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Comparative evaluation of biogas production and process stability in conventional and bioelectrochemical (BES)-assisted anaerobic digestion systems

Valutazione comparativa della produzione di biogas e della stabilità del processo in sistemi di digestione anaerobica convenzionali e assistiti da sistemi bioelettrochimici (BES)

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Abstract

Anaerobic digestion (AD) is a widely established biological process for the stabilization of organic waste and the recovery of renewable energy in the form of biogas. In wastewater treatment plants, anaerobic digestion of waste activated sludge is particularly relevant because it allows part of the organic matter contained in sludge to be converted into methane while simultaneously reducing the amount of residual material requiring further treatment. However, the digestion of sewage sludge is frequently constrained by the limited biodegradability of the substrate, the complex interactions among hydrolytic, fermentative, syntrophic and methanogenic microorganisms, and the sensitivity of the microbial community to changes in substrate characteristics and operating conditions. In recent years, bioelectrochemical systems (BESs) have been investigated as an approach to intensify anaerobic digestion by modifying electron-transfer processes and microbial interactions within the digester. The integration of electrodes into an anaerobic digestion system may provide additional pathways for electron exchange and may influence the conversion of intermediate metabolites towards methane. However, the actual contribution of bioelectrochemical stimulation depends on several interacting factors, including electrode configuration, applied potential, microbial community, substrate characteristics and reactor operating conditions.

The aim of this experimental thesis was to comparatively evaluate the performance and process stability of conventional anaerobic digestion and bioelectrochemically assisted anaerobic digestion of municipal sewage sludge. In particular, the study investigated whether the integration of a BES could improve biogas and methane production and modify the response of the anaerobic process to a substrate-induced perturbation. Two laboratory-scale reactors with a working volume of 10 L were operated in parallel under comparable feeding conditions. One reactor was operated as a conventional anaerobic digestion system, hereafter referred to as AD, whereas the second reactor was equipped with a bioelectrochemical system and is referred to as AD-BES. The BES consisted of two cylindrical stainless-steel mesh electrodes and two carbon-brush electrodes, together with an Ag/AgCl reference electrode. A nominal cell potential of 0.8 V was imposed after the first sludge batch and maintained during the subsequent operation. The carbon-brush electrodes were selected because of their three-dimensional structure and high potential surface area for microbial attachment, whereas stainless-steel mesh was selected for the cathodes because of its conductivity, mechanical resistance, corrosion resistance and open structure.

The reactors were fed with waste activated sludge collected after mechanical thickening from a municipal wastewater treatment plant in Pavia, Italy. Because the sludge could not be stored in sufficient quantities for the entire experimental period, ten different sludge batches were collected at different times. This aspect is important for the interpretation of the results because the composition and physicochemical characteristics of the incoming sludge varied throughout the experiment. Each batch was maintained in the reactors for a variable period according to substrate characteristics, while the reactors were continuously operated according to the same feeding strategy. The working volume was 10 L and each reactor received 0.76 L of sludge three times per week, corresponding to a hydraulic retention time of approximately 30.7 days. Biogas production was measured by a water-displacement system, whereas temperature was monitored before feeding and physicochemical parameters were periodically determined in the influent and digested sludge.

The experimental characterization included several complementary parameters. Biogas production was expressed as Biogas Production Rate (BPR), calculated by normalizing the volume of biogas produced during each sludge period to both the duration of the period and the reactor working volume. Biogas composition was analysed by gas chromatography through collaborating laboratories, with particular attention to methane and carbon dioxide. Organic matter conversion was evaluated through Chemical Oxygen Demand (COD) and Soluble Chemical Oxygen Demand (SCOD), while Total Nitrogen (TN), pH, electrical conductivity and temperature were monitored to characterize the physicochemical environment of the reactors. Selected sludge samples were additionally analysed for volatile fatty acids (VFAs). Finally, the bacterial and archaeal communities were characterized by high-throughput sequencing of the bacterial 16S rRNA gene, allowing changes in the relative abundance of microbial groups to be evaluated before and after BES activation. Because each reactor configuration was represented by a single reactor, no inferential statistical analyses were performed and the results were interpreted through descriptive comparisons and temporal trends.

The first and most relevant result concerned biogas productivity. During the first sludge batch, when the BES was not activated, the two reactors showed very similar BPR values, approximately 0.03 m^3 biogas m^{-3} reactor d^{-1}. This initial similarity provided a useful reference for the subsequent comparison. From the second sludge batch onwards, after activation of the BES at a nominal cell potential of 0.8 V, the AD-BES reactor produced more biogas than the conventional AD reactor in every sludge period. The difference varied according to the sludge batch, indicating that the effect of the BES was influenced by the characteristics of the substrate. The largest relative difference was observed during Sludge 7, when AD-BES reached approximately 14.5 times the BPR of AD. This extreme ratio was partly determined by a pronounced decrease in the performance of the conventional reactor during that period. Considering all sludge batches after BES activation, the average BPR was approximately 1.14 m^3 biogas m^{-3} reactor d^{-1} in AD-BES and 0.46 m^3 biogas m^{-3} reactor d^{-1} in AD, corresponding to an approximately 2.5-fold higher average productivity in the bioelectrochemically assisted configuration.

The higher biogas production in AD-BES represents the principal experimental finding of the study. Nevertheless, it should not be interpreted as direct evidence that the imposed voltage alone increased methanogenic activity. The AD-BES reactor differed from the conventional reactor in both the applied electrical potential and the presence of additional electrode surfaces. Therefore, the experiment demonstrates an improvement associated with the integrated BES configuration rather than allowing the individual contribution of electrical stimulation and electrode surfaces to be separated. This distinction is important because electrode surfaces can provide sites for microbial attachment and may modify the local redox environment independently of the electrical current itself. Consequently, the observed increase in productivity should be described as an effect associated with BES integration rather than as proof of a specific electrochemical mechanism.

The analysis of biogas composition provided a more complex picture. Methane and carbon dioxide concentrations varied among sludge batches and between reactor configurations, demonstrating that the quality of the produced gas was strongly influenced by the substrate used. Available composition data were limited to sludge batches 4–7 because gas composition analyses were performed through external collaborating laboratories and were not available for all ten batches.

Within the available periods, AD-BES generally showed higher volumetric methane production because its BPR was higher, but it did not consistently produce a methane fraction higher than the conventional reactor. Therefore, the principal benefit of the BES configuration was an increase in the quantity of biogas and methane produced rather than a demonstrated and systematic improvement in methane concentration. This distinction is relevant because methane concentration and methane volumetric productivity describe different aspects of biogas performance.

The available gas-composition data were also used to estimate the energy equivalent of methane production. For the four sludge batches for which both BPR and biogas composition data were available, the calculated average energy recovery was approximately 5.2 kWh m^{-3} reactor d^{-1} for AD-BES compared with approximately 0.9 kWh m^{-3} reactor d^{-1} for AD. This difference reflects the substantially higher volumetric methane production observed in the bioelectrochemically assisted reactor during those periods. However, this energy-equivalent calculation represents the theoretical energy content of the methane and should not be confused with net electrical energy efficiency of the BES. A complete energetic assessment would require consideration of the electrical energy supplied to the system, together with the energy associated with mixing, heating and other operational requirements. The present results therefore support an increase in methane energy recovery but do not constitute a complete techno-economic or net-energy assessment.

The comparison of COD and SCOD removal provided an important insight into the mechanism potentially underlying the higher gas productivity. Contrary to the BPR results, the AD reactor generally exhibited higher removal of bulk organic matter. SCOD removal was also higher in AD, with average values of approximately 89.22% compared with 80.23% in AD-BES. Therefore, the greater methane production in AD-BES was not associated with a generalized increase in organic matter degradation. In some of the final sludge batches, this contrast was particularly evident: AD-BES showed decreasing COD removal while its BPR increased, whereas AD increased COD removal without a proportional increase in biogas production. These observations indicate that COD removal alone did not determine the observed gas productivity and suggest that the two systems may have differed in the metabolic fate of the organic fraction available for conversion.

Based on the combined COD, SCOD and biogas results, the most plausible interpretation is that BES integration may have influenced the conversion of fermentation intermediates downstream of hydrolysis rather than accelerating the initial degradation of bulk organic matter. In conventional anaerobic digestion, complex organic matter is progressively hydrolysed and converted through acidogenesis and acetogenesis into intermediates that are subsequently consumed during methanogenesis. The higher biogas productivity observed in AD-BES despite lower COD and SCOD removal is therefore compatible with an increased conversion efficiency of some already-solubilized intermediates towards gaseous products. However, the available measurements do not provide a complete carbon balance and cannot quantitatively establish this mechanism. The result should therefore be regarded as a mechanistic hypothesis supported by the observed combination of datasets rather than as a demonstrated pathway.

VFA measurements provided additional, although temporally limited, evidence for this interpretation. VFA analyses were available only for Sludge 5 and Sludge 6. During Sludge 5, AD-BES contained acetate, propionate, isobutyrate, butyrate, isovalerate and valerate, resulting in a considerably broader VFA profile and a total measured VFA concentration of approximately 0.1 g/L as COD. In contrast, AD showed mainly acetate and propionate and a total concentration of approximately 0.01 g/L as COD. During Sludge 6, the AD-BES VFA profile was reduced mainly to acetate, while AD contained acetate and propionate. Importantly, the higher accumulation and diversity of VFAs observed in AD-BES during Sludge 5 did not coincide with a collapse in biogas production. Instead, BPR continued to increase. This indicates that no evident loss of methanogenic performance was associated with the measured VFA accumulation under those conditions. However, because measurements were available only for two sludge batches, the relationship between VFA accumulation and methane production cannot be generalized to the entire experimental period.

Total nitrogen measurements indicated substantial variability in the incoming sludge, with concentrations ranging from 1.38 to 2.81 g/L and an overall mean of 2.02 ± 0.42 g/L. After digestion, average TN concentrations were 1.75 ± 0.32 g/L in AD-BES and 1.86 ± 0.29 g/L in AD. Although paired inlet–outlet values showed an average decrease of approximately 12% in AD-BES and 6% in AD, these changes should not be interpreted as complete nitrogen removal. Anaerobic digestion primarily promotes the hydrolysis and mineralization of organic nitrogen and its redistribution among different nitrogen pools. The TN results therefore provide evidence that nitrogen concentrations varied with sludge characteristics but do not indicate that nitrogen removal was a major determinant of the different reactor performances.

The physicochemical parameters demonstrated that both reactors maintained generally stable environmental conditions despite differences in gas productivity. pH remained close to neutrality throughout the experiment, with average values of approximately 7.28 ± 0.12 for AD-BES and 7.38 ± 0.07 for AD. No progressive acidification was observed, indicating that the accumulation of fermentation intermediates was not sufficient to cause a major disruption of the acid–base equilibrium. Electrical conductivity increased after digestion in both systems, with somewhat higher average values in AD. This change is consistent with the transformation and mineralization of organic matter and the consequent changes in dissolved ionic species, although conductivity cannot be directly used to determine the fate of individual organic compounds.

Temperature represented an important difference between the two configurations. AD-BES operated at an average internal temperature of approximately 27.0 °C, whereas AD operated at approximately 30.2 °C. Thus, the BES-assisted reactor produced substantially more biogas despite operating at a lower average temperature. Under the experimental conditions, the higher productivity of AD-BES cannot therefore be explained by a higher operating temperature. Nevertheless, the temperature difference remains an experimental confounding factor and may have affected the absolute performance of both microbial communities. The result is consequently best interpreted as evidence that the productivity advantage of AD-BES was not attributable to higher temperature, rather than as evidence that temperature had no influence on the process.

The electrochemical characterization showed that the nominal cell voltage of 0.80 V was maintained, while the average current recorded by the power supply was approximately 0.001 ± 0.0014 A. More pronounced changes were observed in the individual electrode potentials relative to the Ag/AgCl reference electrode. During the early operation, the working electrode remained at positive potentials while the counter electrode showed negative values. During Sludge 5, the WE–RE potential shifted towards negative values and became highly variable, whereas the CE–RE potential shifted towards positive values. This redistribution indicates that the applied cell voltage was distributed differently between the two electrode interfaces during operation. The large standard deviation of the WE–RE measurement during this period also indicates increased variability of the working-electrode environment.

The temporal correspondence between the electrochemical changes and the VFA profile observed during Sludge 5 is noteworthy. Both the highest diversity of measured VFAs and a pronounced redistribution of electrode potentials occurred during the same period. This observation is compatible with an interaction between microbial metabolism, substrate conversion and the electrochemical environment. However, temporal coincidence alone cannot establish causality. Changes in electrode potential may have resulted from changes in microbial activity, biofilm development, substrate composition or local physicochemical conditions, and the available measurements cannot distinguish among these possibilities. The electrochemical results should therefore be considered evidence of a changing electrode–electrolyte environment rather than direct proof of a specific electron-transfer mechanism.

Microbial community analysis provided further information about the biological response to BES activation. Samples corresponding to Sludge 1 and Sludge 2 were analysed before and after BES activation, respectively. Both systems showed an increase in the relative abundance of *Methanobacterium*, a hydrogenotrophic methanogen, reaching approximately 30% in the second sample. This similar increase in both reactors suggests that hydrogenotrophic methanogenesis remained an important component of the microbial community and that part of the observed temporal change could be attributed to adaptation to reactor operation rather than specifically to BES activation.

A more distinct difference was observed in the distribution of *Methanosaeta* and *Methanosarcina*. *Methanosaeta* increased from 35.9% to 42.1% in AD but decreased from 35.2% to 24.6% in AD-BES. Conversely, *Methanosarcina* showed a stronger increase in the AD-BES system. These changes indicate that the methanogenic communities followed different trajectories under the two reactor configurations. The enrichment of *Methanosarcina* is potentially relevant because this genus includes metabolically versatile methanogens capable of utilizing different substrates and tolerating changes in environmental conditions. Nevertheless, because only two sampling points were available, these observations should be regarded as supportive evidence of a different microbial response rather than definitive evidence that a specific microbial shift caused the higher biogas productivity.

The bacterial community also showed configuration-dependent changes. In AD-BES, several bacterial groups increased after BES activation, including *Bacteroidetes_vadinHA17*, *Romboutsia* and an uncultured *Synergistaceae* lineage, while *Syntrophomonadaceae* remained highly represented. In AD, several of the same groups showed different trajectories. These differences are compatible with an effect of reactor configuration on microbial community structure. However, relative abundance data alone cannot establish whether the microorganisms were more metabolically active. Moreover, classical exoelectrogenic genera such as *Geobacter* and *Shewanella* were not detected. Therefore, the sequencing data do not provide evidence for enrichment of these specific exoelectrogenic microorganisms in the AD-BES reactor. At the same time, their absence cannot exclude extracellular or interspecies electron-transfer mechanisms mediated by other microbial populations.

The stability of the two processes was evaluated by analysing their response to the transition from Sludge 6 to Sludge 7, which was considered a substrate-induced perturbation. Both systems experienced a pronounced decrease in BPR, although the magnitude of the decrease differed considerably. AD-BES decreased from 1.63 to 0.85 m^3 biogas m^{-3} reactor d^{-1}, corresponding to a relative change of −47.9%, and retained approximately 52.1% of its pre-disturbance performance. AD decreased from 0.41 to 0.06 m^3 biogas m^{-3} reactor d^{-1}, corresponding to −85.4%, and retained only approximately 14.6% of its previous productivity. Thus, the magnitude of the performance loss was approximately 1.8 times greater in AD than in AD-BES. These results indicate that AD-BES exhibited greater resistance to the identified perturbation.

The subsequent recovery showed a different pattern. AD-BES increased from 0.85 to 1.43 m^3 biogas m^{-3} reactor d^{-1} during Sludge 8, corresponding to 87.7% of its pre-disturbance BPR, and reached 97.5% during Sludge 9. It therefore approached its previous performance within two subsequent sludge batches. AD, in contrast, increased rapidly from 0.06 to 0.84 m^3 biogas m^{-3} reactor d^{-1} during Sludge 8, reaching approximately 205.9% of its pre-disturbance baseline, and remained above the original baseline during the following sludge batches. The comparison therefore suggests a functional trade-off between resistance and resilience: AD-BES experienced a smaller immediate performance loss, whereas AD exhibited a stronger rebound after the disturbance. Importantly, this difference should not be attributed directly to the microbial community because the microbial dataset was collected at a different stage of the experiment and does not provide continuous evidence linking community dynamics to the perturbation response.

Taken together, the results indicate that integration of a BES into anaerobic digestion was associated with a substantial increase in biogas productivity and greater resistance to a transient substrate-related perturbation. The increase in gas production occurred despite lower COD and SCOD removal and despite the lower average operating temperature of AD-BES. These observations suggest that the principal effect of the BES may not have been a generalized acceleration of hydrolysis or bulk organic matter degradation. Instead, the results are compatible with a modification of the conversion efficiency of fermentation intermediates towards gaseous products, potentially involving changes in the methanogenic community and the electrochemical environment. The enrichment of *Methanosarcina*, the persistence of hydrogenotrophic methanogens, the ability to maintain biogas production despite measured VFA accumulation and the temporal redistribution of electrode potentials are all consistent with this interpretation. However, the available datasets were not collected with sufficient temporal resolution to establish direct causal relationships among electrochemical conditions, microbial community structure, intermediate conversion and methane production.

From an applied perspective, the results suggest that AD-BES may represent a complementary strategy for increasing methane recovery from municipal sewage sludge and for improving the robustness of anaerobic digestion against transient changes in substrate characteristics. Its potential advantage appears to lie in process intensification within an existing reactor volume rather than in enhanced bulk substrate degradation. This distinction is relevant for wastewater treatment applications because increasing methane recovery without necessarily increasing the hydraulic retention volume could contribute to more efficient utilization of the energy contained in sewage sludge. Nevertheless, a full assessment of the practical feasibility of BES-assisted digestion would require additional information on electrical energy consumption, electrode durability, scaling effects, operating costs and net energy balance.

Riassunto

La digestione anaerobica (AD) è un processo biologico ampiamente utilizzato per la stabilizzazione della materia organica e per il recupero di energia rinnovabile sotto forma di biogas. Nel trattamento delle acque reflue urbane, la digestione anaerobica dei fanghi di supero rappresenta una tecnologia di particolare interesse poiché consente di stabilizzare il fango e di convertire una parte della sostanza organica biodegradabile in metano. Tuttavia, la digestione anaerobica dei fanghi di depurazione è caratterizzata da diverse limitazioni, tra cui la relativamente bassa biodegradabilità del substrato, la complessità delle interazioni tra microrganismi idrolitici, fermentativi, sintrofici e metanigeni e la sensibilità della comunità microbica alle variazioni nella composizione del substrato e nelle condizioni operative. Negli ultimi anni, i sistemi bioelettrochimici (BES) sono stati studiati come possibile strategia di intensificazione della digestione anaerobica, in grado di modificare i processi di trasferimento elettronico e le interazioni microbiche all'interno del digestore. L'integrazione di elettrodi in un sistema anaerobico può infatti fornire ulteriori vie per il trasferimento degli elettroni e influenzare la conversione degli intermedi metabolici verso la produzione di metano. L'efficacia di tale approccio dipende tuttavia da numerosi fattori, tra cui il potenziale applicato, il materiale degli elettrodi, la configurazione del reattore, le caratteristiche del substrato, la comunità microbica e le condizioni operative.

Lo scopo di questa tesi sperimentale è stato valutare comparativamente le prestazioni e la stabilità del processo di digestione anaerobica convenzionale e di digestione anaerobica assistita da un sistema bioelettrochimico (AD-BES) utilizzando fanghi di depurazione municipali come substrato. In particolare, lo studio ha analizzato se l'integrazione di un BES potesse migliorare la produzione di biogas e metano e modificare la risposta del processo anaerobico a una perturbazione associata alla variazione del substrato alimentato.

Sono stati utilizzati due reattori da laboratorio con volume utile di 10 L, operati in parallelo in condizioni di alimentazione comparabili. Un reattore è stato utilizzato come sistema convenzionale di digestione anaerobica (AD), mentre il secondo è stato equipaggiato con un sistema bioelettrochimico ed è stato indicato come AD-BES. Il BES era costituito da due elettrodi cilindrici in rete di acciaio inossidabile e due elettrodi a carbon brush, oltre a un elettrodo di riferimento Ag/AgCl. Dopo il primo batch di fango, il sistema bioelettrochimico è stato attivato applicando un potenziale nominale di cella di 0,8 V, mantenuto durante il successivo periodo sperimentale. Gli elettrodi a carbon brush sono stati selezionati per la loro struttura tridimensionale e per l'elevata superficie potenzialmente disponibile per l'adesione microbica, mentre la rete in acciaio inossidabile è stata utilizzata per le sue caratteristiche di conducibilità elettrica, resistenza meccanica, resistenza alla corrosione e struttura aperta.

Il substrato utilizzato era costituito da fango attivo di scarto raccolto dopo il trattamento di ispessimento meccanico presso un impianto municipale di trattamento delle acque reflue situato a Pavia. A causa dell'impossibilità di conservare volumi sufficienti di fango per l'intera durata dell'esperimento, sono stati utilizzati dieci differenti batch di substrato, raccolti in momenti diversi. Tale caratteristica sperimentale rappresenta un elemento importante nell'interpretazione dei risultati, poiché le caratteristiche chimico-fisiche del fango variavano nel tempo in funzione delle condizioni operative dell'impianto di provenienza. I diversi batch sono stati mantenuti nei reattori per periodi variabili in funzione delle caratteristiche del substrato. Il volume utile dei reattori era pari a 10 L e ciascun sistema veniva alimentato con 0,76 L di fango tre volte alla settimana, corrispondenti a un hydraulic retention time (HRT) di circa 30,7 giorni. La produzione di biogas è stata misurata mediante un sistema a spostamento d'acqua, mentre la temperatura è stata monitorata prima delle operazioni di alimentazione. I parametri chimico-fisici sono stati determinati periodicamente sia nel fango in ingresso sia nel digestato dei due reattori.

La caratterizzazione sperimentale ha incluso diversi parametri complementari. La produzione di biogas è stata espressa mediante il Biogas Production Rate (BPR), calcolato normalizzando il volume di biogas prodotto durante ciascun periodo sperimentale sia rispetto alla durata del periodo sia rispetto al volume utile del reattore. La composizione del biogas è stata analizzata mediante gascromatografia presso laboratori collaboratori, con particolare attenzione alle concentrazioni di metano e anidride carbonica. La conversione della materia organica è stata valutata attraverso Chemical Oxygen Demand (COD) e Soluble Chemical Oxygen Demand (SCOD), mentre Total Nitrogen (TN), pH, conducibilità elettrica e temperatura sono stati monitorati per caratterizzare l'ambiente chimico-fisico dei reattori. Alcuni campioni selezionati sono stati inoltre analizzati per la determinazione degli acidi grassi volatili (VFA). Infine, la comunità batterica e archeale è stata caratterizzata mediante sequenziamento ad alta produttività del gene 16S rRNA, permettendo di valutare le variazioni nell'abbondanza relativa dei principali gruppi microbici prima e dopo l'attivazione del BES. Poiché ciascuna configurazione sperimentale era rappresentata da un singolo reattore, non sono state eseguite analisi statistiche inferenziali e i risultati sono stati interpretati attraverso confronti descrittivi e andamenti temporali.

Il risultato principale dello studio riguarda la produttività di biogas. Durante il primo batch di fango, nel quale il BES non era ancora attivato, i due reattori hanno mostrato valori di BPR molto simili, pari a circa 0,03 m^3 biogas m^{-3} reactor d^{-1}. Questa condizione iniziale costituisce un riferimento utile per il confronto tra i successivi periodi sperimentali. Dal secondo batch in poi, dopo l'attivazione del BES a un potenziale nominale di 0,8 V, il reattore AD-BES ha prodotto una quantità di biogas superiore rispetto al reattore AD convenzionale durante tutti i batch analizzati. L'entità della differenza è variata in funzione del batch di fango, evidenziando che la risposta del sistema bioelettrochimico era influenzata anche dalle caratteristiche del substrato. La differenza relativa più elevata è stata osservata durante Sludge 7, quando il BPR di AD-BES ha raggiunto un valore circa 14,5 volte superiore a quello di AD. Questo rapporto particolarmente elevato è stato tuttavia determinato anche dal forte calo della produttività del reattore convenzionale durante tale periodo. Considerando complessivamente i nove batch successivi all'attivazione del BES, il BPR medio è risultato pari a circa 1,14 m^3 biogas m^{-3} reactor d^{-1} per AD-BES e 0,46 m^3 biogas m^{-3} reactor d^{-1} per AD, corrispondenti a una produttività media circa 2,5 volte superiore nel sistema bioelettrochimicamente assistito.

L'incremento della produzione di biogas rappresenta quindi il principale risultato sperimentale della tesi. Tale risultato non deve tuttavia essere interpretato come una dimostrazione diretta che il potenziale di 0,8 V abbia determinato, da solo, un aumento dell'attività metanigena. Il reattore AD-BES differiva infatti dal reattore convenzionale non soltanto per la presenza di un potenziale elettrico applicato, ma anche per la presenza delle superfici elettrodiche. L'esperimento permette quindi di dimostrare un miglioramento associato alla configurazione BES nel suo complesso, ma non consente di separare il contributo della stimolazione elettrica da quello della presenza degli elettrodi. Le superfici elettrodiche possono infatti fornire siti di adesione per i microrganismi e modificare localmente le condizioni redox indipendentemente dall'intensità della corrente. Per questo motivo, nella discussione il risultato dovrebbe essere interpretato come un effetto associato all'integrazione del BES e non come prova di uno specifico meccanismo elettrochimico.

L'analisi della composizione del biogas ha mostrato un quadro più complesso. Le percentuali di metano e anidride carbonica sono variate tra i diversi batch di fango e tra le due configurazioni, evidenziando una forte dipendenza della qualità del biogas dalle caratteristiche del substrato. I dati relativi alla composizione del gas erano disponibili soltanto per i batch 4–7, poiché le analisi sono state effettuate presso laboratori esterni e non erano disponibili per tutti i dieci batch. Nei periodi per i quali erano disponibili dati di composizione, il sistema AD-BES ha generalmente prodotto una maggiore quantità volumetrica di metano grazie al maggiore BPR, ma non ha mostrato sistematicamente una percentuale di metano superiore a quella del sistema AD. Pertanto, il principale vantaggio osservato per il BES riguarda la quantità di biogas e metano prodotto, piuttosto che un miglioramento sistematico della concentrazione di metano nel biogas. Questa distinzione è importante poiché la percentuale di metano e la produttività volumetrica di metano descrivono due aspetti differenti della prestazione del processo.

I dati disponibili sulla composizione del gas sono stati inoltre utilizzati per stimare l'equivalente energetico della produzione di metano. Per i quattro batch di fango per i quali erano disponibili sia i valori di BPR sia quelli di composizione del biogas, il recupero energetico medio stimato è risultato pari a circa 5,2 kWh m^{-3} reactor d^{-1} per AD-BES rispetto a circa 0,9 kWh m^{-3} reactor d^{-1} per AD. Tale differenza riflette la maggiore produzione volumetrica di metano osservata nel sistema AD-BES durante questi periodi. Tuttavia, questa stima rappresenta il contenuto energetico teorico del metano e non deve essere interpretata come un'efficienza energetica netta del sistema bioelettrochimico. Una valutazione completa dovrebbe infatti considerare l'energia elettrica fornita al BES, oltre ai consumi associati a miscelazione, riscaldamento e altre operazioni. I risultati attuali supportano quindi un aumento del recupero energetico sotto forma di metano, ma non costituiscono un bilancio energetico o tecno-economico completo.

Il confronto tra COD e SCOD ha fornito uno degli elementi più interessanti per l'interpretazione del meccanismo alla base della maggiore produzione di biogas. Contrariamente ai risultati relativi al BPR, il reattore AD ha generalmente mostrato una maggiore rimozione della materia organica. Anche la rimozione dello SCOD è risultata superiore nel sistema convenzionale, con valori medi pari a circa 89,22% per AD rispetto a 80,23% per AD-BES. Pertanto, la maggiore produzione di metano nel sistema bioelettrochimico non è stata accompagnata da un aumento generalizzato della degradazione della materia organica. In alcuni degli ultimi batch questo contrasto è risultato particolarmente evidente: AD-BES ha mostrato una riduzione della rimozione del COD mentre il BPR aumentava, mentre AD ha mostrato una maggiore rimozione del COD senza un aumento proporzionale della produzione di biogas. Queste osservazioni indicano che la rimozione del COD non è stata sufficiente a spiegare le differenze nella produttività gassosa e suggeriscono che i due sistemi abbiano seguito destini metabolici differenti per la frazione organica disponibile alla conversione.

Considerando congiuntamente i risultati di COD, SCOD e produzione di biogas, l'interpretazione più plausibile è che l'integrazione del BES possa aver influenzato principalmente la conversione degli intermedi di fermentazione prodotti nelle fasi a valle dell'idrolisi, piuttosto che accelerare la degradazione iniziale della materia organica complessa. Nella digestione anaerobica convenzionale la materia organica viene progressivamente idrolizzata e convertita attraverso acidogenesi e acetogenesi in intermedi che vengono successivamente utilizzati durante la metanogenesi. La maggiore produttività osservata in AD-BES nonostante la minore rimozione di COD e SCOD è quindi compatibile con una maggiore efficienza di conversione di alcuni intermedi già solubilizzati verso prodotti gassosi. Tuttavia, i dati disponibili non permettono di costruire un bilancio completo del carbonio e non consentono di dimostrare quantitativamente tale meccanismo. L'interpretazione deve pertanto essere considerata un'ipotesi meccanicistica supportata dalla convergenza dei diversi dataset, e non una dimostrazione diretta del percorso metabolico coinvolto.

Le analisi degli acidi grassi volatili hanno fornito ulteriori informazioni, sebbene limitate a un numero ridotto di campioni. I dati VFA erano disponibili esclusivamente per Sludge 5 e Sludge 6. Durante Sludge 5, il reattore AD-BES presentava acetato, propionato, isobutirrato, butirrato, isovalerato e valerato, con una concentrazione complessiva di VFA pari a circa 0,1 g/L espressa come COD. Il reattore AD presentava invece principalmente acetato e propionato, con una concentrazione complessiva di circa 0,01 g/L come COD. Durante Sludge 6, il profilo di AD-BES era costituito principalmente da acetato, mentre AD mostrava acetato e propionato. È particolarmente interessante osservare che la maggiore concentrazione e diversità degli intermedi osservata in AD-BES durante Sludge 5 non è stata associata a un collasso della produzione di biogas; al contrario, il BPR continuava ad aumentare. Questo risultato indica che, nelle condizioni analizzate, non è stata osservata una perdita evidente della performance metanigena associata all'accumulo misurato di VFA. Tuttavia, poiché i dati sono disponibili soltanto per due batch, non è possibile estendere questa interpretazione all'intero periodo sperimentale.

La concentrazione di azoto totale nel fango in ingresso ha mostrato una considerevole variabilità, con valori compresi tra 1,38 e 2,81 g/L e una media complessiva di 2,02 ± 0,42 g/L. Dopo la digestione, le concentrazioni medie di TN sono risultate pari a 1,75 ± 0,32 g/L in AD-BES e 1,86 ± 0,29 g/L in AD. Sebbene il confronto tra valori in ingresso e in uscita abbia evidenziato una diminuzione media di circa il 12% in AD-BES e del 6% in AD, tale variazione non deve essere interpretata come completa rimozione dell'azoto. La digestione anaerobica favorisce principalmente l'idrolisi e la mineralizzazione dell'azoto organico e la sua redistribuzione tra differenti forme azotate. I risultati relativi al TN mostrano quindi che la concentrazione di azoto variava in funzione delle caratteristiche dei diversi fanghi, ma non forniscono indicazioni sufficienti per considerare l'azoto un fattore determinante della differente produttività dei due sistemi.

I parametri chimico-fisici hanno evidenziato condizioni generalmente stabili nei due reattori nonostante le differenze nella produzione di biogas. Il pH è rimasto vicino alla neutralità durante tutto l'esperimento, con valori medi di circa 7,28 ± 0,12 in AD-BES e 7,38 ± 0,07 in AD. Non è stata osservata una progressiva acidificazione, indicando che l'accumulo degli intermedi di fermentazione non è stato sufficiente a determinare una significativa alterazione dell'equilibrio acido-base dei digestori. La conducibilità elettrica è aumentata dopo la digestione in entrambi i sistemi, con valori medi leggermente superiori nel sistema AD. Tale incremento è compatibile con i processi di degradazione e mineralizzazione della materia organica e con le conseguenti modificazioni della frazione ionica disciolta, anche se la conducibilità non può essere utilizzata direttamente per determinare il destino di specifici composti organici.

La temperatura ha rappresentato una differenza rilevante tra le due configurazioni. Il reattore AD-BES ha operato a una temperatura interna media di circa 27,0 °C, mentre AD ha operato mediamente a circa 30,2 °C. Il sistema bioelettrochimico ha quindi prodotto una quantità significativamente maggiore di biogas pur funzionando a una temperatura media inferiore. Nelle condizioni sperimentali investigate, la maggiore produttività di AD-BES non può pertanto essere attribuita a una temperatura operativa più elevata. La differenza di temperatura costituisce tuttavia una variabile confondente e può avere influenzato la performance assoluta delle due comunità microbiche. Il risultato deve quindi essere interpretato come evidenza del fatto che il vantaggio produttivo di AD-BES non era dovuto a una temperatura più elevata, e non come dimostrazione dell'assenza di un effetto della temperatura sul processo.

La caratterizzazione elettrochimica ha evidenziato che il potenziale nominale della cella di 0,80 V è stato mantenuto, mentre la corrente media registrata dall'alimentatore è risultata pari a circa 0,001 ± 0,0014 A. Variazioni più marcate sono state osservate nei singoli potenziali elettrodici rispetto all'elettrodo di riferimento Ag/AgCl. Durante le prime fasi operative, il potenziale WE-RE si trovava a valori positivi, mentre CE-RE presentava valori negativi. Durante il periodo di alimentazione con Sludge 5, il potenziale WE-RE si è spostato verso valori negativi ed è diventato molto più variabile, mentre CE-RE si è spostato verso valori positivi. Tale comportamento indica una diversa distribuzione del potenziale applicato tra le due interfacce elettrodiche nel corso dell'operazione. L'elevata deviazione standard osservata per WE-RE durante questo periodo evidenzia inoltre un aumento della variabilità delle condizioni all'interfaccia tra elettrodo ed elettrolita.

La contemporanea variazione dei parametri elettrochimici e del profilo dei VFA osservata durante Sludge 5 costituisce un elemento di particolare interesse. Nello stesso periodo in cui sono state rilevate la maggiore concentrazione e la maggiore diversità di VFA sono state infatti osservate anche importanti variazioni dei potenziali elettrodici. Tale corrispondenza temporale è compatibile con una relazione tra metabolismo microbico, conversione del substrato e ambiente elettrochimico. Tuttavia, la sola coincidenza temporale non permette di stabilire una relazione causale. Le variazioni dei potenziali potrebbero essere state determinate da cambiamenti dell'attività microbica, dallo sviluppo del biofilm, dalla composizione del substrato o da variazioni locali delle condizioni chimico-fisiche. I dati disponibili devono quindi essere interpretati come evidenza di una modificazione dell'ambiente elettrochimico del reattore e non come dimostrazione diretta di uno specifico meccanismo di trasferimento elettronico.

L'analisi della comunità microbica ha fornito ulteriori informazioni sulla risposta biologica all'attivazione del BES. I campioni corrispondenti a Sludge 1 e Sludge 2 sono stati analizzati rispettivamente prima e dopo l'attivazione del BES. Entrambi i reattori hanno mostrato un aumento dell'abbondanza relativa del genere *Methanobacterium*, un metanogeno idrogenotrofo, che ha raggiunto circa il 30% nel secondo campione. Il fatto che tale aumento sia stato osservato in entrambi i sistemi suggerisce che la metanogenesi idrogenotrofa abbia mantenuto un ruolo importante in entrambe le configurazioni e che una parte della variazione osservata possa essere attribuita al progressivo adattamento della comunità alle condizioni operative, piuttosto che esclusivamente all'attivazione del BES.

Una differenza più marcata è stata osservata per *Methanosaeta* e *Methanosarcina*. In AD, *Methanosaeta* è aumentata dal 35,9% al 42,1%, mentre in AD-BES è diminuita dal 35,2% al 24,6%. Al contrario, *Methanosarcina* ha mostrato un aumento più marcato nel sistema AD-BES. Questi risultati indicano che le comunità metanigene hanno seguito traiettorie differenti nelle due configurazioni. L'arricchimento di *Methanosarcina* è potenzialmente rilevante poiché questo genere comprende microrganismi metanigeni caratterizzati da una maggiore versatilità metabolica e dalla capacità di utilizzare differenti substrati. Tuttavia, poiché la caratterizzazione microbiologica è stata effettuata soltanto in due momenti, questi risultati devono essere considerati come evidenze a supporto di una diversa risposta microbica e non come prova definitiva che il cambiamento della comunità abbia causato l'aumento della produttività di biogas.

Anche la comunità batterica ha mostrato variazioni differenti nelle due configurazioni. Nel sistema AD-BES, diversi gruppi batterici sono aumentati dopo l'attivazione del BES, tra cui *Bacteroidetes_vadinHA17*, *Romboutsia* e una linea non coltivata appartenente a *Synergistaceae*, mentre *Syntrophomonadaceae* è rimasta altamente rappresentata. Nel sistema AD, alcuni degli stessi gruppi hanno seguito traiettorie differenti. Tali differenze sono compatibili con un effetto della configurazione del reattore sulla struttura della comunità microbica. Tuttavia, i dati di abbondanza relativa non permettono di stabilire direttamente se i microrganismi fossero anche più metabolicamente attivi. Inoltre, non sono stati rilevati generi classicamente associati all'attività esoelettrrogenica, quali *Geobacter* e *Shewanella*. I dati microbiologici non forniscono quindi evidenza di un arricchimento di questi specifici microrganismi nel sistema AD-BES. La loro assenza non permette tuttavia di escludere altri meccanismi di trasferimento elettronico extracellulare o interspecifico mediati da popolazioni microbiche differenti.

La stabilità dei due processi è stata valutata analizzando la risposta alla transizione da Sludge 6 a Sludge 7, considerata una perturbazione associata alla variazione del substrato alimentato. Entrambi i sistemi hanno subito una forte riduzione del BPR, ma l'entità della perdita di performance è risultata molto diversa. AD-BES è passato da 1,63 a 0,85 m^3 biogas m^{-3} reactor d^{-1}, corrispondenti a una variazione relativa del −47,9%, mantenendo circa il 52,1% della produzione precedente alla perturbazione. AD è invece passato da 0,41 a 0,06 m^3 biogas m^{-3} reactor d^{-1}, con una variazione del −85,4%, conservando soltanto il 14,6% della propria produttività pre-perturbazione.

La perdita di performance è stata quindi circa 1,8 volte maggiore nel sistema convenzionale. Questi risultati indicano una maggiore resistance del sistema AD-BES rispetto alla perturbazione individuata.

La fase successiva ha mostrato invece un comportamento differente per quanto riguarda la resilience. In AD-BES il BPR è aumentato da 0,85 a 1,43 m^3 biogas m^{-3} reactor d^{-1} durante Sludge 8, raggiungendo l'87,7% del valore precedente alla perturbazione, e successivamente 1,59 m^3 biogas m^{-3} reactor d^{-1} durante Sludge 9, corrispondente al 97,5% del valore iniziale. Il sistema ha quindi recuperato quasi completamente la performance precedente entro due batch successivi. Il reattore AD ha invece mostrato un recupero più rapido e marcato, passando da 0,06 a 0,84 m^3 biogas m^{-3} reactor d^{-1} durante Sludge 8 e raggiungendo circa il 205,9% del valore pre-perturbazione. La risposta dei due sistemi suggerisce pertanto un possibile trade-off tra resistance e resilience: AD-BES è stato meno influenzato dalla perturbazione iniziale, mentre AD ha mostrato una capacità di recupero più rapida e ha superato il proprio valore di riferimento. Tale differenza non deve tuttavia essere attribuita direttamente alla comunità microbica, poiché i dati microbiologici sono stati raccolti in un momento differente e non consentono di collegare direttamente la dinamica della comunità alla risposta alla perturbazione.

Considerando complessivamente i risultati ottenuti, l'integrazione del BES nel processo di digestione anaerobica è risultata associata a un incremento sostanziale della produttività di biogas e a una maggiore resistance nei confronti della perturbazione osservata tra Sludge 6 e Sludge 7. L'aumento della produzione gassosa non è stato tuttavia accompagnato da una maggiore rimozione del COD o dello SCOD e non è stato associato a un aumento sistematico della percentuale di metano nel biogas. Inoltre, il sistema AD-BES ha operato a una temperatura media inferiore rispetto al sistema AD. L'insieme di queste osservazioni suggerisce che l'effetto principale della configurazione bioelettrochimica non sia stato un'accelerazione generalizzata dell'intero processo di digestione anaerobica, ma piuttosto una possibile modificazione della conversione degli intermedi metabolici verso la produzione di gas. L'arricchimento di *Methanosarcina*, la persistenza dei metanogeni idrogenotrofi, la capacità di mantenere la produzione di biogas nonostante l'accumulo di VFA osservato in uno dei batch e la variazione dei potenziali elettrodici sono tutti risultati compatibili con questa interpretazione.

Tuttavia, i diversi dataset non sono stati raccolti con una risoluzione temporale sufficiente per dimostrare una relazione causale diretta tra condizioni elettrochimiche, composizione microbica, conversione degli intermedi e produzione di metano.

Dal punto di vista applicativo, i risultati ottenuti suggeriscono che l'AD-BES possa rappresentare una strategia complementare per aumentare il recupero di metano dai fanghi di depurazione e migliorare la robustezza del processo nei confronti di variazioni transitorie delle caratteristiche del substrato. Il potenziale vantaggio della configurazione bioelettrochimica sembra essere principalmente legato all'intensificazione della produzione di metano all'interno di un determinato volume di reattore, piuttosto che a un aumento della degradazione complessiva della materia organica. Questo aspetto potrebbe essere rilevante per gli impianti di trattamento delle acque reflue, nei quali l'aumento del recupero energetico dai fanghi potrebbe contribuire a migliorare l'efficienza complessiva del processo. Una valutazione dell'applicabilità su scala maggiore richiederebbe tuttavia ulteriori informazioni relative al consumo elettrico del BES, alla durabilità degli elettrodi, ai costi operativi, agli effetti dello scale-up e al bilancio energetico netto.

1) Introduction

1.1. Introduction to Anaerobic Digestion

Anaerobic Digestion (AD) is a complex biological process in which a consortium of bacteria and archaea, often in syntrophic association, converts biodegradable organic matter into simpler compounds in an oxygen-free environment. The ultimate product is a gaseous mixture, commonly referred to as biogas, which consists predominantly of methane (CH_4), usually up to 60–70%, and carbon dioxide (CO_2), together with other gases in traces.
This process occurs usually in natural environments, like wetlands, aquatic sediments and in the gastrointestinal tract of ruminant. These natural ecosystems provide the biological basis for modern anaerobic digestion technology. Engineered AD systems reproduce and intensify these naturally occurring microbial interactions by concentrating biodegradable substrates and controlling environmental parameters [1-8]

During the twentieth century, anaerobic digestion was used as treatment of sewage sludge and industrial wastewaters, supported by advances in microbiology and reactor engineering [9]. Its application subsequently expanded to a broader range of organic wastes, including agricultural and municipal biowastes [10]. In contemporary applications, anaerobic digestion is increasingly regarded as a waste stabilization process and as a resource-recovery technology, in which biodegradable organic matter is converted into methane-rich biogas that can be recovered as renewable energy in form of biogas [11-13].

A particularly relevant application of anaerobic digestion is the treatment of waste as the sludge generated during municipal wastewater treatment. Wastewater treatment plants (WWTPs) remove organic matter, suspended solids, nutrients and other pollutants from wastewater through a combination of physical, chemical and biological processes, but they generate a waste sludge. Anaerobic digestion can be integrated into the waste sludge treatment line to stabilize this organic material while converting part of its biodegradable fraction into methane-rich biogas [13,14]. WWTPs tend to have a high electricity consumption in order to carry out the biological treatment of the municipal waste water. Improving the recovery of energy contained in wastewater-derived organic matter is an important aspect of the transition from conventional wastewater treatment towards resource-recovery-oriented systems [15,16].

Recently the European Union has written a new directive, Directive (EU) 2024/3019, replacing the previous Directive 91/271/EEC, about urban wastewater treatment. This Directive introduces more stringent requirements for pollutant removal, including nutrients and micropollutants and establishes a progressive objective of achieving progressively energy neutrality at national level by 2045. Its implementation consequently increases the importance of reducing energy consumption, improving energy efficiency and increasing the recovery and production of renewable energy within wastewater treatment systems [17, 18].
Improving the conversion of sewage sludge organic matter into methane and increasing the overall energy recovery from anaerobic digestion have become important research objectives.

Instead of using the raw produced methane-rich biogas as biofuel, it can be upgraded and purified to biomethane which can be used as a better source of energy to generate heat and electricity, as transport fuel or for direct injection into the natural gas supply network [18]. Another product of the AD process is the digestate, which still contains nutrients from the original organic matter, and can also be utilized for agricultural production [19].

1.2 Anaerobic Digestion process

The conversion of organic matter during Anaerobic Digestion occurs through a series of interconnected biochemical pathways that form a delicate balance in which all the microorganisms present, with different needs, are able to cooperate.
Anaerobic Digestion is conventionally divided into four phases (**Figure 1**): hydrolysis, acidogenesis, acetogenesis, and methanogenesis [20].

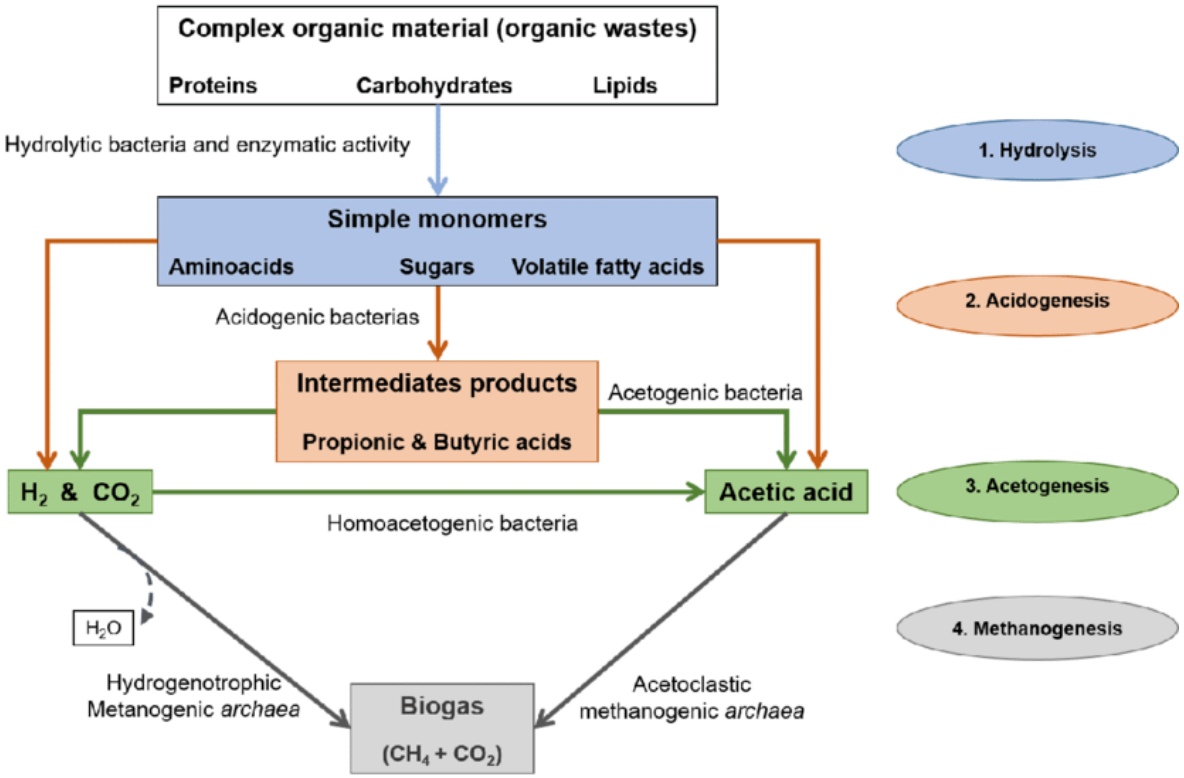

Figure 1: Anaerobic digestion scheme of the different phases with the specific kind of microorganism active in each phase [21]

The hydrolysis phase is the initial step of the Anaerobic Digestion and consists in the extracellular enzymatic transformation of complex molecules, long insoluble or soluble polymers, such as lipids, polysaccharides, proteins, fats and nucleic acids into soluble organic materials suitable for their use as a source of energy or carbon such as monosaccharides, amino acids, long chain fatty acids and other organic compounds. The rate of decomposition during hydrolysis depends largely on the nature of the substrate, and is generally considered one of the rate-limiting steps of anaerobic digestion [14-20].

The second phase of Anaerobic Digestion is the acidogenic process. The products of the hydrolysis of the polymers in the previous phase are used by fermentative acidogenic bacteria and transformed principally into alcohols, CO_2, H_2, ammonia and short-chain volatile fatty acids (VFA) as acetic, propionic and butyric acids.

Those acidogenic phase products need to stay in a fragile equilibrium with the other phases because their accumulation can lead to a methanogenesis inhibition.

Excessive VFAs accumulation can decrease environmental and cellular pH, reducing the activity of methanogens, which are generally more sensitive to acidic conditions than acidogenic microorganisms. [22]
One reason for the accumulation of VFA is the increased partial pressure of hydrogen which inhibits the activity of syntrophic acetogenic bacteria because reactions such as the oxidation of propionate and butyrate become thermodynamically unfavorable, thus methanogenesis is deprived of the necessary substrates. [22]
Free ammonia (NH_3) is another inhibitor of methanogenesis. Its accumulation in cells can alter intracellular pH, ion balance, and cellular energy metabolism of sensitive methanogenic species [23-26].

Acetogenesis is the third phase of the Anaerobic Digestion process and consists in the conversion of the VFAs and alcohols precedently produced into acetate, H_2 and CO_2.
The oxidation of propionate and butyrate is often thermodynamically unfavorable if the products of their conversion, principally H_2, is not continuously consumed by hydrogenotrophic methanogen [1,14, 22, 25, 27, 28].

In the last phase in the anaerobic digestion, the methanogenesis, methane is produced.
Methanogenic archaea, from the Euryarchaeota kingdom of Archaebacteria, are characterized by high physiological specialization and extremely strict anaerobiosis. They can convert acetate, methanol, H_2 and CO_2 to produce cellular energy as ATP while producing CH_4 too. Methane is formed with a complex biochemical process that involves unusual coenzymes, not found in bacteria or eukaryotic organisms [29, 30].

Methanogens can be divided in in three groups based on the substrate they prefer to use to produce methane: hydrogenotrophic methanogens, acetoclastic methanogens and methylotropic methanogens.
Hydrogenotrophic methanogens, known as CO2-reducing methanogens, such as *Methanobacterium* and *Methanobrevibacter*, use H_2 as electron donor and CO_2 as electron receptor through a series of intermediate steps where it is reduced to methyl before becoming methane. Coupled with this process are the electron-transfer reactions which lead to the ATP synthesis.

This group of methanogens have the role as H_2 consumers, maintaining a low hydrogen partial pressure below the limit for the VFA consumption.

Acetoclastic methanogens, such as *Methanosarcina* and *Methanothrix*, cleaves acetate in methane and CO_2.
The third pathway occurs when methanogens use methylated compounds such as methanol as methyl source for the methane production.
The relative contribution of the different pathways is not fixed and depends on substrate composition, pH, temperature, ammonia concentration, hydrogen availability and the composition and physiological state of the archaeal community. In particular, environmental disturbances can cause a shift from acetoclastic toward alternative methane-producing routes. Under high ammonia conditions, for example, acetoclastic methanogens may be inhibited more strongly than hydrogenotrophic populations, potentially favoring syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis rather than direct acetate cleavage. [1, 31-35]

Anaerobic digestion performance is often limited by the sensitivity of the microbial consortium to the substrate and its characteristics. This is primarily due to the availability of nutrients because the sludge can be poorly biodegradable or structurally complex, so hydrolysis can proceed more slowly than the other phases, limiting all the process. To accelerate the digestion and enhance the production of biogas, various pre-treatments can be used to improve the rate-limiting hydrolysis. These treatments include mechanical, thermal, chemical and biological interventions to the feedstock. All pre-treatments result in a lysis or disintegration of sludge cells, thus releasing and solubilising intracellular material into the water phase and transforming refractory organic material into biodegradable species [14].

The development of technologies for improving AD therefore aims not only to increase methane yield, but also to accelerate substrate conversion, increase organic-matter solubilisation and biodegradability, improve process stability, reduce retention time, and enhance the overall energy recovery from the treated substrate.

Mechanical treatments, including grinding, milling, high-pressure homogenisation, hydrodynamic disintegration and ultrasonication, act mainly by reducing particle size, disrupting flocs and damaging cell structures. Thermal pretreatments promote cell lysis and solubilisation through the application of heat, while chemical approaches employ alkaline, acidic or oxidative agents to disrupt complex structures and increase the soluble fraction of organic matter. Biological pretreatments exploit microorganisms or enzymes to selectively degrade complex compounds under comparatively mild conditions [36].

The improvement in methane production must be evaluated together with the energy and environmental costs associated with the pretreatment itself because they may require considerable energy and chemical inputs [37].

A second strategy is to use anaerobic co-digestion, defined as the mixing of two or more substrates. Co-digestion can overcome some limitations associated with the single substrate digestion by combining substrates with complementary physicochemical and nutritional characteristics. In particular, the mixing of substrates can provide a larger spectrum of biodegradable compounds and dilute potentially inhibitory substances [38].

Another enhancement technology involves adding conductive materials to the substrate. Those conductive materials can include activated carbon, biochar, magnetite and other iron-based materials. They can facilitate the direct interspecies electron transfer (DIET)(**Figure 2**), a syntrophic mechanism in which electrons are transferred between microorganisms through conductive materials, rather than through diffusible electron carriers, such as hydrogen or formate, or through the use of specific bacterial pili. Their performance however is dependent on the conductivity of the material, dosage and substrate characteristics [39-42].

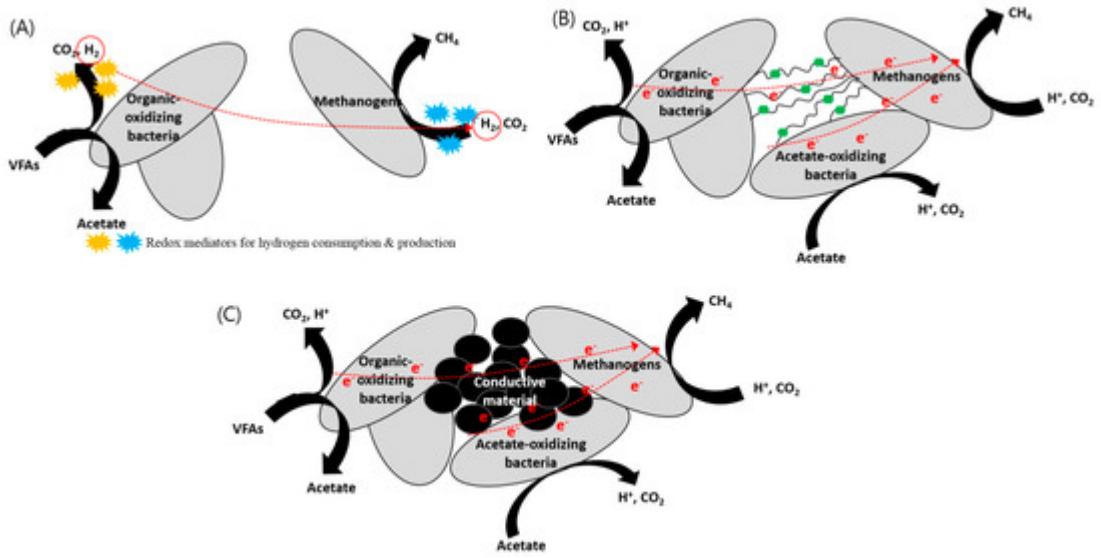

Figure 2: Mechanisms of (A) indirect interspecies electron transfer (IIET) via hydrogen, (B) biological direct interspecies electron transfer (DIET), and (C) conductive material-mediated DIET [43].

A different approach involves bioelectrochemical technologies, when electrodes are incorporated into anaerobic digestion systems to influence the microbial metabolism and the electron transfer in the system.

The presence of electrically connected electrodes provides a medium in which microorganisms can exchange electrons with an external electrical circuit or with other microorganisms, thus allowing for a different DIET and consequently improving the entire process [13, 44-49]

1.3. Bioelectrochemical systems

Bioelectrochemical systems (BESs) are electrochemical devices in which microorganisms participate directly in electrode-associated oxidation or reduction reactions by either donating electrons to an electrode or accepting electrons from it. A typical BES comprises an anode and a cathode, while a reference electrode may additionally be employed when precise control or measurement of the potential of a working electrode is required. Depending on the specific application, the anode and cathode may be located in the same chamber or separated into distinct compartments, with ion-exchange membranes or other separators sometimes employed to control ionic transport, prevent undesirable crossover of compounds, or maintain suitable physicochemical conditions in the two compartments (**Figure 3**).

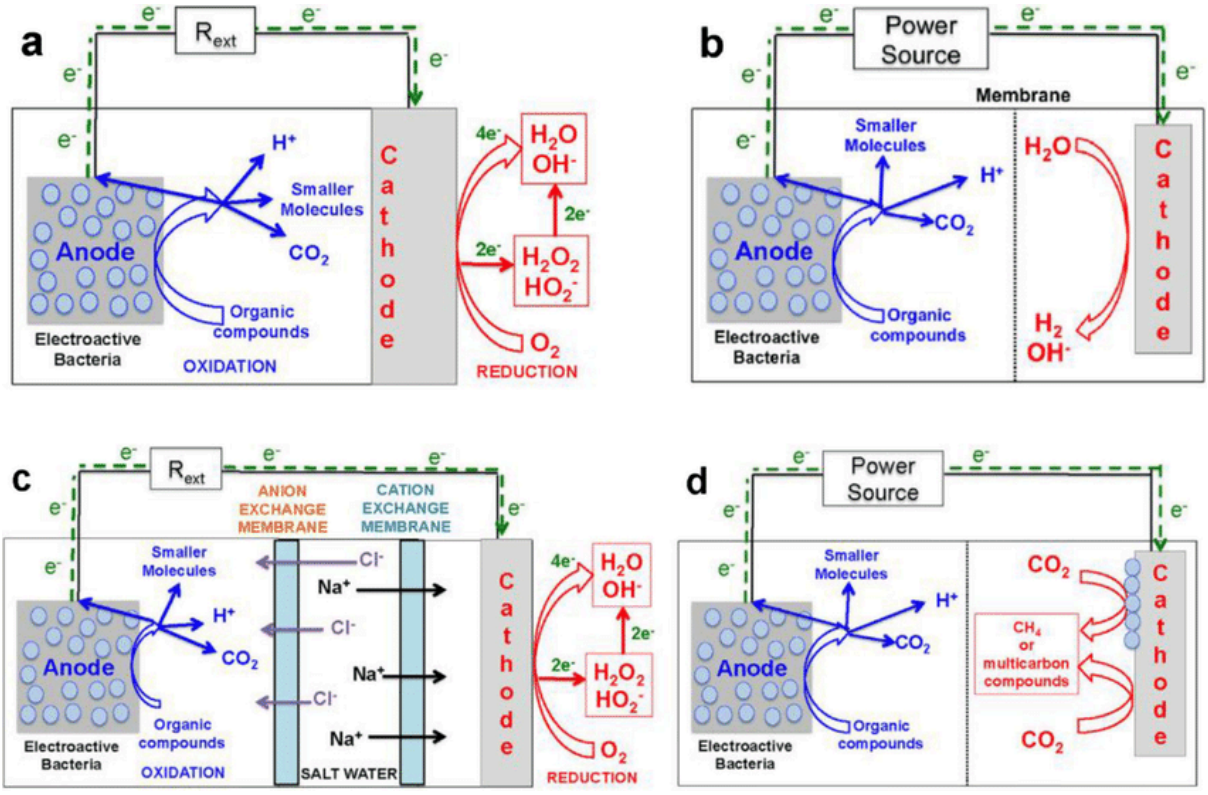

Figure 3: Overview on bioelectrochemical systems (BESs): single chamber, air cathode microbial fuel cell (MFC) (a), microbial electrolysis cell (MEC) (b), microbial desalination cell (MDC) (c), and general microbial electrosynthesis cell (MES) (d) [50].

In the anodic compartment of an anaerobic BES, electroactive microorganisms can oxidise organic compounds and transfer the resulting electrons to the anode through extracellular electron transfer (EET) [51,52].

EET can occur through direct interactions between microorganisms and the electrode, for example through outer-membrane redox proteins or conductive cellular structures, or indirectly through soluble electron mediators. The electrons subsequently travel from the anode towards the cathode through the external electrical circuit, where they can participate in either abiotic electrochemical reactions or microbially catalysed reduction processes. When microorganisms are associated with the cathode, they may accept electrons from the electrode and use them as reducing equivalents to drive reductive metabolic reactions [51,52].

When BES technology is integrated with anaerobic digestion (AD), the electrochemical component can interact with the complex microbial network responsible for the conversion of organic matter into biogas [53,54]. The introduction of electrodes and the establishment of electrochemically controlled conditions can influence electron-transfer processes, redox conditions and microbial interactions within the anaerobic community, potentially facilitating the conversion of intermediate metabolites and affecting methanogenic pathways [53–55].

AD-BES configurations have therefore been investigated as a strategy to improve the performance of anaerobic digestion, but this depends on several factors, including electrode potential, electrode material, reactor configuration, microbial community, substrate characteristics and operating conditions [53–55].

2) Aim of work

The aim of this experimental thesis was to evaluate the effect of the integration of a bioelectrochemical system into a laboratory-scale conventional anaerobic digestion reactor. To establish an improvement the system has been compared with a conventional reactor for anaerobic digestion in the same experimental condition.

This thesis was conducted during the work for the PRIN 2022 PNRR project "AD-MethEnhance - Anaerobic sludge methanation yield improvement by bioelectrochemically/electrically conductive microparticles enhancement" for which the set up was built and operated. This project investigates promising biotechnological solutions to enhance the biogas production of anaerobic digestion.

The quantity and purity of the biogas produced were used as a comparison data between the AD-BES system and the conventional AD system.

3) Materials and Methods

3.1. Materials

Two custom 10 L tank reactors (**Figure 4**), based on an in-house laboratory design (**Figure 5**) and manufactured by T.S. Food Processing S.r.l., were used in this study.

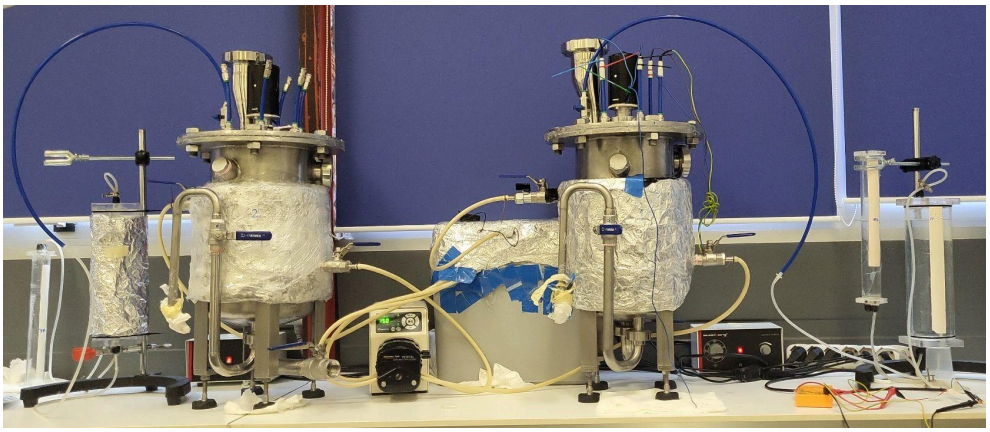

Figure 4: Experimental setup showing the conventional anaerobic digestion (AD) reactor (left) and the bioelectrochemical anaerobic digestion (AD-BES) reactor (right).

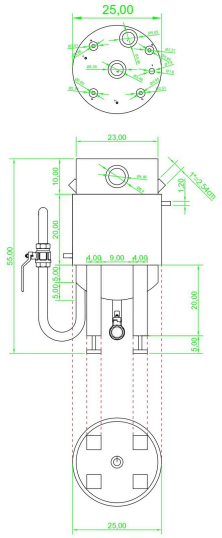

Figure 5: Schematic representation of the laboratory-scale anaerobic digestion reactors used in this study.

3.1.1 Bioelectrochemical System (BES) configuration

The integrated BES in the AD-BES reactor is composed of a single silver/silver chloride (Ag/AgCl) reference electrode (RE), with a potential of +0.222 V compared to the Standard Hydrogen Electrode, two hand-made cylindrical stainless steel mesh for the cathodes, the working electrodes (WE) and two carbon brush for the anode, the counter electrodes (CE), both with a diameter of 3 cm and 10 cm long (**Figure 6**).

Figure 6: Stainless-steel mesh cathode (left) and carbon-brush anode (right) used in the AD-BES reactor.

The electrode materials were selected according to their different electrochemical and structural properties. A carbon brush was employed as the anode because its three-dimensional fibrous structure provides a high effective surface area for microbial attachment and biofilm development, potentially enhancing extracellular electron transfer. A cylindrical stainless-steel mesh was used as the cathode due to its high electrical conductivity, mechanical robustness, corrosion resistance and open mesh structure, which provides a large accessible electrode surface while facilitating mass transfer between the electrode and the anaerobic sludge. The combination of these materials therefore provides a conductive and structurally suitable electrode configuration for the operation of the bioelectrochemical system under anaerobic digestion conditions [73-75]

All of the electrodes were kept connected to an electrical cable and were insulated using heat-shrink tubing and mechanically reinforced with aluminium tubing to ensure electrode stability during reactor operation.

The electrodes have been connected to a Velleman LABPS3005D power supply to apply 0,8 V operating in constant-voltage mode, while a DT830B Digital Multimeter tester was used to read the potential of the cathodes and anodes.

3.1.2 Reactor configuration

The reactors feature a jacket-wall configuration for independent heating, which is filled with water maintained at the desired temperature by a heating bath that circulates the water with a peristaltic pump.

On the reactors cap was installed a Steinberg System Electrical Stirrer (SBS-ER-3000) and it was set for an intermittent mixing of the sludge to avoid the overheating of the instrument.

A valve on the reactors cap was connected to a water-displacement system for biogas quantification.

The water displacement system is composed of two different plexiglas cylinders (2,6 L and 0,6 L) as presented in **Figure 7**, those were connected with the reactor, each other and with a 1L or 10L aluminium foil gas sampling bag (**Figure 8**).

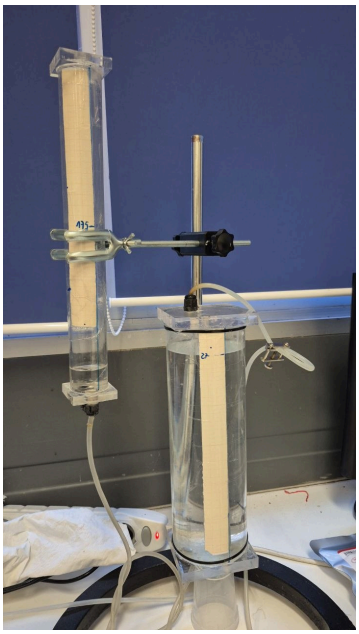

Figure 7 (left) and **Figure 8** (right) respectively the water displaced system composed in two plexiglas tubes with graph paper tape on it and two different size aluminium foil gas sampling bags from AD (left bag) and AD-BES (right bag) from the same sludge period.

The reactors had an inlet port tube that ends in the bottom half of the reactor and two discharge ports to control the level of the sludge inside the reactor.

3.1.3 Sludge source

The feedstock sludge used in this experiment was collected from the wastewater treatment plant operated by ASM Pavia S.p.A., located at Strada Valbona 93, 27100 Pavia, Italy.
Given the impossibility of storing large volumes of sludge due to the limited space of the refrigerator where it was stored at 4°c, the experiment was carried out using 10 different batches of sludge collected at different times. The sludge was collected after the mechanical thickening and its physicochemical characteristics were tested and varied over time depending on plant operating conditions.

3.2. Methods

3.2.1. Reactor operation

Throughout the experimental period (230 operative days), the BES was turned on only after the first batch of sludge used, Sludge 1, until the end of the experiment. A voltage of 0.8 V was applied to the electrodes and their potentials were recorded with the tester before each biogas collection.
Given the volume limits of the quantification system, the measurement of the volume of biogas produced was performed intermittently: once in the morning, but always before feeding the bioreactors, and once in the afternoon, leaving at least 5 hours between the two measurements.
Prior to each feeding, the temperature of each reactor was measured using a digital thermometer to monitor the operating conditions.
The hydraulic retention time (HRT) of the sludge in the reactor was calculated as the ratio between the reactor working volume and the average daily feeding rate.

The reactor had a working volume of 10 L and was fed with 0.76 L of sludge three times per week. The resulting HRT was 30.7 days.

An HRT of approximately 30 days is consistent with the conventional operating range reported in literature for the anaerobic digestion of sewage sludge, for which relatively long retention times are generally required because of the limited biodegradability of the organic matter [56-58].

During feeding, the inlet substrate was fed from the feeding port and an equal volume of digestate is expelled from the reactor through the one of the discharge port, therefore, before feeding, mechanical mixing was temporarily

stopped to allow solids to settle to minimize the mixing between the incoming sludge and the reactor effluent during volume replacement. Agitation was resumed immediately after the feeding procedure and was kept intermittently, mixing for 15 minutes and resting for 15 due to the mixer limits and to avoid overheating and damaging it.

The unsampled portion of the outlet sludge was poured into a special canister which was emptied to the Waste Water Treatment Plant where and when new sludge was taken.

At the end of the experiment the reactors were slowly emptied and cleaned The biofilms attached to the BES reactor were sampled.

3.2.2. Physicochemical analyses

A 40 mL sample was collected weekly from the inlet sludge and from the outlet of both the AD and AD-BES reactors. Samples were stored at 4 °C until analysis.

The collected samples were analysed for pH, electrical conductivity, Chemical Oxygen Demand (COD), Soluble Chemical Oxygen Demand (SCOD), and Total Nitrogen (TN).

The pH and electrical conductivity were measured using calibrated pH and conductivity probes attached to a Hach dual-input digital multi-parameter electrochemistry meter (HQ40D). The instruments were calibrated weekly using pH 4.0, 7.0, and 10.0 standard solutions for the pH probe, while a 1413 µS/cm conductivity standard solution was used for the conductivity probe.

COD and SCOD were determined using the dichromate method, instead Total Nitrogen was measured using the chromotropic acid method. All analyses were performed using HANNA Instruments pre-dosed reagent kits according to the manufacturer's instructions and quantified with a HANNA Instruments Portable Iris Spectrophotometer - HI801

Prior to SCOD determination, samples were filtered through disposable 0.22 μm syringe filters. Samples analysed for COD and Total Nitrogen were diluted 1:10 and 1:30, respectively, to ensure that the measured values fell within the analytical range of the corresponding kits.

3.2.3. Biogas analyses

The composition of the produced biogas was analysed by the Laboratory of Radiation Chemistry and Electron Paramagnetic Resonance Spectroscopy, Department of Chemistry, University of Pavia, under the supervision of Prof. Daniele Dondi, and by the Politecnico di Milano using gas chromatography (GC). Gas samples were collected when the gas bags were filled or before a new sludge was used. The samples were analysed to determine the relative composition of the produced biogas and to assess changes in its quality throughout the experimental period. The analyses performed at the Politecnico di Milano were conducted using a specific calibration procedure developed for biogas analysis, enabling the gaseous components detected by the chromatographic system to be quantified from their analytical response.

The obtained data were used to determine the concentration of the main components of the biogas, CH_4 and CO_2, and to compare the gas composition produced under the different experimental conditions.

3.2.4. Microbial community analyses

To evaluate the effect of BES activation on the microbial community, sludge samples were collected one week before and one week after the activation of the BES system. The samples were sent to the CNR Research Area of Rome for microbiological characterization.

DNA extraction was performed using the DNeasy PowerSoil Pro Kit (QIAGEN, Antwerp, Belgium). DNA concentration and purity were evaluated by 1% agarose gel electrophoresis, NanoDrop 3300 spectrophotometry (Thermo Scientific, Monza, Italy), and Qubit 3.0 fluorimetry (Thermo Fisher Scientific, Waltham, MA, USA). DNA extracts were stored at −20 °C until further analysis.

Microbial community characterization was performed by high-throughput sequencing of the bacterial 16S rRNA gene. The V3–V4 hypervariable region was amplified using primers S-d-Bact-0341-B-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-d-Bact-0785-A-21 (5′-GACTACHVGGGTATCTAATCC-3′), following the protocol described by literature [59]. Sequencing was carried out on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600 cycles) following the manufacturer's standard library preparation protocol with the addition of 20% PhiX control library.

Raw sequence quality was assessed using FastQC as described by literature [60]. Demultiplexing and primer removal were performed using the demux and cutadapt plugins implemented in QIIME2 (version 2025.7). Amplicon Sequence Variants (ASVs) were generated using DADA2 according to the procedure described by literature [61]. Taxonomic assignment was performed using a naïve Bayes classifier trained on the SILVA 138.1 database at 99% sequence similarity.

3.2.5. Volatile fatty acid analyses

Additional sludge samples were analysed by the CNR Research Area of Rome to determine volatile fatty acid (VFA) concentrations.

For VFA determination, 2 mL of the liquid phase were collected from each reactor at the selected sampling times. Samples were filtered through disposable nylon syringe filters (25 mm diameter, 0.22 μm pore size), diluted with deionized water, and acidified by adding oxalic acid to a final concentration of 10% (v/v). Quantitative analysis was performed using external calibration curves.

VFAs were quantified by gas chromatography using an Agilent 8860 gas chromatograph equipped with a flame ionization detector (FID) and an Agilent DB-FFAP capillary column (30 m × 0.53 mm internal diameter, 1.5 μm film thickness). One microlitre of each prepared sample was injected using an SGE liquid microsyringe (Trajan Scientific, Australia). Nitrogen was used as the carrier gas at a flow rate of 10 mL min^{-1}. The injector temperature was set at 200 °C, the interface temperature at 30 °C, and the oven temperature was maintained at 100 °C for 10 min.

3.2.6 Data processing

Experimental data were processed and plotted using Fogli Google (Google LLC, Mountain View, CA, USA). Results are presented as time-course profiles or descriptive summaries. No inferential statistical analyses were performed because each experimental condition consisted of a single reactor, precluding the use of biological replicates for statistical comparison.

4. Results and Discussion

4.1. Biogas and Methane production

Ten different batches of sludge were used as substrate during the operation of the reactor.
Each sludge was fed for a different period based on the sludge quality. On average, the sludge remained inside the reactors for 3 weeks.

4.1.1. Biogas Production Rate

The biogas production rate (BPR) was used to compare the amount of biogas produced between the conventional anaerobic digestion reactor (AD) and the bioelectrochemically assisted anaerobic digestion reactor (AD-BES). This parameter accounts for the different duration of the experimental periods associated with each sludge batch and the working volume of the reactor. For each sludge batch, the BPR was calculated as the total volume of biogas produced during the corresponding experimental period divided by the number of days for which the sludge was used and by the reactor working volume, according to the following equation:

$$\mathbf{BPR = V_{biogas} / (t \times V_R)}$$

Where V_{biogas} is the total volume of biogas produced (m^3), t is the duration of the corresponding sludge batch (days), and V_R is the reactor working volume (m^3).
The BPR was expressed as m^3 of biogas m^{-3} of reactor d^{-1}.

During the operation with Sludge 1 the BES was turned off so this period is considered a reference condition. From Sludge 2 onwards, the AD-BES reactor was operated with an applied cell potential of 0.8 V.
During the first sludge batch the two reactors performed a very similar biogas production with the same approximated BPR values, 0,03 m^3 of biogas m^{-3} of reactor d^{-1} for both AD-BES and AD. From the second sludge batch AD-BES consistently produced more biogas than the conventional AD reactor, as shown in **Chart 1**, outperforming the conventional reactor.

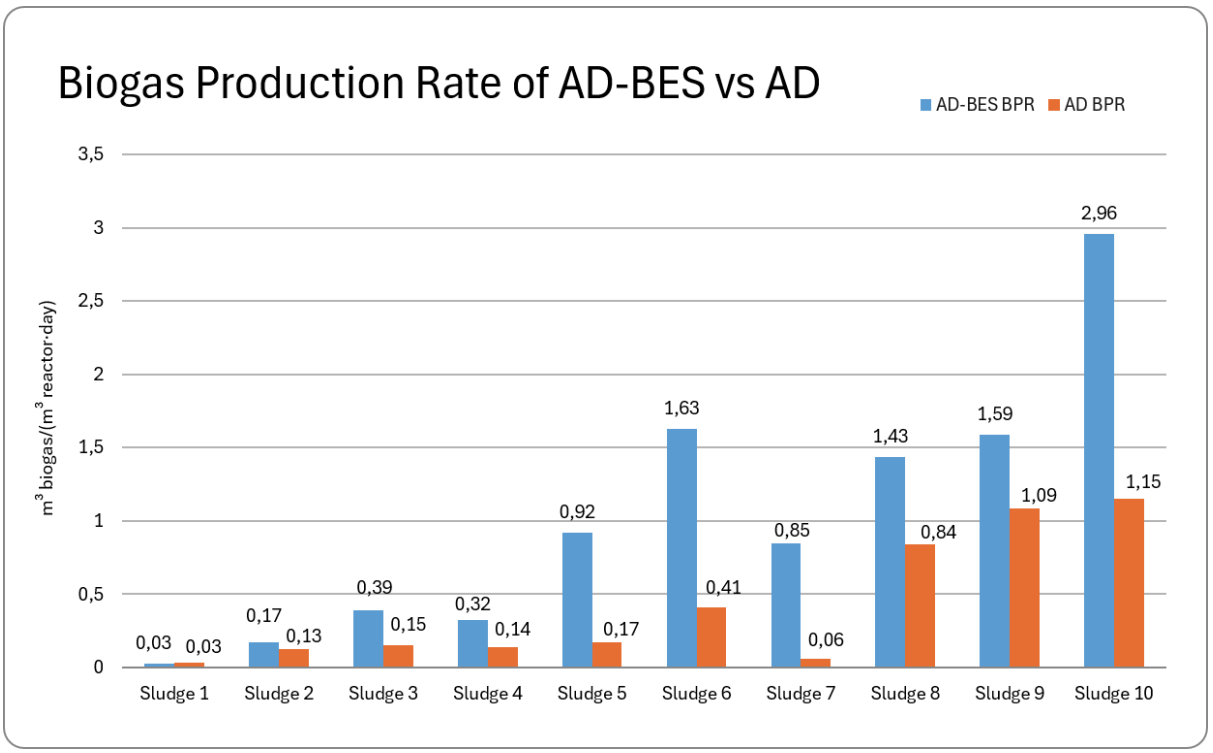

Chart 1: For each sludge was plotted the BPR of the AD-BES and AD, respectively in orange and light blue.

Chart 1 shows that the AD-BES reactor produced more biogas than the AD throughout the experiment period, this difference progressively increases during the experiment up to sludge 7 where the AD-BES produced, 14.5 times more than the AD reactor, as shown in **Table 1**, but this is due to the fact that the conventional reactor decreased the biogas production, to 0.06 cubic m^3 of biogas m^{-3} of reactor d^{-1}, a seventh compared to the BPR of the previous period, comparable to the production of the first control sludge.

This production collapse in both reactors occurred when the new sludge was fed. However, the chemical-physical conditions inside the reactor have not changed enough to be able to attribute a certain cause.

Table 1: AD-BES and AD BPR are shown tabled with the ratio AD-BES/AD BPRs.

	AD-BES BRP	AD BPR	AD-BES/AD
Sludge 1	0,03	0,03	0,88
Sludge 2	0,17	0,13	1,36
Sludge 3	0,39	0,15	2,56
Sludge 4	0,32	0,14	2,37
Sludge 5	0,92	0,17	5,35
Sludge 6	1,63	0,41	3,99
Sludge 7	0,85	0,06	14,54
Sludge 8	1,43	0,84	1,71
Sludge 9	1,59	1,09	1,46
Sludge 10	2,96	1,15	2,57

Considering the entire period following BES activation, the average BPR of AD-BES was approximately 2.5 times higher than that of the conventional AD reactor. The BPR data provide evidence to sustain that the AD-BES configuration enhanced the biogas productivity, but they reveal that it depends also on the sludge batch.

4.1.2. Biogas composition

The composition of the biogas produced is an important fact for evaluating the concentration of methane produced and therefore comparing the two reactors and evaluating whether the presence of the bioelectrochemical system has led to an improvement in the quality of the gas produced. When talking about biomethane production, it must be remembered that downstream of the production system there is an upgrading process to obtain pure biomethane for use as biofuel. If the biogas produced has a high concentration of methane then the cost for upgrading will be lower.

The percentages of the gases analysed from the samples taken, shown in **Chart 2**, are representative of the entire period in which they were produced.

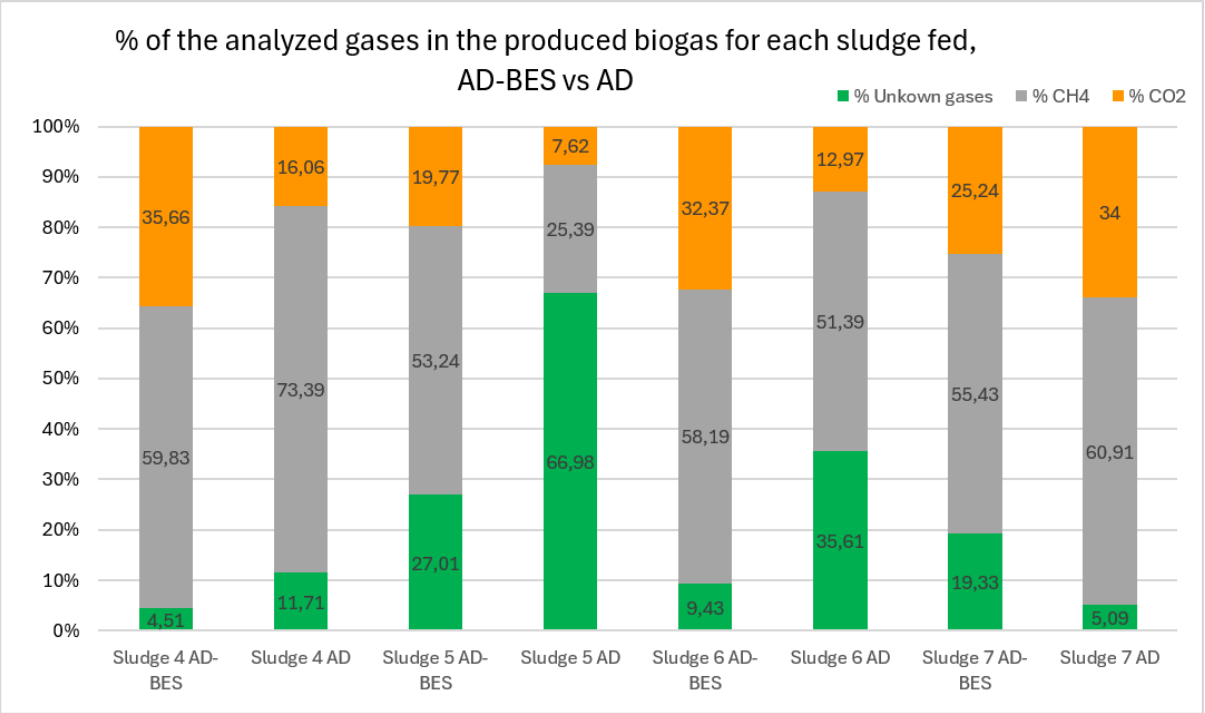

Chart 2: methane, carbon dioxide and other unknown gases concentrations plotted in the biogas produced by AD and AD-BES, side by side, during the analysed sludge batches.

The production of methane, carbon dioxide and other gases of each reactor did not remain constant during the experiment but is dependent on the type of sludge used.

The biogas analysis was performed specifically to quantify methane and carbon dioxide and did not allow for a clear recognition of the fraction represented as unknown, which in one case amounted to over 66% of the biogas produced. This percentage could also be due to an error in the management of gas exchanges causing a gas leak or an error when analyzing the biogas itself.

Thanks to the methane percentages, the liters of biogas produced by the two reactors can be converted into liters of methane and in doing so the AD-BES appears to have produced 2.47 times more methane than the AD reactor, the latter however produced a biogas that reached both the highest and lowest values in the analyzed samples.

Beyond the percentage composition, translating biogas production and quality into an energy-equivalent term is informative given the strategic relevance of energy recovery from sludge digestion for wastewater treatment plants [17, 18]. Using a lower heating value of approximately 9.94 kWh Nm^{-3} CH4 (≈ 35.8 MJ Nm^{-3}) [80], the four sludge batches for which both BPR and biogas composition data were available (Sludge 4-7) show an average energy recovery of approximately 5.2 kWh m^{-3} reactor d^{-1} for AD-BES against approximately 0.9 kWh m^{-3} reactor d^{-1} for AD, corresponding to an average per-batch advantage of roughly 7.7 times, because in these specific batches AD-BES also achieved a methane percentage comparable to or higher than that of AD.

Expressing the result in these terms makes it directly comparable with the energy-efficiency objectives set out in Directive (EU) 2024/3019 for wastewater treatment plants.

However, the present results should not be interpreted as evidence that electrical stimulation alone increased methanogenesis. The AD-BES reactor differed from AD not only because of the applied potential but also because it contained electrode surfaces.

Taking into account the BPR and the concentration of the different gases we can calculate how much of these gases was present in the produced biogas, as shown in **Table 2**.

Table 2: m^3 of biogas m^{-3} of reactor d^{-1} were multiplied by the % of the gases measured in the produced biogas

m^3 of gases m^{-3} of reactor d^{-1}	Sludge 4 AD-BES	Sludge 4 AD	Sludge 5 AD-BES	Sludge 5 AD	Sludge 6 AD-BES	Sludge 6 AD	Sludge 7 AD-BES	Sludge 7 AD
Unknown gases	0,015	0,016	0,248	0,115	0,153	0,145	0,164	0,003
CH4	0,193	0,100	0,490	0,044	0,946	0,209	0,471	0,035
CO2	0,115	0,022	0,182	0,013	0,526	0,053	0,214	0,020

The analysis of biogas composition was restricted to sludge batches 4 to 7, for which analytical data were available. The biogas quality measurements were performed by external collaborating laboratories, and composition data for the preceding and subsequent sludge batches were not made available for the present study.
Therefore, gases concentrations, as well as the corresponding volumetric production rates, were evaluated only for the available sludge batches. This limitation was taken into account when interpreting the results, and no assumptions were made regarding the biogas composition during the remaining experimental periods.
A better analysis would only be possible if more biogas quality data were available with previous and subsequent sludges. This lack reduces the ability to best compare reactor performance and thus evaluate whether the addition of the bioelectrochemical system has led to an overall improvement in biogas production from a qualitative point of view.

4.2. Chemical parameters analysis

4.2.1. COD and SCOD

The Chemical Oxygen Demand (COD), is an indirect way to measure the concentration as g/L of the oxidizable organic matter present in a sample. This organic matter is the organic substrate that can be converted through the Anaerobic Digestion process into biogas.
The COD is also used to evaluate the anaerobic digestion performance as its removal can be used to determine the potential production of methane, but the organic matter has different degrees of biodegradability and a fraction of it can remain not biodegradable with the physico-chemical parameters the process has.

The Soluble Chemical Oxygen Demand (SCOD) is the soluble fraction of the COD and is relevant because during the hydrolysis phase the organic matter is converted into soluble compounds. The chemical composition of the soluble fraction strongly affects its biodegradability, and non-biodegradable compounds may also be soluble.

For the present study, COD removal was calculated according to:

COD removal (%) = ((COD_{in} − COD_{out}) / COD_{in}) × 100

Where COD_{in} and COD_{out} represent the COD concentrations measured in the fed sludge and in the digested sludge, respectively. The same approach was applied to SCOD:

SCOD removal (%) = (($SCOD_{in}$ − $SCOD_{out}$) / $SCOD_{in}$) × 100

The SCOD analysis was not initially planned for the sludge batches preceding sludge 6, as the determination of soluble COD was not part of the original analytical scope of the experiment. During the processing of sludge 6, however, the presence of suspended granular material was observed, consistent with the higher solid content and more heterogeneous physical characteristics of this sludge batch. This observation prompted the inclusion of SCOD analysis to further investigate the soluble fraction of the organic matter and to obtain additional information on substrate availability under these conditions.

Since the samples from the preceding sludge batches had already been discarded at the time this analysis was introduced, SCOD measurements could not be retrospectively performed for sludge batches 1 to 5. Consequently, SCOD results are available only from sludge batch 6 onwards and were interpreted accordingly.

An important result of this experiment is that the higher methane production observed in AD-BES was not accompanied by a proportionally higher COD or SCOD removal, as shown in **Chart 3** and **Chart 4** respectively.

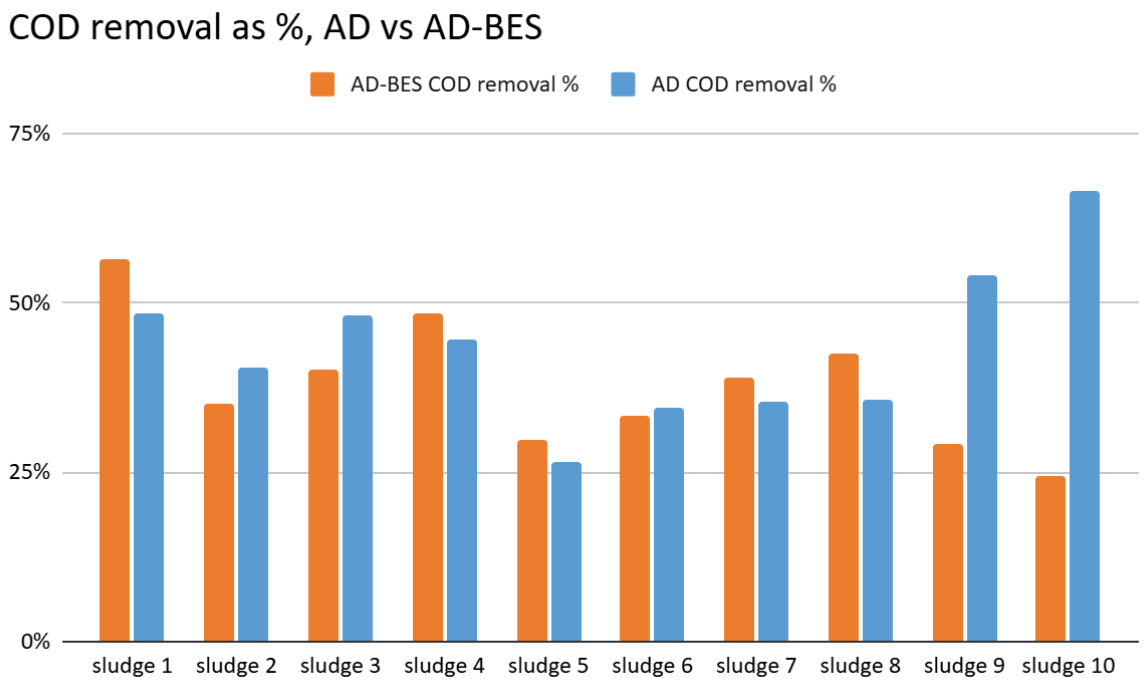

Chart 3: AD-BES and AD COD removal as % for each batch of sludge.

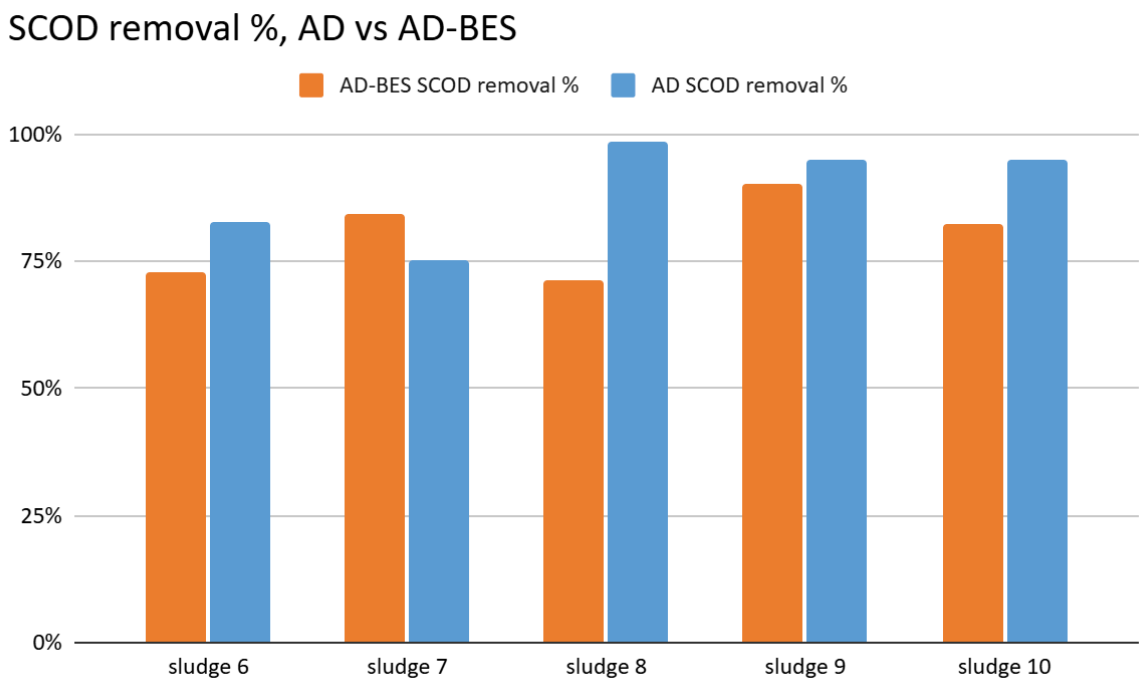

Chart 4: AD-BES and AD SCOD removal as % for each batch of sludge.

In contrast to the chart of the SCOD removal, the COD removal chart shows that the two reactors did not follow the same trend in organic matter removal during all the period, with a diversion from the 7th sludge batch.

In particular, AD-BES in the last two batches recorded a decrease in COD removal, going from 42.53% to 29.09% from sludge 8 and 9 respectively, but biogas production improved, almost doubling during the last sludge compared to the penultimate one.
In contrast, AD from sludge 8 to sludge 9 increased removal, going from 35.83% to 54.06% but without obtaining a particular biogas increase, going from a BPR of 0,84 m^3 of biogas m^{-3} of reactor d^{-1} to 1.09 m^3 of biogas m^{-3} of reactor d^{-1} from sludge 8 to 9 and reaching 1.15 m^3 of biogas m^{-3} of reactor d^{-1} in sludge 10.
Even for the removal of SCOD AD it performs better than AD-BES, removing an average of 0.9 percentage points more (89.22% vs 80.23% respectively).
It is therefore hypothesized that BES electrochemical stimulation does not accelerate hydrolysis and acidogenesis but optimizes the metabolic conversion yield of the organic fraction already hydrolyzed to the methanogenic pathway.

4.2.2. Volatile fatty acids

Volatile fatty acids (VFAs) are key intermediates of Anaerobic Digestion and this class includes acetate, propionate, butyrate and the other branched-chain fatty acids.
They are mainly generated during the acidogenic and acetogenic stages and are consumed by Acetoclastic methanogens to produce methane.
Their concentration and composition can therefore provide information on the balance between acid production and methanogenic consumption and are commonly used as indicators of process stability as their excessive VFAs accumulation can decrease the activity of methanogens [14, 62]

The VFA analyses were performed by the National Research Council of Italy (CNR), Rome. Due to the availability of the external analytical service, it was not possible to analyse all sludge batches collected during the experimental period. Therefore, VFA data were available only for Sludge 5 and 6.

In the Sludge 5 AD-BES contained acetate, propionate, isobutyrate, butyrate, isovalerate and valerate, resulting in a total VFA concentration of approximately 0.1 g/L of COD, whereas AD showed the presence of acetate and propionate, resulting in a total of approximately 0.01 g/L of COD.

Meanwhile in the Sludge 6 AD-BES showed an acetate concentration of 0.01 g/L of COD with no other VFAs detected. The conventional AD reactor showed the presence of acetate and propionate, resulting in a total measured VFA concentration of 0,01g/L of COD and no other VFA detected.

The transition from Sludge 5 to Sludge 6 was characterized in AD-BES mainly by a reduction of the diversity of the detected compounds in the VFA profile, from a mixture of several fermentation intermediates to acetate alone, whereas AD maintained a consistently low and less diverse VFA profile.

This indicates that, in the AD-BES reactor, during the Sludge 5 batch there was a considerably large pool of accumulated fermentation intermediates but this was not sufficient to inhibit the methanogenic activity the BPR in this period rose.

4.2.3. Total Nitrogen

The measurement of total nitrogen (TN) was included to evaluate the nitrogen content of the sludge and to assess its variation during anaerobic digestion. Nitrogen is an essential nutrient for microbial growth and is required for the development and activity of the microorganisms involved in Anaerobic Digestion and is mainly found in proteins and therefore in the cellular biomass.

During the Anaerobic Digestion the nitrogen present in the organic matter can be hydrolysed and mineralised to free ammonium, principally, which exists in equilibrium with free ammonia (NH_3). At sufficiently high concentrations, free ammonia can inhibit microbial activity and therefore the methane production. [14, 23-25, 62]

For this reason the measurement of TN is relevant for evaluating the potential influence of nitrogen on the Anaerobic Digestion performance.

The TN concentration of the incoming sludge varied considerably among the different sludge batches, as shown in **Chart 5**, ranging from from 1.38 to 2.81 g/L, with an overall mean of 2.02 ± 0.42 g/L.

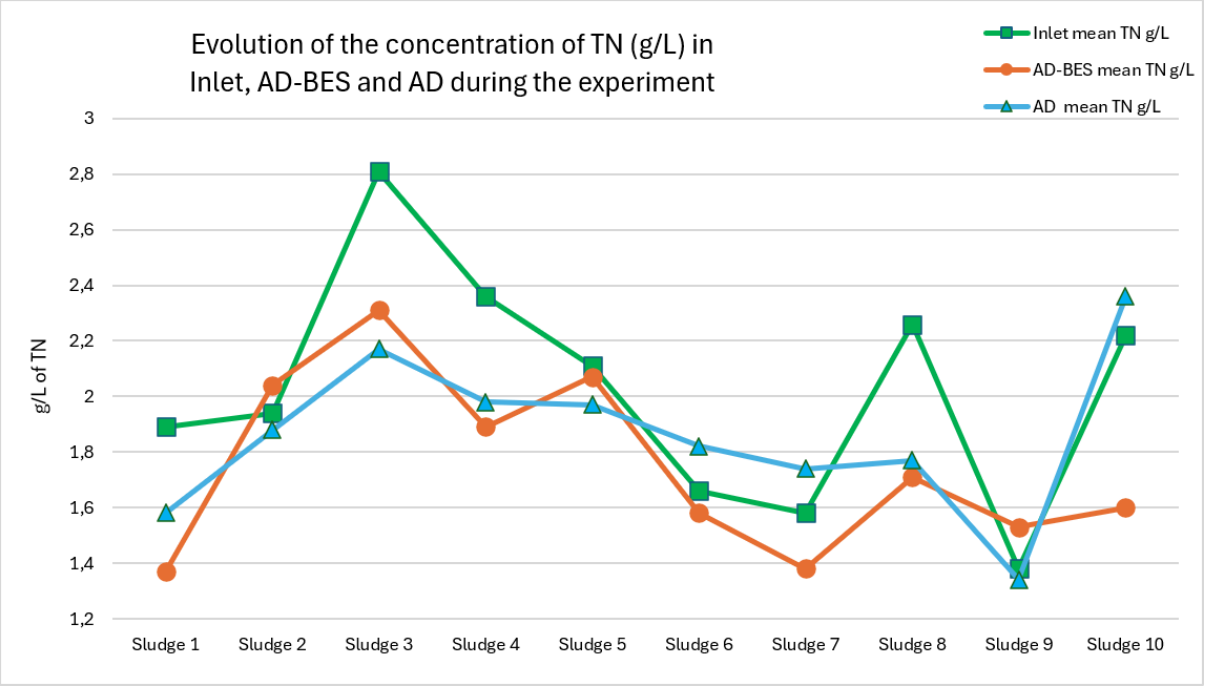

Chart 5: Evolution of the concentration of TN (g/L) in Inlet, AD-BES and AD during the experiment

The TN concentrations measured in the effluent of AD-BES ranged from 1.37 to 2.31 g/L, with a mean value of 1.75 ± 0.32 g/L, whereas AD showed values between 1.34 and 2.36 g/L, with a mean of 1.86 ± 0.29 g/L.

Considering the paired inlet–outlet values, the average decrease was approximately 12% for AD-BES and 6% for AD, but this reduction should not be interpreted directly as a removal, since anaerobic digestion mainly promotes the mineralisation and redistribution of organic nitrogen rather than its complete elimination from the system.

4.3. Physicochemical parameters

The monitoring of physicochemical parameters was performed to assess the stability of the anaerobic digestion process and to identify possible changes in the chemical environment of the two reactors.

In particular, pH, electrical conductivity and temperature were considered because they provide complementary information on the conditions experienced by the microbial community. pH is a critical parameter in anaerobic digestion, particularly during methanogenesis, since methanogenic archaea are generally most active under near-neutral conditions, with optimal values commonly reported around pH 6.8–7.2 [14, 62].

Electrical conductivity, instead, provides an indirect indication of the concentration and mobility of dissolved ionic species in the sludge and can therefore reflect changes in the soluble inorganic fraction during digestion.

Temperature represents a major biological control parameter in anaerobic digestion because it directly affects the growth rate, metabolic activity and community structure of the microorganisms responsible for the four phases of Anaerobic Digestion.

4.3.1 pH

The pH values remained relatively stable in both reactor configurations throughout the experiment as shown in **Chart 6**.

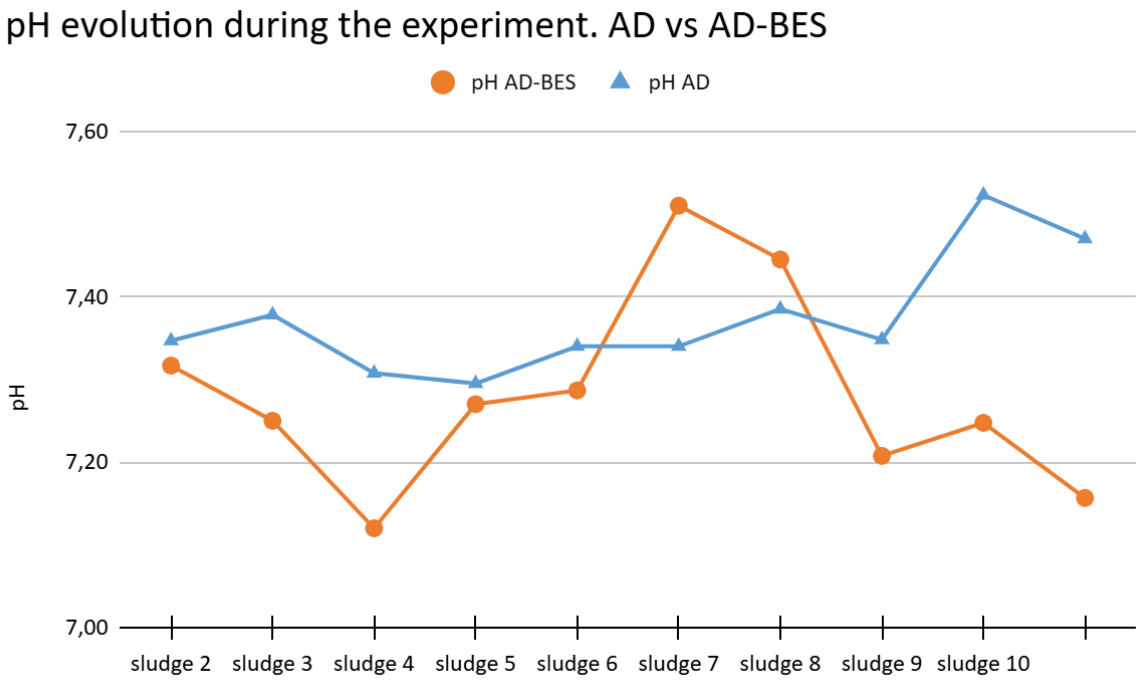

Chart 6: The evolution of AD-BEs and AD pH for each sludge batch.

AD-BES showed values between 7.12 and 7.51, with a mean of 7.28 ± 0.12, whereas AD ranged from 7.30 to 7.52, with a mean of 7.38 ± 0.07. The two reactors therefore remained within a near-neutral pH range throughout the experimental period. No progressive acidification was observed, despite the substantial differences in biogas production observed between the sludge batches and the presence of a large variety of VFAs remained insufficient to cause a significant disturbance of the acid–base equilibrium of the digesters and therefore negatively affected methanogenic activity [14, 62, 63].

4.3.2. Electrical conductivity

Electrical conductivity showed a substantially different behaviour from pH, as shown in **Chart 7**.

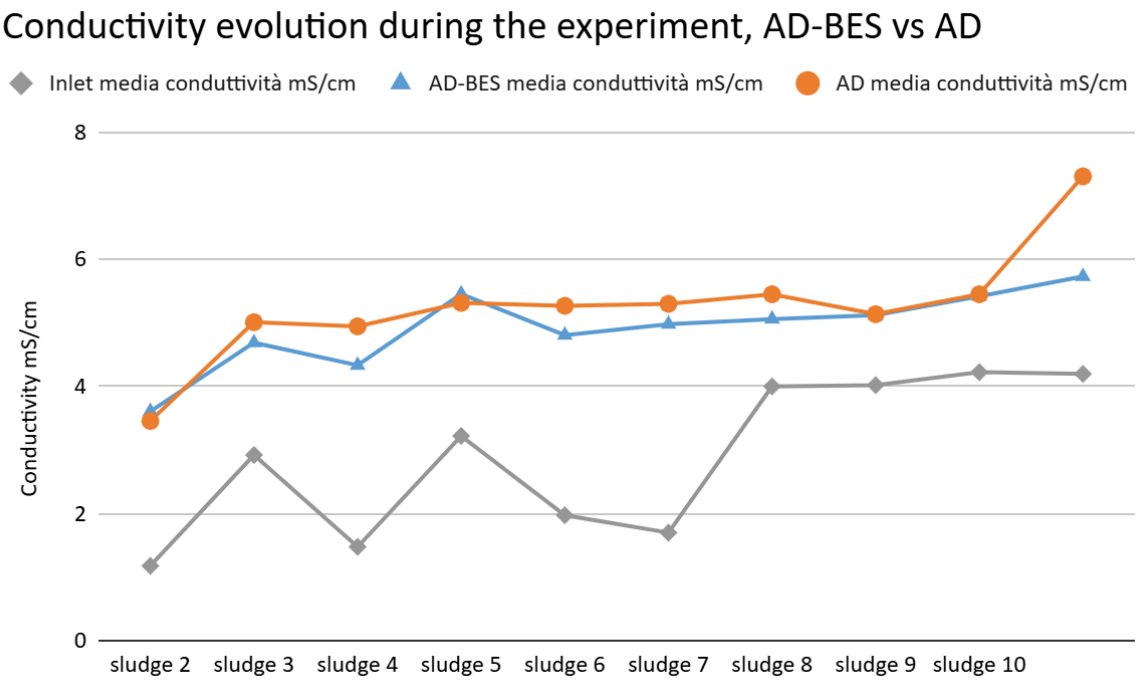

Chart 7: The evolution of AD-BEs and AD conductivity for each sludge batch.

The conductivity of the incoming sludge varied from 1.18 to 4.22 mS/cm, with an average value of 2.89 ± 1.21 mS/cm. After digestion, conductivity increased in both reactors. AD-BES showed values between 3.61 and 5.73 mS/cm, with an average of 4.92 ± 0.61 mS/cm, whereas AD ranged from 3.46 to 7.31 mS/cm, with an average of 5.27 ± 0.92 mS/cm.

The increase in conductivity after digestion indicates a greater concentration or availability of dissolved ionic species in the digested sludge. The simultaneous increase in conductivity can result from the biological degradation of organic matter and the associated mineralisation and solubilisation of inorganic compounds and reflect the changes in the sludge composition.

4.3.3. Temperature

The temperature measured in the two reactors showed a consistent difference throughout the monitored period, as shown in **Chart 8**.

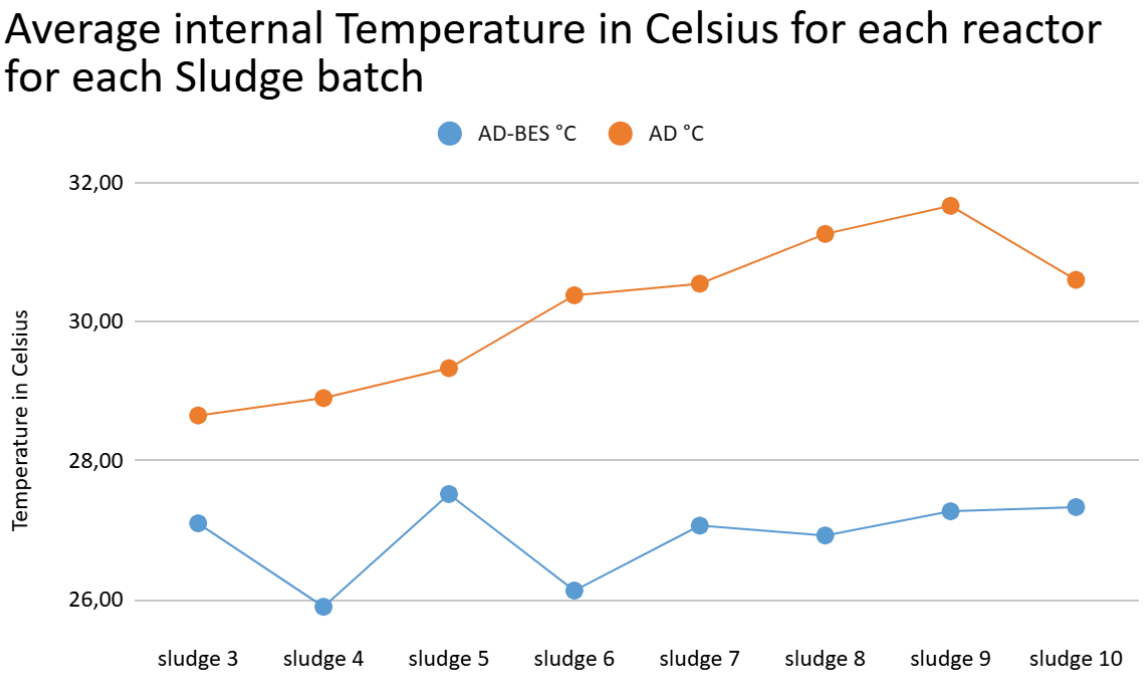

Chart 8: Average internal temperature in Celsius for each reactor for each Sludge batch.

The internal temperature measured in the AD-BES reactor ranged from 25.90 to 27.52 °C, with an average of approximately 27.0 °C, whereas AD ranged from 28.65 to 31.66 °C, with an average of approximately 30.2 °C. Throughout the experiment the internal temperature of the AD-BES reactor remained 3 degrees lower than that of the AD reactor, however both systems operated at temperatures below the conventional mesophilic optimum (35°C) [64, 65].

An abrupt temperature shift can affect microbial community structure and reduce methane production, whereas a stable, even sub-optimal, temperature can favour the establishment of a microbial community adapted to those conditions [1, 65-68].

AD-BES produced substantially more biogas despite operating at a lower temperature than AD. If temperature were the dominant factor controlling reactor productivity under the investigated conditions, the warmer AD reactor would generally be expected to exhibit a kinetic advantage. Instead, the opposite trend was observed, with AD-BES reaching markedly higher BPR values during most of the experiment.

4.4. Electrochemical analysis

In a bioelectrochemically assisted anaerobic digestion system, the applied potential provides an additional electron-transfer pathway that can modify the thermodynamic conditions experienced by electroactive microorganisms and methanogens. The electrode surfaces can also act as sites for microbial attachment and biofilm development, potentially favouring electron transfer between microbial populations and the electrodes.
Therefore the applied cell voltage and the individual electrode potentials are relevant for interpreting the biological performance of the AD-BES reactor.

A nominal cell potential of 0.80 V was imposed with a voltage generator which recorded an average current of 0,001 ± 0,0014 A.
However, the actual electrochemical conditions were evaluated by monitoring both the WE–CE potential and the potentials of the individual electrodes with respect to the Ag/AgCl reference electrode with a dedicated tester.

During the initial measurements, the measured WE–CE potential remained stable and close to the imposed value, but since the Sludge 5 was fed, the WE–CE potential decreased and became more variable, ranging from 432.07 ± 262.93 to 651.42 ± 64.82 mV.

The individual electrode potentials relative to the Ag/AgCl reference electrode showed an even more pronounced evolution. Initially, the WE-RE remained at positive potentials (417.50 ± 82.62 to 494.40 ± 30.31 mV), while the CE-RE showed negative values (−352.80 ± 81.57 to −272.20 ± 30.67 mV).
During the period in which the Sludge 5 was fed, the WE-RE potential shifted towards negative values, reaching −438.07 ± 724.62 mV, while the CE-RE shifted towards positive values, stabilizing approximately 805.47 ± 1.68 mV.

This marked redistribution of the electrode potentials indicates a substantial modification of the electrochemical conditions at the electrode–electrolyte interfaces during reactor operation. However, these changes should not be interpreted as a direct inversion of the functional role of the electrodes, since the applied cell voltage remained imposed between the working and counter electrodes. Rather, the observed potential shifts suggest that the imposed voltage was progressively distributed differently between the two electrode interfaces. The very high standard deviation observed for the WE-RE measurement during the Sludge 5 feeding period also indicates a pronounced increase in the variability of the working-electrode potential, suggesting less stable electrochemical conditions at the corresponding electrode–electrolyte interface.

In bioelectrochemical anaerobic digestion systems, electrode potentials are closely associated with microbial activity and the development of electroactive biofilms. Microbial attachment and extracellular electron transfer can alter the local electrochemical environment at the electrode surface, while changes in the microbial community and biofilm structure can, in turn, affect the measured electrode potential. Therefore, the temporal evolution of the WE-RE and CE-RE potentials observed in the present study may reflect the progressive adaptation of the electroactive microbial community and changes in the electrode–biofilm interface during reactor operation. Nevertheless, because electrode potential is influenced by several concurrent physicochemical and biological processes, the observed potential shifts should be considered as evidence of a changing electrochemical environment rather than as direct proof of a specific electron-transfer mechanism [69].

4.5. Microbial analysis

Microbial community characterization, through the use of high-throughput 16S rRNA gene sequencing, was performed to obtain information on the microorganisms involved in the conversion of organic matter and in the final methanogenic steps of anaerobic digestion.
The microbial characterization in the present study was performed in collaboration with the National Research Council of Italy (CNR), to which Sludge 1 and Sludge 2 (Inlet and outlet samples), corresponding to before and after the BES activation, samples were sent. It was not possible to characterize microbiologically every sludge batch collected during the experimental period.

The archaeal community was dominated by methanogenic genera in all four analysed samples, but the relative distribution of these populations differed between the AD and AD-BES reactors, as shown in **Chart 9**.

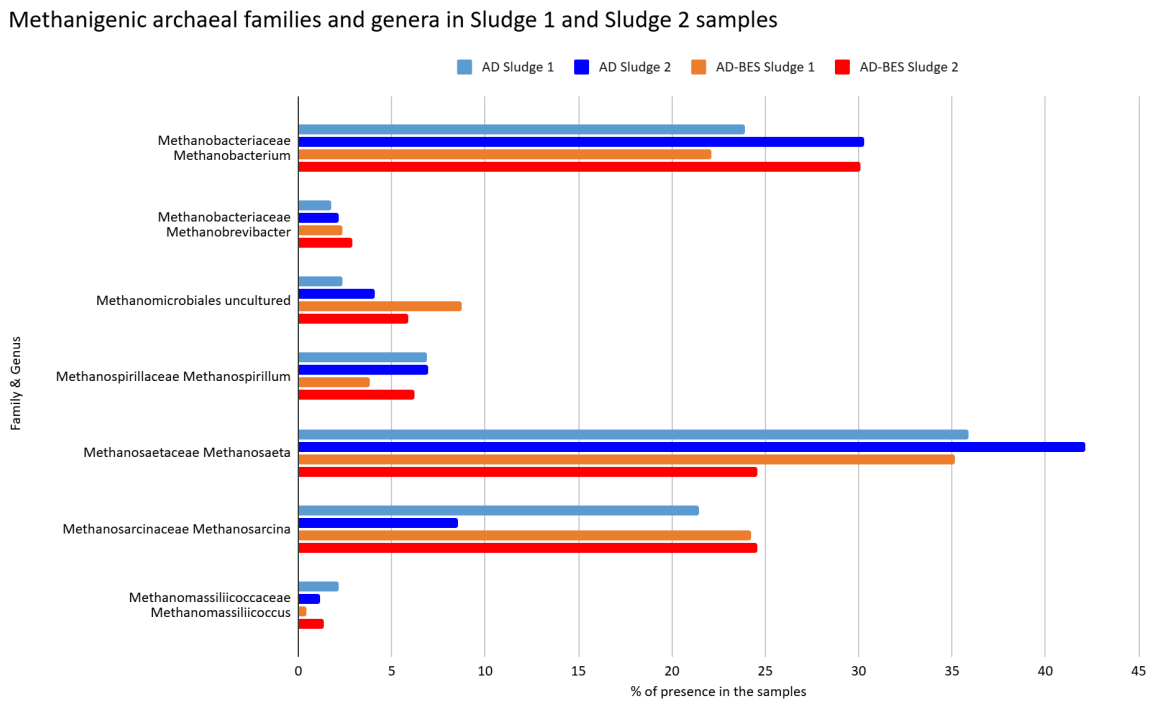

Chart 9: Evolution of the archaeal families and genera from Sludge 1 to Sludge 2, respectively before and after the BES activation in the AD-BES reactor.

The **Chart 9** shows that the two systems exhibit different variation and composition of the methanogenic community between the two sampled sludges.

The changes in *Methanobacterium* were particularly consistent between the two configurations. Its relative abundance increased by approximately 6 percentage points in both AD and AD-BES, reaching approximately 30% in the second sample.

Methanobacterium is a hydrogenotrophic methanogen that uses H_2 and CO_2 for methane formation and contributes to the removal of hydrogen from the anaerobic environment [32].

The presence and enrichment of hydrogenotrophic methanogens is important for syntrophic metabolism because H_2 consumption by methanogens can maintain a low hydrogen partial pressure, favoring thermodynamically constrained reactions that are performed by bacteria in earlier stages of anaerobic digestion [31, 32, 70]

The similar increase observed in both reactors suggests a progressive establishment of a microbial community which has adapted to the chemical and physical conditions during the reactor's operation.

A more distinct difference between the two configurations was observed for *Methanosaeta*. In the conventional AD reactor, its relative abundance increased from 35.9% in AD 1 to 42.1% in AD 2, making it the dominant archaeal genus in the second AD sample. Conversely, in the AD-BES reactor, *Methanosaeta* decreased from 35.2% to 24.6%.

This archaea genus is primarily associated with acetoclastic methanogenesis, converting acetate into methane and carbon dioxide, and its increase in the AD reactor and its decrease in the AD-BES reactor can suggest that the methanogenic community followed a different trajectory in the presence of the bioelectrochemical system.

Another difference between the two configurations was observed for *Methanosarcina*.

In contrast to before, this genus grew more in the AD-BES reactor than in the AD reactor and is optimized that the presence of the bioelectrochemical system has influenced this difference but is unknown why it didn’t grow in the AD reactor.

The bacterial community also showed different temporal patterns between the two reactor configurations, as shown in **Chart 10**.

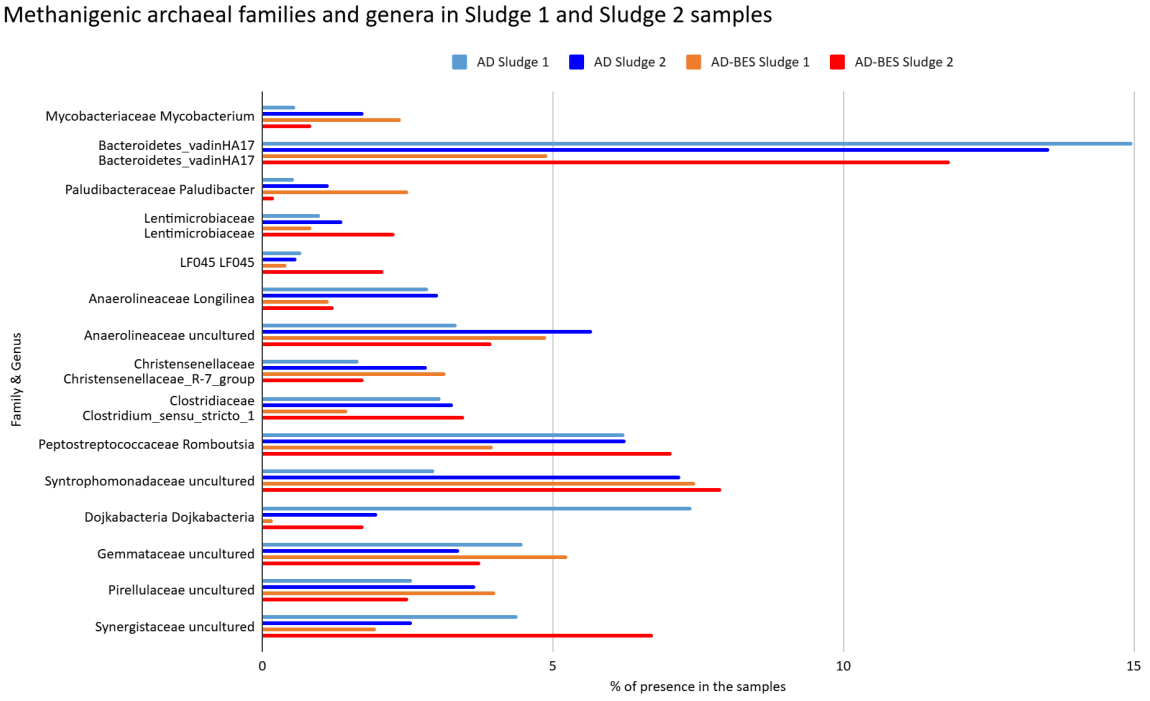

Chart 10: Evolution of the bacterial families and genera from Sludge 1 to Sludge 2, respectively before and after the BES activation in the AD-BES reactor.

In the AD-BES system, several of the main bacterial groups increased in relative abundance in the Sludge 2 batch, after the BES activation, compared to the Sludge 1 batch. *Bacteroidetes_vadinHA17* increased from 4.9% to 11.8%, while *Romboutsia* increased from 4.0% to 7.0% and the uncultured Synergistaceae lineage from 2.0% to 6.7%. Syntrophomonadaceae remained highly represented, with a slight increase from 7.5% to 7.9%.

In contrast, the conventional AD reactor showed a different pattern: *Bacteroidetes_vadinHA17* decreased from 15.0% to 13.5%, Synergistaceae from 4.4% to 2.6%, and *Dojkabacteria* from 7.4% to 2.0%, while *Romboutsia* remained stable at 6.2%. These results suggest that the bacterial communities, as the methanogen's community, during the experiment reactor operation.

None of the classical exoelectrogenic microorganisms, such as Geobacter and Shewanella, were detected among the microorganisms identified in the present samples [71]. Therefore, microbiological results do not provide evidence for the enrichment of a typical exoelectrogenic population in the AD-BES reactor, so it can be hypothesized that the bioelectrochemical setup did not directly select microorganisms involved in extracellular electron transfer (EET) or interspecies direct electron transfer (DIET).

4.6. Process Stability Assessment: Resistance and Resilience

Process stability can be assessed based on the response of a biological system to a disturbance. In this context, two complementary properties are particularly relevant: Resistance, defined as the ability of a system to maintain its performance during a disturbance, and Resilience, defined as its ability to recover its previous functional state after the disturbance. In order to characterize the reactor stability, the magnitude of the performance drop and the time required to return to the condition prior to disturbance is considered [76-79].

The transition from sludge 6 to sludge 7 was considered a disturbance, unexpected, a perturbation hypothesized to be induced by the fed substrate.

Both reactors underwent a marked reduction in biogas production when Sludge 7 was introduced, while the physicochemical conditions did not show sufficient changes to establish a specific cause of the event. Therefore, Sludge 6 was considered the pre-disturbance reference condition, Sludge 7 the disturbance period and the subsequent Sludge batches the recovery phase.

Resistance was assessed using the relative change in the Biogas Production Rate (BPR) between Sludge 6 and Sludge 7 and it was calculated according to:

$$\textbf{Relative change (\%)} = [(BPR_7 - BPR_6) / BPR_6] \times 100$$

For the AD-BES reactor, the BPR decreased from 1.63 to 0.85 m^3 biogas m^{-3} reactor d^{-1}, corresponding to:

$$\textbf{Relative change AD-BES} = [(0.85 - 1.63) / 1.63] \times 100 = -47.9\%$$

Thus, the AD-BES reactor retained approximately 52.1% of its pre-disturbance biogas production during Sludge 7.

The conventional AD reactor showed a substantially stronger decrease. Its BPR decreased from 0.41 to 0.06 m^3 biogas m^{-3} reactor d^{-1}:

$$\textbf{Relative change AD} = [(0.06 - 0.41) / 0.41] \times 100 = -85.4\%$$

Consequently, the AD reactor retained only approximately 14.6% of its pre-disturbance biogas production. The magnitude of the performance loss was therefore approximately 1.8 times greater in AD than in AD-BES. These results indicate that the AD-BES configuration exhibited substantially greater resistance to the Sludge 7 disturbance because it maintained a considerably larger fraction of its previous biogas production.

The response to the disturbance can be further evaluated by examining the subsequent recovery of BPR. Following Sludge 7, the AD-BES reactor increased its BPR from 0.85 to 1.43 m^3 biogas m^{-3} reactor d^{-1} during Sludge 8, corresponding to a recovery to:

Recovery relative to baseline (%) = (BPR_8 / BPR_6) × 100

AD-BES recovery relative to baseline (%) = (1.43 / 1.63) × 100 = 87.7%

The BPR subsequently increased to 1.59 m^3 biogas m^{-3} reactor d^{-1} during Sludge 9:

AD-BES = (1.59 / 1.63) × 100 = 97.5%

Therefore, the AD-BES reactor recovered almost completely to its pre-disturbance performance within two sludge batches.

This recovery pattern indicates that the reactor was able to progressively restore its previous biogas production following the pronounced reduction observed during Sludge 7.

The conventional AD reactor showed a different recovery trajectory. Following the disturbance, its BPR increased from 0.06 to 0.84 m^3 biogas m^{-3} reactor d^{-1} during Sludge 8. Relative to the pre-disturbance value of 0.41 m^3 biogas m^{-3} reactor d^{-1}, this corresponds to:

AD = (0.84 / 0.41) × 100 = 205.9%

Thus, the AD reactor not only recovered its pre-disturbance production during Sludge 8, but exceeded it by approximately 106%. The BPR subsequently increased to 1.09 and 1.15 m^3 biogas m^{-3} reactor d^{-1} during Sludge 9 and Sludge 10, respectively.

Therefore, the results support the interpretation that the BES configuration provided a greater ability to buffer the immediate effect of sludge disturbance 7 but led to a slower recovery likely due to a variation in bacterial population that required more time to readjust to the BES-influenced environment. It is however possible that even just the presence of additional surfaces, the electrodes, influenced the Resilience and Resistance

5. Conclusions

This study investigated the effect of integrating a bioelectrochemical system (BES) into the anaerobic digestion of municipal sewage sludge by comparing a conventional anaerobic digestion reactor (AD) with a BES-assisted reactor (AD-BES) operated in parallel for 230 days and supplied with ten sludge batches. Considering the entire period following BES activation, AD-BES produced, on average, approximately 2.5-fold more biogas than the conventional reactor. This increase in gas productivity represents the main effect associated with BES integration and indicates that the application of an external potential can enhance the conversion of the substrate into gaseous products under the investigated operating conditions.

The increase in biogas production was not accompanied by a corresponding enhancement in bulk organic matter removal. On the contrary, AD consistently showed higher COD and SCOD removal efficiencies than AD-BES, with an average difference in SCOD removal of approximately 9 percentage points (89.22% vs. 80.23%). These results indicate that the higher gas production observed in AD-BES cannot be explained by a generalized improvement in substrate degradation. Rather, the two reactors appear to have differed in the fate of the soluble and readily biodegradable intermediates generated during anaerobic digestion. The results are therefore consistent with the hypothesis that BES integration preferentially enhanced the conversion of available fermentation intermediates towards biogas, particularly methane, rather than accelerating the upstream hydrolysis and degradation of the bulk organic matter.

The available VFA data provide further support for this interpretation. During the Sludge 5 feeding period, AD-BES showed a broader and more concentrated pool of volatile fatty acid intermediates than AD. Despite this accumulation, biogas production continued to increase, indicating that the larger pool of intermediates did not result in an evident inhibition of the methanogenic process. The maintenance of near-neutral and relatively stable pH values in both reactors further indicates that acidification was not sufficient to overcome the buffering capacity of the systems. Taken together, these observations suggest that AD-BES was able to maintain effective conversion of fermentation products despite a greater accumulation of intermediates, although the limited temporal availability of VFA measurements prevents this relationship from being established quantitatively over the entire experimental period.

The microbial community data provide a possible biological explanation for the different behaviour of the two reactors. Both systems showed an enrichment of the hydrogenotrophic genus *Methanobacterium*, indicating that hydrogenotrophic methanogenesis remained an important component of the methanogenic community under both conditions. However, differences were observed among the acetoclastic and metabolically versatile methanogens. *Methanosaeta* increased in AD but decreased in AD-BES, whereas *Methanosarcina* showed a preferential increase in AD-BES. The enrichment of *Methanosarcina* is particularly relevant because members of this genus are generally characterised by broad substrate utilisation capabilities and greater tolerance to environmental fluctuations than strictly acetoclastic methanogens. This community shift is therefore consistent with the ability of AD-BES to sustain elevated biogas production despite the presence of a comparatively larger pool of fermentation intermediates. Nevertheless, the sequencing results represent a limited number of sampling points and should therefore be interpreted as supporting evidence rather than definitive proof that the observed microbial shift caused the increase in productivity.
No classical exoelectrogenic genera such as *Geobacter* or *Shewanella* were detected in either reactor. Consequently, the results do not provide evidence for a direct electron-transfer pathway mediated by these genera.

However, the absence of these taxa cannot exclude other forms of extracellular or interspecies electron transfer, particularly because electron-transfer mechanisms may involve microbial groups that were not resolved at the genus level. An alternative explanation is that the applied potential influenced the methanogenic community indirectly, for example by modifying the availability of hydrogen or other reduced intermediates at the electrode–biofilm interface. Further electrochemical and molecular analyses would be required to distinguish between these possible mechanisms.

The electrochemical measurements revealed a pronounced redistribution of the individual electrode potentials during the Sludge 5 period. The shift in the WE-RE and CE-RE potentials occurred during the same period in which the highest VFA diversity and concentration were observed in AD-BES. This temporal correspondence suggests that changes in the electrochemical state of the reactor may have been associated with changes in the metabolic state of the microbial community.

However, the available measurements do not establish the direction of causality. The simultaneous increase in the variability of the working-electrode potential further indicates that the electrode–electrolyte interface became less stable during this period, potentially reflecting changes in biofilm development, microbial activity, or local physicochemical conditions.

The conductivity measurements provide an additional indication that the two reactors followed different patterns of substrate conversion. Electrical conductivity increased more strongly in AD after digestion, in agreement with its higher COD and SCOD removal. A greater degree of organic matter mineralisation may contribute to the release or accumulation of soluble ionic species in the digestate. In AD-BES, by contrast, the lower organic matter removal together with higher biogas production is consistent with a larger fraction of the available biodegradable intermediates being channelled towards gaseous products rather than remaining within the soluble fraction of the digestate. This interpretation should nevertheless be considered indicative, since conductivity is affected by several ionic and physicochemical processes and cannot be directly used to determine the fate of individual substrate components.

An additional factor supporting the interpretation of the BES effect is the difference in reactor temperature. AD-BES achieved its higher biogas productivity while operating, on average, approximately 3 °C below AD. Thus, the productivity advantage of AD-BES cannot be attributed to a higher operating temperature. Although the temperature difference may have influenced the absolute performance of both systems, the fact that the reactor with the lower average temperature nevertheless exhibited higher biogas production strengthens the hypothesis that factors associated with BES operation, including the applied potential and the associated microbial adaptation, contributed substantially to the observed productivity difference.

The resistance and resilience analysis performed around the Sludge 6–7 disturbance further highlights a functional difference between the two microbial systems. AD-BES retained approximately 52.1% of its pre-disturbance biogas production, compared with only 14.6% for AD, indicating substantially greater resistance to the perturbation. This greater resistance is consistent with the enrichment of *Methanosarcina* and hydrogenotrophic methanogens observed in AD-BES, as a community with broader metabolic capabilities may be better able to maintain methanogenic activity under transient changes in substrate availability and process conditions. The subsequent recovery, however, followed a different pattern. AD-BES progressively recovered to 87.7% and then 97.5% of its pre-disturbance production over the following batches, without substantially exceeding its baseline. AD, in contrast, showed a more rapid rebound and reached approximately 106% of its pre-disturbance production. Although this indicates greater short-term recovery capacity in AD, the absolute biogas production of AD in the subsequent sludge batch remained below that achieved by AD-BES. Overall, the results therefore suggest a trade-off between resistance and resilience: AD-BES was less affected by the initial perturbation but required more time to return to its pre-disturbance state, whereas AD experienced a stronger initial decline but subsequently recovered more rapidly.

Taken together, the chemical, microbial, electrochemical and process-level observations converge on a common interpretation. BES integration increased biogas productivity and improved resistance to a transient operational perturbation, but did not enhance bulk organic matter removal or demonstrably improve biogas quality. The higher productivity therefore appears to have resulted primarily from a change in the conversion of available intermediates rather than from a general acceleration of the entire anaerobic digestion pathway. The concomitant enrichment of *Methanosarcin*a, the persistence of hydrogenotrophic methanogens, the accumulation of fermentation intermediates without an evident loss of methanogenic activity, and the redistribution of electrode potentials are all consistent with a BES-induced modification of the methanogenic stage of the process. However, these relationships remain associative because the different datasets were not collected at sufficient temporal resolution to establish direct causal links.

From an applied perspective, these findings indicate that AD-BES may represent a promising strategy for intensifying methane recovery from sewage sludge within a given reactor volume and for increasing the robustness of the methanogenic process against transient disturbances. Importantly, BES integration should not be regarded as a substitute for strategies specifically designed to increase the hydrolysis and overall degradation of organic matter. Rather, it may represent a complementary intensification strategy that modifies the conversion efficiency of downstream anaerobic intermediates and potentially improves process stability while maintaining the existing reactor configuration.

The conclusions of this study must be considered in light of several experimental limitations. In particular, biogas composition, VFA profiles and microbial community data were only available for a limited subset of the ten sludge batches because these analyses were performed through external analytical services. Consequently, the data were not sufficient to continuously monitor the relationships between substrate degradation, intermediate accumulation, microbial community dynamics, and electrochemical behavior.

The experimental work presented in this thesis is currently being extended through a new experimental phase aimed at further investigating the mechanisms underlying BES-assisted anaerobic digestion. In this ongoing study, two BES-assisted reactors are operated under comparable conditions, with magnetite (Fe_3O_4) added to one of the systems. Magnetite has been investigated as an electrically conductive material capable of promoting direct interspecies electron transfer (DIET) between syntrophic microorganisms, providing an alternative pathway for electron exchange between fermentative bacteria and methanogens [81, 82]. Previous studies have shown that Fe_3O_4 supplementation can reduce the accumulation of volatile fatty acids and enhance methane production during anaerobic digestion, including during the treatment of sewage sludge [81]. Similarly, Fe_3O_4-based conductive materials have been associated with changes in the abundance of electroactive microorganisms and methanogenic populations, together with improved methanogenic performance, supporting a role for magnetite-mediated DIET in anaerobic digestion [82]. Other studies have also reported enhanced methane production and improved conversion of fermentation intermediates following Fe_3O_4 supplementation, although the magnitude of the effect depends on the properties and concentration of the conductive material and on the operating conditions of the digester [83, 84].

The comparison between the magnetite-amended and non-amended BES reactors will therefore provide an opportunity to evaluate whether the addition of Fe_3O_4 can further enhance the performance of an already bioelectrochemically assisted digestion system. In particular, the experiment will allow the effects of magnetite addition on biogas and methane production, volatile fatty acid conversion, and process stability to be assessed. The working hypothesis is that the conductive properties of Fe_3O_4 may facilitate electron exchange between syntrophic partners and reinforce DIET, thereby supporting more efficient transfer of reducing equivalents towards methanogenic microorganisms [81–84]. If confirmed, this approach could provide complementary evidence for the hypothesis emerging from the present thesis that modification of electron-transfer processes, rather than a generalized acceleration of bulk organic matter degradation, may represent an important mechanism through which bioelectrochemical strategies enhance methane recovery.

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