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Accepted Manuscript

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Bioaugmented Reactor

Ammonia Tolerant
Enriched Culture

Bioaugmentation

NH$_4^+$ NH$_4^+$ NH$_4^+$ NH$_4^+$
NH$_3$ NH$_4^+$
NH$_4^+$ NH$_4^+$ NH$_4^+$ NH$_3$
NH$_3$ NH$_4^+$ NH$_3$ NH$_4^+$

Off
On

40% Higher
CH$_4$ Production

Non-bioaugmented Reactor

Enriched ammonia-tolerant methanogenic cultures as bioaugmentation inocula in continuous biomethanation processes

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Abstract

Many ammonia-rich biomass sources, such as manures and protein-rich substrates, are potential inhibitors of the anaerobic digestion (AD) process. It was previously demonstrated that bioaugmentation of *Methanoculleus bourgensis* MS2[T] in an ammonia inhibited process in a continuous stirred tank reactor (CSTR), resulted in up to 90 % recovery of the methane production compared to the uninhibited production. However, cultivation of pure strains has practical difficulties due to the need of special growth media and sterile conditions. In contrast, acclimatized enriched cultures have minor sterility requirements. In the current study, an enriched ammonia-tolerant methanogenic culture was bioaugmented in a CSTR reactor operating under ammonia-induced, inhibited-steady-state. The results demonstrated that bioaugmentation, completely counteracted the ammonia toxicity effect. This indicates that a commercial application of bioaugmentation could improve up to 36 % the methane production, the greenhouse gas reduction efficiency and the gross revenue of ammonia inhibited full scale biogas reactors. 16S rRNA gene sequencing showed that bioaugmentation changed the microbial composition of the reactors resulting in higher bacterial and lower archaeal community diversity. The bioaugmented reactor showed a fourfold increase of the abundance of the bioaugmented methanogens compared to the control reactor. This indicates that ammonia-tolerant methanogens established well in the ammonia-inhibited reactor and dominated over the domestic methanogenic population. Finally, this study showed that the enriched culture alleviated ammonia toxicity 25 % more efficiently than the previously used pure culture.

Keywords

Nomenclature

AD	Anaerobic digestion
ANOVA	Analysis of variance
AOA	Ammonia-oxidizing archaea
CSTR	Continuous stirred tank reactor
HRT	Hydraulic retention time
OD_{600}	Optical density at 600 nm
OLR	Organic loading rate
OTU	Operational taxonomic unit
SAO	Syntrophic acetate oxidation
SAOB	Syntrophic acetate oxidizing bacterium
SRT	Solid retention time
TS	Total solid
VFA	Volatile fatty acid
VS	Volatile solid
μ_{max}	Maximum growth rate

1 Introduction

Vast amounts of ammonia-rich organic wastes are produced yearly from the agricultural and the food industrial sectors (Kovács et al., 2013). Anaerobic digestion (AD) is one of the most effective methods to treat this waste, as it provides energy (methane) and a bio-fertilizer (digestate) (Tampio et al., 2016). Moreover, some manures (e.g. pig, poultry etc.) that are often used as substrates in biogas reactors contain high amounts of urea. Ammonia is a well-known inhibitor of the AD process (Westerholm et al., 2015). It has been widely shown that free ammonia (unionised, NH_3) is the most toxic form of the total ammonia ($NH_4^+ + NH_3$) for

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the methanogenic communities mediating the AD process (Rajagopal et al., 2013). The NH_3 levels depend on the total ammonia concentration in a reactor and on the NH_4^+/NH_3 equilibrium, which is affected by the temperature and the pH (Yenigün and Demirel, 2013).

Many solutions have been proposed to solve the ammonia inhibition problem. Up to now, the two most common methods are lowering the operating temperature and diluting the reactor content with water (Kelleher et al., 2002; Nielsen and Angelidaki, 2008). Nevertheless, these methods can counteract the ammonia toxicity only to a limited extend, are uneconomical and do not provide a permanent solution (Massé et al., 2014).

Europe has currently more than 17,240 biogas plants and most of them use combined heat and power (CHP) units to generate electricity on site, with an average efficiency of 40 % (European Biogas Association, 2015; Herbes et al., 2016). At the same time, it has been found that several of the European biogas reactors are seriously affected by ammonia toxicity, leaving unexploited more than 30 % of their methane potential, while operating in an ammonia induced inhibited-steady-state (Duan et al., 2012; Fotidis et al., 2014a). This suboptimal, but apparently stable process is resulting in severe operational problems with increased CO_2 footprint. In Europe, the minimum price for the electricity produced from biogas (using residual resources as feedstock) is 0.11 € $kWh_{(e)}^{-1}$ and the total installed capacity is 8,333 $MW_{(e)}$ (European Biogas Association, 2015); thus the European biogas industry suffers significant economic losses due to the ammonia toxicity. At present, to secure an uninhibited AD process, biogas plant operators minimize or completely avoid the high ammonia containing feedstocks. Nevertheless, it is imperative to find an alternative and reliable solution for the treatment of the vast amounts of high ammonia containing ammonia-rich substrates and alleviate their ammonia inhibitory effect in continuous biomethanation processes.

It has been previously reported that aceticlastic methanogens are sensitive to ammonia

(Chen et al., 2008; Westerholm et al., 2011; Yenigün and Demirel, 2013). There is however, another metabolic pathway, the syntrophic acetate oxidation (SAO) pathway, where acetate is oxidized by syntrophic acetate oxidizing bacteria (SAOB) to hydrogen and carbon dioxide, followed by hydrogenotrophic methanogenesis (Westerholm et al., 2016). The SAO pathway is much less liable to ammonia inhibition (Moestedt et al., 2016; Westerholm et al., 2011). The use of ammonia-tolerant hydrogenotrophic methanogenic cultures could provide an alternative solution to overcome ammonia inhibition in AD process. Specifically, Costa et al. (2012) have suggested bioaugmentation as the potential method to deliver and establish these ammonia-tolerant cultures in ammonia inhibited continuous anaerobic reactors. Based on that Fotidis et al. (2014b) have successfully bioaugmented an ammonia-tolerant pure culture (*Methanoculleus bourgensis* MS2[T]) in a continuous reactor. Nevertheless, the cultivation of pure cultures to be used as bioaugmentation inocula has specific technical difficulties and is cost-expensive due to required sterile conditions and special growth media (De Roy et al., 2014). Contrary to pure cultures, ammonia acclimatized (enriched) cultures have lower requirements to sterility (Narihiro et al., 2015). Additionally, adaptation of the AD process to high ammonia loads is well established (Rajagopal et al., 2013; Tian et al., 2017). It is hypothesized that due to the acclimation process, these complex communities could be more robust and thus, better biomethanation inocula than a pure strain. Enriched ammonia-tolerant methanogenic cultures have been used before as bioaugmentation inocula in batch reactors with high ammonia levels (Wilson et al., 2013). However, these enriched cultures were never assessed as bioaugmentation inocula in continuous anaerobic reactors where the washout of the inoculated microorganisms poses a big challenge (Han et al., 2016; Sivagurunathan et al., 2015; Westerholm et al., 2016).

Based on the above, three major aims were addressed in the current study. First, to use an enriched ammonia-tolerant methanogenic culture as a bioaugmentation inoculum in a

continuous stirred tank reactor (CSTR) operating under ammonia induced inhibited-steady-state. Second, based on the lab scale results, to define the effect of bioaugmentation on the energy production, greenhouse gas (GHG) emissions and gross revenue of the biogas reactor compared to a reactor operating under ammonia induced inhibited-steady-state. Third, to compare the data extracted from this study with the results derived from a previous study, which has been conducted under the same experimental conditions but with the use of a pure culture as bioaugmentation inoculum (Fotidis et al., 2014b).

2   Materials and Methods

2.1   Ammonia-tolerant enriched culture

The enriched ammonia-tolerant culture used in the bioaugmentation process was derived from a previous experiment (Fotidis et al., 2013), where inoculum from Hashøj Biogas plant (Denmark) was acclimatized to stepwise increased ammonia levels (up to 7 g NH_4^+-N L^{-1}) in batch reactors. Before introduction to the CSTR reactor, the enriched culture was incubated at 5 g NH_4^+-N L^{-1} in batch reactors with BA medium (Zehnder et al., 1980). The pH of the batch reactors was adjusted to 7.0±0.1 using a N_2 and CO_2 gas mixture (4/1 on volume basis). Subsequently, 124.8 mL of H_2 and 31.2 mL of CO_2 were added in the headspace. All the bottles had 118 mL total and 40 mL working volumes and were incubated at 37±1ºC. The enriched culture showed hydrogenotrophic methanogenic pathway at 5 g NH_4^+-N L^{-1} as determined by radio labelled experiments using [2-^{14}C] acetate as reported before (Fotidis et al., 2013). Finally, preliminary high-throughput 16S rRNA gene sequencing analysis of the enriched culture showed that it consisted of a hydrogenotrophic methanogen (*Methanoculleus spp.*) and four primary bacteria (*Tepidimicrobium* spp., *Aminobacterium* spp., *Petrimonas* spp. and *Defluviitoga* spp.).

2.2 Inoculum and feedstock

The inoculum used in the CSTR reactors was retrieved from a full-scale mesophilic anaerobic reactor (Hashøj Biogas, Denmark) fed with pig and cattle manure (>70 %) and organic waste (>10 %). All the dairy slurry derived from Hashøj municipality (Denmark) and was the primary feedstock used in the experiment. The slurry was sieved to remove coarse materials, thoroughly mixed to ensure homogeneity throughout the experiment and kept at 4 ℃ before used in the experiment. The basic characteristics of inoculum and feedstock are depicted in Table 1.

2.3 Experimental setup

The bioaugmentation experiment was performed in two identical glass CSTR reactors (R_{Enc}: Enriched culture bioaugmentation and R_{Ctl}: abiotic augmentation) with 2.3 L total and 1.8 L working volumes. Both reactors had organic loading rate (OLR) of 1.74 g VS L^{-1} d^{-1} and solid (SRT) and hydraulic (HRT) retention times of 24 days. Ammonium chloride was used as additional ammonia source. Each reactor's setup had a feedstock tank, a feeding peristaltic pump, an effluent tank, two magnetic stirrers, a water-displacement gas meter and an electrical heating jacketed system.

The complete bioaugmentation experiment was divided to six distinct experimental periods (P-1 to P-6). Both CSTR reactors started-up (data not shown) with an ammonia level in the feedstock of 1.65 g NH_4^+-N L^{-1} until they established a steady-state, (P-1, days 1-10). Ammonia levels were then stepwise increased in the feedstock at 3 g NH_4^+-N L^{-1} (P-2, days 11-31), 4 g NH_4^+-N L^{-1} (P-3, days 32-62) and 5 g NH_4^+-N L^{-1} (P-4, days 63-96). After ammonia concentration in the feedstock increased at 5 g NH_4^+-N L^{-1} (during P-4), an ammonia induced inhibited-steady-state was established (days 87-96) for both reactors. Addition of 100 mL of the enriched culture (OD_{600}=0.21-0.23 and μ_{max}=0.024 h^{-1}, from a

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batch culture at exponential growth phase) in the R_{Enc} reactor took place twice on P-5 (days 97 and 99). At the same time, the same volume (200 mL in total) of sterile BA medium with 5 g NH_4^+-N L^{-1}, was also introduced in the reactor R_{Ctl}, to apply the same hydraulic effect as the bioaugmentation inoculum (abiotic augmentation). P-6 (days 100-142) of the experiment was defined as the period after the bioaugmentation and abiotic augmentation (i.e. the addition of sterile medium in reactor R_{Ctl} to replicate the hydraulic effect that the bioaugmentation inoculum had on the R_{Enc}) processes took place. Throughout the duration of the experiment, both reactors were operating continuously and the HRT, the OLR and the ammonia levels in the feedstock (corresponding to each period), were kept stable. The maximum organic matter concertation delivered to the reactors during the bioaugmentation and abiotic augmentation, was less than 1 % of the OLR and its effect on the methane production was considered to be statistically negligible. Finally, the total ammonia concentrations of the feedstock and the reactors, throughout the experiment, are depicted in Fig. S1 (Supplementary Material-Part A).

2.4 Analyses

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen, total ammonia and pH were determined according to APHA's Standard Methods (APHA, 2012). The pH was measured with a PHM99 LAB pH meter. The methane content in the biogas produced by the CSTR reactors was measured with GC-TCD (MGC 82-12, Mikrolab a/s, Denmark). The volatile fatty acids (VFA) were determined using gas-chromatograph (HP 5890 series II). The optical density at 600 nm (OD_{600}) was determined with a Spectronic 20D+ Spectrophotometer (Thermoscientific, Soeborg, Denmark).

2.5 DNA extraction and high-throughput 16S rRNA gene sequencing

Both archaea and bacteria high-throughput 16S rRNA gene sequencing was performed in the CSTR reactors before (day 96, P-4) and after (day 121, P-6) bioaugmentation to elucidate

any possible changes in the relative abundance of methanogenic populations. QIAamp DNA

Stool Mini Kit (Qiagen Inc., Mississauga, Canada, Cat No. 51504) was used to treat the

samples for total genomic DNA extraction as has been dictated by the manufacturer's

instructions (Dokianakis et al., 2004). Nested PCR was used for archaea, with archaea-

specific primers 20f (5'-TTCCGGTTGATCCYGCCRG-3') and 958r (5'-

YCCGGCGTTGAMTCCAATT-3') for the first amplification round and ARC-344f (5'-

ACGGGGYGCAGCAGGCGCGA-3') and ARC-519r (5'-GWATTACCGCGGCKGCTG-3')

for the second amplification round. For bacteria, PCR was performed with specific primers

341f 5'-CCTACGGGAGGCAGCAG-3' and 518r 5'-ATTACCGCGGCTGCTGG-3' for

amplification. In all PCR amplifications, Taq PCR Core Kit (QIAGEN) was used with 1 μL

template DNA and 20 pmol of each primer. The PCR conditions for the first and second

amplification were as described by Fotidis et al. (2014b). For purification and removal of the

excess primer dimers and dNTPs from the PCR products, QIAquick spin columns (QIAGEN)

were used. Subsequently, the samples were sent for barcoded libraries preparation and

sequencing on an Ion Torrent PGM apparatus with 316 chip using the Ion Sequencing 200 kit

(all Life Technologies, Inc., Paisley, United Kingdom) according to the standard protocol (Ion

XpressTM Plus gDNA and Amplicon Library Preparation, Life technologies) (Luo et al.,

2013).

2.6   Microbial community data processing

The raw sequence data were deposited at "sequence read archive" database

(http://www.ncbi.nlm.nih.gov/sra) under accession numbers SRS1510840 and SRS1511333-

1511335. Raw reads were analysed using CLC Workbench software (V.8.0.2) with Microbial

genomics module plug in. A trimming procedure was applied to low quality reads according

to default parameters provided by the software. Chimera crossover filter was also performed.

Operational taxonomic units' (OTUs) phylogenetic assignment was performed with Multiple

Sequence Comparison by Log- Expectation on trimmed (240 bp) sequences using Greengenes v13_5 database as reference (clustered at 97 %). OTU similarity cut-off was set at 97 %. The creation of new OTUs was allowed when taxonomy similarity percentage was lower than 80 % and the minimum occurrence was five reads. OTUs were aligned using MUSCLE software implemented in CLC software. Maximum Likelihood Phylogeny (tree) was performed with Neighbor Joining as construction method and with Jukes Cantor as nucleotide substitution model to estimate the evolutionary distance between sequences. Bootstrap analysis was performed with 100 replicates to test the certainty of the evolutionary relation and distance. Alpha diversity was measured based on number of OTUs, Chao 1 bias-corrected and Phylogenetic diversity. Beta Diversity represented as "principal coordinate analysis" of the 16S rRNA gene OTUs was obtained using Bray-Curtis matrix.

3   Theory/calculation

3.1   Growth rate and maximum methane production

The maximum growth rate (μ_{max}) of the enriched culture was calculated from the slope of the linear part of the graph of the batch reactors methane production (natural logarithm) versus time as has been described before (Fotidis et al., 2014a). The methane production of both R_{Enc} and R_{Ctl} continuous reactors during P-1 was considered as the maximum methane production (uninhibited) of the dairy slurry, which used to detect any changes in the performance of the two reactors throughout the experiment.

3.2   Steady-state

As steady-state was defined a quasi-steady-state (Venkiteshwaran et al., 2016), for at least a period of ten successive days with less than 10 % variation in the methane production, VFA accumulation and pH fluctuation (Fotidis et al., 2016).

3.3 Statistics

Analysis of variance (ANOVA) was used to define the statistically significant differences among the different methane productions. ANOVA analysis had a confidence interval of 95 % ($p < 0.05$, i.e., a 5 % significance level). All statistical analyses were made using the Graphpad PRISM 5.0 program (Graphpad Software, Inc., San Diego, California).

3.4 The potential economic and environmental impact of bioaugmentation

In order to define the effect of bioaugmentation on the energy production and the GHG emissions of the biogas reactor compared to a reactor operating under ammonia induced inhibited-steady-state the following assumptions were made:

1. The CO_2 equivalent was calculated as 25 t CO_2 equivalent per t CH_4 (IPCC, 2013).

2. Both reactors had zero GHG emissions.

3. The upper calorific value of methane was used (11.04 kWh m^{-3} CH_4) to evaluate the energy recovery from the bioaugmentation (Schley et al., 2010).

4. The electricity production efficiency from biogas in a typical CHP unit was determined to be 40 % (Uusitalo et al., 2016)

5. The tariff of electrical power produced from a CHP unit combusting biogas in EU is between 0.11 and 0.28 € kWh[1] (European Biogas Association, 2015).

To approximate the increase from the bioaugmentation of the annual gross revenue of a typical full-scale, manure-based biogas reactor operating under inhibited-steady-state, it was assumed that:

1. The reactor's operational size was 5,000 m^3.

2. The feedstock had the same VS content as the feedstock presented in Table 1.

3. The HRT of the reactor was 24 days.

4. The reactor was operating for 330 days per year.

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5. Bioaugmentation improved the biogas production by 36 %.

4   Results and Discussion

4.1   Reactors performance

During P-1, both reactors (R_{Enc} and R_{Ctl}) had a statistically similar ($p>0.05$) average methane production yield (Fig. 1) of 263 mL CH_4 g^{-1} VS, at steady-state. defined as a period of ten successive days with less than 10 % variation in the methane production, VFA accumulation and pH fluctuation (Fotidis et al., 2016). When ammonia levels increased in the feedstock to 3 g NH_4^+-N L^{-1} (P-2) and 4 g NH_4^+-N L^{-1} (P-3) methane production, at inhibited-steady-state, was decreased in both reactors between 30 and 43 % compared to P-1. At the beginning of P-4 both reactors briefly recovered some of their lost methane productivity, due to the acclimation of the methanogenic communities to the increased ammonia levels (Chen et al., 2008). Nevertheless, the methane production was inhibited again, and at the end of P-4 was approximately 31 % less for both reactors (at steady-state) compared to P-1. Immediately after bioaugmentation (P-6), the R_{Enc} reactor demonstrated a significant improvement in methane production rate, which led to a new uninhibited-steady-state (days 131-142). In this new steady-state, the R_{Enc} reactor was operating continuously for approximately two HRTs with approximately 40 % higher methane production rate compared to the inhibited-steady-state at the end of P-4. Surprisingly, the R_{Enc} reactor regained 97.2 % of the methane production rate it had before the introduction of the additional ammonia to the feedstock (P-1), practically completely overcoming the ammonia inhibitory effect. Contrary to R_{Enc}, the control reactor (R_{Ctl}) remained in an ammonia-induced inhibited-steady-state in P-6, slightly increasing (5 %) its methane production compared to P-4. This statistically significant ($p<0.05$), but small improvement in methane production, was due to the slow acclimation of the methanogenic populations to the high ammonia levels (Gao et al., 2015).

Finally, a direct comparison between R_{Enc} and R_{Ctl} during the final steady-state (days 131-142), shows that R_{Enc} had an average of more than 36 % higher methane production rate. Overall, the study showed, for the first time, that an enriched ammonia-tolerant methanogenic culture could be successfully used as bioaugmentation inoculum to completely counteract ammonia inhibition in a CSTR reactor. This remediation approach is alternative to conventional methods (dilution and temperature lowering), used today to alleviate ammonia toxicity in AD reactors (Nielsen and Angelidaki, 2008).

4.2 VFA accumulation and pH fluctuation

The total VFA accumulation pattern (Fig. 2) was in consistence with the biomethanation performance of the two reactors. Specifically, both reactors started with low VFA levels during P-1 and P-2. When ammonia negatively affected the reactors at the end of P-2 and during P-3, led to a maximum VFA accumulation (>2,000 mg HAc L^{-1}). At that point, both reactors had a transient methane production recovery, probably due to microbial acclimation, which initiated the reduction of the VFA levels at the end of P-3. During P-4, VFA levels were stabilized above 1,000 mg HAc L^{-1} for both reactors. After bioaugmentation (P-6), R_{Enc} reactor's VFA dropped below 1,000 mg HAc L^{-1} and were kept stable within the normal limits for continuous AD of dairy slurry until the end of the experiment (Fang et al., 2011). Contrary to the R_{Enc}, VFA accumulation (Fig. 2) in R_{Ctl} verified that the reactor was at inhibited-steady-state (days 121-142) at 5 g NH$_4^+$-N L^{-1}, with VFA levels above the established threshold of 1,500 mg HAc L^{-1} while having a stable daily methane production. Despite the VFA accumulation, pH in both reactors was only slightly reduced throughout the experiment (from 8.15 to 7.7), due to the strong buffering capacity attributed to high ammonia content (Liu et al., 2008).

4.3   Microbial population richness, diversity and dynamicity

In this study, next generation sequencing of 16S rRNA gene hypervariable V3 region was chosen to evaluate microorganisms' richness, to assign microbial taxa and to calculate relative abundances. Microbial richness (number of operational taxonomic units-OTUs, at 97 % similarity level) of R_{Enc} and R_{Ctl} were analysed across two different time points: before (P-4) and after (P-6) bioaugmentation/abiotic augmentation. Quality results of sequencing and complete taxonomic assignment of microbial OTUs are reported in Fig. S2, Table S1 and Data set 1 (Supplementary Material-Part A and Part B).

Alpha and beta diversity indices demonstrated the high dynamicity and diversity in microbial communities most probably due to ammonia toxicity (Fig. 3 and Fig. S3, Supplementary Material-Part A), highlighting opposite trends in archaeal and bacterial communities. Specifically, archaea diversity, after the bioaugmentation (P-6), greatly decreased resulting in a more specialized community. The archaeal community, which was responsible for the biomethanation process, was drastically narrowed its diversity to species more robust to ammonia toxicity. Specifically, a huge reduction in R_{Enc} diversity occurred in P-6 (Fig. 3a), indicating that the bioaugmentation inoculum addition most probably escalated the reduction in archaea diversity.

Contrary to archaea, bacteria samples taken before the bioaugmentation/abiotic augmentation clustered closely, while samples taken in P-6 diverged greatly with a high increase in the diversity, especially in R_{Enc} (Fig. 3b). These findings strongly suggest that microbial acclimation to ammonia, enhanced the number of bacterial species present in the reactors, with an additional increase in R_{Enc} microbial diversity due to bioaugmentation. A possible explanation is that the bioaugmented hydrogenotrophic methanogens decreased the hydrogen partial pressure, thermodynamically allowing the SAO pathway to evolve (Hattori, 2008) and more SAOB species to thrive. Moreover, the increase in bacterial diversity supports

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the assumption that bacteria are tolerant to ammonia toxicity, which mainly is affecting the methanogenic archaea.

4.4 Taxonomy composition and microbial variability following the bioaugmentation effect

A general overview of the microbial community structure showed that *Euryarchaeota* (96 % relative abundance) and *Crenarchaeota* (1 % relative abundance) were the most abundant archaeal phyla. *Firmicutes* (57 % relative abundance)*, Bacteroidetes* (27 % relative abundance) and WWE1 (4 % relative abundance) were by far the dominant phyla of bacteria domain.

The archaeal community was dominated by *Methanosarcina* genus with 94 % relative abundance on average in the different samples. A decreased presence of *Methanosarcina* (from 95 % to 88 % of relative abundance) was evident when comparing P-4 and P-6 in R_{Enc}, while its abundance was quite stable in R_{Ctl} (slightly increase from 96 % to 98 % in P-6). This is a proof that bioaugmentation has rapidly changed the microbial composition in the bioaugmentation reactor; something that the natural acclimation process cannot achieve (if at all) in such short timeframe.

Methanoculleus genus decreased in both reactors after bioaugmentation/abiotic augmentation. Specifically, a drastic decrease in *Methanoculleus* genus abundance was observed (from ~1 % to ~0.1 %) in R_{Ctl}, while the decrease was weaker in R_{Enc} (from ~1 % to ~0.6 %). Analysis showed no difference in *Methanoculleus* genus abundance between R_{Ctl} and R_{Enc} before inocula addition, however, the same comparison after bioaugmentation showed a fourfold increase in abundance of *Methanoculleus* spp. in R_{Enc}. Results obtained at OTUs level indicated the presence of different species of *Methanoculleus* genus with opposite behaviours. It was demonstrated the occurrence of a *Methanoculleus* spp. resident and

abundant in R_{Ctl} and R_{Enc}, before the inocula addition that strongly reduced their abundance after bioaugmentation/abiotic augmentation (from 0.7 % to 0.06 %, on average). On the contrary, *M. bourgensis* increased threefold (from 0.07 % to 0.2 %) its relative abundance only in R_{Enc}, indicating the bioaugmentation positive effect. Moreover, this trend is even more evident comparing R_{Ctl} and R_{Enc} after bioaugmentation/abiotic augmentation where the relative abundance of *M. bourgensis* increased more than 16-fold in R_{Enc} (Fig. 4a). It seems that the microbial changes evolved in the R_{Enc}, were part of the "microbiological domino effect", which has been identified as the main mechanism of a successful bioaugmentation process in anaerobic systems (Fotidis et al., 2014b; Zhang et al., 2015). Since methanogens are known to be crucial players (define the overall process rates) of the SAO pathway (Wang et al., 2015), it seems that in this study methanogens were the bioaugmentation "process steering microbes". As "process steering microbes" are defined the non-dominant microbiological groups that, under specific environmental conditions, can dictate the activity of a microbiological system. Even though, methanogens are non-hub species (i.e. species that are not directly associated with many other species) (Faust and Raes, 2012), it seems that bioaugmentation could give them the pivotal role in determining the overall microbial consortium.

Another interesting finding was the presence of three OTUs of *Candidatus nitrososphaera* genus increasing in abundance of 16-fold in R_{Enc} after bioaugmentation (from 0.2 % to 2.3 %). This genus belongs to phylum *Crenarchaeota*, well known as ammonia-oxidizing archaea (AOA). Moreover, the same trend of increased abundance (27-fold) was found in two OTUs classified only at phylum level as *Crenarchaeota* (from 0.2 % to 1.0 % and from 0.03 % to 0.5 %). It was demonstrated by Tourna et al. (2011) that the AOA *Nitrososphaera viennensis* is able to grow at increased ammonia levels with considerably higher growth rates when was grown in co-culture with bacteria, possibly related to *Hyphomicrobium* spp. and

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Mesorhizobium spp.. It can be postulated that the presence of the bacteria in R_{Enc} (after bioaugmentation), enhances the activity and consequently the abundance of *C. nitrososphaera.*

The bacterial community showed higher diversity when compared with archaeal community, although it was possible to obtain taxonomic assignment at genus level only for 20 to 30 % of relatively abundant microbes. This lack in 16S rRNA databases is because microorganisms are not yet isolated. Overall, six genera were found to be the most abundant (>1 % of relative abundance, Fig. 4b): *Clostridium* spp., *Caldicoprobacter* spp., *Alkaliphilus* spp.*, Candidatus, Cloacamonas*, *Sedimentibacter* spp. and *Syntrophomonas* spp.. All of these genera have members that are known to mediate different processes of the overall AD process (e.g. VFA fermenters, amino acid fermenters, or acetate oxidising bacteria) (Schnürer et al., 1996). It seems that, in this complex anaerobic environment, bacteria were more ammonia-tolerant compared to any methanogenic archaea. This would indicate that, at high ammonia concentrations, in the syntrophic relationship between hydrogenotrophic methanogens and SAOB, the methanogens are the most sensitive partners. The increased abundance of *Caldicoprobacter* spp. has been related before to high ammonia levels (≥ 5 g NH_4^+-N L^{-1}) in batch anaerobic reactors (Poirier et al., 2016). It seems that *Caldicoprobacter* spp., a sugar fermenter (Zamanzadeh et al., 2016), becomes substrate competitive to the other sugar fermenting bacteria, due to its increased tolerance to ammonia toxicity.

Finally, it must be mentioned that the microbiological analysis was performed before (day 96, P-4) and one HRT after (day 121, P-6) bioaugmentation, in order to depict the fast changes in the microbial dynamicity, coupling the increased methane production of the reactors that resulted from bioaugmentation. Therefore, methanogenic communities occurred in both reactors later in the experiment (until day 142) were not analysed. Nevertheless, it would be interesting for the future research to assess the dynamicity of the microbiome of the

bioaugmented anaerobic systems for more HRTs.

4.5 The significance of bioaugmentation for the biogas industry

Based on the lab scale experiments, it was estimated that bioaugmentation will improve up to 36 % the energy production, the GHG reduction efficiency and the gross revenue for every ton VS of substrate treated in an ammonia inhibited biogas reactor (Table 2). This significant overall improvement would take place without any changes in the infrastructure or the operational parameters (HRT, temperature, OLR, ammonia-rich substrate etc.) of the biogas reactor. However, it was not in the scope of the current study to assess meticulously the environmental and economic effects of bioaugmentation. To evaluate all the technical, industrial and economic parameters involved, full-scale experimental assessment must be performed. With this in mind, today manure-based biogas plants are in need of finding more types of biomass to use as co-substrates to increase their methane production potential. However, as it was aforementioned, the biogas industry has no established process to efficiently digest the ammonia-rich substrates that are available in vast amounts. If bioaugmentation is used in the industrial level, will allow new ammonia-rich substrates to be introduced in the reactors, without the corresponding drawbacks from the ammonia toxicity effect. This means, that a typical European full-scale, manure based biogas reactor (with 5,000 m^3 working volume), could have an increase in the annual gross revenue between 100,000 to 240,000 € due to bioaugmentation.

4.6 Bioaugmentation efficiency of enriched versus pure culture

A previous study, performed under the same experimental conditions using a pure methanogenic strain (*M. bourgensis* MS2[T]) as bioaugmentation inoculum, demonstrated a ~90.3 % recovery of the initial uninhibited of methane production (Fotidis et al., 2014b). The comparison between the two studies indicates that the enriched culture alleviated ammonia

toxicity more efficiently (by ~25 %) than the pure culture. Additionally, De Roy et al. (2014) stated that the performance of pure cultures could change when they are co-cultivated with complex microbial communities. This behaviour could have significant implications during the bioaugmentation (e.g. unfavourable physicochemical conditions, washout of the bioaugmented culture etc.), which could lead to process failure (Westerholm et al., 2012). These findings strengthen the hypothesis that ammonia-tolerant enriched cultures could potentially be the proper bioaugmentation inocula to alleviate ammonia toxicity in full-scale biogas reactors.

Finally, except of pure strains and enriched cultures, synthetic microbial communities (i.e. co-cultures of two or more specific microbial populations in a controlled environment) (Pandhal and Noirel, 2014) are currently attracting the interest of scientists for engineered inocula that solve specific biotechnological challenges (Großkopf and Soyer, 2014). Up to our knowledge, ammonia-tolerant, synthetic microbial communities have never been tested as bioaugmentation inocula in continuous anaerobic reactors, which could be considered as a viable solution in future applications.

5 Conclusions

Cultivation of pure strains of methanogens that can be used as bioaugmentation inocula in ammonia-inhibited anaerobic reactors is connected to technical difficulties compared to enriched cultures. This manuscript presents for the first-time the application of enriched cultures for remediation in ammonia inhibited continuous reactors. This novel approach demonstrated a 100 % recovery of the inhibited AD process after bioaugmentation with an ammonia-tolerant methanogenic enriched culture with potential economic and environmental benefits. Since enriched culture improved methane production more than 40 %, it was 25 %

more effective biomethanation inoculum compared to a pure strain (M. bourgensis MS2T).

Finally, the addition of the enriched culture, triggered a shift of the microbial composition,

resulting to lower archaeal community diversity, composed by species more tolerant to

ammonia.

Acknowledgments

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Figure Captions

Fig. 1. Methane production yield of the CSTR reactors. Error bars denote standard deviation from the mean of triplicate measurements of the biogas methane content (n= 3).

Fig. 2. Total VFA accumulation (expressed in acetate equivalent) and pH fluctuation in the CSTR reactors. Error bars denote standard deviation from the mean of triplicate measurements of the VFA (n = 3).

Fig. 3. Principal coordinate analysis describes archaeal a) and bacterial b) communities richness and variability indicated before (Pre) and after (Post) bioaugmentation/abiotic augmentation in R_{Enc} and R_{Clt}. Only the first two principal components are shown.

Fig. 4. Relative abundance identified for the most interesting a) archaea and b) bacteria are represented as a heat map (left part), to evidence folds' change (right part) and indicated before (Pre) and after (Post) bioaugmentation/abiotic augmentation in R_{Enc} and R_{Clt}.

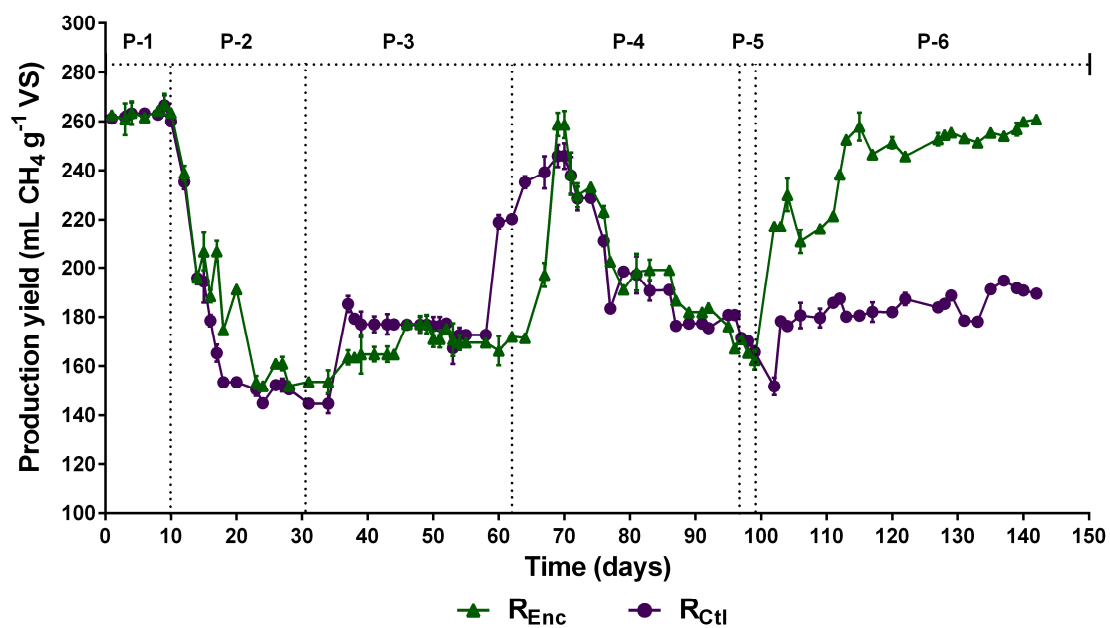**Table 1.** Characteristics of the inoculum and the feedstock used in the CSTR reactors (n=3, SD)

Parameter	Inoculum	Feedstock
	Value ± SD	Value ± SD
TS [a] ($g \cdot L^{-1}$)	29.9±0.2	56.1±0.1
VS [b] ($g \cdot L^{-1}$)	18.5±0.1	41.7±0.2
Total Kjeldahl nitrogen ($g\ N\ L^{-1}$)	4.0±0.2	2.7±0.1
Total ammonia ($g\ NH_4^+\text{-}N \cdot L^{-1}$)	3.2±0.2	1.7±0.1
pH	7.9	7.2
Total VFA [c] ($g\ L^{-1}$)	2.2±0.3	11.0±0.9

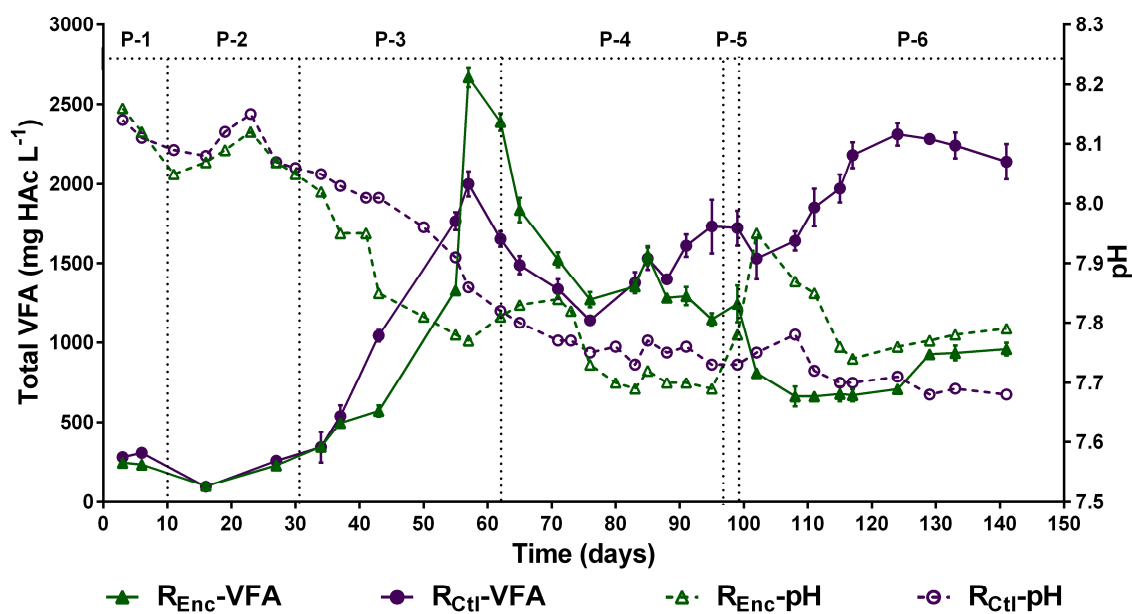[a] Total solids, [b] Volatile solids, [c] Volatile fatty acids

Table 2. Preliminary approximation of the impact of bioaugmentation on the energy production, the GHG reduction efficiency and the gross profit of manure based biogas reactors [a] (n=3, SD)
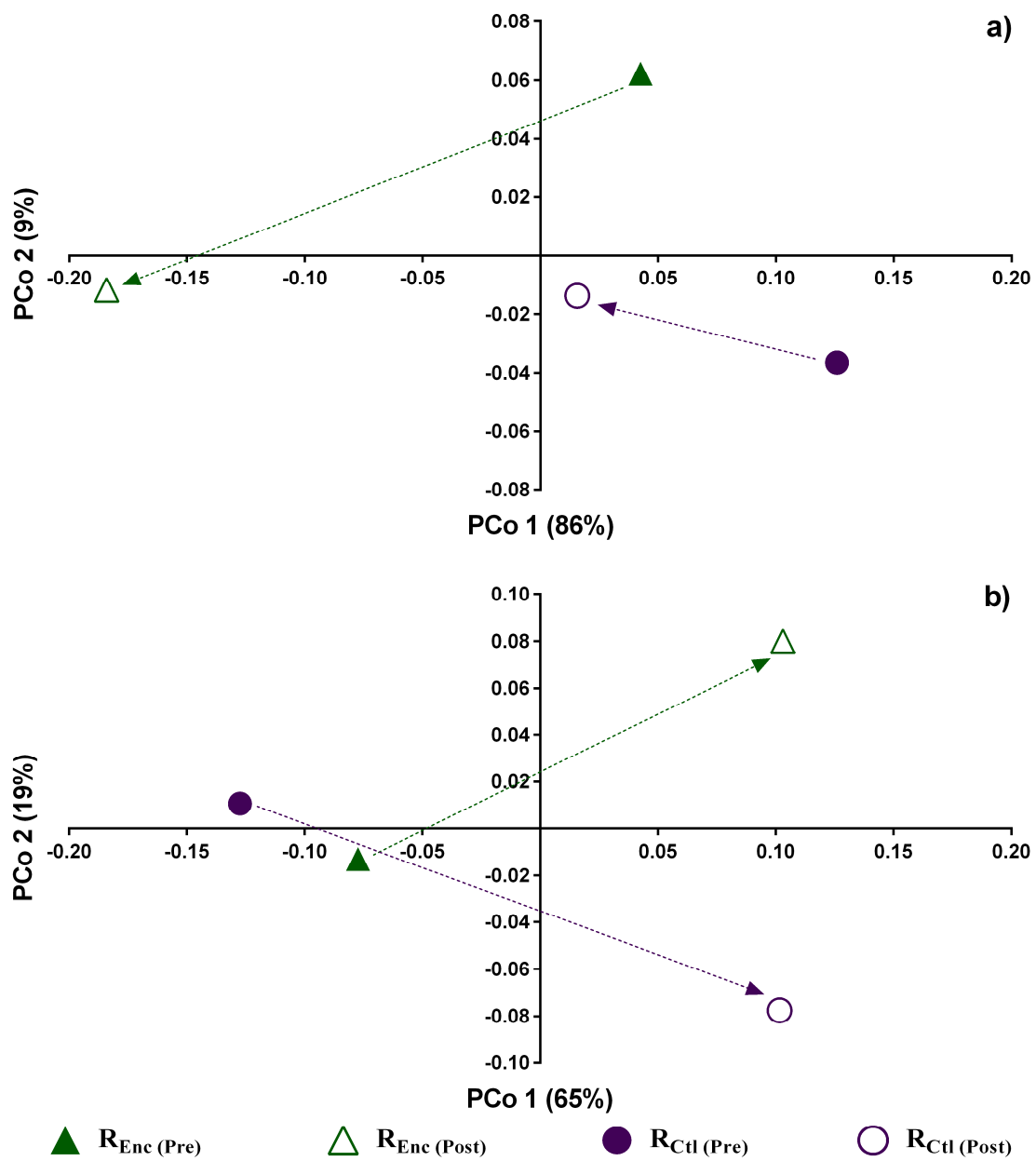
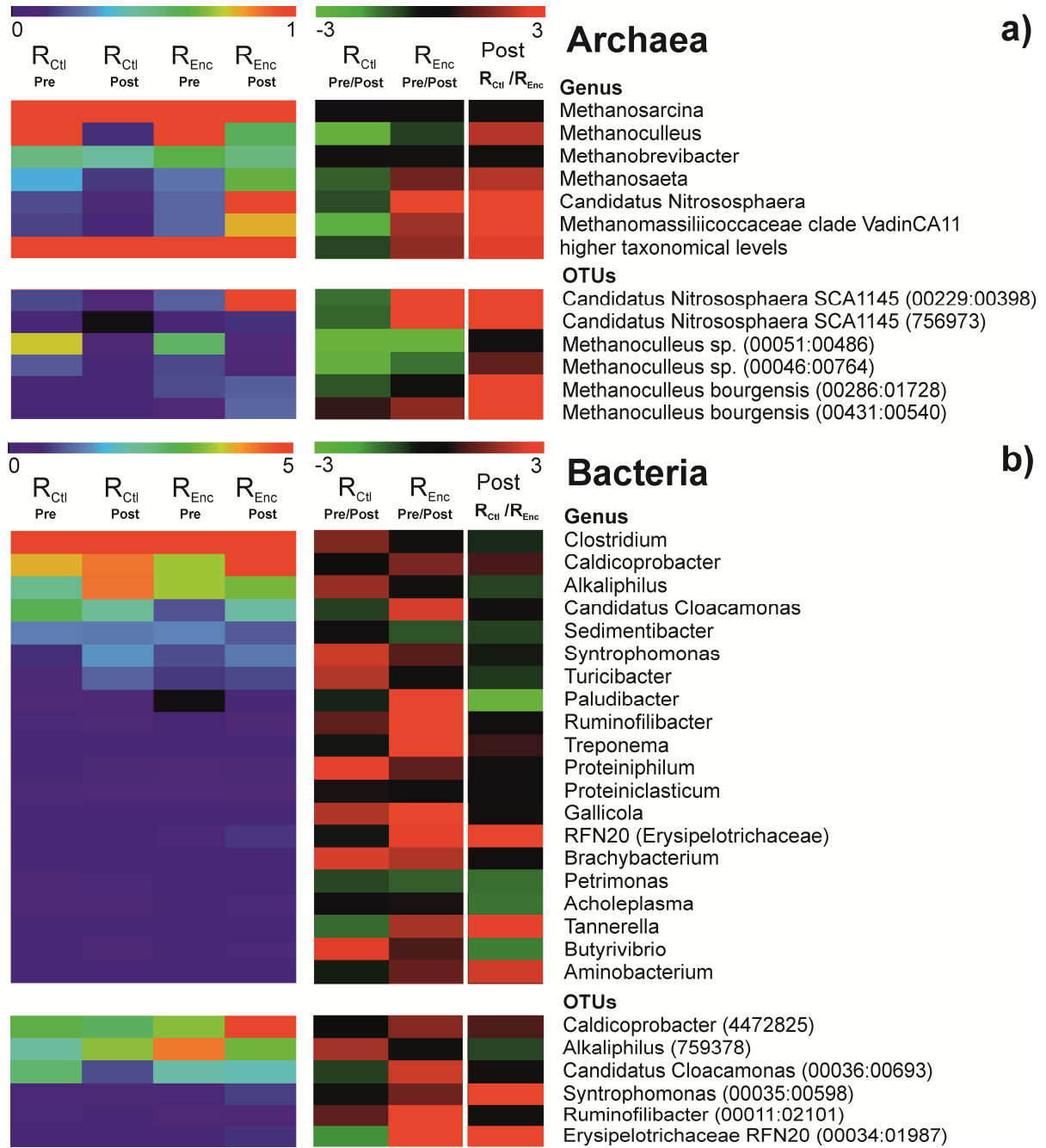|	Bioaugmentation	Without bioaugmentation	Difference
Electrical energy ($kWh_{(e)}$ t^{-1} VS)	1122.9±17.8	824.9±23.6	298±17.1
CO_2 Equivalent (t CO_2 Eq. t^{-1} VS)	4.5±0.1	3.3±0.1	1.2±0.1
Minimum gross revenue (€ t^{-1} VS)	134.7±2.1	99.0±2.8	35.7±2.0
Maximum gross revenue (€ t^{-1} VS)	314.4±5.0	231.0±6.6	83.4±4.8

[a] Overall it was assumed that: 1) the reactor size was 5,000 m^3, 2) the HRT was 24 days, 3) the reactor was operating for 330 days per year, 4) the bioaugmentation improved the production by 36 %, 5) the CO_2 equivalent was 25 t CO_2 equivalent t^{-1} CH_4, 6) the upper calorific value of methane was 11.04 kWh m^{-3} CH_4, 7) the electricity production efficiency was 40 %, 8) the tariff of electrical power was 0.11-0.28 € kWh^{-1} and 9) both reactors had zero GHG emissions.

a)

PCo 2 (9%)

PCo 1 (86%)

b)

PCo 2 (19%)

PCo 1 (65%)

▲ R$_{Enc (Pre)}$ △ R$_{Enc (Post)}$ ● R$_{Ctl (Pre)}$ ○ R$_{Ctl (Post)}$

Highlights

- An enriched culture successfully remediated an ammonia inhibited continuous reactor

- 36 % improvement on energy production, on GHG reduction and on revenue was achieved

- An immediate recovery of the ammonia inhibited biomethanation process was achieved

- An enriched culture was a better bioaugmentation inoculum compared to a pure strain

- Bioaugmentation was performed without excluding the ammonia-rich feedstock