Supporting Information

Inactivation of antibiotic resistant bacteria and resistance genes by ozone: from laboratory experiments to full-scale wastewater treatment

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I. List of chemicals and reagents (purity > 95 %, if not stated otherwise)

LB: NaCl (10mg)	AppliChem, Germany
yeast extract (10 mg)	Becton Dickinson, France
trypton (5 mg)	Becton Dickinson, France
1 L A. dest	
PBS buffer: NaCl (13 mM)	AppliChem, Germany
$Na_2HPO_4 \times 2\ H_2O$ (1 mM),	Sigma Aldrich, Switzerland
KH_2PO_4 (0.2 mM),	Sigma Aldrich, Switzerland
KCl (2.7 mM)	Sigma Aldrich, Switzerland
For experiments PBS buffer was 1:10 diluted in nano pure water, autoclaved and 0.2µm filtered.	
Sulfamethoxazole	Sigma Aldrich
Trimethoprim	Sigma Aldrich
Tetracycline	Sigma Aldrich
Norfloxacin	Sigma Aldrich
Ceftazidime	Sigma Aldrich
Pimaricin (50 %)	ASA Spezialenzyme GmbH**,** Germany
1 mM HCl	Sigma Aldrich
Cinnamic acid (CA)	Sigma Aldrich, recrystalized
tert-Butanol	Sigma Aldrich
SYBR green	life technologies, Switzerland
Propidium iodide	life technologies, Switzerland
RNAse A	Sigma Aldrich
Lysozyme	Sigma Aldrich
Proteinase k	Roche Diagnostics, Switzerland
CIA (49:1)	Sigma Aldrich
Isopropanol	Sigma Aldrich
Na-Acetat	Sigma Aldrich
CTAB	Sigma Aldrich
TRIS	Sigma Aldrich

EDTA	Sigma Aldrich
Glycogen	Roche Diagnostics
Ethanol	Sigma Aldrich

II. Production of ozone gas and –stock solutions

Ozone gas was produced by an ozone generator (Innovatec, Rheinbach model CMG 3-3, Germany) from pure oxygen and bubbled into nanopure water cooled in an ice bath yielding a concentrated ozone stock solution of ~55-65 mg L^{-1}. The ozone concentration was calculated using an absorption coefficient ε_{258} = 3200 M^{-1} cm^{-1}.[1]

III. Preparation and cultivation of *E. coli* J53 (R388, *sul1*) and wastewater bacteria in ozonation experiments

An overnight-culture of *E. coli* J53, which carries the sulfonamide resistance gene *sul1* (and trimethoprim resistance gene *drfB2*) on broad-host-range IncW plasmid R388, was grown in Luria Bertani broth supplemented with sulfamethoxazole and trimethoprim (76 and 4 µg mL^{-1}, respectively). After 12 h shaking at 180 rpm at 37 °C, cells were harvested (3000 rpm, 3 min at room temperature (RT)) and washed 3 times in 1:10 diluted PBS (SI, I). Re-suspended in 1 mL of 1:10 diluted PBS, 2 mL of the initial culture resulted in about 10^9 cells per mL, with more than 80 % membrane intact cells, as determined by flow cytometry (see section 2.5 of the main manuscript) prior to each experiment. The starting cell concentration of *E. coli* J53 for the experiments was ~10^6 cells per mL.

For laboratory experiments, secondary clarified effluent water (SE) was 10µm filtered (∅ 47 mm, nylon-net filter, Merck Millipore, Germany), autoclaved, 0.2µm filtered (∅ 47 mm, cellulose nitrate, Sartorious, Germany) to remove the autoclave-derived background signal and re-inoculated with

E. coli J53. For experiments with wastewater bacteria, SE was either 10µm filtered or used as is for evaluating the influence of flocs on survival of bacteria and damage of intracellular ARG during ozonation (see Table 1 of the main manuscript).

For plate counts (see section 2.6 of the main manuscript), samples from ozonation experiments and from the WWTP were serially diluted in 1:10 diluted PBS and two appropriate dilutions (chosen based on ICC-measurements) per sample were plated. Samples with low expected vital bacteria were plated without dilution. Plates inoculated with *E. coli* were incubated over night at 37 °C, whereas wastewater bacteria were allowed to grow at 25 °C for 72 h. Plates were manually inspected and colonies counted to determine the number of colony forming units (CFU).

IV. Quench-flow experiments

In reaction loops with differing lengths (allowing to cover a wide range of contact times (Table 1 of the main manuscript)) solutions containing suspended bacteria were mixed 10:1 with the ozone stock solution (prepared in sterile-filtered 1 mM HCl, kept at 4 °C) to yield ozone doses as outlined in Table 1 of the main manuscript. The ozone doses were chosen based either on an observed complete inactivation in batch experiments or on practically relevant ozone doses for micropollutant abatement from wastewater. The reaction was stopped at defined contact times by mixing the reaction 11:1 with cinnamic acid (CA, 10 or 100 mM in set-ups with PBS or SE, respectively), which quenches ozone in a stoichiometric reaction leading to benzaldehyde.[2] *tert*-Butanol (final concentration 0.1 mM) was added to experiments with PBS as a scavenger for OH-radicals. For SE *tert*-BuOH was not added to mimic inactivation during ozonation of real wastewaters. It is expected that even in this case, secondarily formed hydroxyl radicals will be of minor importance because of the very fast direct inactivation with ozone and the efficient hydroxyl radical scavenging by the

dissolved organic matter. The ozone concentration in the reaction solution was determined spectrophotometrically at the beginning and end of each experiment. Additionally, blank reaction mixtures of ozone, CA and nanopure water instead of bacteria were run at the beginning (shortest contact time) and end of the experiment (longest contact time). The decay of the ozone in the stock solution over the course of the experiment (1-1.5 h) was 20-30 %. The initial concentration of vital bacteria in quench-flow reactions was checked at the end of each experiment by mixing bacteria, CA and nanopure water instead of ozone solution at the longest contact time. It was assumed, that the bacterial numbers would remain constant over the course of the experiment.

Quenched reaction mixtures were collected in sterile plastic syringes and sub-sampled for HPLC- (determination of residual-ozone by the formed benzaldehyde formed from the 1:1 reaction with cinnamic acid, see SI, V), flow cytometry and plate counts. Sub-samples for DNA-extraction and qPCR were only taken from the 7 highest reaction times.

V. Determination of residual ozone concentrations and ozone exposures in quench-flow experiments

Based on the stoichiometric reaction of ozone with cinnamic acid to benzaldehyde, the residual ozone concentration for each reaction time was determined via High Performance Liquid Chromatography (HPLC) measurements of the formed benzaldehyde. Prior to analysis, all samples were 0.2-µm-filtered (filter type & brand) and cinnamic acid was re-crystallized to be free of benzaldehyde. Benzahldehyde was analysed by a Ultimate 3000 HPLC system (ThermoScientific, Sunnyvale, CA, USA) using a multiple wavelength diode array absorbance detector. A Cosmosil-C18-MS-II column (100 mm × 3.0 mm I.D., 5 µm; Nacalai Tesque Inc., Kyoto, Japan) was used for the separation. Benzaldehyde was detected at 250 nm, the retention time was 3.6 min using an

isocratic mobile phase of 40% Methanol and 60 % 10mM H_3PO_4 with a flow of 0.6 mL/min. 25 to 200 µL were injected depending on the needed method quantification limit. The method quantification limit for benzaldehyde was 0.01 mM with a standard deviation of ± 5%.

Figure S3 summarizes applied contact times, residual ozone concentrations derived from benzaldehyde measurements and calculated exposures for all set-ups.

VI. WWTP Neugut general information and DOC measurement of SE

The WWTP consists of mechanical followed by activated sludge treatment, nitrification, denitrification and phosphorous elimination. After secondary clarification (Figure S1), wastewater is fed into the ozone reactor (530 m^3, average residence time: 33 minutes), which is divided in 6 compartments by horizontal baffles, optimized to avoid shortcuts.[3]

Dissolved organic carbon (DOC) of SE samples was determined using a TOC-VCPH device (Shimadzu, Kyoto, Japan), according to EN 1484.[4]

VII. Identification of multiresistant wastewater bacteria

For sequencing of 16S rRNA gene fragments from strains isolated on AQ-plates supplemented with antibiotics, DNA was extracted as follows: colonies were picked and boiled (99 °C) in 100 µL of nuclease free water and 50 mg mL^{-1} of Chelex 100 for 8 min for rapid DNA extraction. The lysates were centrifuged (20000 g, 5°C, 10 min) and the supernatant was diluted 1:2 in fresh nuclease free water. PCR protocols for 16S rRNA gene amplification, preparation of samples for sequencing and assignment of sequences to phylogenetic units are described elsewhere.[5] Samples which did not amplify for 16S rRNA genes, were cross-checked for fungal 18S rRNA genes using the primers and protocol from [6]. As all samples were negative for 18S rRNA the respective strains and those which

did not reveal good sequencing results were analysed by MALDI-TOF biotyping. Direct smearing of bacterial cells was performed by transferring cell material from the colonies on the agar plate onto the polished stainless steel MALDI target plate (Bruker Daltonics, Bremen, Germany) using a sterile toothpick. The smear was allowed to dry and immediately covered with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid, HCCA, 10 mg mL^{-1} in acetonitrile-water-trifluoroacetic acid (TFA) (50:47.5:2.5); HCCA from Bruker Daltonics). Sample spectra were collected with a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics) using the software flexControl (Version 3.4, Bruker Daltonics) applying parameters suggested by the manufacturer for biotyping: laser frequency of 60 Hz in the positive linear mode with acquisition in a range of m/z = 2000 to 20000. Bacterial Test Standard (BTS) (Bruker Daltonics) was used as mass calibration standard in each run. Final spectra were obtained by 240 laser shots per spot (40 shots per raster spot). The laser intensity was chosen such as to obtain spectra with maximal absolute peak intensities ranging from about 5×10^3 to 10^4 arbitrary units. The spectra were evaluated using the MALDI BioTyper OC software (Version 3.1, Bruker Daltonics). All isolates were analyzed in duplicate and the highest score of a match against the database was used for identification. Score values were assigned according to the Bruker interpretative criteria: score values higher than 2.0 denoted confidence to species level; between 1.7 and 1.99 to genus level and smaller than 1.7 denoted no confident identification.

VIII. Preparation of standards and determination of LOQ and LOD of qPCR assays

The new primer pair for *sul1* was developed based on *sul1*-sequence alignments (downloaded from ARDB[7]) using BioEdit (version 7.2.5)[8] and Primer 3 software version 1.1.4.[9, 10] In order to check for correct amplification of the new primers, *sul1* was amplified in parallel by an established primer set[11] and checked for specificity against non-target DNA (data not shown).

To prepare standards for absolute gene quantification a 965 bp fragment of ARG *sul1* and a 1465 bp fragment of the universal 16S rRNA gene were amplified from control strains using previously published primers and protocols[11, 12] and cloned into a pGEM T-Easy vector in *E. coli* according to manufacturer's guidelines (Promega, Switzerland). Plasmid extracts (QIAprep Spin Miniprep kit, Qiagen, Switzerland) were quantified with qubit and serially diluted (5 to 5×10^7 gene copies) in AE buffer (Qiagen).

All qPCR assays were run in a volume of 10 µL, containing 2 µL of 1:2 or 1:10-diluted DNA-extract (extraction protocols are given in SI, IX), standard or PCR blank (nuclease free water or AE buffer), 1 × master mix (LightCycler 480 SYBR green mix for *sul1_827 bp* or Probes Master hot start reaction mix for 16S rRNA), primer (0.2 µM each for *sul1*_827 bp or 0.9 µM each for 16S rRNA) and for 16S rRNA 0.3 µM TagMan probe. qPCRs were run on a Roche Light Cycler 480 (Roche Diagnostics, Switzerland) using the following thermal programs: 10 min initial denaturing and 45 cycles of 95 °C for 45 s, 60 °C for 30s, 72 °C for 1 min (*sul1*_827 bp) and 95°C for 40 sec, 53°C for 40 sec, and 72°C for 1 min (16S rRNA). Each sample was run in duplicates, standards were run in quadruplicates. Cp-values of generated qPCR data and gene copies in DNA-extracts were determined by the 2nd Derivative Max method in the Light Cycler 480 software version 1.5.1.62.

The limit of detection for each assay was defined by the lowest Cp-value determined in PCR-or extraction blanks. In the SYBR-green assay for *sul1*_827 bp, a sample was also considered negative, if its melting temperature (Tm) deviated by more than 0.5°C from the Tm of standards or if several Tm peaks were detected. The limit of quantitation was determined by the lowest concentrated standard dilution with a standard deviation of quadruplicate Cp-values smaller than 0.5. Moreover, a sample was considered not quantifiable if Cp-values of replicates differed more than 0.5. LOQs and LODs of qPCR assays are summarized in Table S1.

IX. Intra- and extracellular DNA extraction

Bacterial suspensions from experiments (10 mL (quench-flow) - 30 mL (dose-dependent)) and wastewater samples from WWTP Neugut (100-150 mL) were filtered through 0.2µm polycarbonate membranes (∅ 47 mm, Merck Millipore). Wastewater from sampling campaigns was also 5µm pre-filtered (∅ 47 mm, Merck Millipore). Mock extractions with sterile filters served as controls for contamination during extraction. Filters and filtrates were kept at -80 °C until processed. Intracellular DNA from filters was extracted according to [13] with the following modifications: A RNAse A step (50 µg μL^{-1}, 30 min, 37 °C, 300 rpm) was attached to the two-step enzymatic cell-lysis with lysozyme and proteinase K. Two 1 : 1 purification steps with chloroform : isoamyl alcohol (49:1) were conducted only after filters had completely dissolved. DNA was precipitated overnight at -20 °C with 1 volume of ice-cold isopropanol and 0.1 volumes of 3 M sodium acetate (pH 5.2). DNA concentration of intracellular DNA extracts was determined with a Qubit fluorimeter (Thermo Fisher Scientific, MA, USA) and ranged between 0.5-60 ng μL^{-1}.

To determine whether nucleic acids are released by ozonated cells, extra-cellular DNA was extracted from filtrates using a protocol modified from [14]. In brief: DNA- was precipitated with CTAB-solution for 1 hour at room temperature and centrifuged for 30 minutes at 4 °C, 10.000 rpm (Centrikon H-401, Kontron Instruments, Switzerland). Precipitates were dissolved in 900 µl of high-salt TE-buffer and DNA was again precipitated with 900 µL of ice-cold isopropanol and 2 µl of 20 mg L^{-1} glycogen at -20 °C over night. After purification, DNA was again precipitated overnight at -20 °C with 100 % ethanol and glycogen. DNA pellets were washed once with 70% ethanol. Extracellular DNA-concentrations were too low for quantification by Qubit fluorimeter (Thermo

Fisher Scientific, MA, USA) and were thus checked directly for amplifiable DNA using quantitative PCR. Extraction blanks were prepared by mock extractions from sterile 0.1 mM PBS.

X. Data analysis and determination of inactivation kinetics

To establish the relationship between increasing ozone doses or exposures and bacterial or ARG inactivation (expressed by their logarithmic relative decrease $\log\left(\frac{N}{N_0}\right)$), the best fitting inactivation model for the obtained data from batch and quench-flow experiments was chosen by using the Microsoft Excel Addin GInaFiT.[15] This program allows to fit experimental data to nine types of previously published microbial survival curves and enables to extract important inactivation parameters, such as the duration of the lag phases or inactivation rate constants (k) for differing viability or gene disruption indicators. The best fitting model was chosen by the lowest standard error given for these parameter values. Microsoft Excel was used for analysis and plotting of data from flow cytometry, plate counts, HPLC and qPCR. R was used for statistical tests of significance for observations in laboratory- and full-scale experiments.

XI. Identification of multiresistant bacteria from SE, ozonated effluent and SF

To shed some light on the multiresistant genera surviving ozonation and being released from the WWTP to the environment through a sand filtration (SF) as compared to the non-ozonated SE, resistant isolates were identified from the three treatment steps. S/T/T resistant survivors (Table S4) of ozonation, which were also found in SE and SF mainly belonged to the genera *Aeromonas*, *Chryseobacterium*, *Escherichia coli* & *Pseudomonas*. Less prevalent genera were *Stenotrophomonas, Enterobacter cloacae* and *Acinetobacter*. Culturable S/T/T-resistant strains of *Shewanella* were only detected in the SF. Except for *E. coli* and *Enterobacter,* which are typical fecal indicators, the

discussed multiresistant genera leaving the WWTP are both ubiquitous environmental bacteria and important opportunists.[16-19]

Only three strains with N/C resistance from ozonated effluent were successfully identified as *Escherichia coli* (2) and *Acinetobacter* (1), but both genera were absent among SF isolates (Table S5). N/C-resistant genera present in both SE and SF – though apparently absent in ozonated effluent - were mainly identified as flavobacteria and a few others were *Elizabethkingia* and *Microbacteria*. Some flavobacteria are fish pathogens, and they are well adapted to aquatic habitats,[20] whereas microbacteria can cope with various stressors including heavy metals, radioactivity,[21] ozone,[22] can degrade antibiotics including sulfonamides and norfloxacin[23, 24] and have been isolated from clinical specimens.[25] Finally, *Elizabethkingia* is ubiquitous and an emerging nosocomial pathogen in hospitals.[26] Even though the overlap between ARG in WWTPs and clinics is less than 10 %,[27] these strains may provide the bottleneck for resistance spreading through the aquatic cycle.

XII. Selective effects of ozonation and biological post-treatment at WWTP Neugut

It is known that conventional biological treatment of wastewater can select for MRB.[28] Comparing the 2-log higher proportion of MRB in SE to raw influent (IF) indicate such selective effects occur at WWTP Neugut (Figure S4A). Even though less pronounced, also the relative abundance of *sul1* (normalized to 16S rRNA gene copies) increased by 0.4-log units from IF to SE (Figure S4B). These selective effects underline the importance of implementing an additional step to remove MRB and ARG from treated sewage effluent. Ozonation and sand filtration lead to an overall decrease of 0.75- and 0.26-log units of the relative abundance of S/T/T and N/C resistance, respectively, compared to SE-levels. Although the relative abundance of *sul1* slightly increases following ozonation (by 0.36-log units), sand filtration neutralizes this effect, for which SE and SF have similar *sul1* proportions

(Figure S5B). Hence, our data from the WWTP do not indicate, that ozonation followed by biological sand filtration leads to further selection of MRB or ARG. However, the selective effect, which occurred during conventional treatment can likewise not be (fully) reversed. In contrast to our findings, recent studies reported selection for antibiotic resistant *E. coli*,[29] *Staphylococcus* and *Pseudomonas* strains after ozonation, whereas the proportion of resistant *Enterococcus* strains decreased. [30, 31] Post-treatment did however not select for resistant phenotypes and rather reduced their abundance.[30, 31] Therefore, these studies [30, 31] support our finding that sand filtration does not have a selective effect. However, they also highlight that selection may occur on the level of single species and the conditions that lead to such outcomes require further investigation.

XIII. Prevalence of ARG in extracellular DNA extracts

Besides gene disruption, leakage of cellular content including nucleic acids from membrane-damaged bacteria[32, 33] may also contribute to reduced intracellular ARG numbers during ozonation. To account for this effect, extracellular DNA was extracted from 0.2µm filtered samples. Measuring ARG *sul1* in extracellular DNA-extracts revealed its presence in very low amounts (0.5-2 copies mL^{-1}) in ozonated samples, but it was not detected in SE or biological post-treatment (SF) effluents (data not shown). These results suggest that ARG are released during ozonation and can subsequently be degraded or acquired by SF inhabiting bacteria.[32, 34] However, given the low abundance of free ARG in ozonated effluent samples and their remaining undetectable during laboratory experiments (data not shown), the latter effect is assumed to play a minor role in ARG spread during post-treatment.

Table S1. Summary of qPCR parameters (including slope of the standard curve, qPCR-efficiency, limit of detection-LOD and limit of quantitation LOQ) determined for different assays. Given are the averages (and standard deviations) of 2-4 runs per analyzed gene. Note that for LOQ, not even the standard with the lowest gene copy number showed a standard deviation of Cp-values greater than 0.5.

	slope	efficiency	LOD	LOQ
16S rRNA	-3.73 (± 0.24)	1.86 (± 0.07)	33.46 (± 1.92)	< 50 copies
***sul1*_827 bp**	-3.76 (± 0.03)	1.85 (± 0.01)	34.11 (± 1.04)	< 5 copies

Table S2. Summary of dose-dependent (**ozone doses in mg L^{-1}**) parameters for various viability indicators and DNA/gene stability, determined during ozonation experiments with *E. coli* in differing water matrices and natural wastewater bacteria. k = dose-dependent inactivation constant, n.d.= not detected - = not predicted by model or data, starting inoculum < * 2-log or ** 4-log, LOD/? = prediction is insecure as sample values reached LOD of qPCR or no decreasing trend was detected

Measured feature	Medium /bacteria	ozone dose without effect [$mgO_3\ L^{-1}$]	*k* [$L\ mg^{-1}$]	ozone dose [$mgO_3\ L^{-1}$] for inactivation of 2-log	4-log
Cultivability	*E. coli / PBS*	0.02 (± 0.01)	271 (±56)	0.04	0.06
	E. coli / 10-um filtered SE	0.66 (± 0.04)	20 (±3)	0.88	1.12
	10-um filtered SE / SE bacteria	1.29 (±0.27)	4 (±1)	2.5	-**
	non-filtered SE / SE bacteria	1.81 (±4 10^3)	70 (±6 10^6)	-	-**
N/C resistant	10-um filtered SE / SE bacteria	1.28 (±0.04)	8 (±1)	1.85*	2.45**
	non-filtered SE / SE bacteria	0.61 (±0.04)	3 (±2)	-*	-**
S/T/T resistant	10-um filtered SE / SE bacteria	1.61 (±0.04)	10 (±6)	2.05*	2.50**
	non-filtered SE / SE bacteria	1.28 (±0.16)	5 (±1)	-*	-**
Membrane integrity (ICC)	*E. coli / PBS*	0.03 (± 0.01)	154 (±29)	0.06	0.12
	E. coli / 10-um filtered SE	0.67 (± 0.03)	19 (±2)	0.89	1.4
	10-um filtered SE / SE bacteria	1.38 (±0.02)	4 (±0)	2.52	-
	non-filtered SE / SE bacteria	1.01 (±0.18)	3 (±1)	2.66	-
DNA stability (TCC)	*E. coli / PBS*	> 0.2	n.d.	n.d.	n.d.
	E. coli / 10-um filtered SE	1.31 (± 0.02)	15 (±2)	1.61	-
	10-um filtered SE / SE bacteria	2.80 (±0.09)	5 (±1)	3.69	4.56
	non-filtered SE / SE bacteria	2.51 (±0.24)	2 (±1)	4.62	6.67
qPCR *sul1*	*E. coli / PBS*	> 0.2	n.d.	n.d.	n.d.
	E. coli / 10-um filtered SE	1.46 (± 0.05)	16 (±2)	1.73	2.02
	10-um filtered SE / SE bacteria	2.15 (±0.22)	13 (±8)	2.47	LOD
	non-filtered SE / SE bacteria	2.02 (0.18)	5 (±1)	LOD	LOD
qPCR 16S rRNA	*E. coli / PBS*	> 0.2	n.d.	n.d.	n.d.
	E. coli / 10-um filtered SE	1.63 (± 0.04)	22 (±4)	1.82	-
	10-um filtered SE / SE bacteria	1.92 (±0.19)	6 (±1)	2.63	3.43
	non-filtered SE / SE bacteria	1.91 (±0.54)	2 (±1)	?	?

Table S3. Summary of dose-dependent parameters (**specific ozone doses in gO_3 $gDOC^{-1}$**) parameters for various viability indicators and DNA/gene stability, determined during ozonation experiments with *E. coli* in differing water matrices and natural wastewater bacteria. k = dose-dependent inactivation constant, n.d.= not detected - = not predicted by model or data, starting inoculum < * 2-log or ** 4-log, LOD/? = prediction is insecure as sample values reached LOD of qPCR or no decreasing trend was detected

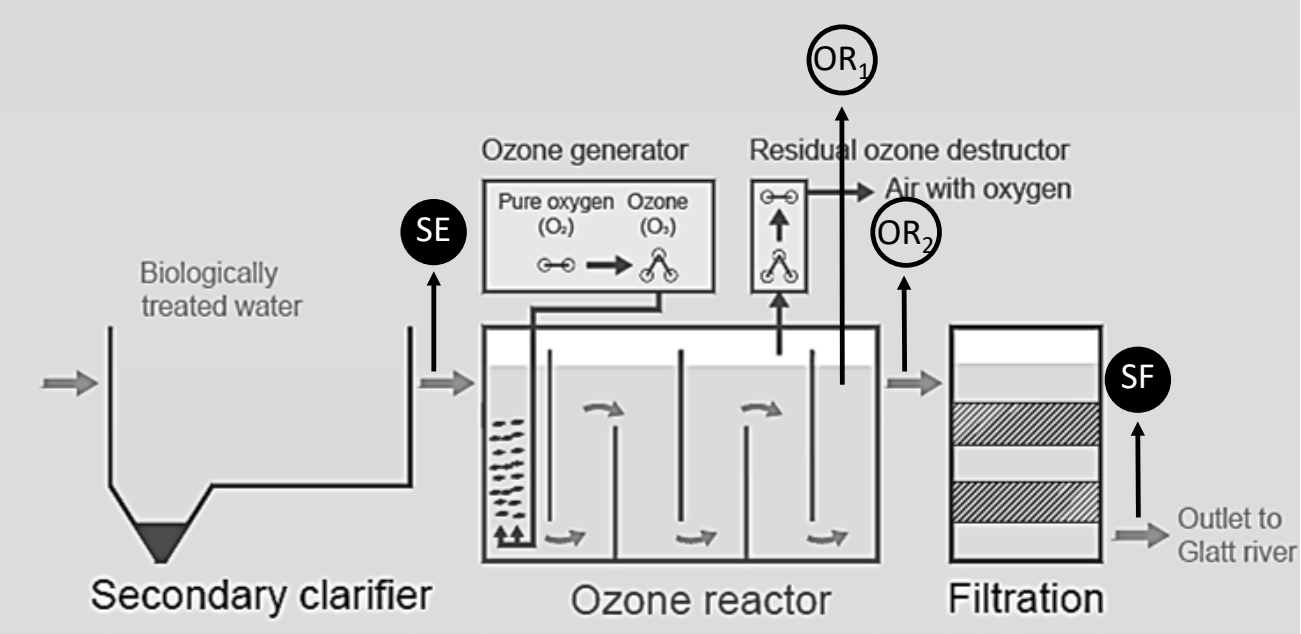
Measured feature	Medium /bacteria	ozone dose without effect [gO_3 g DOC^{-1}]	*k* [g DOC g^{-1}]	ozone dose [gO_3 g DOC^{-1}] for inactivation of 2-log	4-log
Cultivability (PC)	*E. coli / 10-um filtered SE*	0.12 (± 0.03)	50 (±11)	0.21	0.3
	10-um filtered SE / SE bacteria	0.26 (±0.05)	21 (±6)	0.5	-
	non-filtered SE / SE bacteria	0.34 (±126)	329 (±4 10^6)	-	-
NC	10-um filtered SE / SE bacteria	0.26 (±0.01)	40 (±5)	0.37*	0.48*
	non-filtered SE / SE bacteria	0.11 (±0.12)	14 (±3)	-*	-**
STT	10-um filtered SE / SE bacteria	0.32 (±0.01)	52 (±32)	0.41*	0.49**
	non-filtered SE / SE bacteria	0.26 (±0.02)	37 (±11)	-*	-**
Membrane integrity (ICC)	*E. coli* / 10-um filtered SE	0.16 (± 0.01)	82 (±10)	0.21	0.32
	10-um filtered SE / SE bacteria	0.24 (±0.01)	19 (±1)	0.5	-
	non-filtered SE / SE bacteria	0.20 (±0.03)	18 (±3)	0.5	-
DNA stability (TCC)	*E. coli* / 10-um filtered SE	0.31 (± 0.01)	66 (±6)	0.38	-
	10-um filtered SE / SE bacteria	0.56 (±0.02)	27 (±5)	0.74	0.91
	non-filtered SE / SE bacteria	0.47 (±0.05)	12 (±5)	0.87	1.26
qPCR *sul1*	*E. coli* / 10-um filtered SE	0.34 (± 0.01)	69 (±8)	0.40	0.47
	10-um filtered SE / SE bacteria	0.43 (± 0.04)	65 (±42)	0.49	LOD
	non-filtered SE / SE bacteria	0.38 (± 0.03)	25 (±7)	LOD	LOD
qPCR 16S rRNA	*E. coli* / 10-um filtered SE	0.38 (± 0.01)	93 (±17)	0.43	-
	10-um filtered SE / SE bacteria	0.38 (±0.04)	29 (±5)	0.53	0.69
	non-filtered SE / SE bacteria	0.36 (±0.10)	10 (±4)	?	?

Table S4. Abundance (%) and number (n) of identified sulfamethoxazole/trimethoprim/tetracycline resistant genera which were isolated from secondary effluent (SE), ozonated samples (OR_1-reactor/OR_2-ozonated effluent), and sand filter (SF) effluent.

Genus	NKB (n=125)	OR1 (n=59)	OR2 (n=25)	SF (n=123)
Acinetobacter	1.6 % (2)	3.4 % (2)	4 % (1)	0.81 % (1)
Aeromonas	40.8 % (51)	8.5 % (5)	8 % (2)	27.6 % (34)
Bacillus Cereus			4 % (1)	
Chryseobacterium	14.4 % (18)	17 % (10)	24 % (6)	7.3 % (9)
Citrobacter freundii	0.8 % (1)	3.4 % (2)		
Comamonas	1.6 % (2)	1.7 % (1)	4 % (1)	
Elizabethkingia	0.8 % (1)			
Enterobacter cloacae	0.8 % (1)		12 % (3)	0.81 % (1)
Enterococcus	3.2 % (4)	3.4 % (2)		
Escherichia coli	16.8 % (21)	8.5 % (5)	8 % (2)	11.4 % (14)
E. coli / Aeromonas		1.7 % (1)	4 % (1)	1.6 % (2)
Flavobacterium	0.8 % (1)		4 % (1)	
Klebsiella pneumoniae	0.8 % (1)			
Proteus mirabilis		1.7 % (1)		
Pseudomonas	3.2 % (4)	6.8 % (4)	20 % (5)	14.6 % (18)
Serratia marcescens		3.4 % (2)		
Shewanella				5.7 % (7)
Stenotrophomonas	4.8 % (6)	5.1 % (3)		7.3 % (9)
Yersinia enterocolitica				0.81 % (1)
unidentified	13.6 % (17)	32.2 % (19)	8 % (2)	17.1 % (21)

Table S5. Abundance (%) and number (n) of identified norfloxacin/ceftazidime resistant genera, which were isolated from secondary effluent (SE), ozonated samples (OR_1-reactor/OR_2-ozonated effluent), and post-ozonation sand filter (SF) effluent.

Genus	NKB (n=100)	OR1 (n=17)	OR2 (n=7)	SF (n= 39)
Acinetobacter	1 % (1)		14.3 % (1)	
Aeromonas	3 % (3)			
Brevundimonas	1 % (1)			
Cellulosimicrobium cellulans	1 % (1)			
Chryseobacterium	24 % (24)			
Elizabethkingia	2 % (2)			5.1 % (2)
Enterococcus	3 % (3)			
Escherichia coli	2 % (2)	11.8 % (2)		
Flavobacterium	7 % (7)			53.9 % (21)
Leuconostoc mesenteroides	1 % (1)			
Microbacterium	7 % (7)			7.7 % (3)
Microbium sp	3 % (3)			
Pedobacter				2.6 % (1)
unidentified	40 % (40)	88.2 % (15)	85.7 % (6)	28.2 % (11)

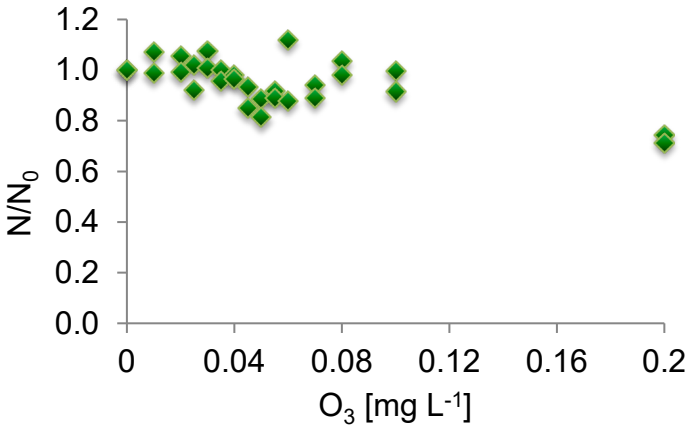


283

284 **Figure S1.** Scheme of the tertiary treatment stage at WWTP Neugut[35] and sampling points of 24-h-integrated samples
285 (filled circles) of secondary effluent (SE) and post-sand-filtered effluent (SF), as well as grab samples from within the
286 ozone reactor (OR_1) and the effluent of the ozone reactor (OR_2)**.**

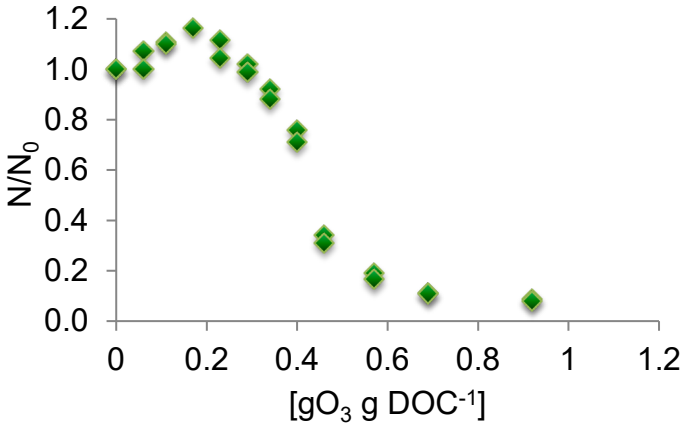
287

288 **A**



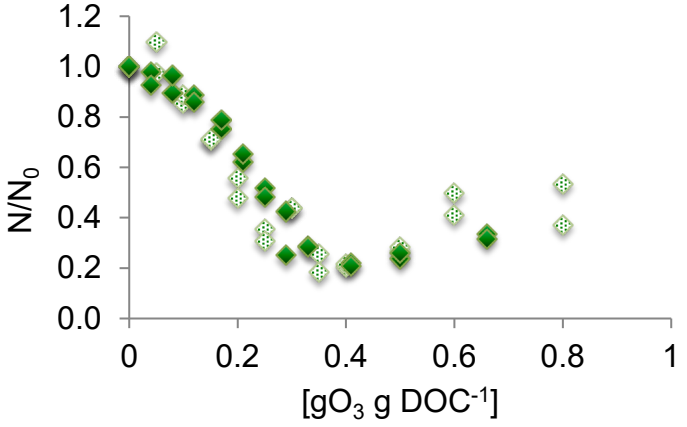
289

290 **B**



291

292 **C**



293

294

Figure S2. Mean fluorescence in flow cytometry data from ozonation experiments. Normalized mean green fluorescence data (collected at 520 nm, mean of all events) of SGI-stained samples from experiments with increasing ozone doses. (A) *E. coli* in PBS: Mean fluorescence remains stable, decreasing slightly at 0.2 $mgO_3\ L^{-1}$ confirming TCC and qPCR results (Figure 1) indicating no major degradation of DNA, (B) *E. coli* in 10µm filtered secondary clarifier effluent (SE): mean green fluorescence decreases at ozone doses > 0.3 $mgO_3\ L^{-1}$ in agreement with TCC results in Figure 2. (C) wastewater bacteria in 10µm filtered SE (filled symbols) and in presence of flocs (shaded symbols). Mean green fluorescence decreases at ozone doses > 0.05 $mgO_3\ L^{-1}$ and follows a dynamic closer to ICC rather than TCC in Figure 3, indicating that TCC may underestimate DNA damage in this case. N_0 = initial mean fluorescent signal (no ozone application)

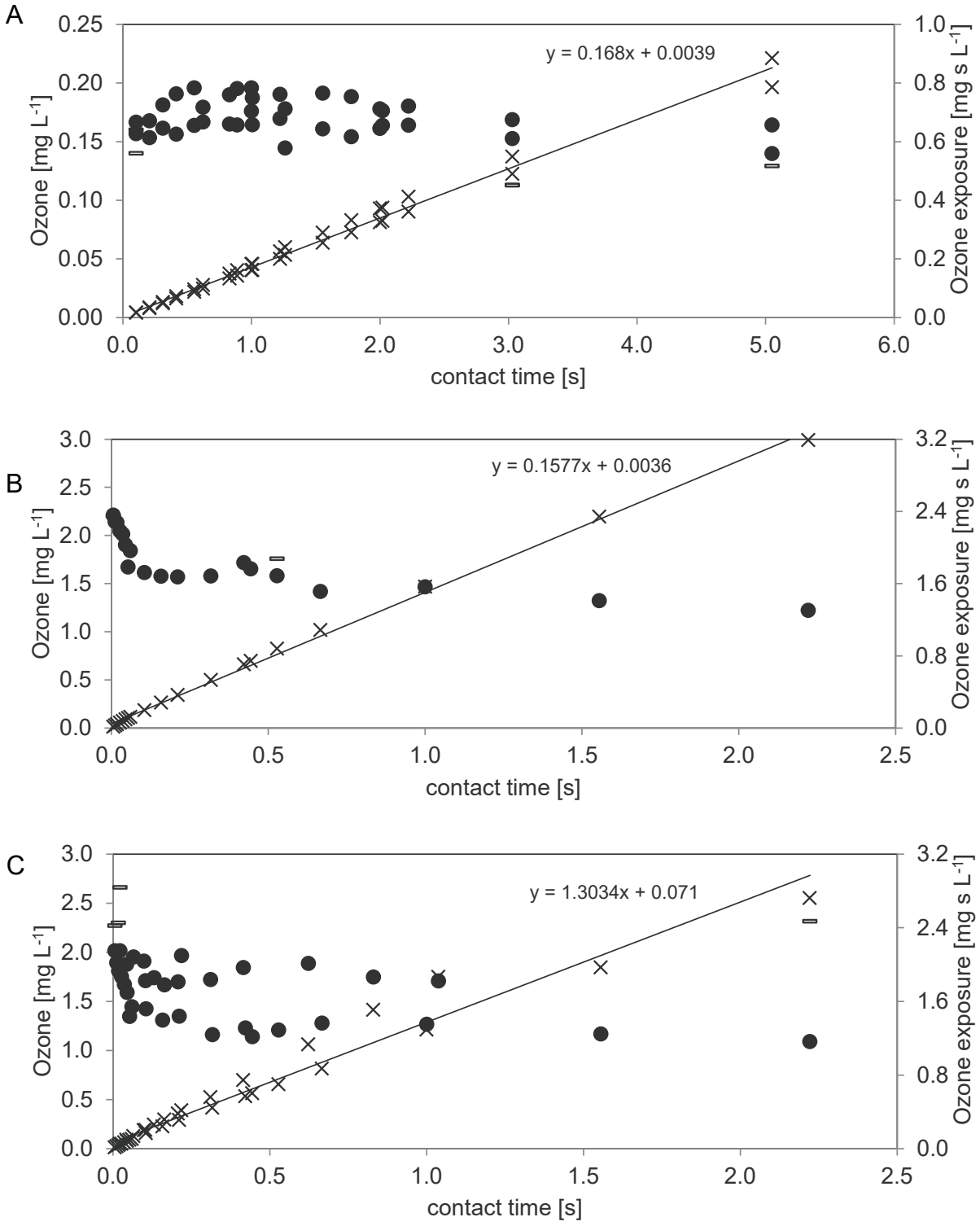


Figure S3. Concentration of residual ozone (circles) and resulting exposure (crosses with linear regression line generated in MS Excel) as a function of the contact time for (A) *E. coli* in 1:10 PBS, (B) *E. coli* in sterile 10µm filtered secondary effluent (SE), (C) 10µm filtered SE. Experiments (A) and (C) were conducted as two individual experiments, experiment B just once. Bars represent blanks for the ozone concentration measured without bacteria at the beginning and end of each experiment. The residual ozone concentration was determined by HPLC-measurement of benzaldehyde, which is formed in a stoichiometric reaction of ozone with cinnamic acid (see IV and V).

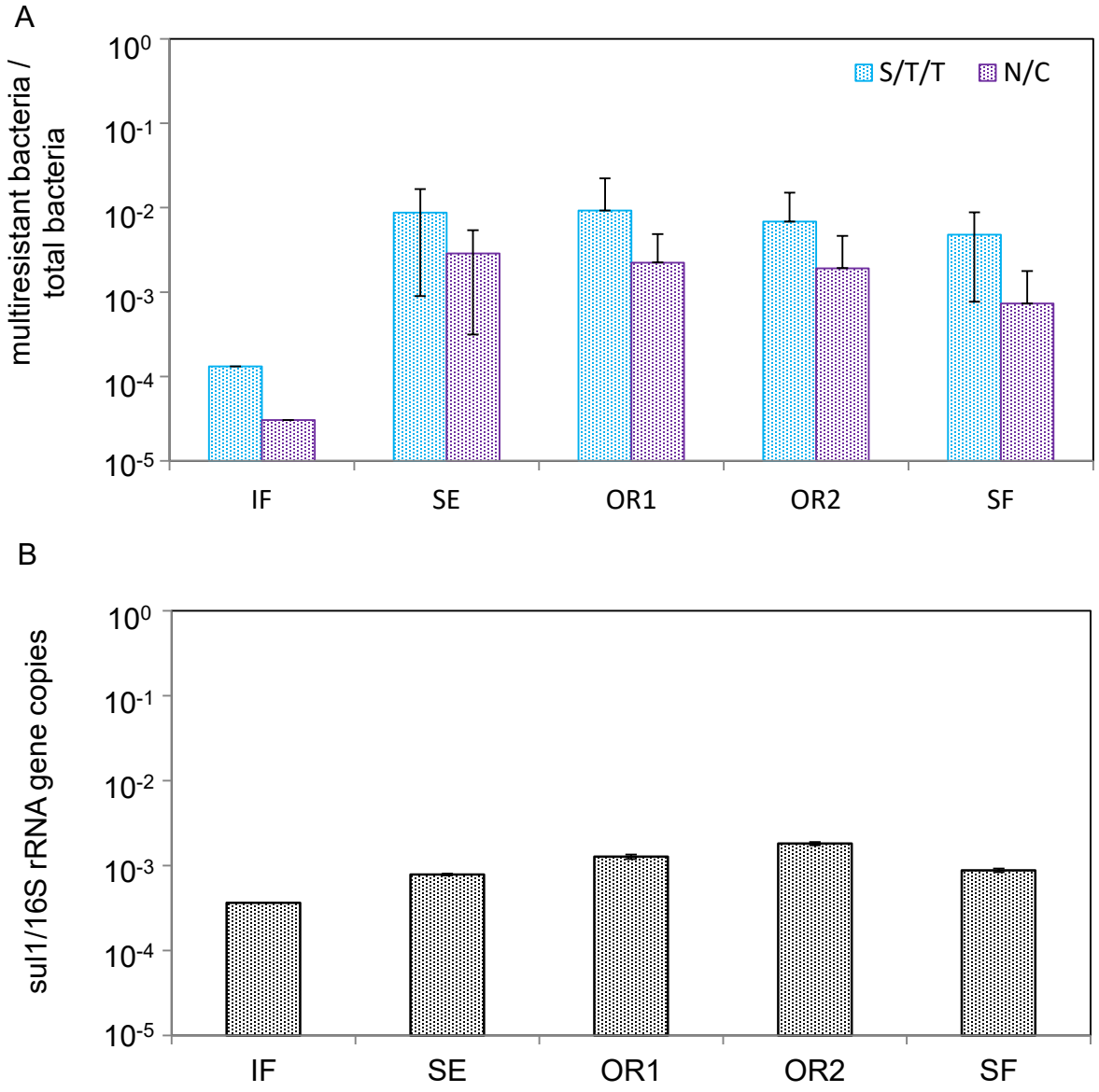


Figure S4. (**A**) Changes of proportions of multiresistant wastewater bacteria and (**B**) proportion of *sul1* at different treatment stages at WWTP Neugut, IF = raw influent, SE = secondary clarified effluent, OR_1 = within the ozone reactor (last compartment), OR_2 = effluent of the ozone reactor, SF = post-sand filter effluent (see Figure S1), N/C or S/T/T = bacteria with resistance to norfloxacin/ceftazidime (violet) or sulfamethoxazole/trimethoprim/tetracycline (turqois) were normalized to total heterotrophic plate counts and *sul1* gene copy numbers were normalized to 16S rRNA gene copy numbers in the respective sample.

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