Article

Improving the Biogas Production and Methane Yield in an UASB Reactor with the Addition of Sulfate

Dimitra Theodosi Palimeri , Konstantina Papadopoulou , Apostolos G. Vlyssides , Anestis A. Vlysidis *

Posted Date: 25 September 2023

doi: 10.20944/preprints202309.1629.v1

Keywords: anaerobic digestion; UASB reactor; COD/SO4-2 ratio; Methane production; Microbial diversity; sulfate reduction; sulfate-rich substrate doi:10.20944/preprints202309.1629.v1

Article

Improving the Biogas Production and Methane Yield in an UASB Reactor with the Addition of Sulfate

D. Theodosi Palimeri [1], Konstantina Papadopoulou [1], A.G. Vlyssides [1] and A.A. Vlysidis [1,*]

[1] School of Chemical Engineering, National Technical University of Athens, Athens, 15780, Greece.
* Correspondence: *: anestisvlysidis@chemeng.ntua.gr

Abstract: Sulfate presence is of great importance for anaerobic digestion as its addition can control the microbial community. In this study, the effect of sulfuric acid addition on the performance of an UASB reactor fed with hydrolyzed starch was investigated. Total Organic Carbon (TOC), Fe, SO_4^{-2} removal and the methane production were monitored under various Chemical Oxygen Demand (COD) to SO_4^{-2} ratios, Hydraulic Retention Times (HRTs) and Organic Loading Rates (OLRs). When HRT was 16 hours and OLR equal to 2 g COD/L_{UASB}-day the methane production was 0.24 L CH_4/L_{UASB}-day. After the sulfuric acid addition, TOC removal reached 95%. At HRT, OLR, and COD/ SO_4^{-2} ratio equal to 16 hours, 2 g COD/ L_{UASB}-d and 3.72 respectively, the methane production was 0.267 L CH_4/L_{UASB}-day. When the OLR was increased to 5.94 g COD/L_{UASB}-day and COD/SO_4^{-2} ratio was equal to 12.5, the methane production was 3 times higher (0.84 L CH_4/L_{UASB}-day) with a methane content in the produced biogas greater than 70% due to the increased amount of *Methanosaeta* sp. and direct interspecies electron transfer. The sulfate addition led to an increased relative abundance of *Desulfovibrio* sp. accounting for 9.9% and an iron accumulation exceeding 98.0%. This study indicates that appropriate sulfate concentrations in the feed in combination with the presence of iron in the UASB lead to enhanced methane yields.

Keywords: anaerobic digestion; UASB reactor; COD/SO_4^{-2} ratio; Methane production; Microbial diversity; sulfate reduction; sulfate-rich substrate

1. Introduction

High- strength wastewaters with high concentrations of sulfate, organic compounds and heavy metals produced during industrial processes, are usually treated via anaerobic digestion. This bioprocess effectively reduces the pollutant load in the wastewater, while simultaneously producing biomethane which is a sustainable alternative energy source. Wastewater generated from potato processing industries contains high amount of starch which has high viscosity and low solubility [1] and for this reason is considered a highly polluted wastewater [2]. Due to its physicochemical characteristics, starch can cause operational harms in industry's wastewater treatment facilities, and thus, it is separated from the wastewater influent stream. After its extraction, this by-product can by hydrolysed and the produced hydrolysates can be effectively treated under anaerobic conditions for energy production in the form of methane [3].

Upflow anaerobic sludge blanket (UASB) bioreactor is one of the most widely used processes in food processing wastewaters with high content of carbohydrates [4] due to its low cost, high organic removal, and recovery of methane which can be used as gaseous biofuel. One of the key factors in the effective operation of upflow anaerobic treatment systems is the generation of sludge in granular form. The latter can efficiently degrade various types of wastewater with high organic loads [5]. Each of these granules consists of many different types of anaerobic bacteria which operate symbiotically and in layers for the successful degradation of substrate. Due to the granular form, bacteria are highly tolerant to the toxic hydrogen sulfide [6].

The last years many studies have focused on UASB systems treating starch wastewater **[5–7]**. He et al. 2019 [8] assessed the energy and economic benefits of three anaerobic bioreactors at various starchy influent COD concentrations. The authors estimated that the annual economic balance for the UASB reactor was achieved when the influent COD was 12,984 mg/L. With the utilization of biogas as source of heat and a COD influent concentration higher than 15 g/L, the system was able to achieve annual energy and economic surpluses. In addition, Lu et al. 2015 [9] observed that when a lab-scale UASB reactor supplied with starch as the sole carbon source at a COD concentration of 1000 mg/L, a total COD removal of 98.7% was achieved with a methane yield of 0.33 L CH_4/g $COD_{removed}$ applying a hydraulic retention time (HRT) of 6 hours and an organic loading rate (OLR) of 4 g COD/L-day.

Although anaerobic digestion has been widespread in recent years in the utilization of industrial wastewaters, there are several challenges that need to be addressed. The content of inhibitors in the substrate, as well as the organic loading rate applied to the anaerobic bioreactor could affect the methane production [10]. According to Lu et al. [9] the OLR for treating low strength starch wastewater should not exceed 8.0 g COD/L-d, with a HRT of over 3 hours. Feeding with excessive starch at a high OLR resulted in a decrease in mass transfer rate and a negative impact on the specific methanogenesis activity of granular sludge. Consequently, the performance of UASB reactor was compromised in terms of both COD removal and biogas production. In addition, Musa et al. 2019 [11] who studied the treatment of high-strength cattle slaughterhouse wastewater in UASB reactors, noticed that the optimum OLR for organic removal efficiency >90% was 5g COD/L-day. They also pointed out that at the highest OLR of 16 g/L-d, an HRT of more than 2 days could be proved adequate to achieve a 90% degradation efficiency of the waste. The methane production is also affected by the physicochemical properties of wastewater such as carbohydrates and fats content, C/N ratio and particle size [12]. Recently, efforts have been directed towards enhancing biogas production by adjusting nutrient content through the use of trace elements (e.g., Fe, Zn, Cu) and employing additives (e.g., biochar) that promote direct interspecies electron transfer (DIET) thereby improving digestibility through increased adsorption potential [13,14].

Wastewater generated from potato processing industries except for high organic load, usually has a high sulfate content which inhibits the production of methane during anaerobic treatment since the presence of sulfate affects the microbial diversity of the reactor [15]. Sulfate-reducing bacteria (SRB) that dominate in environments with high sulfate concentration, use sulfate as electron acceptor reducing sulfate into sulfide which is toxic for anaerobic microorganisms [16]. In addition, SRB compete with methanogens for energy sources such as acetate, starch, and H_2 and in high sulfate environments SRB outcompete methanogens since they can capture electrons more easily. Hence, the COD/SO_4^{-2} ratio affects the microbial community, and consequently, the performance of the anaerobic digester [17]. According to stoichiometry, the COD/SO_4^{-2} ratio must be more than 0.67 for a sufficient sulfate reduction. In addition, Choi et al. [18], observed that SRB and methanogens are very competitive when the COD/SO_4^{-2} ratio is below 2.7. They suggest a COD/SO_4^{-2} ratio of more than 2.0 for a successful anaerobic treatment.

The presence of sulfate in anaerobic digestion has gained significant interest over the last years. Using the Scopus database, we conducted a search with the keywords ("reactor") AND ("sulfur" OR "sulfate") AND ("anaerobic digestion"), resulting in 550 publications since 2000. Notably, there has been an upward trend in research on sulfur presence in anaerobic reactors, with 36% of the total publications emerging in the last 5 years. A graph illustrating the number of publications throughout the last 23 years is available in the SI. Since, the presence of high sulfur content in organic wastes can result in the production of hydrogen sulfide which is toxic during anaerobic digestion, significant research efforts have been dedicated to addressing the removal of H2S emissions **[19–23]**. Although the vast majority of research has focused on the reduction of sulfate during anaerobic digestion, there are few studies examining the biomethane production from high sulfate content-wastewater. The effectiveness of the COD/SO_4^{-2} ratio on methane production depends on many factors such as, among others, the HRT, the OLR and the presence of metals in the influent. Many anaerobic digestion studies using different substrates have reported that a COD/SO_4^{-2} ratio lower than 12 can inhibit the production of methane [24–27]. Lu et al. 2016 [28] examined the treatment of a starchy wastewater in a UASB reactor under gradually decreasing COD/SO_4^{-2} ratio. When the COD/SO_4^{-2} ratio was lower than 2, apart from COD removal which was kept constant, sulfate removal and methane production were decreased significantly at values lower than 21.2% and 0.26 ± 0.37 L/L-day respectively. However, when the ratio was more than 2, biogas production was stable near to 1.15 L/L_{UASB}-day

doi:10.20944/preprints202309.1629.v1

with a total COD removal of 73.5–80.3%. Moreover, Hu et al. 2015 [29] used a synthetic wastewater containing acetate, ethanol, and sulfate as substrate in a UASB reactor. The authors found out that when the COD/SO_4^{-2} ratio decreased from 20.0 to 0.5, the conversion of $COD_{influent}$ to CH_4, dropped from 80.5% to 54.4%. Furthermore, Liu et al. 2015 [30] detected that zero-valent iron can enhance the methane production and sulfate reduction in anaerobic granular sludge reactors only when the COD/SO_4^{-2} ratio is 2- 4.5.

This study aims to improve the methane production and the overall performance of a UASB reactor by adding sulfate in the form of sulfuric acid to the influent. The latter consists of starch hydrolysates. Apart from sulfate, the influent was also enriched with iron ions. The UASB performance was monitored under various conditions examining different OLRs, HRTs, sulfate and iron additions. Moreover, 16S rRNA gene analysis was conducted to examine the microbial community changes before and after the sulfate addition. Our work demonstrates that appropriate sulfate concentrations in the feed, along with the presence of iron, can lead to enhanced methane yields in the UASB reactor. Monitoring the UASB reactor under various feeding conditions can lead to the identification of suitable conditions where not only the pollutant load of the waste is reduced, but also biogas rich in methane is generated. This biogas can be utilized as an alternative form of energy, aiming for economically sustainable and environmentally friendly industrial waste management.

2. Materials and Methods

2.1. Materials

Ten (10) kg of industrial starch were taken from Tasty Foods SA, a potato processing plant located in Athens (Greece) and they were transferred to the Organic Chemical Technology Laboratory at NTUA and stored at 4°C. Molasses was purchased from a local market in Athens and stored at room temperature. $FeSO_4.7H_2O$ and H_2O_2 50% (w/w) that were used for feed pretreatment are described as ACS reagent, ≥99.0% and hydrogen peroxide solution 50 wt. % in H_2O stabilized, respectively. Urea that was added to the substrate is characterized as: powder, BioReagent, for molecular biology, suitable for cell culture. Sulfuric acid that was used as source of sulfates is described as ACS reagent, 95.0-98.0%. Table 1 shows the characteristics of industrial starch as received from the industry.

Table 1. Characteristics of industial starch.

COD [1] (mg/g)	TOC[1] (mg/g)	Dry Weight (% wt)	VS [1] (% wt)	N [1] (% wt)	P [1] (% wt)
0.988± 0.034	0.441 ± 0.024	67.066 ± 0.287	98.872 ± 0.411	0.170 ± 0.019	0.180 ± 0.027

[1] Dry basis.

2.2. Feed pretreatment

The starch was hydrolyzed in order to be biodegradable and suitable for methane production as described previously [3]. Briefly, for its chemical oxidation, 31 g dry weight of starch was suspended in 1L of tap water, $FeSO_4.7H_2O$ and H_2O_2 50% (w/w) were added at concentrations 1 g/L and 0.52 g/L respectively and the suspension was set to 70^0C under stirring for 3 hours.

After the hydrolysis of starch and prior to feeding to the anaerobic digester, the hydrolysates solution was diluted with deionized water for adjusting the Organic Loading Rate (OLR) at the desirable levels. Furthermore, urea, and molasses were also added at concentrations varying from 0.08 to 0.28 g/L, and 0.10 to 0.25 g/L respectively, as source of nitrogen and minerals.

2.3. Operational conditions of the UASB reactor

A cylindrical lab-scale UASB reactor was used for conducting the anaerobic digestion experiments as illustrated in Figure 1. The reactor was manufactured with Plexiglas, with a total and working volume of 11.3 L and 10.2 L, respectively, an internal diameter of 12 cm and a height of 1 m.

The UASB system was also comprised with a recirculation smaller cylindrical tank (total and working volume of 3.28 L and 2.26 L) where all the necessary electrodes (pH, ORP and level electrodes), as well as a thermocouple and a 200 W electric heating device were mounted. The entire system was automated by connecting all electrical sensors to a programmable logic controller (PLC). As biogas is produced, it is accumulated at the headspace of the two vessels and the biogas line pressure is continuously increased. At some point a low pressure switch activates a peristaltic pump, releasing the biogas pressure to a minimum point. The volume of biogas produced per day is measured using the peristaltic pump and a magnetic proximity sensor and it is recorded in the PLC. Air-sampling bags were used for the collection of biogas and the gas composition was examined automatically every 8 hours by using a biogas analyzer (Gas analyzer, GFM 406 series).

As inoculum, 3 L of anaerobic granular sludge derived from a full-scale UASB reactor of the potato processing industry, where the starch was supplied, was used. In order to acclimate the inoculum to the operational conditions, the reactor was initially operated for three days using only water as substrate. There was no need for a longer startup period since the sludge was already activated and acclimated to potato wastewaters.

An upflow velocity of 1 m/h was applied and kept constant during the whole period, in order to avoid the sludge precipitation and achieve better contact between anaerobic bacteria and influent.

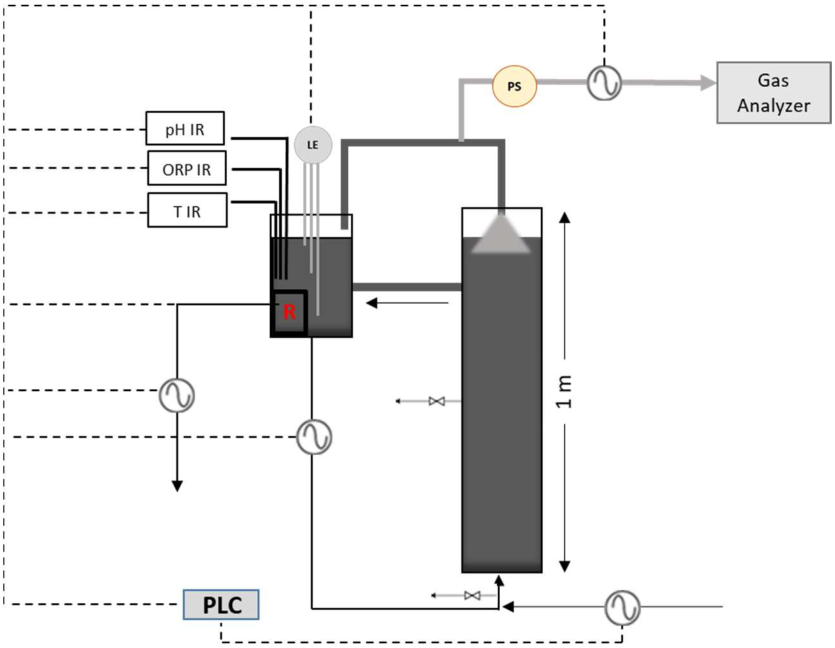

Figure 1. Lab-scale UASB reactor performance testing system.

The UASB system was operated continuously for 170 days and the temperature was maintained constant at 35 ± 2°C for the whole period. During the experiment, several operational conditions were examined at different values such as the Hydraulic Retention Time (HRT), the OLR, the COD/SO_4^{-2} ratio and the addition of iron. To better illustrate the variation of the operational parameters, we have separated the anaerobic digestion into 8 stages as shown in Table 2. Sulfuric acid was added to the influent from 76th to 170th day in order to investigate the role of sulfate on UASB performance. The operational parameters for each stage were adjusted gradually, taking into account the reactor's performance in the previous stage. Samples were obtained from the effluent in a daily basis, whereas samples from the influent were collected periodically after each feed modification. All liquid samples, once collected, were stored at 4°C for further analysis.

Table 2. Operational parameters in each of the 8 stages.

Stage	1	2	3	4	5	6	7	8
Starch (g/L)	0.87	0.91	0.68	1.23	1.32	1.55	2.85	3.95

Urea (g/L)	0.075	0.075	0.100	0.100	0.150	0.200	0.280	0.200
$FeSO_4$ $7H_2O$ (g/L)	0.025	0.023	0.053	0.040	0.033	0.046	0.049	0.120
Molasses (g/L)	0.10	0.10	0.20	0.20	0.10	0.10	0.20	0.20
H_2SO_4 conc. (mL/L)	-	-	-	-	0.20	0.10	0.14	0.18
HRT (hours)	35	32	32	16	16	11	22	16
Days	1-17	18-26	27-44	45-85	86-112	113-130	131-155	155-170

2.4. Analytical methods

Samples from the influent and effluent were filtered through a 0.45-μm glass fiber membrane, and measured in terms of chemical oxygen demand (COD) section 5220 C, sulfate section 4500 C, Fe section 3500 B, NH_4^+ section, total amount of VFAs section 5560 C and alkalinity section 2320 B according to Standard Methods [31] The pH and REDOX potential were measured continuously using the pH electrode HI 3214P from Hanna Instruments, Co. and the ORP electrode H1 2114P from Hanna Instruments, Co. and the results were recorded on a PLC. The TOC of solid starch, the organic carbon in the starch solution as well as the Total Nitrogen (TN) in the substrate, were measured by a TOC analyzer (Shimadzu, SSM-5000A, Shimadzu, TOC-L and TNL-M analyzer).

Molasses characterization was applied in accordance with Standard methods, using the Atomic Absorption Spectrophotometer Shimadzu AA-7000 (Shimadzu, Kyoto, Japan) for Ca, Mg, Fe, Mn, Zn and Cu, and the Sherwood Flame Photometer 410 (Sherwood, Cambridge, UK) for K and Na analysis.

2.5. DNA extraction and PCR Amplification

Before and after the addition of sulfate (85th and 170th day), 5 g samples of the sludge blanket were collected from the bottom of the reactor, washed and centrifuged and the precipitate was stored at -30°C for further analysis. They were analyzed by 16S rRNA sequencing in order to investigate the change in microbial community at genus level. For the microbial DNA extraction, Nucleo-Spin @ Soil Kit (MACHEREY-NAGEL) was used in accordance with the kit manufacturer's instructions. The nano-drop method was used to estimate the concentration and purity of the isolated DNA and the extract was delivered to Novogene Ltd. (Cambridge, United Kingdom), for amplicon pyrosequencing on the Illumina MiSeq platforms. PCR products were mixed in equidensity ratios. Total genome DNA from samples was extracted using CTAB/SDS method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water. Using the primer pair 341F/806R, the V3-V4 region of the bacterial 16S rRNA genes were amplified. According to Novogene, Bioinformatics Technology Co., all PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs) and sequencing libraries were generated using NEBNext Ultra DNA Library Pre ®Kit for Illumina, following manufacturer's recommendations. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina platform and 250 bp paired-end reads were generated. Sequences analysis were performed by Uparse software (Uparse v7.0.1001) [32]. Representative sequence for each operational taxonomy units (OTUs) was screened for further annotation. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in the 2 samples, multiple sequence alignment were conducted using the MUSCLE software (Version 3.8.31) [33]. Alpha diversity was applied in analyzing complexity of species diversity for a sample through indices, including Observed-species, Chao1, Shannon, Simpson, ACE and Good-coverage. All indices were calculated with QIIME (Version 1.7.0) bioinformatics platform software and displayed with R software (Version 2.15.3).

3. Results

3.1. Influent characterization and operational conditions

The TOC of the industrial starch was measured to be 0.441 g/g starch. Consequently, the initial starch suspension had a total TOC of 13.67 g/L. The applied starch hydrolysis conditions lead to a high hydrolysis rate of 99.5% and the hydrolysate can be used for biogas production via anaerobic digestion with a methane yield equal to 0.368 L-biomethane/g-COD as measured in a previous study [3].

The chemical composition of molasses was as follows: Cl, 36.73 mg/g; TN, 12.67 mg/g; Zn, 4.42 μg/g; Cu, 2.99 μg/g; Fe, 71.79 μg/g; Mn, 4.32 μg/g; Ca, 174.91 μg/g; K 3.8 mg/g, P, 0.75 mg/g; SO_4^{2-}, 40.11 mg/g while the urea contained 46% of elemental nitrogen.

The operational parameters, for each stage before and after the addition of sulfate are shown in Tables 2 and 3. At the first 4 stages, which lasted for 85 days, no sulfate was added to the feeding and the concentration of SO_4^{2-} was less than 5.3 mg/L. The composition of the influent contained a TOC ranging from 188.42 ± 13.9 to 835.66 ± 103.12 mg/L, a phosphorus concentration between 0.08 and 0.15 mg/L, a nitrogen content between 30.6 and 46 mg/L and an iron content from 4.58 to 10.68 mg/L. At stage 3, the amount of iron in the influent was doubled in order to study its effect on reactor's performance before the sulfate addition. The OLR and HRT were studied at values varying from 0.5 to 2.3 g COD/L_{UASB}-day and 16-35 h, respectively as shown in Table 3.

On day 86 the influent was enriched with sulfate in the form of H_2SO_4. In the stages from 5 to 8 (i.e., 86-170 days), the composition of the influent contained a COD/SO_4^{-2} ratio varying from 3.72 ± 0.15 to 12.5 ± 0.70 with sulfate concentration more than 300 mg/L, an iron concentration ranging from 6.16 ± 0.08 to 23.07 ± 3.49 g/L, while TOC, phosphorus and nitrogen were 477.71 ± 90.81 - 1551.60 ± 88.24 mg/L, 0.8-0.15 mg/L and 69 - 105.32 ± 22.77 mg/L, respectively. The OLR were equal to 2.1, 3.0, 2.7 and 5.9 g COD/L_{UASB}-day, with respective HRTs equal to, 16, 11, 22 and 16 h as it illustrated in Table 4.

Table 3. Operation conditions applied to UASB without sulfate addition (stages 1 to 4).

Stage	1	2	3	4
TOC (mg/L)	236.47 ± 33.97	296.13 ± 33.68	188.42 ± 13.91	835.66 ± 103.12
SO_4^{2-} (mg/L)	3.34 ± 0.40	2.93 ± 0.01	4.62 ± 1.68	5.02 ± 0.70
OLR (g COD/L-day)	0.54 ± 0.09	0.67 ± 0.02	0.47 ± 0.04	2.02 ± 0.26
Ratio COD/ SO_4^{-2}	239.43 ± 54.62	305.69 ± 10.35	152.82 ± 48.48	271.01 ± 37.55
Fe (mg/L)	5.23 ± 0.15	4.58 ± 0.12	10.68 ± 0.91	7.94 ± 0.93
TN (mg/L)	30.6 ± 0.11	30.6 ± 0.20	46.0 ± 0.17	46.0± 0.15
TP (mg/L)	0.075 ± 0.006	0.075 ± 0.004	0.15 ± 0.009	0.15 ± 0.011
HRT (hours)	35	32	32	16
Days	1-17	18-26	27-44	45-85

Table 4. Operational conditions applied to UASB with sulfate addition (stages 5 to 8).

Stage	5	6	7	8
TOC (mg/L)	477.71 ± 90.81	607.27 ± 44.68	900.55 ± 121.54	1551.60 ± 88.24
SO_4^{2-} (mg/L)	371.9 ± 0.01	371.58	313.01 ± 58.81	317.88 ± 17.60
OLR (g COD/L-day)	2.08 ± 0.21	3.04 ± 0.12	2.65 ± 0.40	5.94 ± 0.02
Ratio COD/ SO_4^{-2}	3.72 ± 0.38	3.72 ± 0.15	7.90 ± 1.54	12.5 ± 0.70
Fe (mg/L)	6.46 ± 0.81	6.16 ± 0.08	9.56 ± 0.49	23.07 ± 3.49
TN (mg/L)	69 ± 0.61	69 ± 0.32	105.32 ± 22.77	92 ± 1.01
TP (mg/L)	0.075 ± 0.009	0.075 ± 0.003	0.150 ± 0.012	0.150 ± 0.009
HRT (hours)	16	11	22	16
Days	86-112	113-130	131-155	155-170

3.2. UASB performance with and without the addition of sulfate

The performance of the UASB reactor for the first 4 stages (until day 85) is demonstrated in Table 5. COD/ SO_4^{-2} ratio was higher than 150, since the only amount of sulfate that observed in the substrate was the one derived from the addition of molasses and ferrous sulfate therefore no sulfate was detected in the effluent. In addition, TOC removal was varying from 60.43 ± 7.32 to 83.27 ± 5.53 %, while the methane percentage was ranging from 42.43 ± 10.71 to 56.25 ± 8.52 %. The highest iron accumulation (87%) was observed at stage 3, despite the high iron influent concentration. The maximum methane production was equal to 0.21 ± 0.07 L CH_4/ LUASB-day and observed at HRT 16 h and OLR 2.02 ± 0.26 g COD/ LUASB- day. At this stage (stage 4), VFAs started to be produced due to the OLR increase.

At stages 5 to 8 (Table 6), COD/ SO_4^{-2} ratio was gradually increased from 3.72 to 12.8 due to the addition of sulfuric acid. The TOC removal was increased from 67.34 ± 5.81 % to 94.81 ± 1.39 %, while the percentage of methane in biogas varied from 69.43 ± 3.27 (stage 6) to 71.86 ± 1.21 % (stage 5). It is noteworthy that the methane content was substantially improved with the sulfate addition from 56.25% in stage 4 to 71.9% in stages 5 and 8. Moreover, the methane production rate was increased by 40% (from 0.268 ± 0.07 L CH_4/ LUASB-day in stage 5 to 0.443 ± 0.135 L CH_4/LUASB-day in stage 6) when the HRT was decreased from 16 to 11 hours and OLR was increased from 2.00 to 3.04 g COD/ LUASB-day. The methane production was further increased at 0.608 ± 0.131 L CH_4/LUASB-day (stage 7) and 0.837 ± 0.073 L CH_4/LUASB-day (stage 8). In the latter stage, COD/ SO_4^{-2} was 12.5 ± 0.70 and the respective HRT, OLR and Fe concentration were equal to 16 h, 5.94 ± 0.16 g COD/ LUASB-day and 23.07 ± 3.49 mg/L. After the addition of sulfate (stages 5 to 8) pH and Redox values remained in satisfactory levels for methane production, despite the accumulation of VFAs. Furthermore, the iron was accumulated in the system in a percentage higher than 95%, while the sulfate removal was ranging from 59.61 ± 10.70 to 74.21 ± 13.09 % in all applied COD/ SO_4^{-2} ratios after the sulfate addition. Both output variables (COD and TOC% removal) seem to have approached their optimal values at COD/ SO_4^{-2} ratios equal to 7.90 ± 1.54 and 12.5 ± 0.70, respectively.

Table 5. Average value of each parameter during UASB operation before the sulfate addition.

stage	1	2	3	4
COD/ SO_4^{-2} ratio	**239.43 ± 54.62**	**305.69 ± 10.35**	**152.82 ± 48.48**	**271.01 ± 37.55**
pH	6.58 ± 0.14	6.75 ± 0.11	7.02 ± 0.46	6.67 ± 0.27
Redox	-455 ± 12	-449 ± 37	-467 ± 13	-438 ± 28
COD removal %	79.14 ± 10.17	82.75 ± 6.24	63.28 ± 5.71	68.40 ± 9.94
Fe accumulation %	63.04 ± 25.9	83.62 ± 10.81	87.69 ± 3.27	84.89 ± 12.07
CH_4%	55.30 ± 6.43	45.78 ± 7.52	42.43 ± 10.71	56.25 ± 8.52
Methane production rate (L CH_4/L-day)	0.027 ± 0.010	0.032 ± 0.017	0.081 ± 0.049	0.212 ± 0.072
SO_4^{-2} removal %	100	100	100	100
VFAs effluent (mg/L)	-	-	-	166.16 ± 25.73
TOC removal %	77.31 ± 10.62	83.27 ± 5.53	60.43 ± 7.32	76.91 ± 6.27

Table 6. Average of each parameter during UASB operation after sulfate addition.

stage	5	6	7	8
COD/ SO_4^{-2} ratio	**3.72 ± 0.38**	**3.72 ± 0.15**	**7.90 ± 1.54**	**12.5 ± 0.70**
pH	7.02 ± 0.18	6.85 ± 0.14	6.90 ± 0.24	6.55 ± 0.12
Redox	-445 ± 7	-445 ± 16	-422 ± 43	-353 ± 21
COD removal %	65.64 ± 6.53	71.86 ± 4.84	90.31 ± 5.55	92.28 ± 1.48
Fe accumulation %	95.17 ± 3.38	96.50 ± 5.45	98.8 ± 0.63	98.33 ± 1.53
CH_4%	71.92 ± 6.67	69.43 ± 3.27	71.04 ± 3.87	71.86 ± 1.21
Methane production rate (L CH_4/L-day)	0.268 ± 0.065	0.443 ± 0.135	0.608 ± 0.131	0.837 ± 0.073
SO_4^{-2} removal %	74.21 ± 13.09	63.19 ± 10.35	59.61 ± 10.70	72.23 ± 4.80
VFAs effluent (mg/L)	247.78 ± 52.13	234.99 ± 57.47	96.10 ± 46.12	111.52 ± 19.54
TOC removal %	67.34 ± 5.70	76.32 ± 4.02	92.56 ± 4.56	94.81 ± 1.39

Figure 2 illustrates the performance of the UASB reactor over the 170 days. Figure 2A demonstrates the TOC removal in each of the 8 stages. After the addition of sulfate, TOC removal was increased. It is observed that at the last two stages where sulfate, iron, and OLR were increased, the removal of the organic load reached its highest values (92.6% at stage 7 and 94.8% at stage 8). The latter was 12.2% higher than the maximum TOC removal from the first four stages obtained at stage 2. The methane yield as L of CH_4/ g $COD_{removed}$ is shown in Figure 2B. A noticeable trend is observed whereby the methane yield was escalated when the OLR was reduced in stage 3 (from 0.67 to 0.47 g COD/L-day) and stage 7 (from 3.04 to 2.65 g COD/L-day). The increase in methane yield can be attributed to the anaerobic bacteria having already acclimated to the OLR from the previous stage promoting the methane production. When the OLR was increased 4.3 times (stage 3 to 4) and 1.46 times (stage 5 to 6) the methane yield was decreased for the first 5-7 days until its rise and stabilization to higher methane yield values. Nevertheless, when OLR reached the highest value of 5.94 g COD/L-day at stage 8 methane yield dropped for 2 days and then started to increase during the first week (149th – 155th day) before it drops to yields at around 0.16 L CH_4/ g $COD_{removed}$.

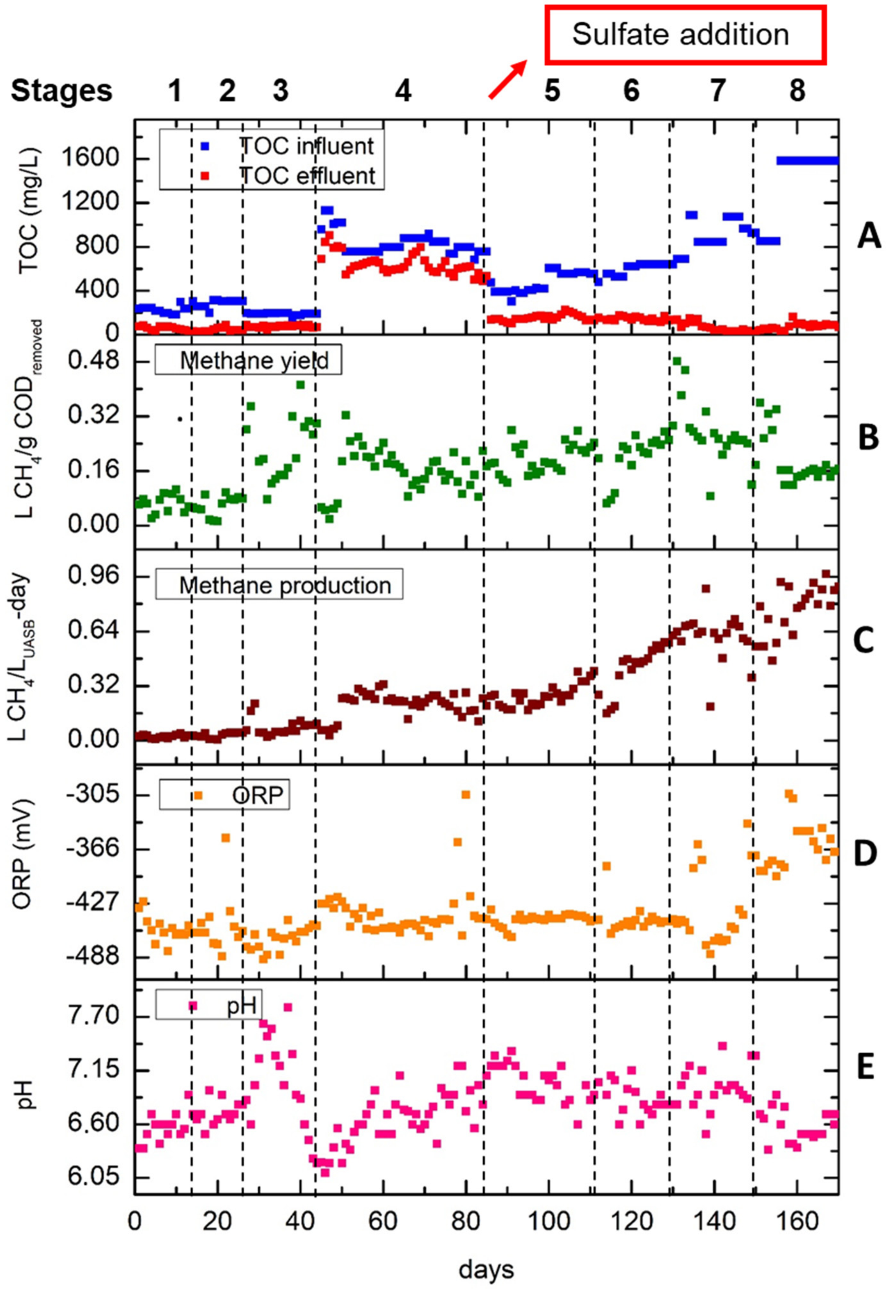

Figure 2. UASB performance in all 8 stages. A) TOC in the influent and effluent in mg/L, B) Methane yield in L CH_4/$gCOD_{removed}$, C) Methane production rate in L CH_4/L_{UASB}-day, D) ORP in mV and E) pH.

According to Figure 2C, after the addition of sulfate the methane production rate (MPR) was gradually increased reaching values higher than 0.96 L CH_4/L_{UASB}-day. MPR of stage 8 is almost 4 times higher than the one obtained in Stage 4. It is observed that when the OLR was risen more than 1 g COD/L_{UASB}-day, the methane production rate was reduced for few days and after that it was sharply increased (e.g., beginning of stage 6).

This behavior is may explained by the fast adaptation of anaerobic sludge to new conditions. Figures 2D and 2E depict the ORP and pH at each stage. Regarding ORP, it is observed that there is no remarkable variance during the first 7 stages where the ORP values, except for isolated cases, are lower than -427 which is adequate for methane production. During the final stage, ORP was increased due to the high OLR of 5.94 g COD/ L_{UASB}-day**.** Regarding Figure 2E, pH values fluctuate between 6.1 to 7.8. It is observed that pH values are strongly related to OLR. The highest pH values are observed in stage 3 where OLR decreased slightly from 0.67 to 0.47 g COD/ L_{UASB}-day, while the sharp increase in OLR to 2 g COD/ L_{UASB}-day at stage 4, resulted in a decrease in pH from 6.6 to 6.1. Generally, the optimal conditions for ORP and pH in anaerobic conditions for methane production are lower than -300 mV and more than 6.8, respectively [34].

Figure 3A represents the sulfate removal as an average of the measurements at each of the 8 stages. As it is mentioned above, until stage 4 the only source of sulfate was molasses and as a result no sulfate was detected in the effluent. After the addition of sulfate in the form of sulfuric acid at stage 5, the sulfate concentration started to increase from 0 (stages 1 to 4) to 80 - 120 mg/L (stages 5 to 8). At stage 7, we decided to decrease the sulfate concentration in the influent from 360 mg/L to 300 mg/L due to the sulfate accumulation in stage 6. This decrease restored the system back to elevated sulfate removal values (72.2% in stage 8 and 74.2% in stage 5). According to Figure 3B, at the first 4 stages the iron was accumulated in the system in a percentage ranging from 60 to 85% while the accumulation of iron approached 100% after the addition of sulfate. Although the influent iron concentration increased in stages 7 and 8, the iron in the effluent did not show any rise. It seems that the presence of sulfates enhanced the retention of iron within the bioreactor.

The percentage of methane in the total biogas produced is shown in Figure 3C. It can be noticed that after the addition of sulfate, the methane percentage has the tendency to be stabilized at a remarkably high value of 71% with the deviation constantly decreasing from ±3.00 (stage 5) to ±0.50 (stage 8). The addition of sulfate into the system, not only increases the methane content but also consolidates the biogas production rate. Finally, Figure 3D represents the total amount of VFAs produced at every stage. VFAs started to accumulate at stage 4 from 0 (stages 1 to 3) to 170 mg/L where OLR was increased to 2.02 from 0.47 g COD/L_{UASB}-day .After the addition of sulfate, VFAs concentration was maintained, initially, at high levels (240-250 mg/L) in stage 5 and 6 before being reduced to low levels (100-110 mg/L) in stages 7 and 8. The low VFAs concentrations at stages 7 and 8 are probably due to the higher iron concentration and COD/sulfate ratio [35]. At the final stages of the experiment, the system was so robust that even the sharp increase of OLR (stage 8) had no negative impact as the VFAs and biomethane content remained at values near to 100 mg/L and 71% respectively.

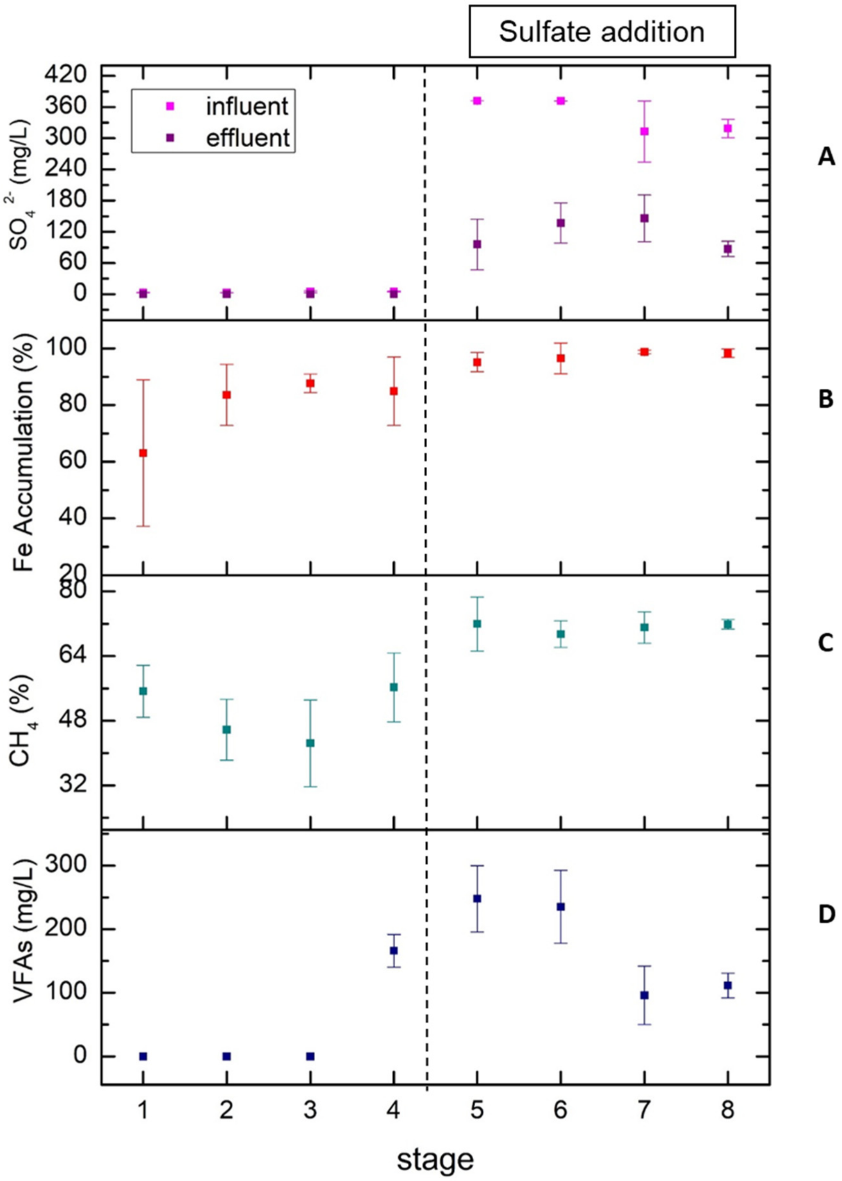

Figure 3. Average values of sulfate in the influent and effluent (A), Fe accumulation (B), Methane percentage in total biogas (C) and VFAs accumulation (D) at each of the 8 stages.

Figure 4 illustrates the nitrogen removal during the operation of the UASB reactor. It is observed that the nitrogen in the effluent was gradually raised as influent nitrogen was increased. During the first two stages, nitrogen was consumed in the high rate of 36.3% and 39.7%, respectively due to microbial acclimation and biomass growth. At stages 3 and 4, nitrogen was accumulated in the reactor. After the sulfate addition, the nitrogen removal was increased despite the increase in the nitrogen supply. More specifically, at stages 5 and 6 where the influent nitrogen concentration was 69 mg/L, the nitrogen removal reached 19.87% and 22.58%, respectively, while at stages 7 and 8 where the influent nitrogen was 103 and 92 mg/L, the respective nitrogen removals had the average values of 24.7% and 14.0%. In addition, at stages 1, 7 and 8, the nitrogen in the effluent was mainly in the form of ammonium, indicating the efficient activity of hydrolytic bacteria and the ammonification process in the reactor. The presence of accumulated ammonium in the UASB reactor can create a conducive environment for the growth of ammonium oxidizing bacteria. At stages 2, 3, 4, 5 and 6 the ammonium concentration was low and ranging between 55% to 63% of the effluent total nitrogen. The accumulation of organic nitrogen in the effluent can be attributed to the limited activity of ammonifying bacteria, high amount of urea in the influent, washout of biomass, or insufficient contact time between the anaerobic bacteria and the feed.

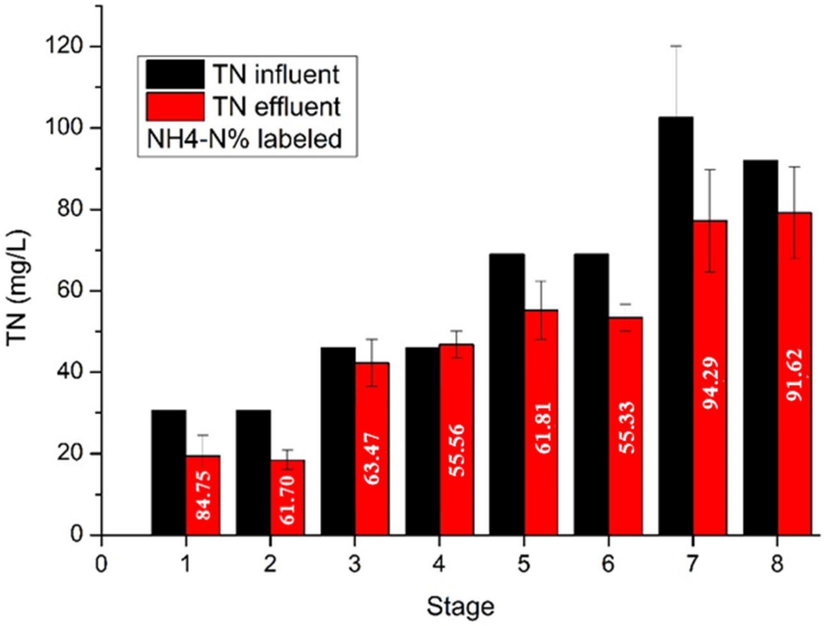

Figure 4. Total Nitrogen in the influent and effluent. The percentage of NH_4-N in the effluent is labeled.

3.2. Microbial community results

16S rRNA gene sequencing was applied to investigate the impact of sulfate addition on microbial diversity within the UASB reactor. Sequence analysis was conducted based on similarity and representation of OTUs [36]. Sequences with ≥97% similarity were assigned to the same OTUs. The number of OTU for sample A and B were 576 and 493 respectively, indicating the well sampling from the UASB reactor. Alpha diversity was used to estimate the richness of microbial species within the sample. Table 7 shows, the Sannon and Simpson index that indicate the diversity within the sample as well as the ACE and Chao1 estimators that reveal the microbial richness of the samples, before and after the sulfate addition. It is observed that microbial diversity and the species richness were decreased after 170 days of operation. According to Schloss et al. 2011 [37], high microbial diversity is observed when Sannon index is high and Simpson has a low value. The Good's Coverage that expresses the ability of 16s rRNA to capture a greater percentage of the microbial diversity including the rare diversity, was 99.9% for the 2 samples indicating that the sampling and sequencing effort conducted were sufficient to capture the majority of the bacterial diversity that was present.

Table 7. Microbial diversity and richness estimators for the 2 samples.

Sample name	observed species	Shannon	Simpson	Chao1	ACE
A	565	4.75	0.881	581.114	588.115
B	469	4.147	0.845	489.204	488.479

Figure 5 illustrates the relative abundances in phylum level for the 2 samples. The phyla that predominate in the samples are Euryarchaeota, Firmicutes, Chloroflexi, Proteobacteria, Bacteroidetes, Caldiserica, Spirochaetes, Acidobacteria, Patescibacteria and Fusobacteria. However, the relative abundances of them, changed before (Sample A) and after the sulfate addition (Sample B). The relative abundance of dominant Euryarchaeota which include methanogenic archaea species, was increased after the addition of sulfate from 32.50% to 35.46%, while the relative abundance of

Chloflexi was decreased from 25.97% to 13.98%. In addition, Proteobacteria were decreased from 18.60% to 11.38%. Furthermore, after sulfate addition the relative abundance of Firmicutes was greatly increased from 6.36% to 27.06%. This phylum can survive in extreme conditions such as strong acidic conditions and plays an important role in acidogenesis [38] . Bacteroidetes increased its relative abundance from 4.72% to 5.02% while Caldiserica had a slightly lower relative abundance in sample B from 3.65% to 2.41%. The other phyla had a relative abundance lower than 1% before and after the sulfate addition, and hence, we do not further comment them. It has been reported that the phyla Euryarchaeota, Firmicutes, Chloroflexi, Proteobacteria, and Bacteroidetes predominate in anaerobic mesophilic reactors [39].

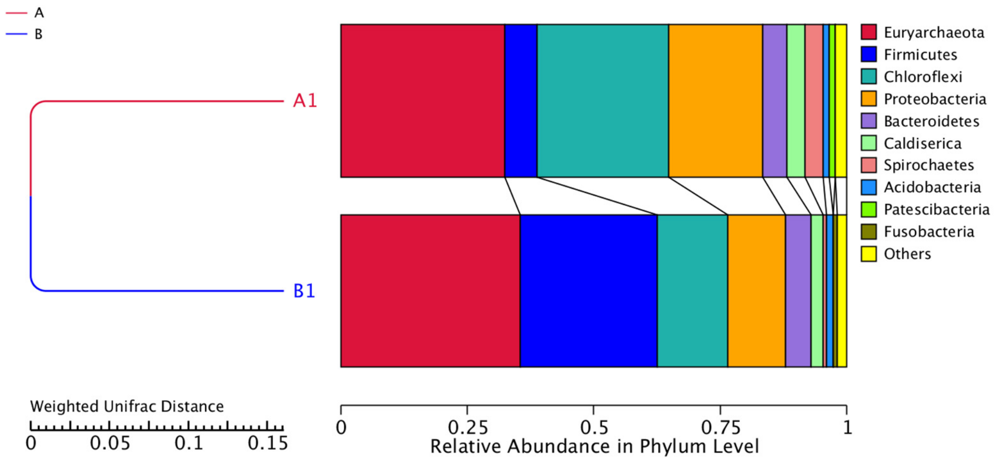

Figure 5. Relative abundance in Phylum level. Samples A1 and B1 were collected on days 85th and 170th, respectively.

Krona graphs in Figure 6 represent the result of taxonomic analysis for each sample depicting the predominant bacteria and archaea [40]. Every different circle in the graph correspond to a different taxonomic rank. Before the addition of sulfate (Figure 6A), the predominant archaea at genus level were, with relative abundance, 27.51% *Methanosaeta* sp. followed by 3.3% *Methanobacterium* sp., 0.49% *Methanospirillum* sp., 0.47% *Methanosarcina* sp. and 0.43% *Methanolinea* sp. Regarding to Bacteria, *Levilinea* sp. dominates with 17.7% while *Propionivibrio* sp. has a high relative abundance of 9.75%. The relative abundance of the *Calsidericum* sp., *Ferribacterium* sp., IheB3-7, Anaerolineaceae_UCG-001 and Deslufovibrio sp. is 3.4%, 2.4%, 1.76%, 1.48% and 1.74%, respectively.

After the addition of sulfate to the substrate (Figure 6B), the predominant archaea are again Methanosaeta sp. but with an increased relative abundance of 32.25% followed by Methanobacterium sp. with 2.25% and *Methanolinea* sp. with 0.54%. From Bacteria, *Ruminococcus* sp. dominates with relative abundance of 20.24% and *Desulfovibrio* sp. which belongs to SRB with 9.89%. Other bacteria with high relative abundance were *Syntrophomonas* sp. (4.02%), *Ignavibacterium* sp. (2.29%), *Caldisericum* sp. (2.25%), *Leptolinea* sp. (2.05%) and toluene-degrading methanogenic consortium bacterium (1.16%).

The distribution of top 100 genera and the comparison between the relative abundance at genus level in microbial community from samples A and B are observed in Figure 7. The red and the orange bars correspond to the microbial abundance before and after the sulfate addition, respectively. In sample A, nine genera were observed to have relatively high abundance. These are *Methanosae-ta, Methanobacterium, Levilinea, Caldisericum, Anaerilineaceae_UCG-001, Propionivibrio, Ferribacterium, IheB3-7*, and *Desulfovibrio*. In sample B, seven observed genera with relatively high abundance were detected: *Methanosaeta, Ruminococcus, Desulfovibrio, Syntrophomonas, Leptolinea, Ignavibacterium*, and *Caldisericum*. After the addition of sulfate, *Methanosaeta, Ruminococcus, Desulfovibrio*, and *Syntrophomonas* showed higher abundance, while *Levilinea, Propionivibrio*, and *Ferribacterium* nearly disappeared.

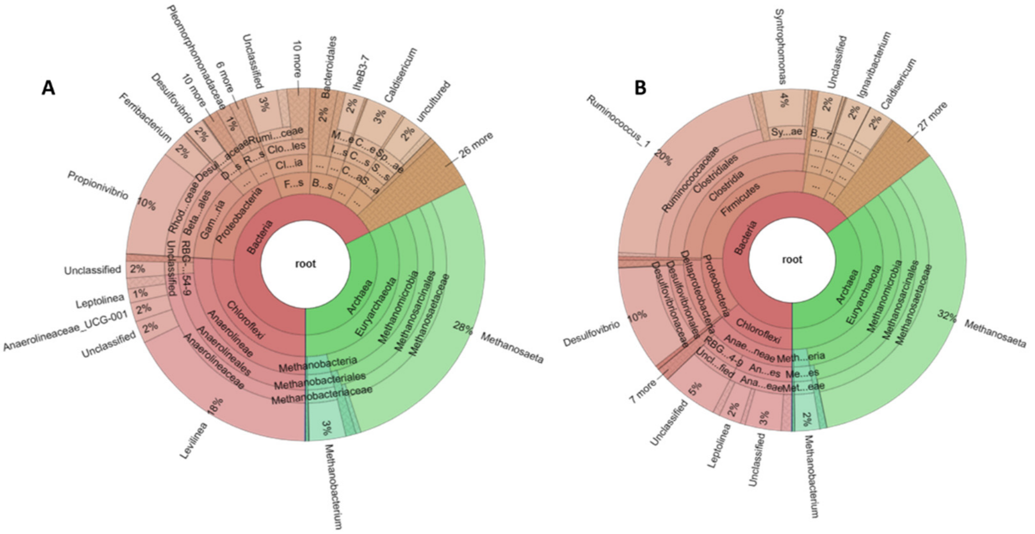

Figure 6. Krona graphs showing microbial community in the reactor. Graph A corresponds to the sample collected before the addition of sulfate to the substrate (day 85th). Graph B corresponds to the sample collected after the addition of sulfate (day 170th).

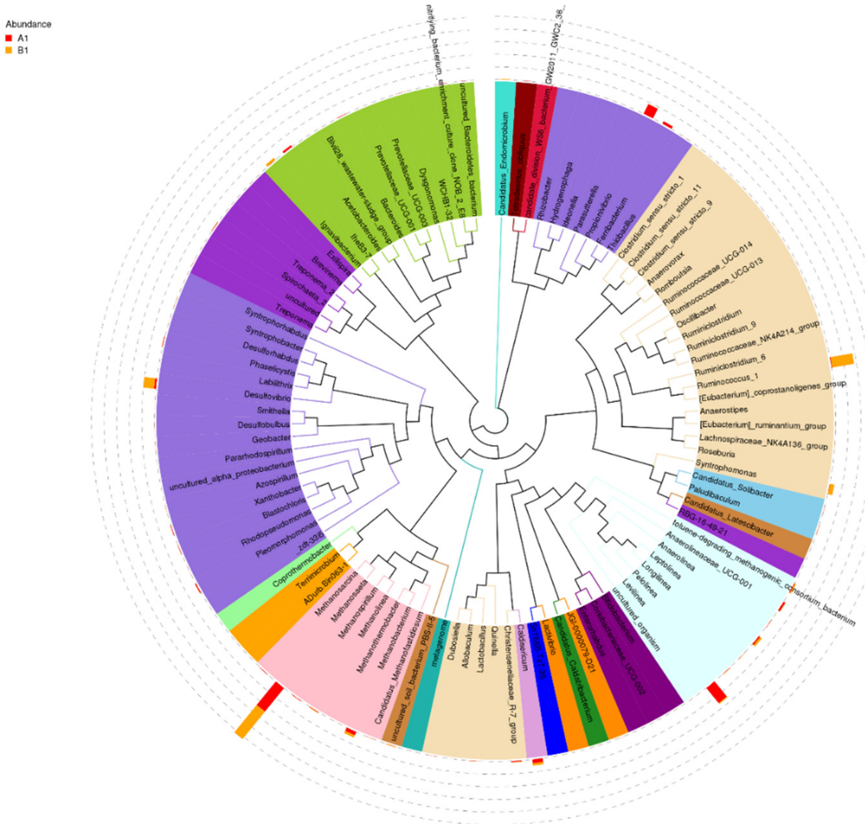

Figure 7. The distribution of the top 100 genera and the relative abundance for the 2 samples. Sample A (labeled with red color) was collected before the sulfate addition. Sample B (labeled with orange color) was collected after the sulfate addition.

4. Discussion

The performance of the UASB reactor showed a relative stability from the first days of its operation. The anaerobic sludge utilized as inoculum had already been activated and acclimated to potato wastewaters since it was obtained from the wastewater facilities of the same industry where the industrial starch was derived. The OLR did not exceed the value of 6 g/L_{UASB}-day during the whole period of experiments in order to avoid a decrease in COD removal efficiency and methane production due to the complex structure of starch [9]. During the first 85 days of the experiment, no considerable fluctuations were observed related to HRT modifications. The increased methane production L CH_4/ L_{UASB}-day at stage 4 was due to the increase in OLR. After the sulfate addition, the COD/SO_4^{-2} ratio was maintained higher than 3, in order to avoid sulfidogenesis [18] and keep the sludge granule integrity [17]. Although in few other studies a low COD/SO_4^{-2} ratio during anaerobic processes can lead to a COD removal lower than 50%, in our study the addition of sulfate and the increase in OLR increased both the COD removal as well as the methane production [41,42]. The increase in COD removal can be explained by the abundance of SRB in the system that utilize organic carbon for the production of new cell metabolites [43]. The stoichiometric ratio indicates that 1200 mg of COD is needed to reduce 1800 mg of sulfate [44]. It has been reported that among different types of anaerobic reactors treating glucose at various COD/SO_4^{-2} ratios, UASB achieved the highest organic matter and sulfate removal level [45]. Although pH in the influent was decreased after the addition of sulfate (ranging from 6.7 to 4.5), the robustness of the UASB system maintained the pH to adequate levels (6.3-7.4) for methane production. It has been reported that SRB can generate alkalinity that allows pH to increase [46,47]. The increased pH values in combination with high alkalinity promote acetogenesis and methanogenesis [48].

The use of sulfuric acid instead of other sulfate salts in this study allows for a more controlled and precise addition of sulfates. It helps maintain an optimal acidic environment minimizing pH fluctuations. Additionally, it is highly soluble in water, ensuring that it can easily react with other substances in the experiment. During the decomposition of H_2SO_4, hydrogen ions released that are not toxic for the anaerobic bacteria -since they can be used for microbial metabolism- and do not interfere with the overall performance of the system. This allows for a more accurate assessment of the specific impact of sulfates on the microbial community without introducing any additional toxic effects that could confound the results. Moreover, sulfuric acid is commonly used in research and industrial applications due to its stability and availability. Several studies have focused on sulfuric acid pretreatment of substrate before anaerobic digestion in order to improve the methane production [49,50]. Lastly, the use of sulfuric acid allows for more precise dosing and easier adjustment of the sulfur content in the AD system. This flexibility is important for optimizing the process and ensuring efficient methane production. It is mentioned that sulfate released from sulfuric acid can undergo reduction by sulfate-reducing bacteria to form sulfide. This dissolved sulfide can then be oxidized to elemental sulfur through the action of ferric ions present in the system. Additionally, the released ferrous ions can react with the sulfide to form precipitates of iron sulfide [51].

The improved performance of the UASB reactor after the sulfate addition that was observed in this study is in agreement with Lu et al. 2016 [11] who stated that at COD/ SO_4^{-2} ratios of 3, 5 and 10, the respective average of methane content was 67.2%, 64.0% and 61.6% in a UASB reactor fed with starchy wastewater at an OLR of 4 g COD/L_{UASB}-day. According to the authors, the improvement is due to the increase in diversity of microbial community that stimulates the hydrolysis-acidification of starch and enhances the degradation of propionate, ultimately resulting in improved acetoclastic methanogenesis. In addition, at COD/SO_4^{-2} ratios between 2 to 10, they found a sulfate reduction of 52.7–77.1% and the sulfide produced, was almost entirely aqueous. Our study demonstrates similar results regarding sulfate reduction (59.61 ± 10.70 to 74.21 ± 13.09%), indicating no significant difference in sulfate removal among the various COD/ SO_4^{-2} ratios. However, when iron addition was significantly increased (stage 8), the sulfate removal was high and more stable due to the precipitation of iron sulfides. Yang et al. 2015 [52] who studied the impact of sulfate addition (0.5-0.8 g/L) on methane production in a mesophilic anaerobic reactor feeding with acetate at an OLR of 4 g TOC/L-day, pointed out that methane production did not affected by sulfate addition, while the sulfate reduction became unstable (23-87%). In contrast with the present study, they noticed that with the increase of sulfate concentration in the substrate, the hydrogenotrophic methanogenesis was promoted and the population of *Methanosaeta* sp decreased. This fact could confirm the importance and necessity of iron in the anaerobic system.

The improved and stabilized performance of the UASB reactor with low amount of VFAs at stages 7 and 8 is due to the long adaptation of microbial community to the starchy substrate, the COD/ SO_4^{-2} ratio values (>7) and the increased iron influent concentration. The absence of high iron concentration in the influent in combination with sulfate addition can play an important role in the increase of methane yield. The high iron accumulation in the UASB system after the sulfate addition might be explained by the fact that in the presence of sulfate, iron precipitates as ferrous sulfide. As it is mentioned above, ferrous iron in the substrate can react with sulfide produced by SRB, resulting in the precipitation of ferrous iron as FeS or FeS_2 and reducing the negative impact of sulfide on methanogenesis [51],[53]. In addition, the iron precipitates have a positive impact on anaerobic granular sludge enriching the microbial community [54,55] and granule stability [56].

The substrate was supplemented with nitrogen to maintain a low but adequate C/N ratio during the whole period of the experiment. Zhang et al. 2022 [57], reported that sulfate in concentrations between 200 mg/L and 1200 mg/L can promote the organic removal and methane production during anaerobic digestion of nitrogenous wastewater. After sulfate addition, nitrogen removal was increased due to the use of ammonium for microbial growth. Several studies have focused on the sulfate-reducing ammonium oxidation process explaining that ammonium is used as electron donor and sulfate as electron acceptor producing nitrogen and elemental sulfur [58,59]. Other recent studies explain the anoxic ammonia removal via Feammox processes where ammonium is converted to N_2 using ferrous iron as electron donor [60,61]. This fact can explain the high nitrogen removal in the last 4 stages, as there was an accumulated amount of iron within the reactor and the amount of iron in the substrate was high. Additional experimental data are required in the current study in order to comprehensively elucidate the significant phenomenon of nitrogen removal. This open field of research is extremely important to our research group and has great potential for further exploration.

Regarding to microbial community it is worth mentioning that the archaeal Euryarchaeota were the most abundant. This phylum participates in carbon conversion during anaerobic digestion and generates methane [62]. Microbial diversity at genus level, indicates that the most of the produced methane is resulted from acetate reduction since the relative abundance of acetoclastic *Methanosaeta* sp. is higher than that of the other methanogens which use CO_2 and H_2 as source of energy [63]. In addition, during the anaerobic fermentation of starch hydrolysates, monosaccharides are converted into acetate from Chloroflexi phylum. It is reported that the syntrophic relationship of Anaerolinae and Clostridia species improves the acidogenesis process. In *Anaerolinaceae* family many species have hydrolytic fermentative properties and can easily degrade glucose molecules. In addition, some of them help in the sludge granulation [48]. It can be noticed that *Levilinea* sp. which had the highest relative abundance among the bacteria genera, as acidogens can convert carbohydrates to organic acids [63,64]. Furthermore, *Rhodocyclaceae* family accounting for 12.23% in this study, is considered a very common microbial group in wastewater treatment processes [66]. *Propionivibrio* sp. that has a high relative abundance accounting for 9.75% utilizes sugars as energy source and produce propionate. In this study, the organic acids produced during acidogenesis may be oxidized by *Ferribacterium* sp. (relative abundance of 2.4%) [67] and ferric iron from the substrate may be used as their electron acceptor. The amount of iron in the substrate was already high due to the $FeSO_4 7H_2O$ addition during the chemical oxidation of starch. In addition, some species from Caldiserica phylum are related to iron reduction in combination with sulfide oxidation [68]. *Calsidericum* sp. with the relative abundance of 3.4% can reduce sulfur compounds such as elemental sulfur, thiosulfate or sulfite that were in the anaerobic system [69].

The low COD/SO_4^{-2} ratio, the high OLR as well as the increased iron concentration led to changes in microbial diversity as it is observed from the genomic analysis of sample B. Regarding to archaea community, the relative abundance of *Methanosaeta* sp. was increased accounting for 32.25% while all the other archaea were diminished or remained at similar levels (Table 8). Consequently, the produced methane after the addition of sulfate derived from the acetate consumption instead of hydrogenotrophic metabolic pathway. *Ruminococcus* sp. that showed the highest relative abundance in bacterial community, belong to acidogenic bacteria and produce H_2 and short chain acids from soluble carbohydrates. Theoretically, 4 mol of H_2 per mol of glucose can be produced [70]. This genus reduces a proton to hydrogen with reduced electron carrier NADH or reduced ferredoxin [71,72]. Iron has a crucial role in electron transfer and hydrogen formation since it is the active site of ferredoxin [73]. Yin et al. 2021 [74] who studied the hydrogen production from sewage sludge, showed that ferrous iron can improve the H_2 yield as ferredoxin hydrogenase activated by Fe^{2+} supplementation. The authors also pointed out that the addition of ferrous sulfate enhances the degradation of the organic compounds. In our study, the cumulative addition of iron seems to affect the acceleration of *Ruminococcus* sp. population. The increase in *Desulfovibrio* sp. relative abundance should be directly related to the addition of sulfate. *Desulfovibrio* sp. bacteria belong to sulfate reducing bacteria (SRB) that reduce sulfate by using H_2 as electron donors [16]. In addition, some species of this genus have iron reduction abilities too [75]. The increase in sulfate removal efficiency, expect for the precipitation as ferrous sulfide, can be also explained from the increase in the SRB's electron acceptors [46]. Paulo et al. 2015 [76] and Colleran et al. 1995 [77] have observed that SRB exhibit a higher affinity for H_2 in comparison with methanogenic bacteria. This characteristic gives to the SRB a competitive edge in environments where sulfates are abundant. On top of that, Paulo et al. 2015 [76], mentioned that hydrogen offers a thermodynamic advantage over acetate as an electron donor for sulfate reduction. Niu et al. 2023 [78] investigated the sulfate removal rate under different influent sulfate concentrations in a UASB reactor treating acid mine drainage and observed that sulfate reduction by SRB is more favorable in communities with higher species diversity.

Furthermore, an increase in *Syntrophomonas* sp. population from 0.3 to 4% was observed. This microbial group produces acetate from fatty acids degradation and it is proved that has a syntrophic role in anaerobic oxidation of butyrate. Zhang et al. 2016 [79], investigated the DIET syntrophic methanogenesis in presence of Fe_3O_4 in lake sediments and observed an acceleration of methane production due to iron. *Syntrophomonas* sp. associates with methanogenic bacteria and bacteria that utilize hydrogen. Direct interspecies electron transfer (DIET) between bacteria that reduce iron and methanogenic archaea can improve the methane production [53]. Moreover, there was a noticeable increase in the relative abundance of *Ignavibacterium* sp., from 0.2% to 2.3%. Ignavibacterium species have been identified as potential participants in the Feammox process [80,81]. These bacteria can use ferric iron as electron acceptor while oxidizing ammonium to nitrogen gas. This fact can confirm that the high nitrogen removal observed is due to N_2 production from *Ignavibacterium* sp. It can be concluded that after the addition of sulfate, *Ruminococcus* sp. and *Syntrophomonas* sp. produce hydrogen and acetate respectively from the carbohydrates, provided by the starch hydrolysates, that can be directly utilized by *Desulfovibrio* sp. The latter outcompete hydrogenotrophic methanogenic bacteria. Therefore, *Methanosaeta* sp. predominates in the microbial community and methane production is derived almost completely from acetate.

In summary, all the conditions applied after the sulfate addition, affect the UASB performance as follows:

- The methane percentage in the total biogas and the methane production rate reached 71% and 0.84 L/L_{UASB}-day, respectively, due to the increased amount of *Methanosaeta* sp. and direct interspecies electron transfer.
- The organic removal efficiency reached 95% due to the high amount of *Ruminococcus* sp. that utilize the soluble carbohydrates from the substrate.
- The amount of VFAs was kept low since some genera such as *Syntorphomonas* and *Desulfovibrio* can effectively use them as substrate.
- The sulfate removal reached 72% due to the reduction of sulfate from *Desulfovibrio sp.*
- Iron accumulation was increased to 98% probably due to precipitation as iron sulfide.
- During stages 5, 6, 7, and 8, the nitrogen removal achieved respective values of 19.87%, 22.58%, 24.66%, and 13.92% probably due to the oxidation of ammonium from *Ignavibacterium sp.*

Table 8. Relative abundance at genus level for Sample A and Sample B.

Relative abudance at genus level %		
	Sample A	**Sample B**
Archaea		
Methanosaeta	27.5	32.3
Methanobacterium	3.3	2.3
Methanospirillum	0.5	0.1
Methanosarcina	0.5	0.0
Methanolinea	0.4	0.5
Bacteria		
Ruminococcus	0.5	20.2
Desulfovibrio	1.7	9.9
Syntrophomonas	0.3	4.0
Caldicericum	3.4	2.4
Ignavibacterium	0.2	2.3
Propionivibrio	9.8	0.2
Levilinea	17.7	1.0
Ferribacterium	2.4	0.1

5. Conclusions

This research reveals that after the addition of sulfate in the influent, the methane content of biogas is increased by 15% and methane production enhances by 3 times. At low COD/sulfate ratios, Fe accumulation in the UASB reactor is increased since ferrous iron reacts with sulfide produced by SRB and precipitates. In addition, after the sulfate addition, *Methanosaeta*'s sp population was increased while other methanogenic bacteria were decreased showing that methane was produced almost entirely from acetatoclastic methanogenesis pathway. Improvement in methane production can be elucidated by the direct interspecies electron transfer (DIET) between methanogens and iron reducing bacteria. Finally, the methane production and organic removal in a UASB reactor treating starch hydrolysates, can be significantly improved by using COD/SO_4^{2-} ratios between 8 to 13, along with an iron concentration ranging from 10 to 23 mg/L. Future recommendations in this field should be directed towards optimizing and analyzing the scalability of the UASB reactor for treating substrates enriched with sulfuric acid in different COD to sulfate ratios. On top of that, future investigations should involve the evaluation of the methane production potential of various organic substrates which already contain high amounts of sulfate such as the organic fraction of municipal solid waste and specific agro-industrial waste streams.

Funding: This research is co-financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432), implemented by the State Scholarships Foundation (IKY).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Li J, Zhou M, Cheng F, Lin Y, Shi L, Zhu PX. Preparation of oxidized corn starch with high degree of oxidation by fenton-like oxidation assisted with ball milling. *Mater Today Commun*. **2020**, *22*, 100793. doi: 10.1016/j.mtcomm.2019.100793
2. Bouchareb EM, Kerroum D, Arikan EB, Isik Z, Dizge N. Production of bio-hydrogen from bulgur processing industry wastewater, *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects,* **2021.** doi:10.1080/15567036.2021.1877853
3. Theodosi-Palimeri D, Papadopoulou K, Vlyssides GA, Vlysidis AA. Hydrolysis optimization of by-products from the potato pro-cessing industry and biomethane production from starch hydrolysates, *Sustainability*, **2023**, *under review*.

4. Daud MK, Rizvi H, Akram MF, Ali S, Rizwan M, Nafees M, Jinet ZS. Review of upflow anaerobic sludge blanket reactor technology: Effect of different parameters and developments for domestic wastewater treatment. *J Chem*. **2018**, *2018*. doi:10.1155/2018/1596319
5. Vlyssides A, Barampouti EM, Mai S. Determination of granule size distribution in a UASB reactor. *J Environ Manage*. **2008**, *86*(4), 660-664. doi: 10.1016/J.JENVMAN.2006.12.018
6. Fang HHP. Microbial distribution in UASB granules and its resulting effects. *Water Sci Technol*. **2000**, *42*(12), 201-208. doi:10.2166/WST.2000.0272
7. Hinken L, Huber M, Weichgrebe D, Rosenwinkel KH. Modified ADM1 for modelling an UASB reactor laboratory plant treating starch wastewater and synthetic substrate load tests. *Water Res*. **2014**, *64*, 82-93. doi: 10.1016/J.WATRES.2014.06.044
8. He H, Ji X, Xie X, Ding X, Wang F, Ding J, Hai Z, Tanget R. Energy and economic evaluation of three generations of anaerobic reactors for starch wastewater treatment. *Env. Pollutants and Bioavailability* **2019**, *31*(1), 252-260. doi:10.1080/26395940.2019.1662734
9. Lu X, Zhen G, Estrada AL, Chen M, Ni J, Hojo T, Kubota K, Li YY. Operation performance and granule characterization of upflow anaerobic sludge blanket (UASB) reactor treating wastewater with starch as the sole carbon source. *Bioresour Technol.* **2015**, *180*, 264-273. doi: 10.1016/J.BIORTECH.2015.01.010
10. Bouaita R, Derbal K, Panico A, Iasimone F, Pontoni L, Fabbricino M, Pirozzi F. Methane production from anaerobic co-digestion of orange peel waste and organic fraction of municipal solid waste in batch and semi-continuous reactors, *Biomass and Bioenergy*, **2022**, 160, 106421. doi: 10.1016/j.biombioe.2022.106421
11. Musa MA, Idrus S, Man HC, Daud NNN. Performance Comparison of Conventional and Modified Upflow Anaerobic Sludge Blanket (UASB) Reactors Treating High-Strength Cattle Slaughterhouse Wastewater. *Water*, **2019**, 11(4), 806. doi: 10.3390/w11040806
12. M. N. I. Siddique MNI, Wahid ZA. Achievements and perspectives of anaerobic co-digestion: A review, *J. Clean. Prod.*, **2018**, 194, 359–371, doi: 10.1016/j.jclepro.2018.05.155.
13. Rasapoor M, Young B, Brar R, Sarmah A, Zhuang WQ, Baroutian S. Recognizing the challenges of anaerobic digestion: Critical steps toward improving biogas generation. *Fuel*, **2020**, 261, 116497. doi: 10.1016/j.fuel.2019.116497.
14. Wang Z, Wang T, Si B, Watson J, Zhang Y. Accelerating anaerobic digestion for methane production: Potential role of direct interspecies electron transfer. *Renew. Sustain. Energy Rev.*, **2021**, 145, 111069. doi: 10.1016/j.rser.2021.111069
15. Kot AM, Pobiega K, Piwowarek K, Kieliszek M, Błażejak S, Gniewosz M, Lipińska E. Biotechnological Methods of Management and Utilization of Potato Industry Waste—a Review, *Potato Res.*, **2020** 63(3), 431–447 doi: 10.1007/s11540-019-09449-6
16. Jing Z, Hu Y, Niu Q, Liu Y, Li YY, Wang XC. UASB performance and electron competition between methane-producing archaea and sulfate-reducing bacteria in treating sulfate-rich wastewater containing ethanol and acetate. *Bioresour Technol*. **2013**, *137*, 349-357. doi: 10.1016/J.BIORTECH.2013.03.137
17. Lu X, Ni J, Zhen G, Kubota K, Li YY. Response of morphology and microbial community structure of granules to influent COD/SO_4^{2-} ratios in an upflow anaerobic sludge blanket (UASB) reactor treating starch wastewater. *Bioresour Technol*. **2018**, *256*, 456-465. doi: 10.1016/J.BIORTECH.2018.02
18. Choi E, Rim JM. Competition and Inhibition of Sulfate Reducers and Methane Producers in Anaerobic Treatment. *Water Sci Technol*. **1991**, *23*(7-9), 1259-1264. doi:10.2166/WST.1991.0577
19. Yang G, Zhang G, Zhuan R, Yang A, Wang Y. Transformations, Inhibition and Inhibition Control Methods of Sulfur in Sludge Anaerobic Digestion: A Review. *Curr. Org. Chem.* **2016**, 20(26), 2780–2789. doi: 10.2174/1385272820666160513152913
20. Lippens C, De Vrieze J. Exploiting the unwanted: Sulphate reduction enables phosphate recovery from energy-rich sludge during anaerobic digestion. *Water Res.* **2019**, 163, 114859. doi: 10.1016/j.watres.2019.114859
21. Visser A, Beeksma I, van der Zee F, Stams AJM, Lettinga G. Anaerobic degradation of volatile fatty acids at different sulphate concentrations. *Appl. Microbiol. Biotechnol.* **1993**, 40(4), 549–556. doi: 10.1007/BF00175747
22. Zhang C, Lu Q, Li Y. A review on sulfur transformation during anaerobic digestion of organic solid waste: Mechanisms, influencing factors and resource recovery. *Sci. Total Environ.* **2023**, 865, 161193. doi: 10.1016/j.scitotenv.2022.161193
23. Zhang L, Schryver P, Gusseme B, Muynck W, Boon N, and W. Verstraete W. Chemical and biological technologies for hydrogen sulfide emission control in sewer systems: A review. *Water Res.*, **2008**, 42(1-2), 1–12. doi: 10.1016/j.watres.2007.07.013
24. Gao M, Guo B, Zhang L, Zhang Y, Yu N, Liu Y. Biomethane recovery from source-diverted household blackwater: Impacts from feed sulfate, *Process Saf. Environ. Prot.*, **2020,** *136*, 28–38. doi: 10.1016/j.psep.2020.01.010

25. Li W, Niu Q, Zhang H, Tian H, Zhang Y, Gao Y, Li YY, Nishimura O, Yang M. UASB treatment of chemical synthesis-based pharmaceutical wastewater containing rich organic sulfur compounds and sulfate and associated microbial characteristics, *Chem. Eng. J.*, **2015**, *260*, 55–63 doi: 10.1016/j.cej.2014.08.085
26. Jeong TY, Chung HK, Yeom SH, Choi SS. Analysis of methane production inhibition for treatment of sewage sludge containing sulfate using an anaerobic continuous degradation process, *Korean J. Chem. Eng.*, **2009**, *26*(5), 1319–1322.
27. Hulshoff Pol LW, Lens PNL, Weijma J, Stams AMJ. New developments in reactor and process technology for sulfate reduction, *Water Sci. Technol.*, **2001**, *44*(8), 67–76. doi: 10.2166/wst.2001.0467
28. Lu X, Zhen G, Ni J, Hojo T, Kubota K, Li YY. Effect of influent COD/SO_4^{2-} ratios on biodegradation behaviors of starch wastewater in an upflow anaerobic sludge blanket (UASB) reactor. *Bioresour Technol*. **2016**, *214*, 175-183. doi: 10.1016/J.BIORTECH.2016.04.100
29. Hu Y. Jing Z, Sudo Y, Niu Q, Du J, Wu J, Li YY. Effect of influent COD/SO42− ratios on UASB treatment of a synthetic sulfate-containing wastewater, *Chemosphere*, **2015**, *130*, 24–33. doi: 10.1016/j.chemosphere.2015.02.019
30. Liu Y, Zhang Y, Ni BJ. Zero valent iron simultaneously enhances methane production and sulfate reduction in anaerobic granular sludge reactors. *Water Res*. **2015**, *75*, 292-300. doi: 10.1016/J.WATRES.2015.02.056
31. Tran T. *Standard Methods for the Examination of Water and Wastewater*, 23nd edition.
32. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods*. **2013**, *10*(10), 996-998. doi:10.1038/nmeth.2604
33. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res***.** **2004**, *32*(5), 1792-1797. doi:10.1093/NAR/GKH340
34. Vongvichiankul C, Deebao J, Khongnakorn W. Relationship between pH, Oxidation Reduction Potential (ORP) and Biogas Production in Mesophilic Screw Anaerobic Digester, *Energy Procedia*, **2017**, 138, 877–882. doi: 10.1016/j.egypro.2017.10.113
35. Li W, Niu Q, Wu J, Luan X, Qi W, Zhang Y, Li YY, Gao Y, Yang M. Enhanced anaerobic performance and SMD process in treatment of sulfate and organic S-rich TMBA manufacturing wastewater by micro-electric field–zero valent iron-UASB, *J. Hazard. Mater.*, **2019**, *379*, 120695. doi: 10.1016/j.jhazmat.2019.05.088
36. Cheng Z, Hu X, Sun Z. Microbial community distribution and dominant bacterial species analysis in the bio-electrochemical system treating low concentration cefuroxime. *Chem Eng J*. **2016**, *303*, 137-144. doi: 10.1016/J.CEJ.2016.05.131
37. Schloss PD, Gevers D, Westcott SL. Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies. *PLoS One*. **2011**, *6*(12), e27310. doi: 10.1371/JOURNAL.PONE.0027310
38. Zhou L, Gao Y, Yu K, Zhou H, De Costa YG, Yi S, Zhuang WQ. Microbial community in in-situ waste sludge anaerobic digestion with alkalization for enhancement of nutrient recovery and energy generation, *Bioresour. Technol*., **2020,** *295*, 122277. doi: 10.1016/j.biortech.2019.122277
39. Magdalena JA, Greses S, González-Fernández C. Anaerobic degradation of protein-rich biomass in an UASB reactor: Organic loading rate effect on product output and microbial communities' dynamics. *J Environ Manage*. **2020**, *274*, 111201. doi: 10.1016/J.JENVMAN.2020.111201
40. Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics*. **2011**, *12*. doi:10.1186/1471-2105-12-385
41. Das BK, Roy S, Dev S, Das D, Bhattacharya J. Improvement of the degradation of sulfate rich wastewater using sweetmeat waste (SMW) as nutrient supplement. *J Hazard Mater*. **2015,** *300*, 796-807. doi: 10.1016/J.JHAZMAT.2015.08.013
42. Li J, Wang J, Luan Z, Ji Z, Yu L. Biological sulfate removal from acrylic fiber manufacturing wastewater using a two-stage UASB reactor. *J Environ Sci*. **2012**, *24*(2), 343-350. doi:10.1016/S1001-0742(11)60772-9
43. Vossoughi M, Shakeri M, Alemzadeh I. Performance of anaerobic baffled reactor treating synthetic wastewater influenced by decreasing COD/SO_4 ratios. *Chem Eng Process Process Intensif*. **2003**, *42*(10), 811-816. doi:10.1016/S0255-2701(02)00107-1
44. Maree JP, Strydom WF. Biological sulphate removal from industrial effluent in an upflow packed bed reactor. *Water Res*. **1987**, *21*(2), 141-146. doi:10.1016/0043-1354(87)90042-X
45. Erdirencelebi D, Ozturk I, Cokgor EU, Tonuk GU. Degree of sulfate-reducing activities on COD removal in various reactor configurations in anaerobic glucose and acetate-fed reactors. *Clean - Soil, Air, Water*. **2007**, *35*(2), 178-182. doi:10.1002/clen.200600027
46. Cunha MP, Ferraz RM, Sancinetti GP, Rodriguez RP. Long-term performance of a UASB reactor treating acid mine drainage: effects of sulfate loading rate, hydraulic retention time, and COD/SO_4^{2-} ratio. *Biodegradation*. **2019**, *30*(1), 47-58. doi:10.1007/s10532-018-9863-8
47. Costa JM, Rodriguez RP, Sancinetti GP. Removal sulfate and metals Fe^{+2}, Cu^{+2}, and Zn^{+2} from acid mine drainage in an anaerobic sequential batch reactor. *J Environ Chem Eng*. **2017**, *5*(2), 1985-1989. doi: 10.1016/J.JECE.2017.04.011

48. Antwi P, Li J, Opoku Boadi P, Meng J, Shi E, Xue C, Zhang Y, Ayivi F. Functional bacterial and archaeal diversity revealed by 16S rRNA gene pyrosequencing during potato starch processing wastewater treatment in an UASB. *Bioresour Technol*. **2017**, *235*, 348-357. doi: 10.1016/J.BIORTECH.2017.03.141
49. Kang X, Lin R, Li L, Wu B, Deng C, O'Shea R, Sun Y, Murphy JD. Assessment of pretreatment and digestion temperature on anaerobic digestion of whiskey byproducts and microbial taxonomy. *Energy Convers. Manag*. **2021**, 243, 114331 doi: 10.1016/J.ENCONMAN.2021.114331
50. Syaichurrozi I, Villta PK, Nabilah N, Rusdi R, "Effect of sulfuric acid pretreatment on biogas production from Salvinia molesta. *J. Environ. Chem. Eng*. **2019**, 7(1), 102857. doi: /10.1016/j.jece.2018.102857
51. Chen L, Zhang Y, Liang J, Li Y, Zhang J, Fang W, Zhang P, Zhang G, Ngo HH. Improvement of anaerobic digestion containing sulfur with conductive materials: Focusing on recent advances and internal biological mechanisms. Chem. Eng. J. **2023**, 472, 144867. doi: 10.1016/j.cej.2023.144867
52. Yang SL, Tang YQ, Gou M, Jiang X. Effect of sulfate addition on methane production and sulfate reduction in a mesophilic acetate-fed anaerobic reactor. *Appl. Microbiol. Biotechnol.* **2015,** 99(7), 3269–3277. doi: 10.1007/s00253-014-6235-0
53. Jin Z, Zhao Z, Zhang Y. Insight into ferrihydrite effects on methanogenesis in UASB reactors treating high sulfate wastewater: Reactor performance and microbial community. *Environ Sci Water Res Technol*. **2020**, *6*(7), 1794-1803. doi:10.1039/D0EW00154F
54. Lin H, Zhu L, Xu X, Zang L, Kong Y. Reductive transformation and dechlorination of chloronitrobenzenes in UASB reactor enhanced with zero-valent iron addition. *J Chem Technol Biotechnol*. **2011**, *86*(2), 290-298. doi:10.1002/jctb.2520
55. Wang SM, Tseng S kung. Dechlorination of trichloroethylene by immobilized autotrophic hydrogen-bacteria and zero-valent iron. *J Biosci Bioeng*. **2009**, *107*(3), 287-292. doi: 10.1016/J.JBIOSC.2008.11.010
56. Yoda M, Nishimura S. Controlling granular sludge floatation in UASB reactors. *Water Sci Technol*. **1997**, *36*(6-7), 165-173. doi:10.2166/WST.1997.0588
57. Zhang H, Zhang X, Chen Z, Liu S, Nai C, Ma Y, Zhanget H. Sulfate affects the anaerobic digestion process treating nitrogenous fertilizer wastewater. *Biochem Eng J*. **2022**, *186*,108550. doi: 10.1016/j.bej.2022.108550
58. Madani RM, Liang J, Cui L, Zhang D, Otitoju TA, Elsalahi RH, Song X. Novel simultaneous anaerobic ammonium and sulfate removal process: A review. *Environ Technol Innov*. **2021**, *23*, 101661. doi: 10.1016/J.ETI.2021.101661
59. Fdz-Polanco F, Fdz-Polanco M, Fernandez N, Urueña MA, Garcia PA, Villaverde S. New process for simultaneous removal of nitrogen and sulphur under anaerobic conditions. *Water Res*. **2001**, *35*(4), 1111-1114. doi:10.1016/S0043-1354(00)00474-7
60. Cisternas J, Rodríguez C, Serrano J, Leiva E. Study of the key biotic and abiotic parameters influencing ammonium removal from wastewaters by Fe3+-mediated anaerobic ammonium oxidation (Feammox). *Chemosphere*. **2023**, *339*, 139463. doi: 10.1016/J.CHEMOSPHERE.2023.139463
61. Desireddy S, Sabumon P.C, Maliyekkal S.M. Anoxic ammonia removal using granulated nanostructured Fe oxyhydroxides and the effect of pH, temperature and potential inhibitors on the process. *J Water Process Eng*. **2020**, *33*, 101066. doi: 10.1016/J.JWPE.2019.101066
62. Ban Q, Zhang L, Li J. Correlating bacterial and archaeal community with efficiency of a coking wastewater treatment plant employing anaerobic-anoxic-oxic process in coal industry, *Chemosphere*, **2022**, *286* part 2, 131724. doi: 10.1016/j.chemosphere.2021.131724
63. Zhang Q, Wang M, Ma X, Gao Q, Wang T, Shi X, Zhou J, Zuo J, Yang Y. High variations of methanogenic microorganisms drive full-scale anaerobic digestion process. *Environ Int*. **2019**, *126*, 543-551. doi: 10.1016/j.envint.2019.03.005
64. Zhang YT, Wei W, Huang QS, Wang C, Wang Y, Ni BJ. Insights into the microbial response of anaerobic granular sludge during long-term exposure to polyethylene terephthalate microplastics. *Water Res*. **2020**, *179*, 115898. doi: 10.1016/j.watres.2020.115898
65. Wu J, Jiang B, Kong Z, Yang C, Li L, Feng B, Luo Z, Xu KQ, Kobayashi T, Li YY. Improved stability of up-flow anaerobic sludge blanket reactor treating starch wastewater by pre-acidification: Impact on microbial community and metabolic dynamics. *Bioresour Technol*. **2021**, *326*, 124781. doi: 10.1016/J.BIORTECH.2021.124781
66. Shi L, Liu N, Liu G, Fang J. Bacterial community structure and dynamic changes in different functional areas of a piggery wastewater treatment system. *Microorganisms*. **2021**, *9*(10), 21-34. doi:10.3390/microorganisms9102134
67. Cummings DE, Caccavo F, Spring S, Rosenzweig RF. Ferribacterium limneticum, gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. *Arch Microbiol*. **1999**, *171*(3), 183-188. doi: 10.1007/s002030050697
68. Rissanen AJ, Peura S, Mpamah PA, Taipale S, Tiirola M, Biasi C, Mäki A, Nykänen H. Vertical stratification of bacteria and archaea in sediments of a small boreal humic lake. *FEMS Microbiol Lett*. **2019**, *366*(5), fnz044. doi:10.1093/femsle/fnz044

69. Mori K, Yamaguchi K, Sakiyama Y, Urabe T, Suzuki KI. Caldisericum exile gen. nov., sp. nov., an anaerobic, thermophilic, filamentous bacterium of a novel bacterial phylum, Caldiserica phyl. nov., originally called the candidate phylum OP5, and description of Caldisericaceae fam. nov., Caldisericales ord. nov. and Caldisericia classis nov. *Int J Syst Evol Microbiol*. **2009**, *59*(11), 2894-2898. doi: 10.1099/ijs.0.010033-0
70. Ntaikou I, Gavala HN, Lyberatos G. Modeling of fermentative hydrogen production from the bacterium Ruminococcus albus: Definition of metabolism and kinetics during growth on glucose. *Int J Hydrogen Energy*. **2009**, *34*(9), 3697-3709. doi: 10.1016/J.IJHYDENE.2009.02.057
71. Zheng Y, Kahnt J, Kwon IH, Mackie RI, Thauer RK. Hydrogen formation and its regulation in Ruminococcus albus: Involvement of an electron-bifurcating [FeFe]-hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative hydrogen-sensing [FeFe]-hydrogenase. *J Bacteriol*. **2014**, *196*(22), 3840-3852. doi:10.1128/JB.02070-14
72. Nishio N, Nakashimada Y, Hydrogen Generation from Food Industry and Biodiesel Wastes. *Food Ind. Wastes*, 1st ed., Chapter 9, Kosseva M , Webb C, Academic Press, **2013**, *1*, 157–170.
73. Wu H, Wang C, Chen P, He AY, Xing FX, Kong XP, Jiang M. Effects of pH and ferrous iron on the coproduction of butanol and hydrogen by Clostridium beijerinckii IB4. *Int J Hydrogen Energy*. **2017**, *42*(10), 6547-6555. doi: 10.1016/j.ijhydene.2017.02.094
74. Yin Y, Wang J. Mechanisms of enhanced hydrogen production from sewage sludge by ferrous ion: Insights into functional genes and metabolic pathways. *Bioresour Technol*. **2021**, *321*, 124435. doi: 10.1016/j.biortech.2020.124435
75. Lovley DR, Roden EE, Phillips EJP, Woodward JC. Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Mar Geol*. **1993**, *113*(1-2), 41-53. doi: 10.1016/0025-3227(93)90148-O
76. Paulo LM. Stams AJM, Sousa DZ., Methanogens, sulphate and heavy metals: a complex system, *Rev. Environ. Sci. Biotechnol.*, **2015**, *14*(4), 537–553. doi: 10.1007/s11157-015-9387-1
77. Colleran E, Finnegan S, Lens P, Anaerobic treatment of sulphate-containing waste streams, *Antonie Van Leeuwenhoek*, **1995**, 67(1), 29–46. doi: 10.1007/BF00872194
78. Niu L, Sun S, Yu F, Zhang X, Fan S, Sun Y, Lin W, Wang B, Zhang X. Performance of Sludge in the UASB Reactor for Treating Sulfate Wastewater: Sulfate Removal and Changes in the Community Structure Before and After Reaction. *Water, Air, Soil Pollut.* **2023**, 2023, 234-246. doi: 10.1007/s11270-023-06267-y
79. Zhang J, Lu Y. Conductive Fe3O4 nanoparticles accelerate syntrophic methane production from butyrate oxidation in two different lake sediments. *Front Microbiol*. **2016,** *7*, 1316. doi: 10.3389/fmicb.2016.01316
80. Iino T, Mori K, Uchino Y, Nakagawa T, Harayama S, Suzuki KI. Ignavibacterium album gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from microbial mats at a terrestrial hot spring and proposal of Ignavibacteria classis nov., for a novel lineage at the periphery of green sulfur bacteria. *Int. J. Syst. Evol. Microbiol.*, **2010,** *60*(6), 1376–1382. doi: 10.1099/ijs.0.012484-0.
81. Chen Y, Jia F, Liu Y, Yu W, Cai W, Zhang X, He H, Yao H. The effects of Fe(III) and Fe(II) on anammox process and the Fe–N metabolism, *Chemosphere*, **2021**, 285, 131322. doi: 10.1016/j.chemosphere.2021.131322