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

Nadine Czekalski[1], Stefanie Imminger[1], Elisabeth Salhi[1], Marjan Veljkovic[1], Karolin Kleffel[1], David Drissner[4], Frederik Hammes[1], Helmut Bürgmann[1*], Urs von Gunten[1,5,6*]

[1]Eawag, Swiss Federal Institute of Aquatic Science and Technology, Ueberlandstrasse 133, CH-8600 Duebendorf or Seestrasse 79, CH-6047 Kastanienbaum, Switzerland

[4] Agroscope, Institute for Food Sciences, CH-8820 Wädenswil, Switzerland

[5]Institute of Biogeochemistry and Pollutant Dynamics, ETH Zurich, CH-8092 Zurich, Switzerland

[6]School of Architecture, Civil and Environmental Engineering (ENAC), Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015, Lausanne, Switzerland

*Corresponding authors:

Helmut Bürgmann, phone: +41 58 765 2165, fax: +41 58 765 2168,

email: helmut.buergmann@eawag.ch

Urs von Gunten, phone: +41 58 765 5270, fax: + 41 58 765 5802, email: urs.vongunten@eawag.ch

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Abstract

Ozone, a strong oxidant and disinfectant, seems ideal to cope with future challenges of water treatment, such as micropollutants, multiresistant bacteria (MRB) and even intracellular antibiotic resistance genes (ARG), but information on the latter is scarce. In ozonation experiments we simultaneously determined kinetics and dose-dependent inactivation of *E. coli* and its plasmid-encoded sulfonamide resistance gene *sul1* in different water matrixes. Effects in *E. coli* were compared to an autochthonous wastewater community. Furthermore, resistance elimination by ozonation and post-treatment were studied in full-scale at a wastewater treatment plant (WWTP). Bacterial inactivation (cultivability, membrane damage) and degradation of *sul1* were investigated using plate counts, flow cytometry and quantitative real-time PCR. In experiments with *E. coli* and the more ozone tolerant wastewater community disruption of intracellular genes was observed at specific ozone doses feasible for full-scale application, but flocs seemed to interfere with this effect. At the WWTP, regrowth during post-ozonation treatment partly compensated inactivation of MRB, and intracellular *sul1* seemed unaffected by ozonation. Our findings indicate that ozone doses relevant for micropollutant abatement from wastewater do not eliminate intracellular ARG.

Keywords: antibiotic resistance genes, ozonation, wastewater, inactivation, kinetics, exposure

1. Introduction

Two of the major current and future challenges in wastewater treatment are micropollutants and multiresistant bacteria (MRB). Micropollutants (substances, including pharmaceuticals (antibiotics), personal care products, food additives, hormones, etc. which occur at μg L^{-1} levels or lower in aquatic environments) can cause adverse ecological effects[1, 2] and may have implications for human health. Multiresistant pathogens have been rated as a global health threat[3] and their antibiotic resistance genes (ARG) as emerging environmental contaminants.[4, 5] Particularly MRB and their (mobile) ARG are of concern for wastewater reuse (e.g., for irrigation of food crops)[6] and for the potential to invade and expand the natural resistance background of receiving waters, which may be directly or indirectly used for drinking water production or irrigation.[7, 8] Conventional wastewater treatment is capable of strongly reducing the MRB load, but may also select for highly resistant phenotypes during biological activated sludge processes.[9, 10] Moreover, only insufficient removal of many micropollutants can be achieved in these processes.[11]

Tertiary treatment for abatement of the micropollutant load from wastewater effluents is currently discussed in many industrialized countries. Switzerland is among the first to implement such treatments in full-scale and plans to upgrade about 100 out of 700 wastewater treatment plants (WWTPs).[12-14] Powdered activated carbon (PAC) or ozonation are currently the two economically feasible options to achieve an abatement of micropollutants by > 80%.[15, 16] Apart from its selective reaction with organic compounds, ozone is a strong disinfectant and already widely applied in drinking water treatment.[17, 18] Thus, abatement of MRB and (transferable) intracellular ARG under conditions optimized for micropollutant abatement could be an additional potential benefit to wastewater ozonation.

The primary cellular targets of ozone are nucleic acids, where damage can range from base lesions to single and double strand breaks.[18] Lesions can lead to more or less compromising point mutations, whereas massive breakdown of DNA is lethal if not repaired.[19-22] Many studies provide evidence that also the cell envelope is affected during ozonation, probably even before severe DNA damage occurs.[23-25] Although not as pronounced as for micropollutant oxidation, the effectiveness of ozone as a disinfectant varies significantly between differing types of bacteria, even on the strain level (reviewed in[17, 18]) and depends on various factors, such as growth stage,[26] cell envelope,[27] efficiency of repair mechanisms,[28] and the type of viability-indicator used (e.g., culture-based vs. alternative approaches). Matrix effects, e.g., concentration and type of dissolved organic material (DOM, measured as DOC) or the presence of flocs or particles, reduce the stability of ozone or can shield microorganisms from its effects, thereby decreasing the disinfection efficiency.[29-32]

Only very few kinetic inactivation studies using ozone exist for selected microbial species[32] (and studies reviewed in[17]). Moreover, so far a lag-phase for inactivation of the highly sensitive *E. coli* could not be resolved, although this feature would be expected based on *E. coli's* repair mechanisms.[22, 33, 34] Within a microbial wastewater community, ozonation may lead to full inactivation of viable MRB and their ARG, but could also select for strains or mutants less sensitive to ozone or other biocides including antibiotics,[35, 36] or it might promote horizontal transfer of ARG released from inactivated MRB to bacteria inhabiting the biological post-treatment and aquatic ecosystems.[23, 32]

To determine the behavior of MRB and their intracellular ARG during ozonation, we conducted bench-scale experiments, representing differing stages of complexity. For measuring the direct effects on bacterial cells and intracellular ARG, the first set of experiments was run in a synthetic water, using *E. coli* J53 and its sulfonamide resistance gene *sul1* (co-located with the trimethoprim

resistance gene *dfrB2* on the conjugative broad-host range plasmid R388) as a controllable, well-studied model system. s*ul1* was chosen as a surrogate for resistance against broad-spectrum antibiotics and because of its ubiquity in Swiss wastewaters and surface waters.[7, 8] The second set of ozonation experiments used the same model system, but took ozonation conditions relevant for micropollutant abatement and matrix effects (parametrized as DOC) into account by performing the experiments in 10-µm-filtered natural secondary clarifier effluent (SE). In the third set of experiments we compared the results obtained with *E. coli* to an autochthonous wastewater community, and in addition studied also the effect of the presence or absence of flocs as an additional matrix effect. Bacterial inactivation at different cellular levels (cultivability, membrane damage and ARG-disruption) was studied as a function of differing ozone doses and inactivation kinetics were determined as a function of the ozone exposure.[18] To verify the predictive potential of the laboratory experiments, Switzerland's first full-scale ozonation WWTP at Neugut near Zurich was sampled over the ozonation and biological post-treatment stages.

2. Materials and Methods

2.1 Chemicals, reagents and preparation of bacteria

Chemicals, reagents, corresponding commercial suppliers, the preparation of ozone stock solutions and of bacteria for experiments are specified in the supporting information (SI, I, II, III).

2.2 Batch experiments

Batch experiments were conducted in 40-mL glass vials, filled with 30 mL of bacterial suspensions according to set-ups and conditions summarized in Table 1. Ozone was transferred with a glass syringe (Hamilton, Switzerland) from the stock solution (see SI section II). Optimal mixing of ozone with bacteria was achieved by continuous stirring for 30 seconds. To determine the dose-dependent inactivation of bacteria and intracellular genes after complete ozone depletion and to preserve samples for downstream analyses, ozonated samples were stored in the fridge for > 30 minutes prior to analyses.

2.3 Inactivation kinetics determined by quench-flow experiments

The stability of ozone may vary significantly depending on the matrix, e.g., 1:10 diluted phosphate buffered saline (PBS, SI, I) vs. secondary clarifier effluent (SE), which influences the disinfection efficiency. Thus, applied ozone doses are not sufficient as a parameter for assessing the efficiency of inactivation of bacteria and intracellular ARG disruption. Instead we used the ozone exposure or *CT*, which takes the matrix derived ozone decay into account. The ozone exposure is the time integral of an ozone decay curve:[37]

$$CT = \int [O_3] \delta t$$

To study fast kinetics of bacterial inactivation and ARG disruption, a quench-flow system (SFM400/Q, Bio-Logic SAS, France) was used in continuous mode (see SI IV and V for details and Table 1 for experimental set-ups).

Table 1. Overview of set-ups and conditions applied in batch and quench-flow experiments (SE = secondary clarifier effluent from WWTP Neugut, n.c. = not conducted). All experiments were run at 22±2 °C, at pH 7.2 for 1:10 diluted phosphate buffered saline (PBS, see SI, I) or 7.8 for SE, respectively. 0.1 mM of *tert*-butanol was added to quench-flow experiments. SS: Suspended solids.

batch experiments								
		DOC	SS	NO_2^-	range of ozone doses			contact times
Microorganism	Matrix	[mg L^{-1}]	[mg L^{-1}]	[mg L^{-1}]	[mg L^{-1}]	[g g DOC^{-1}]	replicates	[min]
E. coli	0.1 × PBS	-	-	-	0-0.2	-	2	> 30
E. coli	sterile 10-μm-filtered SE	4.27	-	0.008	0-7.35	0-1.72	2	> 30
SE bacteria	10-μm-filtered SE	5	-	0.005	0-3.31	0-0.66	2	> 30
SE bacteria	untreated SE	5	4.8	0.005	0-4.24	0-0.85	2	> 30
quench-flow experiments								
		DOC	SS	NO_2^-	initial ozone concentration			contact times
Microorganism	Matrix	[mg L^{-1}]	[mg L^{-1}]	[mg L^{-1}]	[mg L^{-1}]	[g g DOC^{-1}]	replicates	[s]
E. coli	0.1 × PBS	-	-		0.2	-	2	0.104-5.050
E. coli	sterile 10-μm-filtered SE	4.9	-	0.018	2.2	0.45	1	0.006-2.222
SE bacteria	10-μm-filtered SE	4.34	-	0.018	2.5	0.57	2	0.006-2.222
SE bacteria	untreated SE	n.c.	2.4	0.018	n.c.	n.c.	n.c.	n.c.

2.4 Sampling campaigns at a full-scale wastewater ozonation plant

Neugut (Dübendorf), Switzerland's first WWTP equipped with full-scale ozonation (refer to SI VI for further information), is treating municipal wastewater of 105000 inhabitant equivalents ((2-5)×10^4 m^3 d^{-1}). The applied ozone doses during sampling were 0.45-0.55 gO_3 g DOC^{-1} (2.2-2.5 mg O_3 L^{-1}), which meets the Swiss guidelines for an abatement of the micropollutant load of 80 %.[12] Ozonated water is treated by sand filtration (SF, residence time = 20-30 min) for biological post-treatment (SI, Figure S1).[38] Three sampling campaigns were conducted, one in December 2014 and two in January 2015. Automated 24 h-integrated samples (4°C) were taken from SE and after SF. Grab samples were obtained from the 6th compartment of the ozone reactor (OR_1) and its effluent (OR_2) (SI, Figure S1), because 24 h-integrated samples were non-representative for bacterial

139 analyses due to regrowth on the formed assimilable organic carbon (AOC) during the sampling
140 period (data not shown). Samples were kept at 4 °C and processed within 24 h.
141
142 **2.5 Detection of membrane integrity (ICC) and DNA-stability (TCC) by flow cytometry**
143 The loss of membrane integrity as a conservative indicator for cell death[39] and DNA damage in the
144 form of strand breaks and single-stranded DNA, were monitored using flow cytometry (BD Accuri
145 C6 flow cytometer, BD Biosciences, Belgium). For membrane intact cell counts (ICC) samples were
146 stained with SYBR Green I (SGI) and propidium iodide (PI), while total cell counts (TCC) for DNA
147 stability were solely stained with SGI, based on established procedures.[40-44] Flow cytometric density
148 plots were gated to distinguish intact bacterial cells from background signal and damaged cells, using
149 the BD Accuri C6 software. To determine if flow cytometry can provide more highly resolved
150 information on DNA degradation that might be missed by gated cell counts (TCC), changes in mean
151 fluorescence intensity of SGI stained cells were analysed (Figure S2).
152
153 **2.6 Plate counts of *E. coli* and wastewater bacteria**
154 Cultivability is a conventional indicator for viability. However, it depends on the ability of
155 bacteria to grow on artificial media. Plate counts of *E. coli* were conducted on LB agar supplemented
156 with sulfamethoxazole and trimethoprim (see SI, III). Cultivable heterotrophic wastewater bacteria
157 were grown on AQ dry plates (HyServe, Germany) according to the manufacturer's guidelines.
158 Growth of fungi was suppressed by supplementing 25 $\mu g\,mL^{-1}$ of pimaricin. Multiresistant
159 wastewater bacteria were isolated on AQ-pimaricin plates additionally supplemented with two
160 combinations of medically relevant broad-spectrum antibiotics: sulfamethoxazole/trimethoprim/
161 tetracycline (76/4/16 $\mu g\ mL^{-1}$) and norfloxcacin/ceftazidime (16/32 $\mu g\ mL^{-1}$). The concentrations

used conform to CLSI guidelines.[45] Refer to SI, III for sample treatment, growth conditions and SI, VII for identification of selected multiresistant wastewater isolates from SE, OR_1, OR_2 and SF (Figure S1) by MALDI-TOF.

2.7 Quantitative PCR

To detect ozone-induced gene disruption of ARG *sul1,* a new primer set (5'→3' GAC GGT GTT CGG CAT TCT / GAT CTA ACC CTC GGT CTC TGG), covering 827 bp of the 840 bp-sized gene, was developed (SI, VIII). Previously published primers (Bact349F/Bact806R) and probe (Bac516F) were used to quantify a 457 bp fragment of the 16S rRNA gene.[46] Refer to SI, VIII for detailed qPCR protocols, preparation of standards, data analysis and SI, IX for DNA-extraction.

3. Results and Discussion

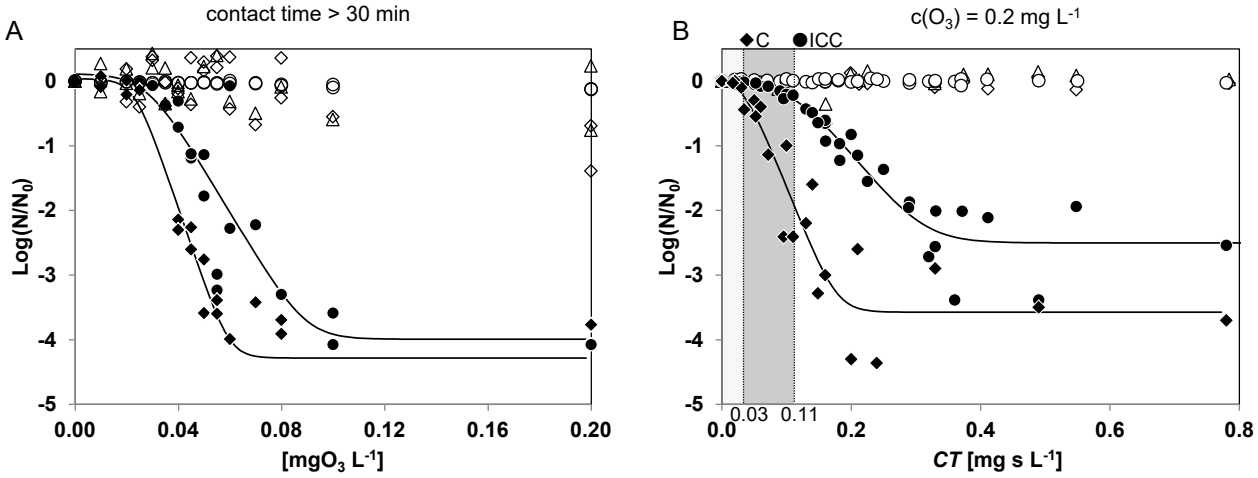
3.1 Inactivation kinetics of *E. coli* and ARG *sul1* in 1:10 diluted PBS

In 1:10 diluted PBS, viability indicators (cultivability and ICC) of *E. coli* diminished about 4 log-units, whereas no severe DNA-damage was detected within the tested range of ozone concentrations (0-0.2 mg L^{-1}) by means of flow cytometric TCC, mean green fluorescence (measure of DNA stability) and gene copy numbers of *sul1* and 16S rRNA (Figures 1A, S2A). Our kinetic experiments confirmed these observations (Figure 1 B). Thus, even though heavy membrane damage and inactivation of *E. coli* occurred at low ozone doses and exposures (Tables 1 and S2), more than 0.2 mg L^{-1} seem to be required for disruption or leakage of intracellular DNA and ARG. The latter supports the suggestion by Dodd,[23] that intracellular ARG are only affected after viability is lost.

Although extremely sensitive towards ozone, *E. coli* is able to tolerate and repair ozone-induced damage at very low exposures.[34, 47] However, previous studies could not provide kinetic evidence for

185 an initial lag-phase (CT_{lag}) neither for *E coli*[25, 48], nor for, presumably slightly more resistant,

186 bacterial communities inhabiting drinking water.[44]



187

188 **Figure 1.** Inactivation of *E. coli* J53 (R388) and its intracellular ARG *sul1* and 16S rRNA gene in 1:10 diluted PBS as a
189 function of (A) varying ozone doses (ozone fully depleted) and (B) ozone exposures (kinetics) at an ozone dose of 0.2
190 mgO_3 L^{-1}, pH 7.2, 22±2°C. 0.1 mM *tert*-butanol was added in experiment B for OH radical scavenging. ◆ Cultivability
191 (C), ● membrane integrity (ICC), ○ DNA stability (TCC), ◇ *sul1*, △ 16S rRNA. Black curves represent fitted
192 inactivation models (GInaFiT, see SI, X). Vertical dotted lines in plot B indicate the CT_{lag} and beginning of log-linear
193 decrease determined for C (light grey) and ICC (mid grey). Determined parameters for dose- and exposure-dependent
194 inactivation kinetics are given in Tables 2 and S2, respectively.

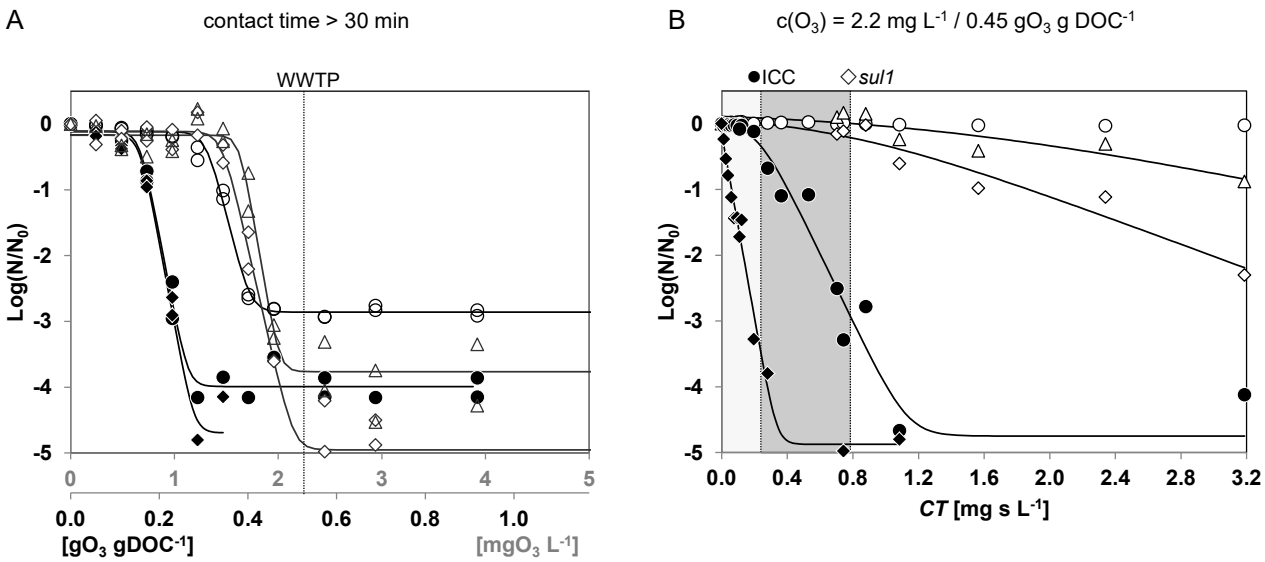
195

196 In Figure 1B kinetic evidence for an initial disinfection delay during the treatment of *E. coli* with
197 ozone is presented. The fitted model (SI, X)[49] predicted a CT_{lag} of 0.03 (±0.03) mg s L^{-1} for
198 cultivability (contact time = 210 ms) and a CT_{lag} of 0.11 (±0.02) mg s L^{-1} for membrane integrity
199 (contact time = 620 ms) for an ozone dose of 0.2 mg L^{-1} (Figure 1B). Thereafter, cultivability
200 decreased faster (k_C = 58 (±12) L mg^{-1} s^{-1}) than membrane integrity (k_{ICC} = 29 (±5) L mg^{-1} s^{-1}). The
201 determined k_C is about 2.3 times lower and the required *CT* for 2-log inactivation (0.12 mg s L^{-1}) is
202 3.3 times higher than previously reported[25, 48] (Table 2). The observed differences are reasonable for
203 kinetic measurements considering the differing *E. coli* strains, cell densities, or vegetative stages.[26, 50]
204 A delayed decrease in ICC compared to cultivability (Figure 1) supports the hypothesis that other
205 cellular constituents, such as proteins or DNA are affected[20-22, 28, 54-56] before severe membrane

damage occurs. However, we cannot rule out that cultivation overestimated the lethal effect of ozone, as cells may switch to a viable but non-culturable state.[51] Hence, the actual cause of ozone-induced cell death may still be destruction of the cell envelope, as suggested previously, [24, 25, 30, 51-53] but cannot clearly be determined in our study.

3.2 Inactivation kinetics of *E. coli* and ARG *sul1* in sterile wastewater

Exposing *E. coli* J53 to increasing ozone doses in sterile 10µm-filtered SE lead to a simultaneous decrease of cultivability and membrane integrity, when exceeding a specific ozone dose of 0.16 gO_3 $gDOC^{-1}$ (0.67 mgO_3 L^{-1}, Figure 2A, Tables S2 and S3). This finding contrasts the experiments in PBS described above and results of a similar study in SE using wild-type (WT) *E. coli* K-12 MG1655.[52] The presence of membrane-destabilizing agents (e.g., surfactants) in the SE sample could have contributed to the observed effect. The threshold ozone dose to affect flow cytometric TCC (1.31 mgO_3 L^{-1} or 0.31 gO_3 $gDOC^{-1}$) and mean green fluorescence (Figure S2B) was more than a factor of 2 higher than for viability indicators. At the TCC-specific threshold ozone dose, inactivation of *E. coli* cells was already nearly completed. Gene copy numbers of *sul1* and 16S rRNA genes had yet slightly higher threshold ozone doses than TCC (1.46 and 1.63 mgO_3 L^{-1}, respectively or 0.34 and 0.38 gO_3 g DOC^{-1}). 2-log abatement of TCC and *sul1* gene copies was achieved at 1.6-1.7 mgO_3 L^{-1} or 0.38-0.40 gO_3 $gDOC^{-1}$. As specific ozone doses of ~0.55 gO_3 $gDOC^{-1}$ are applied at WWTP Neugut for micropollutant abatement, our data from this set of experiments predict that intracellular ARG should be destroyed at the full-scale.



227

228 **Figure 2.** Inactivation of *E. coli* J53 (R388) and its intracellular ARG *sul1* and 16S rRNA gene in sterile SE as a function
229 of (A) differing ozone doses (ozone fully depleted) and (B) ozone exposures (kinetics), pH 7.8-8.0, 22±2°C. ◆
230 Cultivability (C), ● membrane integrity (ICC), ○ DNA stability (TCC), ◇ *sul1*, △ 16S rRNA. Black curves represent
231 fitted inactivation models (GInaFiT, see SI X). Straight dashed lines indicate typical ozonation conditions for
232 wastewaters (A) and the CT_{lag} and beginning of log-linear decrease (B) determined for ICC (light grey) and *sul1* (mid
233 grey). Determined parameters for dose- and exposure-dependent inactivation are given in Tables 2, S2 and S3,
234 respectively.

235

236 Figure 2B shows that the inactivation of *E. coli* as a function of the ozone exposure in sterile SE
237 resulted in a delayed decrease of ICC compared to cultivability, thus confirming the results from
238 experiments in 1:10 diluted PBS and previous studies with *E. coli* WT in SE.[52] Already the lowest
239 contact time (6 ms, (specific) ozone dose = 0.45 gO_3 $gDOC^{-1}$ or 2.2 mgO_3 L^{-1}) resulted in an ozone
240 exposure (0.13 mg s L^{-1}), which affected cultivability of *E. coli*. However, for ICC a CT_{lag} of 0.22
241 mg s L^{-1} was determined (contact time = 20 ms), which is twice as high as the CT_{lag} determined in
242 1:10 diluted PBS (Table 2). Moreover, inactivation rate constants for cultivability (k_c = 32 L mg^{-1} s^{-1})
243 and membrane integrity (k_{ICC} = 12 L mg^{-1} s^{-1}) in SE were a factor of 2 and 3 lower than in 1:10
244 diluted PBS, respectively. Hence, our data indicate that the same ozone exposure in SE is less
245 effective for reducing cultivability and ICC than in 1:10 diluted PBS.

246

Table 2. Summary of kinetic parameters determined during ozonation experiments with *E. coli* J53 (R388) in differing water matrices and in natural wastewater bacteria for various measurements of cellular damage. pH(PBS) = 7.4, pH(SE) = 7.8-8.0

Measured feature	Medium /bacteria	Reference	CT_{lag} [mg s L^{-1}]	k [a] [L mg^{-1} s^{-1}]	Ozone exposure [mg s L^{-1}] for inactivation of 2-log	4-log
Cultivability	PBS / *E. coli*	this study	0.03 (± 0.03)	58 (±12)	0.12	-
	PBS / *E. coli*	[26, 47]	n.d.	130-138	0.04	0.07
	SE / *E. coli*	this study	n.d.	32 (±2)	0.13	0.28
	SE / SE bacteria	this study	n.d.	k_1 = 9 (±2) k_2 = 0 (±1)	-	-
	SE / fecal coliforms/enterococci	[57]	n.d.	k_1 = 133 k_2 = 15 / 3[b]	0.22[c] 1.07[c]	0.53[c] 2.46[c]
Membrane intergity (ICC)	PBS / *E. coli*	this study	0.11 (± 0.02)	29 (±5)	0.28	-
	SE / *E. coli*	this study	0.22 (±0.08)	12 (±2)	0.61	1.01
	SE/ SE bacteria	this study	n.d.	k_1 = 21 (±3) k_2 = 1 (±0)	3.52	11.03
DNA damage (TCC)	PBS / *E. coli*	this study	> 0.78	n.d.	n.d.	n.d.
	SE / *E. coli*	this study	> 3.19	n.d.	n.d.	n.d.
	SE / SE bacteria	this study	> 2.70	n.d.	n.d.	n.d.
Gene damage (qPCR *sul1*)	PBS / *E. coli*	this study	> 0.78	n.d.	n.d.	n.d.
	SE / *E. coli*	this study	0.76 (±0.49)	2 (±0)	2.98	5.15
	SE / SE bacteria	this study	1.03 (±0.10)	6 (±1)	1.74	2.50
Gene damage (qPCR 16S)	PBS / *E. coli*	this study	> 0.78	n.d.	n.d.	n.d.
	SE / *E. coli*	this study	1.43 (±1.05)	1 (±0.6)	5.50	9.35
	SE / SE bacteria	this study	1.14 (±0.19)[d]	5 (±2)[d]	2.09[d]	3.12[d]

n.d.= not detected, - = not predicted by model

[a] In case of SE-bacteria biphasic inactivation kinetics were determined with inactivation rate constant k_1 in the first fast inactivation phase and k_2 in the second slow inactivation phase.

[b] In reference [53] the first value of k_2 refers to fecal coliforms / the second value of k_2 refers to enterococci

[c] Estimated inactivation by GInaFit (SI X) using k- and p-values given in[53] .

[d] Given parameters are uncertain, although selected by the model, from the data itself, the end of the lag-phase is not quite obvious

The reasons for the lower *k*-values in SE compared to the buffered solution remain open. We can only speculate that interactions of the matrix with *E. coli* may be involved, e.g. effects on membrane stability or activation of oxidative stress response systems. The higher pH of SE (7.8) compared to PBS (7.2) can be excluded as the main driver for this effect because the ozone inactivation rate constants for bacteria or bacterial spores are independent of the pH.[25, 54] Nevertheless, these results caution against the transfer of kinetic information on bacteria derived in buffered solutions to real wastewater effluents, a practice that is applicable for micropollutants.[55]

In contrast to 4-log inactivation of cell viability at 0.28 mg s L^{-1} (cultivability) and 1.01 mg s L^{-1} (ICC), TCC did not decrease over the applied exposure range. Intracellular *sul1* copy numbers started to decrease at much higher ozone exposures (CT_{lag} = 0.76 mg s L^{-1}) and the rate of abatement was lower than for viability indicators, with second order rate constants of 2 L mg^{-1} s^{-1} (Table 2). The fitted model for abatement of 16S rRNA gene copy numbers predicted values for CT_{lag} and k (Table 2). However, from the data the beginning of the log-linear decrease is not quite obvious. 2-log inactivation of *sul1* was only achieved for an ozone exposure of 2.98 mg s L^{-1}. The corresponding contact time of 2.0 s and specific ozone dose of 0.27 gO_3 $gDOC^{-1}$ is lower than typical ozonation conditions applied in WWTPs for micropollutant abatement. Thus, based on these laboratory experiments, 2-log abatement of intracellular ARG *sul1* of *E. coli* in SE seems feasible under full-scale conditions.

3.3 Inactivation of native bacteria and intracellular ARG *sul1* from secondary effluent

Dose-dependent inactivation

A major goal of our experiments was to investigate whether disruption of intracellular ARG present in an autochthonous microbial consortium of SE can be achieved under realistic conditions for

281 micropollutant abatement. Compared to *E. coli*, we expected the majority of these bacteria to be
282 more ozone-tolerant and thus likely to require higher (specific) ozone doses and exposures to cause
283 intracellular ARG damage.[53] Indeed, ozone doses twice as high as for *E. coli* were necessary to affect
284 cultivability, ICC and TCC of wastewater bacteria (compare Tables S2 and S3). The lack of
285 cultivability of most wastewater bacteria is illustrated in Figure 3A by plotting[56, 57] plate count, ICC
286 and TCC results as log (N) instead of log (N/N_0). Following similar trends, total cultivable
287 heterotrophs were close to zero at the highest specific ozone dose of 0.66 gO_3 $gDOC^{-1}$, whereas more
288 than 10^4 bacteria survived according to ICC. 2-log abatement of both viability indicators occurred at
289 specific ozone doses similar to those applied at WWTP Neugut (0.5 gO_3 $gDOC^{-1}$) and bacteria with
290 multiple resistance against norfloxacin/ceftazidime (N/C) and sulfamethoxazole/trimethoprim/
291 tetracycline (S/T/T) were not detectable at specific ozone doses > 0.33 gO_3 $gDOC^{-1}$. Hence, though
292 inactivation of autochthonous wastewater bacteria in SE requires higher specific ozone doses than
293 *E. coli*, a considerable reduction may still be achieved during ozonation as implemented at WWTP
294 Neugut.
295 Based on TCC, intracellular DNA damage occurred only when specific ozone doses exceeded 0.56
296 gO_3 $gDOC^{-1}$, with a 2-log decrease predicted at 0.74 gO_3 $gDOC^{-1}$. However, intracellular copy
297 numbers of the 16S rRNA gene started to decrease already at 0.38 gO_3 $gDOC^{-1}$ and were abated by
298 2-log units at 0.53 gO_3 $gDOC^{-1}$. Mean green fluorescence data suggest beginning DNA damage at
299 even lower O_3 doses (Figure S2C). The behavior of *sul1* was difficult to model for wastewater
300 bacteria, as qPCR results beyond 0.4 gO_3 $gDOC^{-1}$ were below the LOD. Based on the obtained
301 Crossing point (Cp)-values we may estimate that copy numbers of this ARG start to decrease at 0.43
302 gO_3 $gDOC^{-1}$ and are abated by 2-log units at 0.49 gO_3 $gDOC^{-1}$. Thus, in contrast to experiments with
303 *E. coli* where TCC, mean fluorescence data and qPCR measurements showed congruent trends, we

obtained somewhat more divergent results for these parameters with the complex wastewater bacterial community. We may speculate that small-scale damage affecting the availability of primer-binding and in case of the 16S rRNA fragment also TaqMan probe-binding sites or strand breaks, occurred more frequently prior to extensive DNA damage observed by TCC in the experiment with wastewater bacteria.

Effect of flocs on inactivation

Wastewater flocs, particles or zooplankton as well as formation of microbial biofilms might have a protective effect on bacteria. [30, 31, 38, 53] Simulations on the diffusion of ozone into various particles in differing wastewater matrixes indicated that ozone can only penetrate 12 µm into particles for ozone doses < 3 mg L^{-1}, if the ozone demand is not exceeded.[58] As indicated by shaded symbols in Figure 3A, the total heterotrophic plate counts did not decrease as pronounced in presence of flocs compared to when flocs were absent (10µm filtered SE). Moreover, viable total and MRB were detected, albeit at low levels, even at the highest specific ozone dose (0.85 gO_3 $gDOC^{-1}$). Flow cytometric data (ICC and TCC) did not show this effect as only suspended bacteria can be measured as single cells, excluding cells associated with flocs (if not dispersed prior to measurement, which was not the case). qPCR analysis of *sul1* and 16S rRNA genes in the presence of flocs do not clearly indicate a linear relationship between ozone dose and intracellular gene disruption. A maximum decrease of 0.8- (16S rRNA) and 1.4-log units (*sul1*) was detected at specific ozone doses of 0.6 and 0.5 gO_3 $gDOC^{-1}$, respectively. The observed differences in qPCR and plate count data suggest that flocs greater than 10 µm can shield wastewater bacteria and their ARG from inactivation, even for specific ozone doses > 0.8 gO_3 $gDOC^{-1}$. A recent study by Pak et al.[32] also reported decreased

disinfection efficiency of ozone on *E. coli* K-12 and its plasmid pB10 with increasing concentrations of suspended solids. Thus, these observations likely have implications for full-scale ozonation.

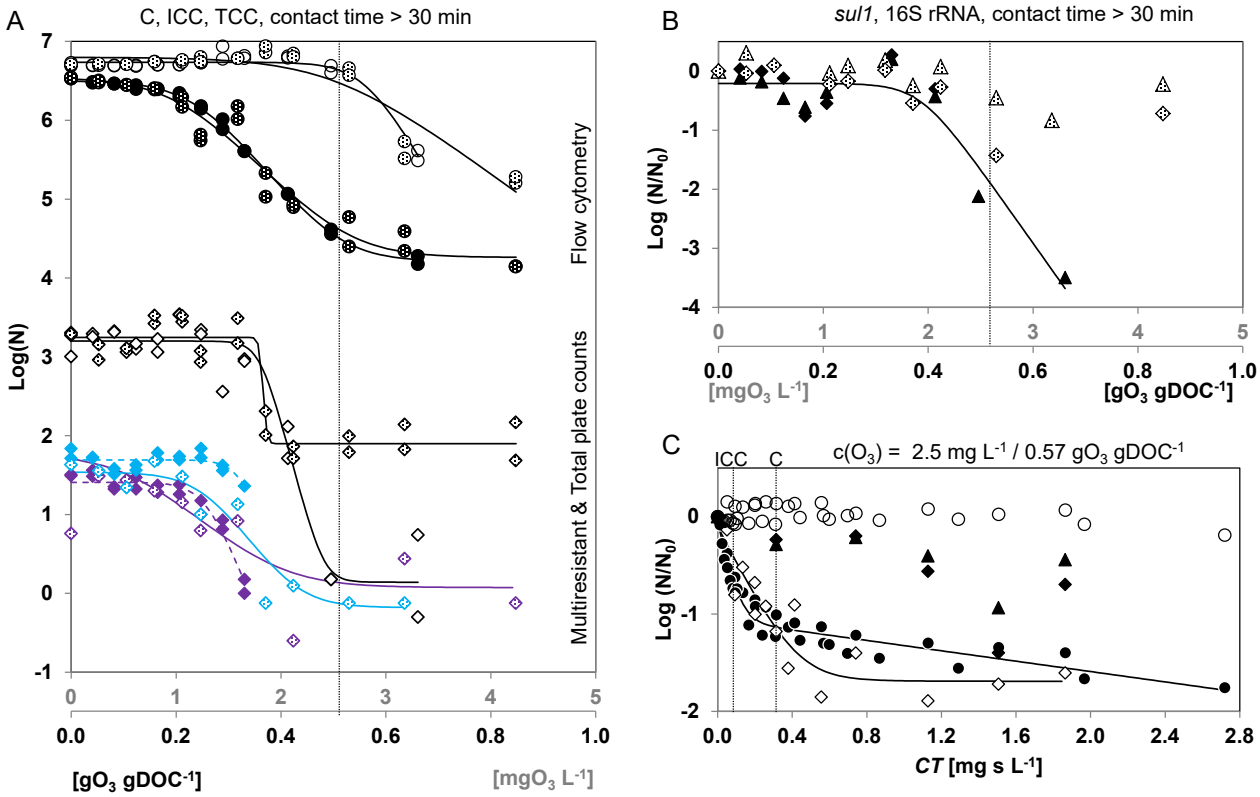


Figure 3. Inactivation of autochthonous wastewater bacteria and intracellular genes for (A + B) differing ozone doses (ozone fully depleted) and (C) exposures in 10µm filtered SE (solid or empty symbols) or in presence of flocs (unfiltered wastewater, shaded symbols). ◆ Cultivability (C) of total (black), norfloxacin/ceftazidime (violet) and sulfamethoxazole/trimethoprim/tetracycline (turquois)-resistant heterotrophs, ● membrane integrity (ICC), ○ DNA stability (TCC), ◇ *sul1*, △ 16S rRNA. Black curves represent fitted inactivation models (GInaFiT, see SI X). Dotted vertical straight lines indicate typical WWTP effluent ozonation conditions (panels A + B) and in (C) mark the end of the initial fast inactivation phase for ICC and C. pH 7.8-8.0

Inactivation kinetics of wastewater bacteria and intracellular genes

The kinetics of the inactivation of the heterogeneous wastewater community and intracellular ARG were measured in 10 µm filtered SE for a specific ozone dose of 0.57 gO_3 $gDOC^{-1}$ (2.47 mgO_3 L^{-1}), which is representative for micropollutant abatement. The resulting inactivation curves for cultivability and ICC (Figure 3C) suggest an incomplete or multiphasic inactivation scenario.

343 Initially (up to CT = 0.14 (ICC) or 0.31 mg s L^{-1} (cultivability)), the most sensitive bacteria (~1-log
344 unit) are rapidly inactivated with an inactivation rate constant k_1 (21 (± 2.8) and 9 (±2.4) L mg^{-1} s^{-1}
345 for ICC and cultivability, respectively). The majority of the wastewater bacteria is more resistant and
346 inactivated much slower (ICC with k_2 = 0.6 (± 0.1) L mg^{-1} s^{-1}) or even reached a plateau
347 (cultivability) where no further inactivation was observed under the experimental conditions of this
348 study. The determined *k*-values of the mixed wastewater community are considerably smaller than
349 those determined previously in a wastewater ozonation pilot-plant for fecal indicators (k_1 = 133 L s^{-1}
350 mg^{-1} and k_2 = 15 or 3 L s^{-1} mg^{-1} for fecal coliforms or enterococci).[53] This confirms that most
351 wastewater bacteria are more ozone tolerant than fecal indicators, which has also recently been
352 stressed by a study of Alexander et al., who identified enterococci as the most ozone-sensitive and
353 pseudomonads among the most ozone-resistant wastewater species.[35] According to the model fitted
354 to ICC data, an ozone exposure of 3.52 mg s L^{-1} would yield a 2-log reduction of the initial
355 wastewater community, which is about 6 times the exposure needed for the same ICC reduction with
356 *E. coli*. Nevertheless, the corresponding specific ozone dose (0.25 gO_3 $gDOC^{-1}$) and contact time (<
357 3s) suggest that a considerable abatement of MRB in full-scale is feasible. Based on TCC, no severe
358 DNA-damage was observed up to the highest ozone exposure (2.7 mg s L^{-1}). The kinetics of
359 intracellular ARG *sul1* and 16S rRNA gene disruption in SE bacteria are difficult to estimate (Figure
360 3C). Particularly due to a fluctuation of the last two data points (1.4-log units reduction, followed by
361 0.7-log units increase for *sul1*) it is not clear, whether or not the initial lag-phase for intracellular
362 gene disruption is exceeded at an ozone exposure of 1.0 mg s L^{-1}. The maximum measured
363 abatements of *sul1* and 16S rRNA genes were 1.4- and 0.9-log units, respectively, at an ozone
364 exposure of 1.5 mg s L^{-1}.
365

3.4 Abatement of MRB and ARG during full-scale ozonation and post-treatment

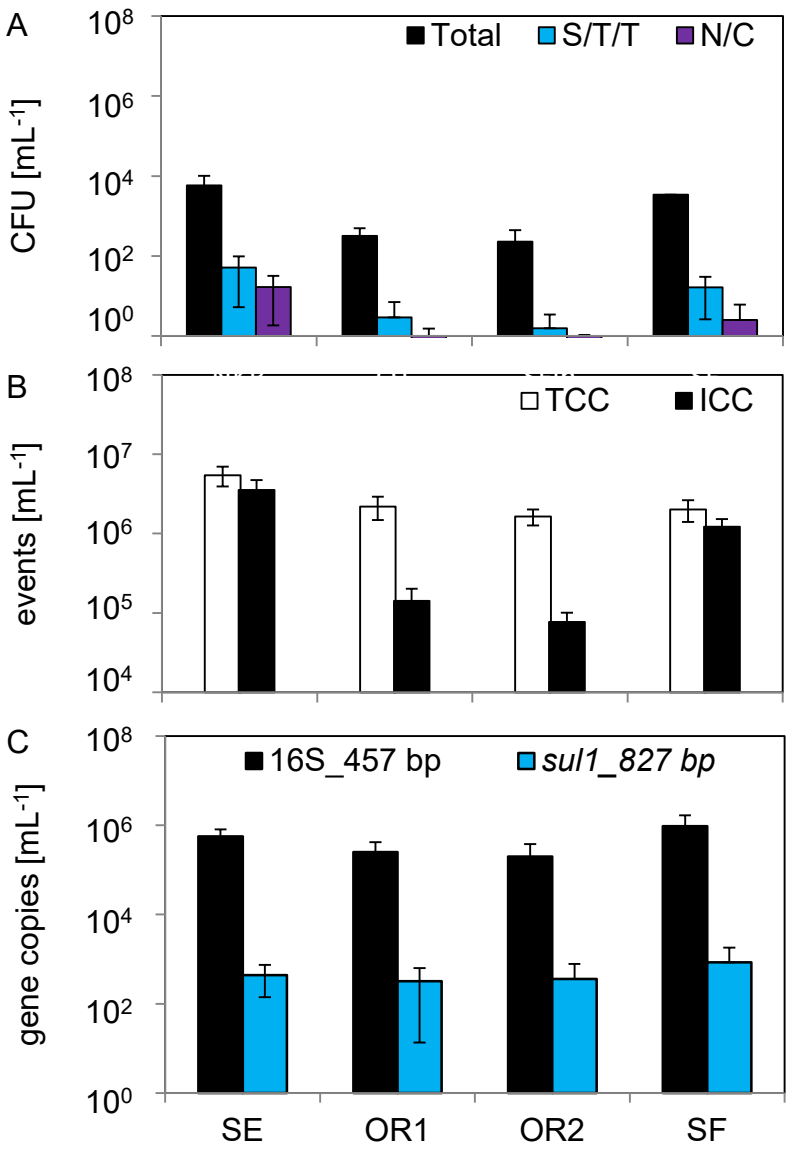
Effect of full-scale ozonation of secondary effluent on abatement of MRB and ARG

Ozonation of SE at WWTP Neugut with a specific ozone dose of 0.55 gO_3 $gDOC^{-1}$ resulted in 1.4-1.6-log abatement of the total and multiresistant cultivable bacteria (Figure 4A (total, S/T/T, N/C)) and membrane integrity (ICC, Figure 4B) in the effluent of the ozone reactor (OR_2) (SI, Figure S1). An overview on identified MRB surviving ozonation is given in SI, XI. The results for plate counts are in good agreement with the laboratory experiments, for which total and MRB were abated by 1.4-1.8-log units for the same specific ozone dose in presence of flocs (Figure 3A). Similar levels of abatement (1.0-1.1-log units) were reported previously for (antibiotic resistant) *E. coli* and enterococci in pilot studies with specific ozone doses of 0.73 and 0.9 gO_3 $gDOC^{-1}$.[35, 36] However, these studies also reported selection for antibiotic resistant species and ARG after ozonation.[35, 36] No selective effects were observed during our campaigns (SI, XII). The abatement of ICC at the WWTP was 0.5-log units lower compared to observations in laboratory experiments (Figure 3A and Table S2 and S3). In contrast to viability indicators, intracellular DNA and ARG were not effectively reduced during full-scale ozonation. Figures 4B and 4C show that TCC and 16S rRNA gene copies only decreased by 0.5-log units, which is again in agreement with laboratory experiments in presence of flocs (Figures 3A and B). In addition, *sul1* gene copies remained nearly unchanged compared to SE (- 0.1-log units, Figure 4C), suggesting negligible disruption or leakage (SI, XIII). Comparably low levels of reduction were reported previously for intracellular imipenem resistance gene *blaVIM* in a pilot-study, even with 10-µm filtered SE at a specific ozone dose of 0.9 gO_3 $gDOC^{-1}$. However other ARG (*vanA*, *ampC*, *ermB*) were abated more efficiently (0.3-2 log units), highlighting that different ARG are affected to various extents.[35] In laboratory experiments, the observed relationship between increasing specific ozone doses and the disruption of intracellular genes in the presence of

389 flocs was not conclusive. Moreover, increasing concentrations of suspended solids were recently
390 reported to decrease the effect of ozone on a transferable ARG-plasmid.[32] Hence, even though a
391 decrease of *sul1* gene copies of up to 1.4-log units had been measured in presence of flocs (Figure
392 3B), the low abatement of intracellular *sul1* in full-scale is not completely unexpected. However, our
393 flow cytometric and PCR based methods may underestimate DNA damage, as ozone is a potent
394 mutagen,[18, 19, 24, 59] and we did not assess sequence changes or transferability[32] of the genes. It should
395 be kept in mind, that the biocidal conditions exerted by ozonation of wastewater, may contribute to
396 mutagenesis[60] and selection of antibiotic resistant and more ozone-tolerant bacterial species in the
397 long term.[32, 35, 36]
398 In summary our results indicate that typical conditions for full-scale ozonation are suitable to
399 inactivate 1-2 log units of viable resistant bacteria without a measurable effect on the abundance of
400 intracellular ARG.
401
402 *Effect of biological post-treatment by sand filtration on MRB and ARG in ozonated effluent*
403 During ozonation, potentially toxic oxidation by-products such as *N*,*N*-nitrosodimethylamine
404 (NDMA) or bromate[37, 38] and considerable concentrations of biodegradable or assimilable organic
405 carbon (BDOC / AOC) may be formed. BDOC/AOC and in part NDMA are typically removed in a
406 biological post-treatment following ozonation.[38] At WWTP Neugut, ozonated effluent is treated by a
407 biological sand filter (SF). This allows bacterial populations to regrow in the partially disinfected
408 water.[38] The analyses of the SF effluent show that not only intact cell counts (ICC) and total
409 heterotrophic cultivable bacteria recover by 1.2-log units, but that also MRB increase significantly
410 (0.8-log units, $p<0.05$ for N/C- and 1-log unit, $p<0.001$ for S/T/T-resistant bacteria, Figures 4A and
411 4B). Thus, the abatement of resistant SE-bacteria achieved during the ozonation step seems to be

412 partly neutralized by the biological post-treatment, resulting in only 0.8- (N/C) and 0.5- (S/T/T) log
413 units abatement ($p<0.001$ for both types). TCC, which were only slightly reduced during ozonation
414 remained unchanged after SF. However, ARG *sul1* and 16S rRNA genes increased by 0.4- and 0.7-
415 log units, thus their concentration is even slightly (but not significantly) elevated compared to SE.
416 Recent pilot-studies, comparing the effect of differing post-treatments (including SF, granular
417 activated carbon and expanded clay) on viable resistant bacteria or bacterial and antibiotic resistance
418 gene markers did not report such an effect.[35, 36] However, in agreement with our observations on the
419 isolates we obtained and identified (SI, XII), these studies also report that differing species are
420 affected to various extents by ozonation and biological post-treatment.[35, 36] It should be noted that the
421 SF at WWTP Neugut treated conventionally purified SE for 18 years before the ozonation step was
422 implemented. Hence, the observed increase in MRB and ARG following SF may potentially be
423 related to the previously established microbial community, which was selected during biological
424 treatment (see SI XII) and experienced elevated micropollutant exposure, potentially including
425 selective effects of sub-lethal antibiotic concentrations.[61-66]
426
427 *Practical implications*
428 As indicated by ozonation experiments, implementing a removal step for flocs >10µm from SE prior
429 to ozonation could lead to more efficient MRB abatement and intracellular ARG disruption. The
430 latter may also be achieved by applying higher specific ozone doses (>0.55 gO_3 $gDOC^{-1}$). As some
431 ARG seem more resistant to ozonation than others,[35] they could be considered as target genes for
432 monitoring in ozonation practice. The related benefits of applying higher ozone doses on ARG may
433 be offset by elevated formation of toxic ozonation by-products, such as bromate or nitrosamines.
434 Such trade-offs should be evaluated individually for WWTPs, which plan to implement an ozonation

435 step.[67] Finally, an ultrafiltration following SF could be implemented to prevent discharge of regrown
436 MRB and their ARG to the aquatic environment. Perhaps, other filter types such as granulated
437 activated carbon (c.f.[35, 36]), or exchanging the filter material with sand not previously exposed to
438 micropollutants or wastewater bacteria, thus eliminating a resident microbial community with
439 already high prevalence of resistance, may be considered.
440 Before additional costly measures at WWTPs as those discussed above can be justified in front of
441 policy makers and stakeholders, more profound research on the environmental effects of the
442 continuous discharge of MRB and ARG from WWTPs is needed and risk assessment addressing the
443 human health impacts needs to be developed.[68]
444



445

446 **Figure 4.** Effects of the full-scale ozonation at WWTP Neugut on various bacterial parameters. SE: secondary clarifier
447 effluent; OR1, OR2: Sampling in and after the ozone reactor (SI, Figure S1), respectively; SF: after biological sand
448 filtration. (A): Inactivation of total viable («Total») bacteria and bacteria multiresistant to
449 sulfamethoxazole/trimethoprim/tetracycline (S/T/T) or norfloxacin/ceftazidime (N/C), (B) membrane integrity (ICC) and
450 DNA stability (TCC) and (C) *sul1* and 16S rRNA gene fragments. pH 7.8-8.0.

451

Supporting Information Available

For additional material and data 23 pages including 5 Tables and 4 Figures are available. This information is available free of charge via the Internet at http://pubs.acs.org.

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