Supplementary Material

Supplementary Material and Methods: Quantification of tobramycin and colistin

Calibrator and sample preparation: Calibrators were prepared by spiking FAB medium with standard solutions of tobramycin and colistin. Calibrator concentrations ranged from 3.13 µM up to 50 µM for tobramycin and from 1.56 µM to 50 µM for colistin A and B. Calibrators were stored in 30 µL aliquots at -20°C. 30 µL of each sample and calibrator were treated with 30 µL of an internal standard solution (2.5 µM streptomycin dissolved in acetonitrile). 10 µL of this mixture were directly injected to the HPLC system.

HPLC-MS/MS analysis: Analysis of colistin and tobramycin was achieved using high performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS). Chromatographic separation was performed using a Shimadzu system (Shimadzu, Duisburg, Germany), consisting of two HPLC-Pumps (LC-30AD), a temperature controlled autosampler (SIL-30AC), a degasser (DGU-20A5), oven (CTO-20AC) and a control unit (CBM-20A). A Nucleodur HILIC column (125 x 3 mm; 3 µm) was purchased from Macherey Nagel (Munich, Germany) and served as chromatographic column for analyte separation in HILIC mode. Mobile phases were 70/30 acetonitrile/water [v/v] (A) and 30/70 acetonitrile/water [v/v] (B), each containing 20 mM ammonium formate and 0.2 % formic acid. For chromatographic separation the column was kept at 40 °C. A gradient was applied increasing from 15 % B to 100 % B within 9 minutes. 100 % B was hold for 2 minutes followed by a 5-minute column reequilibration time. The flow rate was set at 500 µL min^{-1}.

Detection and quantification of colistin and tobramycin was carried out on a QTRAP®5500 mass spectrometer (Sciex, Framingham, Massachusetts) equipped with an electrospray ionization source, operating in positive ionization mode. For SRM detection, the following mass transitions were identified: colistin A, [M+2H]2+: m/z 585.5 → 241.1 (quantifier) and

m/z 585.5 → 223.2 (identifier); colistin B, [M+2H]2+: m/z 578.5 → 227.2 (quantifier) and m/z 578.5 → 202.1 (identifier), tobramycin, [M+H]+: m/z 468.2 → 163.1 (quantifier) and m/z 468.2 → 324.2 (identifier). Streptomycin ([M+H]+; m/z 582.3 → 263.2) served as internal standard for tobramycin as well as for colistin.

Control of the HPLC and MS/MS systems as well as data sampling is performed by Analyst software, version 1.5.2 (Sciex). Data interpretation of the MS/MS signals was carried out by calculating the ratios of the peak areas of the calibrators and samples in relation to the respective peak areas of the internal standard. For colistin the peak areas of the specific mass fragments from colistin A and B were summarized.

Supplementary Table: extra file

Supplementary Figures

47 **Figure S1**

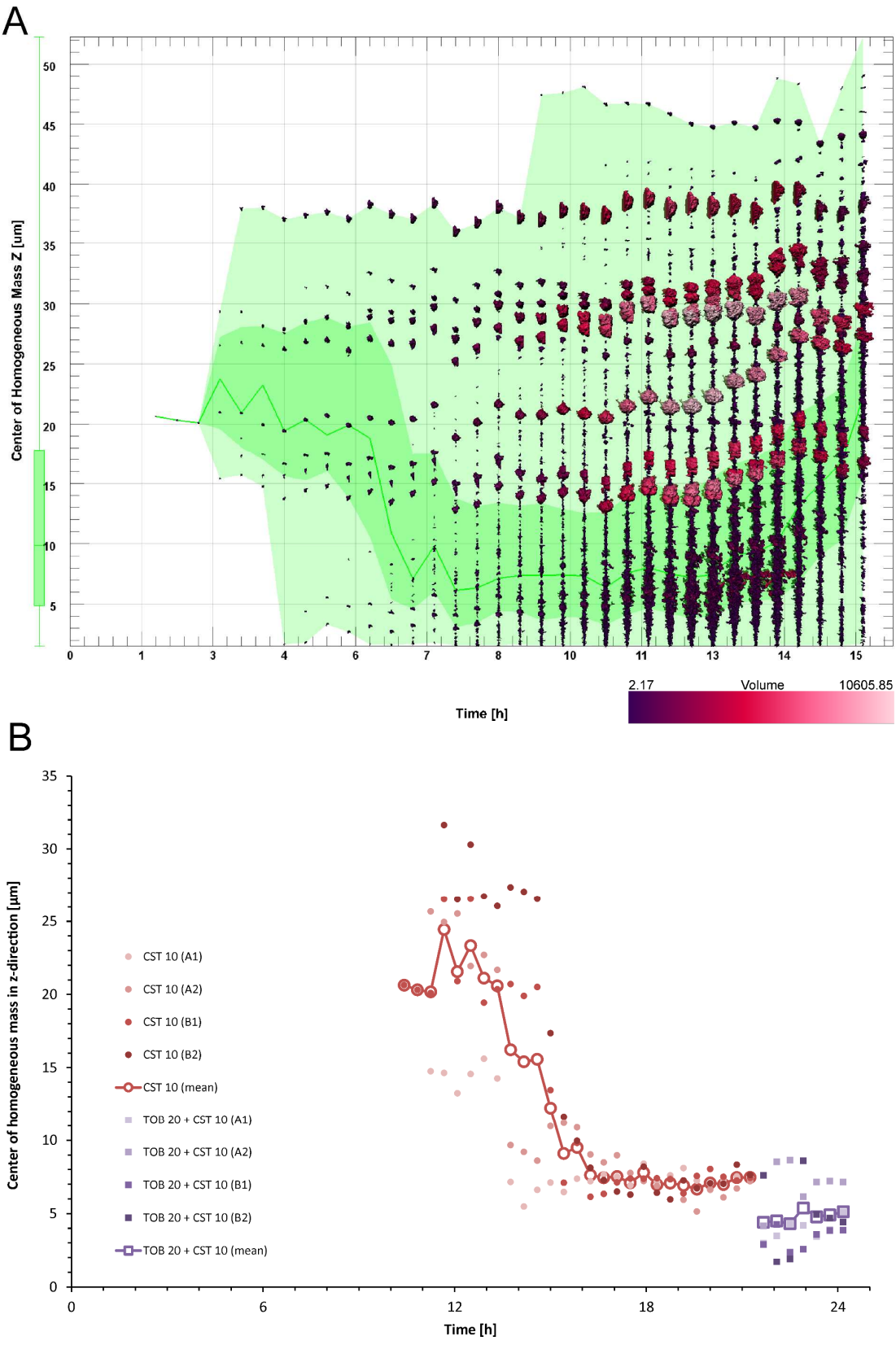

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Fig. S1. Determination of the z-position of regrowth cell clusters in 1 h-treated biofilms. (a) Example for the change in the center of homogeneous mass (CHM) in z of regrowth cell clusters after colistin (10 µg ml^{-1}) treatment plotted over time. The height of detected regrowing cells is estimated by the CHM in z-direction (0 µm = substratum) of cells with strong GFP fluorescence. With increasing time, more and more dividing cells/clusters appear in the biofilm growing over time (visualized by the miniaturized isosurfaces, which increase in size -> pink color code). The mean CHM in z-direction is about 20 µm for approx. 5 h and strongly drops to values below 10 µm due a massive increase of cells close to the substratum. In (a), time point 0 h at the x-axis is equivalent to the time point 8 h after biofilm treatment. (b) Summarized analysis of the CHM in z for 2 biological and 2 technical replicas of colistin and the combination of colistin and tobramycin. Tobramycin alone hardly gave rise to regrowth detectable via strong GFP fluorescence.

Figure S2

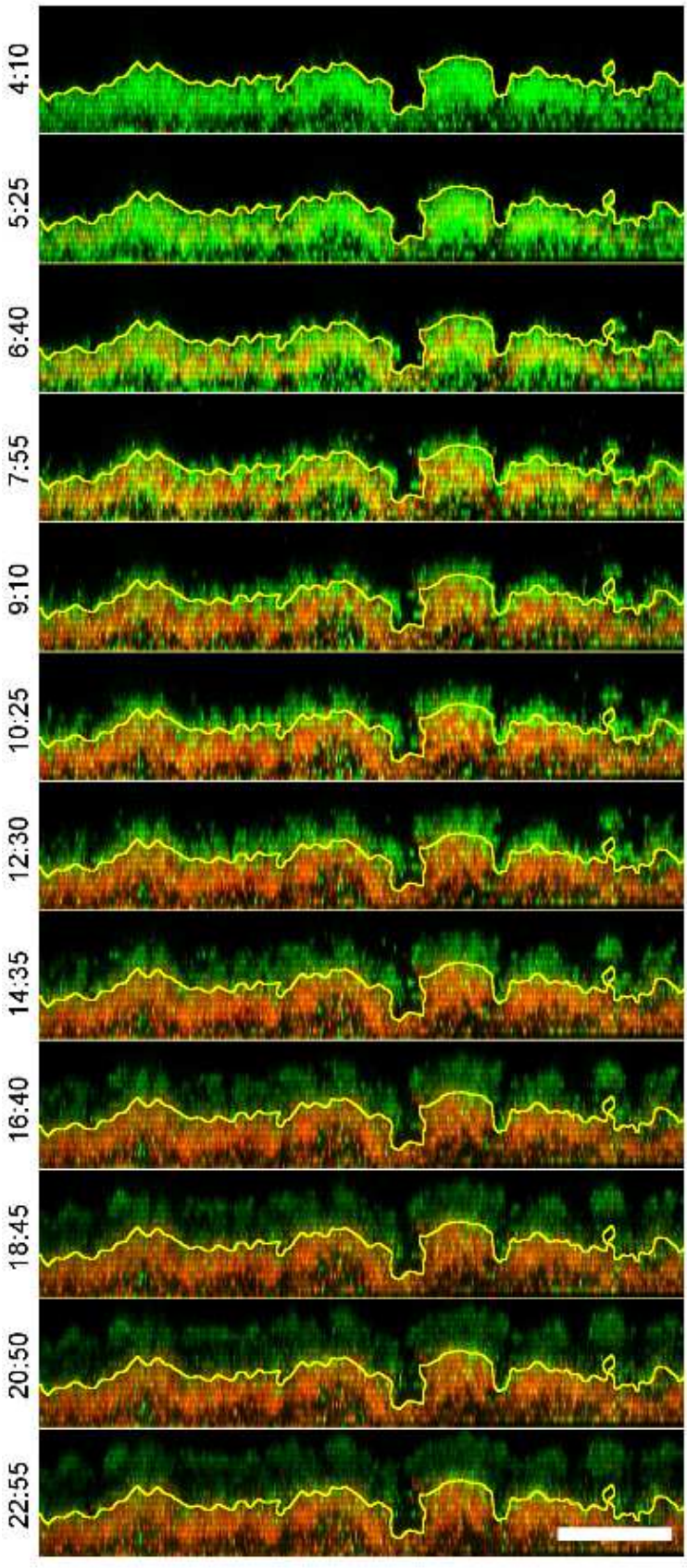

Fig. S2: Killing pattern of a biofilm continuously treated with 2.5 µg ml^{-1} colistin. The biofilm was cultivated for 48 h before treatment. The yellow line within the xz-sectioning projection represents the biofilm surface at time point 4:10 h after antibiotic exposure. Bacterial killing starts below the outer layer, proceeding inwards and – with a slight delay – outwards towards the former surface layer (but not further). Green: GFP fluorescence, red: PI fluorescence. Scale bar 50 µm.

Figure S3

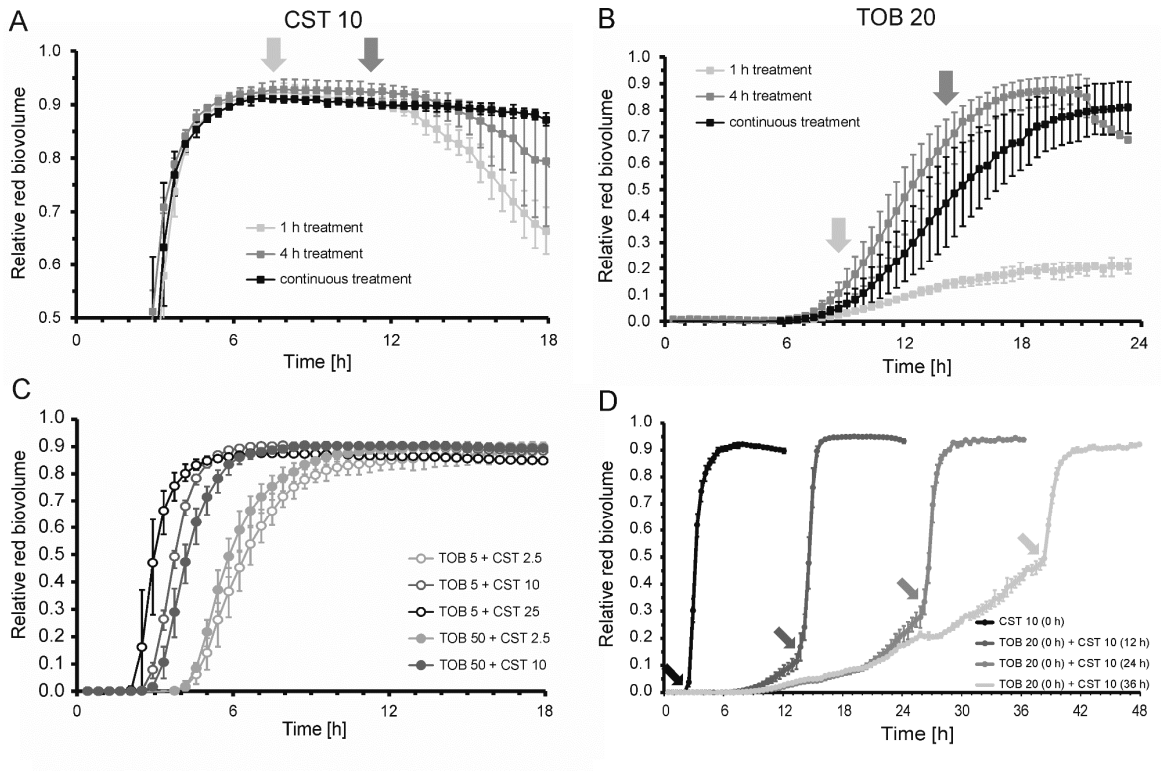

Fig. S3. Killing and regrowth kinetics of 48 h-old PA14 biofilms after variation in antibiotic exposure, combination and sequence. (a-b) Biofilms were exposed to (a) 10 µg ml^{-1} colistin or (b) 20 µg ml^{-1} tobramycin for 1 h, 4 h and continuously. Arrows indicate the time point of regrowth. (c) Biofilms are continuously treated with combinations of colistin (CST) and tobramycin (TOB) at given concentrations (in µg ml^{-1}). Variation of colistin concentration stronger affects killing curve progression than variation in tobramycin concentration. (d) Biofilms were exposed to 10 µg ml^{-1} colistin after no (black), 12 h (dark grey), 24 h (grey) and 36 h (light grey) pretreatment with 20 µg ml^{-1} tobramycin. Antibiotics were given for 1 h in a 12 h interval. Arrows indicate the time point when colistin killing is getting visible. Colistin killing is not influenced by tobramycin pretreatment. Data are mean +- standard deviation of two positions in two independent experiments (n = 4), respectively two technical replicas (2 positions within one lane) for part d. Data of biofilms treated for 4 h / continuously have already been shown in parts in Fig. 2.

Figure S4

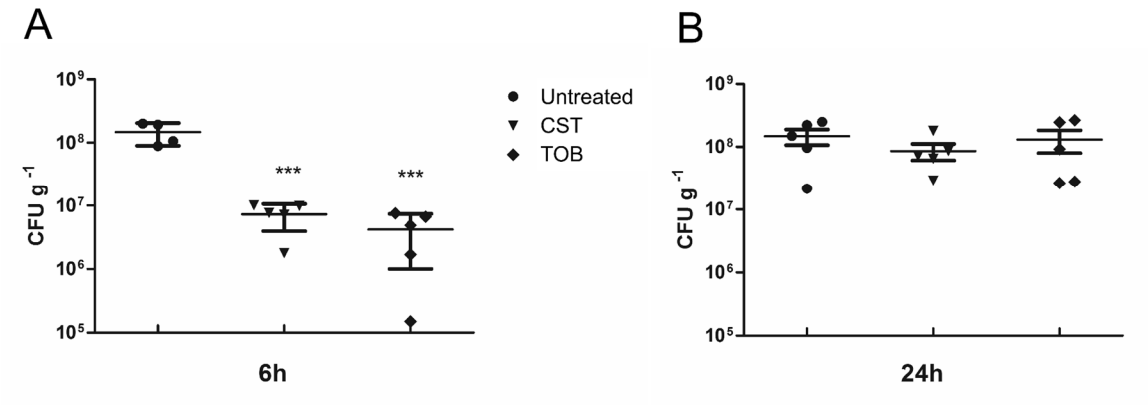

Fig. S4. *In vivo* regrowth of biofilm cells following antibiotic exposure. CFU counts 6 h (a) and 24 h (b) after intra-tumoral injection of 20 mg kg^{-1} tobramycin or 20 mg kg^{-1} colistin, (n = 5).

Figure S5

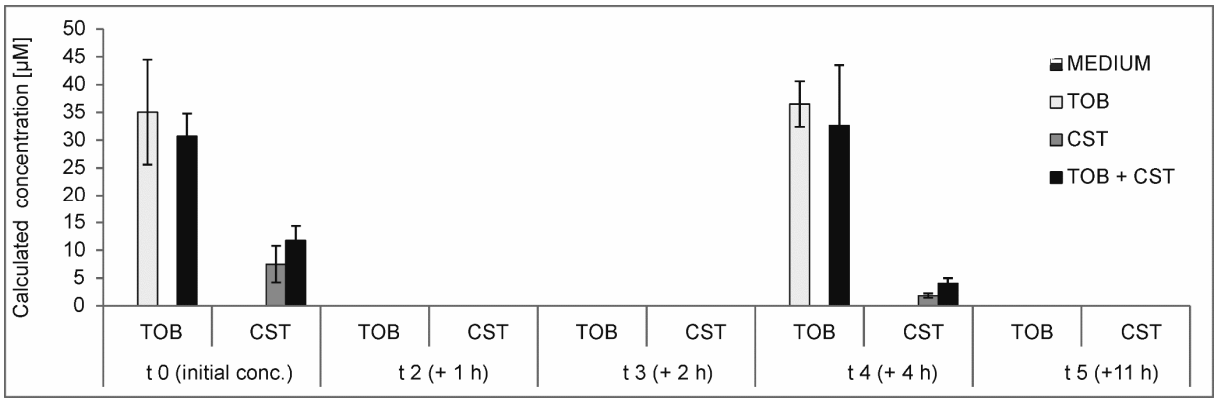

Fig. S5. Antibiotic concentrations in the flow through of treated biofilms. Samples were collected 1 h, 2 h, 4 h and 11 h after flow restart at the outlet tube (approx. 150 cm behind the flow cell). Both antibiotics could be detected at time point t4, 4 h after injection into the system. Samples of medium (non-treated) (black and white), tobramycin (white), colistin (grey) and a combination treatment (black) were analyzed via HPLC and mass spectrometry. n = 2 (biological replica).

Supplementary Movie legends

Video S1. Video sequences of various antibiotic treatments. Biofilms were cultivated for 48 h and treated for a period of 1 h (A, D), 4 h (E-J) or continuously (K-P) with 2.5, 10 and 25 µg ml^{-1} colistin, 5, 20 and 50 µg ml^{-1} tobramycin, and 70 % EtOH (B) (killing control). Video sequences contain biofilm-sectioning projections, which have been acquired every 25 minutes over a period of 24 h. Green: GFP fluorescence, red: PI fluorescence. Scale bar 50 µm.

Video S2. Exemplary visualization of the isosurface generation with the software Imaris for the analysis of biofilm regrowth. The Biofilm was cultivated for 48 h and treated for a period of 4 h with 50 µg ml^{-1} tobramycin. The video sequence contains biofilm 3D maximum intensity projections, which have been acquired every 25 minutes over a period of 24 h. Green: GFP fluorescence, red: PI fluorescence. Grey: Isosurface of the regrown population (strong GFP signal).

Video S3. Visualization of the top layer growth despite antibiotic exposure. The Biofilm was cultivated for 48 h and treated continuously with 2.5 µg ml^{-1} colistin. The video sequence contains a biofilm 3D maximum intensity projections, which has been acquired every 25 minutes over a period of 24 h. Green: GFP fluorescence, red: PI fluorescence.

Video S4. Comparison of two treatment regimens against in vitro biofilms. Biofilms were cultivated for 48 h and four times exposed for 1 h to 10 µg ml^{-1} colistin, 20 µg ml^{-1} tobramycin or a combination of both in two treatment intervals: the first set was treated every 8 h and the second set every 12 h. Video sequences contain biofilm-sectioning projections, which have been acquired every 25 minutes over a period of 48 h. Green: GFP fluorescence, red: PI fluorescence. Scale bar 50 µm.

Table S1:Summary of killing curve analysis

	Sample	replica	1% killing*		5% killing		50% killing		IC50		Half Y		slope		Max Killing†	Start of Regrowth		BAI‡		Doubling Time (isosurface)	
exposure	antibiotic [µg ml^{-1}]	biol + tech	t_{eff1}		t_{eff5}		t_{eff50}		t_{IC50}		rel. red biovolume		Hill Slope		% red biovolume	$t_{regrowth}$		Δ t		Δ t	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	mean	SD	mean	SD	mean	SD
1 h	**TOB 20**	2+2	7.66	0.15	10.22	0.23	no		13.12	0.39	0.12	0.01	0.19	0.01	23%	8.54	0.47	0.89	0.41	2.49	1.72
	CST 10	2+2	2.16	0.02	2.45	0.07	3.22	0.12	3.17	0.12	0.46	0.00	1.24	0.07	92%	7.50	0.42	5.34	0.41	1.46	0.19
	TOB 20 + CST 10	2+2	1.66	0.07	1.90	0.06	3.09	0.09	3.01	0.09	0.46	0.00	0.84	0.02	93%	10.73	0.62	9.07	0.62	0.93	0.10
4 h	**TOB 5**	2+2	10.72	0.86	15.95	0.89	no		20.00	1.06	0.11	0.02	0.10	0.01	22%	*10.83*	0.00	0.44	0.46	2.70	0.80
	TOB 10	1+1	8.27	0.56	10.27	0.02	no		14.06	0.17	0.23	0.00	0.23	0.01	45%	14.79	0.21	6.52	0.35	3.61	1.48
	TOB 20	2+2	6.50	0.24	7.93	0.39	12.39	0.88	11.86	0.65	0.44	0.03	0.26	0.01	88%	14.17	0.93	7.67	0.79	1.19	0.16
	TOB 50	2+2	5.51	0.54	6.68	0.42	11.50	0.39	10.64	0.18	0.42	0.03	0.23	0.01	84%	*16.25*	3.76	10.74	3.43	1.62	0.57
	CST 1	1+1	10.18	0.79	14.21	0.44	no		16.54	0.45	0.08	0.01	0.17	0.01	17%	g.d.		n.d.		n.d.	
	CST 2.5	3+3	4.02	0.21	4.54	0.24	6.10	0.31	5.82	0.25	0.42	0.04	0.80	0.06	83%	*8.10*	0.21	1.45	2.05	n.d.	
	CST 5	1+1	2.80	0.20	2.96	0.01	3.74	0.02	3.74	0.02	0.49	0.00	1.70	0.13	99%	7.08	0.00	4.24	0.11	1.07	0.00
	CST 10	2+2	2.02	0.16	2.25	0.16	3.08	0.19	3.00	0.16	0.45	0.01	1.24	0.05	90%	10.52	1.31	8.50	1.27	0.98	0.02
	CST 25	2+2	1.25	0.04	1.35	0.03	2.03	0.05	1.99	0.05	0.47	0.00	1.00	0.05	94%	15.31	0.62	14.07	0.62	0.99	0.24
	TOB 5 + CST 2.5	2+2	3.84	0.38	4.38	0.51	7.47	1.68	6.28	0.75	0.39	0.05	0.51	0.10	78%	12.09	1.14	8.45	1.08	1.24	0.08
	TOB 20 + CST 10	2+2	2.51	0.05	3.00	0.10	4.76	0.19	4.55	0.19	0.43	0.01	0.69	0.02	86%	21.67	0.00	19.16	0.05	0.68	0.07
	70% EtOH	2+2	1.25	0.00	1.27	0.00	1.46	0.00	1.46	0.00	0.50	0.00	n.d.		99%	n.d.		n.d.		n.d.	
continuous	**TOB 2**	2+2	no		no		no		no		no		0.14	0.03	n.d.	g.d.		n.d.		n.d.	
	TOB 5	3+3	13.50	1.26	16.18	1.40	*24.20*	0.95	20.57	0.54	0.21	0.03	0.17	0.02	42%	n.d.		n.d.		n.d.	
	TOB 20	2+2	7.21	0.35	8.96	0.61	14.84	1.54	13.80	0.96	0.41	0.04	0.20	0.01	81%	n.d.		n.d.		n.d.	
	TOB 50	3+3	5.62	0.32	7.12	0.52	13.61	1.40	12.13	1.02	0.39	0.03	0.18	0.01	78%	n.d.		n.d.		n.d.	
	CST 0.5	2+2	17.35	2.82	18.54	1.55	no		20.11	0.94	0.04	0.03	0.19	0.06	8%	g.d.		n.d.		n.d.	
	CST 1	2+2	8.93	0.32	10.31	0.20	18.33	1.75	13.64	0.23	0.30	0.02	0.23	0.01	60%	g.d.		n.d.		n.d.	
	CST 2.5	3+3	3.88	0.66	4.73	0.51	7.15	0.55	6.59	0.40	0.38	0.02	0.54	0.03	76%	g.d.		n.d.		n.d.	
	CST 10	2+2	2.30	0.24	2.43	0.28	3.05	0.26	3.00	0.27	0.46	0.00	1.22	0.10	91%	n.d.		n.d.		n.d.	
	CST 25	2+2	1.91	0.18	2.04	0.11	2.54	0.10	2.46	0.08	0.44	0.01	1.64	0.10	88%	n.d.		n.d.		n.d.	
	TOB 5 + CST 2.5	5+5	4.06	0.18	4.50	0.15	6.51	0.21	6.21	0.17	0.45	0.01	0.48	0.01	89%	15.00	1.98	10.94	2.07	1.46	0.26
	TOB 5 + CST 10	2+2	2.55	0.03	2.78	0.09	3.77	0.05	3.68	0.04	0.45	0.00	1.06	0.02	91%	n.d.		n.d.		n.d.	
	TOB 5 + CST 25	2+2	2.14	0.16	2.32	0.17	2.97	0.20	2.87	0.20	0.44	0.00	1.28	0.08	87%	n.d.		n.d.		n.d.	
	TOB 50 + CST 2.5	2+2	3.98	0.02	4.39	0.06	5.88	0.23	5.69	0.19	0.45	0.01	0.58	0.02	90%	n.d.		n.d.		n.d.	
	TOB 50 + CST 10	2+2	2.78	0.13	3.13	0.15	4.25	0.16	4.12	0.15	0.45	0.00	0.81	0.03	91%	n.d.		n.d.		n.d.	

* Start of antibiotic activity; † Maximal killing = 2* Half Y; ‡ Biofilm Active Interval; n.d. not detectable; g.d. growth detectable despite treatment; italic: not determined in all samples