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Influence of sewage sludge stabilization method on microbial community and the abundance of antibiotic resistance genes

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ABSTRACT

Municipal sewage sludge (MSS) and other biosolids are of high interest for agriculture. These nutrient-rich organic materials can potentially serve as organic fertilizers. Besides an increase of organic matter in soil, other positive effects were shown after their application. Especially the positive influence on circular economy increased the attention paid to management of MSS in recent years. Unfortunately, the use of sewage sludge has some drawbacks. Biosolids are frequently polluted with heavy metals, xenobiotic organic compounds and industrial chemicals, which may be hazardous for the environment and humans. Here, we investigated the influence of stabilization method and the size of wastewater treatment plant on the structure of microbial communities as well as the abundance of antibiotic resistance genes (ARG) and mobile genetic elements (MGE). All tested ARG and MGE were detectable in almost all of the samples. Interestingly, the presence of MGE as well as particular heavy metals correlated positively with the presence of several ARG. We conclude that the distribution of ARG and MGE in biosolids originated from municipal wastewater treatment plants, cannot be explained by the size of the facility or the applied stabilization method. Moreover, we postulate that the presence of pollutants and long-term impacts should be assessed prior to a possible use of sewage sludge as fertilizer.

1. Introduction

Recently, the recycling and sustainable management of municipal sewage sludge (MSS) has become an issue of growing importance (Cieślik et al., 2015; Fytili and Zabaniotou, 2008; Kelessidis and Stasinakis, 2012; Major et al., 2022). The MSS is a by-product generated during the process of wastewater treatment namely, the primary (physical and/or chemical), the secondary (biological) and the final tertiary (additional to the secondary) treatments (Fytili and Zabaniotou, 2008). Before the recycling, MSS has to be stabilized in order to mitigate the problems related to the presence of human pathogens (viruses, bacteria and parasites), odor and putrescence (Mininni et al., 2015). This can be managed by either biological stabilization performed in aerobic (oxidative stabilization) and anaerobic (fermentation) conditions or by chemical methods, such as the addition of lime (Cieślik et al., 2015; Fytili and Zabaniotou, 2008; Wang et al., 2019). In aerobic stabilization, which is mainly used for lower amounts of MSS and thus small and medium-sized wastewater treatment plants (Kazimierczak, 2012), the residual sludge is hydrolyzed and transformed to biodegradable soluble organic matter. During this process, heterotrophic bacteria convert the organic matter into CO_2, H_2O and active biomass (Song et al., 2017). The anaerobic digestion is one of the most often used procedures for MSS stabilization. This process decreases the amount of MSS solids and produces biogases e.g. CH_4. It is considered as a slow degradation process due to the limited hydrolysis rate where the decomposition of organic matter consists of four steps, hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Tiehm et al., 2001). In regard to lime treatment, an amendment with lime of untreated MSS

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increases the pH to above 12 (or higher), this in turn inactivates viruses, bacteria and other pathogens (Kelessidis and Stasinakis, 2012; Wang et al., 2019). Since MSS is a nutrient-rich organic material it is often valorized through agricultural utilization, contributing to sustainability and circularity aspects of both MSS management and agriculture (Antoniadis et al., 2015; Černe et al., 2022; Horvatić et al., 2021; Miah et al., 1999; Özyazıcı, 2013; Zhang et al., 2015; Heimersson et al., 2017). In the EU, the use of biosolids in agriculture is regulated by the directive 86/278/EEC, which has been implemented now for more than two decades and seems very successful (Rizzardini and Goi, 2014). The agricultural use of MSS is currently one of the most widely available strategies in the EU for its recycling, especially since the conventional disposal (e.g. landfilling) is now restricted (Mininni et al., 2015). Beside the increase of organic matter's content in agricultural soil, several other positive effects like increase in water holding capacity, soil porosity, soil structure, fertility and yield production were observed in MSS-amended soils (Singh and Agrawal, 2008; Tsadilas et al., 1995).

Unfortunately, the use of MSS also has some drawbacks. The MSS are frequently polluted with heavy metals, xenobiotic organic compounds and industrial chemicals, and therefore its land application could lead to an increased risk for human and environmental health (Černe et al., 2021; Eriksson et al., 2008; Mathews and Reinhold, 2013; Singh and Agrawal, 2008; Tangahu et al., 2011; Yang et al., 2014). In a previous study, nine dewatered MSS samples from Croatian wastewater treatment plants (WWTPs) with different stabilization approaches were analyzed in regard to their physicochemical characteristics, presence of macro- and micronutrients as well as trace metals and radionuclides (Černe et al., 2019). The study concluded that except one of the anaerobically stabilized MSS, all other analyzed biosolids were suitable as soil amendments, according to both, Croatian and EU regulation limits for trace metals in MSS. Those biosolids exhibited high P and N contents and posed no apparent risk for human health or for the environment when applied to agricultural soil.

Abiotic characteristics are, however, not the sole factors which could be potentially harmful for human health or other concerned organisms, e.g. crop plants. In addition, the microbiome of a biosolid could pose a potential risk. The presence of human and plant pathogenic microorganisms was documented on several occasions and must be considered before those substances are used as fertilizers in agriculture (Schauss et al., 2016). Another factor is the presence of antibiotic resistance genes (ARGs). Antimicrobial resistance is a growing threat to public health. It challenges the achievements of modern medicine, making a post-antibiotic era a very real possibility for the 21st century (World Health Organization, 2014). Due to the high nutrient availability in WWTPs, microbial cell density as well as their activities are increased. Therefore, WWTPs are considered "hot spots" of horizontal gene transfer (HGT). In addition to the HGT activity, WWTPs and MSS environments are characterized by a plethora of compounds posing a selective pressure to microorganisms, those include residual antibiotics, detergents, heavy metals, and many others. Such conditions promote evolutive changes in the genetic assembly of microorganisms as well as co-selection between different ARGs. They also make for an excellent gateway for the transfer of ARGs between environmental and clinically-relevant bacteria (Jacquiod et al., 2017; Su et al., 2015; Zhang et al., 2011). ARGs are naturally occurring in soil and their presence in WWTPs and or MSS is not surprising (Nesme and Simonet, 2015). However, the constant selective pressure in WWTPs promotes the acquisition of specific resistance feature by different microorganisms, including bona fide or opportunistic human and plant pathogens. The application of MSS to soil as fertilizer could therefore contribute to changes in the soil microbial community composition and introduce antimicrobial resistant bacteria (ARB), ARGs and mobile genetic elements (MGEs) to plant production systems (Wolters et al., 2018). Today, still not much is known about the effects of different stabilization approaches on the MSS-associated microbial communities or the abundance of ARGs and MGEs. How the presence or absence of AGRs and MGEs might be correlated with MSS's origin and the following stabilization processes is yet to be fully understood.

Among the prominent MGEs are broad host range plasmids of the IncQ subgroup. IncQ plasmids were first described in clinical environments, but were also found in manure, biogas digestates, soil and wastewater. All are able to transfer between a remarkable range of putative hosts (Moura et al., 2010; Stalder et al., 2019; Wolters et al., 2015). The non-conjugative but mobilizable IncQ plasmids can be divided into four subgroups, one of them is the IncQ1 subgroup. This subgroup was reported to carry antimicrobial resistance genes and was detected in such human pathogens as for example *Salmonella enterica* (Jibril et al., 2021; Loftie-Eaton and Rawlings, 2012; Oliva et al., 2017). So far, it is not clear whether different stabilization methods would influence the abundance of IncQ1 plasmids in MSS.

To investigate the influence of the method used to stabilize MSS as well as the capacity of the WWTP (number of inhabitant equivalents) on the structure of microbial community, we analyzed the composition of microbial communities in ten dewatered MSS samples from Croatian WWTPs. We analyzed MSS originating from the nine WWTPs described in the study of Černe et al. (2019) and an additional MSS in order to include a third replicate of none-biological stabilization process. The analysis of microbial communities was supplemented in this study with the assessment of the presence of different ARGs and MGEs. We selected ARGs and MGEs based on the known connection to MSS (Liu et al., 2019; Rizzo et al., 2013; Wolters et al., 2019; Wolters et al., 2022).

2. Materials and methods

2.1. Samples

In this study, samples were collected from dewatered MSS from ten Croatian WWTP with different modes of stabilization. The sampling and sample preparation of WWTP 1 to WWTP 9 were already described in the publication of Černe et al. (2019). WWTP 10 was additionally sampled in this study, in order to include a third replicate of no biological stabilization (Table 1). The physicochemical parameters for the WWTP 10 sample were determined according to the publication of and are shown in Table 1.

2.2. DNA extraction and quantitative real time PCR (qPCR) of target genes

Total DNA was extracted from 0.5 g of dewatered sewage sludge using the FastDNA SPIN Kit for Soil and purified with the GENECLEAN SPIN Kit (MP Biomedicals, Heidelberg, Germany), according to the manufacturer's instructions.

Target genes were quantified in total DNA by quantitative real-time PCR 5′-nuclease assays (TaqMan) in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Primers and TaqMan probes as well as PCR conditions used were described previously for sulfadiazine resistance genes *sul1* and *sul2* (Heuer and Smalla, 2007), class 1 and 2 integron integrase genes *intI1* and *intI2* (Barraud et al., 2010), quaternary ammonium compound resistance gene *qacE* and/or its attenuated variant *qacEΔ1* (Jechalke et al., 2014), aminoglycoside resistance gene *aadA* (Walsh et al., 2011), tetracycline resistance genes *tetM* (Peak et al., 2007), *tetW* and *tetQ* (Smith et al., 2004) as well as the *repB* gene variant specific for IncQ subgroup 1 plasmids (Hill et al., 2021). The 16S rRNA (*rrn*) genes were quantified by using the primers BACT1369F and PROK1492R and the probe TM1389F as described in (Blau et al., 2019). Differences in bacterial DNA amount and amplification efficiency between samples were normalized by dividing the target numbers of the respective genes by the *rrn* gene copy numbers and the results were log transformed (relative abundance).

Statistical tests were performed with R version 4.0.4 (R Core Team, 2021). Heatmap of relative abundances was created with the R package "pheatmap" (Kolde, 2019). Comparisons of means of classes "size" and

Table 1
WWTPs characteristics. The WWTPs are later sub-grouped according to their size (big; medium; small) and the applied stabilization treatment (none or lime; aerobic plus anaerobic; aerobic).

	WWTP 1[1]	WWTP 2[1]	WWTP 3[1]	WWTP 4[1]	WWTP 5[1]	WWTP 6[1]	WWTP 7[1]	WWTP 8[1]	WWTP 9[1]	WWTP 10[2]
Inhabitant equivalents (IE)	30,000	100,000	80,000	98,500	100,000	1000	2000	15,000	4000	25,000
Stabilization method	No stabilization	Lime	Aerobic + anaerobic	Aerobic + anaerobic	Aerobic + anaerobic	Aerobic	Aerobic	Aerobic	Aerobic	No stabilization
Size	Medium	Big	Big	Big	Big	Small	Small	Medium	Small	Medium
K [g/kg]	0.8	1.1	4.7	2.4	4.3	2.9	2.5	1.7	4.6	4.7
Ca [g/kg]	26.9	154.0	86.7	74.6	29.9	41.6	58.7	49.8	49.4	86.7
Mg [g/kg]	1.9	3.2	7.4	6.2	9.6	3.4	5.0	5.4	4.6	7.4
P [g/kg]	7.0	7.1	3.2	24.2	25.3	34.0	36.1	21.7	20.7	3.2
Mn [mg/kg]	33.1	38.9	715.0	274.0	308.0	221.0	128.0	101.0	83.5	715.0
Mo [mg/kg]	3.9	3.2	0.4	13.8	3.3	8.0	5.0	6.2	3.7	0.4
Fe [g/kg]	2.9	2.5	27.0	13.9	17.3	29.7	11.7	4.0	4.3	27.0
Cd [mg/kg]	0.8	0.6	0.9	0.7	0.7	1.2	1.0	1.3	0.8	0.3
Cr [mg/kg]	26.4	15.8	55.7	109.0	101.0	39.4	22.9	34.7	24.3	41.6
Cu [mg/kg]	123.0	84.1	660.0	156.0	364.0	185.0	230.0	171.0	159.0	27.0
Hg [mg/kg]	0.9	0.8	1.9	1.9	1.7	0.5	0.4	0.9	1.7	0.1
Ni [mg/kg]	11.0	7.9	41.2	52.6	38.8	23.8	21.2	19.9	17.6	25.2
Pb [mg/kg]	31.9	33.6	49.4	72.1	32.2	30.3	11.8	68.1	57.9	17.0
Zn [mg/kg]	822.0	478.0	1141.0	1254.0	943.0	842.0	1117.0	910.0	801.0	95.7
pH	5.8	12.3	7.1	7.0	7.2	6.9	6.8	6.6	6.7	8.1
EC [ds/m]	1.0	7.4	3.4	2.3	1.6	3.7	4.4	4.0	3.8	0.5
Organic_C [%]	57.4	38.0	33.4	28.4	25.8	42.3	40.7	43.9	44.5	5.7
Total_N [%]	2.6	3.3	5.5	3.9	3.3	7.5	6.0	7.5	7.7	0.4
C/N	21.8	11.7	6.0	7.4	7.9	5.6	6.8	5.9	5.8	13.4
Organic_matter [%]	87.3	55.8	55.7	47.2	44.9	69.4	68.7	73.7	74.5	7.1
Dry_matter [%]	23.3	39.0	18.7	27.3	25.6	15.4	8.3	16.6	94.0	66.8
Moisture [%]	76.7	61.0	81.3	72.7	74.4	84.6	91.7	83.4	6.0	33.2
Ash [%]	12.7	44.2	44.3	52.8	55.1	30.6	31.3	26.3	25.5	92.9

[1] Černe *et al.* (2019).
[2] this publication.

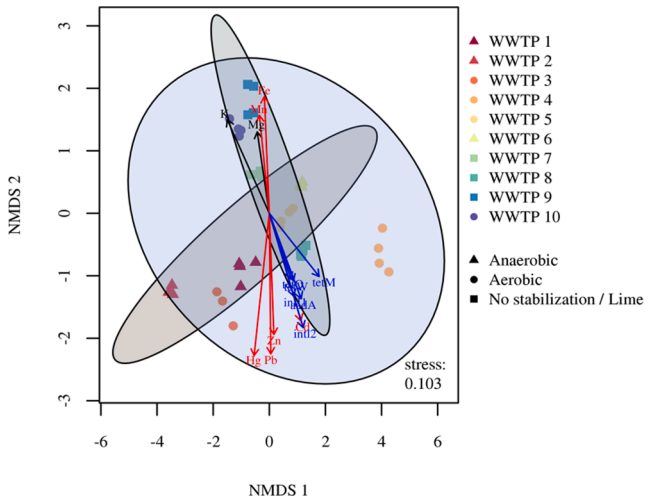

Fig. 1. Composition of the MSS microbial community is not correlated with biosolid's stabilization method. The non-metric multidimensional scaling (NMDS) ordination plot was calculated from the OTU table. Parameters significantly associated with microbial community structure ($p < 0.05$) are plotted as vectors, where the length and direction indicate the contributions to the principal component. Ellipses indicate 95 % confidence interval for treatment groups. Outliers 2.1 and 3.4 were removed before analysis based on Bray-Curtis dissimilarity. WWTP 1 – 10 represent the different wastewater facilities. Triangles represent MMS samples with aerobic and additional anaerobic fermentation. Circles represent samples with aerobic fermentation, squares represent sample with no stabilization procedure but with addition of lime. All variants were analyzed in four replications.

"treatment" were compared by ANOVA and estimated marginal means using the "emmeans" function (Lenth, 2021). A correlation between relative abundances of tested genes and size numbers (inhabitant equivalents), the stabilization method and presence of metals was tested with the function "cor.test" (R Core Team, 2021).

2.3. 16S rRNA sequencing

Four samples per WWTP sample were taken for amplicon sequencing. A 465 bp fragment of the 16S rRNA gene, containing the hypervariable V3 and V4 regions, was amplified using the primers 341F and 806R (Sundberg et al., 2013). In a second PCR reaction step, spacer and adapter barcode tags were added (Nunes et al., 2016) PCR products were purified using HighPrep PCR paramagnetic beads (MagBio Genomics, Gaithersburg, MD, USA) according to the manufacturer's instructions, using a 0.65:1 of beads:PCR reaction volume ratio. Samples were adjusted to equimolar concentrations using SequalPrep Normalization Plate 96 Kit (Invitrogen, Carlbad, CA, USA) and pooled using a 5 µL volume for each sample. High-throughput amplicon sequencing was carried out following manufacturer's instructions on a MiSeq platform (Illumina) using MiSeq V2 kit in paired-end mode (2 × 250 bp). Sequences are available at ENA-SRA under the accession number PRJEB52342.

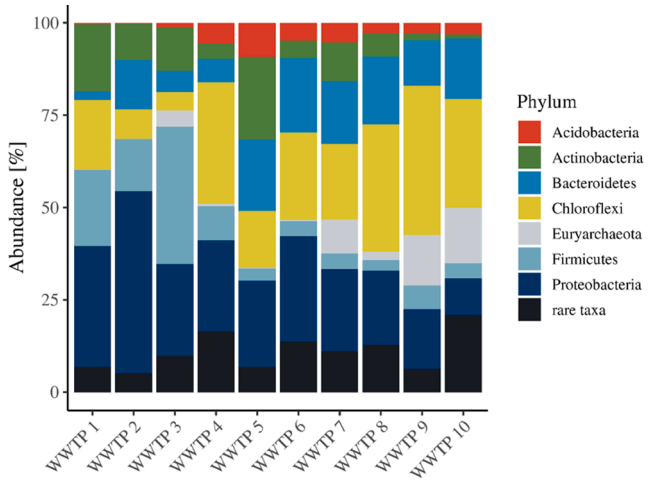

Fig. 2. Relative abundance of phyla identified in MSS samples from the testes WWTPs. Total DNA from dewatered composted sewage sludge was sampled and the relative abundance was calculated as percentage of 16S rRNA gene sequences belonging to a particular phylum in each sample. Each experimental variant was represented by at least three replicates. Outliers 2.1 and 3.4 were removed before analysis based on Bray-Curtis dissimilarity.

2.4. Sequence analyses

Raw sequences were trimmed of primer sequences using cutadapt (Martin, 2011). After merging, the sequences were clustered with 97 % pairwise sequence similarity using UPRASE (Edgar, 2013) and chimeras were removed using UCHIME (Edgar et al., 2011). Taxonomic annotation was performed using mothur "classify.seqs" and "method = wang" with a minimum bootstrap probability of 0.8 (Schloss et al., 2009) against RDP database trainset 16 (Cole et al., 2014). After sequencing 1,943,573 reads and 10,368 OTUs were obtained with an average number of 48,589.32 reads and a minimum of 17,866 per sample.

Alpha-diversity was calculated at the OTU level using the Observed Richness and Shannon Index. Significant differences between treatments were calculated using one-way analysis of variance (ANOVA) with post-hoc Tukey test. Beta-diversity at the OTU level, indicating similarities of the microbial community composition between different treatments, was analyzed by non-metric multidimensional scaling (NMDS) prior to testing significant differences between size and treatment classes a permutation test (10,000 permutations) on OTU level with the R package "vegan" (Oksanen and Guillaume Blanchet, 2017) and the function "adonis" was performed. Differential abundance analysis was performed with DESeq2 (Love et al., 2014) after selecting the method using DAtest (Russel et al., 2018).

3. Results

3.1. Community composition is not depended on the biosolids processing method

In the first step, we analyzed the impact of the processing procedure of wastewater-originated biosolids on their microbial structure. We analyzed MSS from ten different WWTPs. Considering that they differ in stabilization processes, we divided them into three groups: i) aerobic processing; ii) aerobic plus anaerobic stabilization; and iii) no stabilization and with lime addition. In addition, the WWTPs were divided according to the inhabitant equivalent (IE) number into: i) small, with IE below 10.000; ii) medium, with IE between 10.000 and 50.000 and iii) big with>50.000 IE. An ordination by non-metric multidimensional scaling (NMDS) revealed a clear clustering of replicated samples (Fig. 1). At the same time, NMDS showed that the different methods of biosolid processing: no stabilization, aerobic or anaerobic digestion, and addition of lime did not result in similar microbial communities (ANOSIM, R < 0.25). Similarly, the division regarding to WWTP size (small, medium or big) did not result in a clear pattern. Very interesting was that several other factors were associated with the composition of bacterial communities in particular MSS samples. For example, the presence of heavy metals (iron, lead, mercury or zinc) in samples seems to be associated with particular microbial community (Fig. 1).

Similarly unspecific picture could be drawn, when the relative abundance of phyla in different MSSs was taken into account. The most abundant phyla were Proteobacteria, Chloroflexi, Firmicutes and Actinobacteria. In some MSS samples, Acidobacteria and Euryarchaeota

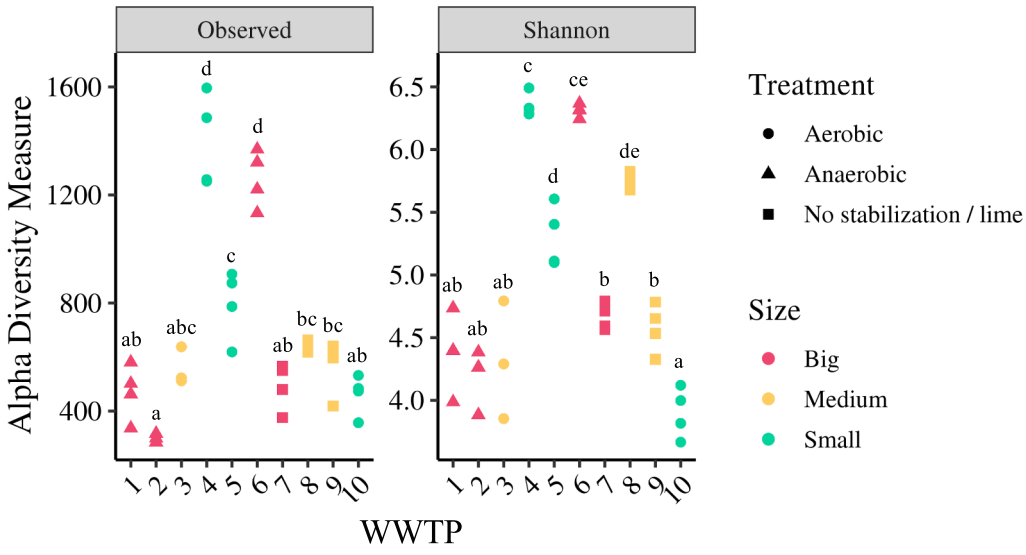

Fig. 3. Differences between the prokaryotic community diversities could not be accorded to the size nor processing technique of WWPTs. All WWTPs were divided into big (IE > 50.000), medium (IE between 10.000 and 50.000) and small (IE < 10.000). Treatments were divided into aerobic, anaerobic (incudes aerobic and anaerobic fermentations), and no stabilization but with lime addition. Observed richness and Shannon index were calculated for all MSS samples. Outliers 2.1 and 3.4 were removed before analysis based on Bray-Curtis dissimilarity, $n = 4$. Differences between samples were identified using ANOVA and estimated marginal means.

were observed. However, we could not identify a pattern, which could be associated with either the processing type used in a particular WWTP or with its size (Fig. 2).

In the next step, we analyzed the observed richness and the alpha diversity of microbial communities in the different MSS samples (Fig. 3). We observed the highest richness in biosolids from WWTP 4, a very small (IE of 2.000) facility with aerobic stabilization process, the lowest was observed in MSS from WWTP 2, which is a big facility (IE of 80.000) with both aerobic and additional anaerobic processing. However, comparing other WWTPs with similar characteristics, for example WWTP 1 (also a big facility with IE of 98.500, using aerobic and additional anaerobic processing), the observed richness was quite different. Similarly, the second highest rate was observed in MSS from WWTP 6, a big facility with aerobic and additional anaerobic processing.

Interestingly, both WWTPs with the highest richness scores, also displayed the highest Shannon index, representing the diversity of the bacterial community. However, neither the processing type nor the sizes of the facility seem to have an impact on the diversity of the bacterial community (Fig. 3).

3.2. MSS samples have specific bacterial communities

Since neither the size of the WWTP nor the processing of the MSS had an apparent influence on the general composition of biosolids' bacterial community, we wondered if specific taxa would be affected. To address that question, we calculated the differential abundance of taxa on genus level, using DESeq2. The heatmap shown in Fig. 4, presents groups with significantly different (adj. $p < 0.05$) abundances in all ten analyzed samples. Interestingly, only eight groups (*Anaerolineaceae*, *Methanosaeta*, *Trichococcus*, *Acinetobacter*, *Romboutsia*, *Clostridium sensu stricto 1*, *Turicibacter*, *Intestinibacter*) were present in all MSS. Among those were *Anaerolineaceae*, which are often associated with sediments, the abundance was enhanced in MSS from WWTP 9 and 4, representing small, aerobic and a medium facility with no stabilization, respectively. Interesting was the enhanced presence of *Methaanosaeta* in MSS from WWTP 10 (small with aerobic treatment), species from this group are some of the most active methanogens, producing an extensive amount of methane. Another soil-related group present in all facilities were *Trichococcus*, those are mesophilic and psychrotolerant bacteria able to utilize different sugars, sugar alcohols and polysaccharides. Quite worrying was the abundance of pathogen-relevant groups in biosolids originated in all facilities. For example, *Acinetobacter* were more abundant in biosolid from WWTP 1, (big facility with aerobic and anaerobic treatment). *Acenitobacter* are important soil organisms, contributing to mineralization of aromatic compounds. However, they are the key source of infection in debilitated patients in the hospital, especially *Acinetobacter baumannii*. Another group present in all samples was *Burkholderiaceae*. This family includes several pathogenic species, such as *Burkholderia mallei* or *Burkholderia pseudomallei*. Even more pronounced was the presence of *Clostridia*. This genus includes several significant human pathogens, including the causative agents of botulism and tetanus and its presence was enhanced in MSS from WWTP 3, a medium size facility with aerobic treatment. In the same samples (MSS from WWTP 3) several other groups related to human intestinal tract, animal guts or feces were present, those include *Romboutsia*, *Turicibacter* and *Intestinibacter*. Remarkably, samples from WWTP 2 and 1, both big facilities with aerobic and anaerobic treatments, exhibited the lack of quite few bacterial groups, otherwise present in the other facilities, for example: *Pedomicrobium*, *Reyranellaceae*, *Anaerolineae*, *Methanobacterium*, *Defluviimonas* or *Syntrophaceae*, among others.

3.3. Presence of antibiotic resistance genes does not depend on size of the WWTP or the treatment

In the next step, we determined the prevalence of several antibiotic resistance genes (ARG) in the MSS samples. A qPCR approach was used to assess the abundance of *qacE* (conferring resistance to quaternary ammonium compounds), the aminoglycoside resistance protein *aadA*, *sul1/2* conferring resistance to sulfonamide, as well as *tetM*, *tetW* and *tetQ* genes, conferring resistance to tetracycline. In addition, we assessed the presence of integrase genes 1 and 2, *intI1/2*, and an IncQ1 plasmid-specific gene, *incQ1*. The mean gene presence in four replicates was presented relative to the abundance of the 16S rRNA gene (*rrn*) (Fig. 5). The genes *aadA* and *sul2* displayed the highest relative abundance with values up to log −1.1, followed by *qacE* (not specifying between *qacE* and *qacEΔ1*) and *intI1* with up to log −2.0 and log −2.4, respectively. Gene specific for plasmids of the IncQ1 subgroup had lower abundance, with log −4.9 until −3.4. No IncQ1-specific genes were detected in MSS samples from WWTP 3, 9 and 10. These results demonstrate fairly high abundance of the tested genes in MSS from all WWTP facilities. Interestingly, the lowest abundance of ARG and MGE genes was detected in the WWTP 9 (a medium facility with no stabilization), (Fig. 5).

Given the observed differences in the prevalence of numerous ARG or MGE, we sought to test whether the differences could be explained by the applied stabilization treatment in the particular WWTPs (aerobic/anaerobic/no stabilization & lime) or with the size of the facility. To this end, we used the Inhabitant Equivalents (IE) and divided all tested WWTPs into, large (IE > 50.000), medium (IE between 10.000 and

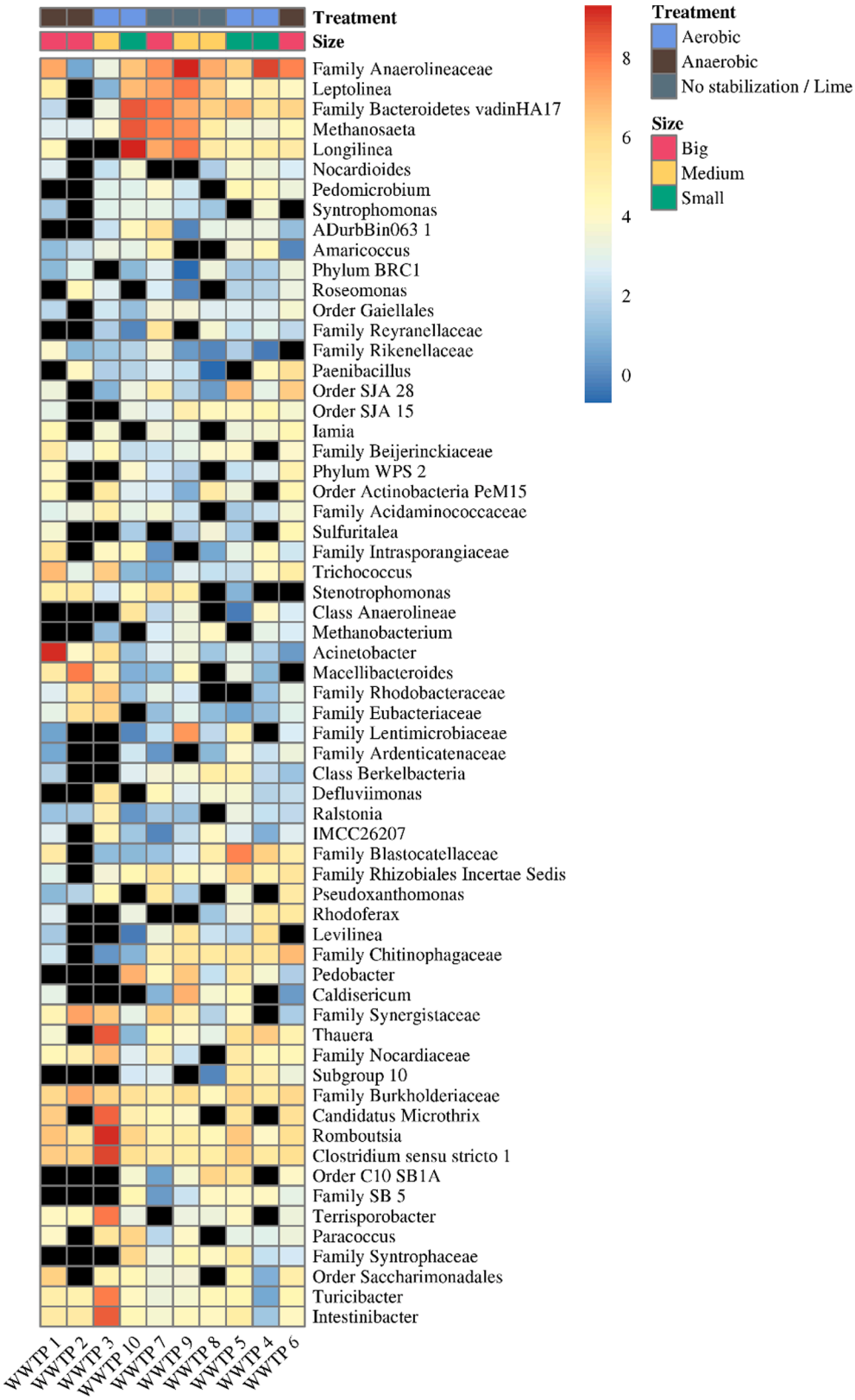

Fig. 4. Heatmap of $\log_{10}$-transformed relative abundance of taxa shows differences between treatment and size based on DESeq2. The differences in relative abundances were calculated using DESeq2. Only differential abundant taxa with an adjusted p value of 0.05 are displayed. Clustering is based on Euclidean distances. Outlier samples 2.1 and 3.4 were removed before analysis based on Bray-Curtis dissimilarity.

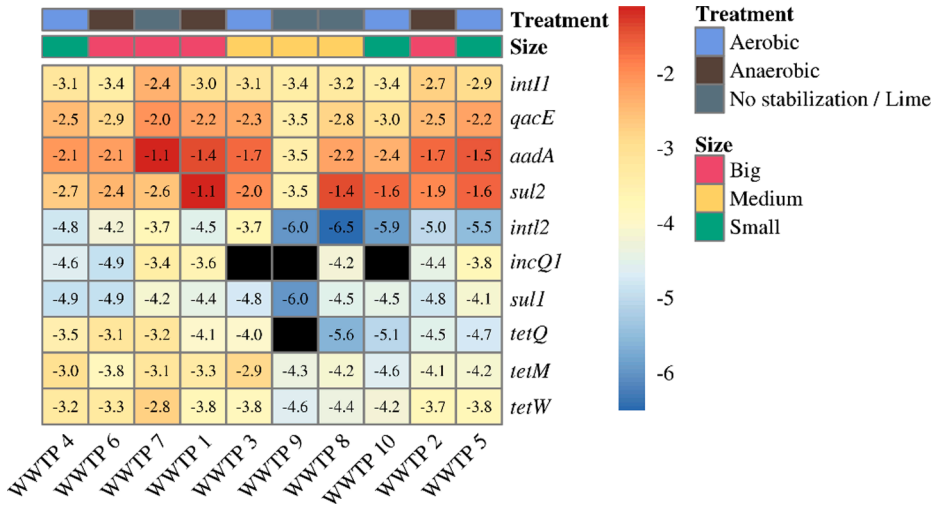

Fig. 5. Relative abundance of particular antibiotic resistance genes and mobile genetic elements. Displayed is the $\log_{10}$ transformed abundance of tested genes relative to the 16S rRNA gene. Clustering is based on Euclidean distances. Black squares indicate values below quantification limit for the respective gene.

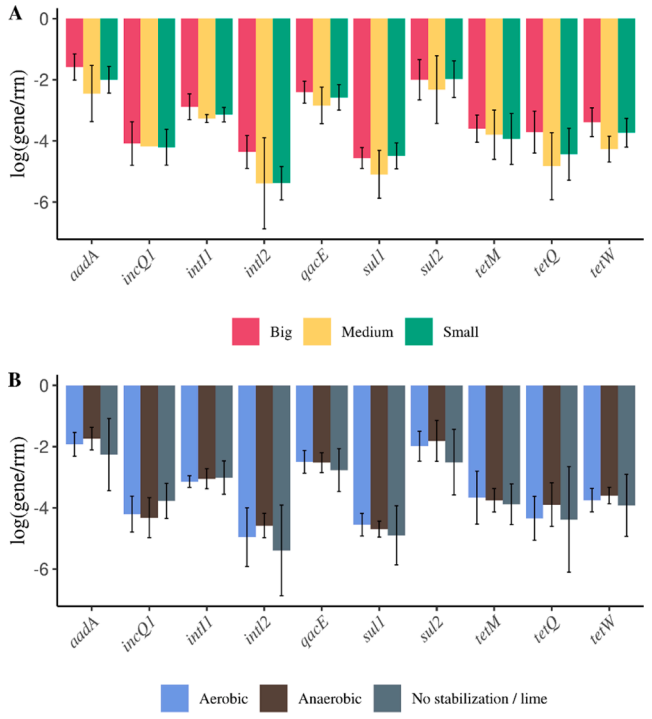

Fig. 6. Presence of antibiotic resistance genes and mobile genetic elements. Displayed is the $\log_{10}$ transformed abundance of tested genes relative to the 16S rRNA gene. For IncQ1 and medium size, no standard deviation is shown because the gene was only detected in MSS from one WWTP of this size. No significant differences were detected between classes "size" (A) or "treatment" (B) within particular gene (Tukey test, $p > 0.05$). The WWTP facility with IE > 50.000 were termed big, medium (IE between 10.000 and 50.000) and small (IE < 10.000). Regarding the treatment, WWTPs without stabilization and with lime application were grouped together (no stabilization / lime); aerobic treatment (aerobic) and a combination of anaerobic and aerobic treatments (anaerobic) were the other treatments groups. No significant differences were detected.

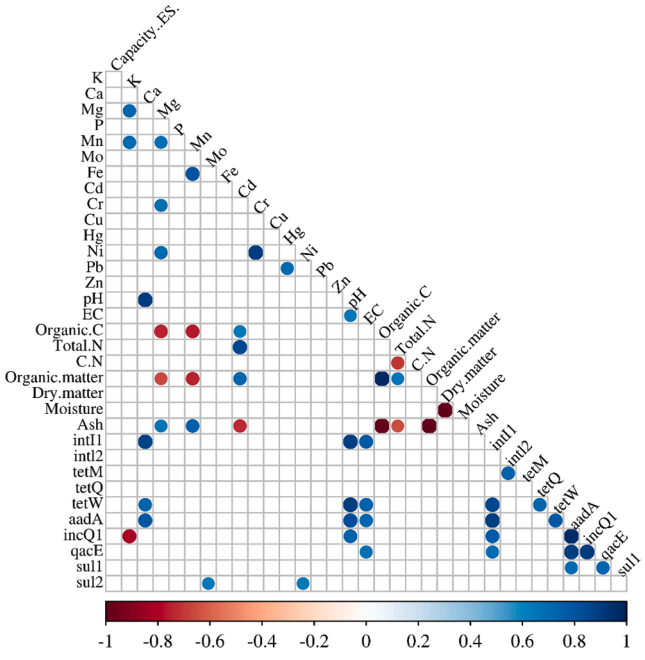

Fig. 7. Presence of several antibiotic resistance genes correlated with the physicochemical properties of the municipal sewage sludge. Pearson's rank correlation coefficient was used to assess potential correlations. The plot shows only correlations with a p value of 0.05 or lower. Negative correlations are shown in red and positive correlations are shown in blue. Correlation with p values above 0.05 were considered as not significant and excluded from the analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

50.000) and small (IE < 10.000), (Table 1). Regarding the treatment, we grouped WWTPs without stabilization and with lime application together, in addition to aerobic treatment and a combination of anaerobic and aerobic treatments. We could observe no differences between the different classes of ARG and MGE and the size (IE) of the facility (ANOVA post hoc Tukey test, $p > 0.05$), (Fig. 6A) or between the treatment type and the presence of ARG or MGE (ANOVA and post-hoc Tukey test, $p > 0.05$), (Fig. 6B).

Subsequently, we wondered if the presence of ARG and MGE correlated with particular MSS features. The correlation between specific soil characteristics were partially already described (Černe et al., 2019). Here, we correlated the abundance of ARG and MGE with the presence of different metals and other elements, pH, organic carbon and other parameters. The Pearson's rank correlation revealed several highly positive correlations as well as some negative (Fig. 7). For example, we could show that the relative abundance of *intI1* gene, class 1 integron-

integrase, positively correlated with the relative abundance of *qacE/qacEΔ1* ($p < 0.05$). The same class 1 integron-integrase coding gene (*intI1*) was positively correlated with *tetW* and *aadA* genes and the IncQ1 plasmid-specific gene. Likewise, the IncQ1 plasmid-specific gene was positively correlated with *aadA*, *qacE* and with electrical conductivity (EC). In contrast, the abundance of the class 2 integron-integrase coding gene, *intI2*, was positively correlated only with *tetM*. A negative correlation was found between the abundance of the IncQ1 plasmid-specific gen and the content of potassium (K). The other ARG *tetW* and *aadA* were on one hand positively correlated with calcium (Ca), pH and EC, and on the other hand *qacE* only with EC. Finally, very interesting was the positive correlation between the ARG *sul2* and the heavy metals molybdenum (Mb) and nickel (Ni).

4. Discussion

In the present study, we investigated the influence of the stabilization method as well as the size of wastewater treatment plants (WWTP) on the structure of microbial communities as well as the abundance of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in municipal sewage sludge (MSS). In a previous study, Černe et al. (2019) analyzed physicochemical characteristics of some of the MSS tested here, and concluded that eight out of nine MSS are suitable replacements for mineral fertilizers in agriculture. Their high content of P and N would benefit crop plants. Since the concentration of trace metals was below the European and Croatian limits, those MSS were considered safe for human health and for environment. In our study, we aimed to increase the knowledge about MSS and complement the previous study with information on ARGs and MGEs. All ARGs and MGEs selected here were typically found in samples from WWTPs and might be involved in the spread of resistance genes in exposed environment and therefore reach humans (Liu et al., 2019; Wolters et al., 2019).

All ARGs and MGEs tested by the qPCR approach were detected in majority of the sewage sludge samples. Genes with the highest abundance were *aadA, sul2, qacE/qacEΔ1* and *intI1*. Especially the class 1 integrons (*intI1*) are widely distributed in clinical settings and are able to acquire, exchange, and express genes embedded in their gene cassettes. In this manner, their bacterial hosts can obtain resistance genes for almost all antibiotic families as well as resistance to disinfectants and heavy metals (Gillings et al., 2015; Jechalke et al., 2014). Due to their properties and mobility, class 1 integrons translocate between a wide variety of pathogenic and nonpathogenic bacteria by horizontal gene transfer. Previous studies suggested even to use class 1 integron integrase genes as a proxy for anthropogenic pollution (Gillings et al., 2015). Indeed, we found a correlation between the class 1 integron integrase gene (*intI1*) and several ARGs (Fig. 7), which would support this intent. Even though, the high relative abundance of *intI1* genes in MSS samples was not surprising, it might present a risk and favor the transfer and spread of ARGs in environments where MSS are applied as fertilizers. The aminoglycoside resistance gene *aadA* is often associated with class 1 integrons in both, environmental and in clinical isolates (Binh et al., 2009; Kiiru et al., 2013). The quaternary ammonium compound resistance gene *qacEΔ1* was associated rather with the class 1 integrons of clinical origin (Gillings et al., 2009). These findings are supported by the significant correlations between *intI1*, *aadA* and *qacE/qacEΔ1* observed in this study. On the contrary, we observed that the sulfonamide resistance gene *sul2,* also with high relative abundance, was not correlated with the *intI1* gene. Interestingly, this gene had higher relative abundance than *sul1*, while in other studies a higher occurrence or concentration of *sul1* was reported in raw sewage and in bacterial isolates from activated sludge (Rolbiecki et al., 2020). Furthermore, *sul2* was associated rather with agricultural systems such as livestock production and aquaculture, it seems therefore, that *sul2* was enriched in MSS because of the applied stabilization method. The plasmids of the incompatibility group IncQ-1 are known to carry diverse antibiotic resistance genes and to transfer efficiently in a broad host range (Loftie-Eaton and Rawlings, 2012). Surprisingly, in the present study, genes specific for plasmids of the IncQ-1 subgroup had low abundance, no IncQ-1-specific genes were detected in MSS samples from WWTP 3, 9 and 10. These findings support the notion that the detected ARGs could be distributed by *intI1*. The size of WWTP and applied treatment had no significant effects on the relative abundance of either of the detected genes, similar to previous reports (Wolters et al., 2022).

Microbial communities of MSS were analyzed by sequencing of 16S rRNA gene fragments. The obtained results revealed that the stabilization method (aerobic, aerobic plus anaerobic, no stabilization or addition of lime) had a significant effect on specific taxa, however, no clear clustering regarding the treatment or size was observed (Fig. 4). Among the phyla with the highest relative abundances were Proteobacteria, Chloroflexi, Firmicutes and Bacteroidetes. Members of Proteobacteria, Chloroflexi and Bacteroidetes are known to have traits related to wastewater treatment; those may include polychlorinated biphenyl degradation or involvement in carbon or sulfur cycles (Fennell et al., 2011; Parnell et al., 2010; Pujalte et al., 2014; Wasmund et al., 2017). In addition, Firmicutes are capable to degrade a large range of substrates present in MSS.

Upon closer inspection on genus level, we could identify several taxa with potentially useful traits. The family *Anaerolineae* within the phylum Chloroflexi was detected in high proportion in MSS samples from WWTP 9, and from other WWTPs of different size and treatment classes. *Anaerolineae* (and the phylum Chloroflexi) were suggested to be syntrophically involved in methanogenic degradation of alkanes (Liang et al., 2015). *Clostridia* were found in high abundance in samples from WWTP 3. This class belongs to the phylum Firmicutes and includes such pathogenic bacteria as *Clostridium perfringens* or *C. difficile.* Both are commonly encountered in raw sewage and are able to survive the treatment process (Cyprowski et al., 2018; Hirata et al., 1991; Xu et al., 2014). Other taxa, including *Anaerolineaceae*, *Methanosaeta* (*Methanomicrobia*), *Methanolinea* (*Methanomicrobia*), *Leptolinea* (*Anaerolineae*) and *Longilinea* (*Anaerolineae*) are known to carry methanogenic traits. These taxa can utilize organic pollutants as source of energy and therefore play a crucial role in waste water treatment. Several additional taxa were found enriched in samples from specific treatments or WWTP sizes however; we could not distinguish a specific clustering pattern. Noteworthy was the presence of several bacteria from the genera *Stenotrophomonas*, *Acinetobacter*, *Ralstonia* as well as the family *Burkholderiaceae*. Those taxa include opportunistic pathogens, which are associated with rhizosphere (Berg et al., 2005) and were very often associated with nosocomial infections and caused severe health problems mostly in clinical environments. Frequent and abundant use of disinfectants was suggested to favor the dissemination of such opportunistic pathogens Wolters et al. (2022). Unfortunately, we have no information on hospitals present in the catchment area of the analyzed WWTPs, which could help to ascertain whether or not the conclusion drawn by Wolters et al. (2022) applies to the present study.

Taken together, our results suggest, that the distribution of ARGs, MGEs or pathogenic bacteria cannot be explained by neither the size of WWTPs nor the applied MSS stabilization method. Since both, the waste water treatment and the MSS stabilization will shape the microbial community and therefore the distribution of ARGs and MGEs, additional studies are required in order to disentangle the impact of specific processes. Nevertheless, caution is advised and the long-term impact assessments of repeated applications are necessary before MSS is used as s soil amendment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data were submitted to ENA-SRA.

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