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Treatment of landfill leachate in intermittently aerated hybrid or conventional SBRs operating at different temperatures

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ABSTRACT

Research on the treatment of leachate from municipal landfill was carried out in three sequential batch reactors (SBRs) and their hybrid variants (HSBR) equipped with biomass carriers made of PU foam cuboids. The reactors were operated at 20°C, 30°C, and 40°C. The operational cycle lasted 24 h, while hydraulic retention time was set at 48 h. In the reactors alternatingly aerobic (DO < 1 mg/L) and anaerobic conditions were maintained. Raw leachate was characterized by COD concentration of 3,278, 672 mg/L of total nitrogen, including 630 mg/L of TAN. Neither nitrate nor nitrite nitrogen was found in the leachate drained from the landfill. The nitrogen loading rate was relatively low in all reactors and amounted to 0.315 kg/m^3 d. The highest removal efficiency of both organics and nitrogen compounds was achieved in HSBR operating at 40°C, and was 78% and 80.8%, respectively. Quantitative analyses of the most common bacteria phyla in the landfill leachate treatment systems were determined after the stabilisation of the outflows by qPCR, and shown a significant decrease of 16S rRNA copies with an increase of temperature.

Keywords: 16S rRNA; *amoA*; HSBR; Landfill leachate; Microbial community; Nitrogen; qPCR

1. Introduction

The leachate composition and quantity may vary, depending on landfill age, climate, amount of precipitation, construction and operational conditions of the landfill and finally the technology of collection, retention, and treatment. Also, the processes taking place in the landfill bed, as organic matter decomposition, gas generation, or settling efficiency have a significant impact [1,2].

High biomass concentration and prolonged age of sludge in the biological reactor allow to make wastewater treatment more effective. The application of biomass carriers causes that sludge is not flushed out from the reactor. This increases the biomass amount and prolongs its age. Research aimed at increasing nitrification efficiency was also carried out with the use of combined methods – active sludge with a biological membrane developing on the fill (movable or immovable carriers) [3].

Fluidized beds and reactors with suspended biomass have been used to increase the biomass concentration and microorganism's age. The tests consisting in the improvement of nitrification efficiency have been conducted using combined methods – activated sludge with biofilm on a packing (moving or fixed) medium. Different biomass carriers are currently available such as, Kaldnes (polyethylene media), Liapor (ceramic media), Linpor (plastic media with high porosity), foam cubes (mainly polyurethane) [4], or BioBall (polypropylene media) [3]. Lim et al. [5] report that suspended biomass plays an important role in ammonia nitrogen oxidation, as the biomass colonizing inner surfaces of polyurethane foam media is used as a carbon source in the denitrification process.

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Lim et al. [4] report that suspended biomass plays an important role in the oxidation of ammonia nitrogen, as the biomass colonizing the carrier's inner space is used as a source of carbon in the denitrification process. Biomass immobilization contributes also to the retention of slower developing nitrifying bacteria in the reactor. In the case of wastewater treatment carried out in reactors with biomass carriers, the amount of the latter is an important factor. The larger surface area of the carriers allows to retain more biomass in the reactor [6]. One of the most important environmental factors affecting the biological treatment process is temperature. It influences the metabolic activity of microorganisms [7] and the rate of biological reactions [8].

Biological treatment of leachates is an economically viable solution as far as nitrogen removal is concerned. It is usually carried out as the nitrification and denitrification process occurring in separate tanks [9]. In order to improve nitrogen removal efficiency processes, such as nitritation–denitritation or simultaneous nitrification–denitrification (SND) [10,11], have been developed. In the nitrification–denitrification process, oxidation of ammonia nitrogen to nitrite occurs followed by its reduction to gaseous nitrogen. In comparison with conventional processes employed for the removal of nitrogen with the use of biological processes, the nitritation–denitritation process allows to reduce aeration by 25% and the carbon needed by 40%. In the SND process, nitritation and denitritation occur at the same time in the same reactor, in identical conditions of the technological process. Therefore, SND is an alternative nitrogen removal technology given a simplification of the process and the possibility to use an internal source of carbon such as active sludge [6,12]. Yan et al. [13] report that it is possible to remove ammonia via the nitrite pathway with dissolved oxygen DO in laboratory sequencing reactors. Moreover, studies [14,15] confirm that the activity of nitrite-oxidizing bacteria (NOB) were suppressed at low oxygen concentrations (0.15–0.18 mg/L), which leads to shorter nitrogen removal with less carbon source demand.

Leachate from young landfills contains high concentrations of organic matter and is characterized by high C/N ratios. Meanwhile, leachate from landfills operated for over 5 y contains less biodegradable organic matter (<2,300 mg/L) and high ammonia (>1,800 mg/L) with a C/N ratio of 1.5, which causes problems in ensuring traditional nitrification and denitrification, where there is a minimal C/N ratio of 2.86 [16]. Although there is a certain amount of data on the conditions that stimulate the simultaneous nitrification–denitrification process in various biological reactor systems treating leachate from municipal landfills, there is a lack of information about the implementation of intermittent aeration conducted in a single hybrid sequencing batch reactor filled with biomass carriers with a highly developed specific surface area. Such layout may be beneficial in starvation conditions typical for mature LL characterized by a high share of refraction organic compounds because could promote endogenous denitration without the addition of costly external carbon source. Additionally, most of the articles that discuss the microbiological dimension of the treatment process using molecular data focus on qualitative measurements which help to recognize its biological basics but do not provide real data concerning quantitative dynamics, which seems to be tremendously useful from an operational point of view.

The use of the SND process is dedicated to such wastewater. In addition, the use of biomass carriers will allow biomass to be retained in the reactor. Also, raising the temperature will contribute to increasing the efficiency of the treatment. Moreover, intermittent aeration with a low concentration of dissolved oxygen (DO <0.5 mg/L) can effectively inhibit the NOB growth and contribute to TAN (total ammonia nitrogen) removal in the azitine pathway. SBRs operating with intermittent aeration were used for the tests. The influence of the presence of filling and different process temperatures on the efficiency of landfill leachate treatment was tested. The study aimed to determine the differences in the influence of the temperature of the landfill leachate treatment process on the efficiency of ammonia nitrogen removal and the abundance of the main components of the microbial community depending on the addition of biomass carriers.

2. Methodology

The study used raw leachate samples from the municipal waste landfill in Kozodrza (south-eastern Poland). Currently, the landfill consists of 12 quarters covering the surface of over 20 hectares and gathers non-hazardous waste in the amount of up to 400 mg/d. The analytical methods and composition of the raw leachate samples are presented in Table 1. The temperature and DO in the SBR were detected using online instruments (Elmetron).

2.1. Experimental layout

The experimental layout consisted of six laboratory-scale reactors with an active volume of 2 dm^3, which were set up in pairs according to the operating temperatures of 20°C, 30°C and 40°C. Each pair, in turn, consisted of a conventional sequential reactor (SBR) operating with active suspended sludge and its hybrid counterpart (HSBR) additionally filled with biomass carriers with dimensions of 10 mm × 10 mm × 10 mm made of reticulated open-cell polyurethane (polyether) foam (PU) with linear porosity of 30 ppi (Fig. 1). The bulk volume of biomass carriers accounted for 10% of the active volume of each HSBR and, according to the methodology described by Koc-Jurczyk and Jurczyk [23], the estimated total and specific surface area of such filling was 332.52 and 5.85 cm^2/cm^3, respectively.

The reactors' operating cycle was 24 h, and consisted of five aerobic phases (DO < 1 mg/L) intermitted by anaerobic conditions (Fig. 2), and hydraulic retention time (HRT) was set at 2 d. The reactors were inoculated with activated sludge taken from nitrification chamber II° of the local municipal WWTP (PE ≈ 200 k) mixed with activated sludge treating LL in the ratio of 4:1, to obtain the initial biomass concentration of 2.3 g/L. The experiment was conducted for 120 d.

2.2. Quantification of the main microbial community components

After the stabilization of TAN concentration in the outflows, 1 mL of suspended activated sludge was sampled

Table 1
Methods used for the analysis of physicochemical parameters and the composition of the raw landfill leachate sample

Parameter (unit)	Mean value	Analytical method compliant with the standard or reference
COD (mg/L)	3,278	Colorimetric. Oxidation of organic substances at 172°C with $K_2Cr_2O_7$ and H_2SO_4 in the presence of Ag_2SO_4 as a catalyst, PN-ISO 6060:2006 [17]
BOD_5 (mg/L)	270	Respirometric measurement (OxiTop®) of negative pressure changes at a constant temperature of 20°C and darkness, in the presence of N-allythiourea as nitrification inhibitor, according to the procedure DIN EN 1899-1/EN 1899-2 [18]
TAN (mg/L)	630	Colorimetric. Distillation in weakly alkaline conditions and titration with Tashiro's indicator, PN-ISO 5664:2002 [19]
NO_2^- (mg/L)	0.1	Spectrophotometric (λ = 520 nm) with sulfanilic acid and N-(1-naphthyl)ethylenediamine dihydrochloride EN 26777/1993 [20]
NO_3^- (mg/L)	0.15	Spectrophotometric (λ = 410 nm) with phenol-2,4-disulfonic acid [21]
FAN (mg/L)	9.32 (20°C)	Calculated according to equations 2, 3 as a derivative of TAN, T and pH [30, 31].
pH	7.58	Potentiometric. PN-EN ISO 10523:2012 [22]

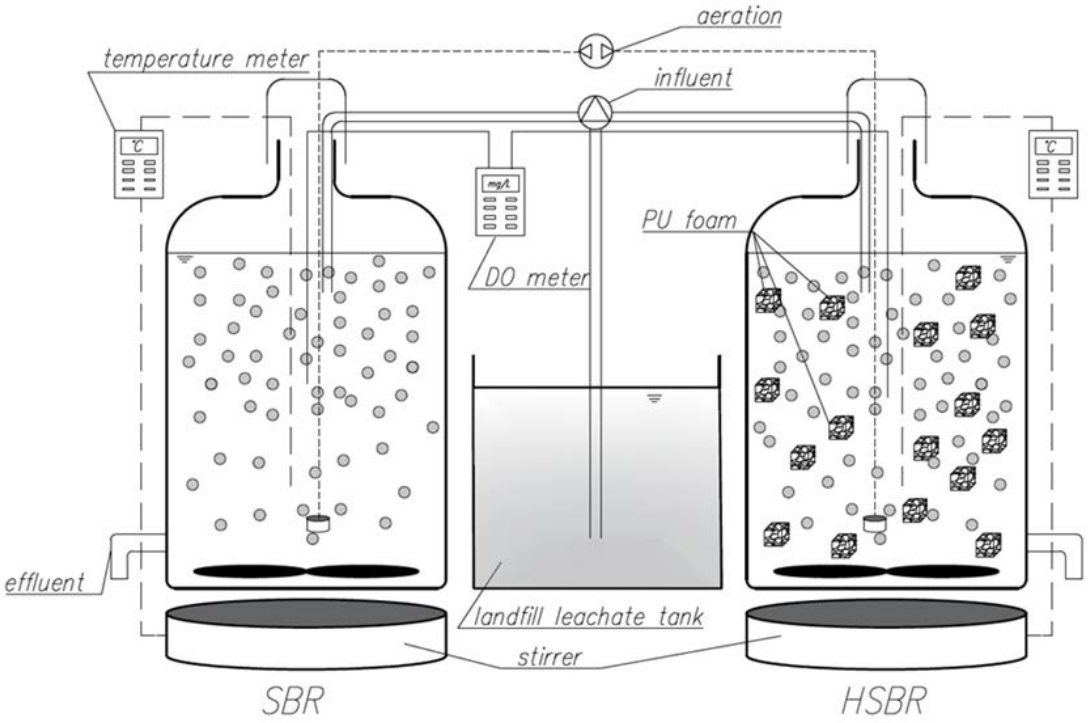

Fig. 1. Schematic diagram of the experimental layout used in this study to compare the efficiency of landfill leachate treatment in different temperatures.

from each reactor during mixing. Samples were centrifuged for 10 min in 6,000 rpm, and the obtained pellet was re-suspended in 1 mL storage buffer (sodium-phosphate 0.0125 M, 5% SDS) and frosted in –20°C for further extraction.

After defrosting, samples were well mixed and 500 μL of each was used for DNA extraction, according to the procedure provided by the kit manufacturer (Genomic Mini AX Bacteria + Spin, A&A Biotechnology, Poland). The DNA was finally eluted to the volume of 150 μL and stored at –20°C for analysis. A set of primers was utilized to amplify fragments of the 16S rRNA genes of the most abundant phyla, according to literature data on landfill leachate treatment systems, and complemented by primers for chosen functional groups. Amplifications were performed in the Roche LightCycler®96 system, and the reaction thermal profiles for each primer pair are shown in Table 2. Single reaction mix consisted of 7.5 μL of RT-PCR Mix SYBR® B (A&A Biotechnology, Poland), 0.5 μM of each primer (Genomed,

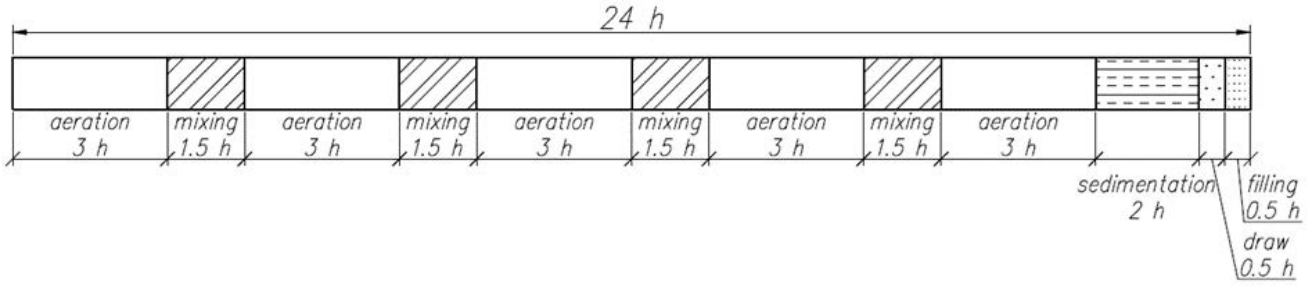

Fig. 2. The operational cycle of hybrid and conventional SBRs.

Table 2
Set of qPCR primers

Primer pairs	Target	Thermal profile
926F/1062R[a]	Universal 16S rRNA	95°C for 300 s, then up to 45 cycles: 95°C for 15 s, 61°C for 15 s, 72°C for 20 s
Cfb798F/Cfb967R[a]	Bacteroidetes 16S rRNA	
Firm 928F/Firm1040R[a]	Firmicutes 16S rRNA	
Act920F3/Act1200R[a]	Actinobacteria 16S rRNA	
Gamma1080F/Gamma1202R[a]	Gammaproteobacteria 16S rRNA	
Beta979F/Beta1130R[b]	Betaproteobacteria 16S rRNA	as above melting 60°C for 15 s
CTO189F/CTO654R[c]	βAOB 16S rRNA (*Nitrosomonas* sp.)	95°C for 300 s, then up to 40 cycles: 94°C for 40 s, 55°C for 30 s, 72°C for 120 s
amoA-1F/*amoA*-2R[d]	Ammonia monooxygenase subunit A	95°C for 300 s, then up to 40 cycles: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s
Amx694F/Amx960R[e]	AnAOB 16S rRNA	96°C for 180 s, then up to 40 cycles: 96°C for 60 s, 55°C for 60 s, 72°C for 60 s
NSR1113F/NSR1264R[f]	*Nitrospira* sp. 16S rRNA	94°C for 300 s, then up to 40 cycles: 94°C for 30 s, 65°C for 30 s, 72°C for 30 s

Taken from: [a]Bacchetti De Gregoris et al. [24], [b]Yang et al. [25], [c]Kovalchuk et al. [26], [d]Rotthauwe et al. [27], [e]Ni et al. [28], [f]Dionisi et al. [29].

Poland), 2 μL of the sample or quantitative standard and nuclease-free water added up to the total volume of 15 μL.

2.3. Calculations

The efficiency of COD and TAN removal was calculated according to:

$$E = \frac{C_{\text{inf.}} - C_{\text{eff.}}}{C_{\text{inf.}}} \cdot 100 \quad (1)$$

where E is the percentage efficiency (%), $C_{\text{inf.}}$ is the concentration (mg/L) of following indicators: COD and TAN in influent, and $C_{\text{eff.}}$ is the concentration (mg/L) of above mentioned indicators in effluent.

Free ammonia concentration (FAN) in effluent was estimated using the following formula [30,31]:

$$\text{FAN} = \frac{\text{TAN}}{1 + 10^{(\text{pKa} - \text{pH})}} \quad (2)$$

where FAN is the concentration of free ammonia (mg/L), TAN is the total ammonia concentration (mg/L), T is the temperature in Kelvin degrees and the dissociation constant (pKa) was calculated from the formula below.

$$\text{pKa} = 0.09108 + \frac{2{,}729.92}{T} \quad (3)$$

The nitrogen load rate (NLR) (kg/m^3 d), nitrogen removal rate (NRR) (kg/m^3 d) and nitrite accumulation ratio (NAR) (%) was calculated using formula:

$$\text{NLR} = \frac{C_{\text{TNinf}} \cdot V}{V \cdot t} \quad (4)$$

$$\text{NRR} = \frac{\left(C_{\text{TNinf.}} - C_{\text{TNeff.}}\right) \cdot V}{V \cdot t} \quad (5)$$

$$\text{NAR} = \frac{C_{NO_2^-}}{C_{NO_2^-} + C_{NO_3^-}} \cdot 100 \quad (6)$$

where $C_{\text{TNinf.}}$ is the concentration of total nitrogen in influent (mg/L), $C_{\text{TNeff.}}$ is the concentration of total nitrogen

in the effluent (mg/L), C_{NO}^{2-} is the concentration of nitrite in the effluent (mg/L), C_{NO}^{3-} is the concentration of nitrate in the effluent (mg/L), V is the working volume of SBR (L) and t is the time (d).

The statistical analyses of the obtained results were carried out by the Kruskal-Wallis nonparametric test and *post-hoc* multiple mid-rank comparisons for all samples (assuming a level of significance of $\alpha = 0.05$) [32].

To quantify the PCR reaction yield, a set of standards was prepared by dilution of the amplification product of the aforementioned genes fragments. The amplicons were purified on silica columns (Clean-Up, A&A Biotechnology, Poland), and the molecular weights were determined by the quantitative electrophoresis in EtBr stained 1.8% agarose gel [33], in comparison to equimolar 100 bp ladder (Carl Roth, Germany). Then, using the formula:

$$N_{DNA} = \frac{C \cdot 6.022 \cdot 10^{23}}{L \cdot 1 \cdot 10^{9} \cdot 650} \quad (7)$$

where C is the quantity of DNA [ng] in the band and L is the length of the amplicon [bp], the estimated number of matrices was calculated. So prepared standards, in a series of 10-, and additionally 2-fold dilutions, were added to the separate reaction mixes in an equal volume as the examined DNA samples, to determine the calibration curve for each primer pair. The resulting data were multiplied by the coefficient taking into account changes in the DNA concentration from sampling to preparation of the reaction.

3. Results and discussion

The leachate used in the research was characterized by a concentration of organic compounds expressed as COD at the level of 3,278 mg/L. The total nitrogen value was 672 mg/L, most of which, 630 mg/L was ammonium nitrogen. No nitrite or nitrate was detected in the raw leachate, thus, the remaining 6.25% was organic nitrogen. Therefore, the ratio of BOD_5/COD and N/COD, which amounted to 0.08 and 0.2, respectively, should be considered as unfavorable from the point of view of biodegradation. Also, the nitrogen loading rate (NLR) was low and amounted to 0.315 kg/m^3 d.

After stabilization of the outflow, defined arbitrary as changes between successive measurements of less than 5%, the efficiency of COD removal was, depending on the reactor, from 35% to 78%. Statistical analysis of the results showed that the presence of a filling, in the form of reticulated open-cell polyurethane foam in the amount of ≈0.17 cm/cm^3 of active volume had an effect on the COD concentration in the outflows from reactors operating at temperatures of 30°C and 40°C (Fig. 3). At 20°C, the COD removal efficiency in the reactor with suspended (R1) and hybrid (R2) sludge was 56% and 50%, respectively. At 30°C, the COD removal efficiency in R3 was 62%, but with the introduction of filling (R4), it decreased to 35%. Increasing the temperature to 40°C resulted in an efficiency of removing organic substances in SBR of 45%, and in HSBR (R6) it increased to 78%.

Analyzing the influence of temperature on the concentration of COD in treated leachate, it was found that in the reactors operating without filling (SBR), a temperature difference between 20°C and 30°C had no effect on COD removal, while statistically significant differences were observed between removal at 40°C and 20°C or 30°C. In reactors operating with filling (HSBR), it was found that the highest concentration of COD in the outflow was obtained in the reactor operating at 30°C. Statistically significant differences were found between the values of COD concentrations from all reactors (Table 3). According to Guo et al. [12] in the low-temperature range, 5°C and 10°C, COD removal from the synthetic wastewater of more than 50% in the first 4 h of the cycle can be achieved, and at 20°C and 30°C even up to 80% in 3 h. Removal of organic compounds is not a priority in the treatment of leachate flowing from landfills operated for more than 10 y; the assimilation of carbon substrates may, however, be of great importance for activated sludge microorganisms, which can then potentially use them as carbon sources for heterotrophic denitrification and nitrification processes.

In the description of the mechanism of removal of nitrogen compounds from leachate, the dynamics of the occurrence of intermediate products is important. In reactors operating at 20°C and 30°C, there were nitrites in the outflow, and the concentration of nitrates did not exceed 6 mg/L (Fig. 3) throughout the process, and these products were present regardless of the presence of filling in the reactors. When analyzing the effect of filling, it was found that for nitrates no differences were found in the outflows, and statistically significant differences occurred between the temperatures of 20°C and 40°C for nitrites (Table 3). On the other hand, when examining the influence of the operating temperature, statistically significant differences in the concentration of nitrites occurred between the lower temperatures (20°C and 30°C) and the higher temperature of 40°C, regardless of whether there was filling in the reactors. Moreover, differences in the concentration of nitrites in the outflows occurred in the case of reactors operating only with activated sludge between the lowest and higher temperatures, in the case of reactors operating with a filling between the intermediate and extreme ones. Irrespective of the presence of filling and the process temperature, no products of the second phase of oxygen nitrification were observed in any of the reactors. Temperatures greater than 25°C may potentially favor an increase in ammonia-oxidizing bacteria (AOB) which becomes dominant over nitrite-oxidizing bacteria (NOB) leading to accumulation of nitrites [34,35].

By analyzing the concentration of mineral forms of nitrogen in the SBR outflow, it was found that in all analyzed variants ammonium nitrogen was present in the outflow. The effectiveness of its removal was, depending on the rector, from 56% to 92%. At all temperatures tested, the efficiency of TAN removal was higher in the reactors with filling compared to the reactors with only suspended sludge. In the assumed experimental set-up, the nitrogen removal efficiency did not have a rectilinear relationship with the temperature; statistical analysis of the results showed that at 20°C and 40°C the average TAN concentrations in the outflow from the reactors operating without filling did not differ significantly from those from the reactors with filling. In the reactors operating at 30°C,

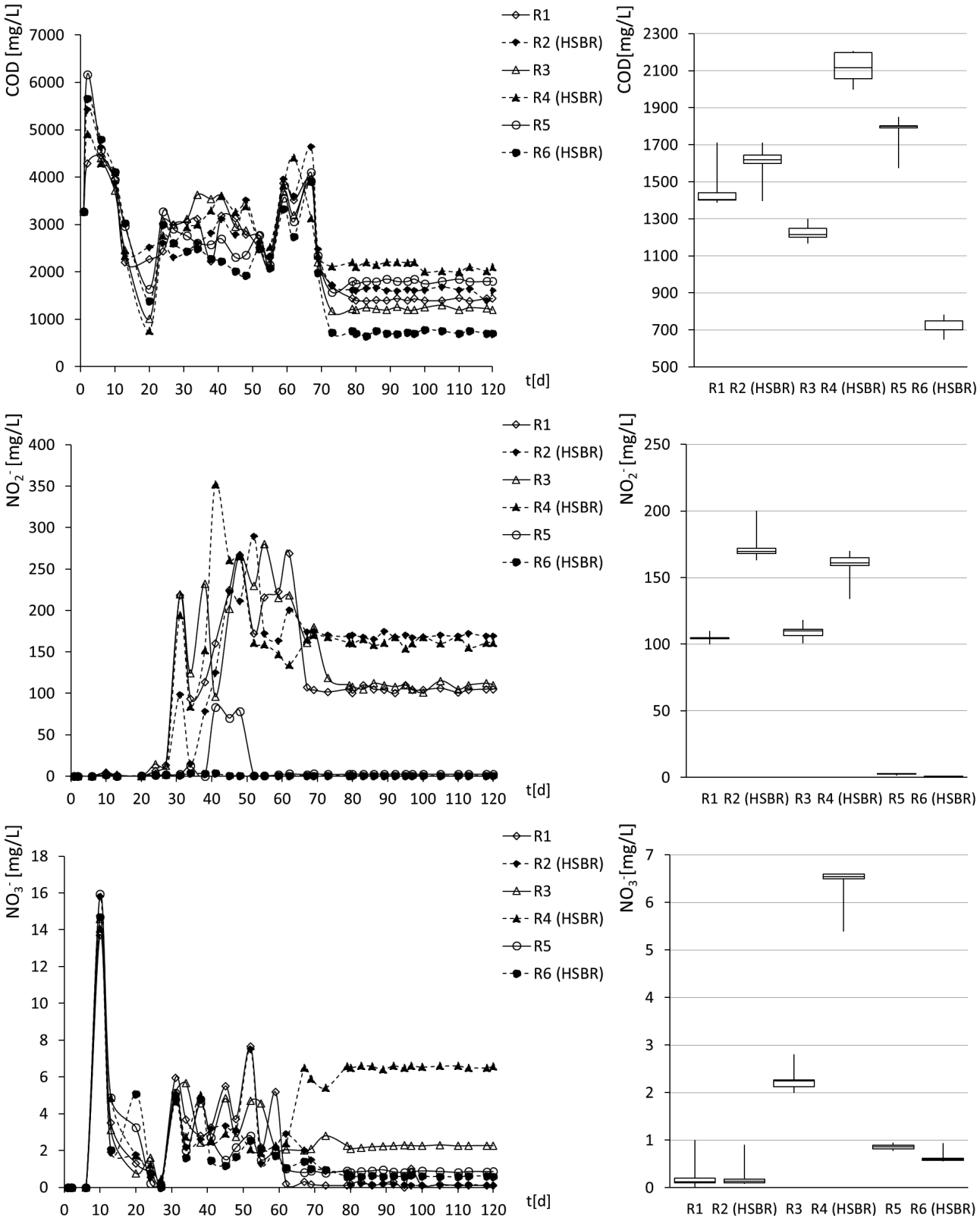

Fig. 3. The dynamics of organic substances expressed as COD, nitrite and nitrate concentration in the outflows from reactors, in subsequent days (d) of the experiment. Box-plot graphs show quartiles (box) and range (whiskers).

Table 3
Kruskal-Wallis nonparametric test and multiple mid-rank comparisons for all samples (assuming a level of significance of $p < 0.05$) for concentration of organic substances expressed as a COD, nitrite and nitrate (statistically significant result marked grey colour)

		R1			R2 (HSBR)			R3			R4 (HSBR)			R5		
		COD	NO_2^-	NO_3^-	COD	NO_2^-	NO_3^-	COD	NO_2^-	NO_3^-	COD	NO_2^-	NO_3^-	COD	NO_2^-	NO_3^-
R2 (HSBR)	COD	3.8^{-01}														
	NO_2^-		2.3^{-05}													
	NO_3^-			8.6^{-01}												
R3	COD	3.8^{-01}			1.1^{-02}											
	NO_2^-		3.4^{-01}			3.2^{-03}										
	NO_3^-			5.9^{-08}			7.2^{-07}									
R4 (HSBR)	COD	5.9^{-05}			1.1^{-02}			4.3^{-09}								
	NO_2^-		1.4^{-02}			2.3^{-01}			1.8^{-01}							
	NO_3^-			8.2^{-13}			3.1^{-11}			2.6^{-01}						
R5	COD	2.6^{-02}			3.8^{-01}			4.3^{-05}			3.8^{-01}					
	NO_2^-		2.9^{-08}			3.4^{-01}			1.7^{-05}			1.4^{-02}				
	NO_3^-			2.9^{-03}			7.6^{-03}			1.9^{-01}			1.1^{-03}			
R6 (HSBR)	COD	1.3^{-08}			1.8^{-05}			5.4^{-14}			3.4^{-10}			1.1^{-02}		
	NO_2^-		1.6^{-01}			4.8^{-12}			1.4^{-02}			3.2^{-07}			1.5^{-16}	
	NO_3^-			2.1^{-01}			2.6^{-01}			3.1^{-03}			2.3^{-05}			3.1^{-01}

the differences were statistically significant (Table 4, Fig. 4). In all temperature variants, the nitrogen removal process stabilized faster in the reactors operating with filling.

The influence of temperature on the efficiency of TAN removal from leachate was found in the case of reactors operating without filling. No statistically significant differences were found between the results obtained in the outflow from the reactors operating at 20°C and 30°C. On the other hand, increasing the temperature to 40°C, both from 20°C and 30°C, resulted in differences in the average concentration of TAN in the outflow. In the case of hybrid reactors, they were statistically significant both when the temperature of the process was decreased and increased from 30°C.

The presence of filling in the reactors had an impact on the FAN concentration in the outflows from the reactors – with an increase in temperature, the FAN values in

Table 4
Kruskal-Wallis nonparametric test and multiple mid-rank comparisons for all samples (assuming a level of significance of $p < 0.05$) for total ammonia nitrogen (TAN), free ammonium nitrogen (FAN) and ammonia nitrogen concentration (statistically significant result marked grey colour)

		R1			R2 (HSBR)			R3			R4 (HSBR)			R5		
		TAN	FAN	NH_4^+	TAN	FAN	NH_4^+	TAN	FAN	NH_4^+	TAN	FAN	NH_4^+	TAN	FAN	NH_4^+
R2 (HSBR)	TAN	3.9^{-01}														
	FAN		3.1^{-03}													
	NH_4^+			2.4^{-08}												
R3	TAN	4.3^{-01}			8.5^{-03}											
	FAN		3.2^{-01}			4.9^{-02}										
	NH_4^+			1.2^{-02}			1.2^{-02}									
R4 (HSBR)	TAN	9.8^{-03}			6.1^{-07}			4.3^{-05}								
	FAN		5.2^{-01}			4.1^{-01}			6.1^{-05}							
	NH_4^+			3.7^{-24}			4.1^{-10}			8.1^{-16}						
R5	TAN	8.5^{-03}			4.3^{-01}			2.2^{-05}			4.7^{-11}					
	FAN		1.1^{-01}			4.4^{-01}			1.1^{-01}			6.3^{-04}				
	NH_4^+			1.3^{-18}			4.3^{-06}			3.6^{-11}			3.3^{-01}			
R6 (HSBR)	TAN	4.3^{-01}			7.8^{-01}			1.1^{-02}			1.2^{-06}			3.9^{-01}		
	FAN		1.1^{-02}			1.2^{-01}			1.1^{-07}			1.1^{-01}			2.2^{-05}	
	NH_4^+			1.2^{-02}			1.2^{-02}			8.8^{-01}			3.8^{-16}			1.9^{-11}

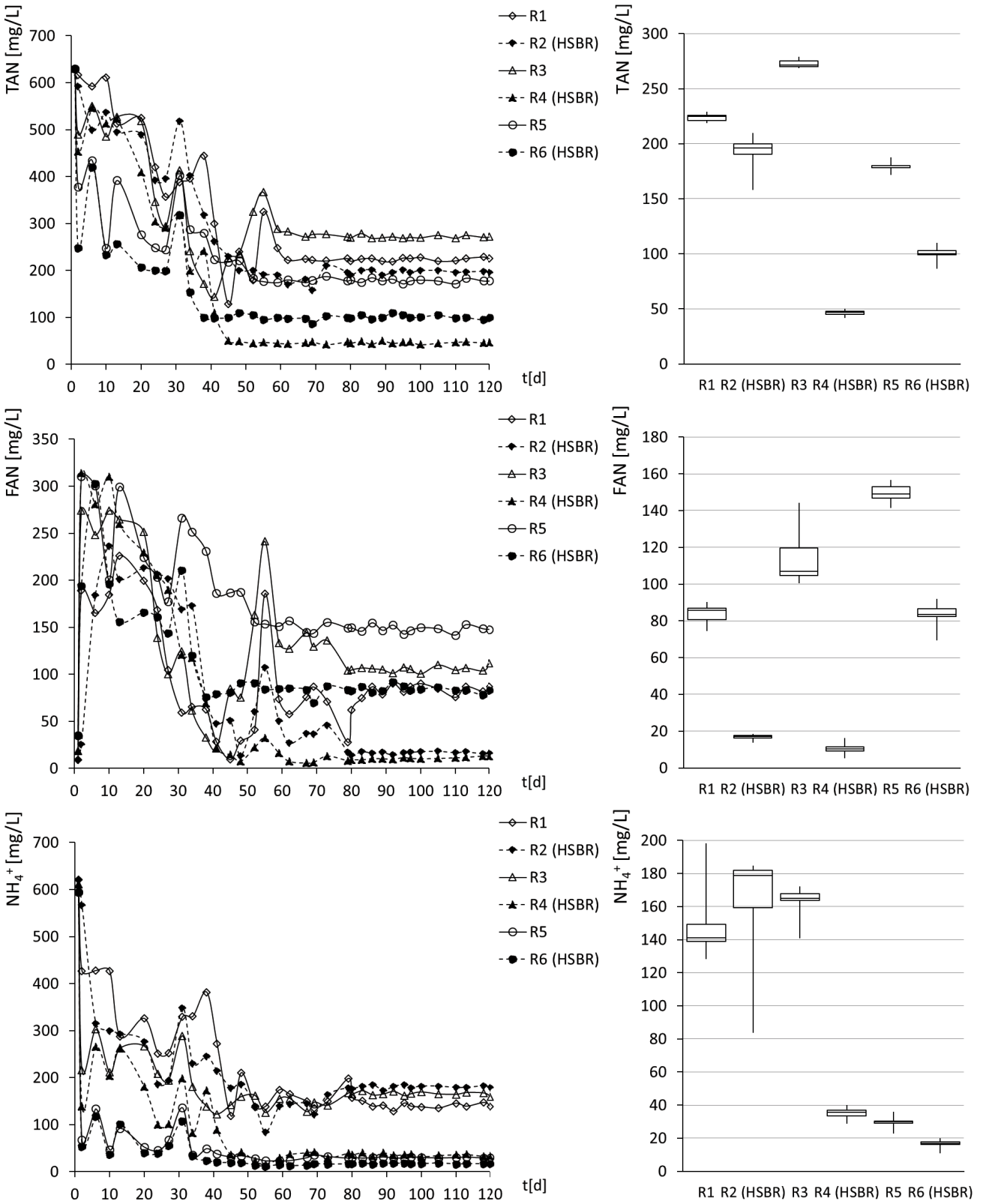

Fig. 4. The dynamics of total ammonia nitrogen (TAN), free ammonium nitrogen (FAN) and ammonia nitrogen concentration in the outflows from reactors, in subsequent days (d) of the experiment. Box-plot graphs show quartiles (box) and range (whiskers).

the outflow of hybrid reactors were 5, 10.9 and 1.8 times lower, respectively, than in the reactors operating without filling, and these differences were statistically significant in the course of the experiment (Fig. 4, Table 4). A high concentration of TAN can, under conditions of high pH, shifting the balance between its two forms, lead to a high concentration of FAN. One of the effects is the accumulation of nitrites [35], due to the inhibition of the activity of nitrifying bacteria, mainly phase II nitrification (NOB). Anthonisen et al. [30] were the first to discover that FAN begins to inhibit the development of AOB and NOB in concentrations of 10–150 mg/L and even 0.1–1.0 mg/L, respectively. In turn, Van Hulle et al. [36] reported that AOB cultures under certain conditions were not inhibited by FAN at a concentration of 70 to even 300 mg/L. Moreover, Vadivelu et al. [37] showed that a concentration of FAN of 16.0 mg/L did not affect either anabolism or catabolism in the studied AOB populations. Meanwhile, Vadivelu et al. [38] also found that an increase in NOB may be inhibited at a concentration of FAN in the range of 6.0–9.0 mg/L. In the present study, the concentration of nitrates in treated leachate did not exceed 6 mg/L, which may indicate inhibition of NOB by FAN. Therefore, the obtained results clearly show that a FAN concentration of 10.34–149.4 mg/L strongly inhibited NOB activity, but did not inhibit AOB activity.

Analyzing the concentration of ammonia nitrogen calculated from the difference between TAN and FAN, it was found that the effect of filling in the reactors was statistically significant for each tested temperature. The influence of temperature on the concentrations of ammonia nitrogen in the effluents from conventional SBRs was statistically significant at all temperature variants, while in the case of HSBR, it was not significant between the reactors operating at 30°C and 40°C (Fig. 4, Table 4).

In the case of the FAN/TAN ratio, it was found that in the outflows from reactors operated at 20°C and 30°C, it did not exceed 42%, and was higher in the SBR than in HSBR by 2.4 and 1.6 times, in 20°C and 30°C, respectively.

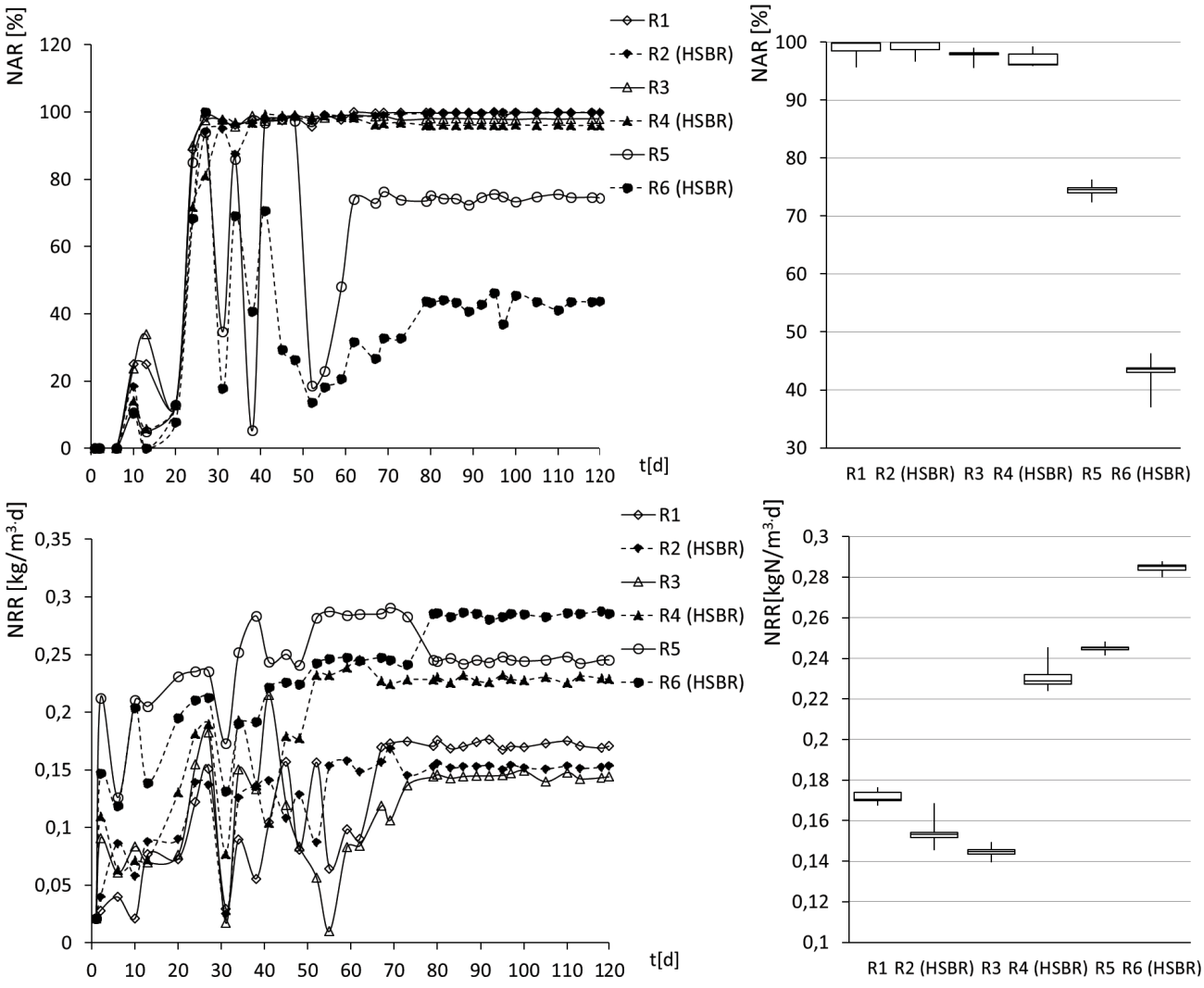

Fig. 5. The dynamics of nitrite accumulation ratio (NAR) and nitrogen removal rate (NRR) in reactors, in subsequent days (d) of the experiment. Box-plot graphs show quartiles (box) and range (whiskers).

Table 5
Kruskal-Wallis nonparametric test and multiple mid-rank comparisons for all samples (assuming a level of significance of $p < 0.05$) for nitrite accumulation ratio (NAR) and nitrogen removal rate (NRR) (statistically significant result marked grey colour)

		R1		R2 (HSBR)		R3		R4 (HSBR)		R5	
		NAR	NRR	NAR	NRR	NAR	NRR	NAR	NRR	NAR	NRR
R2 (HSBR)	NAR	4.8^{-01}									
	NRR		2.1^{-01}								
R3	NAR	2.4^{-01}		1.6^{-02}							
	NRR		5.7^{-03}		3.1^{-01}						
R4 (HSBR)	NAR	2.3^{-03}		3.4^{-05}		2.8^{-01}					
	NRR		1.8^{-01}		2.3^{-04}		5.6^{-07}				
R5	NAR	2.1^{-08}		6.1^{-11}		1.1^{-04}		2.5^{-02}			
	NRR		4.9^{-03}		8.3^{-07}		1.3^{-09}		3.1^{-01}		
R6 (HSBR)	NAR	1.7^{-10}		4.6^{-13}		2.1^{-06}		1.2^{-03}		4.8^{-03}	
	NRR		1.3^{-06}		1.1^{-10}		8.5^{-14}		1.1^{-02}		3.1^{-05}

In the leachate treated at 40°C, FAN accounted for 83% of TAN regardless of the presence of biomass carriers. It follows that simultaneous air stripping can be achieved not only by increasing the pH but also by increasing the temperature of the process. Theoretically, at a temperature of 20°C and a pH of 9, the N-FAN can reach over 40% of N-TAN. However, to ensure effective stripping in practice, the LFL must be brought to pH > 11 and then blown counter-currently with a stream of hot air, which implies a high energy consumption, and moreover demands further pH re-adjustment for the next stage of treatment or discharge [36]. For example, the pH preferred for biological nitrification varies between 8 and 9, with less than 50% of the optimum rate occurring outside the range of 7.0–9.8 [39].

By analyzing the NAR, it was found that in the tested experimental system, in the temperature range of 20°C–30°C, the presence of filling did not affect the rate of accumulation of nitrites. However, regardless of the temperature of the process, in reactors operating with NAR filing, it was lower. The influence of temperature on NAR can be seen primarily in the case of hybrid reactors, where the rate of accumulation of nitrites decreases with its increase. In the case of reactors without filling, these differences do not occur only between reactors operating at 20°C and 30°C (Fig. 5, Table 5). As indicated in the literature, there may be various mechanisms of the influence of temperature on microorganisms from the AOB and NOB groups, inter alia, by inducing the formation of FAN. For example, Balmelle et al. [40] showed that the NOB inhibitory concentration of FAN from 2 to 5 mg/L in the temperature range 10°C–20°C had no negative impact on the efficiency of the process, and no accumulation of nitrites occurred during treatment. Meanwhile, already above 25°C the inhibitory effect on NOB resulted in an accumulation of nitrites. Bougard et al. [41] report that at 30°C the rate of accumulation of nitrites can be inhibited by lowering the temperature. In the presented research, it was shown that increasing the treatment temperature to 40°C may also contribute to the reduction of NAR. The presence of biomass carriers influenced the stabilization time of TAN and nitrite nitrogen concentrations in the outflows Regardless of the temperature, stabilization of outflows has always occurred earlier in hybrid reactors. Temperature is usually an important parameter for achieving nitrification.

The research also revealed the effect of the filling on the stabilization of the NRR value in outflows from reactors in reactors operating only with activated sludge, this happened later than in hybrid reactors (Fig. 5). At a temperature of 20°C, this parameter was slightly lower in R2 compared to R1 and amounted to 0.153 and 0.171 kg/m^3 d, respectively. In hybrid reactors operating at 30°C and 40°C, NRR was higher in the reactors and amounted to 0.23 kg/m^3 d in SBR4 and 0.28 kg/m^3 d in SBR6, and these differences were statistically significant (Table 5). These values were achieved with an NLR of 0.315 kg/m^3 d. It follows that the presence of filling, by creating different conditions for the development of the microbial consortium, can accelerate the stabilization time of the treatment process, which may be of great importance with the inflow of leachate with frequently changing load, as is usually the case with landfill leachate.

In addition, an increase in temperature may result in an increase in NRR. Sobotka et al. [42] achieved NRR at the level of 0.71–3.53 kg/m^3 d, with an NLR not exceeding 3.5 kg/m^3 d. By contrast, Guo et al. [43] at an NLR of 1.2 kg/m^3 d achieved NRR of 0.9 kg/m^3 d, and further respectively for NLR 2.3 kg/m^3 d – NRR 1.9 kg/m^3 d, for 4.6 kg/m^3 d – NRR 3.5 kg/m^3 d, and for 8.9 kg/m^3 d – NRR 6.6 kg/m^3 d, hence the NRR can be from 74% to 82% of the NLR. In the authors' own research, the percentage ratio of NRR to NRL was higher in HSBR than in SBR with suspended sludge. The influence of temperature can also be visible here, because in the reactors operating at 20°C it did not exceed 50%, similarly to R3, while in R4 (30°C, HSBR) and in both reactors operating at 40°C, it exceeded 70%.

In the studies presented here, the anaerobic phase lasted 1.5 h, whereas Zhang et al. [44] obtained NRR of 0.128–0.139 kg/m^3 d depending on the length of the anaerobic phase used in the cycle with intermittent aeration lasting from 2 to 3.5 h. According to Zhang et al. [44], nitrites produced during partial nitrification are consumed by denitrifying bacteria with simultaneous COD removal. In the research, in the technological variant allowing the highest nitrogen

(R4) removal, COD removal was the lowest and amounted to only 35%. The highest COD removal efficiency, of 78%, was accompanied by 84% removal of TAN (R6). The obtained results show that TAN can be efficiently removed from leachate from mature landfills in the SND process in hybrid reactors and at high temperatures.

3.1. Nitrogen concentration profiles in the SBR cycle

Analyzing the profiles of the concentrations of mineral forms of nitrogen in the outflows from the reactors, it was found that in the aeration phase, the concentration of TAN decreased and in the mixing phase it remained at a constant level. In the case of nitrites, the opposite was true and the concentration in the aeration phase did not change, and in the mixing phase, this form accumulated. Nitrates were not found in the reactor operating cycle.

The effect of the filling was also found in the case of the outflow stabilization time in the reactor operation cycle. At a temperature of 20°C in R1, the concentration of nitrites was already constant after 4 h, and in R2 only after 13.5 h. On the other hand, in the case of TAN, in R2 there was more rapid inhibition of the removal process (after 16 h) than in R1 (after 21 h). At a higher operating temperature, the inhibition of the appearance of nitrites in the leachate was still ahead of the point at which the TAN process was stopped. However, regardless of the presence of filling, the nitrite concentration was equalized faster than TAN. This can be explained, for example, by the conversion of ammonium into nitrogen oxides. At a temperature of 40°C, the outflow from the reactors contained mainly TAN and its removal was stopped after 21 h, regardless of the presence of biomass carriers in the reactor (Fig. 6).

The decrease in COD concentration in the reactor operation cycle took place until the concentration of nitrites in the SBR outflow stabilized and could have been caused by the use of carbon by denitrifying bacteria during the anoxic phase. Similar results were obtained by Wang et al. [6]. The authors noted that a decrease in total nitrogen concentration during alternating mixing and aeration could have been caused by two factors: ammonium nitrogen and COD were synchronously degraded during aeration when production of nitrite was removed by denitrifying bacteria before the anoxic phase. Furthermore, total nitrogen could also be removed in the SND process, due to a limited oxygen concentration under aeration. In this experiment, the total nitrogen was removed without the addition of any external carbon sources, organic compounds contained in leachate were utilized in the denitrification. Some of the organic substances adsorbed on the cell walls were used as an internal carbon source by denitrifying bacteria.

3.2. Abundance and role of main microbial components in the process

All samples were pre-tested by PCR in the presence of positive and negative controls to confirm the appearance and quality of the amplification product in the electrophoresis image. These reactions did not show products complementary to the primers for the 16S rRNA *Nitrospira* sp. and AnAOB in all the tested samples. While typical

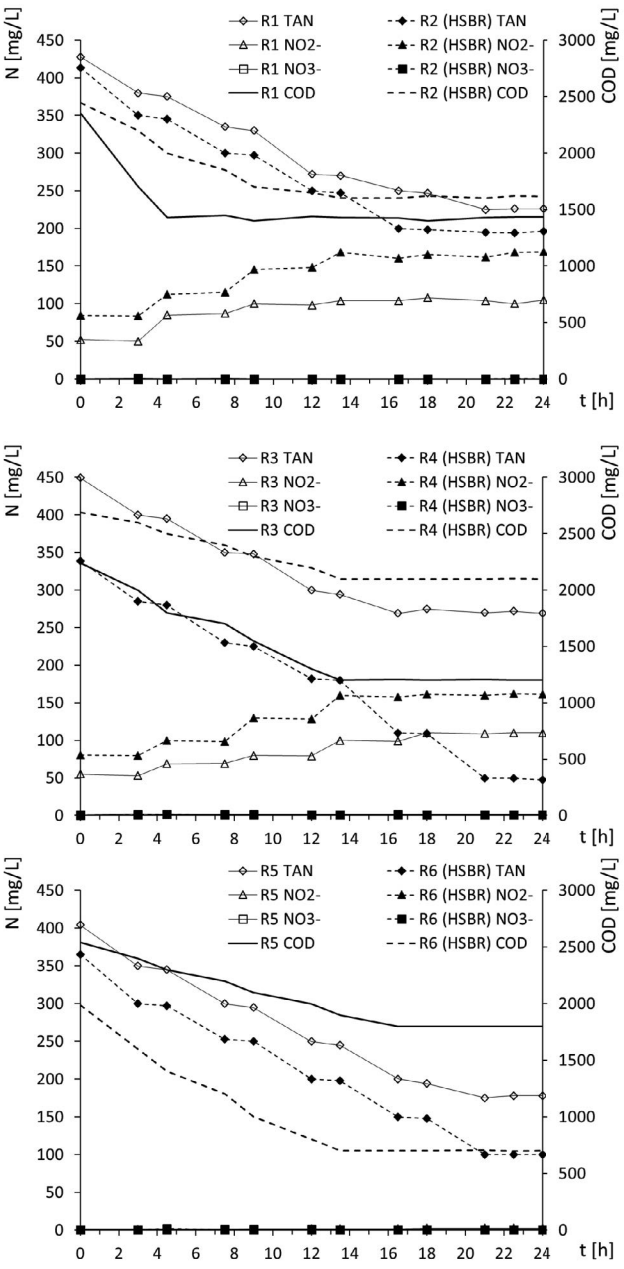

Fig. 6. The change of organic substances expressed as COD, TAN, nitrite and nitrate concentration during the operational cycle.

Anammox reactors are anaerobic, AnAOB representatives are detected under oxygen-restricted conditions, especially in reactors containing biomass carriers of complex structure that helps to maintain a local anaerobic environment. The lack of 16S rRNA AnAOB fragments in the tested reactors at the level of PCR detection may be additionally explained by the short duration of the experiment, as most of the representants have a long time of division.

On the other hand, the absence of *Nitrospira* spp., which is usually one of the main components of NOB in the municipal WWTP, may be explained by the preference for environments with lower ammonium nitrogen content.

In the concentration that is detected in landfill leachate, this group is often suppressed, hence the role of NOB can be played by other bacteria able to maintain the process e.g. *Nitrobacter*. At the same time, a large number of βAOB copies and only a trace of *amoA* were found. As both primer sets were not developed for the RealTime methodology, and both the amplicons lengths and the amplification cycles differ significantly from optimal for this method, although were used many times for quantitative measurements by the competitive PCR method, this study does not compare the results with the quantitative data on phylum level and only shows as a relative ratio to the highest score. The highest number of copies of the βAOB genes was found in R1, and along with the increase in temperature in conventional SBR, this number was half lower at 30°C in R3 (45.61%) and two orders of magnitude lower at 40°C in R5 0.87%. In HSBR, the differences between 20° and 30° were not as significant, they were over 20% and almost 17%, respectively, but there was a significant decrease at the highest temperature of 0.47%. The decrease in the number of βAOB genes depending on the temperature increase could be described by the relationship C = 2.01E + 05T^2 – 3.27E + 07T + 9.91E + 08 for SBR reactors, and in HSBRs this decline was milder C = –2.73E + 05T^2 + 1.23E + 07T – 5.13E + 07. The number of *amoA* gene copies was trace (maximum 1.57E + 03 in R6), and balanced between the reactors (Fig. 7).

Literature data also indicate large inaccuracies in comparing the results of absolute quantitative measurements in functional groups related to nitrogen transformation in the environment and technological systems. Dang et al. [45] by real-time PCR have studied in the sediments of Jiaozhou Bay (China) the abundance of total bacteria, and the *amoA* genes characteristic of the βAOB subgroup or *Nitrosomonas*. The *amoA* number was four orders of magnitude lower than the total bacteria count, and *Nitrosomonas* was even an order of magnitude lower than βAOB. The authors report that the ratio of the βAOB *amoA*/16S rRNA and *Nitrosomonas amoA*/βAOB *amoA* ranging from 0.003 to 0.076 and 2.06 to 47.88, respectively.

Baptista et al. [46] tried to confirm the results of quantitative qPCR measurements by in situ hybridization (FISH). Resulting qPCR measurements of the *amoA* gene turned out to be consistent with the results obtained with the results from FISH, however, the result obtained from measuring the number of copies of the 16S rRNA gene was significantly lower, which was explained by differences in the species composition of the studied bacterial community affecting lower specificity to the primers and lower efficiency of amplification. Also Dechesne et al. [47], analyzed the consistency of the data obtained from two sets of qPCR primers (CTO189/RT1 *amoA*1F/A2R) tracking the AOB number in the high-speed water treatment filters. Such a difference significantly influenced the practical assessment of the number of AOB – in the installation where group 6a was most abundant, the number of *amoA* was estimated to be 50 times smaller than it would appear from the quantitative analysis of the 16S rRNA gene. Sequencing the products of these reactions showed different pictures of AOB biodiversity; the first four clusters included sequences associated with species within the genus *Nitrosospira*, but *Nitrosomonas* was grouped in clusters 5, 6a and b, 7, and 8. In the sequences derived from the amplification with *amoA* primers, group 7 is more frequent than 6a, whilst the 16S rRNA sequences were more diverse and belonged to three different groups including also other bacteria, not belonging to the functional βAOB group.

The relationships between the 16S rRNA copy number specific for the selected phyla were similar to those described above. Absolute quantification of the 16S rRNA copy number of bacteria in the suspended sludge showed that the highest value of 1.17E + 08 occurred in R1, in which the leachate was treated at a temperature of 20°C, in the remaining reactors, the number of DNA copies decreased with increasing temperature, in SBR reactors according to the polynomial model C = 1.96E + 05T^2 – 1.58E + 07T + 3.54 E + 08, and in HSBR according to C = 6.92E + 04T^2 – 5.54E + 06 T + 1.25E + 08. The 16S rRNA gene copy number was lower in reactors with biomass carriers by 35.6%, 36.5%, and 37.9% at 20°C, 30°C, and 40°C, respectively. The relationship between the temperature and number of 16S rRNA gene copies can be represented by the following formulas: C = 3.95E + 04T^2 – 2.94E + 06T + 6.18E + 07 in SBRs, and C = –1.38E + 03T^2 – 6.72E + 04T + 7.20E + 06 in HSBRs for Betaproteobacteria, C = 1.30E + 04T^2 – 9.12E + 05T + 1.82E + 07 and C = –4.93E

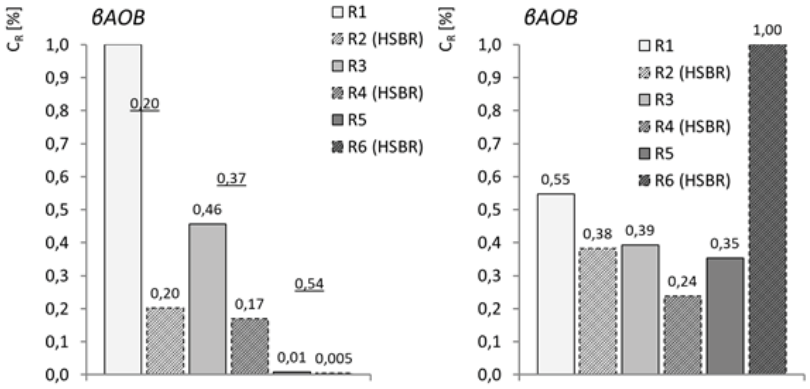

Fig. 7. A number of 16S rRNA copies specifically for the chosen phyla. Numbers above the bars indicate relative proportion of the genomes number to the highest recorded value, and underlined proportion between SBR and HSBR operating at the same temperature.

+ 03T² + 2.84E + 05T – 2.20E + 06 for Gammaproteobacteria, C = 4.69E + 04T² – 4.34E + 06T + 1.05E + 08 and C = –6.00E + 01T² – 3.11E + 05T + 1.64E + 07 for Actinobacteria, C = 1.36E + 04T² – 6.84E + 05T + 1.14E + 07 and C = 1.04E + 04T² – 6.36E + 05T + 1.10E + 07 for Firmicutes and C = 1.61E + 04T² – 1.20E + 06T + 2.26E + 07 and C = –2.57E + 03T²+ 1.40E + 05 – 1.43E + 06 for Bacteroidetes (Figs. 8 and 9).

Literary data on landfill leachate treatment process research points at the presence of constant components of the microbial community in leachate and treatment systems, the share and proportions of which change during the process and depending on its technological parameters. The main components at the phylum level, regardless of aerobic conditions and temperature, include Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria [48–51]. Representants of this phyla are responsible for the hydrolysis of high-molecular refractive or toxic organic compounds and removal of high-strength ammonia concentration. Also, such groups as Firmicutes are able to survive high salinity anaerobic conditions characteristic for landfill retention tanks and systems with recirculation of leachate or reverse osmosis retentate [49, 51].

Currently, most of this data comes from new generation sequencing techniques, that allows to analyze previously inaccessible mass data resources, however, it must be taken into consideration that generally informing about biodiversity as a number of genome variants in the environment, then reveals the actual quantity of organisms in the volumetric unit, which also could be valuable from a technological point of view.

For example, Zhang et al. [50] in concentrated landfill leachate revealed domination of Proteobacteria with 51.1%, when subsequently Bacteriodetes and Firmicutes represented 23.4% and 6.4% of microbial community respectively.

Also in anaerobic systems, such as the Anammox process, similar compositions were revealed. Liang et al. [48] analyzed bacterial community in reactor supplied with low COD (0.3 – 1.0 g L^{-1}) and 0.5–0.9 g L^{-1} and significant concentration of $N–NO_2^-$: 0.4 to 1.0 g L^{-1}, in temperature of 30°C have identified mostly Bacteroidetes and Firmicutes (including such genera as *Bacillus*, *Paenibacillus* or *Staphylococcus*).

Xie et al. [51] in high-temperature Anammox membrane reactor treating LL with high concentrations of organic compounds –13,000 mg/L, containing up to 7,000 mg/L of VFA, and ammonia exceeding 3,000 mg/L revealed that the main components of microflora structure were Firmicutes with 29.7% of relative abundance of, then Bacteroidetes and Proteobacteria with 27.9% and 6.06% respectively.

Also, the bacterial community structure analysis made by Yuan et al. [52] in three-chamber reactor treating synthetic landfill leachate at 35°C with HRT set at 1 d and

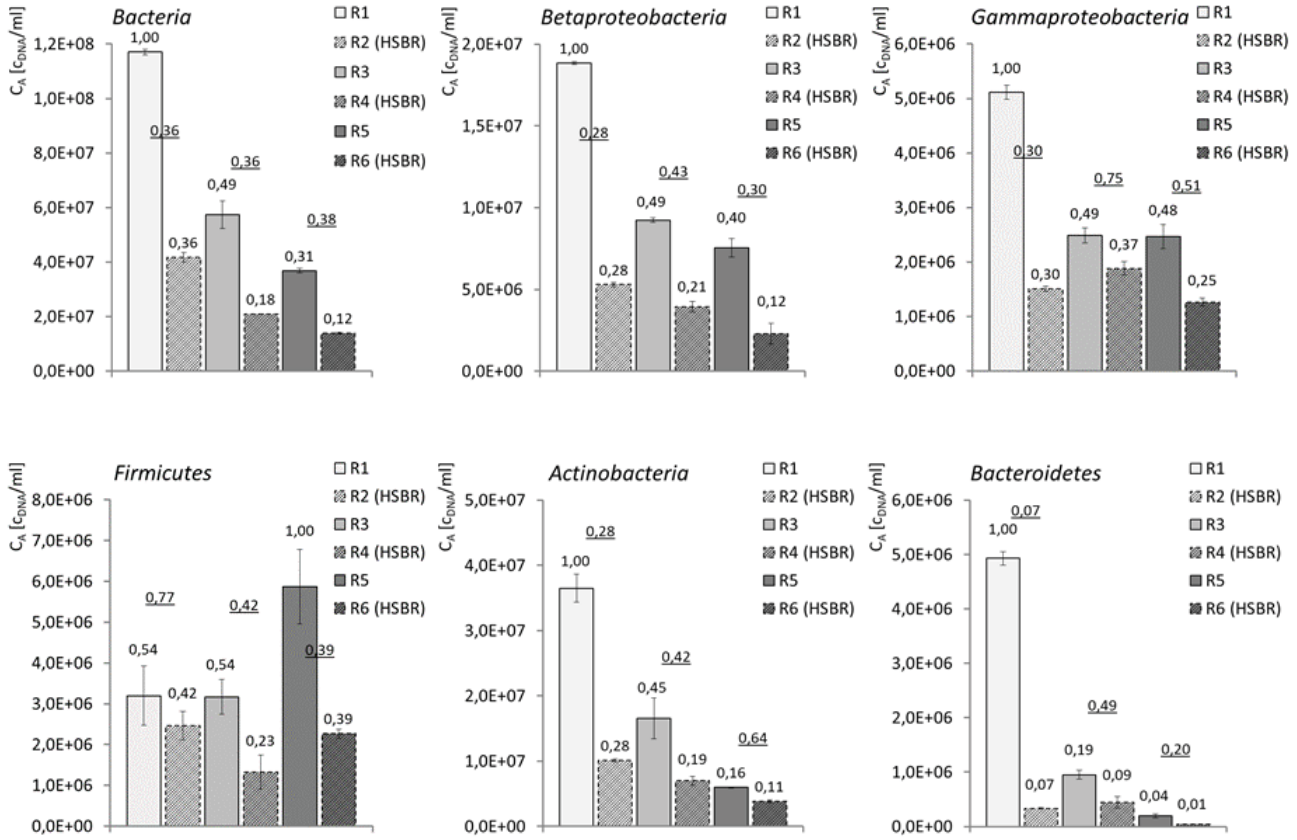

Fig. 8. A number of 16S rRNA copies specific to the chosen phyla. Whiskers indicate the measurement error, numbers above the bars relative proportion of the number of genomes to the highest recorded value, and the numbers underlined proportion between SBR and HSBR operating at the same temperature.

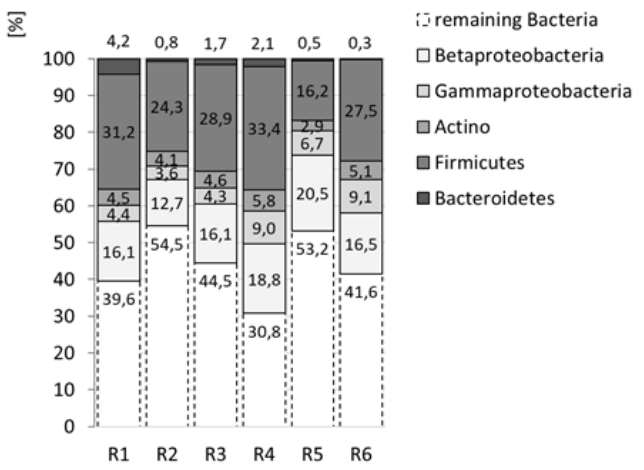

Fig. 9. Proportions of selected phyla in subsequent reactors on the basis of quantitative measurement of total bacteria count.

air expenditure of 0.1 L/min L shown that biofilm was dominated by Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. In raw sludge, Proteobacteria and Bacteroidetes dominate with over 70% share, but also such components as Chloroflexi and Sacharibacteria were significant. Proteobacteria dominates the process growing continuously up to 45% and Bacteroidetes constituted the second component with over 43% of relative abundance). During the treatment Firmicutes, previously not present, rapidly grown to 22% then decreased to 2.69% and were dominated by Actinobacteria. This succession of microbial community structure, bacteria community was explained by the occurrence of aerobic *Saprospiraceae* (Bacteroidetes), which potentially decompose refractory organics to assimilate as a carbon source. On the other hand, denitrifying chemoorganotrophic representants of Betaproteobacteria *Comamonadaceae* and Gammaproteobacteria *Acinetobacter*, had potentially utilized nitrates in nitrogen cycling [53–55]. The authors also report that higher concentration COD promotes Firmicutes, phylum containing anaerobic and heterotrophic bacteria. In turn autotrophic aerobic nitrifying bacteria, as belonging to Betaproteobacteria (βAOB) *Nitrosomonas,* being one of the main identified organisms in municipal wastewater treatment systems, increasing along with decomposition of organic matter, which could be explained also as an influence of toxicity reduction by decomposers.

Partial nitrification reactor treating landfill leachate with BOD/COD = 0.09 and ammonia nitrogen reaching 1,200 g/L operated by Gabarro et al. [56] was settled mainly by Bacteriodetes (52%) and α- and β-Proteobacteria, and AOBs increased significantly during the operation from 5% to 33%, whereas 2.5% of NOBs decreased even to 0.25%.

In 2013, Xie et al. [57] reported that in landfill leachate processed at temperature of 30°C, NLR reaching 2000 mg/kg and limited oxygen access up to 0.5 mg/Proteobacteria dominates the community with abundance reaching 46.4% (including 12.4% of Gammaproteobacteria and 11.3% of Betaproteobacteria) and other components included 7% to 17% of Actinobacteria, 3.0% to 10% Bacteroidetes and 2%–7% Firmicutes. The AOBs did not exceed 2% whilst NOB representatives 0.2% when *Nitrospira* spp was detected in trace amounts.

Silva et al. [58] analyzed microbial community by the activated sludge system treating mature landfill leachate with biodegradability increased by photo-Fenton under high pH up to 8.9 and DO range between 0.5 and 4.0 mg/L. In the nitrification reactor, most abundant phyla were Bacteroidetes (43%), followed by Proteobacteria (32% of which 68% was Betaproteobacteria including less than 7% of *Nitrosomonadaceae*), Firmicutes (11%) and Actinobacteria (2.4%). In the denitrification reactor, Proteobacteria dominates with almost 74% (of which 4.8% was Beta and 17% Gamma), next were Bacteroidetes (16.1%), when Actinobacteria were represented in only 1% and Firmicutes even less.

Soliman and Eldyasti [59] controlled the process of complete PN in SBR by the temperature of 35°C, pH over 8.0 and DO strategy to obtain domination of AOBs over the NOBs, and had implemented CTO654/189, *amoA* and NSR PCR primers to prove biological nature of the observed process, however, they only confirmed the presence βAOB and *Nitrospirae* and excluded *Nitrobacter* spp.

4. Conclusions

Biological methods seem to be the most favorable from both economic and environmental points of view, but become problematic when the installation, which was designed for "young" leachate, still operates on stabilized landfill. The research presented in this publication was focused on the influence of the presence of filling and different process temperatures on the efficiency of landfill leachate treatment were tested. By analyzing the concentration of mineral forms of nitrogen in the SBR cycle, it was found that the stabilization of ammonium concentration in the outflow from reactors operating only with suspended activated sludge took place after 21 h for a temperature of 20°C and 40°C and after 16 h for R3 (30°C). The concentration of nitrite in the outflow from R1 and R3 was stable after 4 and 18.5 h, respectively. The presence of filling influenced the time to stabilize the ammonium concentration in the outflow from the SBRs. As the temperature increased from 20°C to 30°C, this time increased from 16 to 21 h. Further temperature increase did not affect the moment of stabilization of ammonium concentration in the outflow. In the case of nitrite concentration, the outflow stabilized after 13.5 h at both 20°C and 30°C.

A significant abundance of Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria in the examined microbial community was distinctive for the system of landfill leachate treatment. The increase in temperature caused a decrease in the number of gene copies of all examined bacteria groups except the Firmicutes in SBR, but in the case of HSBRs, it was always milder which points to the protective character of biomass carriers in higher temperature inducing an increase of FAN concentration.

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biological system to variable ammonium nitrogen load in the process of landfill leachate treatment (DEC 2018/02/X/NZ9/00097).

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