Insights into the Microbial Community Structure in the Biodegradation Process of High-Strength Ammonia Digestate Liquid Fraction in Conventional Activated Sludge System

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Biodegradation of digestate liquid fraction was performed in the activated sludge system with acetic acid, flume water, and molasses as external carbon sources. High-throughput sequencing was used to gain in-depth insight into the activated sludge microbial community. The type and amount of carbon source in influent (COD/TN ratio) significantly influenced microbial community structure, especially at the genus level, and thus the biodegradation performance of digestate liquid fraction. The highest total nitrogen and chemical oxygen demand removal efficiencies averaging 85.3% and 88.3%, respectively, were achieved in series with acetic acid and flume water and COD/TN ratio of 10.7 and 11.2, respectively. The microbial diversity in these series averaged at 3.08 and 3.65. The dominant bacteria at the phylum level in series with acetic acid were *Proteobacteria* and *Bacteroidota*, and at the genus level *Azospira*, while in series with flume water they were *Bacteroidota* and *Firmicutes*, and *Macellibacteroides*, respectively.

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Keywords: Digestate biodegradation; Biological nitrogen removal; External carbon source; Activated sludge; Microbial diversity

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INTRODUCTION

There is a growing concern about environmental pollution with digestate, which is produced in significant amounts during anaerobic digestion processes (Monfet *et al*. 2018; Świątczak *et al*. 2019). A 1 MW biogas plant generates about 10,000 to 30,000 m^3 of digestate per year, thus around 180 million tonnes of digestate are produced annually in Europe (Gienau *et al*. 2018; European Commission 2019). The currently used method of digestate utilisation, which is application as fertilizer, carries the risk of soil acidification, eutrophication of surface waters, and emission of greenhouse gases. In order to avoid exceeding the fertilization limits of nitrogen and phosphorus, digestate can be used as fertilizer only in the growing season. Due to such restrictions, biogas plants must have adequate facilities with a storage capacity of up to 6 months. This results in high costs compared with digestate fertilizing value, in addition to complicated logistic and transport requirements. Therefore, attempts are still being made to introduce alternative digestate processing technologies (Świątczak *et al*. 2019; Duan *et al*. 2020; Nyang'au *et al*. 2022). The first step of digestate treatment is usually its mechanical separation into a liquid and

solid fraction (Chuda and Ziemiński 2021a). The liquid fraction, containing several thousand mg/L of ammonia nitrogen, can be treated by biological methods, such as advanced nitrogen removal processes (nitritation-denitritation, nitritation-anammox), that have been recently reported (Monfet *et al*. 2018; Świątczak *et al*. 2019). However, because the conventional activated sludge process with autotrophic nitrification and heterotrophic denitrification is still the most common biological method used in on-site wastewater treatment plants, many agro-food factories are considering the possibility of its application for the treatment of the digestate liquid fraction (Yan *et al*. 2018; Yang *et al*. 2020).

Activated sludge technology is based on a complex microbial community, mainly bacteria, including those responsible for the removal of nitrogen and phosphorus compounds, as well as microorganisms devoid of direct bioremediation properties but cooperating with other community members (Cinà *et al*. 2019; Ríos-Castro *et al*. 2022; Wang *et al*. 2022). Therefore, a comprehensive understanding of microbial community structure, especially within specific microbial subpopulations, such as nitrifiers, denitrifiers, and their response to influent composition, is necessary for the efficient operation of wastewater treatment plants (Gao *et al*. 2016; Xu *et al*. 2018). Many techniques have been developed to study and characterize microbial communities over the past decade. Frequently used methods included those based on cultivation, traditional molecular biotechnology techniques, as well as more advanced high-throughput sequencing techniques, which provide high accuracy in the determination of complex microbial communities and their metabolic pathways (Zhang *et al*. 2018; Yang *et al*. 2020). However, despite such work, the relationships between microbial diversity and complex nutrients removal processes in activated sludge systems have not yet been fully explored (Cinà *et al*. 2019). Most studies of microbial community structure focus on activated sludge from municipal wastewater treatment plants, or plants in which a mixture of industrial and domestic wastewater is treated (Gao *et al*. 2016; Zhang *et al*. 2018). Meanwhile, knowledge of the microbial community of activated sludge from industrial wastewater treatment plants remains limited (Yang *et al*. 2020).

The microbial community is influenced by temperature, pH, oxygen concentration, organic and nitrogen loading rate, as well as the composition and COD/TN ratio of influent. If COD/TN ratio is excessively high, heterotrophic organisms use more available ammonia nitrogen compared to nitrifying organisms, resulting in decreased nitrification efficiency (Gao *et al*. 2016; Xu *et al*. 2018, 2019). In contrast, for wastewater with a low COD/TN ratio, such as digestate liquid fraction, the denitrification bacteria, which are mostly heterotrophic, cannot grow, resulting in incomplete denitrification. In such a situation, it is necessary to add an external carbon source to the influent. The most frequently used carbon sources are ethanol, methanol, and acetic acid. This is related to the low molecular weight, which makes them easily broken down by microorganisms and used by denitrifying bacteria. The drawback of these carbon sources is a relatively high price, which significantly increases the operating costs of treatment plants (Xue *et al*. 2018; Yan *et al*. 2018; Lan *et al*. 2022; Liu *et al*. 2022). Therefore, the application of food industry effluents and by-products as alternative carbon sources has recently attracted a lot of attention. The use of these cheap and organic substance-rich products could not only improve the denitrification process, but it also could contribute to their better management, and thus to the creation of a circular economy plant (Rodríguez *et al*. 2007; Xue *et al*. 2018; Yan *et al*. 2018).

The research attempted to determine the impact of the type and amount of external carbon source in the influent on the structure and diversity of the activated sludge microbial community, and thus to fully understand the complex process of biological nitrogen removal from the digestate liquid fraction. The study compared the composition of microorganisms consortium and the efficiency of digestate liquid fraction biodegradation obtained after the use of flume water and molasses, *i.e.*, alternative carbon sources from the sugar factory with those achieved after adding acetic acid, a conventional carbon source, which was a reference point. No studies were found that focused on changes in the microbial community structure in combination with the efficiency of biological treatment of the digestate liquid fraction with the addition of external carbon sources from the sugar industry. These results could contribute to a better understanding of the biodegradation process of high-strength ammonia nitrogen wastewater in a conventional activated sludge system.

EXPERIMENTAL

Substrates Characteristics

Digestate liquid fraction

The study used the liquid fraction obtained after mechanical separation of digestate in the decanter centrifuge type UCD 305-00-32 (GEA, Warsaw, Poland) and stored at 4 °C. The digestate was collected from a biogas plant processing sugar beet pulp located in the Südzucker sugar factory. The chemical composition of digestate and its liquid fraction are presented in Table 1.

Table 1. Characteristics of Substrates

Indicator/Substrate	Liquid Fraction of Digestate	Acetic Acid	Flume Water	Molasses
COD (g O_2/L)	6.78 ± 0.10	913.00 ± 0.00	7.75 ± 0.36	1018.10 ± 9.76
BOD_5 (g O_2/L)	1.47 ± 0.06	538.83 ± 0.16	3.62 ± 0.14	440.00 ± 0.34
TN (g/L)	2.13 ± 0.03	–	0.12 ± 0.01	24.66 ± 0.46
NH_4–N (g/L)	1.84 ± 0.07	–	0.06 ± 0.00	0.52 ± 0.05
pH	7.88 ± 0.12	2.50 ± 0.00	6.97 ± 0.09	7.37 ± 0.07
Total sugars (%)	0.72 ± 0.07	–	21.32 ± 4.32	61.48 ± 5.45
Total VFAs (%)	1.35 ± 0.46	–	37.54 ± 7.54	2.50 ± 0.42

External carbon sources

In the study, acetic acid with a purity of 80% was used. Acetic acid is a conventional carbon source, often added to intensify the denitrification process (Xue *et al*. 2018). Flume water and molasses supplied by the sugar factory were also investigated. Flume water circulates in a closed circuit and is used for hydraulic transport and washing of sugar beet. It is rich in organic matter, which consists mainly of sugars resulting from losses at various points of the manufacturing process (Macarie and Mer 2006). Molasses is a dense, dark brown syrup, the most valuable by-product from the sugar industry with a sugar content of around 50% (Silva *et al.* 2009). The chemical characteristics of the listed carbon sources are summarised in Table 1.

Activated sludge

The activated sludge was taken from an industrial wastewater treatment plant (WWTP) located on the premises of the sugar factory. The wastewater treatment plant operates on the basis of the conventional activated sludge method in a system with preliminary denitrification.

Experimental Set-Up

The biodegradation tests of the digestate liquid fraction were conducted in cooperation with the biogas plant and wastewater treatment plant located in the Südzucker sugar factory. Three laboratory systems were used in the study, containing a separate denitrification chamber (DC), nitrification chamber (NC), and secondary settling tank. The internal recirculation rate between the chambers corresponded to 500% of the inflow. The rate of sludge recirculation from the secondary settling tank to the denitrification chamber was 120%. The concentration of mixed liquor volatile suspended solids (MLVSS) in the activated sludge amounted to 3.2 ± 0.7 g/L, and the age of the sludge averaged 30 days. Influents, consisting of the liquid fraction of digestate diluted with treated wastewater and an external carbon source (ext.), were fed into the denitrification chambers (Fig. 1). Acetic acid was an external carbon source in the first stage of the study involving DAA1 and DAA2 series, flume water in the second stage in DFW1 and DFW2 series, and molasses in the third stage in DMS1 and DMS2 series. The series, which lasted 30 days, differed in the CODext/NO_3–N and COD/TN ratios, and thus in the amount of carbon source in the influent. The composition of the influents is presented in Table 2. During the biodegradation process, the total nitrogen loading rate was constant and amounted to 19.40 ± 1.57 mg N/g MLVSS d, while nitrogen load added with the liquid fraction of digestate accounted for 74.86 ± 6.38% of this value. The COD loading rate depended on the type of carbon source and CODext/NO_3–N ratio and ranged from 172.99 ± 22.82 (DAA1, DFW1, DMS1 series) to 218.89 ± 29.79 mg O_2/g MLVSS d (DAA2, DFW2 and DMS2 series). The method of determining CODext/NO_3–N ratio in the influent, and thus COD/TN ratio, as well as the dimensions and operating parameters of the reactors were presented in detail in the authors' previous article (Chuda and Ziemiński 2021b).

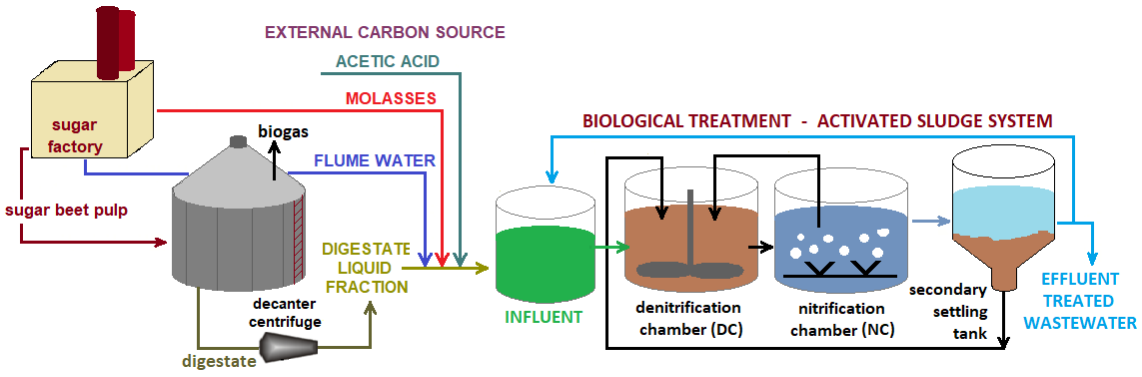

Fig. 1. Diagram with the flow of substrates during biological treatment of the digestate liquid fraction in the activated sludge system

Table 2. Composition of Influents

Stage of Study	I STAGE		II STAGE		III STAGE	
Carbon Source	Acetic Acid		Flume Water		Molasses	
Indicator/Series	DAA1	DAA2	DFW1	DFW2	DMS1	DMS2
COD (mg O_2/L)	1270.92 ± 9.24	1591.31 ± 7.98	1354.29 ± 8.85	1674.53 ± 9.14	1419.86 ± 7.96	1704.65 ± 8.24
BOD_5 (mg O_2/L)	487.06 ± 5.78	669.34 ± 6.98	454.49 ± 6.03	637.76 ± 7.20	464.12 ± 5.52	573.59 ± 6.65
TN (mg/L)	149.43 ± 0.85	148.99 ± 0.81	149.76 ± 0.92	149.99 ± 0.94	148.72 ± 0.75	149.30 ± 0.86
NH_4–N (mg/L)	102.47 ± 0.79	101.89 ± 0.84	107.99 ± 0.69	108.80 ± 0.75	89.83 ± 0.95	85.08 ± 0.78
pH	6.77 ± 0.19	6.85 ± 0.12	7.64 ± 0.25	7.72 ± 0.13	7.87 ± 0.16	7.92 ± 0.23
CODext[a]/NO_3–N	5.20	7.50	6.10	8.70	6.70	8.70
COD/TN	8.50	10.70	9.00	11.20	9.50	11.40

Note: Samples for analysis were taken in triplicate each day during each series, thus composition of the influents in series is average of 90 results ± standard deviation;
[a] ext – external carbon source

Analytical Methods

The methodology for analysing concentrations of total nitrogen (TN), ammonia nitrogen (NH_4–N), nitrate nitrogen (NO_3–N), nitrite nitrogen (NO_2–N), chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), mixed liquor volatile suspended solids (MLVSS), as well as pH and oxygen content measurement methods were described in a previous article (Chuda and Ziemiński 2021b). The concentrations of volatile fatty acids (VFAs) and sugars were determined using the Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) and UV-spectrophotometer (Thermo Scientific Multiskan Go, Thermo Fisher Scientific, Munich, Germany), respectively, following the procedures described by Cieciura-Włoch *et al*. (2021).

Microbial Community Analysis

DNA extraction

Total genomic DNA was extracted from sludge samples (250 g wet weight) using DNeasy PowerSoil Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol with some modifications. The Qubit 2.0 fluorometer (Invitrogen/Life Technologies, Carlsbad, CA, USA) was employed for DNA quantity and quality analysis.

Polymerase chain reaction (PCR) amplification and high-throughput sequencing

The amplification of the V3-V4 region of the 16S rDNA was performed with primers 341F and 785R specific for bacteria (Klindworth *et al.* 2012). The PCR reactions

were performed in triplicate with Q5 Hot Start High Fidelity 2x Master Mix (New England BioLabs Inc., Ipswich, MA, USA) under the following conditions: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 30 s (25 cycles), annealing at 55 °C for 30 s, extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The PCR products were purified by AMPure XP magnetic beads (Beckman Coulter Life Sciences Inc., Indianapolis, IN, USA). The amplicon libraries were indexed using Nextera Index Kit (ThermoFisher Scientific, MA, Waltham, USA) following the manufacturer's protocol.

High-throughput sequencing data analysis

High-throughput sequencing was performed by Genomed S.A. (Warsaw, Poland) on the MiSeq System employing MiSeq Reporter (MSR) v 2.6 software (Illumina, Inc., San Diego, CA, USA) in paired-end technology (PE, 2 × 250 bp). The QIIME 2 bioinformatic platform (https://qiime2.org/) with the SILVA v 138 database was used for the readings classification (Quast *et al.* 2013). The analysis contained: adapter sequences removal (cutadapt program), quality evaluation of the readings and low-quality sequences removal (cutadapt program), connecting paired sequences (SeqPrep algorithm), clustering based on the selected database of reference sequences (uclust algorithm), and sequence chimeras removal (usearch algorithm) (Edgar 2010; Martin 2011). Operational Taxonomic Units (OTUs) were assigned to taxa at an identity threshold of 97%. The diversity and evenness of the microbial community were analysed with alpha diversity estimators including Shannon (H), Simpson's (D), and inverse Simpson's (invD) indices determined using the Mothur program v. 1.30.1 (https://www.mothur.org; Schloss *et al.* 2009). The similarity of samples was presented on Venn diagrams (Venny 2.1, BioInfoGP Service, Centro Nacional de Biotecnologia, Madrid, Spain), based on specific OTUs for each activated sludge sample on genera level. Canonical correspondence analysis (CCA) was employed to determine the influence of operational parameters on the microbial communities. The CCA was conducted using XLSTAT v. 2022.1.1 (Lumivero, Denver, CO, USA) according to the manufacturer's protocol based on datasets for OTU profiles and environmental variables.

Statistical Analysis

To determine the differences between concentrations of NO_2–N, NO_3–N, NH_4–N, TN, COD, removal efficiencies of NH_4–N, TN, COD, percentage of bacteria, and biodiversity indices for all types of carbon sources and COD/TN ratios, an analysis of variance (ANOVA) test followed by Tukey *post hoc* was performed. Samples for analysis were taken in triplicate. In all tests, differences were significant only when $p < 0.05$. All statistical analyses were performed using Statistica 12 program (StatSoft, Krakow, Poland).

RESULTS AND DISCUSSION

Diversity of Microbial Community of Activated Sludge

Following the analysis of the results presented in Table 3, it was found that type and concentration of the external carbon source in the influent affected the microbial community composition and diversity. On average, 108,653 ± 17,409 16S rDNA bacterial sequences were determined in the activated sludge collected from both chambers in series with the lower COD/TN ratios (DAA1, DFW1, DMS1), while it was 102,753 ± 13,180 in

series with the higher COD/TN ratios (DAA2, DFW2, DMS2). Taking into account the carbon source type, the mean number of 16S rDNA sequences in the activated sludge from DAA series, in which acetic acid was used, amounted to 104,506 ± 6,273. In series with the alternative carbon sources, flume water (DFW series) and molasses (DMS series), it was 108,754 ± 26,699 and 103,822 ± 7,645, respectively. The OTUs number in the activated sludge from DAA, DFW, and DMS series averaged at 1683 ± 20, 2036 ± 86, and 1503 ± 118, respectively. The Shannon (H), Simpson's (D), and inverse Simpson's (invD) indices were in the range of 2.71 to 3.73, 0.07 to 0.17, and 6.02 to 14.94, respectively (Table 3).

Table 3. Alpha Diversity Index of the Microbial Community of the Activated Sludge Collected from DC and NC Chambers in All Series Differing in the Type of Carbon Source and COD/TN Ratio

Samples	Sequences Number	OTUs Number	Shannon Index (H index)	Simpson's Index (D index)	Inverse Simpson's Index (invD index)
DAA1 DC	99,162 ± 6240	1694 ± 30	3.40 ± 0.18	0.14 ± 0.02	7.34 ± 0.42
DAA1 NC	112,560 ± 7820	1653 ± 34	3.24 ± 0.21	0.14 ± 0.02	7.00 ± 0.46
DAA2 DC	99,913 ± 6310	1697 ± 31	3.08 ± 0.19	0.16 ± 0.03	6.29 ± 0.38
DAA2 NC	106,387 ± 7530	1689 ± 28	3.07 ± 0.14	0.16 ± 0.02	6.15 ± 0.40
DFW1 DC	85,777 ± 6350	1991 ± 67	3.73 ± 0.31	0.08 ± 0.01	12.43 ± 0.79
DFW1 NC	137,813 ± 8230	2151 ± 75	3.67 ± 0.26	0.07 ± 0.01	14.94 ± 0.87
DFW2 DC	86,379 ± 6080	1953 ± 61	3.65 ± 0.20	0.09 ± 0.02	10.63 ± 0.76
DFW2 NC	125,048 ± 7950	2048 ± 82	3.64 ± 0.24	0.08 ± 0.01	13.08 ± 0.78
DMS1 DC	104,047 ± 7120	1397 ± 82	2.71 ± 0.34	0.13 ± 0.02	7.58 ± 0.70
DMS1 NC	112,560 ± 6940	1405 ± 74	3.00 ± 0.26	0.12 ± 0.03	8.53 ± 0.78
DMS2 DC	93,918 ± 6140	1592 ± 94	3.40 ± 0.31	0.17 ± 0.03	6.02 ± 0.61
DMS2 NC	104,762 ± 6860	1617 ± 86	3.28 ± 0.28	0.15 ± 0.03	6.60 ± 0.55

A high H index, a high invD index, and a low D index indicate greater microbial diversity (Cinà *et al.* 2019; Yang *et al.* 2019). In the current study, the highest biodiversity (H index 3.64 to 3.73, invD index 10.63 to 14.94, D index 0.07 to 0.09) was determined in the activated sludge from both chambers in DFW series. This showed that the usage of flume water rich in a variety of organic compounds enabled the growth of more bacterial genera without causing any of them to dominate. It was noticed that in DFW, as well as DAA series, biodiversity was slightly higher with the lower COD/TN ratio. Chutivisut *et al*. (2018) and Xu *et al*. (2019) also observed that lower C/NO_3^- and COD/TN ratios determined a greater bacteria diversity in the population, which meant that various bacteria had a relatively large share among the dominating ones, while the higher ratios allowed the growth of only certain bacterial groups, but in higher proportions. In the described study, the lowest diversity expressed as Shannon (H) index (2.71 to 3.00) was found in DMS1 series in which the carbon source was molasses, and the COD/TN ratio was 9.5. Similar values of the H index, amounting to 2.39 and 3.29, were noted by Xu *et al*. (2019) during the treatment of synthetic wastewater with glucose and the COD/TN ratio of 10 and 7, respectively (Table 4).

Table 4. Structure and Diversity of Microbial Community in Various Treatment Systems

Treatment System; Wastewater Type	Dominant Phyla of Bacteria Proportion of Total OTUs (%)		Dominant Genera of Bacteria	Shannon Index; OTUs Number	Ref.
Municipal WWTPs with oxidation ditch, A/O[a], A/A/O[b], and two-stage A/O[c] processes; Domestic wastewater	*Proteobacteria*	44.9 to 56.0	*Zoogloea* *Dechloromonas* *Thauera* *Nitrospira* *Arcobacter*	7.71 9345 (mean values)	Gao *et al.* 2016
	Bacteroidetes	15.4 to 37.4			
	Firmicutes	1.7 to 5.0			
	Acidobacteria	0.2 to 6.8			
	Planctomycetes	0.3 to 3.6			
WWTP with A/O[a] process; Municipal wastewater	*Proteobacteria*	51	*Parafilimonas* *Thauera* *Xanthomonadales* *Dechloromonas* *Parafilimonas*	5.61 968	Yang *et al.* 2020
	Bacteroidetes	11			
	Chloroflexi	9			
	Acidobacteria	5			
	Planctomycetes	3			
WWTP with oxidation ditch process; Domestic and industrial wastewater	*Proteobacteria*	26.7 to 48.9	*Flavobacterium* *Dokdonella* *Terrimonas* *Tetrasphaera* *Simplicispira* *Nitrospira*	5.16 858 (mean values)	Xu *et al.* 2018
	Bacteroidetes	19.3 to 37.3			
	Chloroflexi	2.9 to 17.1			
	Actinobacteria	1.5 to 13.8			
	Planctomycetes	1.7 to 4.5			
	Firmicutes	1.4 to 4.8			
	Acidobacteria	0.5 to 5.1			
SBR reactor; Industrial and municipal wastewater	*Proteobacteria*	62	*Dechloromonas* *Hyphomicrobium* *Thauera* *Haliangium* *Halochromatium*	5.27 962	Yang *et al.* 2020
	Bacteroidetes	10			
	Acidobacteria	9			
	Chloroflexi	5			
	Planctomycetes	3			
Deep denitrification in denitrifying biofilter (DNBF); Tail water with acetate	*Proteobacteria*	75.7	*Dechloromonas* *Bdellovibrio* *Sediminibacterium* *Ignavibacterium* *Zoogloea*	4.06 1439	Xue *et al.* 2018
	Bacteroidetes	13.6			
	Acidobacteria	5.3			
	Ignavibacteriae	1.9			
	Firmicutes	0.8			
Cyclic activated sludge system (CASS); Synthetic wastewater with glucose COD/TN ratio of 10	*Proteobacteria*	68.4	not reported	2.39	Xu *et al.* 2019
	Actinobacteria	15.4			
	Bacteroidetes	14.2			
	Firmicutes	1			
Cyclic activated sludge system (CASS); Synthetic wastewater with glucose COD/TN ratio of 7	*Proteobacteria*	43.5	not reported	3.29	Xu *et al.* 2019
	Bacteroidetes	37.9			
	Actinobacteria	5			
	Chloroflexi	4			
	Firmicutes	3			

Note: [a] A/O – anoxic/aerobic; [b] A/A/O – anaerobic/anoxic/aerobic, [c] two-stage A/O – anaerobic/aerobic-anoxic/aerobic

In the current study, the CCA was performed to determine the potential influence of the operational parameters (COD/TN ratio, CODext/NO_3–N ratio, and external carbon source type) on the microbial communities of the activated sludges (Fig. 2). The length of arrows in the ordination plot indicates the strength of the environmental variables associated with the microbial community structure. The filled circles represent the different bacterial communities of activated sludge in both NC and DC chambers. The structure of the microbial community of the activated sludge from both chambers in DMS2 series was strongly related to the type of external carbon source, while in DFW2 series it was related to the COD/TN ratio. The weaker impact of operating parameters on the microbial community was observed in both chambers in DAA series.

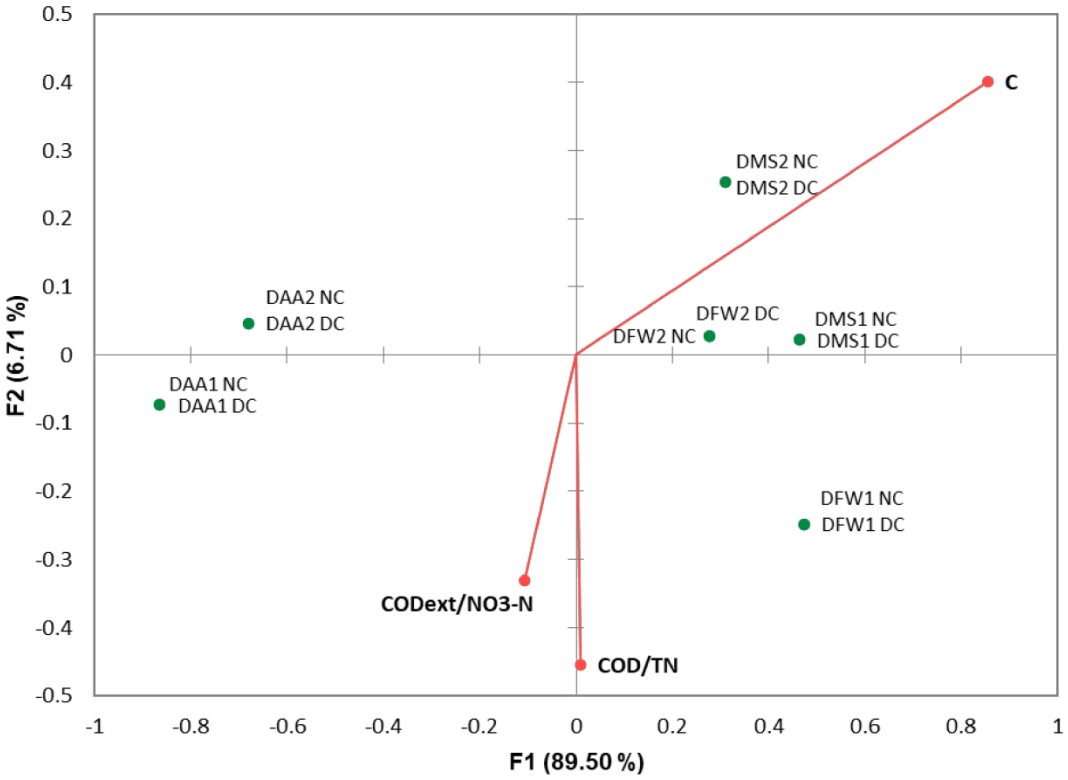

Fig. 2. Canonical correspondence analysis (CCA) of high-throughput sequencing data and operational parameters in the bioreactors

Taxonomic Classification of the Microbial Communities

The composition of microbial communities of the activated sludges is shown in Fig. 3. Bacteria dominated all consortia (90.87 ± 3.23%), with remaining taxa belonging to Archaea. At the phylum level, 26 to 35 different bacterial phyla were identified in the activated sludges, whereas only four phyla of Archaea (*Nanoarchaeota*, *Euryarchaeota*, *Halobacterota*, and *Iainarchaeota*) were detected with a relative abundance being less than 0.05%.

Analysing the results in Fig. 3, it was noticed that *Proteobacteria*, *Bacteroidota*, and *Firmicutes* were the most abundant bacteria at the phylum level in the activated sludges. These bacteria are described in the literature as widely present in conventional

wastewater treatment plants and denitrification environments, playing a significant role in removing organic components and nutrients (Gao *et al.* 2016; Xue *et al.* 2018; Xu *et al.* 2019; Wen *et al.* 2020; Yang *et al.* 2020; Ríos-Castro *et al.* 2022).

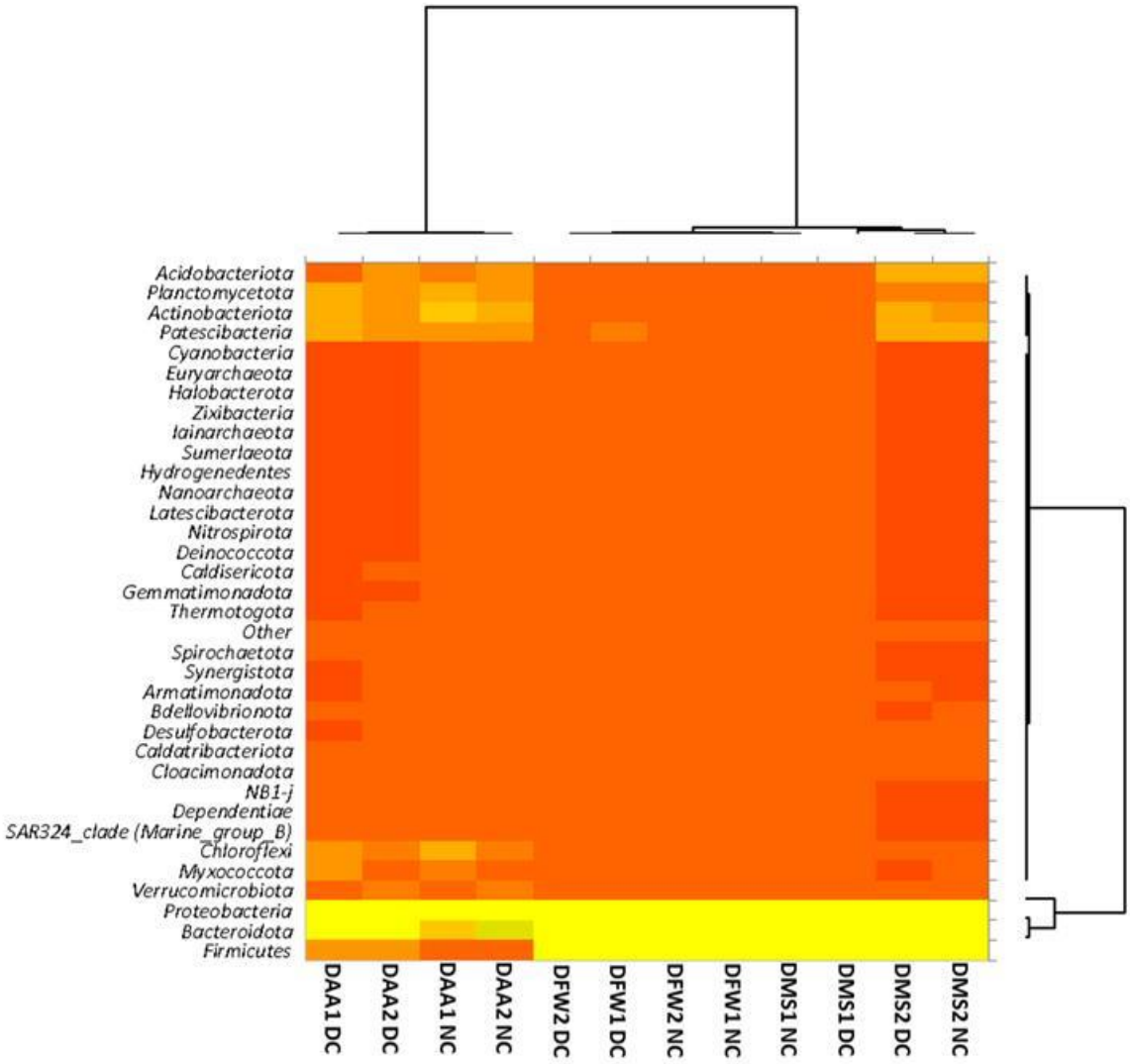

Fig. 3. Heatmap of bacterial phyla in the microbial community of the activated sludge collected in each series from the DC and NC chambers (given in terms of the number of OTUs)

The exact proportion of *Proteobacteria*, *Bacteroidota*, and *Firmicutes* in the microbial community depended on the type of carbon source and the COD/TN ratio in the influents. *Proteobacteria* was the most dominant phylum in the activated sludge in both DAA series in which acetic acid was the carbon source, followed by *Bacteroidota* and *Firmicutes*. The mean proportion of *Proteobacteria*, *Bacteroidota*, and *Firmicutes* amounted to 46.65 ± 2.34, 16.14 ± 1.76, and 4.48 ± 0.51% in the denitrification chambers, and 52.41 ± 2.07, 10.15 ± 1.37, and 1.25 ± 0.48% in the nitrification chambers, respectively. These results were similar to the amounts of these bacteria in the activated sludge during the treatment of domestic and industrial wastewater conducted by Xu *et al*. (2018) in the WWTP and the activated sludge collected by Gao *et al*. (2016) from full-scale municipal WWTP treated domestic wastewater (Table 4).

In DFW and DMS series with flume water and molasses as the carbon source, respectively, the same bacterial phyla were dominant, but their amounts were distributed differently than in series with the addition of acetic acid. The proportion of *Proteobacteria*, *Bacteroidota*, and *Firmicutes* in DFW series averaged at 19.68 ± 2.15, 44.20 ± 2.90, and 29.02 ± 0.09% in the denitrification chambers, and 20.99 ± 0.06, 38.18 ± 3.09, and 33.80 ± 2.35% in the nitrification chambers, respectively. The COD/TN ratio did not have a significant ($p > 0.05$) effect on the populations of mentioned bacteria in DAA and DFW series. It had a more noticeable effect on the microbial community composition in DMS series with molasses. In DMS2 series, in which the COD/TN ratio was 11.4, the proportion of *Proteobacteria*, *Bacteroidota*, and *Firmicutes* in the activated sludge averaged at 14.63 ± 1.02, 29.35 ± 1.12, and 28.05 ± 1.43% in the denitrification chamber, and 11.03 ± 0.98, 33.45 ± 1.54, and 30.47 ± 1.43% in the nitrification chamber, respectively. In DMS1 series, in which the COD/TN ratio was lower and amounted to 9.5, the proportion of *Proteobacteria* was 13.54 ± 1.05% in the denitrification chamber and 17.06 ± 1.12% in the nitrification chamber, *Bacteroidota* 35.26 ± 1.49 and 42.68 ± 1.82%, *Firmicutes* 48.60 ± 1.74 and 36.80 ± 1.56%, respectively. Xu *et al*. (2019) conducting the treatment process of synthetic wastewater with glucose defined the proportion of *Proteobacteria* in the activated sludge at the level of 68.38%, and *Bacteroidetes* at 14.19% with the COD/TN ratio similar to DMS1 series and equal to 10 (Table 4). Analysing the obtained results, it was noticed that *Proteobacteria* in DFW and DMS series occurred in lower amounts than in DAA series, whereas percentages of *Firmicutes* and *Bacteroidota* were significantly ($p < 0.05$) higher. This was probably because of the ability of *Firmicutes* and *Bacteroidota* to break down complex polysaccharides in hydrolytic and fermentative processes. By contrast, *Proteobacteria* comprises a broad variety of aerobic, facultative, and anaerobic bacteria, which can utilise various organic substrates to produce volatile fatty acids (Gao *et al*. 2016; Xue *et al*. 2018; Xu *et al*. 2019; Baskaran *et al*. 2020).

Predominant Bacterial Genera in Biological Treatment of the Digestate Liquid Fraction

The relative abundance of the microbial communities of the activated sludges at the genus level under different external carbon sources and the COD/TN ratios is presented in Fig. 4. In total, 522 genera of bacteria were detected. The article focuses only on the dominant genera and those significant from the point of view of the discussed process.

Analysing the Venn diagram (Fig. 4A through C) showing the similarities of the composition of the activated sludge from the NC and DC chambers at different COD/TN ratios, it was noted that the number of shared OTUs at the genus level in DFW series was the highest at 228 (Fig. 4B). The number of shared genera in DAA (Fig. 4A) and DMS (Fig. 4C) series was lower and amounted to 175 and 108, respectively. The shared OTUs indicated that some bacteria could exist in the activated sludge regardless of the COD/TN ratio and the chamber type. A possible explanation for this observation was the high rate of internal recirculation of oxygenated treated wastewater from the nitrification chamber into the denitrification one. This allowed some aerobic bacteria to survive in the anoxic unit. The unique OTUs specific to the defined environmental conditions were also present in the activated sludges. Their number in DAA series (NC and DC) was 1 to 9, in DFW series 5 to 13, while in DMS series it was in the range of 4 to 15.

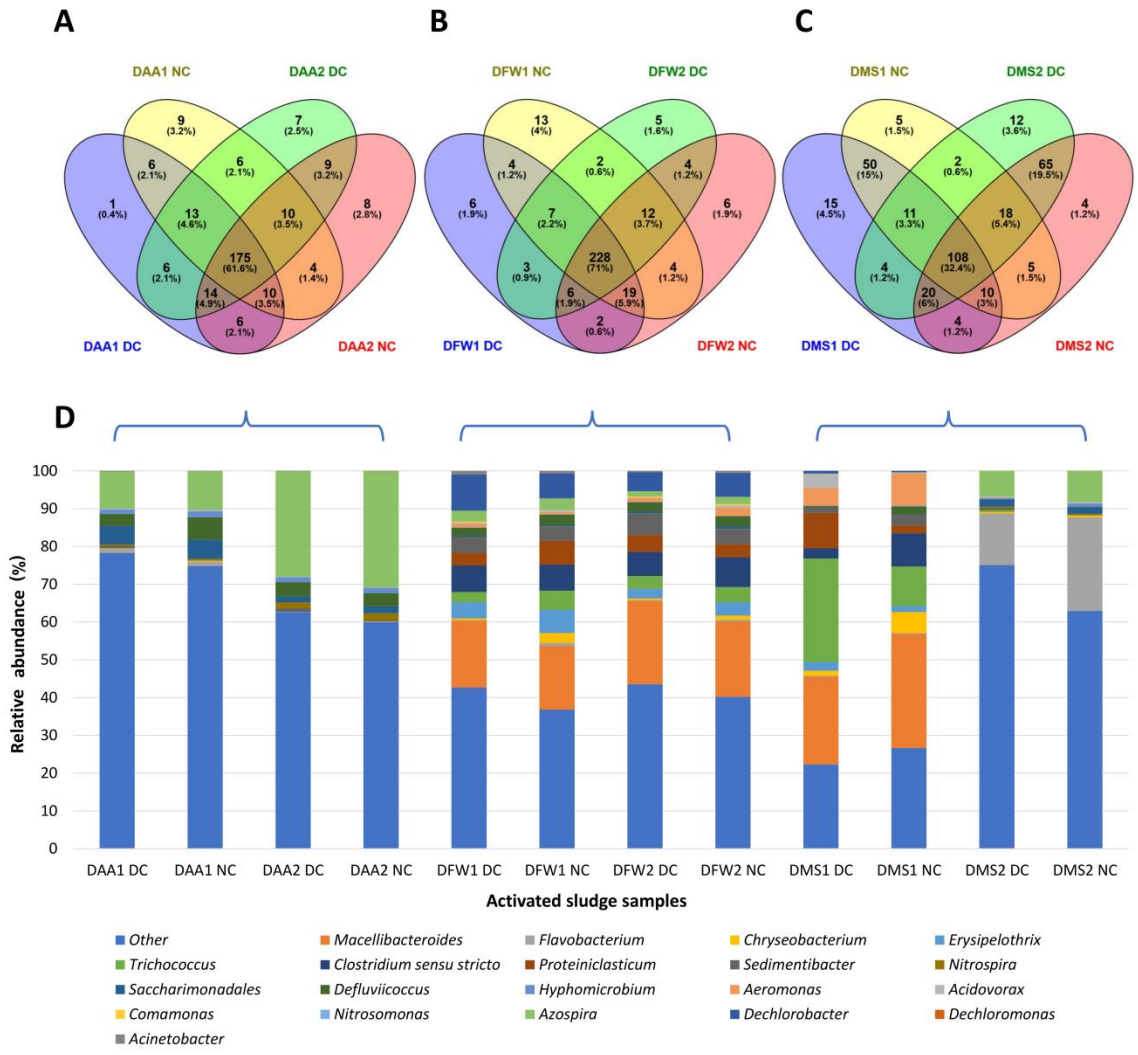

Fig. 4. Venn diagram with the overlap of bacterial genera in the activated sludge collected from the DC and NC chambers in DAA (A), DFW (B), and DMS (C) series; and the relative abundance of major bacterial genera (given in terms of percentage of the total OTUs) in the activated sludge microbial communities (D)

The greater quantity of unique microorganisms in DFW and DMS series was probably because of the more diverse composition of flume water and molasses compared to conventional acetic acid. Combining the relative abundance of the microbial communities with the effluents composition and the removal efficiencies of COD, TN, and NH_4–N shown in Fig. 5, the differences in the microbial community structure at the genus level caused by the addition of various types and amounts of the external carbon sources significantly influenced the performance of biological treatment of the digestate liquid fraction.

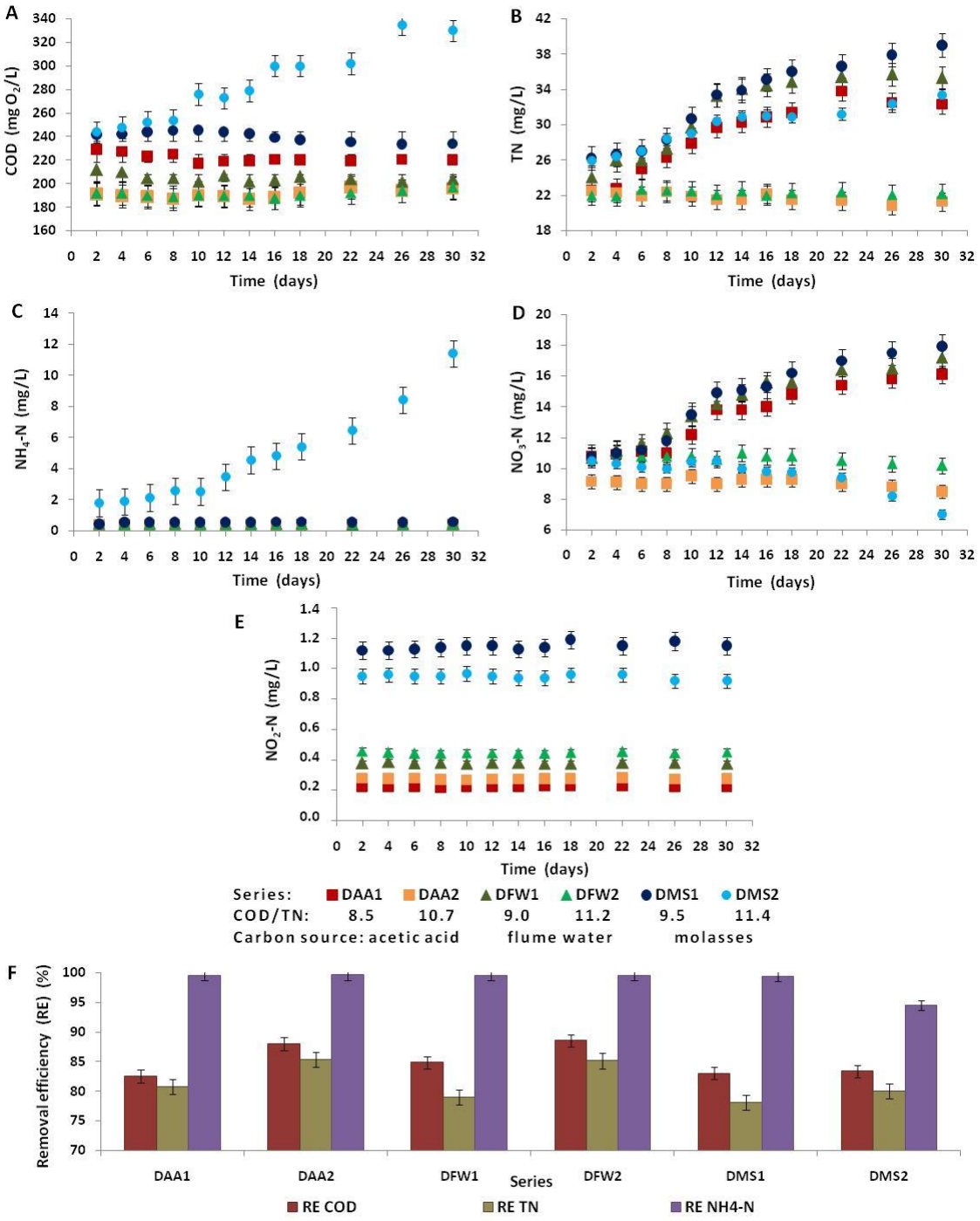

Fig. 5. Concentrations of COD (A), TN (B), NH_4–N (C), NO_3–N (D), and NO_2–N (E) in the effluents and mean removal efficiencies of COD, TN, and NH_4–N (F) during the biodegradation of digestate liquid fraction under different types of carbon sources and COD/TN ratios

The best results were obtained in DAA2 series, in which acetic acid was used as the conventional carbon source, and the COD/TN ratio was 10.7. Under these conditions, the activated sludge microbial community was dominated by *Azospira* (*Proteobacteria*), anaerobic bacteria belonging to heterotrophic denitrifiers with the ability to reduce nitrous oxide (N_2O) (Bae *et al.* 2007; Park *et al.* 2020). The percentage of these bacteria averaged

29.36 ± 2.06% of the total OTUs. Acetate, which is the preferred carbon source for *Azospira*, is easily assimilated into metabolic pathways, as it is degraded directly by the β-oxidation process to acetyl-CoA. Because of this, the nitrogen removal efficiency increases immediately after its addition (Elefsiniotis and Li 2006; Park *et al.* 2020). This is probably why the mean TN and COD removal efficiencies in DAA2 series were high and amounted to 85.49 ± 0.34 and 88.11 ± 0.21%, respectively (Fig. 5F), and the concentrations of NO_3–N, TN, and COD in the effluents were stable and averaged at 9.08 ± 0.26 mg/L, 21.78 ± 0.50 mg/L, and 190.78 ± 3.35 mg O_2/L, respectively (Fig. 5A, B, and D). It is worth mentioning that the use of lower COD/TN ratio equal to 8.5 in DAA1 series contributed to a 3-fold reduction in the amount of *Azospira* in the microbial community (9.81 to 10.20% of the total OTUs). This indicated that the COD/TN ratio determined the presence and percentage of *Azospira* in the activated sludge. The result of the lower proportion of these heterotrophic denitrifiers was an incomplete performance of the denitrification process, and thus an increase in the NO_3–N, TN, and COD concentrations in the effluent to 16.17 ± 0.24 mg/L, 32.30 ± 1.13 mg/L, and 220.23 ± 3.12 mg O_2/L, respectively. These values were significantly higher ($p < 0.05$) than in DAA2 series. The treatment process in DAA series was also supported by *Hyphomicrobium* (*Proteobacteria*), heterotrophic denitrifying bacteria and facultative methylotrophs. These bacteria are most abundant in denitrification systems in which methanol is an external carbon source, therefore their presence in series with acetic acid was unusual (Martineau *et al.* 2015; Wen *et al.* 2020). The similar percentage of *Hyphomicrobium* (1.20 to 1.51%) in both DAA series indicated that the amount of acetic acid did not have a significant ($p > 0.05$) impact on their participation in the microbial community.

The high efficiency of biological treatment of the digestate liquid fraction was also achieved in DFW2 series using flume water as the carbon source and the COD/TN ratio of 11.2. The concentrations of NO_3–N, TN, and COD in the effluents were stable during the process and averaged at 10.70 ± 0.25 mg/L, 22.28 ± 0.26 mg/L, and 191.20 ± 2.50 mg O_2/L (Fig. 5A, B, and D). The mean TN and COD removal efficiencies in DFW2 series amounted to 85.18 ± 0.18 and 88.48 ± 0.15%, respectively (Fig. 5F). It was unexpected that these results were comparable to DAA2 series (COD/TN ratio of 10.7) despite differences in the structure and diversity of the microbial community. In DFW2 series, the dominant genus of bacteria was not *Azospira* as in DAA2 series, but *Macellibacteroides* (*Bacteroidota*), *Clostridium sensu stricto* (*Firmicutes*), and *Aeromonas* (*Proteobacteria*), anaerobic and fermentative bacteria with the ability to ferment simple sugars, active in the denitrification process (Cinà *et al.* 2019; Yang *et al.* 2019; Fernández-Bravo and Figueras 2020; Yang and Wang 2020). Their proportion in the activated sludge from both chambers averaged at 21.10 ± 0.82, 7.90 ± 2.03, and 1.56 ± 0.62% of the total OTUs, respectively. The occurrence of *Macellibacteroides*, *Clostridium sensu stricto*, and *Aeromonas* in the activated sludge was probably related to the fermentation of flume water in evacuation trenches, during which the sugars contained in it, coming from losses in various points of the sugar production process, are converted into volatile fatty acids. It was noticed that with a smaller amount of flume water in the influent in DFW1 series (COD/TN ratio of 9.0), the proportion of these bacteria in the activated sludge was reduced to 16.20 ± 0.78, 6.41 ± 1.25, and 0.86 ± 0.28%, respectively. Meanwhile, the NO_3–N, TN, and COD concentrations in the effluent increased to 17.12 ± 0.43 mg/L, 35.30 ± 0.52 mg/L, and 204.15 ± 2.87 mg O_2/L, respectively. Analysing results in Fig. 5F, in all series with acetic acid or flume water as the carbon source, the removal efficiency of NH_4–N was 99.59 ± 0.02% on average. The COD/TN ratio did not have a significant ($p > 0.05$) impact on the

NH_4–N content, which was approximately 0.42 ± 0.02 mg/L in the effluents in DAA series and 0.48 ± 0.01 mg/L in DFW series (Fig. 5C). The concentrations of NO_2–N amounted to 0.25 ± 0.03 and 0.42 ± 0.03 mg/L, respectively (Fig. 5E). It is worth mentioning that the high efficiency of nitrification was obtained with a relatively low amount of *Nitrospira* (*Nitrospirota*) and *Nitrosomonas* (*Proteobacteria*), *i.e.*, aerobic, autotrophic, ammonia-oxidizing bacteria (Baskaran *et al.* 2020; Wen *et al.* 2020). Their percentage in the activated sludge from both chambers in DAA and DFW series averaged at 1.68 ± 0.28 and 0.07 ± 0.02% of the total OTUs, respectively. This showed that even a small amount of autotrophic nitrifiers in the activated sludge, whose functioning is not disturbed by external factors, allows for achieving high performance of ammonia nitrogen oxidation. This also indicated that knowledge about bacteria with nitrifying abilities is still limited.

The lowest treatment efficiency of the digestate liquid fraction was determined in DMS series using molasses as the carbon source. Analysing the results in Fig. 5A through C, it was noticed that in DMS2 series in which the COD/TN ratio was similar to that of DFW2 series and amounted to 11.4, the NH_4–N, TN, and COD concentrations in the effluent constantly increased and at the end of series reached 11.41 ± 0.53 mg/L, 33.38 ± 2.15 mg/L, and 330.13 ± 5.41 mg O_2/L, respectively. On the other hand, the NH_4–N, TN, and COD removal efficiencies decreased to 86.59 ± 1.72, 77.63 ± 1.95, and 80.64 ± 3.97% (Fig. 5F). The dominant genera in the microbial community in DMS2 series were *Flavobacterium* (*Bacteroidota*) and *Azospira* (*Proteobacteria*). Their percentage in the activated sludge from both chambers amounted to 19.2 ± 0.78 and 7.46 ± 0.86%, respectively. *Flavobacterium* species play a crucial role in the formation of the floc structure of activated sludge and the degradation of organic compounds. An excessive amount of organic material in influent may create conditions for their overgrowth and consequently lead to sludge bulking and foaming (Shchegolkova *et al.* 2016). This phenomenon observed in DMS2 series indicated that the COD/TN ratio and thus the amount of molasses added to the influent was too high. *Flavobacterium* species are known as aerobic denitrifying bacteria that can simultaneously use oxygen and nitrate as electron acceptors in the aerobic denitrification process, thus having a high capacity for nitrate removal. Most aerobic denitrifiers convert nitrates only to nitrous oxide (N_2O) instead of dinitrogen (N_2). The abundance of *Flavobacterium* producing N_2O in the activated sludge probably enabled the growth of *Azospira*, N_2O-reducing bacteria, for which glucose is not the optimal carbon source. *Flavobacterium* species, like many aerobic denitrifiers, are also capable of heterotrophic nitrification (Takaya *et al.* 2003; Baskaran *et al.* 2020; Park *et al.* 2020; Wen *et al.* 2020). These bacteria present in high amounts in the microbial community in DMS2 series probably used more ammonia nitrogen compared to the nitrifying organisms, which resulted in a disruption of the nitrifiers' functioning (Xu *et al.* 2019; Baskaran *et al.* 2020). This was evidenced by the lower nitrification efficiency than that obtained in DAA and DFW series with a similar percentage of *Nitrospira* and *Nitrosomonas* in the activated sludge. It is worth mentioning that *Flavobacterium* and *Azospira* were unnoticeable in DMS1 series with the COD/TN ratio of 9.5, thus a smaller amount of molasses in the influent. The dominant genera of bacteria in DMS1 series were *Macellibacteroides*, *Clostridium sensu stricto*, and *Aeromonas*. Their proportion in the activated sludge from the denitrification and nitrification chamber amounted to 23.22 ± 0.95 and 30.23 ± 1.04%, 2.72 ± 0.23 and 8.83 ± 0.44%, and 4.71 ± 0.36 and 8.52 ± 0.52%, respectively. In DMS1 series, the concentrations of NO_3–N, NO_2–N, and TN in the effluent increased to 17.98 ± 0.78, 1.15 ± 0.02, and 39.10 ± 1.07 mg/L, respectively (Fig. 5B, D, and E). This indicated that the denitrification process was incomplete. The COD and NH_4–

N concentrations in the effluent were stable and averaged at 240.38 ± 4.05 mg O_2/L and 0.28 ± 0.04 mg/L (Fig. 5A and C). Surprisingly, lower amounts of *Macellibacteroides* and *Aeromonas* in the activated sludge in both DFW series with flume water contributed to obtaining higher treatment efficiencies. This was related to the fact that molasses, compared to flume water, contains high concentrations of sugars with carbon chains that are too long to be easily used by microorganisms (Silva *et al.* 2009). In the activated sludge from the denitrification chamber in DMS1 series, *Acidovorax* species (*Proteobacteria*) were also present in the amount of 3.82 ± 0.39%. These aerobic and chemoorganotrophic microorganisms are capable of anaerobic growth and conducting denitrification using nitrate as a terminal electron acceptor and acetate as a carbon source. Some species of these bacteria have the ability to autotrophic growth using the oxidation of hydrogen as an energy source (Heylen *et al.* 2008). The abundance of *Acidovorax* in series with molasses, not acetate, was probably the result of coexistence with *Clostridium sensu stricto*, which produces acetate as one of the soluble metabolites of the hydrogen generation process from various organic substrates, *e.g.*, sucrose and glucose (Yang *et al.* 2019; Yang and Wang 2020).

Analysing the results of this study and those obtained by other authors during the treatment of municipal, domestic, industrial, and synthetic wastewater, included in Table 4, it was noticed that despite the presence of the same bacterial phyla in the activated sludges, the microbial community structure at the genus level was completely different. It is worth mentioning that the genera of bacteria identified in the activated sludge in this study were mostly absent in the sludges analysed by other authors.

CONCLUSIONS

1. The research showed that the type of carbon source and the chemical oxygen demand/ total nitrogen (COD/TN) ratio in the influent significantly affected the activated sludge microbial community, and thus the performance of biodegradation of the digestate liquid fraction.
2. The microbial community structure, especially at the genus level, greatly influenced treatment efficiency more than microbial diversity.
3. In series with acetic acid and flume water, better treatment results were obtained with lower diversity.
4. The variety of organic compounds in flume water positively affected the microbial community and the biological treatment efficiency of the digestate liquid fraction. This shows that wastewater from sugar factories can replace expensive conventional carbon sources, thus complying with the assumptions of a circular economy.
5. The similar biodegradation performance in series with acetic acid and flume water obtained despite differences in the activated sludge composition indicates that a detailed analysis of the microbial community structure is necessary to fully understand the biological process of nitrogen removal from various influents.
6. The described research can fill the gap in the field of alternative processing methods of digestate the amount of which, due to the need to increase renewable energy production, may be significantly larger in the future.

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