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Research Paper

# Advanced wastewater treatment with ozonation and granular activated carbon filtration: Inactivation of antibiotic resistance targets in a long-term pilot study

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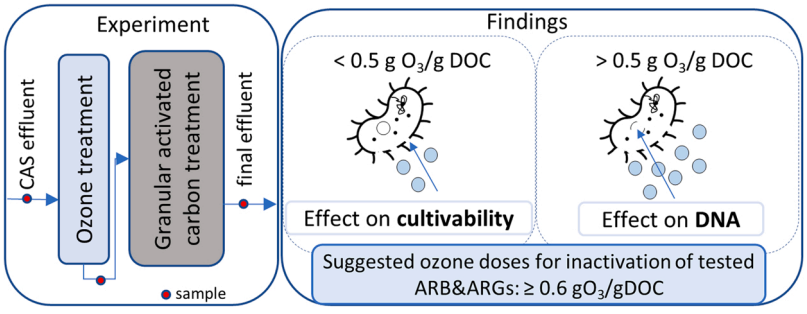
## HIGHLIGHTS

- Ozonation and GAC were evaluated for ARB and ARGs abatement in a long-term study.
- Ozone doses ≥ 0.6 g $O_3$/g DOC were required to affect tested DNA targets.
- Ampicillin resistant *E. coli* was less susceptible to ozonation effects than other ARB.
- A relative abundance of $bla_{TEM-1}$ gene increased after GAC treatment.
- $\Delta UV_{254}$ was tested as a proxy parameter to indicate DNA damage by ozone.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The inactivation of antibiotic resistant bacteria (ARB) and genes (ARGs) in an advanced plant combining ozonation and granular activated carbon (GAC) filtration applied for effluent after conventional activated sludge treatment at a full-scale urban wastewater treatment plant was investigated for over 13 consecutive months. The nitrite compensated specific ozone dose ranged between 0.4 and 0.7 g $O_3$/g DOC with short-time sampling campaigns (0.2–0.9 g $O_3$/g DOC). Samples were analysed with culture-dependent methods for bacterial targets and with qPCR for genes. The log removal values were correlated with a decrease of the matrix UV absorption at 254 nm ($\Delta UV_{254}$) and indicated a range of $\Delta UV_{254}$ that corresponds to a sufficient membrane damage to affect DNA. For trimethoprim/sulfamethoxazole resistant *E. coli*, *sul*1, *erm*B and *tet*W, this phase was observed at $\Delta UV_{254}$ of ~30 % (~0.5 g $O_3$/g DOC). For ampicillin resistant *E. coli* and $bla_{TEM-1}$, it was observed around 35–40 % (~0.7 g $O_3$/g DOC), which can be linked to mechanisms related to oxidative damages in bacteria resistant to bactericidal antibiotics. GAC treatment resulted in a further abatement for trimethoprim/sulfamethoxazole *E. coli*, *sul*1 and *tet*W, and in increase in absolute and relative abundance of *erm*B and $bla_{TEM-1}$.

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## 1. Introduction

Biological wastewater treatment, including conventional activated sludge (CAS) treatment, is only partially effective in the removal of chemical and microbial contaminants of emerging concern (CECs) (Alexander et al., 2016; Hiller et al., 2019; Krzeminski et al., 2019; Kümmerer, 2009). Indeed, residual chemical CECs, e.g., pharmaceutical active compounds like antibiotics and microbial CECs as e.g., antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) were reported to be disseminated into the environment with effluent and excess sludge from municipal wastewater treatment plants (WWTPs) (Berendonk et al., 2015; Hiller et al., 2019; Kümmerer, 2009; Pazda et al., 2019; Vaz-Moreira et al., 2014).

WWTPs act as collection points for ARB, ARGs and antibiotics from a variety of sources like hospitals, households, and veterinary husbandries (Krzeminski et al., 2019). A co-existence of human commensals and pathogens with environmental bacteria under various selection pressures from antibiotics, heavy metals and biocides, and stress factors (nutrient, redox conditions, and temperature) during biological wastewater treatment may facilitate the potential of transfer and proliferation of resistant strains via horizontal or vertical gene transfer (Michael-Kordatou et al., 2018; Poole, 2017). Therefore, biological WWTPs are identified as one of the most important routes for the propagation of antibiotic resistance from humans to the environment (Berendonk et al., 2015; Krzeminski et al., 2019; Pärnänen et al., 2019; Pruden et al., 2013; Rizzo et al., 2013). Moreover, the presence of residual amounts of chemical and microbiological CECs in biologically treated effluents may compromise the reuse and safety of treated wastewater, a practice increasingly applied to address water scarcity globally (Krzeminski et al., 2019; Michael-Kordatou et al., 2018; Rizzo et al., 2020).

Ozonation proved to be an effective and economically feasible option to achieve a further reduction of many chemical CECs by more than 80% (Bourgin et al., 2018; Gerrity et al., 2012; Lee et al., 2013; Rizzo et al., 2019). This technology is also suitable to reduce or inactivate pathogens (Czekalski et al., 2016; Dodd, 2012; Von Gunten, 2003). The mechanisms of bacteria disinfection or inactivation by ozone include the disruption of bacterial cell walls and release of intracellular constituents, the damage of nucleic acids, and the cleavage of carbon-nitrogen bonds in proteins (Alexander et al., 2016; Iakovides et al., 2019; Rizzo et al., 2020). Although the ozone dose applied for effluent ozonation can ensure efficient removal of chemical CECs and inactivation of microorganisms, it may lead to a formation of undesirable organic and inorganic by-products, especially bromate (Wu et al., 2018). Therefore, biological post-treatment is recommended after ozonation (e.g., biological activated carbon filter, BAC) to ensure the degradation of by-products (Rizzo et al., 2020). However, regrowth of ARB in technical filters like BAC may compromise the inactivation efficiency of these biological CECs by biological repair mechanisms and regrowth (Czekalski et al., 2016). Therefore, treatment in BAC filters should be thoroughly investigated for its application for ARB and ARGs abatement from wastewater.

The full-scale application of ozonation for ARB and ARGs inactivation requires technical optimization of the ozonation process considering both the bacterial species and associated ARGs, and the physicochemical properties of the wastewater matrix (Michael-Kordatou et al., 2018). Furthermore, suitable strategies for accurate ozone dosing are needed for achieving desirable abatement of CECs while minimizing the risk of effluent toxicity. To evaluate the ozone treatment efficacy for the reduction of chemical CECs, the relative decrease in UV absorption at 254 nm ($\Delta UV_{254}$) as a difference between the matrix $UV_{254}$ before and after treatment was suggested as an easily accessible surrogate parameter (Wu et al., 2018) suitable for continuous and online ozonation process control (Bahr et al., 2007; Stapf et al., 2016). In this paper, for the first time we were applying the $\Delta UV_{254}$ approach on the assessment of the inactivation of *E. coli* and wastewater autochthonous bacteria as well as the abatement of ARB and ARGs.

Nitrite compensated specific ozone doses between 0.4 and 0.6 g $O_3$/g DOC were found to be suitable for efficient chemical CECs abatement (Bourgin et al., 2018; Hollender et al., 2009; Rizzo et al., 2020). Inactivation of ARB and removal of ARGs under similar ozone doses as chemical CECs would be highly beneficial for the application of ozonation as a wastewater treatment step (Czekalski et al., 2016). The effect of ozonation on ARB and ARGs has not been studied in detail so far (Rizzo et al., 2020), with most of the studies carried out in lab-scale size only (Czekalski et al., 2016; Iakovides et al., 2019; Lamba and Ahammad, 2017; Michael-Kordatou et al., 2017; Moreira et al., 2016; Oh et al., 2014; Sousa et al., 2017; Zheng et al., 2017; Zhuang et al., 2015). Studies treating municipal wastewater in a pilot- or even full-scale are scarce (Table 1) and vary in sampling procedures, operation parameters, examined ARB and ARGs, and methods applied for ARB and ARGs detection/quantification. Though investigations under full-scale and "real life" conditions are of core importance since intrinsic temporal fluctuations in quantitative and qualitative wastewater matrix parameters, varying operation conditions (ozone dose and contact time) and changes in abundance of ARB and ARGs occur under "real-life" conditions and affect the process performance.

In our study, the demonstrator plant for chemical CECs removal from CAS effluent by ozonation followed by GAC filtration was monitored and assessed for ARB and ARGs abatement for 13 consecutive months. The study focused on:

i. Investigating, if the range of ozone doses suggested to be applied for chemical CECs abatement (0.4–0.6 g $O_3$/g DOC) is suitable for ARB and ARGs abatement under "real-life" conditions as well
ii. Correlating ARB and ARGs abatement with $\Delta UV_{254}$ to assess if this parameter could be applied as a surrogate for the elimination of these targets similar to its application for organic CEC removal

**Table 1**
Comparison of pilot-scale ozonation studies targeting ARB and/or ARGs abatement. *The ranges of specific ozone doses were calculated based on the applied flow proportional ozone dose [4 mg $O_3$/L and 6 mg $O_3$/L] and range of measured DOC in wastewater reported by the authors of cited study.

| Treatment scale | Ozone dose | Target ARB | Target ARGs | Reference |
|---|---|---|---|---|
| Pilot-scale | 0.9 ± 0.1 g $O_3$/g DOC | Enterococcus, *P. aeruginosa*, Staphylococcus, Enterobacteria | *van*A, $bla_{VIM}$, *erm*B, *amp*C | (Alexander et al., 2016) |
| Lab-scale and full-scale | 0.45–0.55 g $O_3$/g DOC | *E. coli*, Cultivable heterotrophic bacteria | *sul*1 | (Czekalski et al., 2016) |
| Pilot-scale | 1 g $O_3$/g DOC | *E. coli*, *A. baumannii*, Intestinal enterococci | *sul*1, $bla_{TEM}$, *tet*M, *CTX*-M, *CTX*-M-32, *bla*OXA-48, *bla*VIM, *CMY-2*, *van*A, *mcr-1*, *bla*NDM | (Hembach et al., 2019) |
| Pilot-scale | 0.54–0.72 g and 0.79–1.26 g $O_3$/g DOC* | *E. coli*, Enterococcus | – | (Kirchner et al., 2020) |
| Pilot-scale | 0.5–2.5 mg $O_3$/L | Cultivable heterotrophic bacteria | – | (Li et al., 2018) |
| Pilot-scale | 0.5–11 mg $O_3$/L | *E. coli*, Enterococcus | – | (Luczkiewicz et al., 2011) |
| Pilot-scale | 0.73 g $O_3$/g DOC | *E. coli*, Enterococcus, and Staphylococcus | – | (Lüddeke et al., 2015) |

iii. The differences in the behaviour of tested ARB and ARGs during ozone treatment: may antibiotic resistance mechanisms link to the differences in response to ozonation between bacteria?
iv. Investigating the risk of potential ARGs selection by ozone treatment
v. Assessing a biological activated GAC filter (BAC) for its effects on ARB and ARGs abundance.

## 2. Materials and methods

A comprehensive one–year monitoring of a full–scale ozonation plant coupled with GAC filtration was carried out by applying culture–based and PCR–based methods as suggested in Michael–Kordatou et al. (2018) and Von Sperling et al. (2018). Over 13 consecutive months, samples were analysed to determine changes in ARB&ARGs abundance before and after ozonation, and GAC treatment as described in the following subchapters.

### 2.1. Technical operation

The investigated WWTP is located in the southeast of Austria, has a capacity of 7250 population equivalents and treats municipal wastewater. The WWTP consists of mechanical treatment and a conventional activated sludge (CAS) stage with nitrification and denitrification. The CAS effluent after the secondary clarifier (CAS-OUT) was applied as the feed for the multibarrier demonstrator plant. Over the investigated period, in the CAS-OUT, the average chemical oxygen demand (COD) and dissolved organic carbon (DOC) concentrations were 14.26 ± 2.45 mg/L and 4.26 ± 0.49 mg/L, respectively. The one-year average $NH_4^+$-N, $NO_x$-N, $PO_4^{3-}$-P and suspended solids concentrations in CAS-OUT (n = 13) can be found in Table S1. The three ozone reactors operated in series had a total volume of 12 $m^3$ and the hydraulic retention time varied between 9 and 40 min, depending on the inflow dynamics (a detailed scheme can be found in Mišík et al., 2020). A nitrite compensated specific ozone dose of 0.55 g $O_3$/g DOC was targeted in the automated process control system for routine operation. Since nitrite reacts fast and stoichiometrically with ozone, the ozone consumed for its oxidation (3.43 g $O_3$/g $NO_2$-N) was subtracted from the ozone dose in order to determine the so-called nitrite compensated specific ozone dose. The specific ozone dose control was based on a site-specific UV-DOC correlation model and continuous measurement with an online UV spectrometer probe (s::can, Vienna, Austria). Since nitrite was not measured online, the nitrite compensated specific ozone dose was calculated a posteriori based on laboratory analysis. Due to occasional nitrite peaks in the WWTP effluent, the nitrite compensated specific ozone doses during routine operation ranged between 0.4 and 0.7 g $O_3$/g DOC. Additionally, the wider range of ozone doses was investigated during additional experiments at two of the sampling campaigns (in September and in March). Within these campaigns, 3 additional ozone doses were tested (besides the standard ozone dose) from the wider range of ozone doses between 0.2 and 0.9 g $O_3$/g DOC. Finally, the activated carbon filter was filled with 1.8 $m^3$ of granular activated carbon (GAC), type Epibon A (Donau Carbon, Frankfurt, Germany) and treated a side stream of 8 $m^3$ /h, which resulted in an empty bed contact time of 13.5 min

### 2.2. Sampling scheme

Sampling campaigns were performed between May 2018 and May 2019. Grab samples of CAS effluent (CAS-OUT), effluent after ozonation ($O_3$-OUT), and final effluent after GAC filter (GAC-OUT) were collected on a monthly basis for 13 consecutive months. Therefore, 13 samples were collected from each sampling site (5 L of CAS-OUT, 10 L of $O_3$-OUT and 10 L of GAC-OUT). During September and March sampling, the three extra ozone doses were investigated (besides the standard dose), resulting in additional three $O_3$-OUT samples. The quality of the effluent after CAS treatment regarding tested ARB and ARGs did not fluctuate within a day (data not shown).

Samples from the raw influent wastewater were also collected to monitor the quality of the inflow to the WWTP (data not shown). All samples were transported cooled and processed within 24 h after collection.

### 2.3. Chemical analyses

Collected wastewater samples were analysed for COD (ISO 15705), DOC (EN 1484), $PO_4^{3-}$-P (ISO 6878), $NH_4^+$-N (ISO 11732), $NO_x$-N (ISO 13395), $NO_2^-$-N (ISO 13395) and suspended solids (DIN 38409–2). The UV absorbance at 254 nm ($UV_{254}$) was measured with a lab-photometer (Lamda 35) from Perkin Elmer (USA).

### 2.4. Selection of investigated ARB and ARGs

The selection of *Escherichia coli* as a target microorganism was based on its broad application as an indicator for fecal contamination, its omnipresence in WWTPs and the availability of studies focusing on the inactivation of this organism by ozonation.

The occurrence of four targeted ARGs (*sul*1, *erm*B, *tet*W, $bla_{TEM-1}$) was frequently reported in both influent and effluent of WWTPs (Pärnänen et al., 2019; Pazda et al., 2019). The choice of ARGs was based on their following characteristics: (i) conferring resistance to different types of antibiotics with various modes of action, and (ii) differences in major bacterial hosts in wastewater. Tested genes confer resistance to sulfonamides (*sul*1), macrolide–lincosamide–streptogramin B (MLSB) antibiotics like erythromycin, azithromycin, and clarithromycin (*erm*B), tetracyclines (*tet*W), and type A beta-lactams, like ampicillin ($bla_{TEM-1}$) (Pärnänen et al., 2019). Furthermore, *sul*1 and *erm*B genes were reported to be linked mostly with environment-associated taxa, whereas *tet*W and $bla_{TEM-1}$ genes with human-associated taxa in WWTPs (Yin et al., 2019).

### 2.5. Culture-dependent analysis

Chromogenic coliform agar (CCA) (VWR Chemicals, USA) was used to enumerate total and resistant *Escherichia coli* and coliforms in wastewater. Reasoner's 2 A agar (R2A), conventionally used for microbiological testing of potable waters, was chosen to investigate slow-growing heterotrophs in the effluents (Reasoner and Geldreich, 1985), especially targeting ozonated samples, in which bacteria cells are expected to be damaged and require time to repair and grow (Czekalski et al., 2016). Media were prepared according to the manufacturer's instructions and spiked with the appropriate concentration of antibiotic (to reach final concentrations of 32 mg/L ampicillin (AMP) or 4 mg/L trimethoprim (TMP) together with 76 mg/L sulfamethoxazole (SMX)). Concentrations of antibiotics were chosen to enumerate resistant microorganisms based on information provided by the Clinical and Laboratory Standards Institute CLSI (2017). Serial dilutions of samples were prepared in sterile 0.85% (w/v) NaCl solution in four replicates targeting 10–80 colonies per plate. Prepared dilutions were vacuum filtered through cellulose acetate filters (47 mm Ø, pore size 0.45 µm, Pall Life Sciences, USA). The filter was transferred onto the medium and incubated for 24 h at 37 °C (CCA medium) or for 5 d at 37 °C (R2A medium). Blue (*E. coli*) and pink (coliforms) colonies were counted on CCA medium, and all colonies were counted on R2A plates. The lower and upper quantification limits (LOQ) were estimated as 5 and 150 colonies per plate, respectively. Results outside this range were disregarded, giving a total dataset of n = 13 for total heterotrophs, n = 17 for total *Escherichia coli*, n = 10 for *Escherichia coli* resistant to 32 mg/L ampicillin, n = 11 for *Escherichia coli* resistant to 4 mg/L trimethoprim and 76 mg/L sulfamethoxazole.

### 2.6. DNA extraction

Samples were shaken vigorously and vacuum filtered through

cellulose acetate filters (47 mm Ø, pore size 0.45 μm, Pall Life Sciences, USA) in at least 3 replicates. This type of filter was required to collect sufficient biomass for DNA extraction from samples of GAC-OUT and raw wastewater - influent to the WWTP. Filters were cut into pieces, transferred into Eppendorf tube, and stored at − 20 °C until further processing. Volumes for filtration were chosen individually, depending on the sample's predicted biomass concentration and turbidity to allow maximal DNA yield (0.4–0.8 L of CAS-OUT, 0.8–1.2 L of $O_3$-OUT, 1–2.5 L of GAC-OUT). Total DNA from the filters was extracted with the DNeasy PowerWater Kit (Qiagen, Germany) according to the provided protocol, except for the following modifications: (i) a 2 mL bead beating tube was used instead of a 5 mL tube, (ii) a cell lysis step was performed in a MP Biomedicals™ FastPrep 4™ 5 G Instrument for 2 × 40 s at 6 m/s, (iii) the columns were air-dried for at least 5 min before addition of elution buffer, (iv) the columns were incubated for at least 2 min with elution buffer. The quality and concentration of extracted DNA were measured with an Eppendorf BioSpectrometer® and extracts were stored at − 80 °C until further processing.

### 2.7. Quantitative PCR analysis

Targeted genes: 16 S rRNA gene and ARGs (*sul*1, *erm*B, $bla_{TEM-1}$ and *tet*W) were quantified by TaqMan assays, designed and optimized by Ingenetix GmbH (Vienna, Austria). All reactions were performed in a Roche Light-Cycler 480 (Roche Applied Science, Germany) in a 10 μL reaction mixture, containing 1 x LightCycler® 480 Probes Master (Roche, Germany), 1 x TaqMan assay and 2 μL of a sample, according to the protocols in Table S2. All samples and standards were assayed in triplicates. Standard curves were prepared for each run by 10-fold dilution of the standard, ranging $10^7$–$10^1$ copies for the investigated ARGs and $10^8$–$10^3$ copies for the 16 S rRNA gene. The amplification efficiency ranged 90–105%. LOD and LOQ of qPCR assays are described in Table S3.

### 2.8. Data analysis

The absolute abundance of total and antibiotic resistant bacteria revealed by culture-based methods was presented as colony forming units per mL of the sample (CFU/mL) according to Novo and Manaia (2010). The absolute abundance of 16 S rRNA gene and ARGs was given as copies/mL of the sample. The relative abundance of ARGs was calculated as a ratio of ARGs abundance to 16 S rRNA gene abundance. The log removal values (LRV) of total and antibiotic resistant bacteria, and targeted genes were calculated according to Von Sperling et al. (2018). For ozone treatment, CAS-OUT and O3-OUT values were taken, and for GAC treatment, O3-OUT and GAC-OUT were taken to calculate LRV. Positive LRV represents a reduction of the target, and negative LRV an increase of its abundance after treatment.

R software (version 4.0.4., R Core Team, 2021) was used for statistical analyses and visualization of the outcomes (main packages: *corrplot, pheatmap, drc*, *stats*, *qpcR*, *devtools)*. Regression models for ozone doses, the $\Delta UV_{254}$, bacteria and genes were computed (linear, exponential, logarithmic, logistic and log-logistic with varied numbers of parameters). In addition, Pearson correlations between datasets and heatmaps were also computed and visualized. Targets were considered correlated for $R^2 > 0.5$, and $p < 0.05$. Relatedness with values of $0.5 < R^2 < 0.7$ was considered as a moderate correlation and $R^2 > 0.7$ as a strong correlation.

Data normality was tested with Shapiro-Wilk test and the equality of the variances was evaluated with Leven's test. To test statistical significance between the datasets, ANOVA was used for normally distributed data and Kruskal-Wallis test was used for not normally distributed data. To reveal significantly different groups within datasets, the following tests were applied: Tukey test for normally distributed data and Dunn test for not normally distributed data. T-test was used to determine the significance of the difference between the change in ARGs relative abundance after ozonation at higher and lower ambient temperatures. The used p-value was 0.05.

## 3. Results

### 3.1. The correlation of relative decrease of $UV_{254}$ absorption and nitrite compensated specific ozone dose

As expected, the observed relative decrease of $UV_{254}$ absorption ($\Delta UV_{254}$) was found to strongly correlate with the nitrite compensated specific ozone dose that was compensated for the ozone consumption by nitrite during the monitoring campaign (Fig. 1), thus providing a solid base for further data analysis and correlations. The obtained linear regression was of $R^2 = 0.89$, and p-value = 3.95E-09, and Pearson correlation: $R^2 = 0.94$, p-value = 4.61E-09.

### 3.2. Inactivation of ARB and ARGs

The data for ARB&ARGs abundance collected during the one-year monitoring campaign and two one-day sampling campaigns were correlated with ozone dose and $\Delta UV_{254}$. Regression models were computed in R and the best fit was chosen for each dataset (Table S4). For most of the targets, the adjusted $R^2$ and p-value for linear regression were higher when correlated with $\Delta UV_{254}$ than with nitrite compensated specific ozone dose (except for *erm*B gene). The values were above 0.5 ($R^2$) and below 0.01 (p) for targets except for total heterotrophs, $bla_{TEM-1}$ and *erm*B genes. We suggest that this is a promising result in the direction of application of $\Delta UV_{254}$ as a surrogate parameter in the monitoring of ARB and ARGs during ozonation.

An increase in ozone dose did not result in changes in the log removal of total heterotrophic bacteria, which was between − 0.81–0.73 with no found correlation. The log removal of total *Escherichia coli* and *Escherichia coli* resistant to 32 mg/L AMP, and 4 mg/L TMP with 76 mg/L SMX revealed a correlation with both nitrite compensated specific ozone dose and $\Delta UV_{254}$ in treated effluent (Fig. 2). The log removal value for bacterial targets was increasing until reaching a plateau around 2.50 logs for total *E. coli* (corresponding to $\Delta UV_{254}$ of 30% and nitrite compensated specific ozone dose of 0.5 g $O_3$/g DOC) or 1.99 logs for TMP and SMX resistant *E. coli* (corresponding to $\Delta UV_{254}$ of 25% or nitrite compensated specific ozone dose of 0.4 g $O_3$/g DOC). For AMP resistant *E. coli*, the plateau phase was not as evident, and 2.50 log inactivation was reached with 45% $\Delta UV_{254}$ and 0.9 g $O_3$/g DOC.

The ANOVA test revealed significant differences between log removals of total and antibiotic resistant *E. coli* at the tested ozone doses (p-value = 4.43E-07, Table S5). The Tukey test showed that results for 0.02, 0.18, 0.25 and 0.92 g $O_3$/g DOC (from both monitoring and one-day sampling campaign data) differed significantly from results at other ozone doses (Table S6).

We did not observe fluctuations in the absolute abundance of bacterial targets in CAS-OUT samples; the reported abundance values varied within the same order of magnitude (Table S7). For total *E. coli*, the average absolute abundance in CAS-OUT during the sampling campaign was 187 ± 248 CFU/mL (median = 120 CFU/mL).

Log removal values of targeted genes increased with increasing $\Delta UV_{254}$ (Fig. 3). Moreover, higher $\Delta UV_{254}$ values were required to achieve similar log removal values as for the inactivation of *E. coli*. Data did not show a plateau phase and did not support the estimation of higher asymptotes with statistical relevance. The qPCR signals for *erm*B and $bla_{TEM-1}$ gene were in some cases close to the LOQ value (although above LOQ). The results for observed $\Delta UV_{254}$ significantly varied from each other (p-value = 6.04E-05) Table S8.

### 3.3. Changes in ARGs relative abundance

The absolute abundance of ARGs was related to the absolute abundance of the 16 S rRNA gene to calculate the relative abundance of ARGs

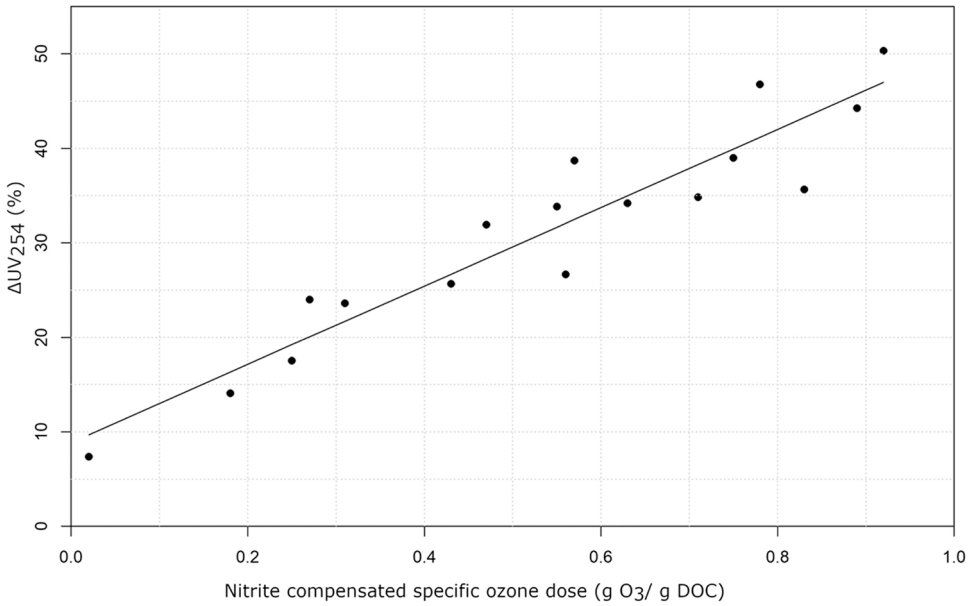


**Fig. 1.** Correlation between nitrite compensated specific ozone dose and $\Delta UV_{254}$ (linear regression $R^2 = 0.89$, p-value = 3.95E-09) for data collected during one-year monitoring including two daily campaigns, n = 18.

indicating their share in bacterial communities of collected samples. For genes *sul*1 and *tet*W, a higher relative abundance was observed indicating that these genes were more abundant within the wastewater bacterial community (in a range of 0 – 0.15 and 0 – 0.20, respectively) than *erm*B and $bla_{TEM-1}$ genes (ranging from 0 to 0.015 and 0–0.08, with one signal at 0.24, respectively).

To visualize how ozone treatment affected ARGs relative abundance, a change in the relative abundance was calculated for each data point (monitoring campaign and one-day sampling campaigns) as a percentage increase or decrease (taking CAS-OUT sample as a starting point for calculation). The average decrease in the relative abundance of ARGs was at a similar level for all tested genes and ranged from 51% (*sul*1) to 68% ($bla_{TEM-1}$), Tab. S9. For *erm*B and $bla_{TEM-1}$ genes, the increase in relative abundance after ozonation was higher than for *sul*1 and *tet*W. The differences between the relative abundance of ARGs at different $UV_{254}$ were not statistically significant (p-value = 0.30, Tab. S10).

Furthermore, the changes in relative abundance were related to sampling dates and average changes in air temperature at the sampling site. We hypothesized that the changes in ARGs relative abundance after ozonation may be related to seasonal changes in bacterial communities. The T-test aimed to estimate the significance of differences between changes in ARGs relative abundance when air temperatures were below or equal to 14 °C (n = 6) versus above 14 °C (n = 5). The 14 °C was the median value for the temperature dataset. The data points from the one-day sampling campaigns for ozone doses outside the standard operating range were excluded to not affect the number of data points at the same air temperature. Obtained p-values were $< 0.05$ for each gene (0.003 for *sul*1, 0.038 for *erm*B, 0.035 for *tet*W) except for $bla_{TEM-1}$ (0.406). Plotting *sul*1 and *erm*B relative abundance changes against sampling dates suggested a seasonality in change of these ARGs abundance after ozonation. Correlation between these changes and air temperature resulted in asymmetrical bell-shape curves with similar centre locations around 7 °C (Fig. 4A-B). For *tet*W and $bla_{TEM-1}$ genes, this correlation could not be observed (Fig. 4C-D).

### 3.4. Correlations between response patterns

Log removals of targeted bacteria and genes for each $\Delta UV_{254}$ were clustered into dendrograms and visualized with a heatmap (Fig. 5). The response patterns clustered into 3 major distinct groups: (i) $\Delta UV_{254}$ between 0 % and 30 % (nitrite compensated specific ozone doses between 0 and 0.5 g $O_3$/g DOC), (ii) $\Delta UV_{254}$ between 30 % and 50 % (0.5–0.9 g $O_3$/g DOC), and (iii) the highest $\Delta UV_{254}$ (equal 50.34 %, corresponding to 0.92 g $O_3$/g DOC) The first cluster (i.e., 0–30 %), corresponds to the majority of nitrite compensated specific ozone doses below 0.5 g $O_3$/g DOC, and the second cluster – above 0.5 g $O_3$/g DOC. The patterns of targeted bacteria and genes removal differed visibly between these two clusters. For the cluster $< 0.5$ g $O_3$/g DOC, log removal values of bacteria and genes were lower than for the second cluster, which was especially visible for the genes. Within the second cluster, the LRV of bacterial targets were at rather similar levels even with increasing ozone dose. However, the LRV for genes increased with increasing ozone dose, which was especially visible for 16 S rRNA gene and *tet*W. The highest $\Delta UV_{254}$ (corresponding to 0.92 g $O_3$/g DOC) was situated on a simplicifolious clade and showed the highest log removal values for all tested genes.

Similarities of response patterns between different genes, as well as total and antibiotic resistant E. coli, suggested that there could be correlations between the behaviour of targeted bacteria and/or genes, which was further investigated. Log removals of the monitored targets, as well as the relative abundance of ARGs, were correlated with each other by Pearson correlations (Fig. 6). The log removal of AMP and TMP/SMX resistant *E. coli* correlated negatively with the relative abundance of *sul*1 gene. This observation suggests a significant relationship between the increase in log removal of resistant *E. coli* and the decrease in the relative abundance of these genes, thus, their share in the bacterial community.

Total and resistant *E. coli* as well as tested genes (except $bla_{TEM-1}$) correlated positively with changes in nitrite compensated specific ozone dose and $\Delta UV_{254}$. Among bacteria, the strongest correlation was found for AMP *E. coli* ($R^2 = 0.93$) and among genes for *tet*W ($R^2 = 0.9$).

### 3.5. GAC filter

In this study, a GAC filter applied in a multibarrier system for CECs abatement as a final step was investigated for its efficiency in reducing ARB and ARGs abundance in the final effluent. The residual abundance of the total, AMP and TMP/SMX resistant *E. coli* after ozone treatment was slightly reduced after GAC filtration (Fig. 7). However, the total number of heterotrophs was up to 1 log higher in the final effluent of the

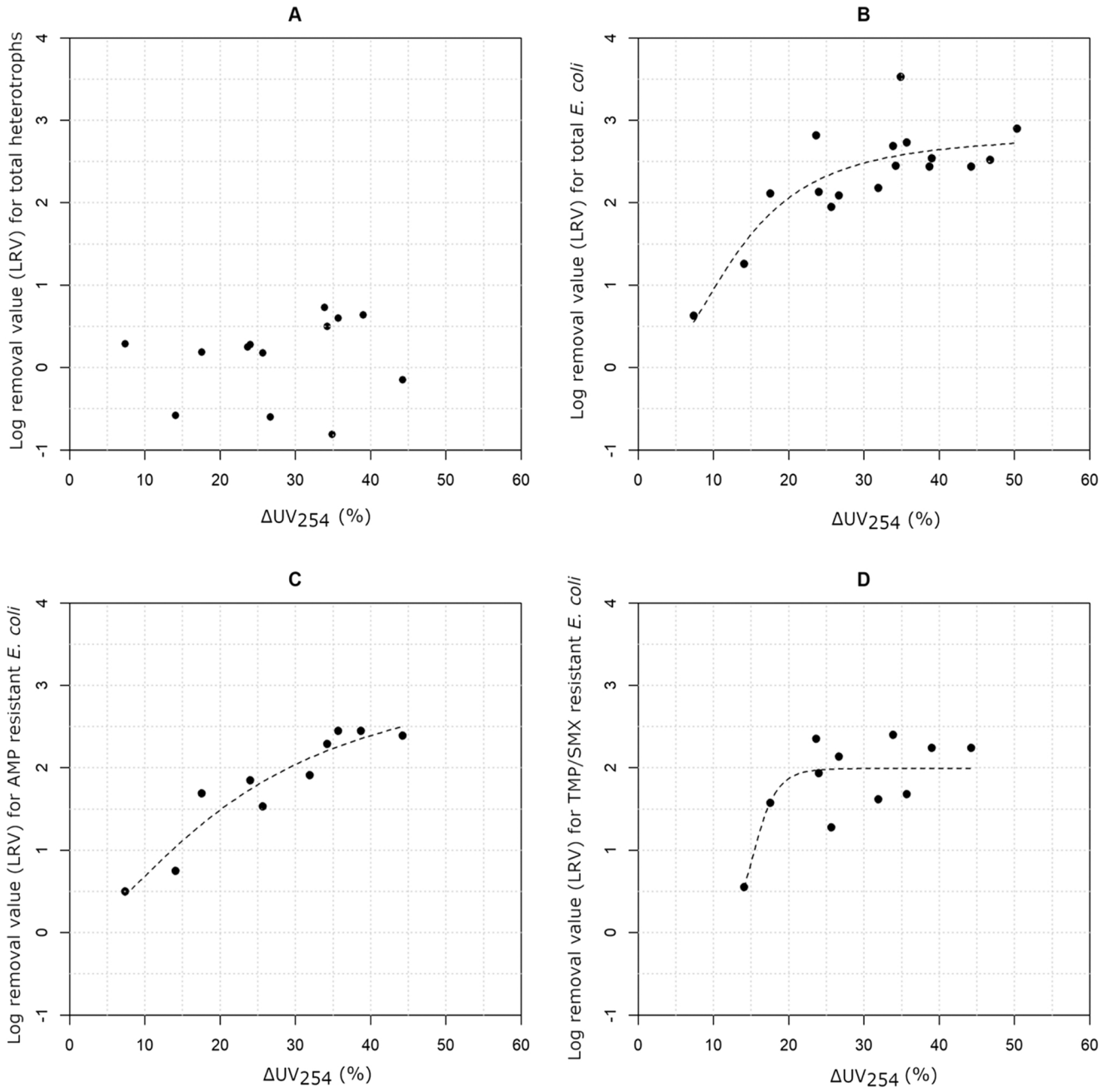


**Fig. 2.** Calculated log removal values for (A) total heterotrophs, n = 13, (B) total Escherichia coli, n = 17, (C) Escherichia coli resistant to 32 mg/L ampicillin, n = 10, and (D) to 4 mg/L trimethoprim and 76 mg/L sulfamethoxazole, n = 11 with regression models (log-logistic). The results outside LOQ range were not considered.

multibarrier system (after GAC) than in ozonated effluent. In general, a reduction in 16 S rRNA gene, *sul*1, and *tet*W ARGs abundance could be observed in GAC-OUT (up to 1 log). Changes in LRV of *erm*B gene varied strongly from close to log 1 reduction and close to 1 log increase. In the case of $bla_{\text{TEM-1}}$ gene, the majority of LRV was negative, suggesting an increase in its abundance in GAC-OUT.

For most sampling points, a reduction in *sul*1 and *tet*W relative abundance was observed in GAC-OUT samples (Tab. S11). However, in the case of *erm*B gene, close to 50% of sampling points showed a reduction in relative abundance at a similar level as for *sul*1 and *tet*W. For the rest of the data points, the average *erm*B relative abundance was approximately 70 times higher than before GAC. This trend was even stronger for $bla_{\text{TEM-1}}$ gene, with only one data point reporting a reduction in its relative abundance and an average increase close to 3000 times.

## 4. Discussion

### 4.1. Application of $\Delta UV_{254}$ as an indicator for ARB&ARGs inactivation by ozonation

Monitoring of ARB and ARGs inactivation in wastewater by ozonation involves costly and time-consuming lab procedures that do not provide an immediate result (e. g. due to bacteria incubation). Therefore, the application of a surrogate parameter is of great benefit in the monitoring of ARB and ARGs abatement by ozonation (Dickenson et al., 2009). Prediction models and correlations of chemical CECs abatement by ozonation with $\Delta UV_{254}$ in wastewater have been previously well

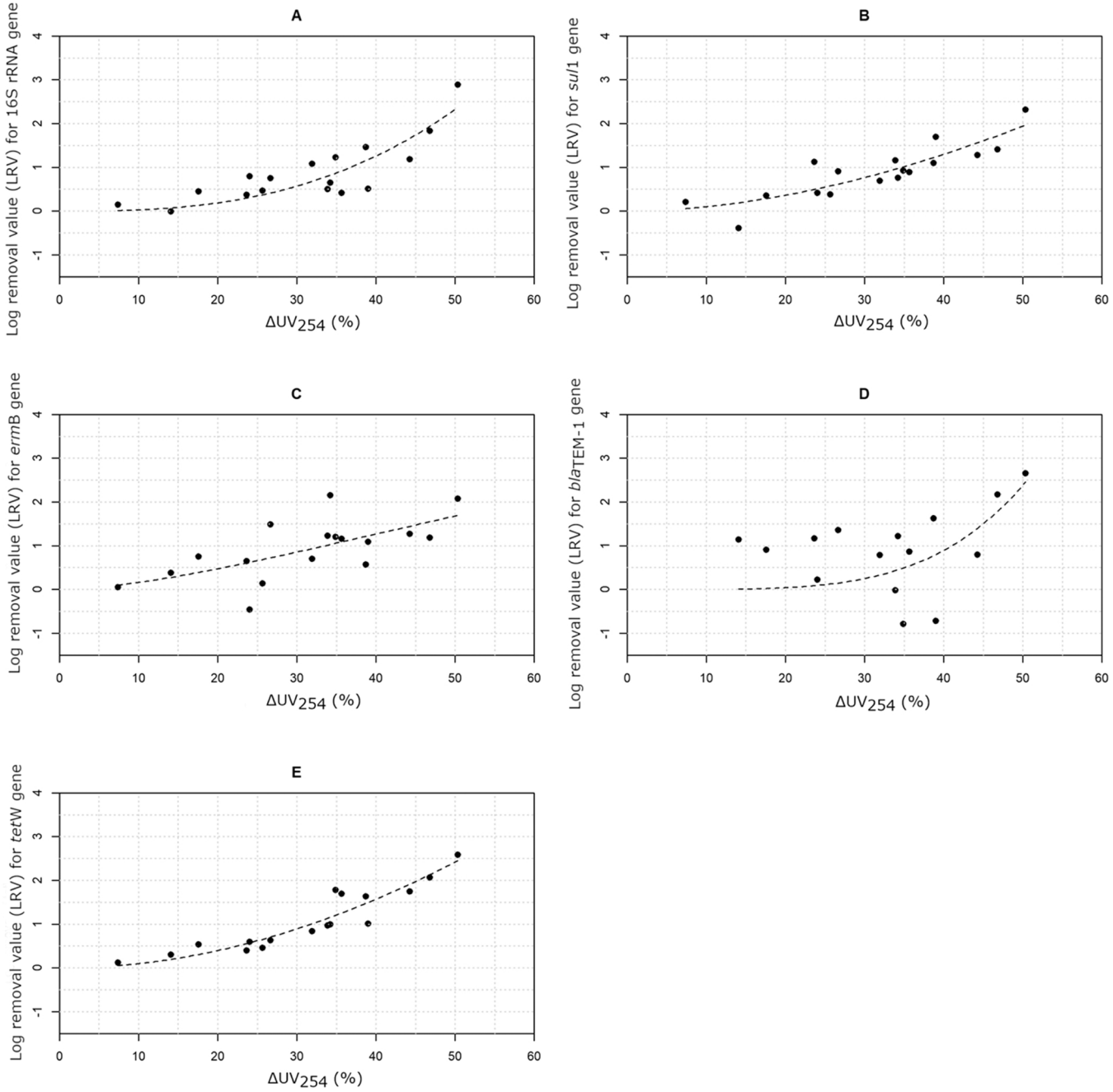


**Fig. 3.** Calculated log removal values for (A) 16 S rRNA gene, n = 17, (B) sul1 gene, n = 17, (C) ermB gene, n = 17, (D) $bla_{TEM-1}$ gene, n = 15, (E) tetW gene, n = 17 with regression models (log-logistic). The results outside LOQ range were not considered.

developed and indicated that $\Delta UV_{254}$ may be used as a surrogate parameter for the elimination of these targets (Gerrity et al., 2012). This parameter was also tested with *E. coli* and other bacteria but not for ARB and ARGs (Gerrity et al., 2012; Lee et al., 2016; Wu et al., 2018). Moreover, $\Delta UV_{254}$ is suggested as a surrogate parameter for estimating ozone exposure in real wastewater systems for chemical CECs (Buffle et al., 2006) and even for some bacteria and viruses (Gerrity et al., 2012; Wu et al., 2018). Since the ARB and ARGs inactivation by ozone depends on ozone exposure (Michael-Kordatou et al., 2018; Rizzo et al., 2020), linking the ARB and ARGs abatement by ozone with $\Delta UV_{254}$ could provide additional information on their response to ozonation. Therefore, we decided to link responses of various target ARB and ARGs at chosen nitrite specific ozone doses with $\Delta UV_{254}$.

In our study, we have observed a strong correlation between nitrite compensated specific ozone dose and $\Delta UV_{254}$ (linear regression: $R^2$ = 0.88, and p-value = 3.95E-09), which was reported before as well (Bahr et al., 2007; Nöthe et al., 2009). The observed correlation between $\Delta UV_{254}$ and total *E. coli* inactivation was similar to one observed by Gerrity et al. (2012) with an $R^2$ value for linear regression comparable to our findings (0.50 in our study compared to 0.47). The plateau phases for bacteria and lag phases for genes caused difficulties with fitting the regression models and resulted in weaker linear regression fits. In general, for tested ARB and ARGs, better fits of regression models and stronger linear and Pearson correlations were obtained with $\Delta UV_{254}$ than nitrite compensated specific ozone dose. This suggests that correlating ARB and ARGs log removal data with the $\Delta UV_{254}$ could better represent these biological CECs exposure to ozone in wastewater, which was also suggested by Wu et al. (2018).

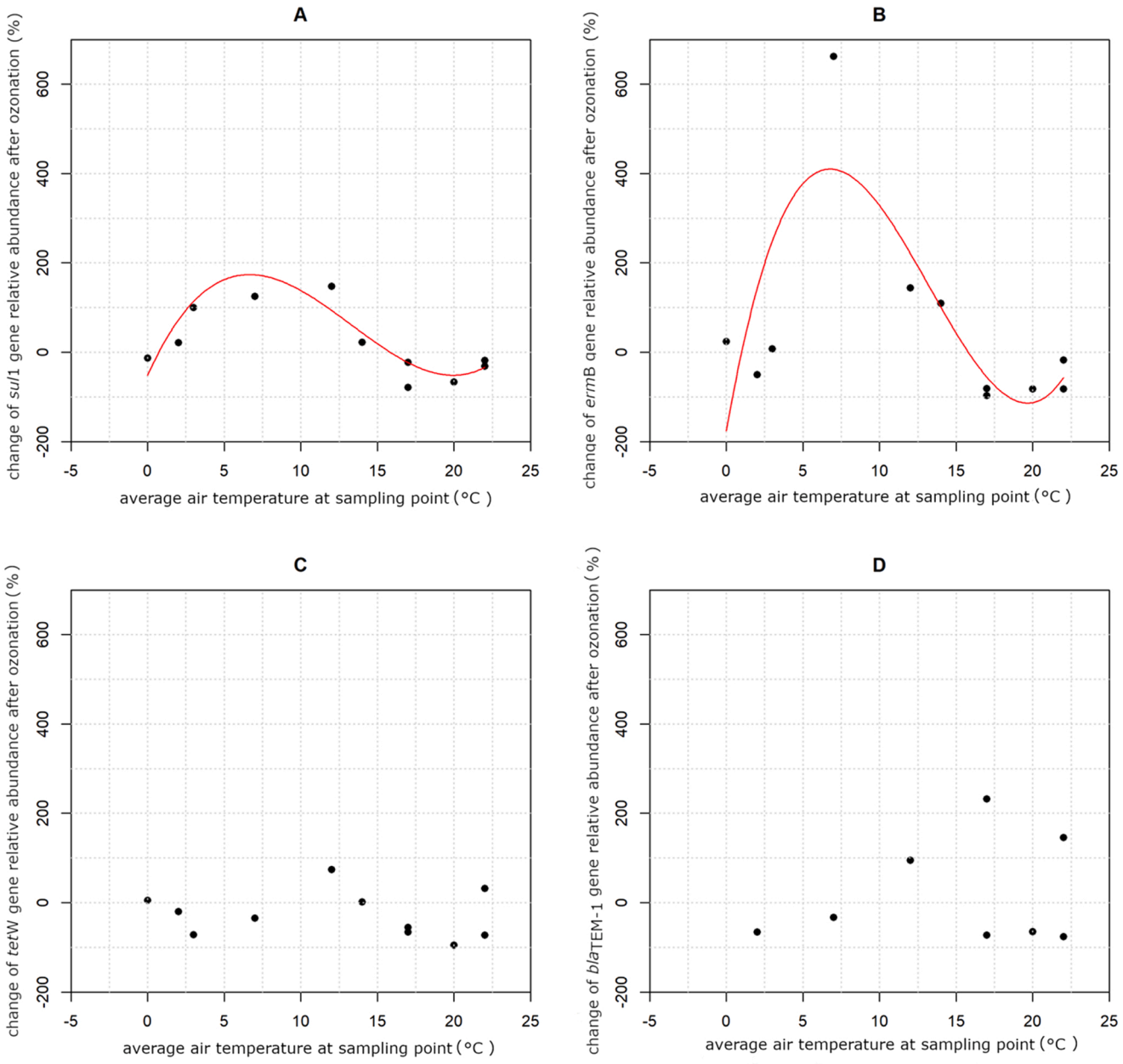


**Fig. 4.** Changes in relative abundance of ARGs during ozone treatment related to average air temperature at sampling location for: (A) sul1 gene with fitted polynomial regression (adjusted $R^2 = 0.71$, p-value = 0.008, 3rd degree), n = 11, (B) ermB gene with fitted polynomial regression (adjusted $R^2 = 0.38$, p-value = 0.10, 3rd degree), n = 11, and (C) tetW gene, n = 11, (D) $bla_{TEM-1}$ gene, n = 8. Negative change represents reduction of the gene abundance by a shown percent.

These observations are promising results in the direction of application of $\Delta UV_{254}$ as a surrogate parameter in the monitoring of ARB and ARGs during ozonation. Further studies in this direction are required and should either involve targeting the range of ozone doses that result in correlations as linear as possible or in multiple long-term studies that would help in generating models predicting the behaviour of target ARB and ARGs at chosen $\Delta UV_{254}$.

### 4.2. Inactivation of ARB and ARGs by ozone treatment

For total and antibiotic resistant *E. coli* and tested ARGs, the log inactivation increased with increasing $\Delta UV_{254}$ (and therefore nitrite compensated specific ozone dose). However, for bacterial targets, above a certain $\Delta UV_{254}$, a further increase in ozone dose did not result in a higher abatement, reaching a plateau phase above which no further reduction of *E. coli* cultivability could be achieved. A similar observation was reported by Gerrity et al. (2012) in their lab-scale ozone disinfection of *E. coli* providing a regression model described by a sigmoidal curve with a plateau phase (approximately 40 % vs. 30% of $\Delta UV_{254}$ in our study). Wu et al. (2018) also observed a steady decrease of *E. coli* cultivability (CFU/mL) up to a specific ozone dose of 0.4 g $O_3$/g DOC (which would correspond to approximately $\Delta UV_{254}$ of 25 % in our study) and no significant further decrease for higher ozone doses.

Even though regression models for total and resistant *E. coli* showed strong similarities in our results, the $\Delta UV_{254}$ required to reach the plateau phase varied between these two targets. For total *E. coli*, the plateau was reached with $\Delta UV_{254}$ of approximately 30 % (0.51 g $O_3$/g DOC), for TMP/SMX resistant *E. coli* with $\Delta UV_{254}$ of 25 % (0.39 g $O_3$/g DOC), and for AMP resistant *E. coli* with 45% $\Delta UV_{254}$ (0.87 g $O_3$/g DOC). Alexander et al. (2016) suggested that these differences may result from varying levels of susceptibility against oxidative stress between the bacteria resistant or susceptible to different types of

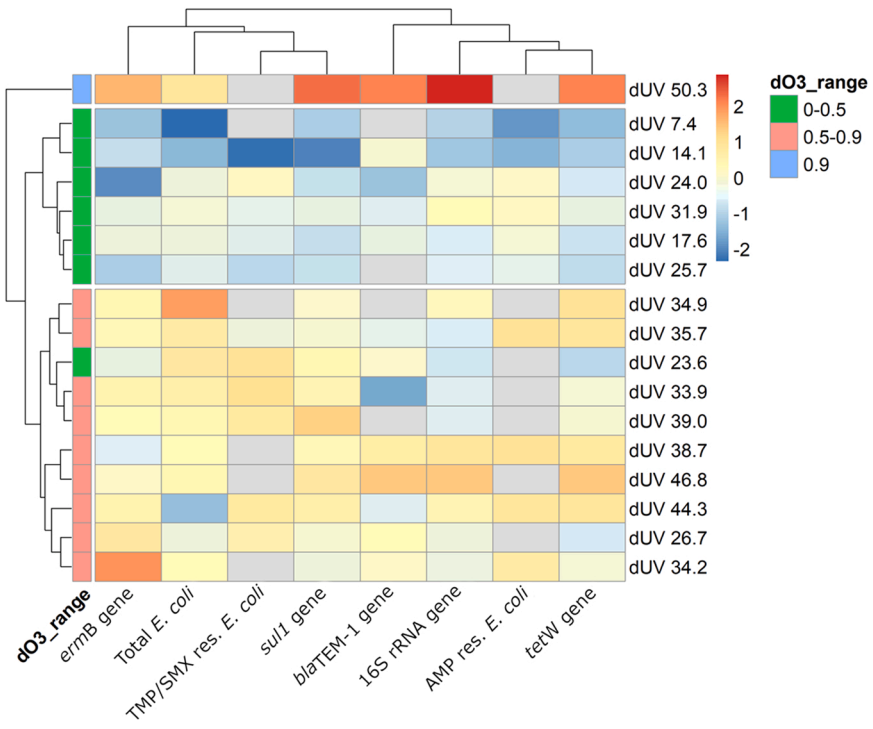


**Fig. 5.** Heatmap with dendrogram for data collected for each $\Delta UV_{254}$. Log removal values for total and resistant E. coli as well as for ARGs and 16 S rRNA gene were clustered against $\Delta UV_{254}$ (presented in the Figure as dUV). Each $\Delta UV_{254}$ corresponds to the nitrite compensated specific ozone dose applied in the study. The applied ozone doses were divided into three ranges (dO3_range: 0–0.5, 0.5–0.9, and 0.9), and corresponding samples were marked with colours to visualize the differences in responses between the ranges, especially for doses below and above 0.5 g $O_3$/g DOC. Analysed targets were total Escherichia coli, n = 17 ("Total E. coli"), Escherichia coli resistant to 32 mg/L ampicillin, n = 10 ("AMP res. E.coli"), and to 4 mg/L trimethoprim and 76 mg/L sulfamethoxazole, n = 11 ("TMP/SMX res. E.coli"), 16 S rRNA gene, n = 17, sul1 gene, n = 17, ermB gene, n = 17, $bla_{TEM-1}$ gene, n = 13, and tetW gene, n = 17.

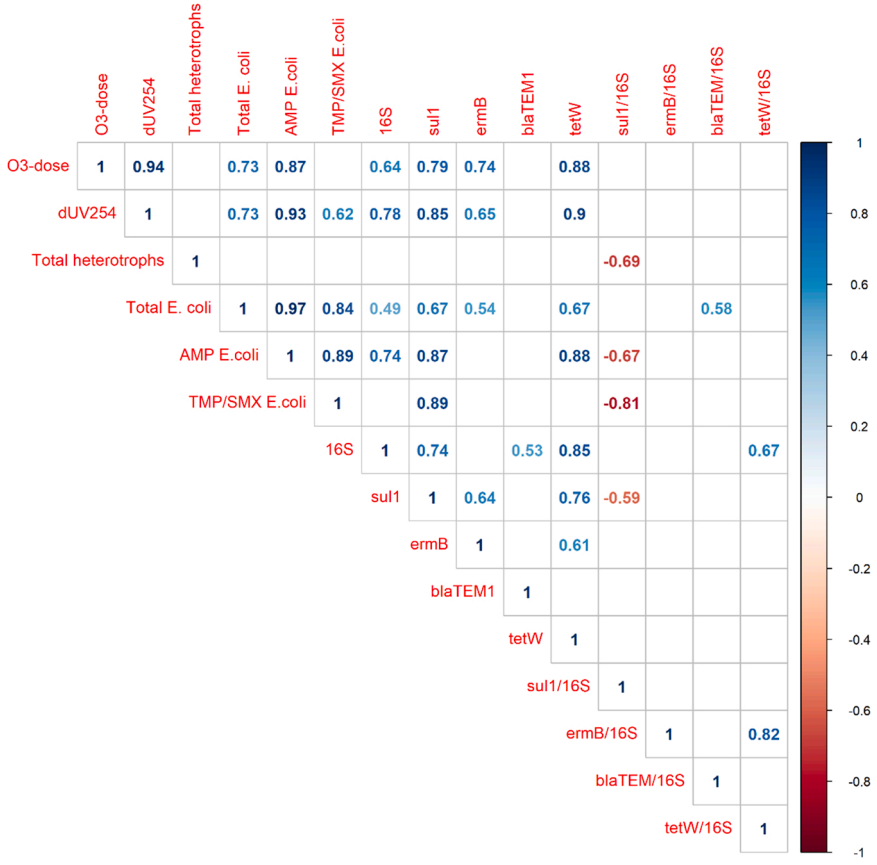


**Fig. 6.** Pearson correlations between monitored targets (only significant ones are shown, p-value > 0.05) for data collected during the one-year monitoring campaign (13 consecutive months) and two one-day sampling campaigns. $\Delta UV_{254}$ was presented in the Figure as dUV and applied in the study nitrite compensated specific ozone doses as O3-dose. AMP E. coli represents AMP resistant E. coli and TMP/SFX E. coli - TMP/SFX resistant E. coli.

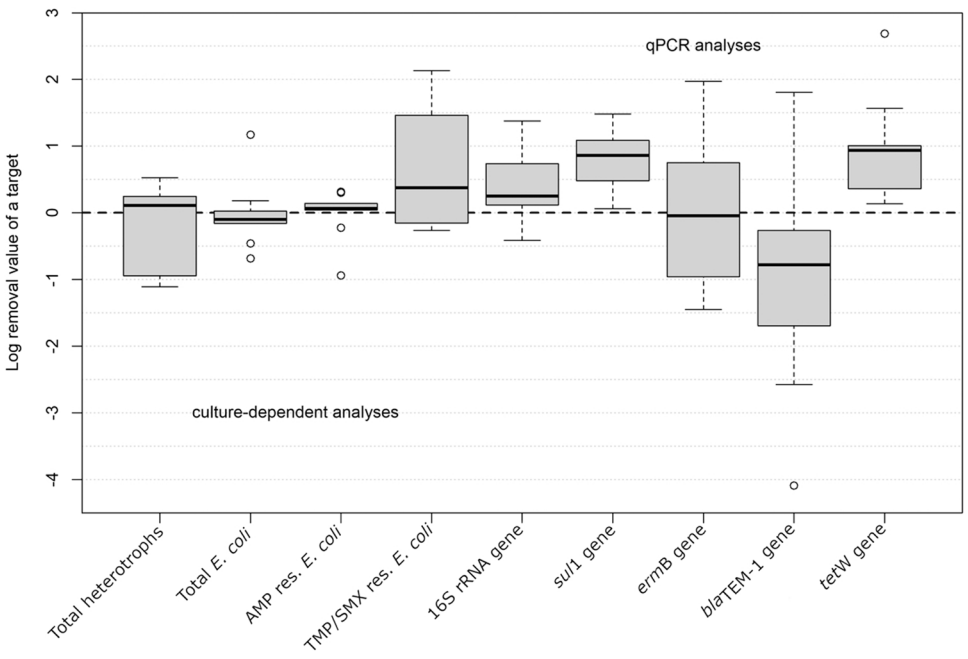


**Fig. 7.** Box plot with whiskers from minimum to maximum, with the displayed median value for log removal values calculated for GAC treatment (taking O3-OUT and GAC-OUT as before and after treatment). Analysed targets were total heterotrophs, n = 9, total Escherichia coli, n = 11 ("Total E. coli"), Escherichia coli resistant to 32 mg/L ampicillin, n = 9 ("AMP res. E. coli"), and to 4 mg/L trimethoprim and 76 mg/L sulfamethoxazole, n = 4 ("TMP/SMX res. E. coli"), 16 S rRNA gene, n = 11, sul1 gene, n = 11, ermB gene, n = 11, $bla_{TEM-1}$ gene, n = 9, and tetW gene, n = 11. Positive log removal represents the reduction of the gene abundance by a shown log.

antibiotics. The oxidative stress may be induced by reactive oxygen species (ROS) generated by the interaction of bactericidal antibiotics with their classical targets and activated ROS protective responses (Acker and Coenye, 2017; Kohanski et al., 2007). The trace abundance of antibiotics (and potentially their transformation products formed during ozonation (Szabó et al., 2016)) could induce the antibiotic resistance adaptation response as well as sub-lethal stress response. These mechanisms would activate anti-oxidative and oxidative damage-associated responses against produced ROS (e.g., recA response) in ARB (Alexander et al., 2016). Therefore, ARB that can effectively induce these responses have an advantage over non-resistant bacteria in dealing with the damages caused by ozonation. Our observation of reaching the plateau phase for cultivability of AMP resistant *E. coli* at $\Delta UV_{254}$ higher than for TMP/SMX resistant *E. coli* would support this hypothesis (AMP is a bactericidal antibiotic in contrast to TMP and SMX, which are bacteriostatic).

The log removal values of targeted genes increased with increasing ozone dose and did not reach a plateau phase. Moreover, higher ozone doses were required to achieve similar LRV of ARGs as compared to ARB (e.g., $\Delta UV_{254}$ of 30 % resulted in 2 logs inactivation of AMP resistant *E. coli* and approximately 1 log abatement of *sul*1, *tet*W and *erm*B gene). The need for a higher ozone dose to achieve DNA damage or leakage from attacked bacteria cells was also observed by other authors (Czekalski et al., 2016; Lee et al., 2016; Wu et al., 2018). At lower ozone doses, a certain lag phase could be observed, except for *erm*B (up to 20–25% $\Delta UV_{254}$, thus, 0.4 g $O_3$/g DOC for 16 S rRNA gene, *sul*1 and *tet*W, and approximately 35 % $\Delta UV_{254}$ for $bla_{TEM-1}$, thus 0.6 g $O_3$/g DOC). Furthermore, the observed lag phases seem to correspond to the plateau phases for bacterial targets. Cho et al. (2010) and Wu et al. (2018) reported that the lag phase was finished when sufficient membrane damage was reached to cause DNA leakage or for ozone to penetrate into cell plasma and cause further DNA damage. This would suggest that $\Delta UV_{254}$ of 30% (0.5 g $O_3$/g DOC) at the link between the start of plateau phases for bacteria and the end of lag phases for genes could correspond to reaching sufficient membrane impairment to cause DNA damage. Previous works (Czekalski et al., 2016; Wu et al., 2018) reported significant lag phases for DNA damage until approx. 0.4 g $O_3$/g DOC. The higher lag phase observed for $bla_{TEM-1}$ gene may be correlated with higher $\Delta UV_{254}$ required to reach a plateau phase for AMP resistant *E. coli*, suggesting higher robustness of ampicillin resistant bacteria against ozone.

The abundance of total heterotrophic bacteria (based on CFU results) oscillated between 1 log reduction and 1 log increase after treatment. The applied medium would be suitable for bacteria thriving in both bulk and flocs. The abundance of flocs, which are three-dimensional high cell-density structures, result in difficulties in a reliable enumeration of total heterotrophic bacteria directly from wastewater samples on agar plates, as one CFU can represent several bacteria within an activated sludge floc that may be split up to several smaller units and thus CFUs in ozonation. Therefore, total heterotrophs seem to not be suitable for an indicator of the efficiency of total bacteria disinfection by ozone treatment, and changes in 16 S rRNA gene abundance should be used instead. Since *E. coli* is rather associated with bulk (not flocs), the relationship between $\Delta UV_{254}$ and changes in CFU could be observed. It is worth noting that the presence of particles can protect microorganisms: bacteria abundant at $> 12$ μm into the floc were reported to be protected from ozone treatment (Czekalski et al., 2016).

### 4.3. Recommended ozone doses for ARB&ARGs abatement in wastewater

Specific ozone doses between 0.4 and 0.6 g $O_3$/g DOC (nitrite compensated) were reported to be efficient for chemical CECs abatement from wastewater (Rizzo et al., 2020). Doses above 0.5 g $O_3$/g DOC were suggested as feasible to achieve significant levels of microbial inactivation (Von Sonntag and Von Gunten, 2012). Inactivation of ARB and ARGs at similar ozone doses as required for chemical CECs abatement would be highly beneficial for the operation of wastewater ozonation and its application in real-life conditions. Since wastewater is a complex matrix, which composition fluctuates over time, the control and adaptation of the effective applied ozone dose are vital for the efficient abatement of targets.

In our study, a significant difference between the pattern in bacteria and gene responses to ozone treatment at $\Delta UV_{254}$ below and above 30% (0.5 g $O_3$/g DOC) was observed. The loss of cultivability of bacterial targets was already reached below 0.5 g $O_3$/g DOC while a significant log removal of genes could be observed above 0.5 g $O_3$/g DOC. This corresponds to the overlap of plateau phases for bacterial targets and lag phases for genes around $\Delta UV_{254}$ of 30 %. This would implicate that for tested targets (except for AMP resistant *E. coli* and $bla_{TEM-1}$) a sufficient membrane impairment resulting in significant DNA damage or leakage

would be reached at around 0.5 g $O_3$/g DOC. Czekalski et al. (2016), Lee et al. (2016) and Wu et al. (2018) suggested a similar scheme of bacteria cell inactivation: the effects on cultivability (CFU) were followed by the effects on membrane permeability leading to significant DNA damage.

The chosen ozone dose should ensure not only membrane but also DNA damage to (i) minimize the abundance of intact ARGs that may remain within the resulting cell debris even after the loss of ARB viability (Dodd, 2012), and (ii) maximize the negative effects on cell components and thus minimize the effectiveness of possible repair mechanisms induced by oxidative stress. Moreover, the bacteria resistant to bactericidal antibiotics may exhibit higher robustness towards ozone since the overlap between the plateau and lag phases for ampicillin resistant *E. coli* and $bla_{\text{TEM-1}}$ resistance gene occurred at higher $\Delta UV_{254}$ than for other targets (> 0.6 g $O_3$/g DOC). Our findings suggest that the ozone doses required for inactivation of tested ARB and ARGs correspond to more than ~35% $\Delta UV_{254}$ or ~0.6 $gO_3$/gDOC. These values are similar to the findings of Wu et al. (2018).

### 4.4. ARGs selection during ozone treatment

A few studies have reported an increase in abundance after ozone treatment for various ARGs (e.g., of *van*A and $bla_{\text{VIM}}$ by Alexander et al., 2016, of *sul*1 and *tet*G by Zhuang et al., 2015) suggesting a possible selection during ozone treatment for bacteria possessing these genes. The selection of bacteria carrying ARGs alongside with the general reduction of bacterial community diversity may lead to a proliferation of ARB in emptied niches in treated wastewater and their further dissemination into receiving environments. Therefore, we monitored changes in relative abundance after ozonation at tested nitrite compensated specific ozone doses to evaluate if (i) selection of specific ARGs occurs, and (ii) if there may be a correlation between ozone dose and selection of specific genes.

Changes in ARGs relative abundance after ozonation varied strongly between the genes. The increase in the relative abundance of tested ARGs after ozone treatment was observed for up to 45% of datapoints (depending on the gene) and was higher for $bla_{\text{TEM-1}}$ and *erm*B than for *sul*1 and *tet*W genes. These observations suggest that factors favouring especially the first two ARGs' bacterial hosts could be of importance. The observed increases and decreases of relative abundance did not significantly correlate with $\Delta UV_{254}$ or suspended solids. Therefore, we further investigated if these changes may be connected with seasonal shifts in bacteria type and abundance resulting from changes in bacterial community structure at WWTPs. Such changes were reported to occur twice a year and repeatedly assemble into seasonal steady states (LaMartina et al., 2021). For genes: *sul*1 and *erm*B, we could observe seasonal shifts in response to ozone treatment: a decrease in relative abundance clustered from May to December and an increase from January to May next year. These shifts correlated with changes in air temperature with a maximum increase in relative abundance occurring around 7 °C and no changes or decrease at extrema: temperature around 0 and 20 °C. Those trends could not be observed for $bla_{\text{TEM-1}}$ and *tet*W genes, which would suggest that these genes are carried by different groups of hosts in the WWTPs bacterial community. According to Yin et al. (2019), the abundance of *sul*1 gene in activated sludge was strongly correlated with α-Proteobacteria and γ-Proteobacteria like *Pseudomonas aeruginosa*, and *erm*B gene was correlated with δ- and γ-Proteobacteria. Beta-lactam and tetracycline resistance genes were linked to Actinobacteria, the former also with *Enterobacteriaceae* and the latter with Firmicutes. Furthermore, seasonal shifts in WWTPs bacterial community were reported to be driven mostly by environment-associated taxa, whereas human-associated taxa seemed to not follow seasonal trends (LaMartina et al., 2021). Thus, since $bla_{\text{TEM-1}}$ and *tet*W genes are rather linked to human-associated taxa (Firmicutes, *Enterobacteriaceae* and other γ-Proteobacteria), the effects of hosts abundance seasonality on changes in their relative abundance after ozone treatment could not be observed, on the contrary to *sul*1 and *erm*B genes.

### 4.5. Multibarrier approach: GAC filter as a post-treatment after ozonation

Activated carbon (AC) treatment has been widely investigated for the removal of chemical CECs from wastewater (Rizzo et al., 2020) and was suggested as a post-treatment after ozonation to minimize potentially formed by-products and maximize the further removal of chemical CECs (Ravasi et al., 2019). The impact of AC application on ARB and ARGs abatement from the effluent is not yet clear with different studies reporting limited removal or even increased abundance after treatment by various adsorption/filtration technologies (Hiller et al., 2019). In our study, the GAC filtration resulted in a further abatement for TMP/SMX *E. coli, sul*1 and *tet*W genes, and a slight decrease or no effects on 16 S rRNA gene, AMP *E. coli*, and total *E. coli* abundance. Czekalski et al. (2016) observed that the application of sand filtration as a post-treatment resulted in the increase of *sul*1 and 16 S rRNA genes abundance after passing the sand filter. In our study, an increase in the both absolute and relative abundance of *erm*B and $bla_{\text{TEM-1}}$ genes could be observed after passing the GAC filter, and it was significantly higher for the latter gene. Lüddeke et al. (2015) also reported that filter passages of ozonated effluent partially caused an increase in the resistance level. The significant increase in $bla_{\text{TEM-1}}$ gene relative abundance follows the previous observation of higher robustness of AMP resistant *E. coli* and this corresponding ARG against ozone. This may suggest that bacteria less susceptible to stress caused by ozone treatment may prevail in the GAC filter and be abundant in the final effluent.

## 5. Conclusions

The multibarrier demonstrator plant combining ozonation and GAC filtration for chemical CECs removal from CAS effluent was monitored and assessed for its suitability for ARB and ARGs abatement during 13 consecutive months of routine operation.

- For total *E. coli*, TMP/SMX resistant *E. coli* and ARGs: *sul*1, *erm*B, and *tet*W, sufficient membrane damage leading to significant DNA impairment or leakage from affected cells was observed at around $\Delta UV_{254}$ of 30% (corresponding to 0.5 g $O_3$/g DOC). For AMP resistant *E. coli* and $bla_{\text{TEM-1}}$ gene, the overlap was observed at a slightly higher $\Delta UV_{254}$ of around 35–40% (corresponding to around 0.7 g $O_3$/g DOC). After Alexander et al. (2016), we hypothesized that for these targets, the lower susceptibility towards ozone may be connected with sub-lethal stress response mechanisms in ARB resistant to bactericidal antibiotics. This hypothesis could be investigated in future studies bringing more insight into the mechanisms of ARB reaction to stress conditions.
- Reaching the ozone dose resulting in DNA damage is vital for maximizing the efficiency of ARB and ARGs abatement by ozone. The results suggest that the ozone doses required for inactivation of tested ARB and ARGs correspond to more than ~35% $\Delta UV_{254}$ or ~0.6 g $O_3$/g DOC, to ensure reaching sufficient DNA damage.
- The increase of ARGs relative abundance after ozonation varied between genes and was higher for $bla_{\text{TEM-1}}$ and *erm*B than for *sul*1 and *tet*W. The changes did not significantly correlate with $\Delta UV_{254}$ or suspended solids. However, for *sul*1 and *erm*B, these changes appeared to be seasonal with a decrease in relative abundance from May to December and an increase from January to May, which we hypothesize to be strongly dependent on major hosts of targeted genes, their origin, and seasonal abundance fluctuations.
- The GAC filtration resulted in a further abatement of TMP/SMX *E. coli, sul*1 and *tet*W genes, and a slight decrease or no effects on the 16 S rRNA gene, AMP *E. coli*, and total *E. coli* abundance. The significant increase in $bla_{\text{TEM-1}}$ gene relative abundance follows the previous observation of higher robustness of AMP resistant *E. coli* and this associated ARG against ozone.

- $\Delta UV_{254}$ is considered a surrogate parameter for chemical CECs abatement and ozone exposure in real wastewater systems. Our observations indicate that correlating ARB and ARGs log removal data with the $\Delta UV_{254}$ gives a better representation of these biological CECs exposure to ozone in wastewater.

## Environmental Implication

The worldwide increase of antibiotic resistance (AR) levels is one of the biggest threats to global health and food security. Wastewater treatment plants (WWTPs) are identified as one of the most important AR propagation routes from humans to the environment. AR is only partially removed in biological wastewater treatment, which indicates a need for advanced treatment. We investigate the removal of AR targets from WWTPs effluent by ozonation followed by granular activated carbon filtration in a long-term pilot-scale study. Our results are of a practical relevance to WWTPs operators, for future legislations, and help to address AR dissemination problems.

## CRediT authorship contribution statement

**K. Slipko**: Conceptualization, Methodology, Formal analysis, Investigation, Software, Writing – original draft, Writing - review & editing, Visualization; **D. Reif**: Conceptualization, Methodology, Investigation, Writing – review & editing; **H. Schaar**: Conceptualization, Methodology, Investigation, Writing – review & editing; **E. Saracevic**: Methodology, Investigation, Resources; **A. Klinger:** Methodology, Investigation; **L. Wallmann**: Methodology, Investigation; **J. Krampe**: Resources, Writing – review & editing, Supervision; **M. Woegerbauer**: Resources, Supervision, Project administration; **P. Hufnagl**: Resources, Supervision; **N. Kreuzinger**: Conceptualization, Resources, Writing – review & Editing, supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

## Acknowledgments and disclaimers

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129396.

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