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Assessment of diversity and composition of bacterial community in Sludge Treatment Reed Bed systems

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Abstract

Due to their low emission of odours and lack of the need to apply additional chemical agents, sludge treatment reed beds (STRBs) constitute an economically feasible and eco-friendly approach to sewage sludge management. Correctly designed and operated STRBs ensure effective reduction of the dry matter content coupled with the mineralisation of organic compounds. Successful operation of STRBs relies on complex interactions between the plants and microorganisms responsible for the decomposition of organic matter and nutrient cycling. While the biocenoses of wetland systems dedicated to wastewater treatment have been intensively investigated, in the case of sludge treatment applications, there is a deficit of available microbial data. The aim of this study was to explore the diversity and spatial distribution of the bacteria in three distinct STRBs which differ in maturation and feeding patterns. Analyses of the dry mass and organic matter content showed the general trend of the sludge stabilisation processes advancing through the bed depth, with the best performance in the Matured Continuous Feed (MCF) bed being noted. Samples from the MCF bed showed the statistically greatest biodiversity in relation to the other beds. Moreover, increased biodiversity of microorganisms was observed on the surface of the STRBs and the bottom zone of the MCF equipped

with a passive aeration system, which proves the application of such solutions in order to enhance the performance of the process. The results of 16S rRNA gene sequencing revealed that *Bacteroidetes*, *Proteobacteria* and *Firmicutes* contributed approximately 80% of all identified sequences read. Network analysis revealed dominant role of *Bacteroidetes* in the formation of interspecies co-existence patterns. *Nitrospira* was the most abundant organism responsible for nitrogen metabolism in the STRBs.

Keywords: 16S rRNA high-throughput Illumina sequencing; diversity; sludge treatment reed bed (STRB)

1. Introduction

Sludge treatment reed beds (STRBs) are a type of constructed wetlands, i.e. engineered systems that combine the physiological activity of the higher plants and microorganisms with the physicochemical processes specified for soil ecosystems to treat sewage sludge. STRBs are considered to be an environmentally friendly technology of sludge management, which provides integrated dewatering and stabilisation of surplus activated sludge without the use of additional chemical agents (e.g. coagulants or polyelectrolytes) at low operating costs. In STRBs, reeds grow on the mineral subsoil with overlying layers of sludge. The system consists of a number of beds where the bottom of the bioreactor is isolated from the ground by a waterproof layer of concrete or another membrane. To drain off rejected water produced during sludge dewatering, the bottom zone of the bioreactor is equipped with a drainage system, which performs the additional function of providing residual sludge aeration (Nielsen, 2003; Kołecka and Obarska-Pempkowiak, 2013; Kołecka et al., 2017, 2018). This method is dedicated to long-term sludge management, where the operational life is planned to be a dozen years (10–15 years, most commonly). The operation of STRBs consists of three periods: commissioning, full operation, and emptying (Kołecka and Obarska-Pempkowiak, 2008, 2013; Nielsen, 2003, 2011). In the commissioning period (which lasts for about 2 years), the dose of dry matter of the sludge should be lower than 60 kg/m^2/year. During the full operation period, STRBs are

periodically irrigated with sewage sludge with a low dry matter content (from 0.5% to 1.5%) in feeding/resting mode. Raw sewage sludge feeding is provided for a few days (3 to 7 days) followed by a longer resting period. The resting periods (breaks in sludge supplying) may last for several days (usually 20 to 50 days), depending on the weather conditions, the age of the system, the dry matter content in the sludge, as well as the thickness of the accumulated sludge (Nielsen, 2003).

The data from the literature (Kołecka and Obarska-Pempkowiak, 2008, 2013; Matamoros et al., 2012; Nielsen, 2011) shows that the sludge dewatered in STRBs over a long period can reach a dry matter content of over 30%. Therefore, the effective increase of dry matter content results in a significant decrease of sludge volume. At the same time, the STRB ensures suitable conditions for the organic matter stabilisation process. Previous studies by Kołecka, et al. (2017) showed that the degree of mineralisation in reed bed systems ranged from 31.4 to 38.6%, and tended to decrease when the system was overloaded with raw sludge. Since mainly aerobic transformation of organic matter takes place, unpleasant odours are not emitted, and STRBs blend very well into the landscape (Kołecka and Obarska-Pempkowiak, 2013). The final product from STRBs can be safely disposed or used for agricultural purposes (Brix, 2017), and thus this technology is consistent with the assumptions of a circular economy.

Although STRBs in theory are relatively simple systems that rely on natural processes, experience has shown that it is very important to understand and respect the operational requirements (Brix, 2017). Microbe-mediated processes in STRBs are mainly dependent on the hydraulic conditions, wastewater properties, including substrate/nutrient quality and availability, filter material or soil type, applied plants, and other environmental factors (Truu et al., 2009).

Despite technological concerns, the overall potential of pollutant removal and stabilisation of sewage sludge in STRBs relies on the activity of, and interspecies interactions between, plant roots and distinct microbial groups of fungi and bacteria. Numerous papers have indicated that the activity of the microorganisms existing around plant roots in constructed wetlands is the main factor responsible for organic matter stabilisation and nitrogen removal (Balcom et al., 2016; Stottmeister et al., 2006; Guan et al., 2015). It was also proven that microbial diversity in constructed wetlands is necessary for the stable functioning of the system (Usharani, 2019). However, available references are focused on the

microbial communities of conventional, constructed wetlands designed for wastewater treatment, where the operation and feeding substrate are substantially different from STRBs.

In terms of the literature dedicated to STRBs, most of the attention has been paid to technological and process performance issues such as the quality of the processed sludge in the context of organic matter content (Nielsen and Bruun, 2015, Mennerich et al, 2017); detection of specified contaminations – heavy metals (Boruszko et al., 2017; Caicedo et al., 2015), pharmaceuticals (Kołecka et al., 2019), antibiotics (Ma et al., 2019); as well as carbon footprint and life cycle assessments (Larsen et al., 2018, Uggetti et al., 2012) and greenhouse gas emissions (Gómez-Muñoz et al., 2017). Despite the significant scientific interest in STRBs, the availability of species-specific data on microbial communities in such systems is scarce. Therefore, it is impossible to draw any general conclusions about which bacteria groups play a potentially dominant role in the decomposition of the organic matter, nutrient removal, or other metabolic activities. Further development of sludge management via STRBs can be achieved by expanding of our knowledge of the composition of the bacterial communities in such systems and their waste degradation functions.

Currently, only a few reports dedicated to the characterisation of a biocenosis of STRBs, using modern microbial tools, are available. In one of them, a predominance of the *Proteobacteria* (38.3%) and *Ascomycota* (77.5%) phyla in the bacterial and fungal kingdoms, respectively, was detected. However, due to the unoptimised data analysis procedure used, only six bacterial genera were successfully identified. Moreover the study was limited to a single sample, without examination of the microbial spatial distribution through the filter bed (Usharani, 2019). In another reference, Arroyo et al., (2018) applied 16S rRNA gene high-throughput sequencing to characterise the composition of the bacterial community in STRBs treating swine slurry. The authors found that the composition of the bacterial communities varied between the STRBs depending on the applied feed (treated and untreated slurry). In terms of the STRBs supplied with untreated slurry, the community was predominated by members of the *Firmicutes* phylum. In the systems where treated slurry was applied, *Proteobacteria* became the most abundant bacterial group. The authors identified pH as an important predictor of the composition, diversity and organisation of the bacterial community. By considering nitrogen metabolism, ammonification and assimilatory nitrate reduction were established as the key nitrogen pathways in

the treated swine slurry. The presented studies provide valuable insight into the bacterial community structure and diversity of untreated and treated swine slurry, however in terms of STRBs treating sewage sludge, such data was missing.

Another aspect that has to be considered is the occurrence of pathogens in the STRB systems. Only a few references related to this issue are available (i.e. Gibbs et al., 1995; Nielsen 2007). Moreover, previous studies were limited to the detection and enumeration of the specified pathogenic bacteria groups i.e. *Escherichia coli*, enterococci and *Salmonella* sp. To fully assess the epidemiological risk connected to the operation of STRBs, more advanced approaches have to be applied.

Currently, the analysis of a metagenome – i.e. the set of genomes of all organisms present in a specific environment – enables precise characterisation of the total microbial community (Ciesielski et al., 2011). This powerful approach provides the possibility to obtain a significant number of DNA sequence reads in a relatively short time, which makes it possible to identify particular microbial groups and to assess their abundances. In such applications, the 16S rRNA gene is the most commonly applied molecular marker. Determination of taxonomic diversity based on 16S rRNA sequencing has been used in the study of microorganisms from various environments, including: constructed wetlands (Pan et al., 2020), seawater (Krolicka et al., 2019; Adyasari et al., 2019), as well as activated sludge (Wu et al., 2019; Pan et al., 2020).

The aim of the presented study was to determine the phylogenetic structure and spatial distribution of the bacterial communities in three beds of an STRB which differed in terms of maturation and feeding patterns: Young Continuous Feed (YCF), Matured Continuous Feed (MCF) and Matured Fed Batch (MFB). The genetic data was obtained via high-throughput Illumina sequencing of the V3–V4 region of the 16S rRNA gene, followed by bioinformatic data processing and network analysis. The presented results provide complex information and insight into the biodiversity of the bacterial communities in STRBs, which have not been characterised in detail yet. Moreover, the identification of potentially pathogenic microorganisms as well interactions between bacterial species in terms of metabolic pathways have been provided.

2. Materials and methods

2.1. Analysed facility and sample collection

The research was conducted in the STRB located in the wastewater treatment plant in Gniewino (Pomerania Voivodeship, Poland (54.72N, 17.99E)). The system has been in operation since 2011. The STRB has a total area of 2400 m^2 and is divided into 6 beds, which began operation at different times. Until 2017, the reed bed system in Gniewino was operated continuously. However, due to maintenance problems, gradual modernisation of some beds was necessary. For the microbial investigations, samples from three beds were collected, which were selected based on their distinct operating ages and feeding patterns. The following beds were selected for the studies: (i) a bed that had been in continuous operation for 6 years (Matured Continuous Feed — MCF), (ii) a bed that had not been in operation for the previous 3 years due to operational problems leading to the decay of the reeds (Matured Fed Batch — MFB), and (iii) a new bed that had been in operation continuously over the previous year (Young Continuous Feed — YCF). The schemes of the beds are presented in Figure 1.

Figure 1. Scheme of the Matured Continuous Feed (MCF), Matured Fed Batch (MFB) and Young Continuous Feed (YCF) beds. 1 – raw sludge distribution system, 2 – drainage/leachate collection, 3 – passive aeration system, 4 – reed culture.

The one-time volume of sludge discharge into the beds was about 150–180 m^3. The sludge was poured evenly onto the reed bed surface. Their solids remain on the surface of the bed, while the reject water is recycled to the beginning of the treatment plant. The reed beds in the analysed sewage treatment plant are exploited only from March to December, while in the winter, the sludge is dewatered on a press and collected in the yard to be supplied to the reed beds during plant vegetation period. A year before sampling, the load of raw sludge discharged to the MCF was about 75 kg of DM/m^2/year. The

bed was fed for one to two days and then, depending on the weather conditions, the rest period lasted from 14 to 21 days. The MCF bed was equipped with an aeration system which increased the oxygen concentration in the lowest layers of sludge. The raw sludge was discharged to the bed 2 months before sludge sampling.

The MFB was the first bed constructed in the Gniewino STRB, and was not equipped with an aeration system. Due to problems with stable operation, the MFB had not been fed for 3 years and was left for sludge stabilisation. The YCF feeding pattern was similar to the MCF, with a lower loading rate of about 25 kg of DM/m^2/year. Raw sludge was discharged to the bed 6 months before sludge sampling. The average temperatures at the WWTP area during the experimental period were: 0.2℃ for winter, 8.4℃ for spring, 18.8℃ for summer and 10.3℃ for autumn. In the same periods, the average rainfall was: 128.4 mm, 98.1 mm, 158.4 mm and 101.4 mm, respectively (data available at https://www.meteoblue.com).

All of the samples for microbial analysis were collected on the same day during the summer period (June 2019), along a vertical profile using a dedicated probe for soil core sampling at six random points. The thickness of the MCF was 100 cm, MFB — 75 cm, and YCF — 25 cm. In order to determine the spatial distribution of the bacterial species along the bed profiles, the sample cores were divided into 25 cm segments. The sludge samples from each of six repetitions, collected from the same bed and derived from the same depth, were mixed together in order to be averaged. The samples prepared according to this procedure were stored at -25℃ prior to microbial analysis.

2.2. Analytical methods

In all of the samples of sewage sludge, the basic parameters such as dry (DM) and organic matter (OM) were determined. Additionally, the nitrogen and phosphorus content was examined in the MFB along the bed profile. The analyses were carried out according to Polish Standards (PN-EN 12879:2004, PN-EN 12880:2004) along with recommendations from the American Public Health Association (APHA, 2005).

2.3. DNA extraction, amplicon sequencing and bioinformatic analysis

DNA extraction from 100 mg of the sludge samples was performed in duplicate with the use of a FastDNA™ Spin Kit for Soil (MP Biomedicals, USA), in accordance to the manufacturer's protocol. DNA matrices from two repetitions were mixed together. The microbial communities in the analysed samples were examined by sequencing of the V3–V4 region of the 16S rRNA gene. The 16S rRNA gene fragment was amplified with the PCR primers recommended for the Illumina technique. The primers were developed by adding the Illumina adapter overhang nucleotide sequences primers given by Klindworth et al. (2013) to the PCR. Amplicons were indexed using a Nextera® XT Index Kit according to the manufacturer's instructions. The DNA was sequenced in Illumina MiSeq in 2 × 250 paired-end mode. The sequencing results were saved in FASTQ files and uploaded to the MetaGenome Rapid Annotation Subsystems Technology (MG-RAST) server for analysis (Meyer et al., 2008). Each file underwent quality control (QC) which included quality filtering (removing sequences with ≥5 ambiguous base pairs) and length filtering (removing sequences with a length ≥2 standard deviations from the mean). The Illumina metagenomic datasets are available at MG-RAST under accession numbers from 4866336.3 to 4866341.3.

Taxonomic differences between the metagenomes were analysed using Statistical Analysis of Metagenomic Profiles (STAMP v. 2.1.3) (Parks and Beiko, 2010). Statistically significant differences between amplicon datasets were identified by G-Test + Fisher's exact test and the multivariate Anova test I. A correlation matrix was developed by calculating all possible pairwise Spearman's rank correlations among the 50 bacterial genera with the highest abundances. A correlation between two items was considered statistically robust if the Spearman's correlation coefficient (ρ) was ≥ 0.8 and the p value was ≤ 0.01 (Junker and Schreiber, 2008). The genetic distance between the samples was tested at the phylum level with the ANOVA test, followed by the post-hoc Tukey-Kramer test at 0.95 significance. The unweighted pair group method with the arithmetic mean algorithm (UPGMA) was applied in order to group the samples. The analysis was conducted with the STAMP software to visualise the data output in the form of a PCA plot.

The robust pairwise correlations formed co-occurrence networks. Network analyses were visualised and explored to identify their topological properties (i.e., clustering coefficient, shortest average path length, and modularity) in Gephi (Bastian et al., 2009) using the Fruchterman-Reingold layout. The

biodiversity of the microbial communities was assessed with the Shannon (H) and Simpson diversity indexes calculated by the following formulas:

Shannon's diversity (H)

$$H = -\sum_{i=1}^{R} pi \ln pi \quad (1)$$

Simpson's diversity (D)

$$D = 1/\sum_{i=1}^{R} p^2 \quad (2)$$

where: *pi* – the ratio between the number of DNA sequences assigned to the particular i^{th} genus to the total number of DNA sequences obtained from the sample.

Correlations between the parameters of the biomass stabilisation process in relation to the biodiversity of the microbial communities, and the abundances of the individual bacterial groups were calculated using the formula:

$$r_{XY} = \frac{\sum(x_i-\bar{x})(y_i-\bar{y})}{\sqrt{\sum(x_i-\bar{x})^2 \sum(y_i-\bar{y})^2}} \quad (3)$$

where: r_{xy} – correlation coefficient of the linear relationship between the variables x and y; x_i – the values of the x-variable in a sample; $\bar{x}$ – the mean of the values of the x-variable; y_i – the values of the y-variable in a sample, $\bar{y}$ – the mean of the values of the y-variable.

3. Results and discussion

3.1. Sludge stabilisation process parameters from STRB

The dry and organic matter content in the sewage sludge samples along the depth profiles in the three analysed reed beds from the Gniewino STRB are presented in Table 1, with the addition of the nitrogen and phosphorus content profiles for the MFB.

Table 1. Basic characteristics of the sewage sludge treated in the analysed STRB.

In the analysed MCF and MFB, the highest content of dry matter was found in the bottom layer, and was 17.0±1.9% and 20.9±2.1%, respectively. In the MFB, a strong positive correlation was found in terms of the increase of dry matter with the depth. Although there were no reeds on the MFB, the content of dry matter was relatively high. This was connected with the fact that raw fresh sludge had not been discharged for 3 years, while the remaining sludge was stabilised due to microbial activity. In terms of the sludge samples collected from the surface of the MCF, the dry matter content was higher in comparison to the layer directly below (25–50 cm). This indicates that the bed was clogged, which caused slow filtration of the reject water, which instead persisted in the middle section of the bed. On the other hand, the stabilisation process on the surface was performing at higher rate due to the greater availability of oxygen. The DM content in the deeper layers of the MCF showed a similar trend to the MFB, i.e. it tended to increase with depth. The lowest dry matter content (11.8±2.4%) was found in the YCF, which clearly indicated that time has a significant impact on the efficiency of the dewatering process. This is also consistent with the research results of Matamoros et al. (2012), who identified long storage time and variable composition of the fresh sludge in the past as the main factors influencing the dry matter content through the bed profile.

Analyses of the organic matter (OM) content showed a general decrease of this parameter along the bed profile, which in relation to dry matter, confirms the advance of the sludge stabilisation processes with the depth. The lowest content of organic matter (65.2±5.7%.) was in the bottom layer of the MCF, which was most overgrown with reeds, even though raw sludge was being systematically discharged there. In the MCF, the decrease of organic matter was related to the bed depth, except in the surface layer. In the YCF and MFB, the content of organics was similar in the bottom layers, and amounted to 67.1±7.5% and 67.2±5.8, respectively. This proves that the presence of the reeds improves the effectiveness of the stabilisation process, in contrast to long term operated beds without plants.

The nitrogen and phosphorus content at the following depths of the MFB showed opposite trends. While the share of nitrogen in the dry mass showed a strong negative correlation with the sampling

depth, the phosphorus content tended to increase. The explanation of such a state is connected with the metabolic activity of the microbial community, which tends to nitrify $N\text{-}NH_4^+$ to NO_3^- in the area of the STRB where aerobic conditions dominate, and then finally reduces them to gaseous products in the deeper layers along with the depletion of the oxygen supply. As a consequence, this leads to nitrogen losses in the system. The increase of the phosphorus content with the depth was instead related to the progress of the sludge stabilisation process. Similar results related to spatial DM, OM and the distribution of other stabilisation process indicators have been reported by Nielsen (2011), as well as Kołecka and Obarska-Pempkowiak (2013). They showed that the abovementioned factors were affected by surface feeding of the STRBs with fresh sludge.

3.2. Biodiversity of the bacterial communities

Values of the Shannon's (H) and Simpson's (D) indices, which describe the biodiversity of the microbial communities in the analysed beds, are summarised in Table 2.

Table 2. Values of the Shannon's and Simpson's biodiversity indices along the depth profile in the analysed beds with correlation matrix with depth, DM and OM parameters.

The MCF showed the statistically greatest biodiversity in relation to the other beds, as evidenced by the highest average of the Shannon's and Simpson's indices. Along the vertical profile of the MCF, the Shannon's and Simpson's biodiversity indices reached relatively high values in the surface sample, then dropped in the 25–50 cm layer, and tended to increase to reach the highest values in the bottom zone. In the case of the MFB bed, the largest genetic diversity of microorganisms was observed in the surface layer, and along with the increase in the sampling depth, the H index values decreased. In the case of the youngest bed (YCF), both biodiversity indices were statistically similar to the MFB. The greatest biodiversity of microorganisms in the surface zones of the beds can be explained by the method of top-down bed supply, and the availability of oxygen and fresh OM. Therefore, in the surface layer, bacteria gain access to optimal concentrations of nutrients, which limits inter-species

competition and creates an ecological niche that ensures the development of a wider number of distinct microbial groups. In addition, due to the presence of oxygen, the microorganisms can activate more effective metabolic pathways, which promotes the growth of the biomass of each individual species. The greatest species diversity observed in the long-term operated bed with reeds (MCF) in relation to the new system (YCF) and the matured system without plant growth (MFB) may be due to the fact that the composition of microorganism assemblies is formed as a result of long-term interspecies interactions and environmental selection processes (Joshi, et al. 2016). Due to the continuous feed into the MCF, there were more substrate resources available to maintain the growth of diverse bacterial groups compared to the MFB, while the bacterial community in the YCF seemed to have not yet reached maturity. The secondary increase in the value of the biodiversity indices in the deeper layers of the MCF, and the lack of a positive correlation to OM, were potentially due to the complex metabolic relationships formed between particular microorganisms and plant roots over the long system operating time (Lv et al., 2013). Such a relationship enabled the metabolism of more complex substrates and promoted biodiversity despite the limited availability of OM. An additional factor conducive to the development of diverse groups of microorganisms in the bottom zone of the MCF was potentially the use of a passive aeration system arranged in the drainage layer. Some differences in terms of the biodiversity trends over the depth profiles reflected by Shannon's H and Simpson's D values, were connect with the principal assumptions of the methods used for biodiversity estimations. While Shannon's indicator values basically rely on the general biodiversity of the organisms presented in the environment, Simpson's indicator is more sensitive to the occurrence of some dominant groups (Dedys, 2011). This suggests that the relatively low D values in the deeper layers of the MFB were connected with high abundances of the selected bacteria which outcompeted the environmental niche.

3.3. General structure of the microbial communities

The results of the multivariate Anova test used to understand the composition of the microbial community between the analysed samples and beds are presented in Figure 2.

Figure 2. Differences between microbial community composition in samples from the distinct beds and collected at the different depths (in the range of 0 to 100 cm) at the phylum level, ANOVA test for eight samples was conducted using STAMP ($P < 0.05$). Unweighted pair group method with the arithmetic mean algorithm (UPGMA) was applied in order to group the samples.

The samples from the MFB formulated a compact cluster, which indicates a significant similarity between them despite the depth. This suggests that long term bed operation, without being supplied with fresh sludge, allowed the composition of the microbial community to stabilise. On the other hand, the bacterial assemblages in the MCF reflected greater variability. While the samples derived from 0–25, 50–75, and 75–100 cm were grouped relatively close and showed some similarities in terms of the samples that originated from the MFB bed, the bacterial community analysed at the depth of 25–50 cm was significantly different. However, it matched the sample obtained from the young bed (YCF), thus their bacterial composition was probably affected by the regular supply of fresh sewage sludge. Such an observation suggests that even though the beds are fed from the top, in terms of beds operated for a long time and reflecting elevated depths, the fresh supply of sludge affects the deeper layers of the bed rather than the surface. At those depths, the diverse microbial community is potentially induced by the substrate availability which changes over time due to the irregular bed feed patterns, the deliveries of 'new' microorganisms with the fresh sludge, and the mixed anaerobic/aerobic conditions. The availability of oxygen at 25–50 cm, and the YCF may reflect the highest variability because of the oxygen depletion from the microbial activity connected with the basic metabolism and decomposition of the organic matter, and limited oxygen permeability through the deeper bed layers.

Over 95% of the 16S rRNA sequences obtained during taxonomic identification were assigned to the *Bacteria* domain. Twenty two phyla were identified, of which 21 belonged to bacteria and 1 to archaea. At the phylum level, *Bacteroidetes*, *Proteobacteria* and *Firmicutes* contributed approximately 80% of all obtained sequences reads (Table 3., Figure S1).

Table 3. Percentage of microbial community representatives at the phylum level. MCF – Mature Continuous Feeding; MFB – Mature Fed Batch, YCF – Young Continuous Feeding. Only phyla showing an abundance higher than 1% in at least one sample are shown. Correlation matrix of individual phyla against depth, DM and OM parameters.

Table 4 Percentage of microbial community representatives at the genus level. Only genera showing an abundance higher than 1% in at least one sample are shown. Correlation matrix of individual phyla against depth, DM and OM parameters. Representatives of particular bacterial phyla are distinguished by different colours. MCF – Mature Continuous Feeding; MFB – Mature Fed Batch; YCF – Young Continuous Feeding.

3.3.1. Members of the *Bacteroidetes* phylum

Excluding the YCF, and the 50–75 cm layer in the MCF, representatives of *Bacteroidetes* predominated the bacterial communities in the analysed samples. Their abundance showed a moderate-to-strong negative correlation in terms of depth, and a positive correlation in relation to the OM content. Bacteria affiliated with *Bacteroidetes* belonged mainly to the *Bacteroidia*, *Flavobacteria* and *Sphingobacteria* classes (Figure S2). Of the 24 most abundant genera detected in the matrix samples, 12 belonged to *Bacteroidetes* (Table 4), where members of *Saprospira*, *Prolixibacter* and *Porphyromonas* accounted separately for at least 3% of the total bacterial population in all samples in the MCF and MFB. Representatives of *Saprospira* and *Prolixibacter* reflected variable abundance ratios in each of the analysed systems. In the MCF, both genera reflected a similar share, in the young system (YCF), *Saprospira* prevailed, while in the MFB representatives of *Prolixibacter* gained advantage. Members of *Saprospira* are obligate aerobes, which explains their elevated numbers in the surface layers of the analysed beds, as well as in the MCF bed where oxygen was supplied by the passive aeration system. The mentioned bacterial group was characterised due to their active capability to hydrolyse proteins in activated-sludge waste treatment plants and many other environments, as well as their predation upon other bacteria by ixotrophy to obtain nutrients (Saw et al., 2012), which underlines the potential dominant role of *Saprospira* in the decomposition of the organic matter in analysed STRBs. In terms of *Prolixibacter*, it was found that representatives of this genera are commonly detected denitrifying bacteria in soil ecosystems (Iino et al., 2015), which proves the significance of *Bacteroidetes* in STRBs not only for the biodegradation of organic compounds, but also in nitrogen metabolism.

The individual genera (Table 4) reflected distinct correlations between the analysed factors, which resulted in general indications for the *Bacteroidetes* phylum. Such an observation is connected with the high versatility of the *Bacteroidetes* phylum which consists of over 7000 different species, where individual genera show a wide range of metabolic activity, often distinct from each other. For instance, the *Bacteroidetes* phylum includes strict anaerobes and aerobes (Gibiino et al., 2018). *Bacteroidetes* are considered, together with *Firmicutes*, to be a main component of the human gut microbiota, where

their share was estimated at about 50% (Eckburg et al., 2005). Therefore, they end up in wastewater treatment plants and ultimately in sewage sludge in large quantities via faeces and other metabolites. Based on this, the most probable source of the *Bacteroidetes* in the STRB was fresh sludge supplies. Representatives of *Bacteroidetes* are known as organisms with a dominant heterotrophic metabolism, capable of recognising and metabolising dozens of plant and mammalian polysaccharides and proteins (Gibiino et al., 2018). Due to their metabolic versatility *Bacteroidetes* rapidly adapt to changes in nutrient availability, and form complex cooperator and competitor relationships with other microbial groups. Thus, they constitute the core of bacterial communities in versatile environments (Lee et al., 2013). Elevated numbers of DNA sequences derived from *Bacteroidetes* in the analysed samples indicated the dominant role in the degradation of organic matter of this bacterial group in STRBs, especially in matured systems.

3.3.2. Members of the *Proteobacteria* phylum

In contrast to the matured beds, where *Bacteroidetes* prevailed, *Proteobacteria* (40.9%) were the most numerous phyla in the YCF bed, while in the MCF and MFB, their average share accounted for 28.7% and 31.4%, respectively. In the MCF and MFB beds, a relatively proportional share of representatives of particular classes was observed between particular layers (Figure S2), with a predominance of *Betaproteobacteria* and *Gammaproteobacteria* which contributed from 8.6 to 16.6%, and 5.8 to 10.6%, respectively, of the total bacterial community in all of the analysed samples. The exceptions were the MCF layer at 25–50 cm, and the YCF, which reflected the highest abundances of *Epsilonproteobacteria* with 13.7% and 20.7%, respectively. The elevated *Epsilonproteobacteria* occurrence seems to be related to the degree of maturation of the given system and the continuous feed pattern. This leads to the conclusion that, under stable conditions and in matured biocoenoses, as in the MCF and MFB, balanced development of all proteobacterial classes took place, while in systems under development and variable conditions, such as the YCF, *Epsilonproteobacteria* were able to gain an environmental advantage. At the genus level *Arcobacter*, *Coxiella* and *Methylomonas* constituted an abundant component of the microbial community in the MCF system (Table 4, Figure S4), while

the mentioned bacterial groups occurred only scarcely in the MFB and YCF systems. Special attention should be paid to the *Coxiella* genus which is exclusively formed by a single species, *C. burnetii*, known as an obligate intracellular bacterial pathogen which causes Q fever (Shaw and Voth, 2018). A notable number of DNA sequences specified for *Coxiella* were identified in the surface layer of the MCF bed (Table 4), which indicated the transfer of this potentially pathogenic microorganism with contaminated sludge. The presence of members of *Methylomonas*, capable of aerobic methane oxidation, reveals the methanogenic activity in STRBs during the sludge stabilisation process (Nguyen et al., 2017). In a direct comparison of the MFB with the MCF and YCF beds, greater abundances of *Nitrosomonas* and *Rhodanobacter* were found. Representatives of *Rhodanobacter* constituted approx., 1.9% of the bacterial community in the MFB system, three times greater in relation to other beds, and showed a strong positive correlation with the OM content. Green et al. (2012) revealed denitrifying activity within representatives of the *Rhodanobacter* genus, which predominated the specified waste site with a lowered pH. Bacteria affiliated to *Nitrosomonas* are considered to be key ammonium oxidising microorganisms in natural and artificial ecosystems. In this study, their share reached 1.2% at a depth of 25–50 cm only in the MFB bed, while in the MCF and YCF beds, *Nitrosomonas*-related microorganisms constituted less than 0.3% of the bacterial consortium. This finding suggests a dominant role of other bacterial groups in the nitrification process, while the growth of *Nitrosomonas* was potentially inhibited by the high loads of OM supplied to the STRBs. This supposition is in accordance with Martens-Habbena et al. (2015).

3.3.3. Members of the *Firmicutes* phylum

The share of *Firmicutes*, the third most-represented group of bacteria in all of the samples, was at a similar level (on average, it ranged from 12.2 to 13.5% of the total bacterial community) and showed no significant differences between particular depths (Figure S3). However, there were some differences at the class level. The *Clostridia* class dominated in all layers except for the 50–75 cm layer of the MCF bed, where *Bacilli* were more numerous. Most representatives of the *Bacilli* and *Clostridia* classes are saprophytic organisms found in many types of ecosystems, in particular soil

(Graham et al., 2018). The main difference between these classes is related to oxygen sensitivity. While *Bacilli* show oxygen tolerance, the representatives of the *Clostridia* class belong to obligatory anaerobes. The presence of both considered classes in the surface layers of STRBs, with the dominance of *Clostridia*, proves the occurrence of anaerobic zones. Sludge supply should be considered to be the main source of *Firmicutes* in STRBs, which together with *Bacteroidetes* constitutes the main component of the human gut microbiota (Thursby and Juge 2017). However, probably due to their lower adaptability and sensitivity to oxygen compared to *Bacteroidetes*, they accounted for approx. three to four times less in the total microbial community of the analysed beds. Out of the whole group of *Firmicutes*, three genera predominated: *Clostridium*, *Aneurinibacillus* and *Desulfitobacterium*, where *Clostridium*-related organisms prevailed in the MCF and YCF systems, and the remaining two genera occurred in noticeable numbers in the MFB (Table 4, Figure S4). This leads to the conclusion that fresh sludge supply promotes the growth of representatives of the *Clostridium* genus, primarily due to the occurrence of more anaerobic zones formed by intensive oxygen consumption during OM decomposition. Meanwhile, in matured systems without fresh sludge resupply, *Clostridium*-related organisms could be outcompeted by other *Firmicutes*, which were better adapted to the limited nutrient availability.

3.3.4. Members of the *Verrucomicrobia* phylum

Representatives of *Verrucomicrobia* constituted a stable component of the bacterial biocenosis, accounting for an average of 4.4% to 4.7% of all analysed samples (Table 3). The occurrence of *Verrucomicrobia* reflected a weak positive correlation with the OM content. Bacteria belonging to the *Verrucomicrobia* group are considered to be a natural component of soil biocoenoses and fresh waters, and a natural resident on the skin of humans and animals. Their main nutrient substrates are simple and complex sugars. Moreover, only in the case of *Verrucomicrobia* and certain groups belonging to *Proteobacteria*, the ability to grow using methane as the only carbon source was identified (Sharp et al., 2014). The ecosystem of a STRB, due to the presence of various zones related to the oxygen and

OM content, creates the ideal conditions for these bacteria. On the one hand, because of its capability of aerobic metabolism, *Verrucomicrobia* is able to grow effectively when oxygen is available.

3.3.5. Members of the *Nitrospirae* phylum

In all of the analysed samples, representatives of the *Nitrospirae* phylum were an important component of the bacterial community. This phylum includes aerobic bacteria of the *Nitrospira* genus, responsible for the second phase of nitrification (nitrite oxidation), whose share ranged from 1.8 to 6.3%. Because *Nitrospirae* reflects mainly aerobic metabolism, their abundance in individual layers is a good marker of the occurrence of aerobic conditions in the profile of the beds. At the same time, it allows the effectiveness of the aeration system to be assessed (Palomo et al., 2016). Therefore, it can be concluded that microaerobic conditions were present in all of the analysed beds at all depths. In terms of the YCF, *Nitrospirae* represented the fourth most abundant bacterial phylum. In the MFB, which was not equipped with an aeration system in accordance with expectations, *Nitrospirae* were most abundant in the surface layer, while their number reduced significantly in the deeper layers (Figure S3). The opposite trend was observed in the case of the MCF, where the highest *Nitrospirae* abundances were observed in the two deepest layers. This fact was probably related to effective operation of the passive aeration system applied in the mentioned bed, which effectively transferred oxygen to the bottom zones. The *Nitrospirae* phylum was almost exclusively represented by the nitrite oxidising genus *Nitrospira*, which is considered to be a K-strategist with a high affinity for nitrite and dissolved oxygen. Moreover, some representatives of *Nitrospira* possess the ability to perform complete ammonia oxidation (comammox) and to metabolise organic compounds such as glycerol and formate (Mehrani et al., 2020). All of the abovementioned attributes of *Nitrospira* provide metabolic versatility to representatives of this genus, resistance to variable conditions, and ecological advantage over other microorganisms involved in nitrogen metabolism (Daims and Wagner, 2018). In this study, due to the lack of DNA sequences specified for the *Nitrobacter* genus, as well as the low contribution of typical ammonium oxidising bacteria from the betaprotebacterial *Nitrosomonas* genus (average 0.4% in all samples) in the total bacteria community (Table 4), members of *Nitrospira* seem to be the

dominant microorganism responsible for the nitrogen cycle in the beds. Based on this, the role of *Nitrospira* in the analysed STRBs was not limited to canonical nitrite oxidation, but some of their representatives were also responsible for the comammox process.

3.3.6. Members of the *Actinobacteria* phylum

Another notable group of microorganisms were representatives of the *Actinobacteria* phylum, characterised by a Gram-positive cell wall, which are typical for soil ecosystems (Servin et al., 2008). In all of the analysed samples, the share of *Actinobacteria* representatives was at least 2.4%, mainly belonging to the *Pseudonocardiaceae*, *Intrasporangiaceae* and *Streptomycetaceae* classes (Figure S3). Excluding the 50–75 cm layer of the MCF, where representatives of *Actinobacteria* accounted for 5.0% of the total bacterial community, their share in the remaining layers and beds was stable in the range of 2.4 to 3.3% (Figure S3). In terms of the MCF, a strong positive correlation between *Actinobacteria* abundance and OM content was observed, which suggests their participation in the degradation of the organic compounds supplied with the fresh sewage sludge.

3.3.7. Members of the *Planctomycetes* phylum

The averaged share of *Planctomycetes* in the STRB accounted for 2.3 to 3.7% of the total bacterial community. Especially in the MFB, there were strong correlations between the abundance of *Planctomycetes* and depth and DM (both negative), and OM (positive) (Table 3, Figure S3). Similar correlational trends were found in the MCF, however no significant correlation with depth was noted. Moreover, an about 3 times higher abundance of *Planctomycetes* was detected at the depth of 25–50 cm in the MCF compared to the other layers. At the same depth, the highest OM content was noted, which suggests heterotrophic preferences of the analysed *Planctomycetes.* Members of the *Planctomycetes* phylum mainly inhabit aquatic ecosystems, but are also commonly identified in samples from wetlands (Dedysh and Ivanova, 2019). The discussed type includes bacteria capable of carrying out the anammox process, i.e. anaerobic oxidation of ammonia in the presence of nitrites, which in light of new literature reports on specific ecosystems, may play a greater role in the nitrogen

cycle than conventional nitrification-denitrification processes (Trimmer et al., 2013). In this study, using the criteria of 97% similarity in order to assign a DNA sequence to a given genus, it was not possible to clearly determine the phylogenetic affiliation of the detected representatives of the *Planctomycetes* phylum. For this reason, the presence of typical bacteria capable of carrying out the anammox process, such as *Candidatus* Brocadia or *Candidatus* Kuenia, has not been confirmed. The discussed DNA sequences were grouped together with representatives of other *Planctomycetes* genera — *Isosphaera*, *Pirellula* and *Planctomyces*, with the dominance of the last genera (Figure S4). Unlike typical anammox bacteria, the discussed groups of microorganisms reflect the dominant heterotrophic metabolism (Bauer et al., 2004, Göker et al., 2011). On this basis, it can be concluded that the representatives of *Planctomycetes* present in the STRBs play a greater role in the metabolism of carbon compounds than nitrogen.

3.3.8. Representatives of other bacterial phyla

The other phyla of microorganisms in general were characterised by a share not exceeding 1%, especially in the YCF system. A few exceptions were related to the occurrence of *Tenericutes* and *Spirochaetes* in the first two layers of the MCF and MFB. *Tenericutes* accounted approx. 2% and 1% in both beds, respectively, while *Spirochaetes* accounted for 1.5% in the 0–25 cm layer of the MFB, and the 25–50 cm layer of the MCF. Moreover, members of *Synergistetes* were detected at 1.7% in the total bacterial community at the surface of the MCF (Table 3, Figure S3). The *Tenericutes* phylum includes saprotrophic as well as parasitic microorganisms, such as the *Candidatus* Phytoplasma detected in this study. Thus, the discussed phylum, despite its contribution to the removal of organic compounds, reflected a potential pathogenic interaction with the reeds (Derickx and Antunes, 2013). The *Spirochaetes* and *Synergistetes* phyla (mainly heterotrophic chemoorganotrophs, commonly found in wastewater treatment plants) participated in the anaerobic decomposition of OM (Militon et al., 2015, Silva-Bedoya et al., 2016).

However, the most profound exceptional increase of abundance was observed in the case of the *Acidobacteria* phylum, which on average contributed 0.5% in most of the analysed samples. On the

other hand, in the sample from the bottom of the MFB, their share was 20 times higher (10.29%), which makes them the fourth most numerous group of bacteria in this layer, almost as numerous as the *Firmicutes* (Figure S3). Most of the DNA sequences classified as *Acidobacteria* derived from the *Candidatus* Solibacter genus, known for its capability to break down organic compounds and participate in nitrate and nitrite reduction (Pearce et al., 2012). The relatively high abundance of the abovementioned genera representatives in the bottom zone of the MFB may suggest their preference for systems with lower loads of organics, and the potentially the low pH of this layer, which favours the growth of *Acidobacteria*.

3.4. Co-occurrence network

A correlation network was constructed based on the 16S rRNA reads assigned to the 50 genera occurring with the highest abundances (Figure 3).

Figure 3. Network analysis revealing co-occurrence patterns among bacterial genera. Nodes were coloured by modularity class. The edges present the correlation between two nodes. A connection represents a strong (Spearman's correlation coefficient $\rho > 0.8$) and significant (P-value > 0.01) correlation. The size of each node is proportional to the number of connections.

The correlation networks consisted of 32 nodes of significant ($P \leq 0.01$) positive and negative correlations. Generally, 11 modules were identified, however among them, two main modules ("pink" and "green") should be highlighted. The pink module was composed of six genera belonging to the *Bacteroidetes: Cytophaga*, *Leeuwenhoekiella*, *Parabacteroides*, *Saprospira*, *Terrimonas*, and *Tissierella;* three genera of *Firmicutes*: *Ethanoligenens*, *Alkaliphilus*, and *Acholeplasma*, and single genera of other phyla: *Ralstonia* (*Betaproteobacteria*), *Nitrospira* (*Nitrospirae*), *Opitutus* (*Verrucomicrobia*), and *Candidatus* Phytoplasma (*Tenericutes*). On the other hand, the green module was made up of three genera belonging to the *Bacteroidetes: Elizabethkingia*, *Prolixibacter*, and *Tenacibaculum*, two genera of *Firmicutes*: *Desulfitobacterium*, as well as single genera of *Candidatus*

Magnetobacterium (*Nitrospira*), *Streptomyces* (*Actinobacteria*), and *Coxiell*a (*Gammaproteobacteria*). In both modules, heterotrophic microorganisms and those involved in nitrogen metabolism prevailed in the formation of the co-occurrence networks, thus OM and nitrogen availability seem to be the important factors that shape the microbial community structure in STRBs. Moreover, along with the highest abundances, representatives of *Bacteroidetes* have to be considered to be a key bacterial group in such systems, in terms of organic compound co-metabolism another interspecies relations.

4. Conclusions

In this study, a first insight into the complex structure of the microbial communities of STRBs has been provided in terms of bacterial spatial distribution, maturation stage of the system as well distinct feeding patterns. The investigations revealed predominant abundances of *Bacteroidetes*, *Proteobacteria* and *Firmicutes* phyla in STRBs. Samples from the MCF bed showed statistically the greatest Shannon's (H) and Simpson's (D) indices values in relation to other beds, which leads to conclusion about main role of system maturation stage and continuous feeding pattern as main factor promoting biodiversity. Highly divers bacterial communities and the presence of aerobic microorganisms in the bottom zone of the MCF, which was equipped with a passive aeration system, prove the application of such solutions in order to enhance sludge stabilisation process performance. Representatives of *Bacteroidetes* are recognised as a key bacterial group in the formation of interspecies co-existence patterns. In terms of the nitrogen cycle, significantly higher abundances of *Nitrospira* in relation to *Nitrosomonas* genus reveals the potential occurrence of comammox activity in STRBs. The detection of DNA sequences specified for *Coxiella* genus indicates STRBs as a potential reservoir of Q fever pathogens.

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CRediT Authors Statement

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Table 5. Basic characteristics of the sewage sludge treated in the analysed STRB.

Layer depth (cm)	Dry matter (%)			Organic matter (% d.m.)			TN (% d.m.)	TP (% d.m.)
	MCF	MFB	YCF	MCF	MFB	YCF	MFB	
0--25	15.8±2.4	12.9±1.4	11.8±2.4	65.4±6.4	69.6±5.9	67.1±7.5	5.59±0.01	2.56±0.10
25–50	14.5±2.2	14.8±2.5	-	69.6±4.8	68.1±7.1	-	5.57±0.07	2.60±0.06
50–75	15.9±3.1	20.9±2.1	-	66.0±6.6	67.2±5.8	-	5.08±0.13	2.85±0.86
75–100	17.0±1.9	-	-	65.2±5.7	-	-	-	-
correlation with depth	0.63	0.96	-	-0.89	-0.99		-0.88	0.92

MCF – Matured Continuous Feed; MFB – Matured Fed Batch; YCF – Young Continuous Feed.

Table 6. Values of the Shannon's and Simpson's biodiversity indices along the depth profile in the analysed beds with correlation matrix with depth, DM and OM parameters.

Depth (cm)	Shannon's diversity (H)			Simpson's diversity (D)		
	MCF	MFB	YCF	MCF	MFB	YCF
0–25	4.01	3.87	3.80	28.4	22.6	20.4
25–50	3.90	3.77	-	23.7	14.4	-
50–75	3.99	3.75	-	26.6	16.9	-
75–100	4.11	-	-	28.9	-	-
Average	4.00 ± 0.09	3.80± 0.09	3.80	26.9 ± 2.36	18.0 ± 4.21	20.4
correlation with depth	0.59	-0.95	-	0.23	-0.68	
correlation with DM	0.98	-0.82	-	0.91	-0.43	-
correlation with OM	-0.85	0.99	-	-0.97	0.77	-

MCF – Matured Continuous Feed; MFB – Matured Fed Batch; YCF – Young Continuous Feed.

Table 7. Percentage of microbial community representatives at the phylum level. MCF – Mature Continuous Feeding; MFB – Mature Fed Batch, YCF – Young Continuous Feeding. Only phyla showing an abundance higher than 1% in at least one sample are shown. Correlation matrix of individual phyla against depth, DM and OM parameters.

Correlation heat map ■ strong negative (-1.0 : -0.7 :); ■ moderate negative (-0.6 : 0.4); ■ moderate positive (0.4 : 0.6); ■ strong positive (0.7 : 1.0)

Table 8. Percentage of microbial community representatives at the genus level. Only genera showing an abundance higher than 1% in at least one sample are shown. Correlation matrix of individual phyla against depth, DM and OM parameters. Representatives of particular bacterial phylum were distinguished by different colours. MCF – Mature Continuous Feeding; MFB – Mature Fed Batch; YCF – Young Continuous Feeding.

Correlation heatmap ■ strong negative (-1.0 : -0.7 :), ■ moderate negative (-0.6 : 0.4); ■ moderate positive (0.4 : 0.6); ■ strong positive (0.7 : 1.0)

Highlights

- First complex characteristics of bacterial community of the STRBs were studied
- OM and DM patterns reflect advance of sludge stabilization process in the STRBs
- The main bacterial phyla in STRBs were *Bacteroidetes, Proteobacteria* and *Firmicutes*
- *Nitrospira* is the most abundant organism responsible for N metabolism in STRBs
- *Bacteroidetes* have a dominant role in formation of interspecies coexistence patterns

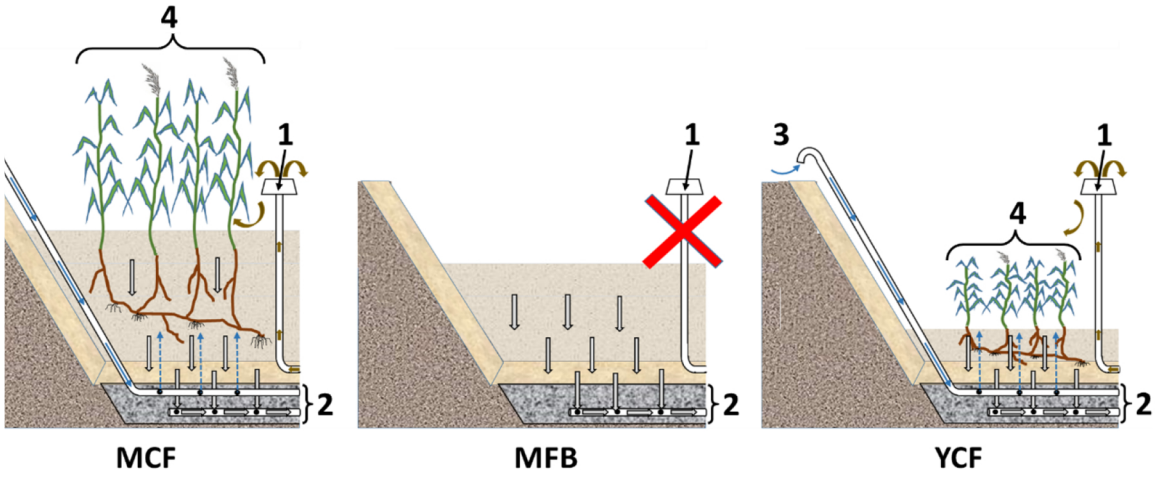

Figure 1

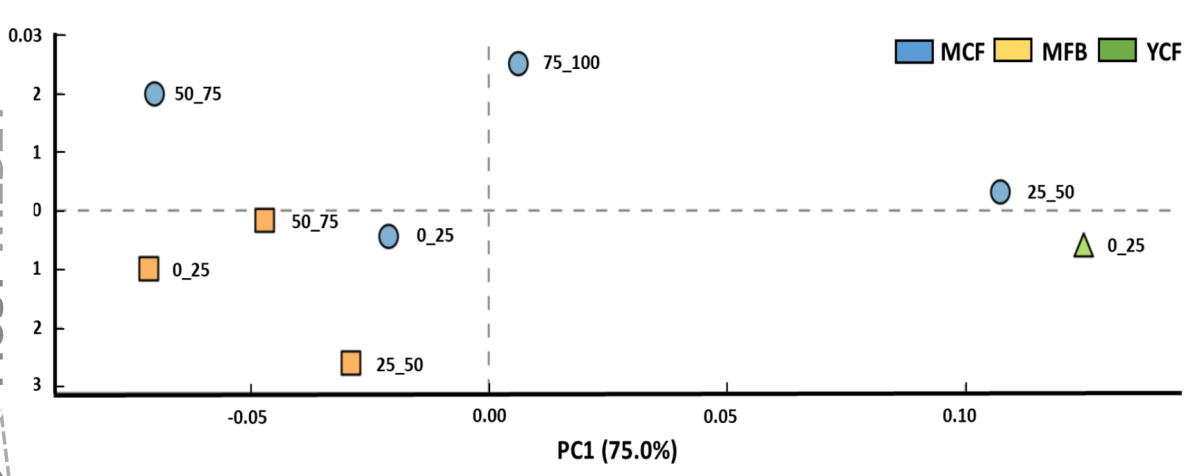

Figure 2

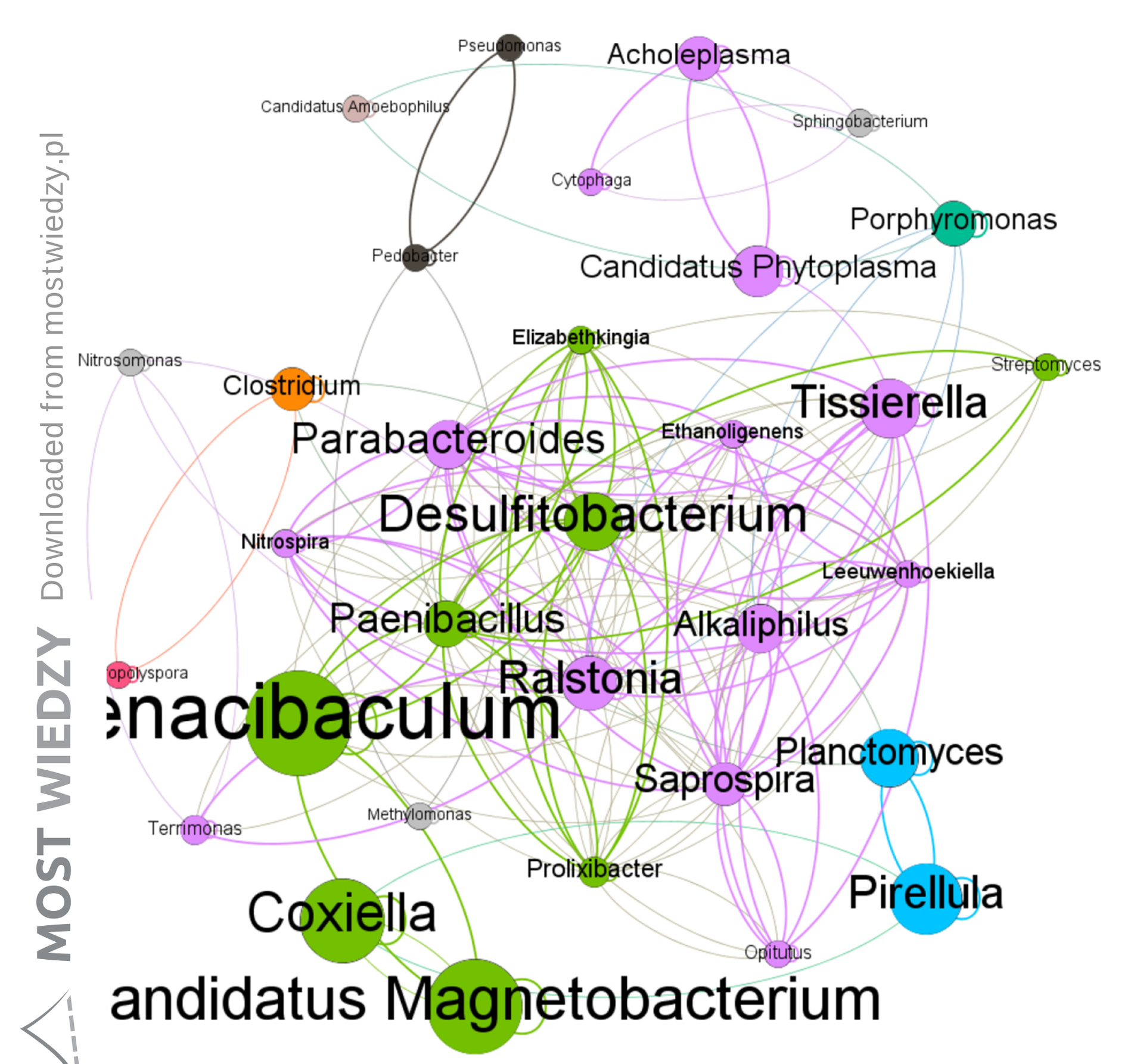

Figure 3