Regueiro et al., 2012; Yenigün and Demirel, 2013).

To overcome these limitations, several strategies to enhance PoM treatment were evaluated, such as anaerobic co-digestion (ACoD) or pretreatments prior AD. ACoD compensates the lack of appropriate characteristics of PoM for AD, such as the

humidity content and the C/N ratio (Abouelenien et al., 2014; Li et al., 2014; Sun et al., 2016). Within the agroindustrial waste treatment framework, pig manure (PM) is a very suitable substrate for ACoD due to its high humidity and excellent buffering capacity (Regueiro et al., 2012). Nevertheless, the proportions of the substrates should be adequately balanced to ensure the stable operation and also to provide the highest methane production . In order to define the optimal mixture of substrates for ACoD, several procedures have been applied: blending of substrates based on trial-and-error with different proportions of co-substrates in batch experiments (Alatriste-Mondragón et al., 2006), response surface methodologies (Wang et al., 2014) or linear programming to maximize methane production (Álvarez et al., 2010). The latter was further improved by García-Gen et al. (2015) combining both optimization and control principles in order to establish blends that maximise methane production while keeping a suitable reactor performance.Pretreatment of slowly biodegradable substrates prior to AD or ACoD is often encouraged in order to speed up and/or increase their methanisation potential either by making accessible some organic material or removing toxic compounds for the anaerobic process (Monlau et al., 2012). Accordingly, different methods were employed to improve the degradation of PoM such as a) thermochemical pretreatment (Costa et al., 2012), consisting of applying a temperature of 90ºC, an alkali dose of 0.2 $g_{lime}/g\ TS_{waste}$ and a pressure of 1.27 bar; b) chemical pretreatment (Ardic and Taner, 2005) by the the addition of NaOH (20% of the total solid content) at high temperature (boiling temperature of water); and c) biological co-treatment (Costa et al., 2012) by the bioaugmentation with *C. cellulollyticum*, *C.thermocellum* and *C. saccharolyticus*. The different methods applied to PoM resulted in the improvement of the organic matter solubilisation of PoM, however,

the nitrogen concentration remained at high levels probably limiting the methane yield improvement, thus being necessary complementary or alternative methods to diminish ammonium content (Zhang et al., 2012). In this line, several strategies have been used in literature such as struvite precipitation or ammonia stripping (Rajagopal et al., 2013). The former achieves an appropriate performance when liquid streams are considered and when a high fraction of phosphorus is present, such as leachates, urine or swine wastewater (Kumar and Pal, 2015). Similarly, ammonia stripping was successfully proved (ammonia removal efficiencies higher than 80%), but mainly with low solid concentration streams either prior to anaerobic digestion (Bonmatí and Flotats, 2003; Laureni et al., 2013) or during AD process (Abouelenien et al., 2009). However, enhancing the anaerobic digestion of complex substrates such as poultry manure leads to the application of methods combining ammonia removal, co-substrates blending optimisation and solubilisation of organic matter.

Therefore, the aim of this study was to enhance anaerobic treatment of poultry manure through co-digestion with pig manure: i) by determining the optimum poultry-pig mixture by linear programming methods, and ii) by applying a thermochemical pretreatment based on the application of both high temperature and pH combined with ammonia stripping to enhance anaerobic biodegradability and remove nitrogen.

2. MATERIALS AND METHODS

2.1 Wastes and inoculum

PoM was collected from a poultry farm with a 4-replacement cycle ratio per year of 20,000 chickens and it consisted of a mixture of manure and bedding material, composed by sawdust and straw. After collection, PoM was stored at 4ºC throughout the experimental period. PM was taken from a pig fattener farm with a total herd of 3,000 heads. Several batches of PM, which were stored at 4ºC, were needed along the experimental period.

Both wastes were characterised in triplicate in terms of total (TS, g TS/kg) and volatile (VS, g VS/kg) solids content, total and soluble chemical oxygen demand (COD_t and COD_s, g O_2/kg), total Kjeldahl nitrogen (TKN, g N-TKN/kg), total ammonium nitrogen (TAN, g N-TAN/kg), pH, alkalinity (g $CaCO_3$/kg) and biomethane potential (L CH_4/kg_{VS} and L CH_4/kg_{waste}). Furthermore, the lignocellulosic content of the PoM was determined in terms of lignin, hemicellulose and cellulose concentrations.

The inoculum used in both biochemical methane potential (BMP) tests and anaerobic reactors was flocculent biomass (15 g VS/kg) coming from a mesophilic digester treating sewage sludge of a municipal wastewater treatment plant.

2.2 Determination of optimum mixture and operational conditions

In order to determine the optimum PM:PoM mixture and operational conditions, the ACoD of pig and poultry manure was simulated using the Optiblender tool developed at University of Santiago de Compostela (ES2156615, 2014), which consists of three steps: determination of optimum mixture (Blender) according to the procedure developed by García-Gen et al. (2014), the simulation of the co-digestion process (virtual plant) using the "ADM1-based ACoD model" developed by García-Gen et al.

(2015) and the optimization of the process based on two diagnosis variables (Optimiser).

The blending protocol (Blender) is based on a linear programming optimisation software, which calculates the mixture that maximises methane production and the optimum hydraulic retention time (HRT) mantaining a stable operation according to a set of linear restrictions based on the heuristic knowledge of the process. From the feeding composition and the HRT, the organic load rate (OLR) is determined. In addition, blender also informs about the active restriction, that is, the boundary that limits the optimisation towards a new potential optimum.

The anaerobic co-digestion of this mixture is then simulated in the Virtual Plant and the results are evaluated. For diagnosis in Optimiser, two variables are used: reactor stability, which is determined by alkalinity ratio (relation between intermediate alkalinity, associated with volatile fatty acids (VFAs), and the total alkalinity) and methane yield. The values given by the virtual plant are compared to the target values (set-point), 0.3 and 15 L CH_4/L d for alkalinity ratio and methane productivity (García-Gen et al., 2015), respectively, and the result of this comparison will allow reformulating the boundaries of the active restrictions, deriving in the calculation of a new optimum mixture, HRT and OLR.

In summary, a closed loop is proposed: blending formulation, simulation of the co-digestion process and diagnosis, modification of active restriction boundaries and again blending formulation. The inputs required for the application of Optiblender are the physico-chemical characterisation and the BMP of wastes, the initial restrictions (Table 1) and a simulation time (200 days were considered in this study). The outcome is a mixture producing the highest methane possible under safe conditions, which was referred as max(PM:PoM) mixture, and the optimum HRT.

2.3 Pretreatment systems

The pretreatment experiments, where a thermochemical process consisted of increasing temperature and pH and ammonia stripping simultaneously took place, were conducted in two different units: Unit 1 (U1) used for the determination of the optimal operational conditions and Unit 2 (U2) employed for pretreating the reactor feeding throughout the continuous reactor performance.

2.3.1 Batch pretreatment unit (U1)

U1 consisted of a glass-jacketed column with an internal diameter of 4.5 cm and a height of 20 cm equipped with an aeration system with a continuous airflow of 11 L/h (Figure 1). The temperature of the unit was controlled by recirculating hot water from a temperature-controlled water bath. The airflow was supplied at the bottom of the column and the outlet gas (mainly air plus ammonia desorbed) was delivered to an absorber vessel containing a sulphuric acid solution (5M) where ammonia was precipitated as ammonium sulphate.

Nine batch pretreatment assays were performed in triplicate at different temperatures (70, 80, 90ºC) and pH values (8, 9, 10, modified by NaOH 10 M addition). The mixture tested was the max(PM:PoM) mixture and the retention time was 2 h. Nitrogen removal (expressed as the difference of TKN concentration before and after the experiment), organic matter solubilisation (determined as the soluble/total COD ratio after the experiment) and the methane potential of pretreated mixture were evaluated.

2.3.2 Batch pretreatment unit (U2)

The layout of U2 was similar to U1 but with a volume of 5 L. The pretreatment was carried out once a week, approximately, at a temperature of 90ºC and pH of 10 for 2 h with an airflow of 11 L/h.

2.4 Biochemical methane potential (BMP)

Biochemical methane potential (BMP) tests were carried out to determine the biodegradability of both raw wastes and the max(PM:PoM) mixture and also to assess the impact of the pretreatment on anaerobic biodegradability.

BMP were performed in 500 mL bottles (375 mL of working volume) in triplicate following the protocol described by Rodriguez-Verde et al. (2014b). Inoculum was transferred with an in-reactor concentration of around 5 g VS/L and the substrate was adjusted in order to achieve an inoculum-to-substrate ratio of approximately 2 g VS/g VS. Accumulated methane production was monitored over time (depending on the biogas flow produced, sampling was proceeded once, twice or three times per week) in order to determine the COD fraction converted into methane and the biochemical methane potential.

2.5 Anaerobic digesters

Two continuous stirred tank reactors (CSTR) made of stainless steel with a working volume of 14 L were operated in mesophilic conditions (37ºC). Both reactors were inoculated with an initial in-reactor biomass concentration of 15 g VS/L and their performance was divided into four operational periods:

2.5.1. Period 1 (days 0-100). Biomass adaptation. An initial 98PM:2PoM (%v/v) mixture and a HRT of 28 days was considered according to the simulation results. During this period, the OLR was around 1 g COD/L d in both reactors, fluctuating due to COD variation in PM stocks. Reactors were operated under these conditions during 100 days in order to ensure biomass adaptation to the substrate because acclimation to PoM requires long adaptation periods (Nasir et al., 2012).

2.5.2 Period 2 (days 101-190). Organic loading rate increase. The Optiblender described in section 2.3 was employed to define how and how much the OLR of the anaerobic digesters can be boosted till reaching the maximum OLR given by the

max(PM:PoM) mixture. The difference is that diagnosis was made using the experimental results obtained during the reactor performance instead of the simulated values given by the virtual plant. Each blend was evaluated for 4-12 days, depending on the stability of the reactor (indicated by the alkalinity ratio). Again, a constant HRT of 28 days was indicated by Optiblender for the whole period.

2.5.3. Period 3 (days 191-265). Influence of thermochemical pretreatment. One reactor (R1) was fed with pretreated feeding, while the second one (R2) remained as control (non-pretreated feeding). The OLR, the HRT and the feeding composition stayed constant at 3 g COD/L d, 28 days and the max(PM:PoM) mixture, respectively.

2.5.4 Period 4 (days 266- 360). Influence of HRT. Since the optimum mixture and operational conditions (HRT and OLR) obtained through Optiblender did not consider the effect of the thermochemical pretreatment, HRT was modified in order to evaluate the treatment capacity of PoM under anaerobic co-digestion. Accordingly, it was gradually decreased in both reactors from 28 to 20 days, thus increasing the OLR from 3 to 4 g COD/L d maintaining the feeding composition of period 3.

Biogas production was measured on-line and biogas composition twice a week. Mixed liquor samples were taken twice a week for determining the main performance parameters (COD, NH_4^+, pH, alkalinity). The differences between both reactor were assessed through a statistical analysis based on Student's t test, considering significant P values <0.05. Furthermore, one sample of biomass of each reactor (R1 and R2) was taken on day 337 in order to assess whether the pretreatment has an effect or not on the anaerobic microbial community.

2.6 Analytical methods

pH, COD, TS, VS, alkalinity, NH_4^+, TAN and TKN were measured according to Standard Methods (Apha, 2005). VFA was analysed through gas chromatography with

flame ionization detection (FIC, HP 5890A). Biogas production was measured with a pressure transducer (Centrepoints electronics) in BMP tests and by Ritter millligascounters (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany) in the reactors. Biogas composition was determined by gas chromatography (HP 5890 Series II).

The content of lignin, hemicellulose and cellulose was assessed following the protocol adapted by (López-Abelairas et al., 2013) where lignin was separated from the sugar fraction by acid hydrolysis. In the sugar fraction, total reducing sugars and glucose were analysed to determine cellulose and hemicellulose content.

2.7 Microbiological Analysis

2.7.1 DNA extraction

Biomass samples were sonicated for 1 minute (UP200s, Dr. Hielscher) and total genomic DNA was extracted according to the phenol-chloroform protocol (Alonso-Gutiérrez et al., 2009). DNA was suspended in 50 µL of milliQ water and kept at -20°C until PCR amplifications. DNA concentration was determined using a fluorimetric method with Quant-IT PicoGreen reagent (Thermo Fischer) in a Quantifluor ST fluorometer (Promega).

2.7.2 Illumina

The sequence of the V3-V4 region of 16S rRNA gene was used as the taxonomic basis to estimate bacterial populations present in the samples. DNA concentration was determined in the samples using a fluorimetric method with Quant-IT PicoGreen reagent (Thermo Fischer) in a Quantifluor ST fluorometer (Promega). Afterwards, DNA samples were diluted to 1,5 ng/µl and 2 µl of each diluted sample were used to amplify the V3-V4 region of 16 S rRNA gene using specific primers for 16S rRNA with the following: (i): S-D-Bact-0341-b-S-17, 5′-CCTACGGGNGGCWGCAG-3′ and S-D-

Bact-0785-a-A-21, 5′-GACTACHVGGGTATCTAATCC-3 (Klindworth et al., 2012). These specific primers were used as fusion primers respectively linked to CS1 and CS2 sequences (Fluidigm) useful for subsequent barcoding. Positive amplification was evaluated by gel electrophoresis of PCR products which showed a marked and clean band of a size around 440 pb in most of the samples. Therefore, a second PCR of low number of cycles was applied to add the individual barcode to each of the samples, as well as to incorporate Illumina-specific sequences in the amplicon libraries. Individual libraries were analyzed using a Bioanalyzer 2100 (Agilent) to estimate the concentration of the specific PCR products and a pool of samples was made in equimolar amounts. The pool was further cleaned, quantified and the exact concentration of the library was measured by real time PCR, using Illumina specific primers (Kapa Biosystems). Finally, samples were denatured and prepared at 12 pM to be seed into a Miseq flowcell (Illumina) and run under a 2x280 pair end sequencing procedure (Unidad genómica de Cantoblanco, Parque Científico de Madrid). A total amount of > 100,000 reads were obtained for each samples. After quality filtering and demultiplexing, data were analysed using the 16S-Metagenomics Illumina pipeline (Base Space, Illumina). MSR software (MiSeq Reporter v 2.4) was used for the analysis. Bioinformatic assays were performed with predominant operational taxonomic units (OTUs), i.e. with abundance above 1% of the total observed OTUs, in order to study the taxonomic profiles.

3. RESULTS AND DISCUSSION

3.1 Characterisation of wastes

Main physico-chemical characteristics of PM and PoM are collected in Table 2. The COD value for PoM was very high (783 g/kg) compared to PM (24 g/kg), and only 20% was in soluble form. In addition, PoM showed a significant concentration of lignin in comparison to both hemicellulose and cellulose (163 g/kg vs 17 g/kg and 20 g/kg, respectively), which might cause an impediment to the proper biodegradability of the substrate (Triolo et al., 2011). PoM also had a high nitrogen content (22.5 g N-TKN/kg)with a low fraction of TAN (0.77 g N-TAN/L). Overall, the characteristics of PoM were in the range reported by other studies (Costa et al., 2012; Flotats and Sarquella, 2008; Güngör-Demirci and Demirer, 2004).

As expected, the anaerobic biodegradability of PM (48.8 ± 2.5%, Figure 2A) was higher than PoM (28.5 ± 3.4%, Figure 2B) resulting in methane potentials of 356 ± 18 L CH_4/kg_{VS} and 159 ± 19 L CH_4/kg_{VS}, respectively. The presence of a significant fraction of lignin (163 g/kg, Table 2) explains the relative low value of PoM. These results are in agreement with previous studies (Güngör-Demirci and Demirer, 2004; Regueiro et al., 2012; Triolo et al., 2011).

3.2 Determination of optimum mixture and operational conditions

ACoD of PM and PoM was simulated during 200 days to determine the composition of the optimal mixture at the optimal HRT (Table 3, Figure 3). Optiblender suggested to stepwise increase the percentage of PoM in the mixture from 2 to 24%, volume basis, in a total of 140 days, increasing the OLR from 1.00 to 2.98 g COD/L d. The OLR increase was wider during the 6 first cycles (days 0-60, Figure 3A) in accordance to the low alkalinity ratio simulated (Figure 3B). From day 60 to 140, alkalinity ratio was higher (0.15-0.20, Figure 3B) which derived in smoother increases in OLR to fully

guarantee the stability system (Figure 3A). The max(PM:PoM) mixture was obtained on day 140 and it was composed of 76% of PM and 24% of PoM (v/v%) (Table 1).

Until day 140, OLR became the active restriction for all the control cycles (Table 3). From this day on, TS was set as the active restriction and no further performance improvement could be experimented because the total solid content of the mixture (80 g TS/L or 88 g TS/kg) reached the top boundary of total solids restriction (set at 80 g/L, volume fraction). Under this perspective, the Optiblender system is programmed to be not further modified to avoid operative problems with digester equipment, which are commissioned to perform at high levels of humidity. As simulation achieved the steady state from day 140 on, the selected simulation time of 200 days was considered appropriate.

The simulation also reported information about the methanisation yield of the mixture with an average of 0.40 L CH_4/ L d at steady state after 140 days of simulation(Figure 3B). This value corresponds to a biodegradation of 25%, similar to the experimental BMP (Figure 2C, 24.1 ± 4.0%). However, this value differed from the theoretical one calculated from the BMP of each substrate (33%). These differences can be explained either by the heterogeneity of PoM or by the high levels of ammonia in the simulated continuous operation, which can hamper the biodegradation, especially in short-term periods (Rajagopal et al., 2013).

3.3 Pretreatment experiments

In total, nine experiments were performed varying temperature and pH (Table 4). The higher the temperature and pH, the higher the nitrogen removal, the COD solubilisation and the BMP. Nitrogen removal was mainly affected by pH (almost 10-fold increase when pH rose from 8 to 10), and slightly by temperature. Guštin and Marinšek-Logar

(2011) also observed that pH had a higher effect on nitrogen removal than temperature during the stripping process. They changed the pH from 8.5 to 10.5 and the ammonium removal efficiency increased from 27 to 93%, while varying temperature from 303 K to 343 K derived in a slight improvement from 80 to 92%. In contrast, COD solubilisation was driven by temperature, rather than by pH, resulting in an increase in the solubilised fraction from 0.20 to 0.38 and 0.43 at 70ºC and 90ºC, respectively. A higher temperature improves the hydrolysis step in anaerobic degradation process since solubilisation of organic matter is enhanced (Costa et al., 2012). Accordingly, BMP value of the max(PM:PoM) mixture was slightly improved at higher temperatures (39-40% compared to 24% of raw mixture, Table 4). Therefore, pH of 10 and temperature of 90ºC were selected as the optimal conditions to pretreat feedstock for continuous anaerobic co-digestion experiments, foreseeing the performance improvement by lowering ammonia content and increasing the biodegradation of wastes.

3.4 Anaerobic co-digestion continuous performance

3.4.1. Period 1 (days 0-100). Biomass adaptation.

As expected (same operational conditions), during this period both reactors did not displayed significant differences (Figures 4, 5) ($P>0.05$). Although OLR was fluctuating due to the variations of PM, methane production was pretty constant averaging 0.49 ± 0.06 and 0.50 ± 0.05 g COD/L d in R1 and R2, respectively, leading to a COD removal efficiency of 43 and 44%, respectively (Table 5). The simulation evidenced a COD removal of 45% at an OLR of 1 g COD/L d treating the mixture 98PM:2PoM (v/v%). This consistency demonstrates the applicability of the Optiblender software and its calibration accurateness in order to simulate the anaerobic co-digestion of PM and PoM. Ammonium concentration in the reactors remained constant at around 1.4 g N-NH_4^+/L (Figure 4B, 5B) and pH ranged between 7.8 and 7.9 (Table 5), resulting in free

ammonia levels of 116 ± 38 and 132 ± 20 mg $N\text{-}NH_3$/L in R1 and R2 (Table 5), respectively. Moreover, no VFA were detected, pointing out the stability of the reactor and an appropriate adaptation of the microorganisms to the substrates.

3.4.2 Period 2 (days 101-190). Organic loading rate increase

During this period, the feeding mixture was modified until the max (PM:PoM) was reached providing an OLR of 3 g COD/L d. OLR was the factor limiting the operation towards a new potential optimum with a higher methane production. Accordingly, Optiblender allowed expanding the top boundary of the OLR restriction and higher values could be achieved. The alkalinity ratio, the stability factor selected, was always below the set point established (0.3), recording values ranging from 0.10 to 0.25, pointing out the stability of the reactors during the whole period.

Methane production increased from 0.42 to 0.82 g COD/L d (Figure 4A) and from 0.50 to 0.89 g COD/L d (Figure 5A) and no significant differences were observed between both reactors ($P>0.05$) with average values of 0.61 ± 0.18 and 0.61 ± 0.17 g COD/L d for R1 and R2 (Table 5), respectively. Although OLR was 3-fold augmented, methane productivity was only doubled, because COD removal efficiency decreased (average COD removal of 31%, Table 5), reporting similar values than the theoretical one (33%) considering the BMP of PM and PoM (Figure 2A, 2B). The lower COD removal efficiency is explained by the fact that most of the COD of the mixture was provided by PoM (almost 80% at the end of the period, Figure 4A, 5A), the waste showing the lowest BMP (28.5%). Borowski et al. (2014) observed that a mixture composed of swine manure, sewage sludge and poultry manure, the latter representing 10% of the mixture (weight basis), derived in lower methane production than the anaerobic co-digestion of the mixture without poultry manure (from 400 to 336 L CH_4/kg_{VS}). They concluded that the reason of the decrease in the methane yield was the inhibition of the

methanogenesis step by free ammonia. Similar insights were confirmed by Sun et al. (2016) pointing out that the specific methane potential decreased by increasing the percentage of chicken manureduring the co-digestion with maize silage. Ammonium concentrations in the reactors (3.34 and 3.63 g N-NH_4^+/L for R1 and R2, respectively, Table 5) derived in high free ammonia levels (up to 430 mg N-NH_3/L) (Figure 4B, 5B), which might drive to inhibition episodes and decrease methane production (Zhi and Zhou, 2011). However, despite ammonia levels increased, no VFA accumulation was observed.

3.4.3 Period 3 (days 191-265). Influence of thermochemical pretreatment

From day 191 on, R1 was fed with pretreated feeding, while R2 remained as control with unpretreated feeding. The pretreatment improved the biodegradability (Figure 4A) and lowered the ammonium levels (Figure 4B), but adversely, COD content slightly decreased. That explains the slightly lower OLR in R1 (2.50 ± 0.17 g COD/L d, Figure 4A) in comparison to R2 (2.79 ± 0.04 g COD/L d, Figure 5A).

Despite the lower OLR, the methane production was significantly higher ($P=1.6x10^{-17}$) in R1 (0.87 ± 0.09 g COD/L d, Figure 4A) than in R2 (0.71 ± 0.10 g COD/L d, Figure 5A). In accordance, COD removal efficiency in R1 was higher (35 ± 5% in R1 vs. 25 ± 4% in R2, Table 5), which meant an improvement of 40%. The COD removal efficiency attained in R2 was similar to the BMP of the mixture max(PM:PoM) (24%, Figure 2C). While the pretreatment allowed to lower the ammonium concentration from 3.34 N-NH_4^+/L to approximately 1 g N-NH_4^+/L in R1 (day 265, Figure 4B), the levels in R2 were much higher (average of 3.55 ± 0.32 g NH_4^+/L), resulting in free ammonia concentrations up to 600 mg N-NH_3/L. The latter values were already reported by several authors as limiting factors for the proper anaerobic digestion of wastes (Bujoczek et al., 2000; Nie et al., 2015; Rajagopal et al., 2013).

3.4.4 Period 4 (days 266- 360). Influence of HRT

In this period, the treatment capacity of the system was assessed by gradually lowering the HRT during 21 days from 28 to 20 days (thus increasing the OLR from 2.8 to 3.9 and 4.0 g COD/L d in reactors R1 and R2, respectively). The reactors were operated under these conditions during 72 days in order to achieve the steady state regime.

The methane production in R1 increased and stabilised at an average value of 1.39 ± 0.03 g COD/L d (Figure 4A), but COD removal efficiency remained constant at 37 ± 1%. Similarly, R2 showed higher methane production (1.04 ± 0.06 g COD/L d) (Table 5), but equal COD removal efficiency (27 ± 1%) than in period 3. Nitrogen levels remained low in R1 (around 1 g $N\text{-}NH_4^+$/L and 100 mg $N\text{-}NH_3$/L, Figure 4B), while relatively high values were observed in R2 (3 g $N\text{-}NH_4^+$/L and 300 mg $N\text{-}NH_3$/L, Figure 5B); however, the system did not show evidences of inhibition possibly due to the adaptation to ammonia in a long term performance (Chen et al., 2014). This indicates that a higher treatment capacity of the mixture PM:PoM was affordable in both reactors regardless of the application of the pretreatments, that is, decreasing the HRT up to 20 days is possible without compromising the removal efficiency. In this study, HRT was limited to 20 days because, according to BMP results (Figure 2), times lower than 20 days would limit the biodegradation of the mixture PM:PoM. Most studies dealing with anaerobic co-digestion of PoM with different co-substrates were performed at high HRT (between 30 and 60 days) in order to provide enough time to degrade the poultry manure (Abouelenien et al., 2014). However, the application of methods to enhance the treatment of PoM such as ammonia stripping or co-digestion with readily biodegradable wastes can derive in the reduction of the HRT, thus increasing the methane capacity production of the system.

3.5 Influence of thermochemical pretreatment coupled to ammonia stripping on anaerobic microbiome

As a complementary evaluation, a comparison between both reactors was conducted at microscopic level. A biomass sample of each reactor (R1 and R2) was taken on day 337 in order to confirm the effect of the pre-treatment on the anaerobic microbial community. Figure 6 shows the most abundant bacterial populations at phylum (A) and order (B) level of the two samples.

Firmicutes and *Bacteroidetes* represented the major phyla (Figure 6A, more than 60% of the total abundance) and *Clostridales* (Figure 6B) the most abundant order in both reactors. Therefore, the pretreatment had no clear influence in the microbiome composition, since the clear similarities in the population profile seems to be related with the substrate used, which was the same in both reactors (Regueiro et al., 2014a). However, some differences are appreciated, which reflect the susceptibility of certain micro-organisms and the resistance of others to high ammonium concentrations. *Firmicutes* and *Synergistetes* (Figure 6A) seem to be more important working at high ammonia concentrations, with *Clostridiales* and *Synergistales* as most abundant orders (Figure 6B). The shift from *Bacteroidetes* to *Firmicutes* has been previously observed (Alsouleman et al., 2016) and *Synergistetes* species have been pointed out as users of the syntrophic acetate degradation (SAO) route combined with hydrogenotrophic Archaea at high ammonium levels (Briones et al., 2014; Regueiro et al., 2014b).

Despite the Archaeal abundance was minimal in both samples (0.07% in R1 and 0.11% in R2, data not shown), clear differences were observed in the dominant methanogenic communities. *Methanobacterium*, a hydrogenotrophic Archaea, was the most abundant genus in R2 while *Methanosaeta* genus, an acetoclastic Archea, was the most abundant in R1 (data not shown). The latter indicates that bacterial acetate

oxidation followed by hydrogenotrophic methanogenesis seems to be the primary pathway for acetate consumption at high ammonium levels.

4. CONCLUSIONS

The blending based optimisation was an useful tool to determine the mixture between pig and poultry manure, 24% and 76% (volume basis), respectively, which maximised methane production maintaining the stability of the system achieving an OLR of 4 g COD/L d with a HRT of 20 days. Moreover, instead of using isolated treatments to increase degradability of poultry manure, combined application of thermochemical pretreatment coupled to ammonia stripping to the pig-poultry mixture drove towards a higher methane production and a more stable reactor environment compared to anaerobic co-digestion of unpretreated mixture. Therefore, the combination of blending optimisation methods with pretreatment technologies are feasible strategies to enhance the anaerobic treatment of solid- and nitrogen-rich substrates.

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Table Legends

Table 1. Initial set of linear restrictions considered for the Optiblender.

Table 2. Physico-chemical characterisation of pig and poultry manure and the max(PM:PoM) mixture composed of 76% of pig manure and 24% of poultry manure (n=3).

Table 3. Determination of the optimum mixture and operational conditions by using Optiblender.

Table 4. Nitrogen removal (expressed as total Kjeldahl nitrogen (TKN) concentrations after pretreatment), organic matter solubilisation (expressed as CODs/CODt ratio) and methane potential of the pretreated mixtures.

Table 5. Operational parameters of the anaerobic digesters R1 and R2 during the different operational periods.

Figure Captions

Fig.1. Layout of the thermo-chemical and stripping pretreatment unit.

Fig.2. Biomethane potential test of pig manure (**A**), poultry manure (B) and the mixture max(PM:PoM) (76% pig manure, 24% poultry manure, volume basis, **C**).

Fig. 3. Results of the simulation of the anaerobic co-digestion of poultry manure and pig manure using the Optiblender: percentage of substrates in the mixture (bar chart; : poultry manure; : pig manure) (COD basis) and resulting organic loading rate (—♦—) (A) and alkalinity ratio (—**)** and methane flow (—**)** (B).

Fig. 4. Organic loading rate (OLR, —), including the contribution from pig manure ( ), poultry manure ( ) and the pretreated feeding ( ), and methane production (CH_4, —) (A) and ammonium (—) and free ammonia (—) concentration (B) in R1.

Fig. 5. Organic loading rate (OLR, —), including the contribution from pig manure ( ) and poultry manure ( ),and methane production (CH_4, —) (A) and ammonium (—) and free ammonia (—) concentration (B) in R2.

Fig. 6. Relative abundance of the *Bacteria* communities at phyla (A) and order (B) level in reactors R1 and R2 on day 337 (only communities representing more than 1% appear in the figure).

Table 1

Linear restriction	Minimum	Maximum
OLR (g COD/L d)	0	1
HRT (d)	20	30
TKN (g N-TKN/L)	0.2	4
TS (% vol)	0	8
Alkalinity (g $CaCO_3$/L)	2	10
Na^+ (g/L)	0	3
K^+ (g/L)	0	3
Biogas quality (ppm H_2S)	0	10,000
Digestate quality* (g COD/L)	0	6

* Expressed as volatile fatty acids concentration

Table 2

Parameter	PM	PoM	max(PM:PoM)
pH	7.4 ± 0.2	9.5 ± 0.1	7.8 ± 0.1
Density	1.00	0.350	0.93
CODt (g O_2/kg)	24.3 ± 0.4	783 ± 15	93 ± 4
COD_s (g O_2/kg)	10.4 ± 0.0	154 ± 20	13.9 ± 0.4
TS (g/kg)	17.3 ± 1.5	806 ± 59	88 ± 4
VS (g/kg)	11.7 ± 1.8	490 ±61	55 ± 2
Alkalinity (g $CaCO_3$/kg)	7.6 ± 0.0	15.2 ± 0.1	8.2 ± 0.3
TKN (g N-TKN/kg)	3.3 ± 0.6	22.5 ± 1.4	4.9 ± 0.2
TAN (g N-TAN/kg)	3.1 ± 0.4	0.8 ± 0.2	2.7 ± 0.1
Lignin (g/kg)	n.m.	163	n.m
Hemicellulose (g/kg)	n.m.	17	n.m
Cellulose (g/kg)	n.m.	20	n.m
BMP (L CH_4/kg_{VS})	356 ± 18	159 ± 19	142 ± 31

CODt: total chemical oxygen demand; COD_s: soluble chemical oxygen demand; TS: total solid content; VS: volatile solid content; TKN: total Kjeldahl nitrogen; TAN: total ammonium nitrogen; BMP: biochemical methane potential; n.m.: not measured.

Table 3

Time (d)	PM (%vol)	PoM (%vol)	OLR (g COD/L d)	Active restriction Parameter	New boundary	Units
0-10	98.42	1.58	1.00	OLR	1.36	g COD/L d
10-20	94.45	5.55	1.36	OLR	1.79	g COD/L d
20-30	89.69	10.31	1.79	OLR	2.16	g COD/L d
30-40	85.55	14.45	2.16	OLR	2.43	g COD/L d
40-50	82.54	17.46	2.43	OLR	2.60	g COD/L d
50-60	80.57	19.43	2.60	OLR	2.71	g COD/L d
60-70	79.30	20.70	2.71	OLR	2.79	g COD/L d
70-80	78.47	21.53	2.79	OLR	2.84	g COD/L d
80-90	77.89	22.11	2.84	OLR	2.88	g COD/L d
90-100	77.47	22.53	2.88	OLR	2.91	g COD/L d
100-110	77.14	22.86	2.91	OLR	2.93	g COD/L d
110-120	76.88	23.12	2.93	OLR	2.95	g COD/L d
120-130	76.65	23.35	2.95	OLR	2.97	g COD/L d
130-140	76.44	23.56	2.97	OLR	2.98	g COD/L d
140-200	76.31	23.69	2.98	TS	80	g/L

Table 4

Experiment	Temperature (ºC)	pH	N-TKN* (g/kg)	Solubilised fraction (COD_s/COD_t)	BMP (%)
Non-pretreated	-	-	22.5 ± 1.4	0.20 ± 0.02	24.1 ± 4.0
E1	70	8	4.72 ± 0.09	0.38 ± 0.05	28.3 ± 1.8
E2	70	9	4.46 ± 0.04	0.38 ± 0.02	28.9 ± 2.9
E3	70	10	2.08 ± 0.08	0.39 ± 0.12	29.6 ± 3.1
E4	80	8	4.83 ± 0.05	0.40 ± 0.01	33.1 ± 2.5
E5	80	9	4.05 ± 0.08	0.39 ± 0.01	33.3 ± 5.3
E6	80	10	1.97 ± 0.08	0.40 ± 0.01	33.2 ± 11.0
E7	90	8	4.77 ± 0.14	0.41 ± 0.00	35.9 ± 5.7
E8	90	9	3.43 ± 0.03	0.42 ± 0.02	39.6 ± 0.1
E9	90	10	1.45 ± 0.07	0.43 ± 0.02	39.9 ± 8.2

*** Concentration at the end of the experiment**

Table 5

Reactor	Period (days)	HRT (d)	OLR (g COD/L d)	PM:PoM (v/v%)	CH_4 production (g COD/L d)	COD removal (%)	pH	NH_4^+ (g N-NH_4^+/L)	NH_3* (mg N-NH_3/L)
R1-Pretreated	1 (0-100)	28	1	98:2	0.49 ± 0.06	43 ± 6	7.8 ± 0.2	1.45 ± 0.1	116 ± 38
	2 (101-190)	28	1→3	98:2→76:24	0.42 → 0.82	38 → 29	7.8 ± 0.1	1.25 → 3.34	100 → 430
	3 (191-265)	28	2.50 ± 0.17	76:24	0.87 ± 0.09	35 ± 5	8.0 ± 0.1	2.42 ± 1.01	261 ± 126
	4 (266-360)	28→20	3→4	76:24	1.39 ± 0.03	37 ± 1	7.9 ± 0.1	1.13 ± 0.09	114 ± 14
R2-Control	1 (0-100)	28	1	98:2	0.50 ± 0.05	44 ± 6	7.9 ± 0.1	1.43 ± 0.15	132 ± 20
	2 (101-190)	28	1→3	98:2→76:24	0.50 → 0.89	40 → 30	7.8 ± 0.1	1.25 → 3.63	124 →334
	3 (191-265)	28	2.79 ±0.04	76:24	0.71 ± 0.10	25 ± 4	7.9 ± 0.1	3.55 ± 0.32	377 ± 100
	4 (266-360)	28→20	3→4	76:24	1.04 ± 0.06	27 ± 1	7.9 ± 0.1	3.00 ± 0.16	285 ± 39

*Calculated taking into account the NH_4^+-NH_3 equilibrium (Regueiro et al., 20

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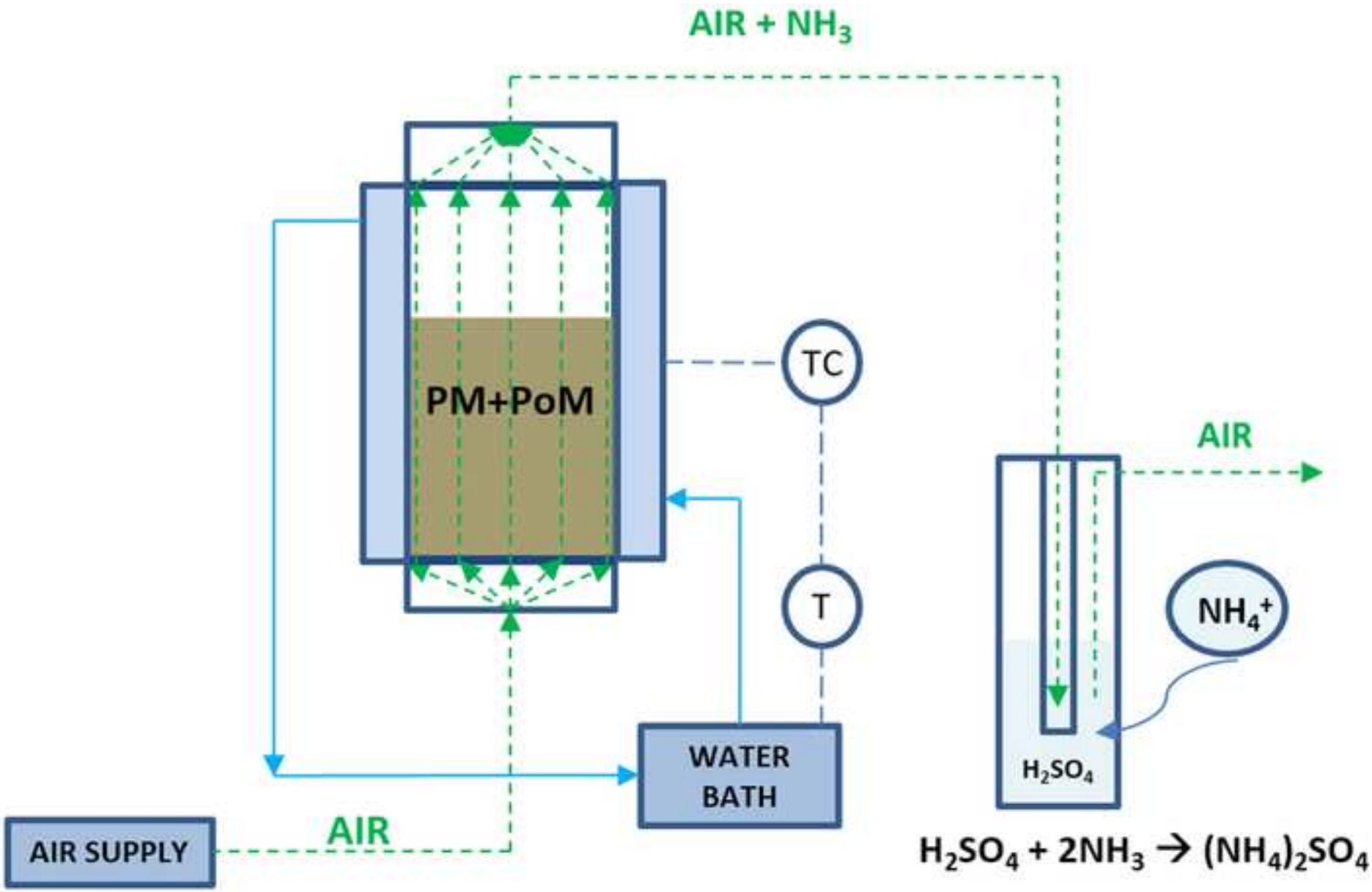

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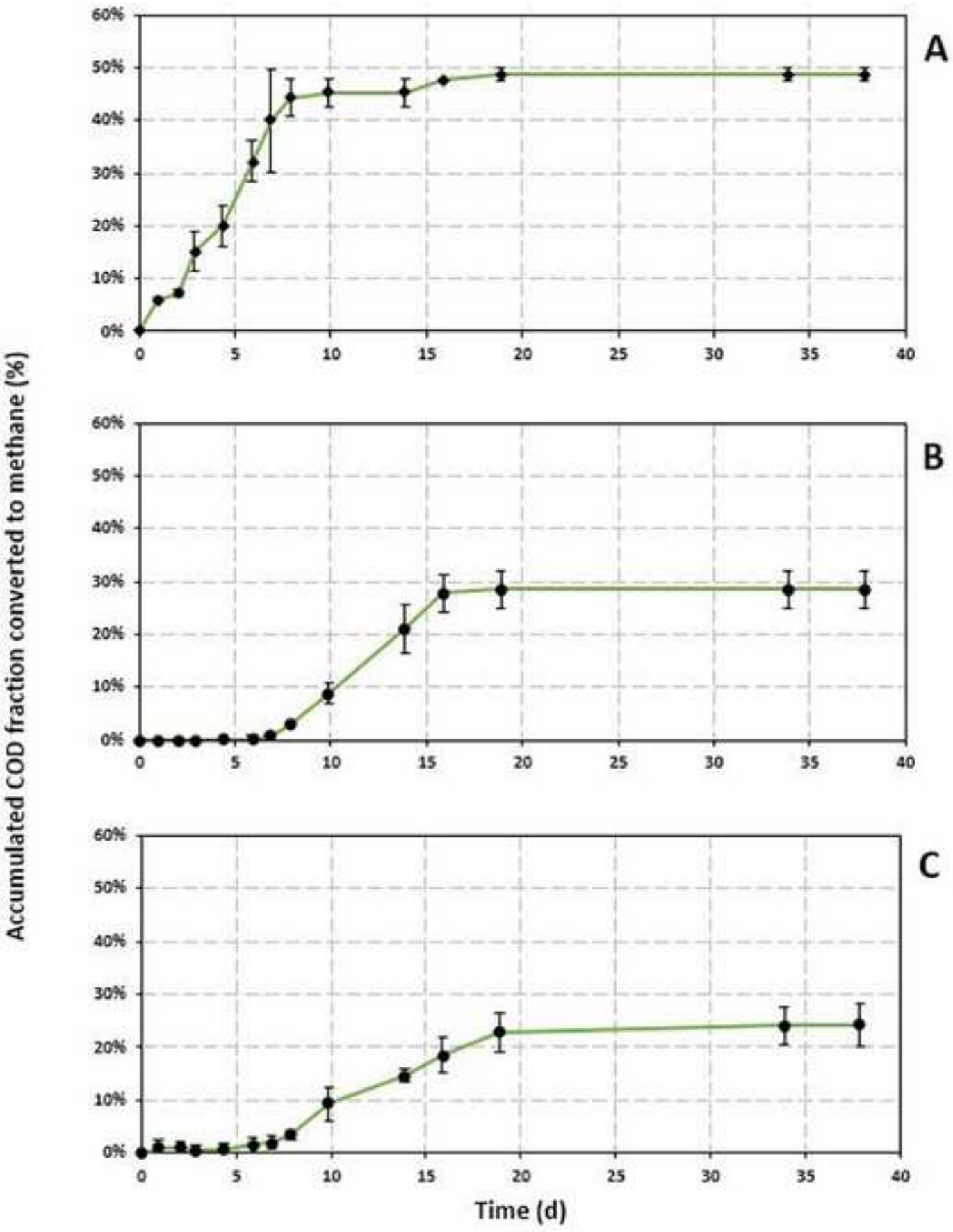

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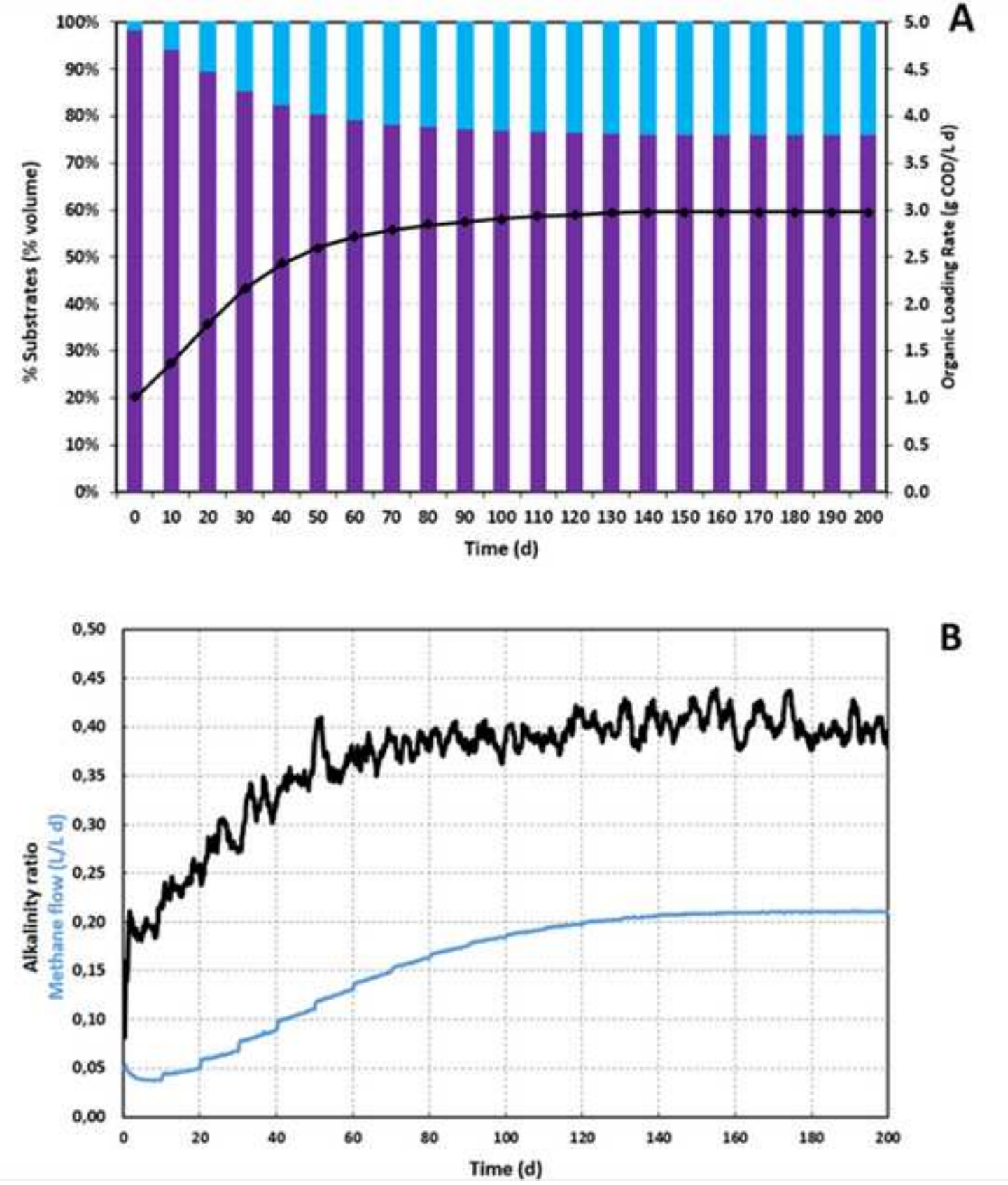

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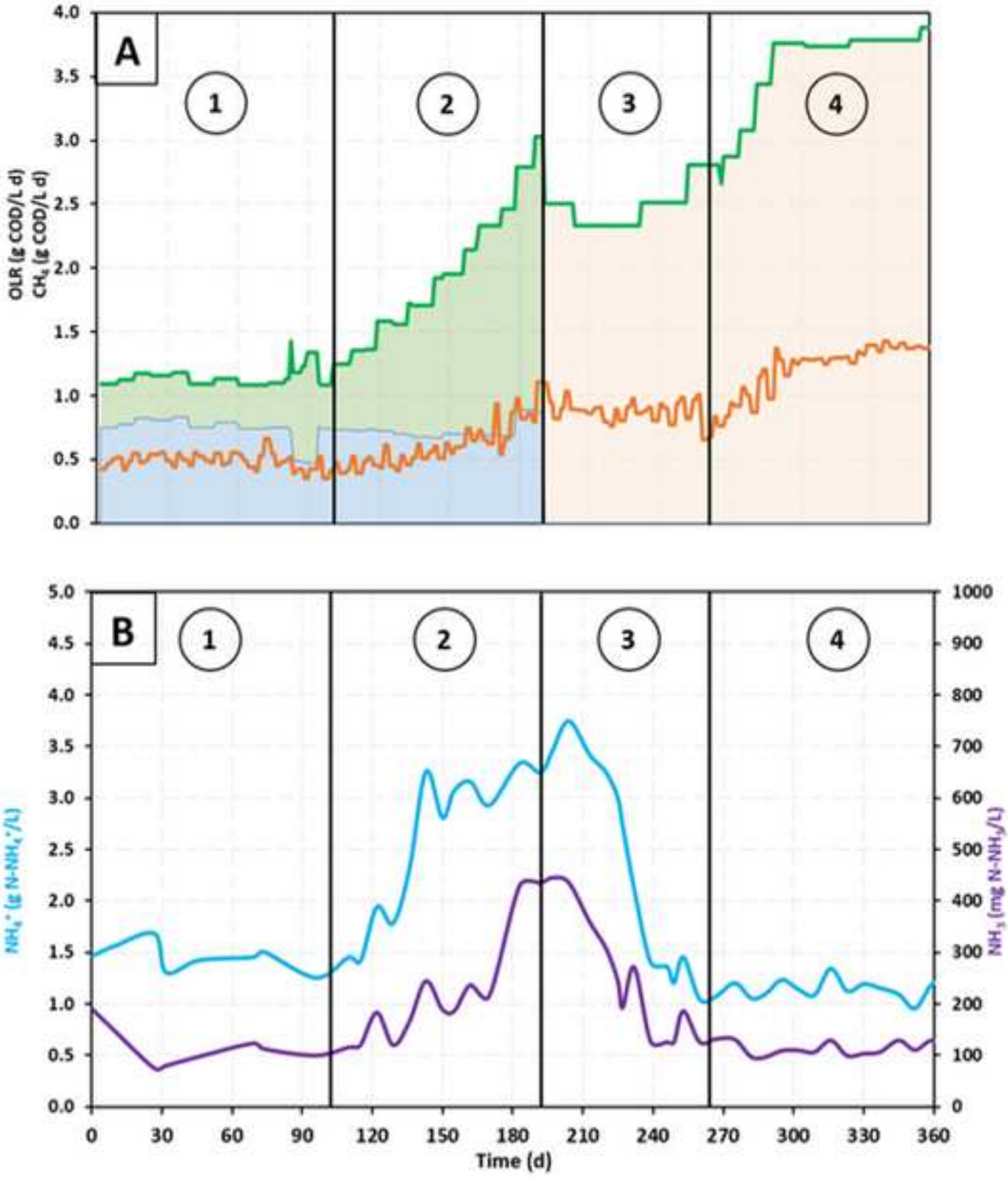

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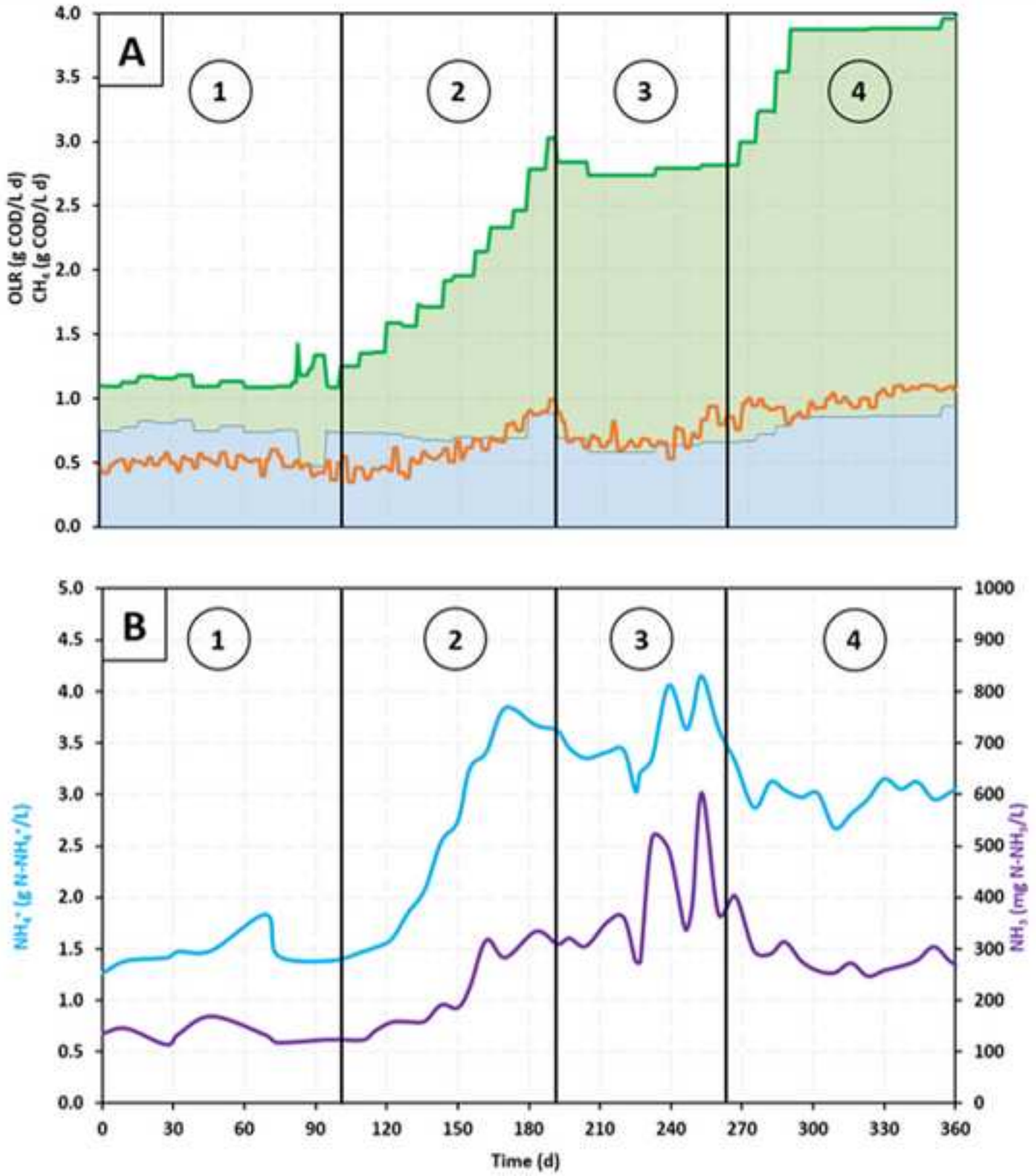

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