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The Effect of Antibiotics on Mesophilic Anaerobic Digestion Process of Cattle Manure

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Supplementary to Materials and Methods

S1. Anaerobic Digestion and Methane Fermentation

Reactors with a volume of 250 mL were filled with 25 g of substrate and 175 g of inoculum for an initial organic loading of 5 g VS L^{-1}. Mixing in the reactors run for 30 seconds each 10 minutes. Rotating speed was 100 rpm. Anaerobic conditions were achieved by continuous flushing of pure nitrogen through the sludge. The Automatic Methane Potential Test System (AMPTS) II (Bioprocess Control, Lund, Sweden) was used to measure the quantity of produced CH_4. The quality of biogas was measured by using a gas chromatograph connected with thermal conductivity detector (GC-TCD) (Agillent 7890 A, Lund, Sweden). In the samples before and after fermentation the volatile fatty acids (VFAs) content, the pH, the FOS/TAC ratio (The FOS/TAC is the ratio of the concentration of volatile fatty acids (FOS) to the basicity (i.e., buffer capacity) (TAC) in the fermentation chamber), the TS, and VS, and the TN and TP content were determined (Table S1). The methane production from bioreactors followed first-order kinetic (Figure S1).

S2. Analyses of Antibiotic Concentration

S2.1. Samples Preparation, Standards and Chemicals

Chemical standards of pharmaceuticals: metronidazole (MET), amoxicillin (AMO), ampicillin (AMP), enrofloxacin (ENR), sulfamethoxazole (SMX), oxytetracycline (OXY), chlortetracycline (CHLOR) and tetracycline (TET) were purchased from Sigma–Aldrich (Saint Louis, MO, USA).

Acetonitrile and water were HPLC grade and were purchased from Merck (DarMstadt, Germany) as well as formic acid. Acetonitrile, methanol, ethyl acetate and ammonium hydroxide solution 25% (all of analytical reagent grade) were obtained from CHEMPUR (Piekary Śląskie, Poland). Acetic acid was provided by POCH S.A. (Gliwice, Poland) and citrate buffer (pH = 4.0) by Merck (Darmstadt, Germany) (both of analytical reagent

grade). Digestate samples were first centrifuged (20 min, 4000 rpm), and the supernatant was then decanted to separate the solid phase from the aqueous phase.

Liquid samples were filtered through a 0.22 μm syringe filter and analysed by LC–MS/MS. If the amounts of pharmaceuticals were above the calibration curve, the samples were diluted before analysis.

Lyophilized and homogenized digestate sample (0.2 g) was accurately weighed and extracted using different mixture of solvents. For extraction of OXY, TET and CHLOR mixture of citrate buffer (pH = 4.0) and acetonitrile (1:1, *v*/*v*). Two steps procedure (2% acetic acid in ethyl acetate and 2% acetic acid in methanol) was applied for extraction of AMO and AMP. Extraction of SMX, MET and ENR was performed in three steps using 2% ammonium hydroxide in methanol, 2% acetic acid in methanol and methanol, respectively. The digestate with 10 mL of solvents (listed above) was successively shaken for 1 h at 750 rpm (Vibramax 100, Heidolph Instruments GmbH & Co., Schwabach, Germany). Then, the extracts were filtrated, supernatants were combined and evaporated. The dry residue was dissolved in 15 mL of 5% methanol in water and then treated by the SPE procedure.

The SPE clean-up (CHROMABOND® SPE-system, Macherey–Nagel, Germany) was performed on an Oasis HLB cartridge (500 mg per 6 mL, Waters, Milford, MA, USA). The cartridge was preconditioned successively with 6 mL methanol and 6 mL water prior to sample load. After sample passage, the cartridge was dried under vacuum for 15 min. The analytes were then eluted with 6 mL of 0.1% acetic acid in methanol. The eluate was evaporated to just dryness. The sample was then reconstituted in 1 mL of 0.1% acetic acid, filtered through a 0.22 μm syringe filter and analysed three times by Liquid Chromatography—Mass Spectrometry (LC–MS/MS).

S2.2. Liquid Chromatography—Mass Spectrometry

Pharmaceutical determination was performed on an Dionex ultra high-performance liquid chromatography 3000 system (Dionex Corporation, Sunnyvale, CA, USA) coupled to a 4500 QTrap triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA).

Chromatographic separation was achieved on a ZORBAX SB-C3 column (150 mm × 3.0 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA). The column temperature was kept at 30 °C and the injected volume was 2 μL. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). For separation of the selected pharmaceuticals, elution started at 10% B, increased to 45% B in 3 min (flow rate 0.8 mL per min), to 90% B in 0.1 min, increased to 100% B in 2 min, back to the initial condition within 0.1 min and held for 3 min (flow rate 1 mL per min). The analyses were performed using the TurboV® (Waltham, MA, USA) ion spray in positive ionization mode (ESI+). The operating conditions for the analysis were the following: ion spray voltage, 4000 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 60 and 50 psi, respectively; probe temperature (TEM), 500 °C. Nitrogen served as the nebulizer and collision gas. Multiple reaction monitoring (MRM) experiments were performed to obtain the maximum sensitivity for the detection of target molecules. The optimization of MS parameters as declustering potential (DP), collision energy (CE) and entrance potential (EP) and collision cell exit potential (CXP) was performed by flow injection analysis (FIA) for each compound (Table S5). Analyst® version 1.4 software (Applied Biosystems/AB Sciex, https://sciex.com/content/SCIEX/na/us/en/products/software/analyst-software.html) was used to control all components of the system and also for data collection and analysis.

S2.3. Calculation of the Solid-Liquid Distribution Coefficient and the Removal Efficiency of the Selected Antibiotics

A measure of the affinity of a given substance for the solid or liquid phase, respectively, can be the parameter known as the solid-liquid distribution coefficient (K_d), which in an equilibrium state may be calculated using the Equation (1) [73]:

$$K_d = \frac{X_p}{S} = \frac{X}{X_{SS} \times S} \quad (1)$$

where:
K_d—solid-liquid distribution coefficient, L g_{SS}^{-1};
X_p—concentration of the compound sorbed onto suspended solids, per amount of dry matter of suspended solid, μg g_{SS}^{-1};
S—concentration of the compound dissolved in the liquid phase, μg L^{-1};
X—concentration of the compound sorbed onto suspended solids, per unit of reactor volume, μg L^{-1};
X_{SS}—suspended solids concentration in the reactor per L, g_{SS} L^{-1}.

Under equilibrium conditions, the total compound concentration in the reactor (C) can be expressed as (2) [73]:

$$C = X + S = S \times (1 + X_{SS} \times K_d) \quad (2)$$

where:
C—total compound concentration in the whole reactor, μg L^{-1}.

The parameters determined by means of Equation (2) may be used to calculate the total removal efficiency (R) of a given compound during the digestion process, according to the Equation (3):

$$R = \frac{C_0 - C_t}{C_0} \times 100 \quad (3)$$

where:
R—total removal efficiency, %;
C_0—initial total compound concentration in the reactor, μg L^{-1};
C_t—total compound concentration in the reactor after the digestion process, μg L^{-1}.

The abovementioned equations were used to calculate the corresponding parameters which are discussed later in the manuscript.

S3. Analyses of Genes Concentration

S3.1. Genomic DNA (gDNA) Isolation

The anaerobic digestion mixture samples (2 g) were transferred to centrifuge tubes with a capacity of 2 mL and centrifuged for 10 min at 8000 rpm. The supernatant was removed and pellet was stored in −20 °C for gDNA isolation. The gDNA was extracted from previously obtained pellets, using a Fast DNA Spin Kit for Soil® (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The total gDNA were eluted with 75 μL of provided DES (DNAse pyrogen free water) solution. Concentration and quality of extracted gDNA were determined with a spectrophotometer (Multiskan Sky, Thermo Scientific™, Waltham, MA, USA) and stored at −20 °C for further analysis.

S3.2. Quantitative Real-Time Polymerase Chain Reactions (qPCR)

A LightCycler® (Roche Diagnostics, Mannheim, Germany) was used to identify genes. All qPCR reactions were performed using 15 μL reaction with the master mix SYBR GREEN (Roche Diagnostics, Mannheim, Germany). The qPCR reaction mixture contained 0.8 μL of DNA template, 0.4 μL of each 10 μM primer, 7.5 μL of SYBR GREEN, and 5.9 μL of RNase-free water. The qPCR analysis was conducted according to the following settings shown in Table S3. The reactions were repeated in triplicate. Melting curves were generated and analysed to detect nonspecific amplification. The fluorescent signal data were processed using LightCycler ® software (version 1.5.0, https://lifescience.roche.com/en_de/products/light-cycler14301-480-software-version-15.html).

S3.3. Illumina MiSeq Sequencing

To identify the microbiome the high-throughput sequencing of the hypervariable region V3–V4 of 16S gene was performed with the Illumina MiSeq instrument (Seoul, Korea) (Table S3), using MiSeq Reagent v3 Kit (Macrogen, Seoul, Korea) with 2 × 250 bp paired-end reads and primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG-NGGC WGCAG) and 785R (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG-GACTACHVGGGTATCTAATCC) designed by Klindworth et al.,[74]. The quality control was performed using both FastQC software version 0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Qualitative Insights Into Microbial Ecology (QIIME2) version 2019.4 (https://qiime2.org/). Adapter trimming and quality filtration were carried out by using both Trimmomatic version 0.38 (http://www.usadellab.org/cms/?page=trimmomatic) [75] and QIIME2 and denoising, merging reads, and chimera removal were done by using DADA2 tool [76] in QIIME2 environment. The sampling depth was set to 17,200 reads per sample. The operational taxonomic units (OTUs) were classified basing on the predefined 99% OTUs Greengenes database version 13.8. A representative sequence of each OTU has been deposited in European Nucleotide Archive (ENA) database under the accession numbers PRJEB40682 (EMBL–EBI service, https://www.ebi.ac.uk/).

S4. Statistical Analyses

Data were analysed using Statistica 13.1 software (https://www.statsoft.pl/statistica_13/). Spearman's rank correlation coefficient was calculated to determine the correlations between the analysed genes. The distribution of variables was compared by the Kruskal–Wallis test. Network analysis based on the Spearman's Correlation analysis between ARGs and the bacterial community composition (based on OTUs) was determined using the Gephi 0.9.2 (https://gephi.org/) platform. Circos diagrams were generated using the online software Circos©2004-2016 (http://circos.ca/). Heatmap was created in GraphPad Prism 8.4 (https://www.graphpad.com/scientific-software/prism/).

Table S1. The characteristics of cattle manure used as substrate in AD process and anaerobic sludge used as inoculum.

	TS [a] g_D^{-1} (mg)	**VS [b] g_D^{-1} (mg)**	**pH**	**TP [c] g_{TS}^{-1} (mg)**	**TN [d] g_{TS}^{-1} (mg)**
Substrate	150.0 ± [e] 10.8	123.1 ± 14.5	8.2 ± 0.5	1.6 ± 2.4	5.3 ± 2.8
Inoculum	38.8 ± 5.2	25.2 ± 3.8	8.1 ± 0.5	0.9 ± 0.4	5.5 ± 1.9

[a] TS—total solids; [b] VS—volatile solids; [c] TP—total phosphorus; [d] TN—total nitrogen; [e] ±—standard deviation.

Table S2. The concentrations of antibiotics used in the investigations, per unit of reactor feed volume.

Class of Antibiotics	**Antibiotics**	**Concentration of Antibiotics (μg mL^{-1})**
Nitroimidazole derivatives	Metronidazole (MET)	512
Beta-lactams	Amoxicillin (AMO)	1024
	Ampicillin (AMP)	1024
Fluoroquinolones	Enrofloxacin (ENR)	1024
Sulfonamides	Sulfamethoxazole (SMX)	512
Tetracyclines	Oxytetracycline (OXY)	1024
	Chlortetracycline (CHLOR)	1024
	Tetracycline (TET)	1024
-	Substrate without antibiotic addition (SA)	-

Table S3. Primers sequences and parameters use for qPCR analysis.

Target Gene	Primer Sequence (5′-3′)	Annealing Temperature (°C)	Amplicon Size (bp)	References
*bla*TEM	AGTGCTGCCATAACCATGAGTG	58	431	[77] (Kim et al., 2009)
	CTGACTCCCCGTCGTGTAGATA			
*bla*OXA	ATTATCTACAGCAGCGCCAGTG		296	
	TGCATCCACGTCTTTGGTG			
*cfx*A	TGACTGGCCCTGAATAATCT	55	312	
	ACAAAAGATAGCGCAAATCC			
*erm*F	TAGATATTGGGGCAGGCAAG	58	126	[78] (Eitel et al., 2013)
	GGAAATTGCGGAACTGCAAA			
*lin*A	CTGGGGAGTGGATGTCTTGT	60	230	
	AGTTGGCTTGTTTGGAAGTG			
*mef*A	ATACCCCAGCACTCAATTCG	56	346	
	CAATCACAGCACCCAATACG			
*qep*A	CCAGCTCGGCAACTTGATAC	58	570	[79] (Li et al., 2012)
	ATGCTCGCCTTCCAGAAAA			
aac(6′)-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	52	482	[80](Park et al., 2006)
	CTCGAATGCCTGGCGTGTT			
*tet*A	GCTACATCCTGCTTGCCTTC	53	211	
	GCATAGATCGCCGTGAAGAG			
*tet*M	GTGGACAAAGGTACAACGAG	55	406	[81] (Nawaz et al., 2006)
	CGGTAAAGTTCGTCACACAC			
*tet*Q	TTATACTTCCTCCGGCATCG	55	904	[82] (Ng et al., 2001)
	ATCGGTTCGAGAATGTCCAC			
*sul*1	CGCACCGGAAACATCGCTGCAC	52	163	
	TGAAGTTCCGCCGCAAGGCTCG			
16S *r*RNA	AGAGTTTGATCATGGCTCAG	60	904	[83] (Nadkarni et al., 2002)
	GGTACCTTGTTACGACTT			
*intI*1	CCTCCCGCACGATGATC	54	280	[84] (Goldstein et al., 2001)
	TCCACGCATCGTCAGGC			
*intI*2	TTATTGCTGGGATTAGGC	54	233	
	ACGGCTACCCTCTGTTATC			
MSC	GAAACCGYGATAAGGGGA	60	408	[85] (Luton et al., 2002)
	TAGCGARCATCGTTTACG			
MST	TAATCCTYGARGGACCACCA	60	164	
	CCTACGGCACCRACMAC			
*mcr*A	GGTGGTGTMGGATTCACACARTAYGCWACAGC	95	467	
	TTCATTGCRTAGTTWGGRTAGTT			
V3-V4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	72	185	[74] (Klindworth et al., 2013)
	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC			

Table S4. The FOS/TAC ratio, concentration of nitrogen and phosphorus in biomass, and pH value in digestate samples from methane fermentation of cattle manure with the addition of selected antibiotics and in bioreactor with substrate without antibiotic supplementation (SA).

	FOS [a]/TAC [b] ratio	Nitrogen (mg g_{TS}^{-1})	Phosphorus (mg g_{TS}^{-1})	TS [c] (mg TS g^{-1})	VS [d] (mg VS g^{-1})	pH
MET	0.17 ± 0.02	2.6 ± 0.2	0.7 ± 0.3	46.5 ± 5.3	31.3 ± 7.4	8.7 ± 0.1
AMO	0.26 ± 0.00	3.0 ± 0.3	0.3 ± 0.4	62.4 ± 9.2	45.5 ± 6.3	8.6 ± 0.1
AMP	0.16 ± 0.02	2.6 ± 0.1	0.2 ± 0.5	51.1 ± 5.6	35.3 ± 8.3	8.7 ± 0.1
ENR	0.16 ± 0.02	2.7 ± 0.2	0.8 ± 0.5	31.0 ± 9.7	16.9 ± 8.0	8.7 ± 0.0
SMX	0.17 ± 0.03	2.3 ± 0.1	0.7 ± 0.3	45.8 ± 7.2	30.6 ± 4.9	8.7 ± 0.1
OXY	0.17 ± 0.02	2.6 ± 0.2	0.2 ± 0.5	43.2 ± 4.3	28.9 ± 6.2	8.7 ± 0.0
CHLOR	0.16 ± 0.02	2.4 ± 0.3	0.1 ± 0.2	47.7 ± 7.1	32.3 ± 6.9	8.7 ± 0.0
TET	0.18 ± 0.03	2.6 ± 0.4	0.2 ± 0.4	47.7 ± 4.8	32.2 ± 5.5	8.7 ± 0.0
SA	0.17 ± 0.02	2.7 ± 0.1	0.2 ± 0.3	48.4 ± 3.2	31.8 ± 5.2	8.7 ± 0.0

[a] FOS—volatile organic acids; [b] TAC—buffer capacity; [c] TS—total solids; [d] VS—volatile solids; ± indicates standard deviation.

Table S5. LC-MS/MS parameters for the investigated compounds.

Analyte	tR [a] (min)	Q1 [b] (m/z)	Q3 [c] (m/z)	DP [d] (V)	EP [e] (V)	CE [f] (V)	CXP [g] (V)	LOD [h] (ng g^{-1})	LOQ [i] (ng g^{-1})
MET	2.34	172.1	128.1	76	7	21	8	1.23	3.70
			82.1			33	6		
AMO	1.59	365.9	349.1	56	7	13	10	6.57	19.7
			114.1			31	8		
AMP	2.52	350.0	106.1	81	7	31	6	2.63	7.90
			160.2			19	14		
ENR	3.11	360.8	316.2	101	7	29	8	10.0	30.0
			245.2			27	10		
SMX	3.70	253.9	108.1	96	7	37	8	8.37	25.1
			92.1			43	6		
OXY	2.67	461.1	426.0	106	7	29	20	6.10	18.3
			443.0			17	14		
CHLOR	3.28	479.1	444.1	111	7	29	20	6.57	19.7
			462.0			23	18		
TET	2.89	445.1	410.2	106	7	27	12	5.97	17.9
			427.2			19	18		

[a] tR—retention time; [b] Q1—precursor ion; [c] Q3—fragment ion; [d] DP—declustering potential; [e] EP—entrance potential; [f] CE—collision energy; [g] CXP—cell exit potential; [h] LOD—limit of detection; [i] LOQ—limit of quantification.

Table S6. Total compound concentration at the beginning of the experiment (C_0) and in the equilibrium conditions (after the process) (C_t), per unit of reactor volume, μg L^{-1}.

Compound(s)	C_0 (μg L^{-1})	C_t (μg L^{-1})
MET	512,000	2.1 *
AMO	1,024,000	7.0 *
AMP	1,024,000	8.6 *
ENR	1,024,000	176,950.0
SMX	512,000	28.0
OXY	1,024,000	443,058.4
CHLOR	1,024,000	19,734.0
TET	1,024,000	370,339.0

* Calculated according to the limit of detection (LOD).

Table S7. Concentrations, sorption parameters, and removal efficiency of the test substances during the anaerobic digestion.

Compound	Xp (μg g^{-1})	S (μg L^{-1})	log Kd	R (%)
MET	0.02 ± 0.001	<LOD	n.a.	≈100
AMO	<LOD	<LOD	n.a.	100
AMP	<LOD	14.3	n.a.	≈100
ENR	4350.0 ± 254.56	42,100.0 ± 1272.79	2.0	83
SMX	0.24 ± 0.08	25.6	1.1	≈100
OXY	962.0 ± 43.08	401,500.0 ± 3535.53	0.4	57
CHLOR	163.5 ± 7.78	11,935.0 ± 2920.35	1.1	98
TET	5070.0 ± 1725.34	128,500.0 ± 13,435.03	1.6	64

Xp—concentration of the compound sorbed onto suspended solids; S—concentration of the compound dissolved in the liquid phase; LOD—limit of detection; log Kd—log of the calculated value of Kd (expressed in L kg^{-1}); n.a.—not available; ± indicates standard deviation.

Table S8. Hydration of samples after the anaerobic digestion process.

Compound(s)	Hydration (%)
MET	95.4
AMO	93.8
AMP	94.9
ENR	96.9
SMX	95.4
OXY	95.7
CHLOR	95.2
TET	95.2

Table S9. Relative abundances (RAs) of genes specific for *Archaea* antibiotic resistance genes, and integrase genes in digestate with antibiotic addition and substrate without antibiotic addition. The relative abundances (RAs) of the ARGs were calculated as: copy number of ARG/copy number of 16S rRNA.

	Genes Specific for *Archaea*			MLS Group		Fluoroquinolones			Tetracyclines		Sulfonamides			Beta-Lactams		Integrase Genes	
	*mcr*A	*MSC*	*MST*	*erm*F	*lin*A	*mef*A	*qep*A	*aac6′-Ib-cr*	*tet*A	*tet*M	*tet*Q	*sul*1	bla_{TEM}	bla_{OXA}	*cfx*A	*int*I1	*int*I2
MET	1.10×10^{-3}	7.51×10^{-4}	3.17×10^{-5}	2.67×10^{-4}	3.61×10^{-5}	4.00×10^{-3}	8.33×10^{-5}	1.20×10^{-4}	2.14×10^{-2}	1.40×10^{-1}	9.02×10^{-3}	5.97×10^{-3}	2.32×10^{-5}	0	1.77×10^{-4}	6.89×10^{-4}	4.08×10^{-3}
AMO	2.29×10^{-4}	2.05×10^{-4}	2.46×10^{-5}	1.28×10^{-4}	3.31×10^{-5}	4.42×10^{-4}	5.45×10^{-2}	8.74×10^{-5}	1.98×10^{-3}	1.40×10^{-2}	1.16×10^{-2}	1.19×10^{-3}	2.26×10^{-6}	2.79×10^{-5}	1.57×10^{-4}	1.35×10^{-4}	5.88×10^{-4}
AMP	8.47×10^{-5}	1.04×10^{-4}	2.61×10^{-5}	6.03×10^{-5}	1.70×10^{-5}	3.55×10^{-4}	8.45×10^{-3}	6.74×10^{-5}	4.92×10^{-3}	2.50×10^{-2}	6.29×10^{-3}	1.72×10^{-3}	5.05×10^{-7}	0	1.51×10^{-4}	2.46×10^{-4}	9.98×10^{-4}
ENR	3.10×10^{-2}	3.70×10^{-3}	2.69×10^{-4}	9.24×10^{-5}	7.10×10^{-6}	3.92×10^{-4}	7.99×10^{-2}	7.48×10^{-5}	9.67×10^{-2}	9.08×10^{-2}	1.30×10^{-2}	1.82×10^{-3}	4.88×10^{-7}	3.47×10^{-5}	2.26×10^{-4}	3.02×10^{-4}	9.94×10^{-4}
SMX	2.19×10^{-2}	1.18×10^{-3}	3.41×10^{-4}	7.70×10^{-4}	2.55×10^{-5}	4.17×10^{-4}	2.40×10^{-2}	1.00×10^{-4}	1.69×10^{-2}	1.60×10^{-2}	1.20×10^{-2}	1.89×10^{-3}	2.78×10^{-5}	0	7.42×10^{-4}	2.46×10^{-4}	1.43×10^{-3}
OXY	1.17×10^{-2}	6.92×10^{-4}	1.73×10^{-4}	3.32×10^{-4}	3.40×10^{-5}	1.86×10^{-4}	5.38×10^{-2}	7.86×10^{-5}	3.65×10^{-3}	9.93×10^{-3}	3.56×10^{-3}	2.56×10^{-3}	3.16×10^{-6}	0	1.35×10^{-4}	2.14×10^{-4}	5.81×10^{-4}
CHLOR	2.87×10^{-2}	2.66×10^{-3}	6.28×10^{-4}	3.84×10^{-3}	1.35×10^{-5}	6.17×10^{-4}	3.58×10^{-5}	1.09×10^{-4}	3.93×10^{-2}	4.96×10^{-2}	5.06×10^{-3}	2.96×10^{-3}	2.76×10^{-6}	0	1.06×10^{-4}	4.32×10^{-4}	1.02×10^{-3}
TET	2.57×10^{-2}	1.05×10^{-3}	1.99×10^{-4}	7.78×10^{-3}	7.48×10^{-6}	4.88×10^{-4}	3.01×10^{-2}	4.92×10^{-5}	5.44×10^{-2}	4.02×10^{-2}	3.00×10^{-3}	1.78×10^{-3}	1.17×10^{-6}	0	1.71×10^{-4}	3.30×10^{-4}	8.05×10^{-4}
SA	2.89×10^{-2}	9.98×10^{-4}	3.48×10^{-4}	1.37×10^{-2}	2.52×10^{-5}	4.33×10^{-4}	6.32×10^{-2}	6.56×10^{-5}	2.37×10^{-2}	1.21×10^{-2}	3.55×10^{-3}	1.97×10^{-3}	3.56×10^{-6}	0	1.47×10^{-4}	1.80×10^{-4}	5.85×10^{-4}

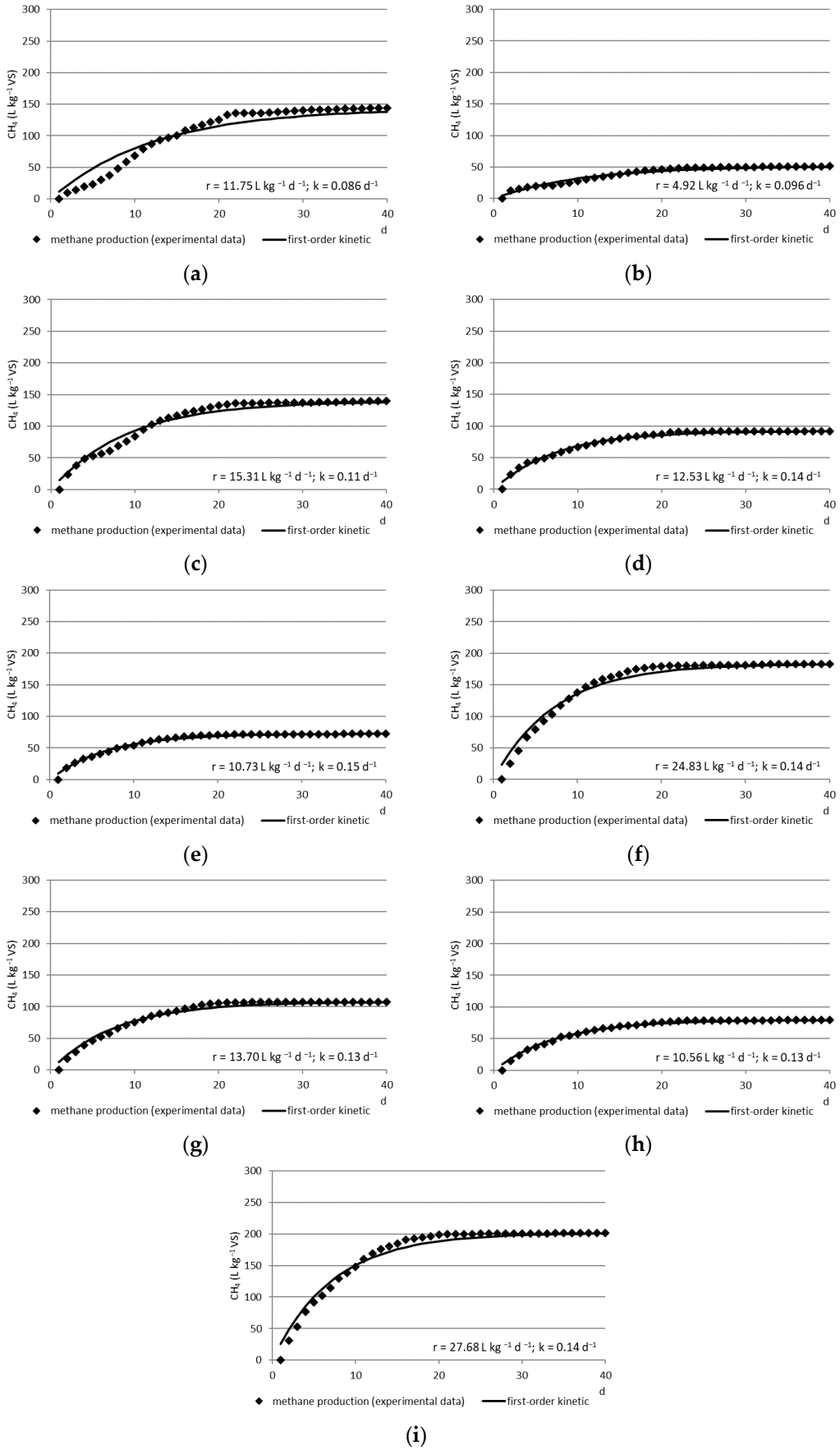


Figure S1. Average values of specific methane production during anaerobic digestion in bioreactors with (**a**) MET, (**b**) AMO, (**c**) AMP, (**d**) OXY, (**e**) ENR, (**f**) SMX, (**g**) CHLOR, (**h**) TET supplementation, and (**i**) control reactor (SA).

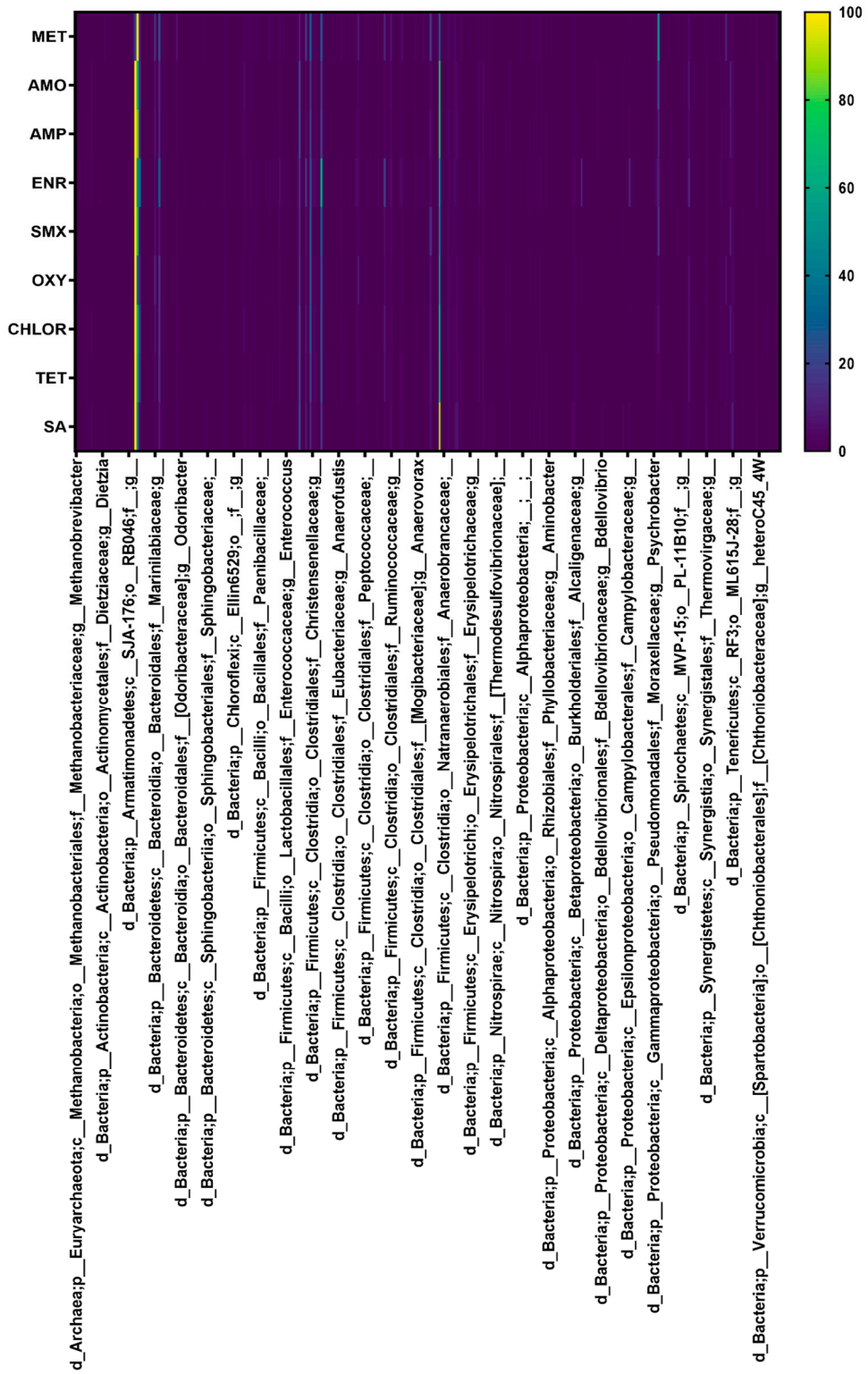
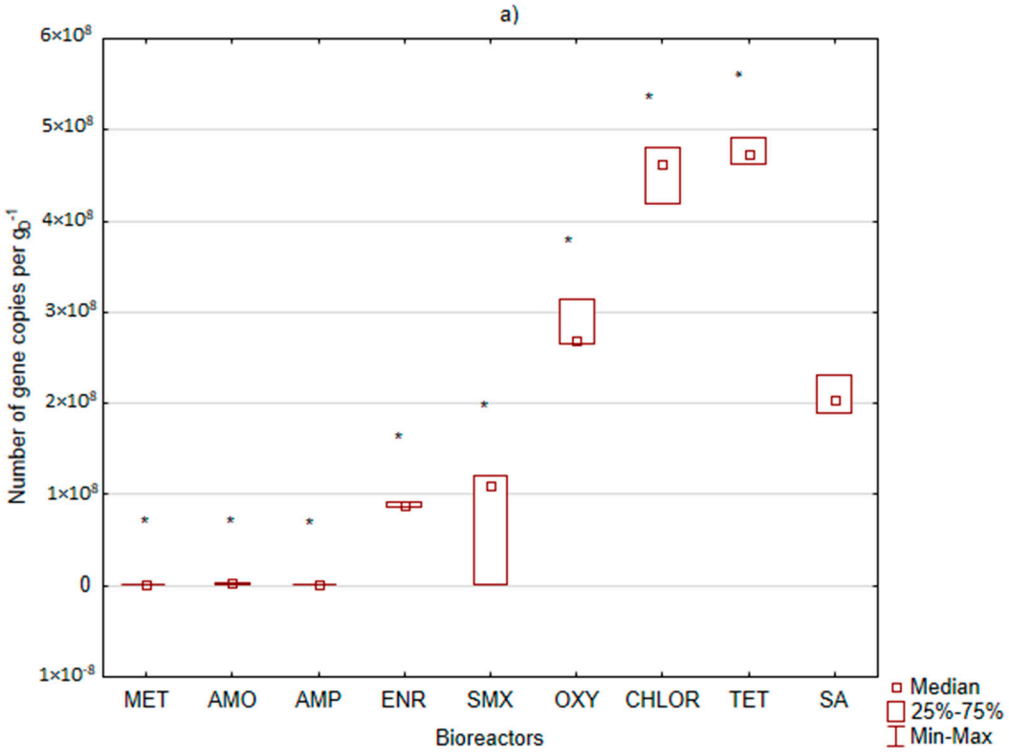
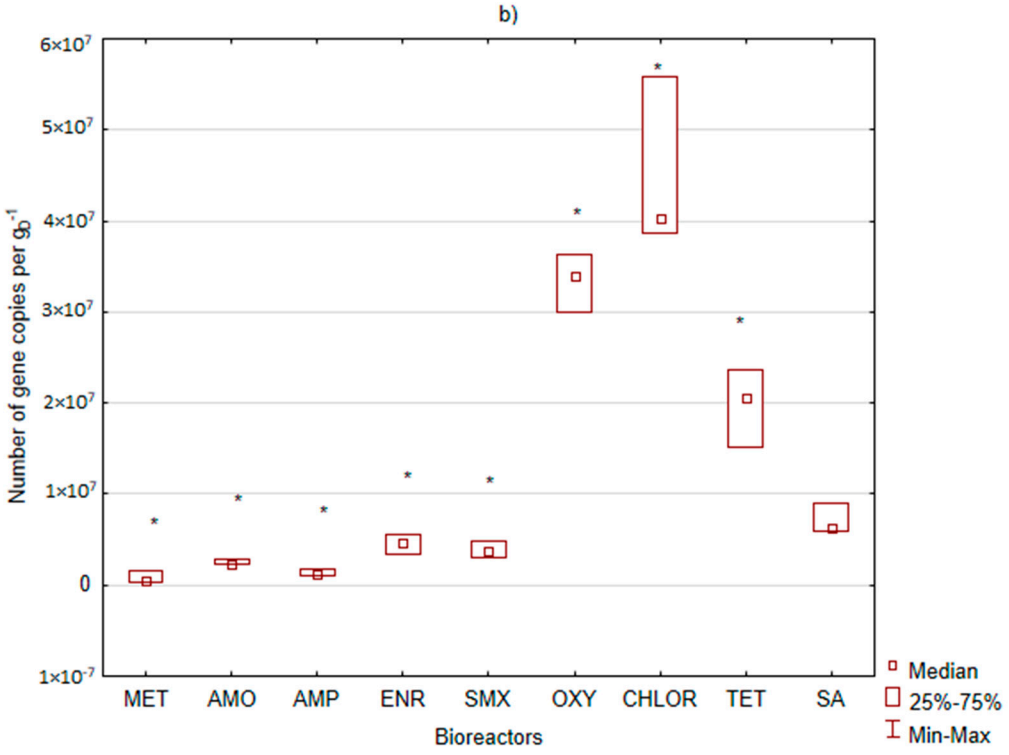


Figure S2. Detailed taxonomy and community structure of *Archaea* and bacteria (based on the OTUs) up to species in digestate with supplementation of selected antibiotics and in bioreactor with substrate without antibiotic supplementation (SA).


a)
6×10^8
5×10^8
4×10^8
3×10^8
2×10^8
1×10^8
0
1×10^-8
Number of gene copies per g_D^-1
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max


b)
6×10^7
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
1×10^-7
Number of gene copies per g_D^-1
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max

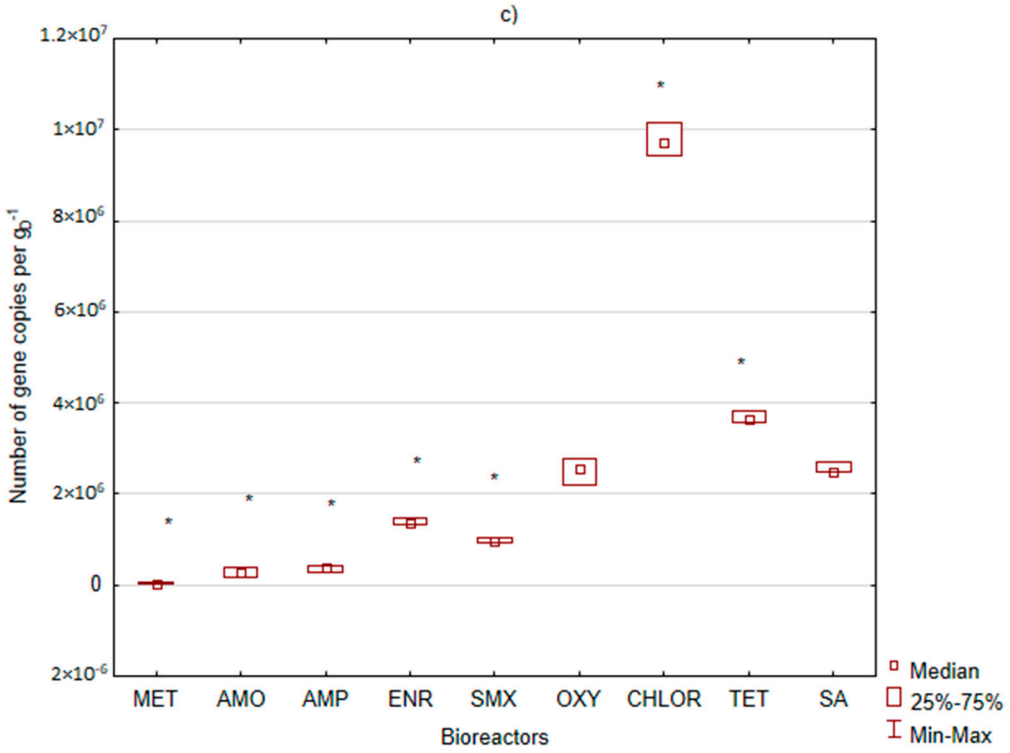
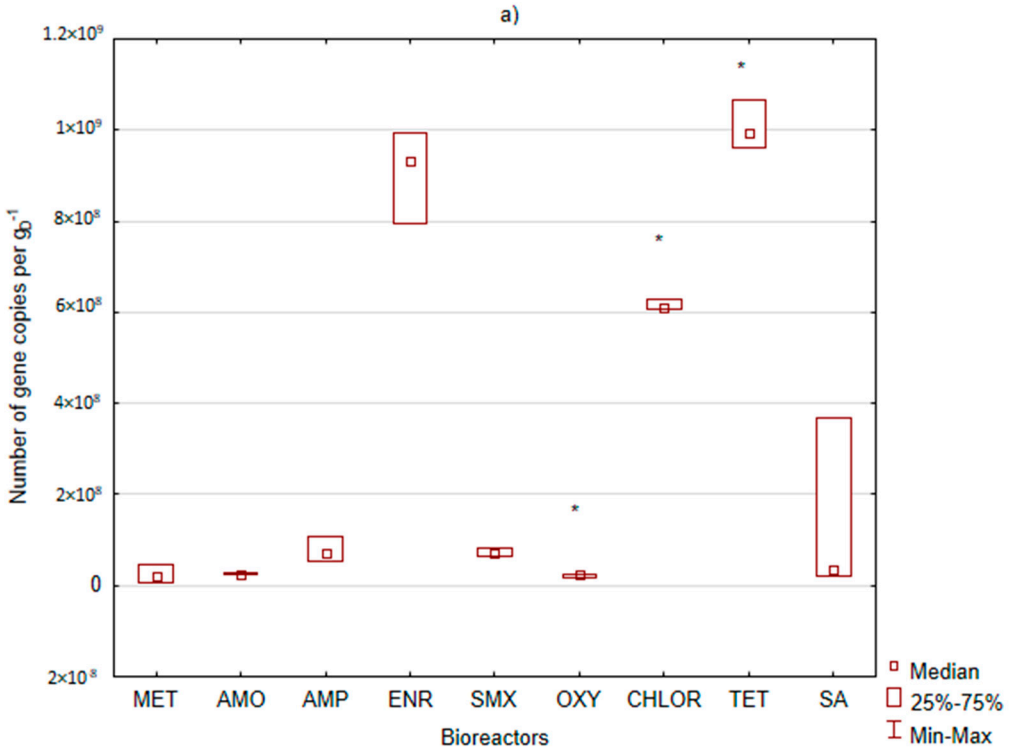


Figure S3. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of genes specific for *Archaea* like (**a**) *mcr*A gene, (**b**) *MSC* gene, (**c**) *MST* gene are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant).



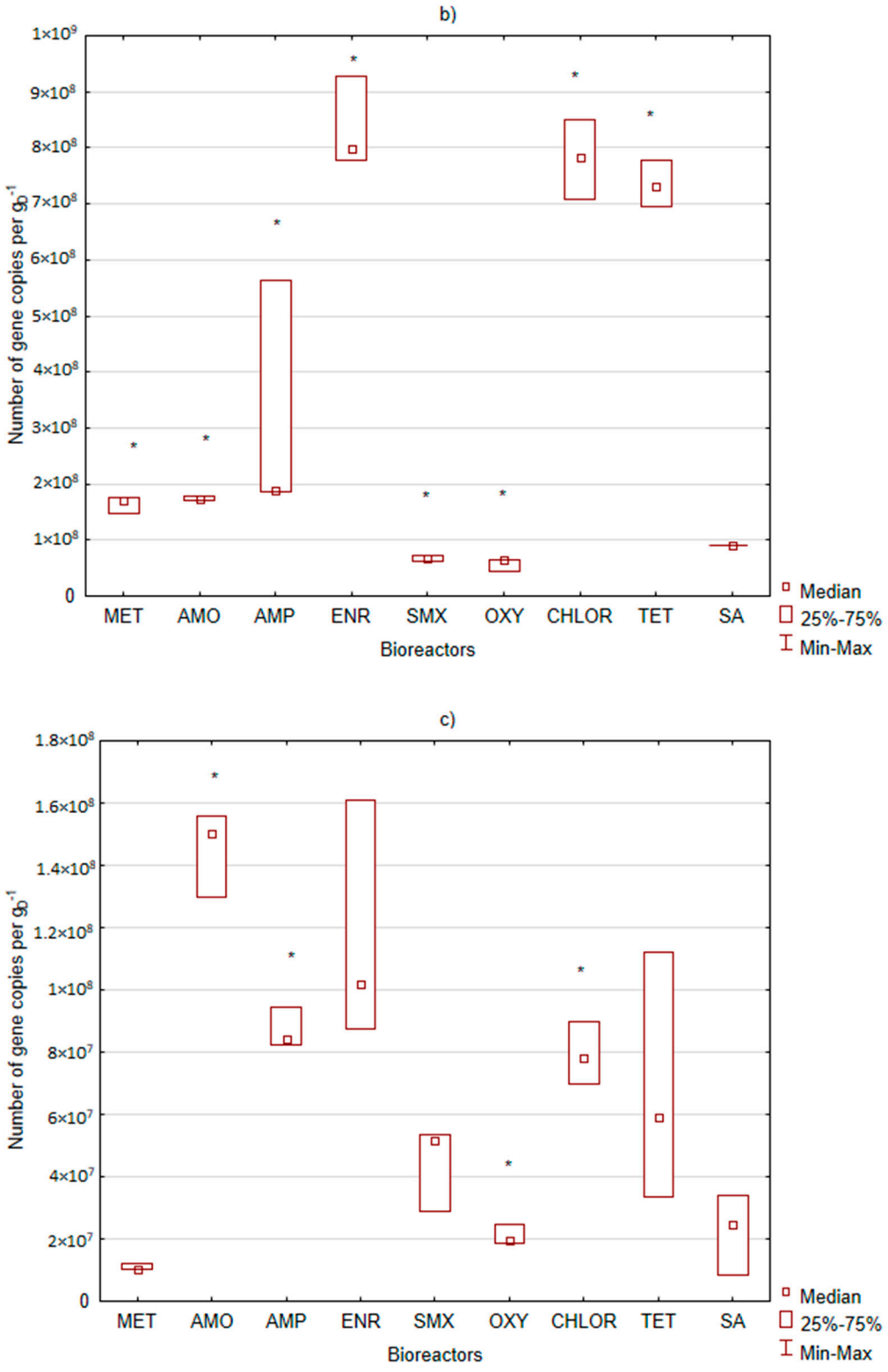
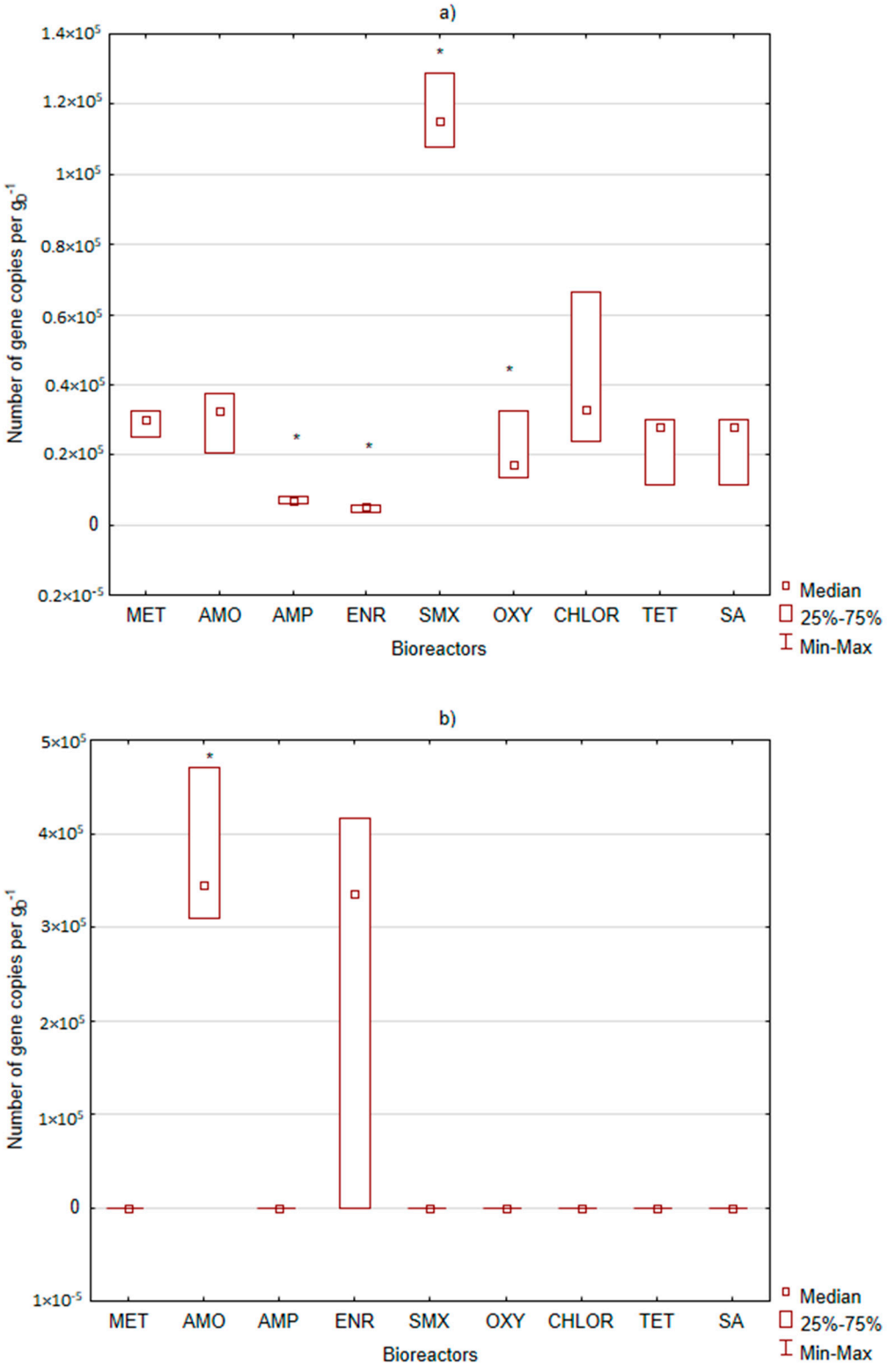


Figure S4. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of *tet* genes like (**a**) *tet*A, (**b**) *tet*M, (**c**) *tet*Q are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant).


a)
1.4×10^5
1.2×10^5
1×10^5
0.8×10^5
0.6×10^5
0.4×10^5
0.2×10^5
0
0.2×10^-5
Number of gene copies per g_D^-1
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max
b)
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
1×10^-5
Number of gene copies per g_D^-1
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max

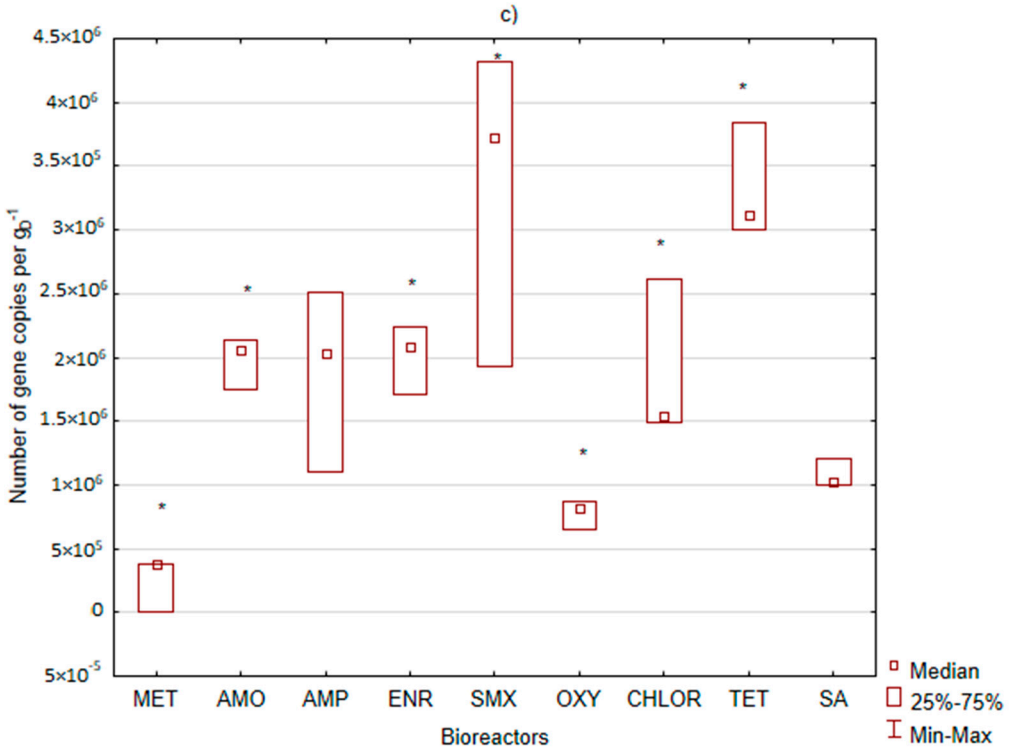


Figure S5. Frame-mustache charts showing the results of the Kruskal-Wallis test (ANOVA). Statistically significant changes in the concentration of *bla* genes like (**a**) *bla*TEM, (**b**) *bla*OXA, (**c**) *cfx*A are marked with an asterisk (*) (p values <0.05 were considered statistically significant).

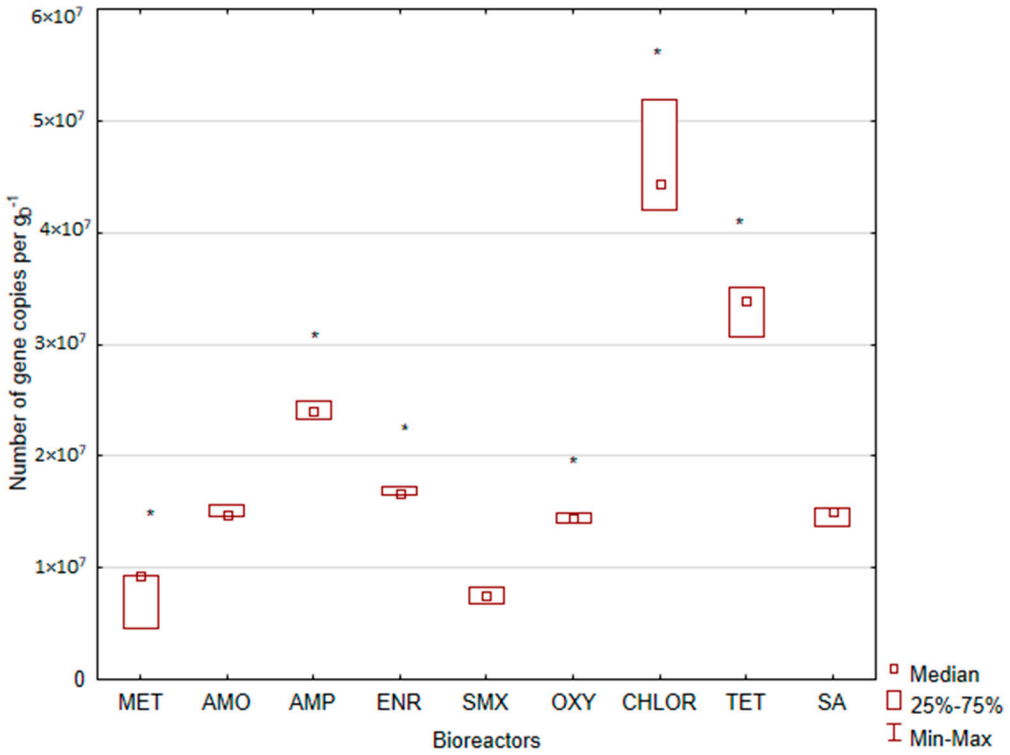
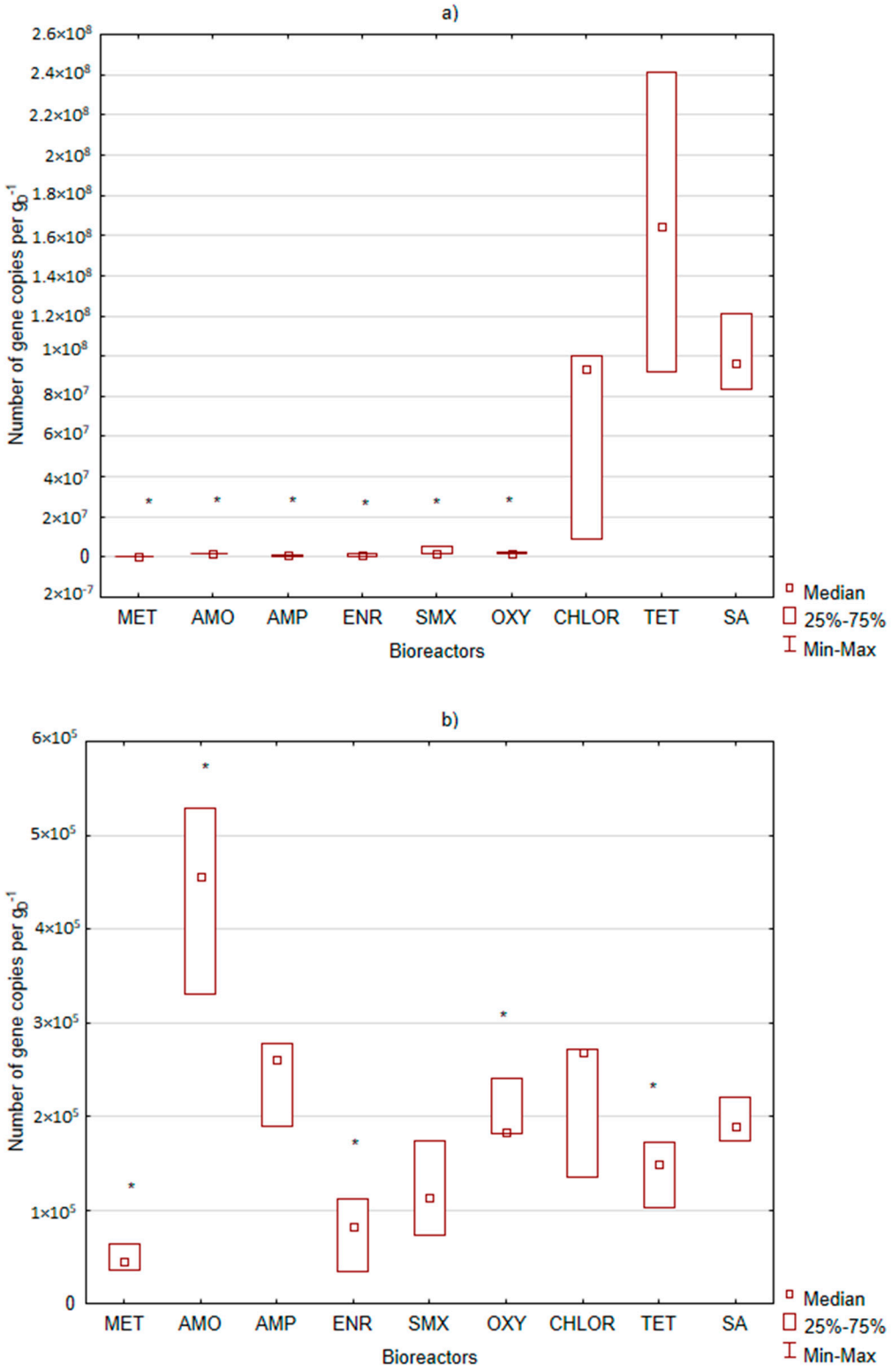


Figure S6. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of *sul*1 genes are marked with an asterisk (*) (*p*-values < 0.05 were considered statistically significant).


a)
Number of gene copies per gDS-1
2.6×10^8
2.4×10^8
2.2×10^8
2×10^8
1.8×10^8
1.6×10^8
1.4×10^8
1.2×10^8
1×10^8
8×10^7
6×10^7
4×10^7
2×10^7
0
2×10^-7
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max
b)
Number of gene copies per gDS-1
6×10^5
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
MET
AMO
AMP
ENR
SMX
OXY
CHLOR
TET
SA
Bioreactors
Median
25%-75%
Min-Max

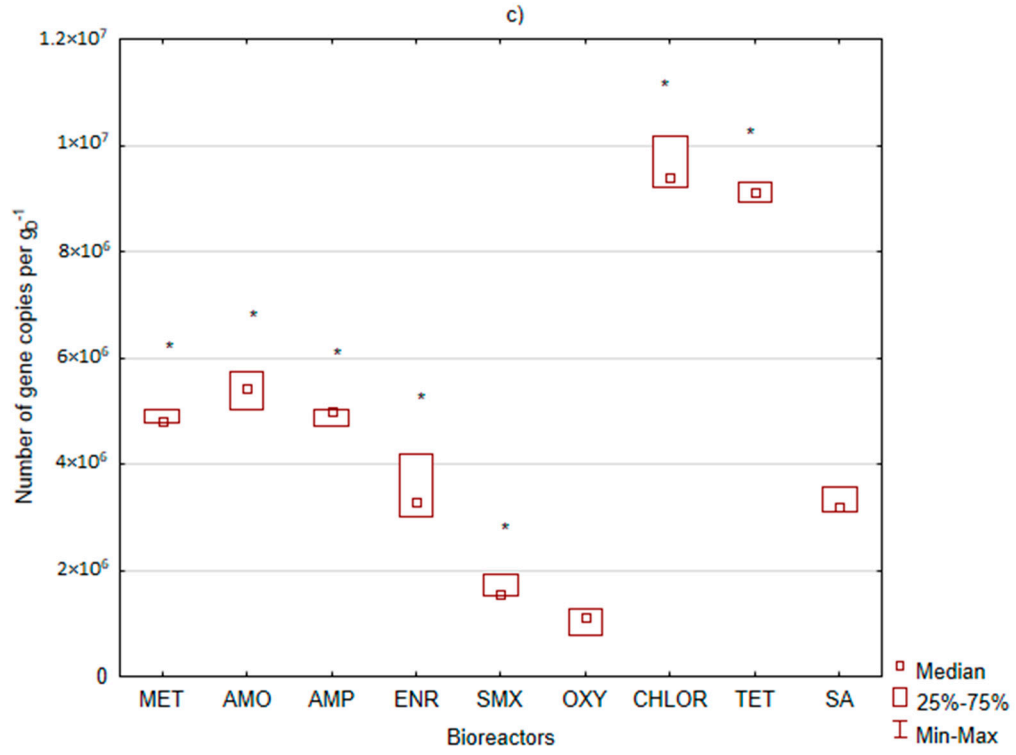
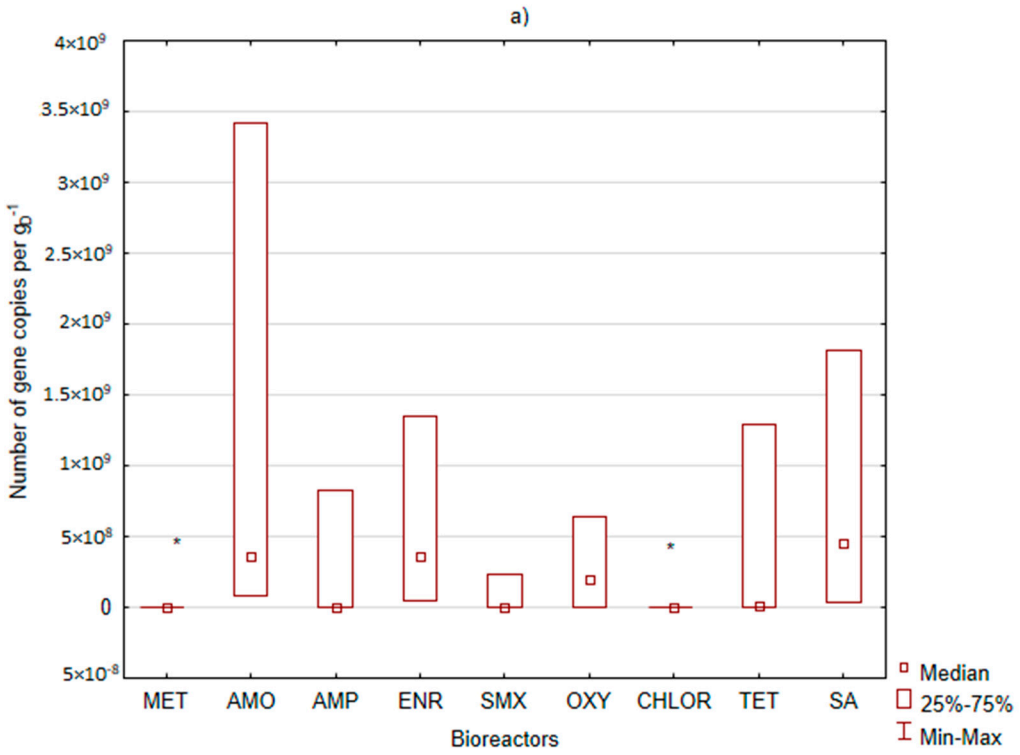


Figure S7. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of MLS group genes like (**a**) *erm*F, (**b**) *lin*A, (**c**) *mef*A are marked with an asterisk (*) (p-values < 0.05 were considered statistically significant).



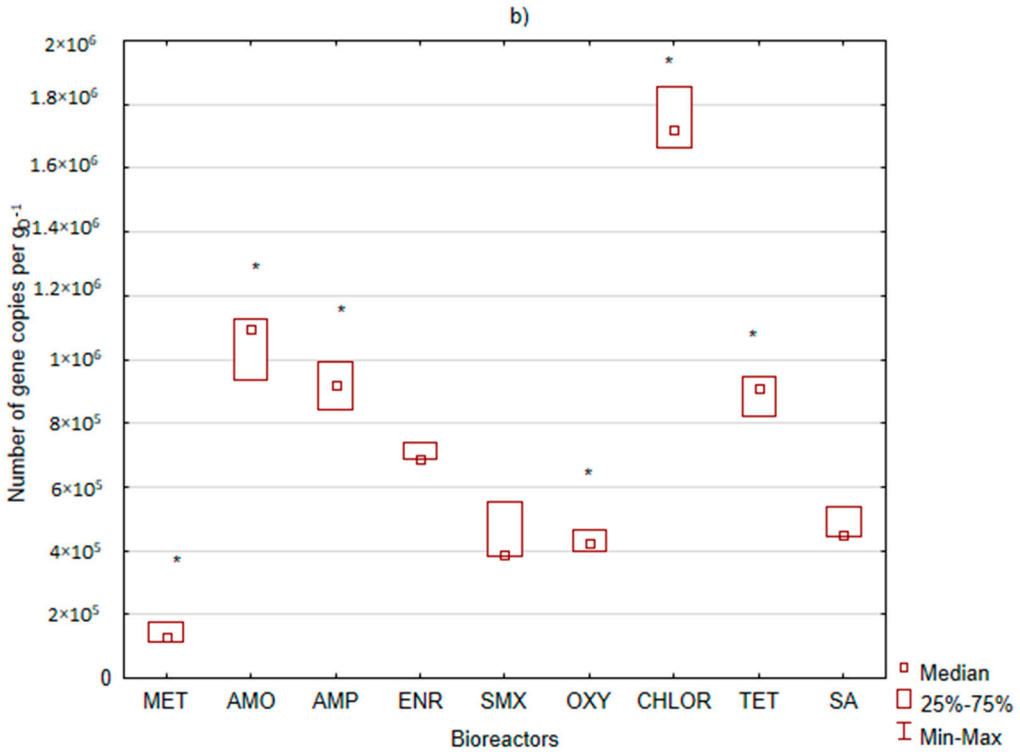
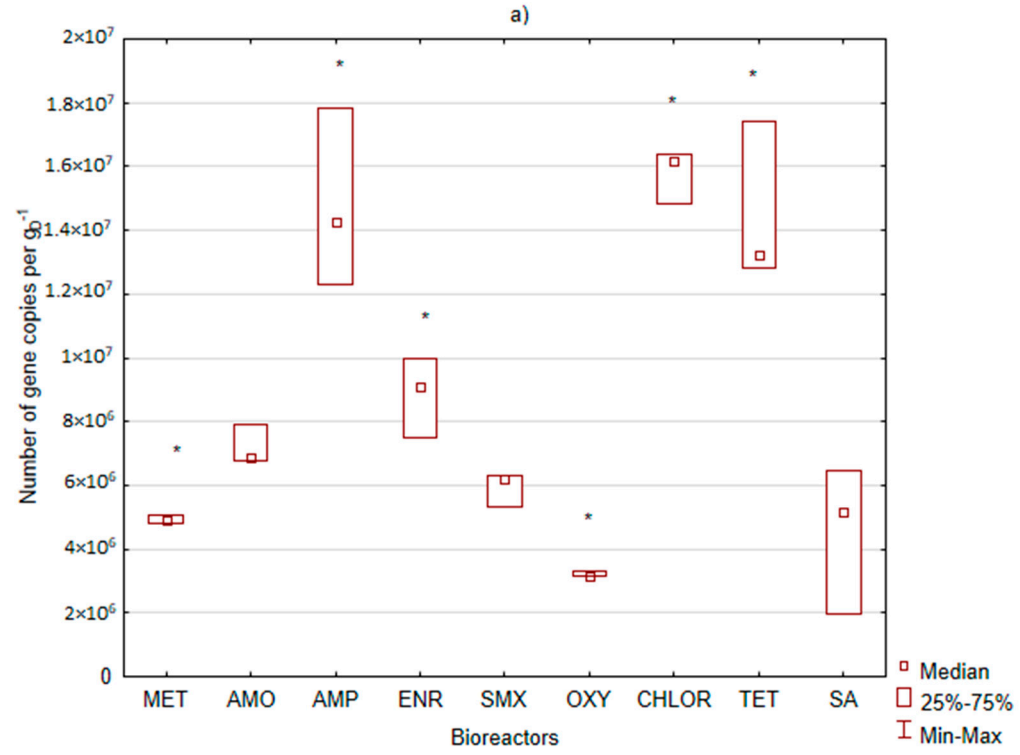


Figure S8. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of fluoroquinolones genes like (**a**) *qep*A and (**b**) *aac-(6′)-Ib-cr* are marked with an asterisk (*) (*p*-values < 0.05 were considered statistically significant).



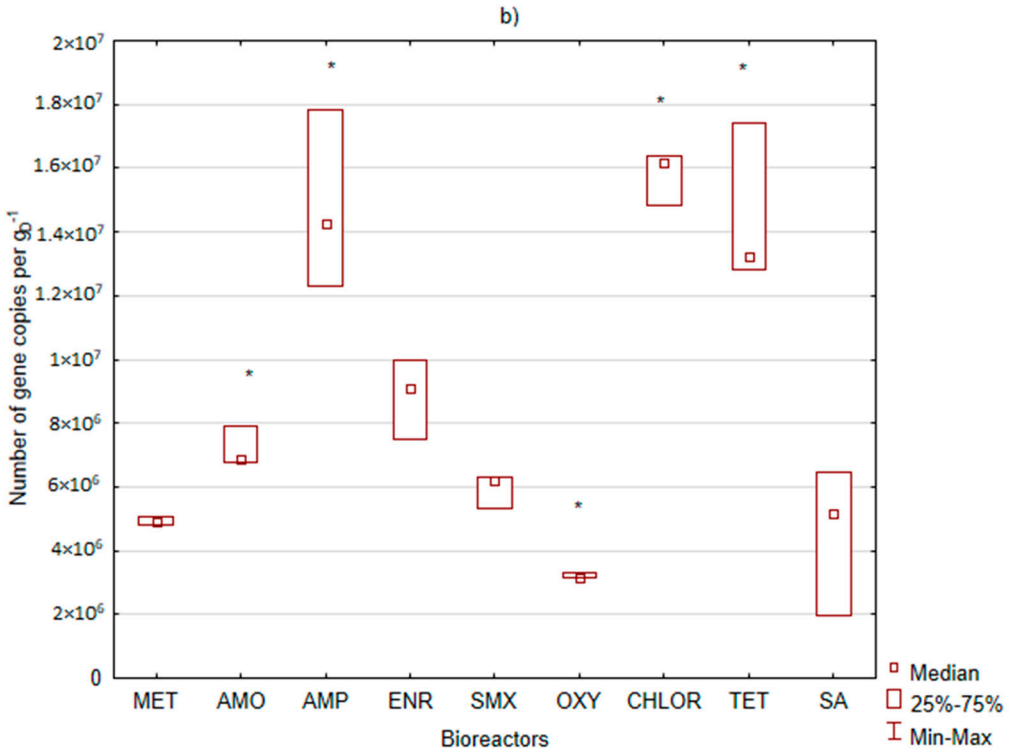


Figure S9. Frame-mustache charts showing the results of the Kruskal–Wallis test (ANOVA). Statistically significant changes in the concentration of integrase genes like (**a**) *int*I1, (**b**) *int*I2 are marked with an asterisk (*) (*p*-values < 0.05 were considered statistically significant).