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Microbiome Characterization after Aerobic Digestate Reactivation of Anaerobically Digested Sewage Sludge

Pascal Otto [1,†], Mozhdeh Alipoursarbani [2,†], Daniel Torrent [3], Adriel Latorre-Pérez [3], Thomas Paust [4], Alfred Albert [4] and Christian Abendroth [2,*]

[1] Institute of Waste Management and Circular Economy, Technische Universität Dresden, 01796 Pirna, Germany; pascal.otto@tu-dresden.de
[2] Brandenburgische Technische Universität Cottbus-Senftenberg, 03046 Cottbus, Germany; alipours@b-tu.de
[3] Darwin Bioprospecting Excellence, S.L. Parc Científic de la Universitat de Valencia, 46980 Paterna, Spain; dtorrent@darwinbioprospecting.com (D.T.); alatorre@darwinbioprospecting.com (A.L.-P.)
[4] PRO-Entec East GmbH Bio Engineering, 16928 Gerdshagen, Germany; info@pro-enteceast.de (T.P.); alfredalbert@pro-entec.de (A.A.)
* Correspondence: christian.abendroth@b-tu.de
† These authors contributed equally to this work.

Abstract: A demonstrator plant of a recently patented process for improved sludge degradation has been implemented on a municipal scale. In a 1500 m^3 sewage sludge digester, an intermediary stage with aerobic sewage sludge reactivation was implemented. This oxic activation increased the biogas yield by up to 55% with a 25% reduction of the remaining fermentation residue volume. Furthermore, this process allowed an NH_4-N removal of over 90%. Additionally, 16S rRNA gene amplicon high-throughput sequencing of the reactivated digestate showed a reduced number of methane-forming archaea compared to the main digester. Multiple ammonium-oxidizing bacteria were detected. This includes multiple genera belonging to the family Chitinophagaceae (the highest values reached 18.8% of the DNA sequences) as well as a small amount of the genus *Candidatus nitrosoglobus* (<0.3%). In summary, the process described here provides an economically viable method to eliminate nitrogen from sewage sludge while achieving higher biogas yields and fewer potential pathogens in the residuals.

Keywords: anaerobic digestion; anaerobic microbiomes; aerobic sludge activation; 16S rRNA sequencing; water treatment

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1. Introduction

Anthropological activities often result in large quantities of waste in the form of sewage, which poses a significant risk to human health, soil health and aquatic systems [1,2]. The production of large quantities of sewage has presented difficulties in effective management and efficient treatment [3]. Most sewage treatment techniques rely on both physical and biological processes [4]. As part of this process, large amounts of semisolid material, referred to as sludge, are produced. The accumulation of this sludge in the environment poses a significant risk of pollution to both soil and water [5]. Multiple technologies are already applied for the treatment of residual sludges, such as biodrying [6], composting [7] and anaerobic digestion [8]. Even the combination of these technologies has been proposed. For example, it has recently been shown that the addition of digestate helps to stabilize biodrying [9]. Although these methods are generally regarded as efficient, cost-effective and environmentally friendly, they are not always applicable. For example, composting of sewage sludge continues to decrease in Germany, since the resulting fertilizer can no longer meet the requirements of the German Fertilizer Ordinance (DüV) (e.g., heavy metals). If combined with energy and phosphorous recovery, incineration is a promising alternative to composting that can be combined with anaerobic digestion or biodrying.

In the project presented here, a municipal sewage treatment plant uses incineration to dispose of the fermentation residues from sewage sludge digestion. The sewage treatment plant that served as a test object in the present study was interested in reducing the amount of digestate and the associated disposal costs. They further wanted to increase the biogas yield from the anaerobic digestion of sewage sludge. To achieve these goals, the wastewater treatment plant from the present study was looking for a way to increase the degradation rate of the applied anaerobic digestion process. Anaerobic digestion is a process that allows the production of methane from biomass due to bacterial degradation [10]. Although the process is usually carried out under anaerobic conditions, there are a few sources that investigate the impact of oxic treatment on anaerobic digestion processes. There is evidence that oxygen in limited amounts can have a positive impact on the process. In 2011, a review article compiled evidence showing that the adverse effect of oxygen on anaerobic processes is less toxic than originally thought [11]. Some studies even reveal that oxygen can improve anaerobic digestion processes. To give here an example, applying 0.01–0.25 L O_2 $L_{feed}{}^{-1}$ d^{-1} to an up-flow microaerobic sludge blanket (UMSB) resulted in an increase in the hydrolysis of organic matter and enabled removal efficiencies for chemical oxygen demand (COD) of up to 85% [12].

Apart from applying oxygen directly to the anaerobic sludge, there is also the possibility of using oxygen for the pre-treatment of substrates. This possibility should first be distinguished from the process of composting. Oxygen is also used in composting to break down biomass, and the end product is a sanitized fertilizer [13]. In contrast to anaerobic digestion, however, composting does not allow the production of biogas for electricity generation. To increase the biogas yield, some researchers are experimenting with the possibility of adding small amounts of oxygen. These should increase the oxidation power to produce larger amounts of biogas. However, the oxygen must be dosed carefully to break down substrates better, but not to break them down completely. In this regard, a recent article addresses the impact of aerobic pre-treatment prior to anaerobic digestion. The authors found that aerobic pre-treatment improves the degradation of paper residues, probably due to better accessibility of lignin due to oxygen [14]. Another recent study showed that aeration of sewage sludge within anaerobic digesters can improve the anaerobic digestion process as well. They used 0.02 vvm (air volume per liquid volume per minute) and an aeration time of 40 h, which increased methane production by 221% [15].

The possibility of the targeted digestion of biomass by oxygen could be used to post-treat anaerobic sludge. In this regard, the so-called $PEGA_{KA}$-process can be highlighted, which was patented in 2015 by one of the authors of the present study [16]. $PEGA_{KA}$ is a German abbreviation for "PRO-Entec digestate treatment for sewage treatment plants". Aerobic post-treatment could make substrate fractions that remain undigested, accessible for further degradation. Although there are countless articles, which already describe manifold configurations in respect to denitrification/nitrification systems, there are very few articles about microaerophilic conditions in anaerobic digesters. Apart from articles on microaerophilic conditions, the authors did not find any article addressing the possibility of intermediate oxic treatment to reactivate anaerobically digested sewage sludge. In the $PEGA_{KA}$ process presented here, the oxic treatment refers to digested sludge that has already undergone denitrification/nitrification before its digestion. It has been shown before that in conventional wastewater treatment, up to 50% of the COD fraction is turned into biogas [17]. The remaining COD fraction remains inaccessible. To fall below this limit in the process presented here, the anaerobic sludge is passed through an aerobic process stage for a short intermediate oxic treatment. This makes the remaining sludge fraction accessible again. Some of the poorly degradable substrates become accessible because of the oxygen activation and can thus be turned into methane. From February 2019 to November 2022, the authors of this work connected a demonstrative sludge reactivation stage ($PEGA_{BB}$) to the main digester of a municipal wastewater treatment plant. Prior to this, the process was tested and certified during a 10-month trial in a semi-technical 1 m^3 prototype of the $PEGA_{BB}$ reactor (verified TÜV-Süd Industrie Service GmbH, München, Germany). The

sewage sludge digester of the respective reference plant yielded 67% more biogas and 36% less digestate. While this is a huge improvement, there is a lack of knowledge about the underlying microbiome. Therefore, this paper focuses on investigating the microbiome in a municipal $PEGA_{KA}$ system consisting of aerobic digestate reactivation stage ($PEGA_{BB}$) connected to a sewage sludge digester. Thus, conclusions were drawn on the increased nitrogen elimination and efficient substrate utilization.

2. Materials and Methods

2.1. $PEGA_{KA}$-Process and Sampling

The presented process, shown in Figure 1, is a modified version of the $PEGA_{KA}$ process, which is detailed in the German patent DE 10 2015 118 988 B4 [16]. The company Pro-Entec recently built its first prototype on a municipal scale with a population equivalent value of 48,043 inhabitants' average value between 2019 and 2021 (designed for 35,000 inhabitants). The system was implemented for a period of 46 months for demonstrational reasons. According to the plant operator, the system achieved a 55% increase in biogas yield with a 25% reduction in the sludge volume at the end of the process. The $PEGA_{KA}$ process involves the activation of already digested sludge followed by recirculation and re-digestion in the main digester. The main digester had a size of 1500 m^3. With a total organic load in volatile solids (VS) of Φ 2514.6 kg VS d^{-1}, the organic loading rate (OLR) amounts to 1.68 kg VS m^{-3} d^{-1}. The hydraulic retention time (HRT) was 41 d. According to the plant operator, the pH in the main digester was 7.68 on average. The activation occurred in an aerated reactor entitled $PEGA_{BB}$-reactor. An amount of Φ 23 m^3 d^{-1} of digestate was reactivated in the $PEGA_{BB}$ reactor daily. The $PEGA_{BB}$ reactor was implemented in a former sequencing batch reactor (SBR), which had a volume of 860 m^3. Although digestate reactivation with pure oxygen is described in the patent, ambient air was used in the demonstrator due to technical limitations. A 46 kW compressor (Aerzener Maschinenfabrik GmbH, Aerzen, Germany) was used for air injection (10 h/d). When the compressor is switched off, two 5 kW agitators are switched on to keep biological material in suspension. Residual water from the final mechanical sludge dewatering with a chamber filter press, shown in Figure 1, was treated in the reactor for the digestate reactivation. After the oxic reactivation, static and mechanical thickening occurred on a band press, and following this, the sludge was returned to the sewage sludge digester. The $PEGA_{BB}$-reactor was equipped with automated sensors for NH_4-N, NO_3-N, redox values, O_2 and TS (Hach Lange GmbH, Berlin, Germany). Measurements took place every 15 min from June 2020 until December 2022 (Figure 2C). Additionally, NH_4-N was measured occasionally before and after the $PEGA_{BB}$ stage using the Nanocolor photometer (Machery-Nagel, Düren, Germany). For the manual ammonia measurements, between 31 and 47 samples were taken from the anaerobic digester, filtrate at the chamber filter press and filtrate at the band press (Figure 2B). The process was realized with small modifications compared to the original patent. The reuse of a previous SBR reactor allowed only a batch process. On 23 August 2022, samples were taken from the main digester and as well as from the $PEGA_{BB}$ reactor to carry out taxonomic analyses using 16S rRNA gene amplicon sequencing. Three independent samples were taken from the main digester and the $PEGA_{BB}$ stage.

2.2. DNA Extraction and Quantification

Sludge samples were extracted as described recently in [18]. An amount of 200 µL of each sample was washed in 750 µL of PBS. The samples were then centrifuged, and the supernatant was discarded. This step was repeated until the supernatant was completely clear. Subsequently, DNA from all the pre-processed samples was extracted by using the DNEasy Power Soil Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions, but incubating at 65 °C after the addition of C1. Qubit x1 dsDNA HS Assay kit (Qubit 2.0 Fluorometer, Thermo Fisher, Waltham, MA, USA) was used for DNA quantification.

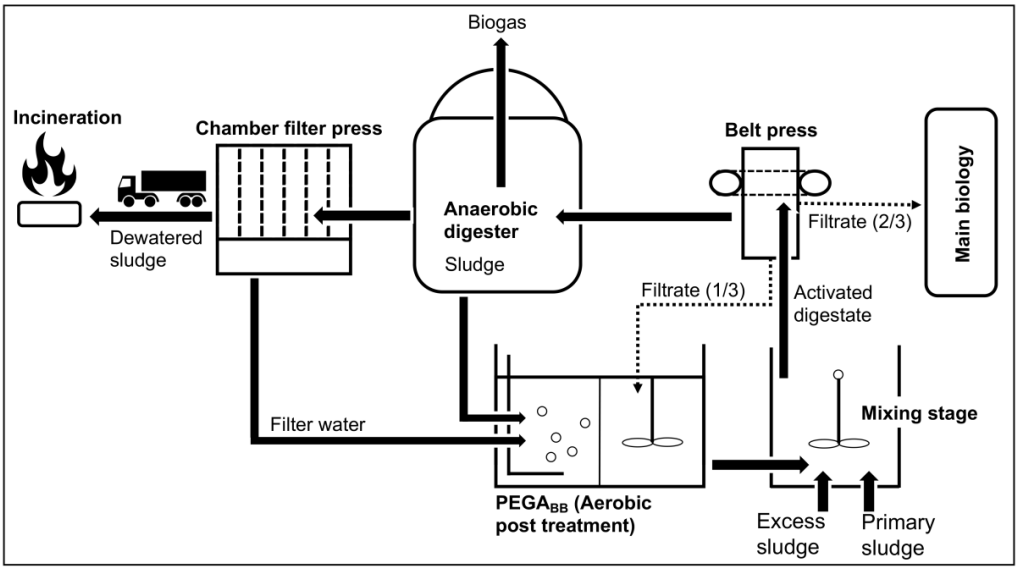

Figure 1. Schematic illustration of the modified PEKA$_{KA}$ process. Digestate from the digestion tower is reactivated in the PEGA$_{BB}$ stage using oxygen in the form of ambient air. After thickening on a belt filter, the activated digestate is treated a second time in the digestion tower. The biogas yield can thus be increased by 55% and the fermentation residue to be disposed of can be reduced by 25%.

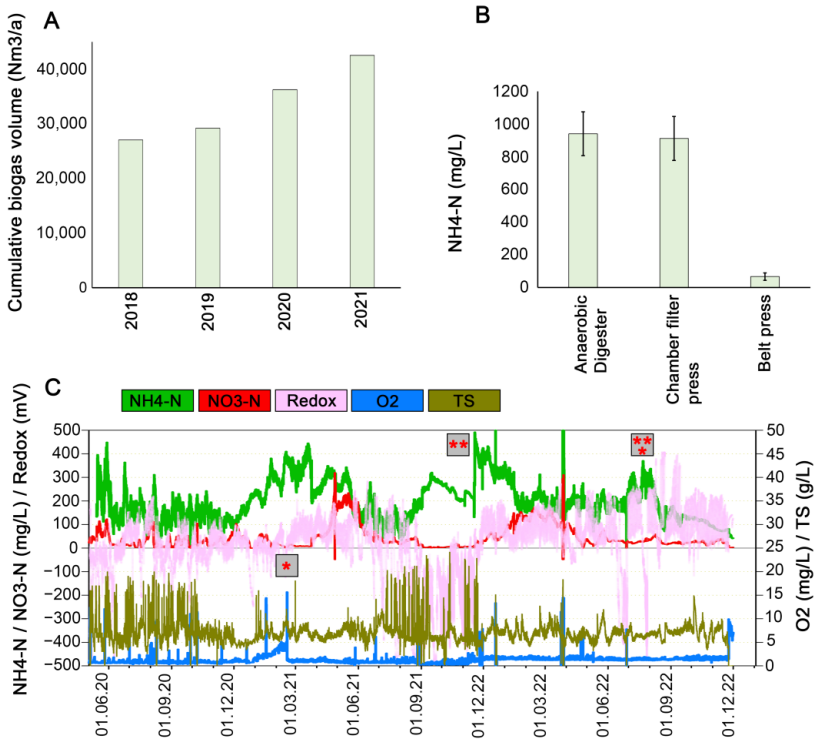

Figure 2. Process chemical parameters of the modified PEKA$_{KA}$ process. Biogas formation before (2018) and after the implementation (2019–2021) of the modified PEGA$_{KA}$ concept (**A**). NH_4-N was measured manually to compare the NH_4-N content before digestate reactivation in the main digesters and afterwards at the belt press (**B**). The PEGA$_{BB}$ stage was equipped with automated sensors for NH_4-N, NO_3-N, redox values, O_2 and TS, which performed measurements every 15 min. Some sensors were recalibrated during the experiment. This was the case for TS *, NH_4-N ** and Redox *** (**C**).

2.3. Amplification and Sequencing of the 16S rRNA Gene

The forward and reverse primers used to amplify the conserved regions V3 and V4 of the 16S rRNA gene were 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3′ and 5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C -3′, respectively. Library preparation was performed following the Illumina standard protocol. Amplicons were then sequenced with the Illumina MiSeq platform (2 × 300 pb). A complete description of the protocol followed for amplification and library preparation is provided by [19].

2.4. Bioinformatic and Statistical Analysis

Raw Illumina sequences were analyzed using Qiime2 (v. 2021.2.0) [20]. The quality of the reads was assessed with the Demux plugin, and the sequences were subsequently corrected, trimmed and clustered into amplicon sequence variants (ASVs) via Dada2 (q2-dada2). The taxonomy of each sequence variant was assigned by applying the classify-Sklearn module from the feature-classifier plugin. SILVA (v. 138) [21] was used as the reference database for the 16S rRNA assignment. It is worth highlighting that SILVA's taxonomic nomenclature was followed (i.e., Bacteroidota was used instead of Bacteroides). The Phyloseq package (v. 1.30.0) [22] and Vegan (v. 2.6.4) [23] were used for analyzing the data. All the α-diversity tests were carried out using ASVs, and a t-test was applied to analyze the existence of significant differences between the treated and untreated samples in terms of α-diversity. A PERMANOVA test was used to verify the existence of significant differences between bacterial composition of both groups. In addition, a DESeq2 test (v. 1.26.0) [24] was used to determine differences in the relative abundances of taxa between the treated and untreated groups. It must be noted that all the sequences assigned to chloroplasts, mitochondria or eukaryotic species were removed from the analysis.

3. Results and Discussion

3.1. Chemical Process Parameters of the PEGA$_{BB}$-Reactor

A municipal wastewater treatment plant was equipped with a new type of reactor for oxygen-initiated digestate reactivation (the $PEGA_{BB}$ reactor), as shown in Figure 1 and described in the Materials and Methods, Section 2. The $PEGA_{BB}$ reactor was implemented in 2019. The cumulative gas volume produced in the main digester increases stepwise (Figure 2A). In 2019, the gas volume increased from 270,096 $Nm^3\ a^{-1}$ to 291,867 $Nm^3\ a^{-1}$. One year later, it reached 362,233 $Nm^3\ a^{-1}$, and finally, in 2021, 425,044 $Nm^3\ a^{-1}$ were produced. Comparing the biogas volume between 2018 and 2021, an increase of 57.40 % has been observed. This tremendous increase in biogas productivity cannot be explained by an increase in population equivalent (pe), as the pe only increased about 4 % and the sewage treatment plant recorded no additional inflows from industries. Although the implementation of the $PEGA_{KA}$ concept began in February 2019, it took until September 2019 for the system to be fully operational. This explains why in 2019, only a small increase in gas production was observed.

The $PEGA_{BB}$ stage was equipped with automated sensors for NH_4-N, NO_3-N, redox values, O_2 and TS (Figure 2C). Despite air injection, the oxygen concentration was relatively low (1.2 mg L^{-1} on average), which shows intense O_2 consumption due to the involved microbes. Although 1.2 mg L^{-1} is very low, there are recent research articles showing that nitrification at such low oxygen levels is possible. For example, a recent study highlighted nitrification at oxygen levels of 0.5 mg L^{-1} [25]. The authors of this study highlight that such low oxygen levels reduce the operational costs and improve the carbon footprint. In order to be able to better understand the nitrification in the present process in detail, additional data on the nitrite content would be important. Therefore, subsequent studies are recommended, in which the nitrite content is also measured.

Total solids had an average of 6.7 g L^{-1}. There was a high fluctuation in the TS, reaching up to 24.2 g L^{-1}. The relatively low concentration of TS is explained due to the chamber filter press, which thickens the sludge, but only the residual water enters the $PEGA_{BB}$ reactor. The

solid fraction is transported to an incinerator. Most of the time, NO_3-N was in a range of <100 mg L^{-1}, and NH_4-N was cycling between 100 and 400 mg L^{-1}. NH_4-N was additionally verified by manual measurements. Measurements were taken regularly over the entire project period in the anaerobic digester, in the downstream chamber filter press and in the belt press. The values from the chamber filter and belt press allow a comparison of the ammonium values in the outlet of the digestion tower and the $PEGA_{BB}$ reactor. More than 30 values were measured for each of the three measuring points. The comparison of these values showed a NH_4-N removal of 92.70% on average (Figure 2B).

3.2. Taxonomic Profiles and Microbial Diversity

To assess the underlying microbiome, DNA samples were collected from the main digester and as well as from the $PEGA_{BB}$ stage. Applying 16S rRNA gene amplicon high-throughput sequencing, samples from both stages, the main digester and the $PEGA_{BB}$ stage, were compared (Figure 3; Supplementary Table S1). The main phyla detected in both the main digester and $PEGA_{BB}$ were the same, although with differences in their relative abundances. Proteobacteria, Bacteroidota and Patescibacteria were the most abundant phyla in all samples (Figure 3A). These phyla represent the main component of the microbiome of sewage sludge, and they are considered [26,27]. However, while Proteobacteria and Bacteroidota abundances were higher in the main digester, the presence of Patescibacteria was greater in oxygen-activated digestate. A previous study has already observed a high abundance of Proteobacteria and Bacteroidota phyla in samples of wastewater treatment [28]. To determine if these and other differences observed were statistically significant, differential abundance analyses at the phylum level were performed (DESeq2 test). For the activated digestate samples, these tests confirmed the major prevalence of Proteobacteria and Bacteroidota together with Actinobacteriota, Campilobacterota, Cloacimonadota and Marinimicrobia (SAR406 clade) (FDR-adjusted p-value < 0.05). In contrast, Chloroflexi, Verrucomicrobiota, Acidobacteriota, Desulfobacterota and Caldisericota, among others, were less abundant after the treatment.

The microbial profiles between the samples from the main digester and the $PEGA_{BB}$ reactor differed significantly (Figure 3B; Supplementary Table S2). However, a high degree of homogeneity was detected within each group, which was confirmed by means of replicate measurements. No genus exceeded 10% relative abundance in any of the samples, which goes hand in hand with the high microbial α-diversity detected (Figure 3C). In the main digester, an uncultured genus from the *Anaerolineaceae* family, and *Dojkabacteria, Arcobacter* and *Saccharimonadales* were the most prevalent genera. In contrast, *Chujaibacter*, *Alcanivorax, Ferruginibacter* and an uncultured genus from *Chitinophagaceae* showed higher abundances in the activated digestate. In total, 224 genera showed a significantly different abundance between the two types of samples (FDR-adjusted p-value > 0.05).

For instance, the abundance of *Chujaibacter* increased from the main digester to the oxygen-activated sludge from an average of 0.8% to 8.4%. This confirms recent results, which showed that *Chujaibacter* has a significant effect on the nitrogen cycle, especially in aerobic systems [29]. The abundance of *Alcanivorax* increased the most from the main digester to the oxygen-activated sludge, from an average of 0.06% to 8.3%. This genus was discovered to be an important player in the nitrification of sewage sludge under aerobic conditions [30]. *Ferruginibacter* increased from the main digester to the oxygen-activated stage from 0.4% to 7.1%. This genus is associated with aerobic conditions, denitrification and higher COD utilization [31]. Another genus from the same family, *Chitinophagaceae*, which could not be cultivated yet, was not found in the main digester but showed an abundance of 4.9% in the $PEGA_{BB}$ reactor. *Chitinophagaceae* can oxidize ammonium [32], and some representatives are even xenobiotic, which may contribute to reducing hazardous anthropogenic compounds in sludge. It has even been reported that *Chitinophagacaea* in co-culture with microalgae had a negative effect on the presence of waterborne pathogens [33].

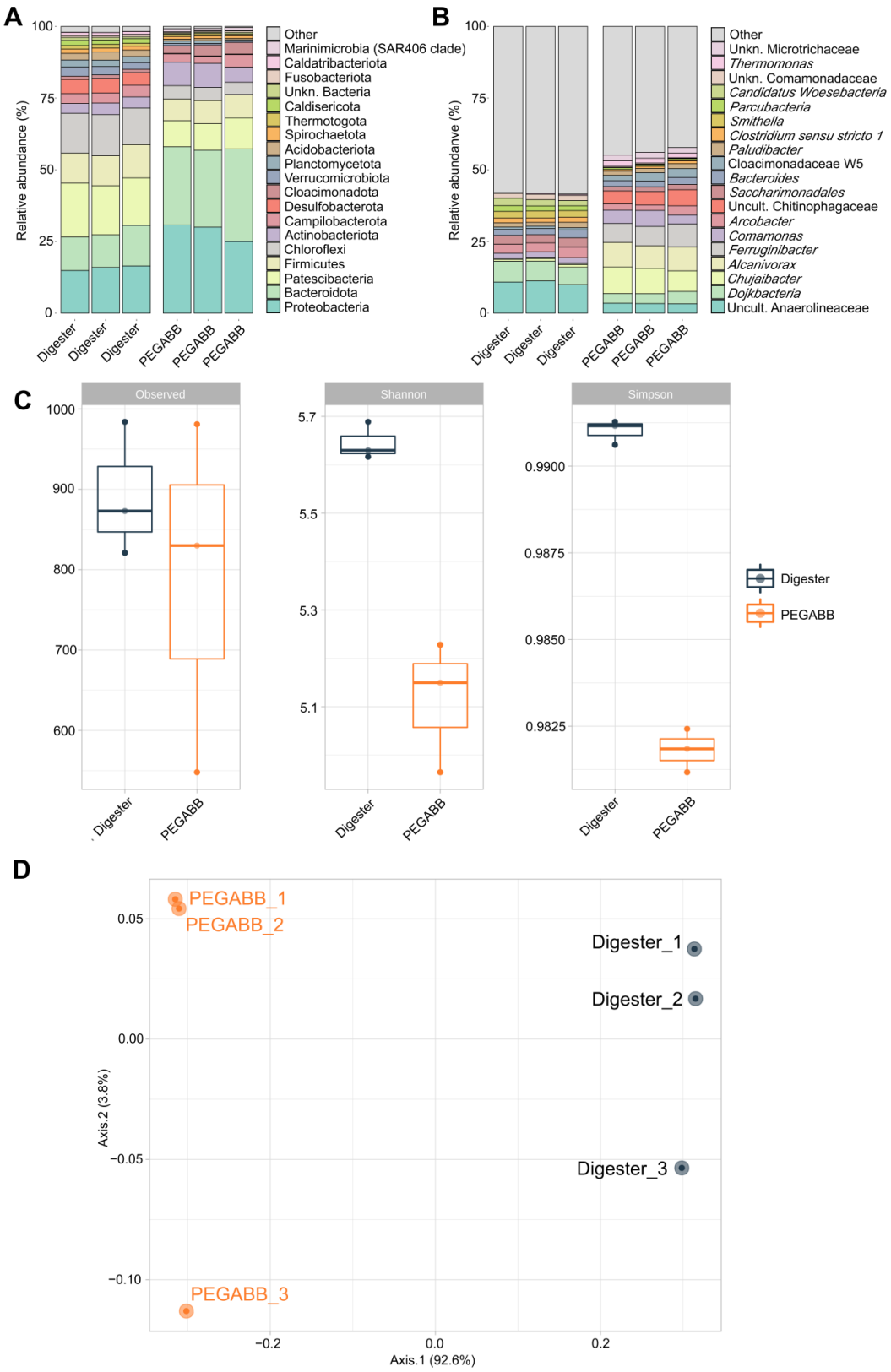

Figure 3. Comparison of taxonomic profiles found in the main digester and the $PEGA_{BB}$ stage. Taxonomic profiles were analyzed on the phylum (**A**) and genus (**B**) levels. The data from 16S rRNA gene amplicon sequences were also used to assess the alpha diversity (**C**). Beta diversity shown based on a principal coordinates analysis (PCoA) using the Bray–Curtis dissimilarity metric (**D**).

For that reason, the presence of pathogenic genera whose abundances were reduced in the treated sludge samples was examined in detail. Some of the best-known human pathogens which could be present in wastewater (such as *Escherichia, Shigella, Salmonella, Brucella, Staphylococcus* or *Legionella*) were virtually absent from all samples. However, other potential pathogens such as *Uruburuella*, *Laribacter* and *Pseudomonas* showed significantly lower abundances in the oxygen-activated samples. In addition, other potentially pathogenic genera showed a non-significant reduction of their relative abundances in the treated group. This is the case for *Arcobacter* and *Streptococcus*. All these genera can affect human health. Therefore, reducing their presence in the output of wastewater treatment is of great importance to prevent possible diseases. For example, *Uruburuella* is associated with respiratory infections [34] and *Laribacter* with infectious diarrhea [35]. As for *Pseudomonas*, not all species belonging to this genus are pathogenic. However, further sequence homology analysis of the major amplicon sequence variants (ASVs) that were less present in the treated samples indicated that the species was likely to be *P. aeruginosa*. This is an opportunistic pathogen that infects patients with previous disorders [36]. Furthermore, *Arcobacter* is an emerging food-borne zoonotic pathogen, which is very frequent in sewage [37] and can cause serious infections in humans and animals [38]. Finally, the species of *Streptococcus* found in this study's samples were *S. salivarius, S. parasuis* and an unassigned species closely related to *S. sanguinis, S. Sinensis* and *S. porcorum porcorum* in terms of sequence homology. All these species are potentially pathogenic to human health and were reduced due to the influence of oxygen [39].

Nevertheless, there were also a few potentially pathogenic genera which showed significantly higher abundances in treated samples. This was the case for *Mycobacterium*, *Prevotella* and *Actinomyces.* It was not possible to determine with certainty which species belonging to these genera were present in the analyzed samples. *M. minnesotense* was the closest species according to sequence homology. The most abundant *Prevotella* ASV was classified as *P. paludivivens*, with no associated risk for humans. Regarding *Actinomyces*, sequence homology analysis did not retrieve an accurate taxonomy at the species level, so it was not possible to check whether the microorganisms detected are potentially pathogenic or not. To further enhance the anti-pathogenic effects, supplements could be applied as well. For example, it has recently been shown that the addition of calcium oxide [40] or silver particles [41] favors sanitization in biological treatments.

The resulting taxonomic profiles were also used to assess the microbial α-diversity (Figure 3C). Both Shannon and Simpson indices were significantly reduced in the oxygen-activated samples (p-value < 0.05; t-test), while the number of ASVs observed (i.e., richness) did not show significant differences, as shown in Figure 3D (p-value > 0.05; t-test). These results show that oxic activation of the digestate reduced the bacterial diversity in the sludge samples. This is in line with expectations, as the microbiome of digested sewage sludge contains a large proportion of obligate anaerobic microorganisms, most of which might be affected negatively by oxic activation. Apart from analyzing the α-diversity, the β-diversity was also studied (Figure 3D). This analysis showed that the bacterial composition of the sludge samples was highly altered by the oxic activation of the digestate. As shown in the PCoA (Figure 3D), the samples were clearly grouped according to whether they had been activated or not (Axis.1). A PERMANOVA test confirmed that the activation of the digestate significantly affected the microbial profile of the samples in terms of β-diversity (p-value < 0.05).

3.3. Methanogenic Archaea

The resulting 16S rRNA gene amplicons were manually screened for methanogenic archaea. The genera *Methanosaeta (Methanothrix)*, *Methanospirillum* and *Methanobacterium* could be identified (Figure 4A). Overall, the number of methanogenic archaea involved was very small in both systems. While the ratio of methanogenic archaea in biogas plants is sometimes very small, high proportions of methanogenic archaea are often found in sewage sludge digesters from wastewater treatment [28]. In the present study, the proportion of

methanogenic archaea in the examined digester was <0.5% and fell to <0.1% in the PEGA-reactor. The results indicate that methanogenic archaea are inhibited by the PEGA method. Due to the low frequency in the digester, the question arises as to whether the oxygen that gets into the digester through the PEGA process has contributed to a reduction in the number of methanogens. It is well known that oxygen inhibits methanogenic archaea. Nevertheless, it was published in 2018 that several cases of increased oxygen resistance have now been observed. In the meantime, genes for antioxidants have also been found for methanogens [42]. These findings fit with the results presented here, since the anaerobic digester remained operational despite a slight reduction in methanogenic archaea and even showed increased biogas formation.

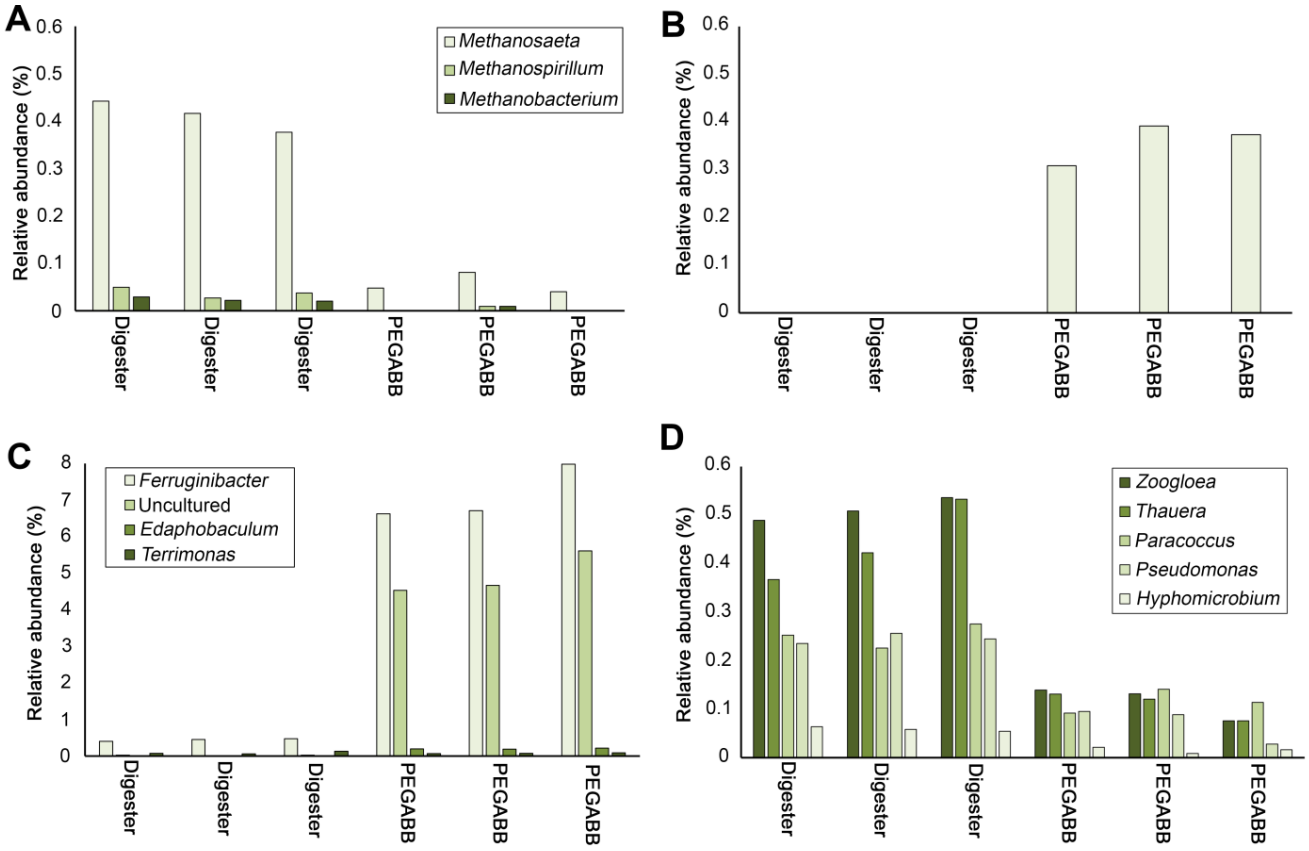

Figure 4. Relative proportion of methanogenic archaea (**A**), the relative proportion of the ammonium-oxidizing microorganisms *Candidatus nitrosoglobus* (**B**) and different genera belonging to the family *Chitinophagaceae* (**C**) as well as the abundance of denitrifying genera (**D**).

Interestingly, no AOBs or AOAs were found in the digestion tower without an oxygen activation. This shows that *Candidatus nitrosoglobus* growth is stimulated exclusively by the PEGA process. In fact, there is reflux from the PEGA process into the digester, which means that *Candidatus nitrosoglobus* is regularly returned to the digestion tower but cannot establish itself there. Thus, *Candidatus nitrosoglobus* is only active in the PEGA-reactor and not in the digester.

3.4. Ammonium Oxidation

The involved holders of the patent for the PEGA-reactor were concerned about whether the formation of nitrogen oxides might take place. Although the PEGA-reactor has already been implemented industrially, no chemical assessment of gaseous emissions has been performed so far. Therefore, indicators for nitric and nitrous emissions were searched for in the produced set of 16S rRNA gene amplicon sequences.

The basis for the release of nitrogen oxides is that ammonium is oxidized. Therefore, the search was initially focused on ammonium-oxidizing microorganisms. Known ammonium-oxidizing bacteria (AOBs) belong to the genera *Nitrosomonas*, *Nitrosovibrio*, *Nitrosoglobus*, *Nitrosospira*, *Nitrosococcus* [43] and as well to the family of *Chitinophagaceae* [32].

In addition to the AOBs, there are also ammonium-oxidizing archaea (AOAs). Known genera are *Candidatus Crenarchaeum*, *Candidatus Nitrosotalea*, *Candidatus Nitrosocosmicus*, *Nitrospheara*, *Candidatus Nitrosotenius*, *Candidatus Nitrosopelagicus*, *Nitrosopamilus*, *Nitrosarchaeum*, *Candidatus Nitrosocaldus*, *Nitrosomarinus*, *Candidatus Nitrosococsmicus*, and *Candidatus Nitrosocosmicus* [44–46]. All genera were searched for in the generated DNA sequences. Of all the AOBs and AOAs mentioned, multipe genera were found: *Candidatus Nitrosoglobus* (see Figure 4B) and genera belonging to the family of Chitinophagaceae (see Figure 4C).

3.5. Anammox Bacteria

The presence of *Candidatus nitrosoglobus* indicates ammonium oxidation, which could yield nitrite. The nitrite (NO_2^-) formed during this process might be used by anammox bacteria for the anaerobic oxidation of ammonium (NH_4^+). To investigate this possibility, the generated 16S rRNA gene amplicon sequences were specifically searched for anammox bacteria. A recent review article gives an overview of the known anammox genera [47]. The following genera are known: *Candidatus anammoxoglobus*, *Candidatus brocadia*, *Candidatus jettenia*, *Candidatus kuenenia*, *Candidatus scalindua* and *Candidatus anammoximicrobium*. Some of the genera mentioned above are further divided into several species. However, none of the genera listed could be found in the generated 16S rRNA gene amplicons. The results, therefore, indicate that despite the introduction of oxygen, no known anammox bacteria were involved.

3.6. Formation of NO and N_2O

Nitric oxide (NO) and nitrous oxide (N_2O) are potent greenhouse gases. Therefore, it was of particular interest to determine whether emissions of these gases need to be considered when applying the $PEGA_{KA}$ process. It is very likely that nitric oxide and nitrous oxide are produced, although the present results provide no insight into the extent of it. The regular change between anaerobic (digester) and aerobic ($PEGA_{BB}$) treatments will likely allow the formation of NO and/or N_2O. In this regard, a work from 2018 highlights that environments which fluctuate regularly between aerobic and anaerobic conditions tend to produce N_2O [48]. Nevertheless, the possibility of NO and N_2O release based on the available sequences should be discussed in more detail at this point.

Multiple microbial groups can be involved in the release of NO or N_2O. One of them is the earlier-mentioned group of AOBs [49]. An important intermediate produced by this group is hydroxylamine (NH_2OH). NO and N_2O are formed by oxidation of NH_2OH due to chemical reactions with oxygen or NO or by biochemical oxidation due to AOBs (nitrifier nitrification) [32,50]. Apart from this, it is also possible that heterotrophic nitrifiers [32], as well as autotrophic or heterotrophic denitrifiers [50], are involved. As NH_2OH is a common intermediate during ammonia oxidation [51], it can already be assumed that it is produced in the $PEGA_{BB}$ reactor, but further investigations should validate this assumption. N_2O is likely released at least in minor quantities, since [48] describe that it can be released from NH_2OH via chemical conversion due to a reaction with NO_2^- or O_2. As one AOM genus (i.e., *Candidatus nitrosoglobus*) was found in the present set of 16S rRNA gene amplicon sequences, it might also be that this microorganism contributes to the release of NO and N_2O.

To analyze whether nitrifier nitrification took place, the produced sequences were further searched for autotrophic nitrite-oxidizing bacteria (NOBs). Thus far, there are seven known genera belonging to NOBs. These are *Nitrobacter*, *'Candidatus nitrotoga'*, *Nitrococcus*, *Nitrospira*, *'Nitrolancetus'*, *Nitrospina* and *'Candidatus nitromaritima'* (*Nitrospinae*) [52], and none of them were found in the produced set of sequences. This indicates that further processing of NO_2^- is not performed by autotrophic nitrifiers, but by heterotrophic nitrification. At this point, the analysis becomes complicated just based on 16S rRNA sequences as those groups are rather widespread and insufficiently investigated. For example, [36] high-

lighted that the diversity of heterotrophic nitrifiers in different soil types is not well known. Nevertheless, heterotrophic denitrifiers might be involved in the release of NO/N_2O as well. A 2014 published study came to the conclusion that denitrification capability is widespread, not only among bacteria but also among archaea. The authors described further that although 16S rRNA gene-based studies indicated a high diversity in denitrifying bioreactors, bacterial isolates were mostly related to *Hyphomicrobium*, *Paracoccus*, *Pseudomonas* and *Comomonas* [53]. It must be considered that culturing might not reflect the microbes that really dominate the denitrifying communities within water treatment plants. It was found that based on DNA, *Azoarcus* and *Zoogloea* are found abundantly in water treatment plants, as well as *Thauera*, *Methylophaga*, *Accumulibacter* and *Acidovorax* [53]. Searching for these microorganisms, five of them were detected in the present set of samples (Figure 4D). *Zoogloea* and *Thauera* were the most abundant (>0.5% of sequences in the digester), followed by *Parococcus* and *Pseudomonas* (between 0.2% and 0.3% of the sequences in the digester). The least abundant genus was *Hyphomicrobium* (less than 0.1% of the sequences). Interestingly, all genera were reduced tremendously in the $PEGA_{BB}$ reactor, with less than 0.2% in all three samples. However, this reduction should be taken with caution, as the abundance of these genera was all less than 1%.

Even if no measurements of NO or N_2O took place, the generated taxonomic profiles point out that the release of these gases is conceivable. The analytical proof as well as the actual extent should be examined in further studies. The first research articles are already reporting on the possible effects of an N_2O tax [54]. Any NO or N_2O emissions are therefore of considerable importance for the $PEGA_{KA}$ system and for sewage treatment plants in general.

3.7. Profitability of the $PEGA_{KA}$ System

It needs to be highlighted that the aeration and stirring in the additional $PEGA_{BB}$ reactor stage are related to a high energy consumption. In-depth details on the economic concept and technical details were recently presented on the German water and wastewater portal, "gwf" [55]. Nevertheless, at this point it should be briefly explained why the planned oxic sludge activation is economically viable. The system is aerated with air by an AERZENER blower with an output of 46 kW. This covers an oxygen demand of 70.60 kg O_2 d^{-1}. With 12 h d^{-1} of operation (according to the operations manager), this corresponds to 552 kWh d^{-1} or 201,480 kWh a^{-1}. There are also two agitators, each with an output of 5.5 kW, which are used at least in the non-aerated phases. The agitators cause an additional energy consumption of 132 kWh d^{-1} or 48,180 kWh a^{-1}. In total, 249,660 kWh a^{-1} must be considered. With the last known electricity costs of EUR 0.211 per kWh, this amounts to EUR 52,678.26 a^{-1}. When using pure oxygen instead of air, it would be possible to reduce the cost of the oxygen supply tremendously. With loading costs of 0.15–0.20 EUR kg^{-1} and an annual requirement of 25.769 kg a^{-1}, this results in costs of just EUR 3865.4–EUR 5147.8 per year. Charging with ultrapure oxygen would therefore be far cheaper than using oxygen from the air. However, another field experiment with ultrapure oxygen is still pending. Based on statements by the plant operator, the sludge volume was reduced by about 25% due to better digestion. The annual residual sludge volume decreased by about 500 t a^{-1}. The plant operator calculates EUR 108.09 for the disposal of 1 t of residual sewage sludge. With 500 t less residual sludge, this amounts to savings of EUR 54,045. This shows that the sludge reduction alone justifies the monetary outlay for operating the $PEGA_{BB}$ reactor. Further savings result from increased energy yields in the coupled CHP plant. According to the plant operator, the methane content is approximately 60%. With a biogas yield of 425,044 Nm^3 a^{-1}, this relates to 255,026 Nm^3 a^{-1} of CH_4. With 9.97 kWh per m^3 of methane and a CHP efficiency of η_{el} = 32% and η_{th} = 55%, this corresponds to an increased energy yield of 813,635 kWh_{el} a^{-1} and 1398,435 kWh_{th} a^{-1}. With EUR 0.211 $kWh_{el}{}^{-1}$, this yields another EUR 171,677. At this point, it must be emphasized that the full energetic potential of the biogas produced was not exhausted, since no additional CHP was installed. Only 436.26 kWh_{el} could be produced with the existing CHP. When implementing the $PEGA_{KA}$ concept, an expanded use of gas must therefore be considered. It should be emphasized that the assumed electricity revenues

reflect the purchase price of the sewage treatment plants in 2021. The calculation is therefore only valid under the premise that the energy is used to cover the energy needs of the sewage treatment plant; thus, it buys a reduced amount of energy. The thermal energy generated was not considered in monetary terms but can also be viewed as a positive side effect. Another positive side effect is the improved nitrogen removal. Water from the sludge dewatering is returned to the main biology of the sewage treatment plant (denitrification/nitrification). As a result, the $PEGA_{BB}$ reactor significantly reduces the nitrogen content, which in turn relieves the main biology and increases the capacity of the wastewater treatment plant. Overall, it can be assumed that the oxic sludge activation does not result in any financial disadvantages.

4. Conclusions

A new, already patented process ($PEGA_{KA}$) increases the anaerobic digestion efficiency of sewage sludge due to the reactivation of fermentation residues from the digesters of wastewater treatment plants. Additionally, 16S rRNA gene amplicon high-throughput sequencing revealed that the number of methane-forming archaea was significantly affected, and seemingly reduced in the methane stage. Ammonium oxidation was indicated due to the presence of multiple genera belonging to the family Chitinophagaceae (up to 13.8% of the DNA sequences) as well as a minor amount of the genus *Candidatus nitrosoglobus* (<0.3% of the DNA sequences). Several denitrifying genera were found: *Zooglea*, *Thauera*, *Paracoccus*, *Pseudomonas and Hyphomicrobium*. Denitrifying microorganisms and *Candidatus nitrosoglobus* might be engaged in the release of nitric and nitrous oxide, which is supported by recent literature. β-diversity analysis showed that the treatment stage caused large changes in the bacterial and archaeal communities of the sludges. Finally, the $PEGA_{KA}$ process reduces the number of potential pathogens, which goes hand in hand with a reduction in biodiversity. Overall, the $PEGA_{KA}$ system has proven to be a promising system that increases the biogas yield, reduces the residual sludge and improves the nitrogen removal.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9050471/s1. Table S1: Results from DESeq2 test at the phylum level. Table S2: Results from DESeq2 test at the phylum level.

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