sustainability

MDPI

Article

Tracking of Antibiotic Resistance Genes in Sludge from Treatment Plants for Organic Fraction of Municipal Solid Waste

Elena Franchitti *, Antonino Mafrica and Deborah Traversi

Department of Public Health and Pediatrics, University of Turin, Piazza Polonia 92, 10126 Turin, Italy; antonino.mafrica@edu.unito.it (A.M.); deborah.traversi@unito.it (D.T.)
* Correspondence: elena.franchitti@unito.it

Abstract: Bacteria quickly acquire the ability to survive or grow in the presence of an antibacterial agent that should be able to inhibit or kill them, leading to increased mortality caused by infective diseases. The digestate from the anaerobic digestion (AD) of the organic fraction of municipal solid waste (OFMSW) is spread on soil, but the knowledge on the presence and persistence of the antibiotic resistance genes (ARGs) is limited. Thus, this study aims to evaluate the presence of seven ARGs (bla_{TEM}, bla_{OXA}, *ermB*, *qnrB*, *sulI*, *sulII*, *tetA*, and *tetW*) in the OFMSW and their persistence after the AD, using the innovative droplet digital Polymerase Chain Reaction (ddPCR), not yet used on this matrix. A total of 31 samples were collected from the influent and effluent of the AD in two plants located in Northern Italy. ARG concentration ranged between 4 and 9 Log gene copies/kg of sludge. A limited reduction in the concentration of antibiotic resistance targets given by AD was observed in the study (<1 Log). The persistence of ARGs after mesophilic digestion of the OFMSW suggests a risk of horizontal transmission when directly spread on soil. Further evaluations are needed for safe and sustainable reuse of such sludges.

Keywords: antimicrobial resistance; antibiotic resistance genes; ddPCR; anaerobic digestion; organic fraction of municipal solid waste; waste management; circular economy

Citation: Franchitti, E.; Mafrica, A.; Traversi, D. Tracking of Antibiotic Resistance Genes in Sludge from Treatment Plants for Organic Fraction of Municipal Solid Waste. *Sustainability* **2024**, *16*, 10922. https://doi.org/10.3390/su162410922

Academic Editor: Carlos A. Teixeira

Received: 27 September 2024
Revised: 25 November 2024
Accepted: 9 December 2024
Published: 13 December 2024

1. Introduction

Nowadays, antibiotic resistance is an increasingly alarming problem for human health. Antibiotic resistance consists of the adaptation of certain microorganisms that become able to survive or grow in the presence of a specific concentration of an antibacterial agent, usually sufficient to inhibit microorganisms of the same species. This phenomenon is a consequence of the misuse of antibiotics not only in human health care, but also in veterinary and agricultural practices, also producing an environmental accumulation [1,2]. Infections of resistant microorganisms cannot be treated with standard therapies (as they are ineffective). As a consequence, the following may occur: prolongation of disease, occurrence of complications, increased risk of death, and increased risk of epidemic outbreaks. In addition, health care costs also increase [3]. Antibiotic resistance also develops because some bacterial genes can shift between chromosomal and extra-chromosomal DNA, even between bacteria of different species and genera. Gene transfer can occur through several mechanisms: transformation, transduction, and conjugation [4].

Antibiotics can persist for a long time in the environment. They are excreted from the human body, the most important reservoir, with resistant bacteria or with biological samples such as food waste and fomites [2]. Recently, antibiotic compounds have been included in the watch list of the emerging compounds for environmental pollution mitigation [5]. The EU commission highlighted the necessity of better addressing the role of the environment in the "One Health Action Plan against AMR", with a focus on environmental monitoring [6]. Municipal solid waste dumps can also be considered as reservoirs of antibiotics and a vehicle for the spread of antibiotic resistance genes as they contain a great amount of food

waste from both animal and vegetal origins [1]. The amount of the organic fraction of the municipal solid waste (OFMSW) has increased over time due to the improvement of waste sorting, reaching an annual production of 71 million of tons in the EU, with 35% of such organic materials treated in plants by anerobic digestion (AD). Anaerobic digestion is a green biotechnology carried out in extreme conditions, such as absence of oxygen and high temperatures, in which a microbial community decomposes the organic matter present in the sludge to produce biogas. The biogas is mainly composed of methane (CH_4) and carbon dioxide (CO_2), commonly used to produce heat and electricity. New or updated treatment plants with higher capacities were installed during recent decades all over the world, and their number could be above 11,600 plants by 2035, two times the number of plants already present [7]. However, the occurrence and the removal efficiency of AMR in OFMSW is not well known. Various articles showed an effective reduction of the resistant bacteria and a reduction of some AMR genes (ARGs) and an increment of others [8]. Despite these findings, an exhaustive risk assessment is missing. Antibiotics have also been included in various research studies to increase the biogas yield of anaerobic digestion through bacterial selection [9,10], but the use of such compounds must be carefully evaluated when not aimed at sanitary purposes. Therefore, proper organic waste management is crucial to avoid environmental spread of resistant microbes but also of AMR genetic elements that can potentially be transferred to other microbes. However, the health risk related to the presence of ARGs in the effluent of the OFMSW treatment is not yet fully known and, generally, culture methods are used to gather information on the potential pathogenicity and infectivity. Non-cultural methods based on the detection of AMR genes are now also applied to the sludge coming from the AD process. Among these, generally, real-time quantitative Polymerase Chain Reaction (qPCR) has been used, although its application is hindered by technical disadvantages, such as the presence of inhibitors, including metals and humic acids, of the reaction [11]. New quantification methods based on PCR, which are rarely used on this kind of matrices, can help to overcome some limits. The technical development of biomolecular methods is very fast and laboratory evidence has also been produced starting from digestate samples [12].

This study aims to screen the occurrence and persistence, after AD, of the most prevalent ARGs in organic waste sludges, in full-scale settings, using innovative biomolecular methods.

2. Materials and Methods

2.1. OFMSW Plants and Samplings

Two of the main OFMSW treatment plants of Northern Italy were involved in this study. The OFMSW-A receives 60,000 tons/year, OFMSW-F 45,000 tons/year. In such technologically advanced plants, an AD phase is included. The sampling activities covered a 6-month period from November 2021 to April 2022, in which both implants provided their samples consecutively each week. From the OFMSW plants, 16 samples from the influent of the digester and 16 digestate samples were collected by the plants' workers. Chemical and physical data on the samples, including T (temperature), pH, percentage of Total Suspended Solids (%TSS), and percentage of Volatile Suspended Solids (%VSS), were provided by the involved plant with standard internally validated methods and are reported in Tables 1 and S1.

Table 1. OFMSW plant. Physical and chemical characteristics of the sludge and the concentration of DNA extracted from the samples.

	OFMSW-A		OFMSW-F	
	Medio	**Dev. St.**	**Medio**	**Dev. St.**
pH	6.83	1.55	6.93	1.21
Temperature	42.34	3.78	40.90	0.24
%TSS	5.86	2.98	4.61	1.18
%VSS	4.54	2.98	3.20	1.19
DNA ng/µL	55.97	47.54	66.61	43.51

Once collected, samples were delivered to the Environmental Hygiene Section of the Department of Public Health and Pediatrics of the University of Turin in one-liter containers.

2.2. DNA Extraction and ARGs Quantification

The extraction of nucleic acids from the samples took place within 24 h of sampling. Nucleic acid extraction from samples was conducted in duplicate using a DNeasy Powersoil Pro Kit (QIAGEN, Venlo, The Netherlands) following the manufacturer's instructions. The chosen extraction methodology was based on column filtration. The samples' cells were lysed through chemical and mechanical homogenization, the lysis buffer was added to a mixed zirconium bead tube containing the 250 µL of sample and vortexed. The supernatant was then subjected to inhibitor removal for clean-up. Then, purified lysate was mixed with an equal volume of DNA binding solution and passed through a silica spin filter membrane. That membrane was washed with a two-step washing regime. The DNA bound to the silica was then eluted using a 10 mM Tris elution buffer. DNA was eluted in 100 µL of elution solution and quantified using a TECAN Infinite M200 Pro spectrophotometer, a NanoQuant Plate (Tecan Group Ltd., Männedorf, Swizerland), and i-Control™ software (version 1.11.10). Extracts were stored at −80 °C for subsequent biomolecular analysis. The heterogeneity of the matrix was high so the extraction was not always optimal for biomolecular analysis, even though the sample was homogenized. To select extracts on which to conduct further studies, excluding very poor extractions, a cut-off value for DNA of 5 ng μL^{-1} was set [13], finally obtaining 22 samples.

In this paper, a target-specific analysis was chosen to select the genes of interest, and two criteria were followed: (i) it should be one of the ARGs that induce a resistance ability against the most prescribed groups of antibiotics in Italy according to the European Centre for Disease Control (ECDC) [14] (Table 2), and (ii) the literature should include a solid PCR protocol with primers published in more papers. Consequently, seven ARGs were selected: bla_{OXA}, bla_{TEM}, *ermB*, *qnrB*, *sulI*, *sulII*, *tetA*, and *tetW*. *BlaOXA*, which encodes for carbapenemases, results in resistance to carbapenems and penicillins, both subclasses of β-lactam antibiotics. The carbapenemases, which are members of the β-lactamases, destroy the β-lactam ring. *BlaTEM*, which gives resistance to ampicillin and penicillin G through β-lactamase production, acts similarly to *BlaOXA*. *ErmB* and *qnrB* confer resistance toward macrolides and fluoroquinolones through a ribosomal protection mechanism. *SulI* and *sulII* both carry resistance to sulfonamides through a process of enzymatic inactivation that alters the chemical structure of the antibiotic molecules. Finally, *tetA* and *tetW* both bring resistance toward tetracyclines, but by two different mechanisms: *tetA* through increased expression of efflux pumps that expel the antibiotic from the bacterial cell, while *tetW* through a ribosomal protection mechanism. According to the most recent data from the European Surveillance of Antimicrobial Consumption Network, the most consumed antimicrobials in Italy in 2023 were penicillins and β-lactam antibiotics (399.03 tonnes per year), followed by macrolides, lincosamides, and streptogramins (40.49 tonnes per year), and, finally, other β-lactam antibiotics (58.20 tonnes per year) and quinolones (30.04 tonnes per year), sulfonamides (50.05 tonnes per year), and tetracyclines (3.31 tonnes per year) [14]. The gene encoding the *16S rRNA* V3-V4 region was added to evaluate the bacterial presence. Primers were selected by comparing more than three papers and after Primer BLAST alignment. After a screening phase of the positivity with traditional PCR, conducted by amplifying the ARGs with a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) and evaluated by the presence of bands in gel electrophoresis, antimicrobial resistance was measured using the biomolecular method of the droplet digital PCR (ddPCR, Bio-Rad, Hercules, CA, USA) on the DNA extracts.

Table 2. List of antibiotic resistance genes. The primers and the thermal protocols are listed.

Antibiotic Resistance Gene	Primers	Annealing Temperature	Amplicon Size (bp)	Reference
16S rRNA	F:5′-ACTCCTACGGGAGGCAGCAG-3′ R:5′-ATTACCGCGGCTGCTGG-3′	61 °C	2736	[15]
blaOXA	F: 3′-TATCTACAGCAGCGCCAGTG-5′ R: 3′-CGCATCAAATGCCATAAGTG-5′	61 °C	199	[16]
bla_{TEM}	F: 3′-CATTTTCGTGTCGCCCTTAT-5′ R: 3′-GGGCGAAAACTCTCAAGGAT-5′	61 °C	167	[16,17]
ermB	F: 3′-AAAACTTACCCGCCATACCA-5′ R: 3′-TTTGGCGTGTTTCATTGCTT-5′	61 °C	139	[18]
qnrB	F:3′-GCGACGTTCAGTGGTTCAG-5′ R: 3′-TGTCCAACTTAACGCCTTGTAA-5′	61 °C	148	[19]
sulI	F: 3′-CGGCGTGGGCTACCTGAACG-5′ R: 3′-GCCGATCGCGTGAAGTTCCG-5′	60 °C	433	[20]
sulII	F: 3′-GCGCTCAAGGCAGATGGCATT-5′ R: 3′-GCGTTTGATACCGGCACCCGT-5′	61 °C	293	[20]
tetA	F: 3′-GCGCGATCTGGTTCACTCG-5′ R: 3′-AGTCGACAGYRGCGCCGGC-5′	62.5 °C	164	[21]
tetW	F: 3′-GAGAGCCTGCTATATGCCAGC-5′ R: 3′-GGGCGTATCCACAATGTTAAC-5′	61 °C	168	[22]

The thermal protocol used was 95 °C for 5 min, 94 °C for 1 min, annealing temperature for 1 min, and 72 °C for 3 min, for 35 cycles, followed by a post-cycling step at 4 °C for 5 min. The detailed annealing temperatures are listed in Table 2. The LOQ for each target is included in Table 3.

Table 3. LOQ calculated for each target. The percentage of the samples in which the level was above the LOQ before and after the AD for total bacteria (*16S rRNA*), for each target, and samples positive for at least 6 and for all the included targets. The difference between influent and effluent, as well as its statistical significance, is shown in the last two columns.

		% Samples > LOQ			
	LOQ (Log Gene Copies/mg Sludge)	Influent	Effluent	Δ(in-ef)	χ^2 *p*-Value
16S rRNA	6.60	100%	100%	0%	-
bla_{OXA}	4.54	63.6%	90.9%	−27%	0.127
bla_{TEM}	5.74	100%	100%	0%	-
ermB	5.28	100%	100%	0%	-
qnrB	3.92	100%	90.9%	9.1%	0.306
sulI	6.12	100%	100%	0%	-
sulII	4.74	100%	81.8%	18.2%	0.138
tetA	5.45	100%	100%	0%	-
tetW	5.43	72.7%	81.8%	−9.07%	0.611
Positivity to at least 6 ARGs		100%	90.9%	−9.1%	0.306
Positivity to all the ARGs		45.5%	63.6%	−18.1%	0.392

2.3. Statistical Analyses

Statistical analysis was performed using IBM SPSS Statistics (version 29.0). ARG concentrations were expressed as Log of gene copies per kg of sludge and as ratios of the Log of AMR gene copies on the 16S rRNA copies. The data, adjusted considering the bacterial presence, were then used in all statistical analyses. A descriptive analysis of all the variables was performed. We reported categorical variables as absolute numbers and percentages and as mean and standard deviations for continuous variables. Statistical analyses for categorical variables were conducted using the χ^2. Differences between gene concentrations and chemical and physical parameters were analyzed using nonparametric tests (Mann–Whitney U test and Kruskal–Wallis) for independent samples by ranks, comparing the plant type and the type of sludge. Statistical significance was evaluated with an ANOVA test. The Spearman rank-order correlation coefficient was determined. A p-value of $p < 0.05$ was considered significant, a value <0.01 was considered highly significant.

3. Results and Discussion

The OFMSW plants involved in the study provided the physical–chemical analyses, including pH (6.1 ± 1.4), temperature (41.9 ± 3.2 °C), TSS (5.5 ± 2.6%), and VSS (4.1 ± 2.6%). Overall, no significant differences were observed between the two plants. However, the main difference was the temperature of the anaerobic digestion, whereby the OFMSW-A plant produces a digestion oriented towards thermophilicity. As can be observed in Table 1, the temperature was, on average, 2 degrees higher but variable (Mann–Whitney U test, $p > 0.05$).

Higher concentration of solids (>8%) was inversely proportional to the hydrolysis velocity. This presents benefits such as lower water and working volume requirements, however, it can only be achieved under constant thermophilic conditions. High solids anaerobic digestion can also produce a higher amount of biogas, but often fails due to the rapid buildup of volatile fatty acids (VFAs) and the associated drop in pH, producing a severe acidosis (pH < 4) of the digester and the necessity to stop the plant working to remove the materials from the digester. As a consequence, the OFMSW plants generally choose a lower but more easily manageable organic load for the digester.

In the Table 3, the percentage of positive samples for each included gene (>LOQ) is shown, including both in influents and effluents from the AD. Moreover, the percentage of samples positive for more than one ARG are included. Several studies indicate that multidrug resistance is ubiquitous in environmental matrices [23], with a relatively high abundance, increasing the risk of multi antimicrobial resistance transmission.

The average concentration observed in the organic fraction prior to the anaerobic digestion was estimated at 9.45 ± 0.78 Log *ermB* copies/kg of sludge, 8.01 ± 0.49 for *sulII*, 7.66 ± 0.53 for *sulI*, 7.62 ± 0.57 for *tetA*, 7.23 ± 0.37 for bla_{TEM}, 7.11 ± 0.53 for *qnrB*, 6.03 ± 4.01 for *tetW*, and 4.11 ± 3.30 for bla_{OXA}. Significant differences were observed for *ermB*, *sulI*, *sulII*, *tetA*, *qnrB*, and bla_{TEM} (Mann–Whitney U test, $p = 0.04$, $p = 0.01$, $p = 0.007$, $p = 0.004$, $p < 0.001$, and $p < 0.001$, respectively), but the reduction caused by the AD observed was limited (<1 Log unit) (Figure 1). On the other hand, bla_{OXA} and *tetW* seemed not to be affected by the process (Figure 1). In line with the literature, at higher temperatures (40–50 °C) *tetW*—which encodes for an efflux pump—increased [24–26].

In order to evaluate the viability of the bacteria present in the sludge, *16S rRNA* was also measured at a concentration of 10.41 ± 0.56. The AD seemed to have no impact on the concentration of the total bacteria, as in the digestate, the concentration of *16S rRNA* was 10.57 ± 0.22. The *16S rRNA* gene was also included as internal control of the analytic process, the concentrations of the ARGs were normalized to the internal control dividing the Log ARGs copies/kg of sludge by Log *16S rRNA* copies/kg of sludge. Statistical analyses on normalized data (with respect to *16s rRNA*) produced quite similar results to those for sludge mass unit (Table 4).

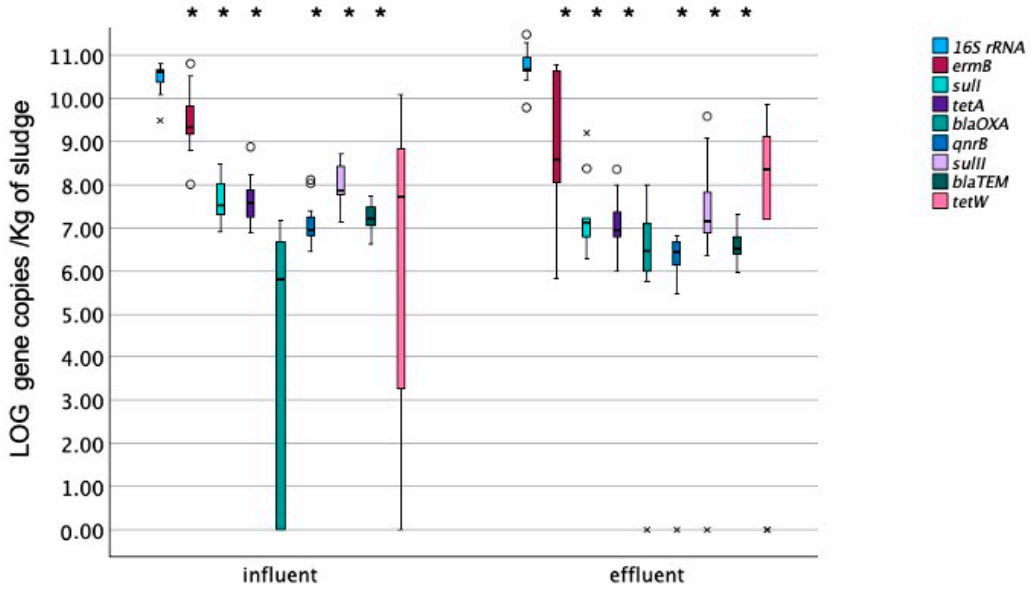

Figure 1. Effect of the AD on the concentrations of the total bacteria and ARGs. Boxplot of the total bacteria and ARG concentrations. Circles show the outliers and x shows the extreme values. Significant differences (Mann–Whitney U test, $p < 0.05$) between the influent and the digestate are marked with *.

Table 4. Effect of the AD on the ARG reduction. The concentration is reported as the ratio between the Log ARG copies/kg of sludge and the Log *16S rRNA* copies/kg of sludge. The effect of the AD is quantified as the difference between the influent and the effluent of the digester. Significant differences (*) were evaluated using the Mann–Whitney U test, $p < 0.05$.

	Influent		**Effluent**			
	Medium	**Dev. St.**	**Medium**	**Dev. St.**	**Δ(in-ef)**	***p*-Value**
bla_{OXA}	0.40	0.32	0.57	0.21	−0.17	0.27
bla_{TEM}	0.69	0.06	0.62	0.04	0.07	<0.001 *
ermB	0.91	0.08	0.81	0.13	0.10	0.04 *
qnrB	0.68	0.06	0.54	0.19	0.14	<0.001 *
sulI	0.73	0.06	0.67	0.07	0.06	0.01 *
sulII	0.77	0.06	0.58	0.30	0.19	0.007 *
tetA	0.73	0.61	0.66	0.05	0.07	0.004 *
tetW	0.58	0.40	0.63	0.32	−0.05	0.898

As already mentioned, the main difference between the plants is the temperature in the digester. Analyzing the data divided by plant, the significant differences between influent and effluent were observed just for the OFMSW-A: *ermB*, *sulI*, *tetA*, *qnrB*, *sulII*, and *blaTEM* (Mann–Whitney U test, $p = 0.07$, $p = 0.002$, $p = 0.001$, $p < 0.001$, $p < 0.001$, and $p = 0.002$, respectively) (Figure 2). Thermophilic conditions have higher hygienic potential, reducing the presence of pathogens and ARGs [27,28]. According to the literature, temperature is a key factor in the reduction of potential biological health threats. Pathogenic spore-forming bacteria, such as Clostridium perfringens or Bacillus cereus, seem less affected by the extreme conditions of the digester and they could be found at high concentrations also in the digestate [29,30]. Flores-Orozco et al. suggested that the temperature induces a selection of the ARGs based on their mechanisms of action; indeed, genes encoding for antibiotic inactivators are reduced in mesophilic conditions. The thermophilic conditions reduce the ARGs concentration but the level of total ARGs remaining are similar [29].

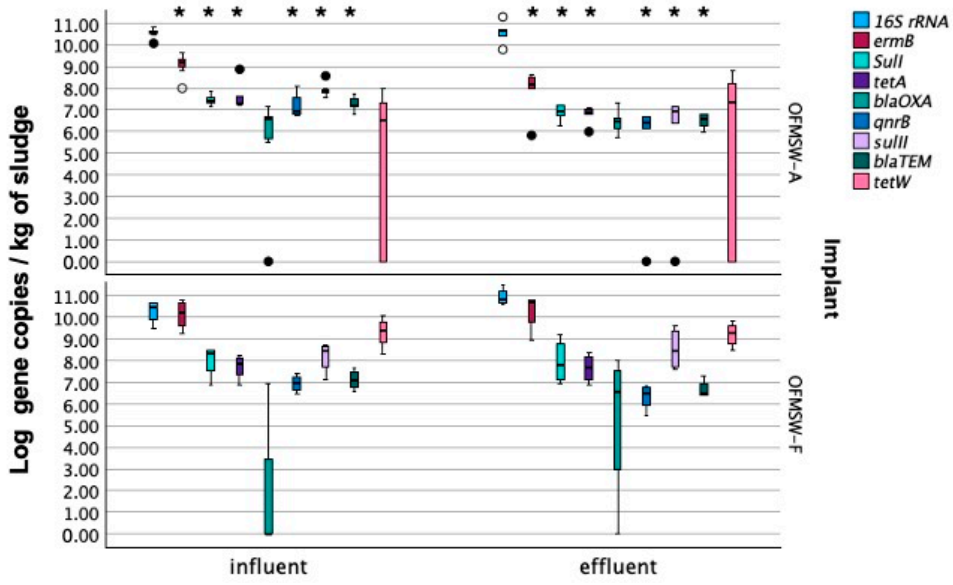

Figure 2. Temperature is a key factor in ARG concentration. Boxplot representing ARG concentration expressed in Log gene copies per kg of sludge. Significant differences between influent and digestate (Mann–Whitney U test, $p < 0.05$) are marked with *. The white circles represent the outliers, the black ones the extremes.

The %VSS is a driven parameter for the amount of biological molecules in the sludge. According to the literature, the solid component of the wastewater, due to its physical–chemical composition, shows a higher affinity for the biological compound [31,32]. In this study, a positive correlation among ARGs like *ermB*, *tetA*, *qnrB*, *sulI*, *sulII*, *blaTEM*, and %VSS is reported (Spearman's Rho, *ermB*: 0.582, $p = 0.007$, *tetA*: 0.731, $p < 0.001$, *qnrB*: 0.743, $p < 0.001$, *sulI*: 0.622, $p = 0.003$, *sulII*: 0.699, $p < 0.001$, *blaTEM*: 0.753, $p < 0.001$).

The persistence of the ARGs in the digestate implies a potential risk of horizontal transmission to the bacteria present in the soil. Although the decision making has admitted the relevance of the pollution linked to the presence of pharmaceuticals in the environmental matrix, only few references are present for water and referring to the watch list compounds, in which three antibiotics are listed [6]. A lack of regulation for limiting antibiotics and the presence of ARGs in the digestate destined for agricultural use has been reported in the European Union and other developed countries. According to the latest regulation on organic fertilizers [33], even though references to biological targets are present (for example *Salmonella* spp.), no specific statements are reported for antibiotics or ARGs.

The OFMSW is mainly composed of fruit and vegetable leftovers, as well as animal waste from meat and fish products. The main antibiotics used on crops are streptomycin and oxytetracycline [34]. According to the European Medicine Agency, in 2022 in the food-producing animals, the most administered antibiotics were penicillin and tetracyclines [35]. Due to the broad-spectrum properties of the above-mentioned antibiotics and their use in the agricultural setting, it is worth noticing that in this paper, ARGs against the most-used antibiotics in agriculture were the three most present antibiotics (5.74, 5.45, 5.43 Log gene copies/Kg of sludge for *blaTEM*, *tetA* and *tetW* respectively) (Table 3).

Biochar, eggshell, and biochar-based composites as other porous materials are able to reduce ARG transfer, decreasing mobility of heavy metals and antibiotics through adsorption and lowering the selection pressure. Moreover, bacteriophage therapy improvement and diffusion can also be an alternative to inhibit the proliferation and diffusion of ARGs [36]. However, more effort is needed to investigate the AMR removal mechanisms, and the interactions between both environmental matrices, to inhibit the proliferation and diffusion of ARGs.

According to the literature, the use of organic fertilizers (compost or digestate) favors vegetable growth [37]. Spreading contaminated digestate, on agricultural soil, due to the

limited effect of the AD, could increase the pollution in soil, water, and food destined for human consumption. The soil microbial community, especially the rhizosphere environment, can mitigate the dissemination of ARGs [37,38]; on the other hand, the bacteria present in the soil can acquire the ARGs from the digestate, developing multi-drug resistance [38]. The limited reduction of the ARGs in the digestate can be partially overcome by the subsequent destination of the effluent. Frequently, the final product has two potential destinations: (i) dehydration for storing and spreading on the soil or (ii) dehydration followed by the aerobic composting plant for further treatment. The OFMSW plants tested in the experiment send the dehydrated digestate to the aerobic composting plants. This additional step, due to the prolonged high temperature reached in the process, inactivates more ARGs and pathogens, boosting its reuse in agriculture [39,40].

Both biomolecular and culture methods have been previously generally employed in AMR detection. However, the cultural methods require a lot of time, and they are able to detect only a fraction of the potential antibiotic resistance risk. In this study, biomolecular analyses have been conducted using the ddPCR to screen for the presence of AMR genes. This method accounts for several advantages, including higher sensitivity and sensibility, reduced effect of the chemical inhibitors presents in the environmental matrix, and high throughput without requiring standard curves. This last point is crucial for ARG research. Moreover, the use of the ddPCR allows a quantitative assessment of the occurrence and potential horizontal transfer of ARGs.

4. Conclusions

The detection and quantification of the antibiotics and the characterization of their behavior in the environment are limited and mainly refer to water. On the other hand, ARGs can cross environmental and species boundaries. An effective one health approach is crucial for health management in landfill systems. The one health pillars are the monitoring of human, veterinary, and environmental dissemination of ARGs. Surveillance of the first two is already ongoing, but few matrices are tested for the third one, with wastewater being the most well-known. This paper focused on the sludge as an environmental matrix with a high potential biological risk. Although the misuse of antibiotics in human and animal health is widely recognized in the literature that, it is only in recent documents from European institutions, such as WHO Europe, the Council of the European Union (EU), the European Commission, and the European Environment Agency (EEA), that the agricultural dimension of the problem has been addressed. In the "A European One Health Action Plan against Antimicrobial Resistance (COM/2017/0339)", references to the agricultural sector were limited to livestock and aquaculture [6]. However, the "Council Recommendation on stepping up EU actions to combat antimicrobial resistance in a One Health approach (2023/C 220/01)" indicated a notable advancement in 2023 by recognizing the importance of monitoring agricultural residues to better understand the role of microbial contaminants in the environment [41]. While the recommendation emphasizes the prudent management of manure and sewage sludge, it does not provide detailed guidelines and instead highlights the need for further data to fully assess the issue. Similar considerations are also reflected in national plans, such as the Italian National Action Plan on Antimicrobial Resistance (PNCAR) 2022–2025, which highlights the importance of fostering cooperation between the veterinary and agricultural sectors to develop effective strategies for reducing antimicrobial consumption [42]. Further general indications are provided in the "Roadmap on Antimicrobial Resistance for the WHO European Region (2023–2030)", which focuses on the risks of antifungal use in agriculture, particularly those similar to compounds critical for human health. The roadmap also identifies agricultural workers, including those in food animal production and meat-processing sectors, as a high-risk group for AMR exposure [43]. The availability of validated monitoring data could support future regulatory decisions.

The ddPCR presents several advantages, including the possibility to not include standards for the quantification of the target of interest, but also the lower sensitivity to

inhibitors present in complex matrices like sludge. These biomolecular methods produce reliable datasets for further analyses.

From a sustainability perspective, this study provides new knowledge on the presence and persistence of the ARGs in the digestate for safe reuse of the organic wastes. The extreme conditions of the anaerobic process led to a significant reduction of the ARGs, observable only in thermophilic conditions. The reuse of the digestate in agriculture shows promising advantages as a soil amendment or fertilizer [44]. This practice mitigates the impact of the OFMSW on the environment following a circular economy approach. In this study, when the AD was conducted under thermophilic conditions, it seemed to be responsible for ARG spread, even if a significant reduction was observed. Further studies are required for an exhaustive risk assessment for human health linked to the residual presence of the antibiotics and AMR microbials in the digestate, as well as the potential horizontal transmission of the ARGs from OFMSW to the environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su162410922/s1, Table S1: Dataset for data availability.

Author Contributions: Conceptualization, D.T.; methodology, E.F. and D.T.; software, E.F.; validation, D.T.; formal analysis, A.M.; resources, D.T.; data curation, A.M. and E.F.; writing—original draft preparation, A.M. and E.F.; writing—review and editing, D.T.; visualization, A.M. and E.F.; supervision, D.T.; project administration, D.T.; funding acquisition, D.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Turin in the context of the finalized local research program.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the Supplementary Material. Further inquiries can be directed to the corresponding author(s).

Acknowledgments: The authors would like to thank the plants involved and their workers for collaboration on this project.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Haenni, M.; Dagot, C.; Chesneau, O.; Bibbal, D.; Labanowski, J.; Vialette, M.; Bouchard, D.; Martin-Laurent, F.; Calsat, L.; Nazaret, S.; et al. Environmental Contamination in a High-Income Country (France) by Antibiotics, Antibiotic-Resistant Bacteria, and Antibiotic Resistance Genes: Status and Possible Causes. *Environ. Int.* **2022**, *159*, 107047. [CrossRef]
2. Miłobedzka, A.; Ferreira, C.; Vaz-Moreira, I.; Calderón-Franco, D.; Gorecki, A.; Purkrtova, S.; Bartacek, J.; Dziewit, L.; Singleton, C.M.; Nielsen, P.H.; et al. Monitoring Antibiotic Resistance Genes in Wastewater Environments: The Challenges of Filling a Gap in the One-Health Cycle. *J. Hazard. Mater.* **2022**, *424*, 127407. [CrossRef]
3. Ministero della Salute Antibiotico-Resistenza nel Settore Umano. Available online: https://www.salute.gov.it/portale/antibioticoresistenza/dettaglioContenutiAntibioticoResistenza.jsp?lingua=italiano&id=5282&area=antibiotico-resistenza&menu=vuoto (accessed on 19 September 2024).
4. Arnold, B.J.; Huang, I.T.; Hanage, W.P. Horizontal Gene Transfer and Adaptive Evolution in Bacteria. *Nat. Rev. Microbiol.* **2022**, *20*, 206–218. [CrossRef] [PubMed]
5. Gomez Cortes, L.; Marinov, D.; Sanseverino, I.; Navarro Cuenca, A.; Niegowska, M.; Porcel Rodriguez, E.; Stefanelli, F.; Lettieri, T. *Selection of Substances for the 4th Watch List under the Water Framework Directive*; Publications Office of the European Union: Luxembourg, 2022.
6. European Committee. *A European One Health Action Plan against Antimicrobial Resistance (AMR)*; European Committee: Brussels, Belgium, 2017.
7. Gilbert, J.; Siebert, S. *Ecn Data Report 2022 Compost and Digestate for a Circular Bioeconomy*; European Compost Network ECN e.V: Bochum, Germany, 2023.
8. Tong, J.; Lu, X.; Zhang, J.; Angelidaki, I.; Wei, Y. Factors Influencing the Fate of Antibiotic Resistance Genes during Thermochemical Pretreatment and Anaerobic Digestion of Pharmaceutical Waste Sludge. *Environ. Pollut.* **2018**, *243*, 1403–1413. [CrossRef] [PubMed]

9. Wu, Q.; Zou, D.; Zheng, X.; Liu, F.; Li, L.; Xiao, Z. Effects of Antibiotics on Anaerobic Digestion of Sewage Sludge: Performance of Anaerobic Digestion and Structure of the Microbial Community. *Sci. Total Environ.* **2022**, *845*, 157384. [CrossRef]
10. Xiao, L.; Wang, Y.; Lichtfouse, E.; Li, Z.; Kumar, P.S.; Liu, J.; Feng, D.; Yang, Q.; Liu, F. Effect of Antibiotics on the Microbial Efficiency of Anaerobic Digestion of Wastewater: A Review. *Front. Microbiol.* **2021**, *11*, 611613. [CrossRef]
11. Li, S.; Ondon, B.S.; Ho, S.H.; Jiang, J.; Li, F. Antibiotic Resistant Bacteria and Genes in Wastewater Treatment Plants: From Occurrence to Treatment Strategies. *Sci. Total Environ.* **2022**, *838*, 156544. [CrossRef] [PubMed]
12. Wybraniec, C.; Cournoyer, B.; Moussard, C.; Beaupère, M.; Lusurier, L.; Leriche, F.; Fayolle, K.; Sertillanges, N.; Haudin, C.S.; Houot, S.; et al. Occurrence of 40 Sanitary Indicators in French Digestates Derived from Different Anaerobic Digestion Processes and Raw Organic Wastes from Agricultural and Urban Origin. *Front. Microbiol.* **2024**, *15*, 1346715. [CrossRef] [PubMed]
13. Botes, M.; De Kwaadsteniet, M.; Cloete, T.E. Application of Quantitative PCR for the Detection of Microorganisms in Water. *Anal. Bioanal. Chem.* **2013**, *405*, 91–108. [CrossRef] [PubMed]
14. European Centre for Disease Prevention and Control ECDC AMC2 Dashboard. Available online: https://qap.ecdc.europa.eu/public/extensions/AMC2_Dashboard/AMC2_Dashboard.html#eu-consumption-tab (accessed on 10 April 2024).
15. Harbison, J.E.; Roth-Schulze, A.J.; Giles, L.C.; Tran, C.D.; Ngui, K.M.; Penno, M.A.; Thomson, R.L.; Wentworth, J.M.; Colman, P.G.; Craig, M.E.; et al. Gut Microbiome Dysbiosis and Increased Intestinal Permeability in Children with Islet Autoimmunity and Type 1 Diabetes: A Prospective Cohort Study. *Pediatr. Diabetes* **2019**, *20*, 574–583. [CrossRef] [PubMed]
16. Wang, Y.; Wang, C.; Song, L. Distribution of Antibiotic Resistance Genes and Bacteria from Six Atmospheric Environments: Exposure Risk to Human. *Sci. Total Environ.* **2019**, *694*, 133750. [CrossRef]
17. Markowicz, A.; Bondarczuk, K.; Wiekiera, A.; Sułowicz, S. Is Sewage Sludge a Valuable Fertilizer? A Soil Microbiome and Resistome Study under Field Conditions. *J. Soils Sediments* **2021**, *21*, 2882–2895. [CrossRef]
18. Knapp, C.W.; Dolfing, J.; Ehlert, P.A.I.; Graham, D.W. Evidence of Increasing Antibiotic Resistance Gene Abundances in Archived Soils since 1940. *Environ. Sci. Technol.* **2010**, *44*, 580–587. [CrossRef]
19. Chen, Z.; Li, Y.; Ye, C.; He, X.; Zhang, S. Fate of Antibiotics and Antibiotic Resistance Genes during Aerobic Co-Composting of Food Waste with Sewage Sludge. *Sci. Total Environ.* **2021**, *784*, 146950. [CrossRef]
20. Kerrn, M.B.; Klemmensen, T.; Frimodt-Möller, N.; Espersen, F. Susceptibility of Danish Escherichia Coli Strains Isolated from Urinary Tract Infections and Bacteraemia, and Distribution of Sul Genes Conferring Sulphonamide Resistance. *J. Antimicrob. Chemother.* **2002**, *50*, 513–516. [CrossRef]
21. Aminov, R.I.; Chee-Sanford, J.C.; Garrigues, N.; Teferedegne, B.; Krapac, I.J.; White, B.A.; Mackie, R.I. Development, Validation, and Application of PCR Primers for Detection of Tetracycline Efflux Genes of Gram-Negative Bacteria. *Appl. Environ. Microbiol.* **2002**, *68*, 1786–1793. [CrossRef]
22. Aminov, R.I.; Garrigues-Jeanjean, N.; Mackie, R.I. Molecular Ecology of Tetracycline Resistance: Development and Validation of Primers for Detection of Tetracycline Resistance Genes Encoding Ribosomal Protection Proteins. *Appl. Environ. Microbiol.* **2001**, *67*, 22–32. [CrossRef] [PubMed]
23. Song, L.; Yang, S.; Gong, Z.; Wang, J.; Shi, X.; Wang, Y.; Zhang, R.; Wu, Y.; Wager, Y.Z. Antibiotics and Antibiotic-Resistant Genes in Municipal Solid Waste Landfills: Current Situation and Perspective. *Curr. Opin. Environ. Sci. Health* **2023**, *31*, 100421. [CrossRef]
24. Ma, Y.; Wilson, C.A.; Novak, J.T.; Riffat, R.; Aynur, S.; Murthy, S.; Pruden, A. Effect of Various Sludge Digestion Conditions on Sulfonamide, Macrolide, and Tetracycline Resistance Genes and Class i Integrons. *Environ. Sci. Technol.* **2011**, *45*, 7855–7861. [CrossRef] [PubMed]
25. Wang, J.; Chu, L.; Wojnárovits, L.; Takács, E. Occurrence and Fate of Antibiotics, Antibiotic Resistant Genes (ARGs) and Antibiotic Resistant Bacteria (ARB) in Municipal Wastewater Treatment Plant: An Overview. *Sci. Total Environ.* **2020**, *744*, 140997. [CrossRef] [PubMed]
26. Burch, T.R.; Firnstahl, A.D.; Spencer, S.K.; Larson, R.A.; Borchardt, M.A. Fate and Seasonality of Antimicrobial Resistance Genes during Full-Scale Anaerobic Digestion of Cattle Manure across Seven Livestock Production Facilities. *J. Environ. Qual.* **2022**, *51*, 352–363. [CrossRef] [PubMed]
27. Sun, W.; Qian, X.; Gu, J.; Wang, X.J.; Duan, M.L. Mechanism and Effect of Temperature on Variations in Antibiotic Resistance Genes during Anaerobic Digestion of Dairy Manure. *Sci. Rep.* **2016**, *6*, 30237. [CrossRef]
28. Mortezaei, Y.; Williams, M.R.; Demirer, G.N. Effect of Temperature and Solids Retention Time on the Removal of Antibiotic Resistance Genes during Anaerobic Digestion of Sludge. *Bioresour. Technol. Rep.* **2023**, *21*, 101377. [CrossRef]
29. Flores-Orozco, D.; Levin, D.; Kumar, A.; Sparling, R.; Derakhshani, H.; Cicek, N. Metagenomic Comparison of Effects of Mesophilic and Thermophilic Manure Anaerobic Digestion on Antimicrobial Resistance Genes and Mobile Genetic Elements. *Environ. Adv.* **2024**, *15*, 100472. [CrossRef]
30. Lorine, D.; Céline, D.; Caroline, L.M.; Frédéric, B.; Lorette, H.; Julie, B.; Laure, M.; Christine, Z.; Typhaine, P.; Sandra, R.; et al. Influence of Operating Conditions on the Persistence of E. Coli, Enterococci, Clostridium Perfringens and Clostridioides Difficile in Semi-Continuous Mesophilic Anaerobic Reactors. *Waste Manag.* **2021**, *134*, 32–41. [CrossRef] [PubMed]
31. Bai, W.; Tang, R.; Wu, G.; Wang, W.; Yuan, S.; Xiao, L.; Zhan, X.; Hu, Z.H. Role of Suspended Solids on the Co-Precipitation of Pathogenic Indicators and Antibiotic Resistance Genes with Struvite from Digested Swine Wastewater. *J. Hazard. Mater.* **2023**, *459*, 132235. [CrossRef] [PubMed]

32. Cerrada-Romero, C.; Berastegui-Cabrera, J.; Camacho-Martínez, P.; Goikoetxea-Aguirre, J.; Pérez-Palacios, P.; Santibáñez, S.; José Blanco-Vidal, M.; Valiente, A.; Alba, J.; Rodríguez-Álvarez, R.; et al. Excretion and Viability of SARS-CoV-2 in Feces and Its Association with the Clinical Outcome of COVID-19. *Sci. Rep.* **2022**, *12*, 7397. [CrossRef] [PubMed]
33. *Official Journal of the European Union Regulation (EU) No 2019/1009 of the European Parliament and of the Council Laying down Rules on the Making Available on the Market of EU Fertilising Products and Amendig*; The Publications Office of the European Union: Luxembourg, 2019.
34. Haynes, E.; Ramwell, C.; Griffiths, T.; Walker, D.; Smith, J. *Review of Antibiotic Use in Crops, Associated Risk of Antimicrobial Resistance and Research Gaps*; Department for Environment, Food and Rural Affairs (Defra) & The Food Standards Agency (FSA): London, UK, 2020.
35. European Medicines Agency. *European Surveillance of Veterinary Antimicrobial Consumption Sales of Veterinary Antimicrobial Agents in 31 European Countries in 2022*; European Medicines Agency: Luxemburg, 2022.
36. Wang, F.; Fu, Y.H.; Sheng, H.J.; Topp, E.; Jiang, X.; Zhu, Y.G.; Tiedje, J.M. Antibiotic Resistance in the Soil Ecosystem: A One Health Perspective. *Curr. Opin. Environ. Sci. Health* **2021**, *20*, 100230. [CrossRef]
37. Yang, J.; Xiang, J.; Goh, S.G.; Xie, Y.; Nam, O.C.; Gin, K.Y.H.; He, Y. Food Waste Compost and Digestate as Novel Fertilizers: Impacts on Antibiotic Resistome and Potential Risks in a Soil-Vegetable System. *Sci. Total Environ.* **2024**, *923*, 171346. [CrossRef]
38. Zhang, R.; Gong, C.; Li, J.; Zhuang, H.; Lan, L.; Zhou, L.; Shan, S.; Wang, Y. Tracing the Transfer Characteristics of Antibiotic Resistance Genes from Swine Manure to Biogas Residue and Then to Soil. *Sci. Total Environ.* **2024**, *912*, 169181. [CrossRef]
39. Gurmessa, B.; Milanovic, V.; Foppa Pedretti, E.; Corti, G.; Ashworth, A.J.; Aquilanti, L.; Ferrocino, I.; Rita Corvaglia, M.; Cocco, S. Post-Digestate Composting Shifts Microbial Composition and Degrades Antimicrobial Resistance Genes. *Bioresour. Technol.* **2021**, *340*, 125662. [CrossRef]
40. Wang, M.; Shen, W.; Yan, L.; Wang, X.H.; Xu, H. Stepwise Impact of Urban Wastewater Treatment on the Bacterial Community Structure, Antibiotic Contents, and Prevalence of Antimicrobial Resistance. *Environ. Pollut.* **2017**, *231*, 1578–1585. [CrossRef] [PubMed]
41. European Council. *Council Recommendation on Stepping up EU Actions to Combat Antimicrobial Resistance in a One Health Approach*; European Council: Brussels, Belgium, 2023.
42. *Ministero della Salute Piano Nazionale di Contrasto dell'antibiotico-Resistenza*; Ministero della Salute: Rome, Italy, 2017; pp. 1–80.
43. *Regional Commitee for Europe Roadmap on Antimicrobial Resistance for the WHO European Region 2023–2030*; Elsevier Ltd.: Astana, Kazakhstan, 2023; Volume 7.
44. Fernández-Rodríguez, M.J.; Mancilla-Leytón, J.M.; Jiménez-Rodríguez, A.; Borja, R.; Rincón, B. Reuse of the Digestate Obtained from the Biomethanization of Olive Mill Solid Waste (OMSW) as Soil Amendment or Fertilizer for the Cultivation of Forage Grass (Lolium Rigidum Var. Wimmera). *Sci. Total Environ.* **2021**, *792*, 148465. [CrossRef]