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Challenges in Treatment of Digestate Liquid Fraction from Biogas Plant. Performance of Nitrogen Removal and Microbial Activity in Activated Sludge Process

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Abstract: Even thoughdigestate, which is continually generated in anaerobic digestion process, can only be used as fertilizer during the growing season, digestate treatment is still a critical, environmental problem. That is why the present work aims to develop a method to manage digestate in agricultural biogas plant in periods when its use as fertilizer is not possible. A lab-scale system for the biological treatment of the digestate liquid fraction using the activated sludge method with a separate denitrification chamber was constructed and tested. The nitrogen load that was added tothe digestate liquid fraction accounted for 78.53% of the total nitrogen load fed into the reactor. External carbon sources, such as acetic acid, as well as flume water and molasses, i.e., wastewater and by-products from a sugar factory, were used to support the denitrification process. The best results were obtained using an acetic acid and COD (Chemical Oxygen Demand)/NO_3–N (Nitrate Nitrogen) ratio of 7.5. The removal efficiency of TN (Total Nitrogen), NH_4–N (Ammonia Nitrogen) and COD was 83.73%, 99.94%, 86.26%, respectively. It was interesting to see results obtained that were similar to those obtained when using flume water and COD/NO_3–N at a ratio of 8.7. This indicates that flume water can be used as an alternative carbon source to intensify biological nitrogen removal from digestate.

Keywords: anaerobic digestion; biological digestate treatment; activated sludge; nitrification/denitrification; external carbon source; COD/N ratio; microbial activity

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1. Introduction

The increase in organic waste generated in food industry plants observed in recent years and the emphasis on sustainability has significantly contributed to the growing interest in anaerobic digestion as a method for waste management. Apart from biogas, digestate is generated as the main by-product of anaerobic digestion (AD) [1–3]. A 1 MW biogas plant produces about 20,000–30,000 m^3 of digestate per year [4]. Digestate, which constitutes 90–95% of the substrate volume fed into a reactor, has become a major bottleneck in the development of the methane fermentation industry [1]. Many industrial biogas plants struggle with digestate processing on account of its large amounts. digestate is often used in land spreading as a fertilizer with a high nutrient content. Digestate, which is continually generated through the AD process, can only be used as a fertilizer during the growing season or during vegetative growth in order to avoid nutrient infiltration into the groundwater, soil acidification as well as the eutrophication of surface waters [2,5]. For this reason, biogas plants must have proper facilities with enough storage capacity for a 3- to 6-month period. This generates problems for biogas plants, as ammonia nitrogen is emitted during storage [6]. Therefore, alternative methods for digestate treatment, in particular nitrogen removal, are still being sought after. The first step in such a process is typically mechanical separation into a solid and liquid fraction [5]. Separation technologies allow better digestate management but do not change the total amount of nitrogen in the liquid

fraction [7,8]. The treatment of the digestate liquid fraction can be performed by ammonia stripping, membrane filtration, evapo-concentration, or drying [5,6,9]. However, due to the moderate effectiveness and high costs of these methods, which can range between 5.4–7.0 EUR/m^3 of digestate for membrane drying and stripping, their full-scale applications are limited [7,9]. This is the reason why if the agricultural management of digestate is not possible, then its biological treatment, which is regarded as the most economical process for nitrogen removal, may be a rational solution [7,10]. Currently, researchers are attracted to technologies that can be used for anaerobic ammonium oxidizing (anammox), which enables the treatment of wastewater with a high NH_4–N content [11]. However, the problems associated with the fragile resistance of anammox bacteria to environmental changes, the low growth rate of these bacteria, and limits placed on maximum nitrogen removal efficiency constitute major obstacles to the wide range application for these processes [12]. For this reason, the most commonly used biological method in industrial wastewater treatment plants is still a conventional activated sludge process that involves autotrophic nitrification and heterotrophic denitrification [13–15]. The stability of this process and the treatment outcome depend on the microbial community structure, the amount of functional groups belong to the activated sludge (denitrifiers, nitrifiers, heterotrophic microorganisms), their activity (Nitrate Utilization Rate—NUR, Ammonia Utilization Rate—AUR, Oxygen Utilization Rate—OUR), and the ratio of AOB (Ammonia–Oxidizing Bacteria) to NOB (Nitrite–Oxidizing Bacteria) [14,16,17], while the microbial activity is influenced by operating parameters, such as pH, temperature, and oxygen concentration as well as the nitrogen and COD loading rate and COD/TN ratio in the influent [1,13,15,18]. The increase in biological nitrogen removal efficiency in wastewater with a low COD/TN ratio can be achieved by adding an external carbon source [18]. The most frequently used carbon sources are easily biodegradable organics, such as ethanol [19,20], methanol [20–22] and acetic acid [15,20,21,23]. However, due to their high prices, the extensive use of these carbon sources on an industrial scale significantly increases operating costs [18,20]. Hence, many researchers are currently investigating the use of various types of organic waste from the agro–food industry as alternative external carbon sources [23–27]. This may allow them to not only enhance denitrification but to also reduce the amount of industrial waste that is subject to treatment [24]. Several factors have to be considered when selecting a carbon source, including the costs, denitrification rate, degree of utilization, sludge production, and adaptation time of the activated sludge [19,20].

The present study attempted to biologically treat the digestate liquid fraction in a conventional activated sludge system to help better manage digestate during periods when it cannot be used as a fertilizer. This was verified using flume water and molasses, so the industrial wastewater and the by–products generated in sugar factories can be used as alternative carbon sources to intensify biological nitrogen removal from digestate. The aim was to create a system where organic waste would be converted into energy in an anaerobic digestion process and where digestate can be biologically treated in an on–site wastewater treatment plant. These results can bring new ideas to digestate management practices.

2. Materials and Methods

2.1. Substrates Characteristics

2.1.1. Digestate

The liquid fraction of the digestate obtained after its mechanical separation in the UCD 305–00–32 decanter centrifuge (GEA, Warsaw, Poland) was used for test purposes. The digestate was taken from an agricultural biogas plant where sugar beet pulp (SBP) is a substrate for biogas production. The biogas plant is located on the property of the sugar factory belonging to the Südzucker company. The liquid fraction was stored at 4 °C. Its chemical composition is summarised in Table 1.

Table 1. Substrate characteristics.

Indicator	Liquid Fraction of Digestate	Flume Water	Molasses	Acetic Acid
COD ($g\ O_2/L$)	7.96 ± 0.55	6.38 ± 0.28	1014.00 ± 3.22	913.00 ± 0.00
SCOD ($g\ O_2/L$)	1.97 ± 0.05	4.32 ± 0.16	650.64 ± 0.86	913.00 ± 0.00
BDCOD ($g\ O_2/L$)	2.03 ± 0.02	5.13 ± 0.12	709.06 ± 0.94	912.84 ± 0.15
BOD_5 ($g\ O_2/L$)	1.18 ± 0.08	2.97 ± 0.10	410.00 ± 0.35	527.83 ± 0.26
TN (g/L)	2.32 ± 0.06	0.08 ± 0.00	25.38 ± 0.36	–
NH_4–N (g/L)	1.95 ± 0.07	0.04 ± 0.00	0.53 ± 0.03	–
TP (mg/L)	23.12 ± 1.59	2.20 ± 0.07	68.40 ± 1.13	–
pH	8.07 ± 0.16	6.83 ± 0.09	7.33 ± 0.03	2.50 ± 0.00

2.1.2. Activated Sludge

The activated sludge was obtained from an industrial sugar wastewater treatment plant (WWTP) that uses the activated sludge method in a system with preliminary denitrification. The wastewater treatment plant was located on the premises of the Südzucker sugar factory.

2.1.3. External Carbon Sources

Various external carbon sources were subject to tests. Their chemical characteristics are summarised in Table 1.

Acetic Acid

In the study, 80% acetic acid was used with a COD concentration of 913 g O_2/L, which is an easily biodegradable, conventional carbon source that is frequently used to intensify the denitrification process [18,21,24].

Flume Water

The flume water used for hydraulic transport and sugar beet washing was tested. It was supplied by the Südzucker sugar factory. During a sugar campaign, about 4 m^3 of flume water is produced per 1 ton of sugar beet, which accounts for about 72% of the total wastewater volume that is generated in the sugar factory [28].

Molasses

Thick, dark brown molasses syrup that was obtained after centrifuging crystallized sugar from concentrated beet juice was used. The molasses was also supplied by the Südzucker sugar factory. The processing of 100 kg of beet produces approx. 2.5–4 kg of molasses.

2.2. Experimantal Set-Up

The research on the biodegradation of the digestate liquid fraction was conducted in cooperation with the biogas plant and wastewater treatment plant located next to the Südzucker sugar factory. Studies were conducted under lab–scale conditions using the activated sludge method in three parallel systems (Figure 1). Each of them reflected the layout of the wastewater treatment plant in the sugar factory and consisted of a denitrification chamber with a volume of 0.013 m^3 and a diameter of 0.24 m, a nitrification chamber with a volume of 0.033 m^3 and a diameter of 0.39 m, and a secondary settling tank with a volume of 0.002 m^3 and a diameter of 0.1 m. Individual chambers were cylindrical

in shape. A mechanical stirrer was placed in the denitrification chamber, allowing the chamber content to mix at an intensity of 200 rpm. The nitrification chamber was aerated using Akwatech 50 PG membrane diffusers connected to a HIBLOW HP–80 air blower. The dissolved oxygen (DO) concentration in the nitrification chamber was maintained at 3.0 ± 0.2 mg O_2/L. The rate of internal recirculation between the nitrification and the denitrification chambers was 500% in relation to the inflow rate into the system. This was determined in previous unpublished studies by using Equation (1) [29]:

$$R = \left[\frac{(NH_3 - N)_o - (NH_3 - N)_e}{(NO_3 - N)_e} \right] - 1, \quad (1)$$

where R—rate of internal recirculation;

$(NH_3–N)_o$—NH_3–N concentration in the influent (g NH_3–N/m^3);

$(NH_3–N)_e$—NH_3–N concentration in the effluent (g NH_3–N/m^3);

$(NO_3–N)_e$—NO_3–N concentration in the effluent (g NO_3–N/m^3).

Influents containing the digestate liquid fraction, external carbon source, and treated wastewater were fed into the denitrification chambers. The aim of adding treated wastewater was to maintain the nitrogen loading rate at the assumed level of 21 mg N/g MLVSS d. The nitrogen load added to digestate accounted for 78.53 ± 8.81% of the total nitrogen load fed into denitrification chambers. Three study stages were conducted that differed in terms of the type of external carbon source that was used. In the first stage, including series DAA1, DAA2, and DAA3, the external carbon source was acetic acid; in the second stage in series DFW1, DFW2, and DFW3, flume water was used as the carbon source; and in the third stage in series DMS1, DMS2, and DMS3, molasses was used as the carbon source. The test series differed in terms of the COD/NO_3–N ratio, and each series lasted 30 days. The composition of the influents was analysed using the methods described in Section 2.3. on each day during each series. Each influent sample was taken in triplicate. The data shown in Table 2 are the mean of the 90 results obtained in the given series. The study began with series DAA1, DFW1, and DMS1, in which the COD/NO_3–N ratio was determined from the Equation $2.86/(1 - Y_{HD})$ [30,31] by taking into account the heterotrophic anoxic growth yield (Y_{HD}) that is equal to 0.45 for acetic acid as a carbon source [32], 0.53 for flume water (wastewater) [33], and 0.57 for molasses (glucose) [32]. The nitrate concentration to be denitrified was calculated from the equation described in [34]. The demand for the external carbon source necessary to reduce nitrate nitrogen may be higher than the values calculated from the equation [25]. Therefore, in subsequent series the COD/NO_3–N ratio, and thus the COD/TN ratio, was gradually increased until a stable course of the denitrification process was obtained during the research. The COD/NO_3–N and COD/TN ratios used in the studies are presented in Table 2. The adaptation period for the activated sludge to laboratory conditions and carbon sources in series DAA1, DFW1, and DMS1 lasted 30 days. During the adaptation period, the load of the organic matter and nitrogen was increased by approximately 33% every 10 days. For the first 10 days, the average COD loading rate was at 60.17 ± 10.15 mg COD/g MLVSS d, and the average, total nitrogen loading rate was at 6.65 ± 0.76 mg N/g MLVSS d. After the first series of tests, for 14 days, the activated sludge was adapted to higher COD/NO_3–N ratios, and thus to the changed composition of the influents in series DAA2, DFW2, and DMS2 and next in DAA3, DFW3, and DMS3. In order to maintain a constant concentration of mixed liquor suspended solids (MLSS) at 5.0 ± 0.4 g/L in the reactor, 120% external recirculation was applied between the secondary settling tank and the denitrification chamber. The mixed liquor volatile suspended solids (MLVSS) were at 3.0 ± 0.5 g/L, and the sludge age was at 30.00 ± 0.10 days. The temperature in the reactors was 25 ± 1.56 °C. The influence of the type of external carbon source and the COD/NO_3–N ratio on the treatment efficiency of the digestate liquid fraction is shown in Figure 2.

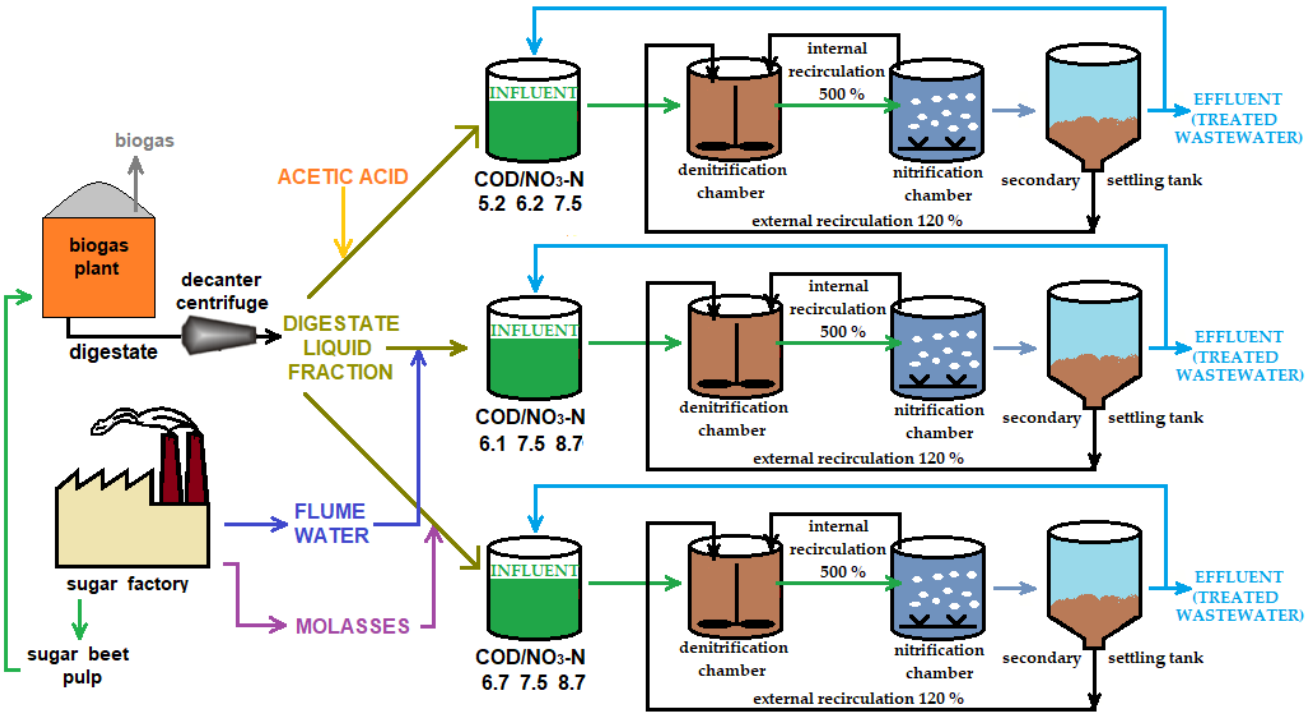

Figure 1. Schematic view of the laboratory wastewater treatment plant.

Table 2. Composition of influents.

Stage of Study	I Stage			II Stage			III Stage		
External Carbon Source	Acetic Acid			Flume Water			Molasses		
Indicator/Series	DAA1	DAA2	DAA3	DFW1	DFW2	DFW3	DMS1	DMS2	DMS3
COD (mg O_2/L)	1268.38 ± 8.54	1440.98 ± 8.02	1592.54 ± 8.08	1348.78 ± 8.08	1519.53 ± 9.02	1663.01 ± 11.02	1423.74 ± 5.86	1555.49 ± 6.43	1699.82 ± 6.81
BDCOD (mg O_2/L)	821.70 ± 8.12	955.32 ± 6.48	1126.39 ± 7.25	757.07 ± 6.78	916.49 ± 8.09	1037.08 ± 9.23	737.79 ± 7.56	811.95 ± 5.42	926.90 ± 5.95
BOD_5 (mg O_2/L)	475.14 ± 7.02	552.40 ± 7.00	651.32 ± 7.21	437.76 ± 8.19	529.94 ± 9.02	599.67 ± 9.50	426.62 ± 6.03	469.50 ± 5.69	535.96 ± 6.00
TN (mg/L)	149.19 ± 0.75	149.22 ± 0.72	149.30 ± 0.70	149.72 ± 0.90	148.83 ± 0.95	148.82 ± 0.92	149.81 ± 0.81	149.58 ± 0.88	149.73 ± 0.92
NH_4–N (mg/L)	107.95 ± 0.68	107.74 ± 0.64	107.34 ± 0.68	106.84 ± 0.81	106.68 ± 0.85	106.57 ± 0.81	87.67 ± 0.90	85.06 ± 0.95	82.61 ± 0.85
TP (mg/L)	1.76 ± 0.02	1.75 ± 0.02	1.75 ± 0.02	1.68 ± 0.01	1.72 ± 0.02	1.75 ± 0.02	1.49 ± 0.02	1.47 ± 0.03	1.45 ± 0.03
pH	6.71 ± 0.21	6.72 ± 0.19	6.75 ± 0.28	7.56 ± 0.20	7.61 ± 0.14	7.65 ± 0.10	7.79 ± 0.17	7.83 ± 0.24	7.89 ± 0.15
CODext */NO_3–N	5.20	6.20	7.50	6.10	7.50	8.70	6.70	7.50	8.70
BDCODext */NO_3–N	5.10	6.10	7.50	4.80	6.00	7.00	4.70	5.20	6.10
COD/TN	8.50	9.70	10.70	9.00	10.20	11.20	9.50	10.40	11.40

* ext—external carbon source.

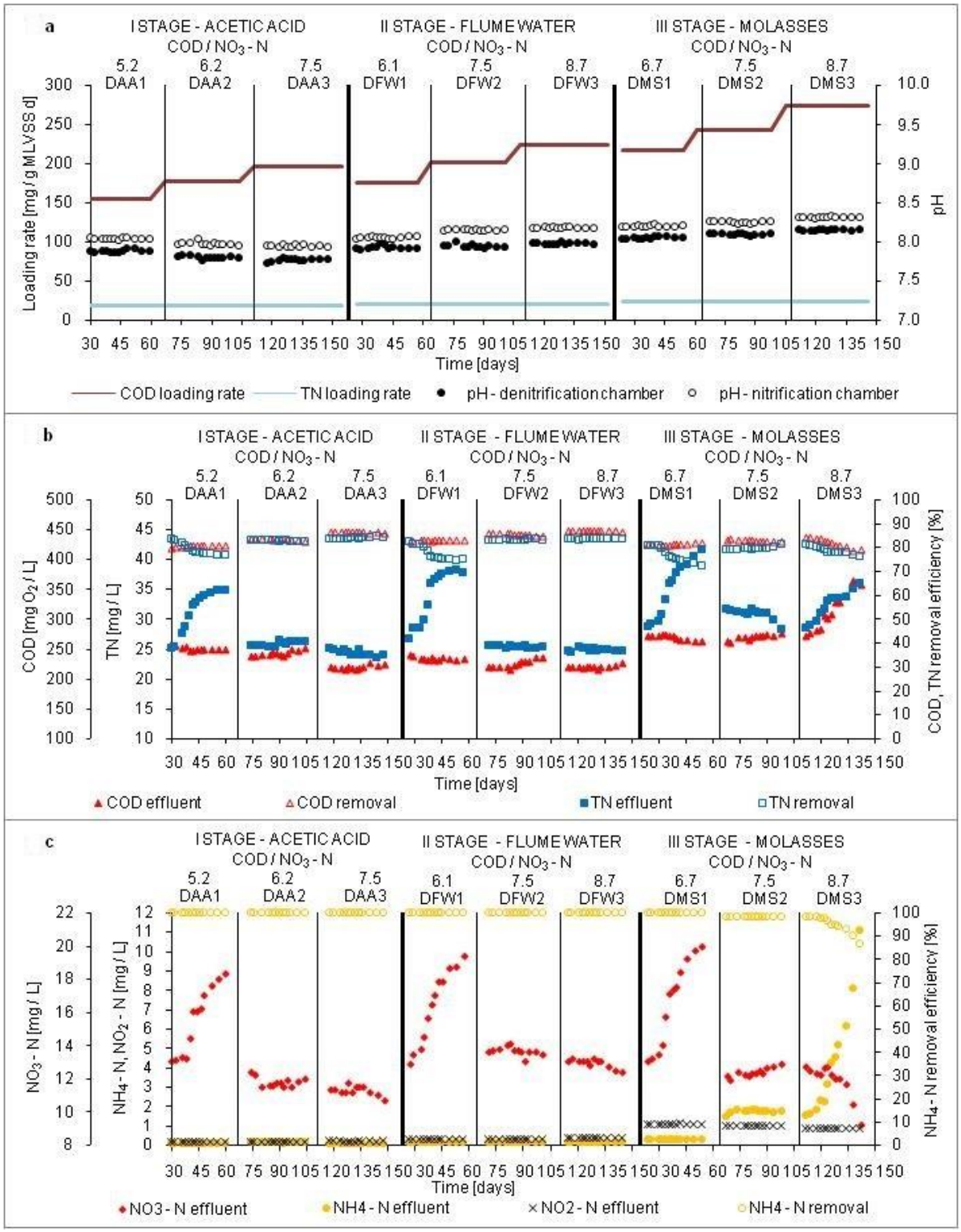

Figure 2. Operating parameters of reactors (**a**); COD and TN concentration in effluents and COD and TN removal efficiency (**b**); NH_4–N, NO_3–N, and NO_2–N concentration in effluents and NH_4–N removal efficiency (**c**).

2.3. Analytical Methods

The concentrations of COD, TN, NH_4–N, total phosphorus (TP), and nitrate and nitrite nitrogen (NO_3–N, NO_2–N) were measured using HACH tests (Hach–Lange, DR 6000 UV–VIS Spectrophotometer). The analytical procedures adopted by Hach Lange GmbH (Düsseldorf, Germany) followed the Standard Methods [35]. The concentration of

the biochemical oxygen demand (BOD_5) was analysed using the OxiTop system, WTW. The COD fractional composition was determined based on the ASM1 model [36] as well as on the methodology presented by [37]. According to these methods, soluble COD and biodegradable COD can be defined as:

$SCOD/COD_{mf}$—soluble COD from raw wastewater filtrated after coagulation with zinc chloride using 0.45 µm membrane filters;

BDCOD—biodegradable fraction of COD; $BDCOD = BOD_{tot}/(1 - f_{BOD})$ with the correction factor $f_{BOD} = 0.15$;

BOD_{tot}—total Biochemical Oxygen Demand assumed as $1.47 \times BOD_5$.

The concentrations of VSS, MLSS, and MLVSS in the reactors were determined following the procedures described in the Standard Methods [35]. The pH was measured with a CPI–505 pH meter (ELMENTRON, Poland). The concentration of oxygen in the reactor was measured using a CO–411 oxygen meter (ELMETRON, Poland).

2.4. Batch Tests (NUR, AUR, OUR)

The activity of the functional groups of the activated sludge (denitrifiers, nitrifiers, heterotrophic microorganisms) was determined by performing biochemical tests on the 30th day of each series: the specific denitrification rate (SNUR test–Specific Nitrate Utilization Rate), the specific nitrification rate (SAUR test–Specific Ammonia Utilization Rate), and the specific oxygen utilization rate (SOUR test–Specific Oxygen Utilization Rate). The methodology described by [17,30,38] was used in the tests.

The volumetric denitrification rates r_D and specific denitrification rates of the SNUR were calculated from Equations (2) and (3), respectively:

$$r_D = \Delta\ NO_x\text{–}N/\tau\ [mg\ N/L\ h], \quad (2)$$

$$SNUR = r_D/X_V\ [mg\ N/g\ MLVSS\ h], \quad (3)$$

where $NO_x\text{–}N = NO_3\text{–}N + 0.6\ NO_2\text{–}N$—the sum of nitrate and so-called nitrite–nitrate equivalent, which is also the sum of oxygenated nitrogen compounds, which is reduced to gaseous nitrogen (mg N/L);

τ —time of test (h);

X_V—volatile activated sludge concentration (g MLVSS/L).

The volumetric nitrification rate r_N (mg NH_4–N/L h) was calculated from the slope of the resulting ammonia utilization curve. The specific nitrification rate SAUR was calculated from Equation (4) by dividing the volumetric nitrification rate by the sludge concentration X_V:

$$SAUR = r_N/X_V\ [mg\ N/g\ MLVSS\ h], \quad (4)$$

The volumetric total oxygen utilization rate $r_{O2,tot}$(mg O_2/L h) was calculated from the slope of the resulting oxygen utilization curve. The specific oxygen utilization rate SOUR was obtained by Equation (5) by dividing the $r_{O2,tot}$ by the concentration of VSS in the batch experiment:

$$SOUR = r_{O2,tot}/X_V\ [mg\ O_2/g\ MLVSS\ h], \quad (5)$$

The specific oxygen utilization rate in the presence of $NaClO_3$ and ATU (the nitrification inhibitors) is an indicator of heterotrophic oxygen activity.

2.5. Molecular Studies

The activated sludge in the reactors was characterised by determining the total amount of bacteria, the amount of nitrifying bacteria, including AOB and NOB bacteria, and the growth balance between AOB and NOB as well as the number of denitrifying bacteria.

Genomic DNA from the activated sludge samples collected directly from separately working denitrification and nitrification chambers on the 30th day of each series was isolated using the Genomic Mini AX Bacteria kit (A&A Biotechnology) in accordance with

the manufacturer's protocols. A Real–Time PCR reaction was set up for each sample of the isolated DNA. Target genes were 16SrDNA (corresponding to the total bacterial DNA) and the *amoA* gene (AOB characteristic gene), *nxrA* gene (NOB characteristic gene), and the *nirS* and *nirK* genes (two nitrite reductase genes *nirS* and *nirK* characteristic of denitrifying bacteria). The Real–Time PCR reactions were performed in a Stratagene Mx3000P thermocycler (Agilent Technologies) using SYBR Green dye as the fluorochrome (A&A Biotechnology). Prior to setting up the reaction, the isolated DNA samples were diluted to 10 ng/µL; thus, a total of 10 ng of each DNA extract was used as the template in each reaction mixture. The oligonucleotide sequences of the primers, the composition of the reaction mixtures, and the description of the PCR programs are shown in Table 3. For each target gene, a melting curve was determined by measuring the fluorescence at each temperature (65 °C -> 95 °C). The efficiencies of the real–time PCR reactions in the amplification of the analysed genes were from 90 to 100%, and the correlation coefficient of the determined curves was higher than 0.997. The results of the analyses are shown in Figure 3.

Table 3. Primers and PCR programs.

Target	Primer	Sequence (5′—3′)	The Composition of Reaction Mixture		PCR Program	References of Primers Seguence
Bacterial 16SrDNA	1055F	ATGGCTGTCGTCAGCT	1 µL 10 µL 0.4 µL 0.4 µL 8.2 µL	DNA template (10 ng/µL) Real Time 2xRT–PCR Mix SYBR A (A&A Biotechnology) Primer 1055F (10 µM) Primer 1392R (10 µM) Nuclease–free water	3 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C; 65 °C → 95 °C	[39]
	1392R	ACGGGCGGTGTGTAC				
AOB—*amoA* gene	amoA–1F	GGGGTTTCTACTGGTGGT	1 µL 10 µL 0.4 µL 1.0 µL 7.6 µL	DNA template (10 ng/µL) Real Time 2xRT-PCR Mix SYBR A (A&A Biotechnology) Primer amoA–1F (10 µM) Primer amoA–2R (10 µM) Nuclease–free water	3 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C; 65 °C → 95 °C	[40]
	amoA–2R	CCCCTCKGSAAAGCCTTCTTC				
NOB—*nxrA* gene	nxrA–RT–F	GTGGTCATGCGCGTTGAGCA	1 µL 10 µL 0.4 µL 0.4 µL 8.2 µL	DNA template (10 ng/µL) Real Time 2xRT–PCR Mix SYBR A (A&A Biotechnology) Primer nxrA–RT–F (10 µM) Primer nxrA–RT–R (10 µM) Nuclease–free water	3 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C; 65 °C → 95 °C	[41]
	nxrA–RT–R	TCGGGAGCGCCATCATCCAT				
Denitrifying bacteria—*nirS* gene	nirS 1f	TACCACCCSGARCCGCGCGT	1 µL 10 µL 0.1 µL 0.1 µL 8.8 µL	DNA template (10 ng/µL) Real Time 2xRT–PCR Mix SYBR A (A&A Biotechnology) Primer nirS 1f (10 µM) Primer nirS 3r (10 µM) Nuclease–free water	3 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C; 65 °C → 95 °C	[42]
	nirS 3r	GCCGCCGTCRTGVAGGAA				
Denitrifying bacteria—*nirK* gene	nirK 876	ATYGGCGGVCAYGGCGA	1 µL 10 µL 0.1 µL 0.1 µL 8.8 µL	DNA template (10 ng/µL) Real Time 2xRT–PCR Mix SYBR A (A&A Biotechnology) Primer nirK 876 (10 µM) Primer nirK 1040 (10 µM) Nuclease–free water	3 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C; 65 °C → 95 °C	[42]
	nirK 1040	GCCTCGATCAGRTTRTGGTT				

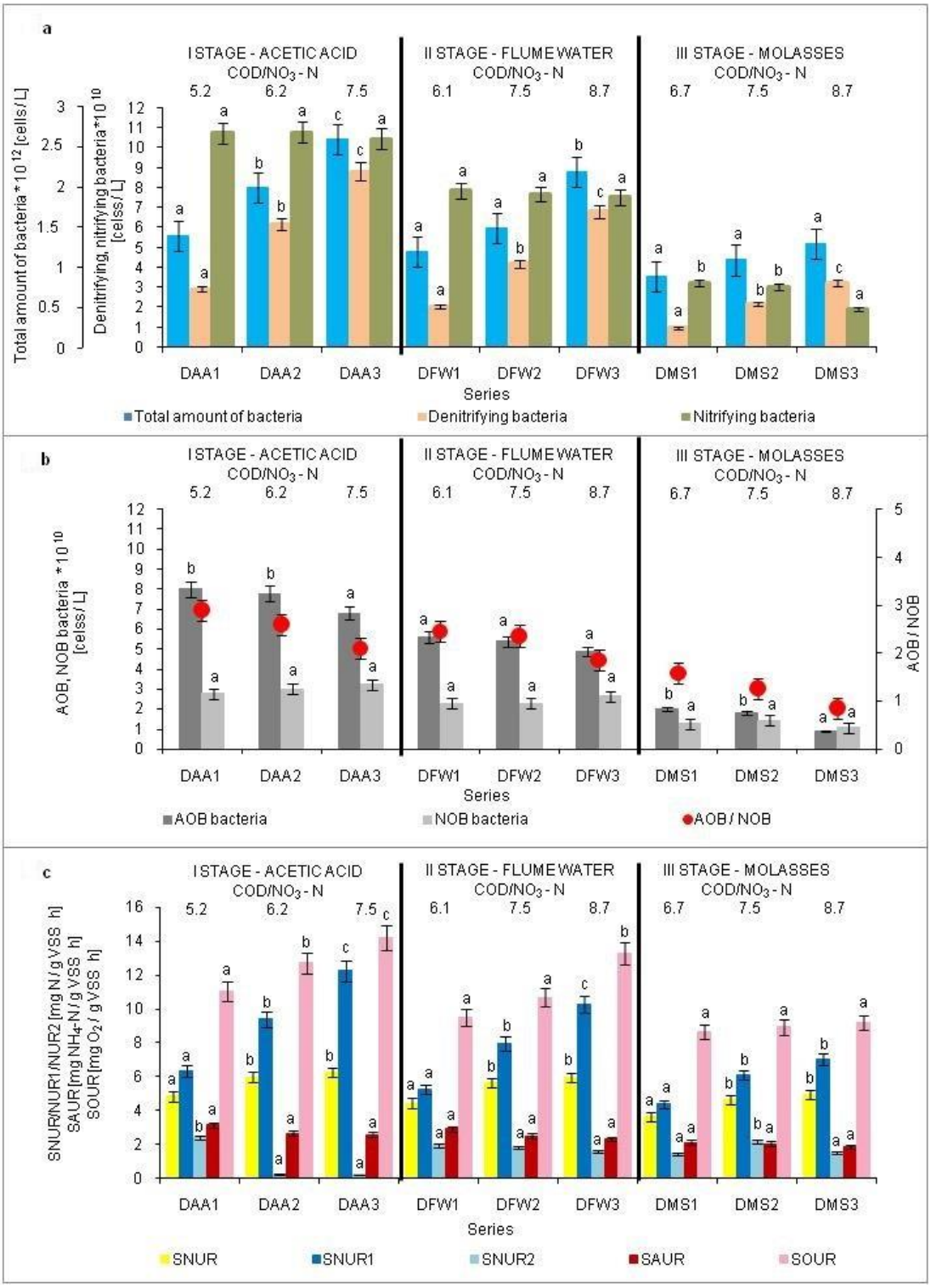

Figure 3. The amount (**a**,**b**) and activity (**c**) of bacteria in the activated sludge depending on the type of external carbon source and the COD/NO_3–N ratio. Means (n = 3) within each kind of bacteria and each batch test and each kind of external carbon source followed by the same letter are not statistically different according to Tukey's test.

2.6. Statistics

In order to verify the difference between the COD, TN, and NH_4–N removal efficiencies and the NO_3–N concentrations for different COD/NO_3–N ratios, an Anova test followed by Tukey's post hoc test was conducted. For all of the tests, the differences

were only considered significant if $p < 0.05$. All statistical analyses were conducted using Statistica 12 (StatSoft, Krakow, Poland).

3. Results and Discussion

3.1. Treatment Efficiency of the Digestate Liquid Fraction

The treatment efficiency results of the digestate liquid fraction in a conventional activated sludge system with the addition of external carbon sources are presented in Figure 2a–c. The studies were conducted with a constant TN loading rate, which was 20.49 ± 2.31 mg N/g MLVSS d (Figure 2a), while the COD loading rate was dependent on the type of carbon source and rose when the COD/NO_3–N ratio increased (Figure 2a). The pH in the biological reactors in the DAA and DFW series was within the optimal range for biological wastewater treatment, which is equal to 6.0–8.0 in the denitrification chamber and to 7.5–8.5 in the nitrification chamber [43]. The pH in the denitrification chambers in the DMS series slightly exceeded the values demonstrated by Meng et al. [43], standing at 8.09 ± 0.05 (Figure 2a). Analysing the results presented in Figure 2b,c, it was observed that the COD/NO_3–N ratio and the type of carbon source had a significant impact on the treatment efficiency of the digestate liquid fraction. The best results were obtained in the DAA3 series, in which acetic acid was used as a conventional carbon source and where the COD/NO_3–N ratio was 7.5. Comparable results were achieved in the DFW3 series when using the flume water as a carbon source when the COD/NO_3–N ratio was 8.7. The removal efficiency of TN and COD in the DAA3 series was 83.73 ± 0.34% and 86.26 ± 0.21%, respectively, and in DFW3 series at 83.35 ± 0.18% and 86.83 ± 0.15% (Figure 2b). The mean concentrations of NO_3–N in the effluent in the DAA3 and DFW3 series remained stable at 11.28 ± 0.26 mg/L and 12.90 ± 0.25 mg/L, respectively (Figure 2c). These values were significantly lower ($p < 0.05$) than those obtained in the DAA1 and DFW1 series at the COD/NO_3–N ratios equal to 5.2 and 6.1, respectively. Nitrate concentrations in the effluent in DAA1 and DFW1 series reached 18.30 ± 0.65 and 19.40 ± 0.79 mg/L. The mean TN and COD removal efficiencies in the DAA1 series were 79.16 ± 2.42% and 80.31 ± 0.28%, respectively, and in DFW1 series, they were 77.39 ± 2.94 and 82.71 ± 0.24%. Analysing Figure 2c, it can be noted that in all of the test series in which acetic acid and flume water were used as the carbon sources, the NH_4–N removal efficiency averaged at 99.89 ± 0.13%. Changes in the COD/NO_3–N ratio in the range of 5.2–7.5 and 6.1–8.7, respectively, did not significantly ($p > 0.05$) affect the NH_4–N concentrations in the effluents, which in the DAA series, averaged at 0.09 ± 0.02 mg/L, and in DFW series, averaged at 0.15 ± 0.03 mg/L. The NO_2–N concentrations were 0.16 ± 0.03 mg/L and 0.32 ± 0.03 mg/L, respectively. The lowest treatment efficiency of the digestate liquid fraction was determined in the DMS series using molasses as an external carbon source. In the DMS3 series, in which the COD/NO_3–N ratio was identical to that of DFW3 series and amounted to 8.7, lower COD and TN removal efficiencies equal to 81.67 ± 1.86% and 78.46 ± 1.59%, respectively, were obtained (Figure 2b). Subjecting the results presented in Figure 2c to an analysis, it was observed that in the DMS3 series, the concentration of NH_4–N in the effluent increased to 11.10 ± 0.67 mg/L, and thus its removal efficiency significantly ($p < 0.05$) decreased to 86.56 ± 0.89%. In contrast, in the DMS1 series in which the COD/NO_3–N ratio was 6.7, the NH_4–N removal efficiency was higher and amounted to 99.69 ± 0.56%. The NH_4–N concentration in the effluent was 0.27 ± 0.08 mg/L, and NO_2–N was 1.05 ± 0.24 mg/L. When conducting the process of synthetic wastewater treatment with glucose as a carbon source in an Integrated Fixed–Film Activated Sludge reactor, Machat et al. [44] also found that an increase in the C (carbon)/N (nitrogen) ratio contributed to a reduction in the nitrification process efficiency. The authors found that the NH_4–N removal efficiency was equal to 96.54 ± 2.44% when the C/N ratio was 10. Increasing the C/N ratio to 12 resulted in the reduction of the NH_4–N removal efficiency to 88.20 ± 10.84%.

No literature data were found on the biological treatment of the digestate liquid fraction in a conventional activated sludge system with a separate denitrification chamber.

However, Dosta et al. [45], when carrying out a treatment process for centrifuged reject water from an anaerobic digester of a WWTP with the addition of acetate as a carbon source in a lab–scale SBR (Sequencing Batch Reactor) seeded with the activated sludge, obtained a nitrogen removal efficiency of almost 100%. Obaja et al. [15], using acetic acid as a carbon source during the treatment of the liquid fraction after centrifuging digested piggery wastewater in a lab–scale SBR reactor filled with activated sludge, obtained NH_4–N, NO_3–N, and COD removal efficiencies equal to 99.7%, 99.9%, 64.1%, respectively. Yan et al. [18], when treating manure landfill leachate with the addition of acetate as a carbon source in a lab–scale membrane bioreactor, determined TN, NO_3–N, and COD removal efficiencies of 90%, 94.6% and 95.1%, respectively. However, when the centrifuged liquids fermented from food waste were used as a carbon source, the removal efficiencies were 92.8%, 99.9%, and 96.5%, respectively. The results obtained in the DAA3 and DFW3 series are similar to those described in the literature, which indicates the possibility of using a conventional activated sludge system with a separate denitrification chamber for digestate treatment.

3.2. Amount and Activity of Denitrifying Bacteria

The type of carbon source and the C/N ratio influence not only the efficiency of the nitrification and denitrification processes but also influences the amount and activity of the bacteria in the activated sludge [13,19,46]. In our study, following the analysis of the results presented in Figure 3a,c, it was found that the amount and activity of denitrifying bacteria in the activated sludge increased as the COD/NO_3–N ratio rose in the influents at each stage of the research. Furthermore, it was noted that not only the COD/NO_3–N ratio, but also the biodegradability of the carbon source used, is essential in the denitrification process. The activity of denitrifiers and thus the denitrification rate were defined as the SNUR. The curves for the changes in the NO_x–N concentrations used to calculate the SNUR values are shown at Figure 4a. The observed SNUR1 rates were associated with the utilization of soluble, readily biodegradable organic compounds and the SNUR2 rates with slowly biodegradable organic compounds [26].

The highest amount of denitrifying bacteria and the highest SNUR were determined in the DAA3 series, in which the COD/NO_3–N ratio was 7.5 and where the carbon source was acetic acid characterized by a biodegradable fraction (BDCOD) accounting for 99.98 ± 0.02% of the total COD (Table 1). In DAA3 series, the amount of denitrifying bacteria was equal to 8.8 × 10^{10} cells/L (3.4% of the total bacteria amount), and the SNUR was 6.28 ± 0.85 mg N/g VSS h. Due to high biodegradability of acetic acid, the SNUR1 in the DAA3 series was the highest at 12.26 ± 1.06 mg N/g VSS h, and the SNUR2 was 0.25 ± 0.08 mg N/g VSS h (Figure 3c). In the DFW3 series in which the carbon source was flume water and where the COD/NO_3–N ratio was 8.7, a lower amount of denitrifying bacteria was determined than it was in the DAA3 series, which was equal to 6.8 × 10^{10} cells/L (3.1% of the total bacteria amount). The reason for this could have been the lower content of the BDCOD fraction in the flume water than in acetic acid, which is equal to 79.88 ± 1.17% of the total COD (Table 1). As a consequence of this, the SNUR1 was 15.99 ± 0.87% lower than it was in the DAA3 series and stood at 10.30 ± 1.14 mg N/g VSS h, and the SNUR2 was 1.59 ± 0.48 mg N/g VSS h (Figure 3c). Analysing the obtained results, a clear correlation was noted between the amount and the activity of the denitrifying bacteria and efficiency of the denitrification process. The amount of denitrifying bacteria and their activity determined in the DAA3 and DFW3 series ensured a high efficiency of the reduction of NO_3–N and COD concentrations in effluents (Figure 2b,c). In our study, the lowest amounts of denitrifying bacteria were obtained in the series in which molasses was used to enhance the denitrification process. Molasses was characterized as being the lowest among the analysed carbon sources, with a BDCOD fraction accounting for 69.92 ± 1.45% of the total COD (Table 1). The SNUR1 and SNUR2 in the DMS3 series at the COD/NO_3–N ratio of 8.7 were 7.03 ± 0.94 and 1.53 ± 0.36 mg N/g VSS h, respectively (Figure 3c), while the amount of denitrifying bacteria was 3.4 × 10^{10} cells/L (2.6% of the

total bacteria amount). The SNUR was 4.97 ± 0.87 mg N/g VSS h and was 16.47 ± 0.82% lower than it was in the DFW3 series, which also used the COD/NO_3–N ratio of 8.7. In the DMS1 series at the COD/NO_3–N ratio of 6.7, the amount of denitrifying bacteria and the SNUR were the lowest among those determined in the study and were at 9.9 × 10^9 cells/L (1.1% of total bacteria amount) and 3.7 ± 0.81 mg N/g VSS h, respectively. This resulted in an increase of the NO_3–N concentration in the effluent (Figure 2c).

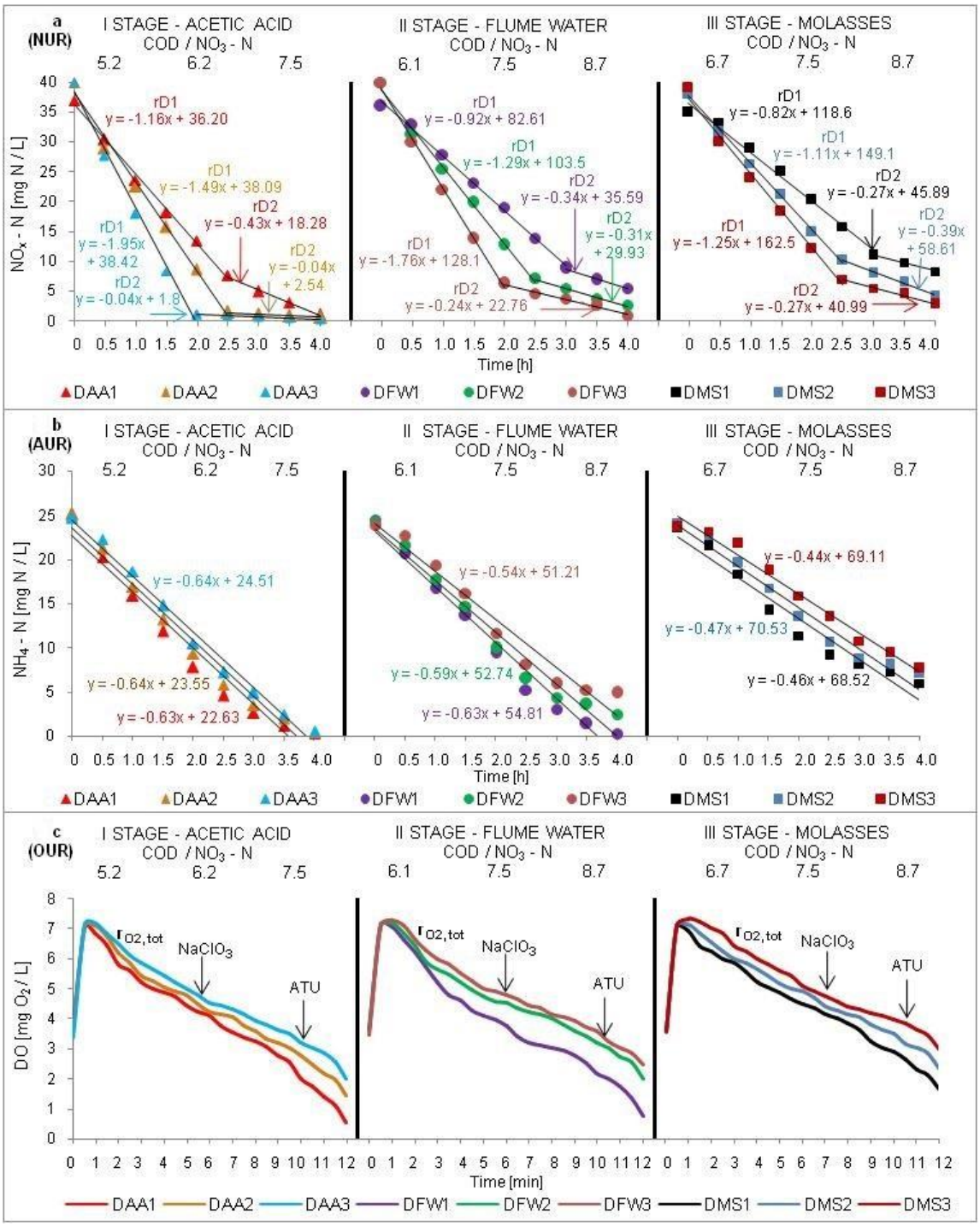

Figure 4. Profiles of changes in concentrations of NO_x–N (**a**), NH_4–N (**b**), and DO (**c**) used to calculate SNUR, SAUR, and SOUR values, respectively; where, r_{D1} is the denitrification rate on S_s (the readily biodegradable COD fraction), X_s (the particulate COD fraction), and endogenous respiration r_{D2} is the denitrification rate on X_s and endogenous respiration.

The impact of the biodegradability of the carbon source on the denitrification process was also observed by comparing the results of the DAA3, DFW2, and DMS2 series in which the COD/NO_3–N ratio was 7.5. The highest SNUR was obtained in the DAA3 series. By contrast, in the DFW2 and DMS2 series, the SNURs were lower by 9.87 ± 1.12% and 26.11 ± 1.43%, respectively. These results show that the denitrification process mainly uses readily biodegradable organic matter. Choubert et al. [47] also pointed out that the concentration of biodegradable COD is among the main factors affecting the denitrification process. Quan et al. [26] found that the highest denitrification rates are obtained when readily biodegradable organic matter is used.

The results of the NUR tests obtained by other authors are presented in Table 4. The SNURs described in the literature with the addition of acetic acid or acetate range from 3 [48] to 40 mg N/g VSS h [45]. The differences in the rates probably result from the type of reactors, sludge sources, and environmental factors. The SNURs determined in the study in the DAA1 series (4.9 mg N/g VSS h) were similar to those determined by Rodriguez et al. [25] when using acetate as a carbon source in a lab–scale SBR reactor. The highest rates, which were equal to 31.1 and 40 mg N/g VSS h, were determined by Obaja et al. [15] and Dosta et al. [45], respectively, by dosing acetic acid and acetate directly into the anoxic chambers of lab–scale SBR reactors. Such high rates may confirm the good adaptation of the activated sludge to the carbon source. The SNURs obtained with acetic acid or acetate are higher than those obtained in the case of alternative external carbon sources. Cappai et al. [24] and Rodriguez et al. [25], using wastewater from the sugar industry as a carbon source in lab–scale SBR reactors, determined SNURs of 2.7 mg N/g VSS h and 1.75 mg N/g VSS h, respectively. By contrast, Quan et al. [26], using hydrolyzed molasses as a carbon source in an SBR reactor and at a COD/NO_3–N ratio of 5, determined an SNUR of 3.6 mg N/g VSS h. This value was comparable to the one obtained in the DMS1 series at the COD/NO_3–N ratio of 6.7. This shows that the hydrolysis improved the uptake of molasses by bacteria, so similar SNURs were obtained at a lower COD/NO_3–N ratio. A low SNUR value of 0.43 mg N/g VSS h was determined by Yu et al. [49] when treating synthetic domestic wastewater containing glucose in a lab–scale SBR reactor. Zhao et al. [50], analysing the influence of three carbon sources on nitrogen transformation in an aerobic granular sludge system, also found that bacteria hardly used saccharides, whereas the nitrogen removal rate was the highest when using sodium acetate.

Table 4. Specific denitrification and nitrification rates for different substrates and different reactors type in the literature.

Carbon Source	Denitrification Rate SNUR	Nitrification Rate SAUR	Reactor Type	Reference
	[mg N/g VSS h]			
octan	3–4	–	full–scale reactor removing N and P	[48]
octan	4.7	–	lab–scale SBR reactor	[25]
acetic acid	31.1	27.5	lab–scale SBR reactor	[15]
octan	40	19	lab–scale SBR reactor	[45]
wastewater from beet—sugar factory	2.7	2.82	lab–scale SBR reactor	[24]
wastewater from beet—sugar processing	1.75	–	lab–scale SBR reactor	[25]
hydrolyzed molasses	3.6	–	lab–scale SBR reactor; COD/NO_3–N ratio of 5	[26]
glucose	2.4–3.1	–	full–scale reactor removing N and P	[48]
glucose	0.43	1.65	lab–scale SBR reactor	[49]
acetic acid	4.9–6.3	2.6–3.2	lab–scale conventional activated sludge reactor	This study
flume water from beet—sugar factory	4.5–6.0	2.3–2.9	lab–scale conventional activated sludge reactor	This study
molasses	3.7–5.0	1.9–2.1	lab–scale conventional activated sludge reactor	This study

3.3. Amount and Activity of Nitrifying Bacteria

Data on the amounts of nitrifying bacteria, the activity of nitrifiers (SAUR) as well as the heterotrophic activity (SOUR) are plotted on Figure 3a–c. Curves of changes in NH_4–N and the DO concentrations used to calculate the SAUR and SOUR values are shown at Figure 4b,c, respectively. In order to fully characterise the nitrification process, apart from the amount of nitrifying bacteria, the interaction of the AOB and NOB bacteria was also determined (Figure 3b). The AOB bacteria activity in the activated sludge is closely related to the NH_4^+–N removal rate [51]. Maintaining the proper interaction of these bacteria plays a key role in optimizing the nitrification process in a biological wastewater treatment plant [46,52].

Upon the analysis of the results shown in Figure 3a–c, it was found that the highest amount of nitrifying bacteria and their highest activity were determined in the series in which acetic acid was used as a carbon source. In the DAA1 series, in which the COD/NO_3–N ratio was 5.2, the nitrifying bacteria accounted for 7.7% of the total bacteria amount (10.8×10^{10} cells/L), with the amount of AOB bacteria being equal to 8.0×10^{10} cells/L and NOB 2.8×10^{10} cells/L. The AOB/NOB ratio was 2.9, which was higher than the theoretical value, which should be 2 in a balanced nitrifying system, which is in accordance with thermodynamics and electron transfer [52]. The SAUR was 3.17 ± 0.25 mg NH_4–N/g VSS h, and the SOUR was 11.05 ± 1.12 mg O_2/g VSS h. In the DFW series in which flume water was used to enhance the denitrification process, lower amounts of nitrifying bacteria and their lower activity were determined. In the DFW1 series, at the COD/NO_3–N ratio of 6.1, the amount of AOB bacteria was 5.6×10^{10} cells/L, NOB 2.3×10^{10} cells/L, and the AOB/NOB ratio was 2.5. The total amount of nitrifying bacteria was 7.9×10^{10} cells/L (6.6% of the total bacteria amount), the SAUR was 2.92 ± 0.34 mg NH_4–N/g VSS h, and the SOUR was 9.52 ± 1.02 mg O_2/g VSS h. The nitrification rates determined in the DAA and DFW series as well as the amounts and interactions of AOB and NOB bacteria were sufficient for an efficient and stable nitrification process, which was evident from the comparatively low concentrations of NH_4–N and NO_2–N in the effluents (Figure 2c). The lowest amounts of the nitrifying bacteria were obtained in the DMS series during the treatment of the digestate liquid fraction with molasses as a carbon source. In the DMS1 series at the COD/NO_3–N ratio of 6.7, the nitrifying bacteria accounted for 3.6% of the total bacteria amount (3.3×10^{10} cells/L), and the SAUR was 2.14 ± 0.37 mg NH_4–N/g VSS h. The amount of AOB bacteria was 2.0×10^{10} cells/L, whereas the NOB was 1.3×10^{10} cells/L. In the DMS3 series, at the COD/NO_3–N ratio of 8.7, the amount of nitrifiers decreased to 2.0×10^{10} cells/L (1.5% of the total bacteria amount), including the AOB bacteria to 9×10^9 cells/L and NOB to 1.1×10^{10} cells/L. The AOB/NOB ratio was 0.9, the SAUR was 1.87 ± 0.52 mg NH_4–N/g VSS h, and the SOUR was 9.20 ± 1.09 mg O_2/g VSS h. According to Nielsen et al. [53], the proportion of the nitrifying bacteria should be 4–6% of the total bacterial biomass in the efficient nitrification process. The amounts determined in the DMS series were too low, resulting in increased concentrations of NH_4–N and NO_2–N in the effluents (Figure 2c).

The analysis of the results led to the observations that in all of the test series, an increase in the COD/NO_3–N ratio in the influents resulted in a decrease in the amounts of the nitrifying bacteria and the SAURs and an increase in the SOURs. This was probably caused by a weaker ability of the nitrifiers to compete for oxygen with heterotrophs at high COD concentrations. This was evidenced by the increase in the SOUR, which constituted an indicator of heterotrophic oxygen activity. Ma et al. [54] point out that at higher C/N ratios in the influents, the inhibition of the nitrifying bacteria occurs, with AOB amounts decreasing more intensively than NOB. Carrera et al. [55] claim that increasing the COD/N ratio in the influent from 2.6 to 3.4 in a biological nitrogen removal process involving nitrification and denitrification resulted in a reduction of the amount of autotrophic bacteria from 2 to 1.5%. Mota et al. [56] and Sepehri and Sarrafzadeh [57] pointed out that the low C/N ratio in the influent to the wastewater treatment plant is probably the most important

factor that has an influence on the high proportion of the nitrifying bacteria in the activated sludge and thus on the stability of the nitrification process.

The results of the AUR tests obtained by different authors are summarized in Table 4. These values range from 1.65 [49] to 27.5 mg N/g VSS h [15]. The highest SAURs of 27.5 and 19 mg N/g VSS h were obtained by Obaja et al. [15] and Dosta et al. [45] during the treatment of wastewater with a high ammonia nitrogen content, liquid fraction of digested piggery wastewater, and centrifuged reject water from an anaerobic digester in lab–scale SBR reactors. This means that a high concentration of ammonia nitrogen in the influent stimulates high nitrifier activity in the activated sludge [49,58]. By contrast, Cappai et al. [24], when conducting a municipal wastewater treatment process with the addition of wastewater from a sugar factory as a carbon source, and Yu et al. [49], when treating synthetic domestic wastewater containing glucose in lab–scale SBR reactors, obtained SAURs equal to 2.82 and 1.65 mg N/g VSS h, respectively. These values were comparable to those determined in the described studies for the DFW1 and DMS3 series, which amounted to 2.92 and 1.87 mg N/g VSS h, respectively.

4. Conclusions

Studies have indicated that the digestate liquid fraction can be treated in a conventional activated sludge system. This has been found that flume water and molasses, so industrial wastewater and by–products generated in a sugar factory can be used as alternative carbon sources to intensify the biological nitrogen removal from digestate. It has been shown that the type of external carbon source, and especially its biodegradability as well as the COD/NO_3–N ratio, had a significant impact on the amount and activity of activated sludge bacteria and thus on the nitrogen removal efficiency and organic compound removal. The best results for the treatment of the digestate liquid fraction were obtained when the acetic acid was a carbon source and when the COD/NO_3–N ratio was 7.5. Comparable results were achieved in the DFW3 series using flume water as a carbon source and at the COD/NO_3–N ratio of 8.7. The use of molasses as an alternative carbon source resulted in lower nitrification and denitrification efficiency compared to acetic acid and flume water.

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Abbreviations

The following abbreviations are used in this manuscript:

AD	Anaerobic Digestion
AOB	Ammonia Oxidizing Bacteria
BDCOD	Biodegradable COD/biodegradable fraction
BOD_5	Biochemical Oxygen Demand
C/N	Carbon/Nitrogen
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
MLVSS	Mixed Liquor Volatile Suspended Solids
MLSS	Mixed Liquor Suspended Solid
NH_4–N	Ammonia Nitrogen
NOB	Nitrite Oxidizing Bacteria
NO_2–N	Nitrite Nitrogen
NO_3–N	Nitrate Nitrogen
SAUR	Specific Ammonia Utilization Rate/Specific Nitrification Rate
SBP	Sugar Beet Pulp
SBR	Sequencing Batch Reactor
SCOD	Soluble COD
SNUR	Specific Nitrate Utilization Rate/Specific Denitrification Rate
SOUR	Specific Oxygen Utilization Rate
TN	Total Nitrogen
TP	Total Phosphorus
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

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