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Communication

The Development of Anammox and *Chloroflexi* Bacteria during Composting of Sewage Sludge

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Abstract: The C/N ratio is an extremely important parameter in the composting process, which is directly responsible for the growth of microorganisms. A low C/N ratio contributes to higher emissions of greenhouse gases and odorous substances, such as ammonia (NH_3), which is formed by nitrogen mineralization. Due to the highly toxic effects of ammonia, it is a particularly unwanted by-product that can disrupt the composting process because it poisons microorganisms and cause environmental issues. The activity of anammox bacteria, so far analyzed only in wastewater treatment processes, is a particularly efficient method of nitrogen removal, having an advantage over the conventional methods used previously. Our study proofs the presence of anammox bacteria during composting, what gives an opportunity to improve the process and reduce its impact on atmospheric pollution. Despite the aerobic nature of this process, the composted mass of waste presents conditions conducive to the development of these ammonia oxidizing bacteria, as well as other strains of microorganisms cooperating with them. This makes it possible to compost at a low C/N ratio; in addition, there is no need for additional energy supply through aeration, as the processes carried out by anammox bacteria do not require oxygen.

Keywords: anammox bacteria, composting, sewage sludge, low C/N ratio, nitrogen balance, ammonia emissions

1. Introduction

Composting is a microbiological process that involves the biochemical conversion of organic matter into humic substances that can be used in agriculture as soil fertilizers [1]. This complex physical and chemical process is characterized by a dynamic course. The most important parameters that affect the process are temperature, pH, presence of microorganisms, aeration, and C/N ratio [2]. In the composting process, there is a strong relationship between these; at the same time very often the biochemical processes that occur in the compost pile change from the ambient conditions, the composition of the composted material and the microbial community inside [3].

The C/N ratio is an extremely important parameter in the composting process, which is directly responsible for the growth of microorganisms. Basic knowledge about composting indicates that the optimum C/N value for composting material containing organic compounds susceptible to biological oxidation is 20÷35 [4–6]. A low C/N ratio contributes to higher emissions of greenhouse gases and odorous substances, such as ammonia (NH_3), which is formed by nitrogen mineralization. Due to the highly toxic effects of ammonia, it is a particularly unwanted by-product that can disrupt the composting process because it poisons microorganisms and cause environmental issues [2,7].

The essence of biological decomposition involves a sequence of complex biochemical reactions in the presence of catalysts (enzymes), resulting in the partial conversion of organic compounds into so-called humus during the humification process, and partial

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oxidation to mineral compounds (such as CO_2, H_2O, NH_3, NO_x) in the mineralization process. The final effect of humification and mineralization makes the organic matter decomposition method applicable in the transformation of municipal waste containing high amounts of compounds susceptible to biological decomposition [8].

Enzymes produced by bacteria metabolism are crucial in the process of nitrogen transformations during composting. These transformations include several processes such as ammonification, nitrification, anaerobic ammonia oxidation and denitrification [9]. The direction and sequence of nitrogen transformation during composting can be presented in the following way:

$$NH_3(gas) \leftrightarrow NH_3\,(aq) \leftrightarrow NH_4^+ \leftarrow \text{organic nitrogen} \rightarrow NH_4^+ \rightarrow NO_2^- \leftrightarrow NO_3^-$$

Those process are fundamental in the global nitrogen cycle and must be sustained in engineered and natural environments, including those with extreme conditions. Currently, ammonification, nitrification and denitrification are the three main processes considered for measures to prevent N loss in composting, but anaerobic ammonia oxidation (anammox) has recently been recognized as an alternative microbial metabolic pathway of denitrification involved in N_2 emissions [10][10]; therefore, it is seen as the most important way to reduce N loss during low C/N composting.

In composting process high temperature (~70°C) plays crucial role in reduction of pathogens [2], but such thermal conditions can be harmful for anammox bacteria [10]. In this part of composting process *Chloroflexi* as putative nitrite-oxidizing bacteria plays an important role in nitrification (nitrite oxidation) [11].

There are many examples in scientific literature describing composting of mixtures of waste within broad scope of C/N=15÷28.9 ratio [8,12–14] but none of it explains the reason of providing the process that promote the risk of environmental harmful emission and loss of nitrogen in final product. Additionally, many studies have analyzed the composition and dynamics of nitrifier and denitrifier communities in compost but only a few confirm the presence of anammox [15,16] or *Chloroflexi* bacteria [11,17,18]. However, their relevance and specific activity conditions in composting haven't been well explored so far. Therefore, the aim of this article was to evaluate the presence of microbial community during composting of the substrate with low C/N ratio. The particular emphasis was given to anammox bacteria and environmental conditions that affect their role in nitrogen balance during the process.

2. Materials and Methods

2.1 Material characteristic and experiment configuration

Field research was performed at the site of wastewater treatment plant in Goleniów (West Pomeranian Voivodeship, Poland) producing ca. 5500 Mg of mechanically dehydrated sewage sludge. Composting proceeds in roofed piles approximately 70 m long, with dimensions of trapezoid transversal cross-section being 3 m bottom base width and 1.5 m height. The piles are mechanically overturned; during the first three weeks of composting twice a week, and, in subsequent weeks, once per week. Composting takes 4–5 months depending on the external conditions. After this period, the compost is used for agricultural purposes [1].

Samples were collected in March 2022 from 3 different composting piles, during different stage of composting: 1st week of composting (sample A), after 2 weeks of composting (sample B) and after 4th week of composting (sample C) (Fig. 1). In each samples basic analysis has been done pH, moisture content (MC), volatile solids (VS), elemental composition (C, H, N content), C/N ratio, and microbial community analysis by PCR analysis. During the composting process the changes of temperature in all piles were monitored.

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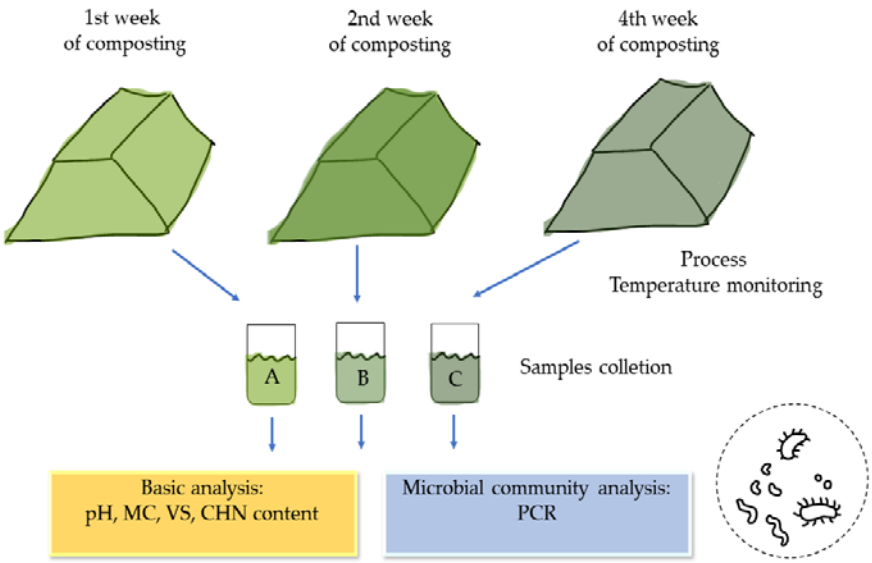

Figure 1. Experimental set-up of experiment

2.2 Methods

2.2.1 Determination of physicochemical properties of compost samples

Each sample (A, B, C) of compost was characterized by moisture content (MC) after sample drying at 105°C to a constant mass, volatile solids (VC) in 550°C in muffle furnace. Elemental composition (C, H, N), according to PN-EN ISO 11885:2009, spectrometer ICP-AES iCAP 7400 Thermo Scientific.

2.2.2 Microbiology community analysis

Fresh composting samples were collected after manual turning of composting materials to track dynamics of microbial community structures. Briefly, this analytical method involved in DNA extraction using GeneMatrix Environmental DNA/RNA Extraction kit (Eurx, Gdańsk, Poland), amplification of V3 – V4 hypervariable region of bacterial 16S rRNA gene with primer pairs of:

- 16S_V3-F 357F: (5-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG- CCTAC-GGGNGGCWGCAG -3) and
- 16S_V4-R785R: (5-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG- GAC-TACHVGGGTATCTAATCC-3)

by polymerase chain reaction (PCR), and high-throughput sequencing on the Kapa HiFi PCR Mix (Roche) [19]. The extracted DNA was processed to 1% agarose gel electrophoresis using a spectrophotometer (Qubit 3.0 (Thermo Scientific) to evaluate the integrity, purity, and concentration.

The reaction was carried out in 25 µl volumes containing 2.5 µl (5 ng/µl) genomic DNA, 5 µl (1 µM) of each primer, and 12.5 µl of 2× KAPA HiFi PCR Mix (Roche). PCR reaction mix containing 2.5 µl distilled water instead of genomic DNA was used as a negative control. Amplification was performed using the following program: initial denaturation at 95 °C for 2 min, followed by 25 cycles of 95 °C for 15 s, 55 °C for 10 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min before cooling to 10 °C. PCR products (~450 bp) were purified with Agencourt AMPure XP beads (Beckman Coulter genomics) to remove free primers and primer dimer species.

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Next generation sequencing was carried out using Illumina MiSeq platform utilizing using MiSeq Reagent kit v3 (600 cycles). The sequences with ≥ 97% similarity were all gathered into operational taxonomic units (OTUs) by usearch software (V10). Alpha diversity of bacterial community (i.e., Observed species, Chao1 and Shannon index) and Beta diversity was calculated with QIIME2. After quality filtering, sequences with ≥ 97% similarity were assigned to the same OTU to represent a species, which were annotated for taxonomic information (set the confidence threshold to default to ≥ 0.5) to get the OTU taxonomy synthesis information table for the final analysis using silva (https://www.arb-silva.de/) database [20] .

The VOS viewer software was used to create a network map among the syntropic bacteria from the study. The relative abundance of each bacterium served as basis for the occurrence and link in the map. Tab delimited file using notepad was used to prepare the code for all bacteria including those that of relatively low abundance serving as both source and type for bibliographic data of the Web of Science. Further, the type of analysis employed was based on the cooccurrence of bacteria which the software read as coauthors. Full counting with 1 as the threshold for the minimum occurrence of each bacterium was more appropriate in the generation of the network map. The circle represents each bacterium and the size indicate the occurrence which is based on the relative abundance. Whereas curved line was chosen to indicate the link between the bacteria and the relative size represents links. Cluster coloring was used to distinguish group of bacteria that are most abundant, slightly abundant and from those that are not abundant.

3. Results and discussion

3.1. Compost characteristics and temperature changes during process

The composting samples used is these experiments had 8,93-8,99 of pH, 63,1-66,7% of MC, 77,4-84,0% of TS and are characterized by 34–44%, 4.8–6.3, 1.1% of C, H, N, respectively (by dry mass base) (Table 1). Such results are typical for composting process [3]. The addition of wood chips extremely changed the C/N ratio of sewage sludge from 5-10 [1,21] to 36-44. Use of woodchips helps improving the C/N ratio, but also gives the structure necessary in composting for to provide an air to the pile. However, the C and N from wood chips does not contribute to the composting process due to low biodegradability. Therefore, the realistic C/N ratio should be considered as for raw sewage sludge ~5 [1]. But even in the system with a good aeration and structure, there are a still possibilities of exiting a place with a low oxygen and high temperature place – hot spots and otherwise a place with a low temperature [22]. Such places are an ideal environment for growing anammox bacteria (cold spot [16]) or *Chloroflexi* (hot spots [18]).

During the process internal temperature has been measured (Fig. 2). The information about temperature during the sampling where highlighted using the red line. It was observed that in each place thermophilic temperatures were dominant (>50 °C).

Table 1. Properties of composting samples

Sample	Moisture content, %	Volatile solids, % d. m.	pH	C, % d.m.	H, % d.m.	N, % d.m.	C/N
A	66.3	78.7±3.3	8.93	34±7	4.8±1.0	1.1±0.2	36.3
B	63.1	77.4±1.8	8.98	40±8	5.8±1.2	1.1±0.2	43.6
C	66.7	84.0±1.6	8.99	44±9	6.3±1.3	1.2±0.2	42.7
Sewage sludge*	-	-	-	-	-	-	5

* According to [1], d.m. – dry mass

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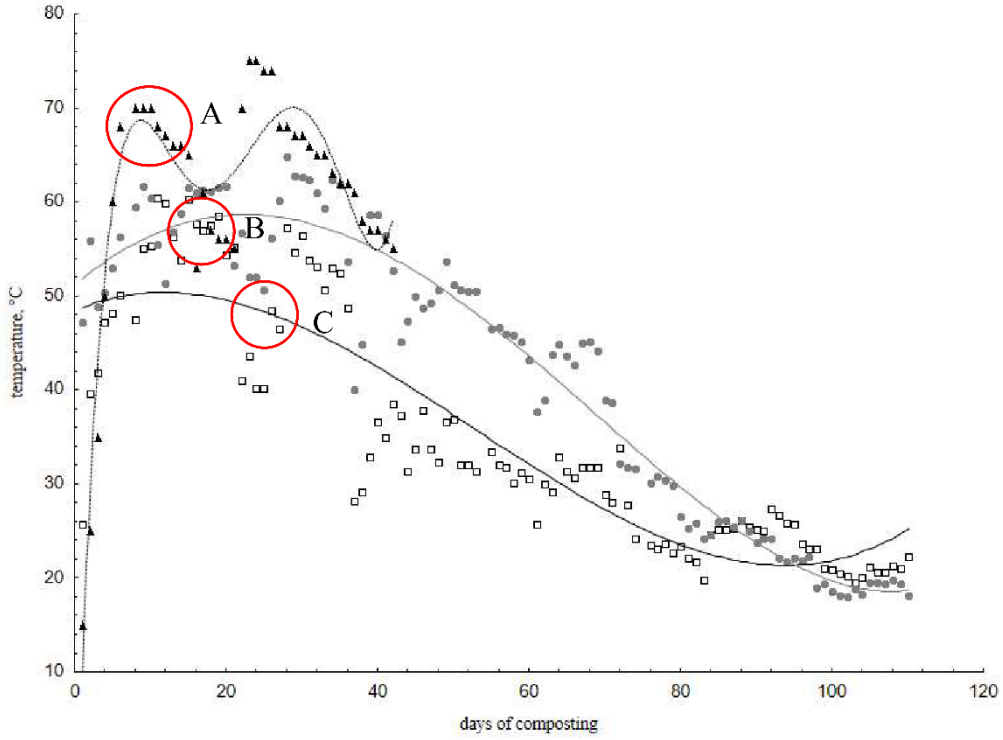

Figure 2. Temperature during the composting: 1st week of composting (sample A), after 2nd weeks of composting (sample B) and after 4th week of composting (sample C)

3.2. Community of bacteria structure changes

Since the nitrogen transformation process in composting is reported as a complex one, including several pathways like ammonification, nitrification, denitrification and anaerobic ammonia oxidation, it is crucial to get to know and describe the mechanisms of the last process, conducted by anammox bacteria [1,2]. Anammox bacteria has been found in different ecosystems, including stream sediments, wetlands and groundwater [3–5] but there's only a few sources describing the potential participation of these microorganisms during aerobic waste treatment process. The lack of research in this area makes it impossible to determine a quantitative link between functional gene groups and nitrogen transformation, and consequently the issue of this compound changes during composting remains an unexplored gap in the literature [2]. When analyzing the presence of anammox bacteria in the composting process, it is therefore necessary to refer to the wastewater treatment and anaerobic processes for which research is carried out more frequently and in a broader scope.

Few sources investigating the presence of anammox bacteria in the composted mass of waste show that their activity is characteristic of the thermophilic phase of the process [6]. The reports of Byrne et. al and Rysgaard et. al [7,8] agree with this, according to which these bacteria are characterized by high resistance to functioning in extreme environments where the temperature exceeds 85°C or is lower than -2°C. Their presence was recorded for instance in the mid-Atlantic Ridge vent fields and hot springs in California and Nevada, where thermal conditions ranged from 52 to 80°C [7,9]. This is in line with the results presented in this paper; the temperature of the composting process during the collection of each sample was in the thermophilic range (~50-70°C, Fig. 2). Greater diversity of anammox bacterial groups under these conditions Wang et. al. [6] connected with the availability of nitrogen compounds. According to the authors, the precondition for the presence of these microorganisms is the coexistence of ammonium and nitrite nitrogen under anaerobic conditions; the concentration of the former peaks precisely during the thermophilic

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phase of composting. In turn, the source of nitrite for these microorganisms is the nitrification process, the activity of which was recorded at temperatures close to 70°C [10]. Additionally, due to the progressive decomposition of simple organic matter, which in turn results in a rapid increase in temperature and oxygen depletion, anaerobic areas may arise in this phase of the composting process, favoring the growth of anammox bacteria.

However, it was reported that in anammox reactors, in addition to typical anammox bacteria, there are microorganisms that interact with each other through enzymatic processes, creating an environment conducive to anaerobic ammonia oxidation processes. Figure 3 prepared in VOSviewer software shows the syntropic relationship, through the link lines, among the microorganisms reported in the research at phylum level. The size of the circle represents the relative abundance of each bacterium. Those that are connected through the lines are considered syntrophic bacteria as they were relatively abundant and enriched. Other bacteria, not linked in the map, were only detected at the end of the decomposition process with relative abundance lower than 1.0% (*Acidobacteria*, *Armatimonadota*, *Cyanobacteria*, *Thermogota*, and *wps-2*) and from this reason they are not considered as a part of syntropy of the community.

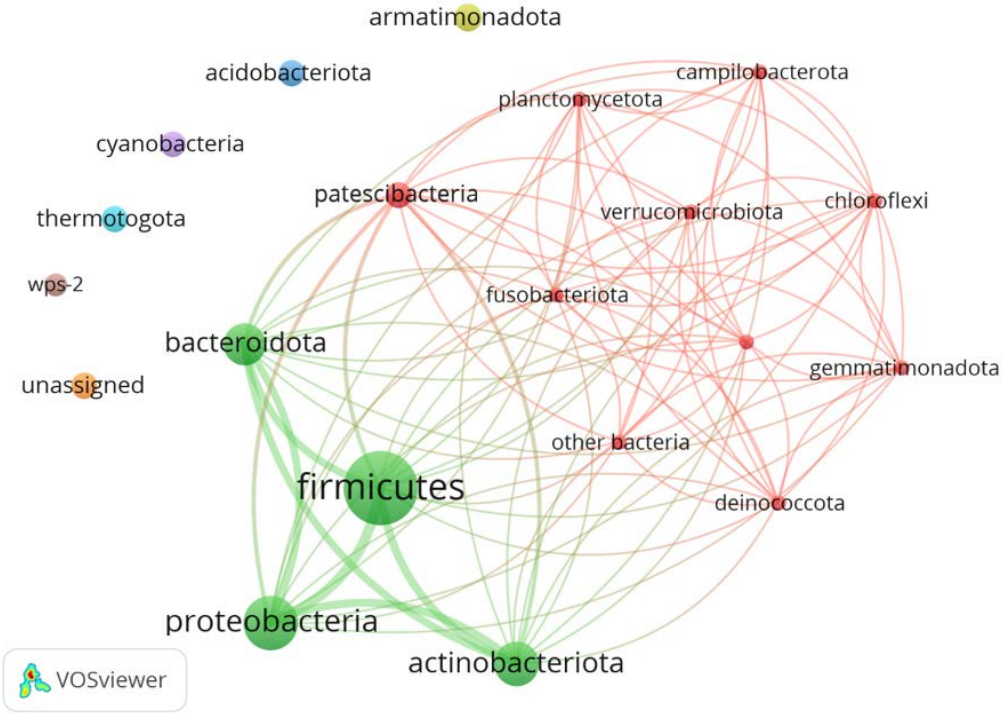

Figure 3. Bacterial community at phylum level showing the syntropic relationship represented by the link lines and the relative abundance through the size of the circle

In the early stage of the composting process (sample A), *Firmicutes* was the most abundant constituting around 48% of the total population followed by *Proteobacteria* (16.8%), *Actinobacteria* (14%) and *Bacteroidota* (12%, Fig. 4). In the later stage of decomposition (sample B), *Chloroflexi* showed the highest percentage increase from 0% to 1.1% (2068% increase) followed by *Deinococcota* from 0.8 to 11.3% (1352.7% increase) and *Myxococcota* from 0.1 to 0.8% (603%). Firmicutes and Proteobacteria were slightly suppressed toward the end of the decomposition (sample C). The presence of these groups is also confirmed by other authors; in each of the analyzed sludge samples by Chen et al. the microorganisms discovered came from the major phyla of *Proteobacteria*, *Chloroflexi*, *Planctomycetes*, and *Bacteroidetes* [11]. In addition, the authors also noted the increase in *Chloroflexi* community with the duration of the process that was observed here; they explained this situation by the fact that the development of these group is based on the use of cellular compounds derived from already dead microorganisms and their metabolites [12].

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Although the ecophysiology of these bacteria has not been understood so far, several studies have been carried out on the role of this group of microorganisms in anammox reactors. In addition to using organic compounds from lysed cells, they take part in the granulation of the sludge during its processing, and are also a carrier that enables the transfer and development of other microorganisms [13,14]. So far, it has been reported that the *Anaerolineae* and *Caldilineae* classes were distinguished from among the *Chloroflexi* present in anammox reactors [15–17], of which representatives of the former were also observed in the studies described here (supplementary material Table S1). A similar role to *Chloroflexi* regarding the granulation of the sludge was also discovered for *Bacteroidota*, which in the studies described here were the fourth most numerous groups of microorganisms in the composting process. It was investigated that after sticking to the outer layer of sludge particles, they form spiderweb-like structures, thus building granules [14]. However, the role of these microorganisms in anammox reactors has not been fully explored; although the studies conducted so far have shown that they possess genes responsible for nitrogen binding, thus they act in the nitrogen cycle, and may even be able to bind N_2 [18]. However, it is worth emphasizing that they can also have a toxic effect on anammox bacteria due to their phosphate removal; additionally, they compete with each other in terms of denitrification of nitrite [19,20]. Therefore, it is possible that anammox bacteria were not present in the analyzed compost samples due to earlier elimination by the *Bacteroidota* noted here. An additional argument here is the fact that they can work in both aerobic and anaerobic conditions, thus gaining an advantage in the process of aerobic waste treatment [21].

It is also worth mentioning that the found so far genera of anammox bacteria that have been described in the literature belong to *Planctomycetes*. In this study, the presence of five representatives of this phylum was noted (orders *Pirellulales*, *Isosphaerales*, *Tepidisphaerales*, *Planctomycetales*, supplementary material Table S1). Although the order *Brocadiales*, into which the currently known anammox bacteria are classified, was not discovered here, there is an indication that such bacteria could be present at an earlier stage of the process, and then after the composted mass reached unfavorable conditions (temperature exceeding the optimal value of 40°C) they were lysed; related microorganisms from the same phylum could appear in their place. The pH of composted waste may also be included in the conditions unfavorable for the further development of anammox bacteria. The samples analyzed here were characterized by an alkaline pH close to 9, while the ideal range for them ranges from 6.7 to 8.3 [23].

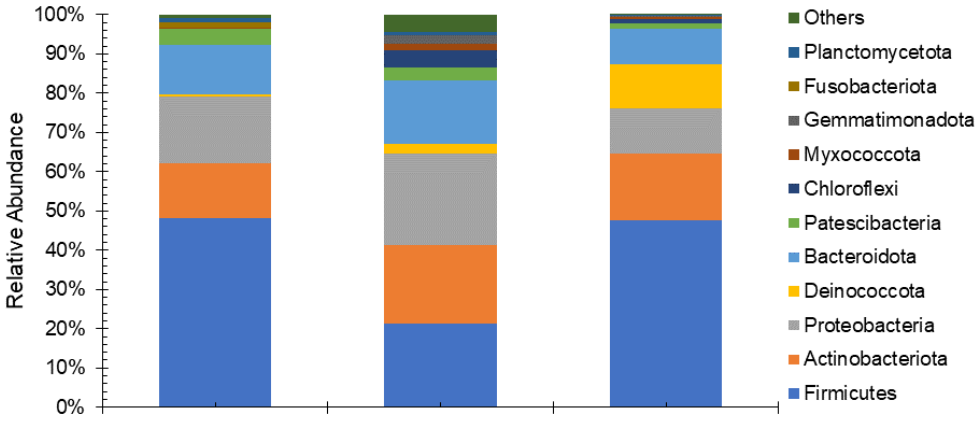

Figure 4. Relative abundance of the bacterial community at phylum level detected from the composting samples measured at different weeks of the operation; after week 1 (A); after week 3 (B); and after week 4 (C)

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Considering the large group of *Proteobacteria* in the analyzed samples, it should be noted that two classes of microorganisms dominated within it – *Alphaproteobacteria* and *Gammaproteobacteria*, for which 18 and 16 representatives were identified, respectively (Fig. 4). A similar observation was made by Gao et. al. [24]. During their analysis of the role of long- and short-chain N-acyl-l-homoserine lactones on microbial community dynamis in activated sludge, they distinguished various groups of ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB) participating in ammoniacal nitrogen removal process and indicated *Alphaproteobacteria* and *Gammaproteobacteria* as the main representatives.

One of the factors limiting the use of anammox bacteria in the treatment of sewage sludge is their slow growth. Due to the long doubling time, they are susceptible to external factors, such as the previously mentioned complex ecosystem, the introduction of other microorganisms that are their competitors, or sudden changes in the conditions prevailing in the reactor, e.g. during accidental leaching of sediments [25]. Doubling times of anammox bacteria range from 1.8 to 11 days [26], and the associated delays in reactor start-up were noticed by other researchers [27].A similar effect was observed in the experiment described here. For many microorganisms from the predominant in the sample's phyla *Proteobacteria, Bacteroidota, Chloroflexi, Acidobacteriota* and *Planctomycetota*, the highest abundance was recorded in the second week of composting, 14 days after starting the process (in case of 16, 7, 5, 2 and 1 families, respectively, supplementary material Table S1). On the other hand, the lower presence of these bacteria in the samples taken at 4th week can be explained by the competition effect, as well as the gradual depletion of substances necessary for their growth.

4. Conclusions

The activity of anammox bacteria, so far analyzed only in wastewater treatment processes, is considered to be a particularly efficient method of nitrogen removal, having an advantage over the conventional methods used previously. The benefits of using them are not only operational, but also environmental. Based on the analyzes carried out so far, it has been shown that in addition to reducing the costs of the wastewater treatment process by up to 90%, the necessary infrastructure and space are also reduced. Considering the environmental aspects, an important element is the lack of production of additional CO_2, which is simultaneously used by anammox bacteria, preventing the process from contributing to an increase in the amount of greenhouse gases.

The discovery of the presence of anammox bacteria during composting becomes not only a new research issue, but also an opportunity to improve the process and reduce its impact on atmospheric pollution. Despite the aerobic nature of this process, the composted mass of waste presents conditions conducive to the development of these ammonia oxidizing bacteria, as well as other strains of microorganisms cooperating with them. This makes it possible to compost at a low C/N ratio; in addition, there is no need for additional energy supply through aeration, as the processes carried out by anammox bacteria do not require oxygen.

However, the preliminary research carried out so far does not fully explain this phenomenon. It is necessary to implement experiments explaining the mechanisms of anammox bacteria activity in the composting process, describing optimal conditions for their development, considering the further effectiveness of the process and the production of a valuable end product. For this purpose, it is necessary to simultaneously monitor the growth of these bacteria and the processes of ammonia oxidation and nitrite reduction, as well as the use of molecular biology techniques.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: title; Microbial community in sewage sludge compost samples

Author Contributions: "Conceptualization, A.B. S.S-D. and R.S.; methodology, S-S-D; and R.S.; software, M.V.; validation, A.B.; formal analysis, S.S-D, and K.S.; investigation, S.S-D and R.S.; resources, S.S-D, K.S. and R.S.; data curation, S.S-D and A.B.; writing—original draft preparation, S.S-D., K.S., M.V and J.R.; writing—review and editing, S.S-D., A.B., K.S., M.V and J.R.; visualization, S.S-D, M.V.; supervision, A.B. and R.S.; project administration, S.S-D and A.B.; funding acquisition, S.S-D and A.B.

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