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Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts

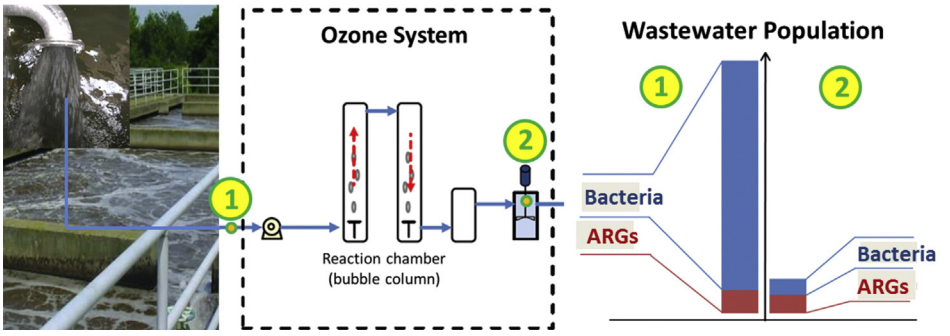
Johannes Alexander [a], Gregor Knopp [b], Andreas Dötsch [a], Arne Wieland [c], Thomas Schwartz [a,*]

[a] Karlsruhe Institute of Technology (KIT) - Campus North, Institute of Functional Interfaces (IFG), Microbiology at Natural and Technical Interfaces Department, P.O. Box 3640, 76021, Karlsruhe, Germany
[b] Technische Universität Darmstadt, Institute IWAR, Wastewater Technology, Franziska-Braun-Straße 7, 64287, Darmstadt, Germany
[c] Xylem Services GmbH, Boschstraße 4 – 14, 32051, Herford, Germany

HIGHLIGHTS

- Ozone treatment selects vancomycin- and imipenem-resistant bacteria.
- Ozone impact depends on bacterial species.
- Strong population shifts in the spectrum of living bacteria.
- Ozone treatment reduces bacterial diversity.
- Ozone treatment selects bacteria with GC-rich genomes.

GRAPHICAL ABSTRACT



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ABSTRACT

An ozone treatment system was investigated to analyze its impact on clinically relevant antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs). A concentration of 0.9 ± 0.1 g ozone per 1 g DOC was used to treat conventional clarified wastewater. PCR, qPCR analyses, Illumina 16S Amplicon Sequencing, and PCR-DGGE revealed diverse patterns of resistances and susceptibilities of opportunistic bacteria and accumulations of some ARGs after ozone treatment. Molecular marker genes for *enterococci* indicated a high susceptibility to ozone. Although they were reduced by almost 99%, they were still present in the bacterial population after ozone treatment. In contrast to this, *Pseudomonas aeruginosa* displayed only minor changes in abundance after ozone treatment. This indicated different mechanisms of microorganisms to cope with the bactericidal effects of ozone. The investigated ARGs demonstrated an even more diverse pattern. After ozone treatment, the erythromycin resistance gene (*ermB*) was reduced by 2 orders of magnitude, but simultaneously, the abundance of two other clinically relevant ARGs increased within the surviving wastewater population (*vanA*, *bla*VIM). PCR-DGGE analysis and 16S-Amplicon-Sequencing confirmed a selection-like process in combination with a substantial diversity loss within the vital wastewater population after ozone treatment. Especially the PCR-DGGE results demonstrated the survival of GC-rich bacteria after ozone treatment.

1. Introduction

The need for additional wastewater treatment processes to reduce contaminations of adjacent water systems is subject of many

* Corresponding author.
E-mail address: thomas.schwartz@kit.edu (T. Schwartz).

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discussions worldwide. The occurrence of newly emerging chemical and microbiological contaminants in the aquatic environment has become an issue of increasing environmental concern. Different wastewater treatment processes were developed to achieve an adequate wastewater quality based on chemical reference values not mentioning the microbiology status. Especially the dissemination of clinically relevant bacteria is of high priority to public health (bathing waters or surface waters containing purified wastewater). Regulations concerning wastewater quality which are currently based on chemical discharges underestimate the risk of a microbiological contamination.

Recent studies found increased abundance of clinically relevant antibiotic resistant bacteria in the sediments near the outlets of wastewater treatment plants (WWTPs) and adjacent downstream aquatic habitats (Schwartz et al., 2003; Czekalski et al., 2012; Rizzo et al., 2013). In addition to secondary and tertiary wastewater treatment processes, which were primarily designed to remove nitrogen, well biodegradable organic compounds, ammonia, nitrate, phosphate, another treatment step is needed to efficiently reduce micropollutants and microbiological contaminants.

Ozonation is an efficient process to remove organic micropollutants and also considered adequate to reduce or inactivate pathogens via in situ production of highly reactive radicals (e.g.Dodd, 2012; Zimmermann et al., 2011; Hollender et al., 2009; Lüddeke et al., 2014; Zhuang et al., 2014). The disinfection mechanism includes the destruction of the bacterial cell walls followed by leakages of cellular constituents out of the cell, damages of nucleic acids (breaking aromatic structure), and disruption of carbon-nitrogen bonds of proteins leading to depolymerization. The efficiency of inactivation depends on the susceptibility of the target organism, contact time, and concentration of the radicals. Concerns about toxic effects of emerging transformation products on aquatic and terrestrial organisms were recently analyzed by Michael et al. (2013); Ternes et al. (2015), and Funke et al. (2015). Recent studies indicated a removal capacity of only two ARGs (*sul*1, *tet*G) with 1.68 and 2.55 log at elevated ozone concentrations of 177.6 mg L^{-1} (Zhuang et al., 2014). However, selection of only two widespread antibiotic resistance genes is not adequate to discuss the hygienic relevance of the results. Furthermore, the applied ozone concentrations were much higher compared to those of European wastewater plant systems. In addition, a culture-based study showed that antibiotic resistant strains of *Escherichia coli*, enterococci, and staphylococci are more likely to survive ozonation, in comparison to their respective antibiotic sensitive versions (Lüddeke et al., 2014). Although this study was conducted under real conditions at a German wastewater treatment plant with adequate ozone concentrations, knowledge about the impacts of ozonation on ARBs and ARGs is limited to cultivable bacteria not mentioning the whole wastewater community. A number of studies only covered the impacts of ozone on cultivable indicator bacteria like *E. coli*, fecal coliform bacteria or fecal streptococci in reals systems or on the pilot scale without taking into account antibiotic resistances (Mezzanotte et al., 2007; Blatchley et al., 2012; Ostoich et al., 2013). Hence, consideration of whole community is very important to assess the load of the aquatic system with clinically relevant ARGs, since a number of ARGs are located on mobile genetic elements, which can pass the species or genus barriers. This horizontal gene transfer contributes to the increasing widespread of antibiotic resistant bacteria in clinics as well as in aquatic environment (Bellanger et al., 2014; Bennett, 2008)

Therefore, the present study covers more opportunistic bacteria (enterococci, *Pseudomonas aeruginosa*, staphylococci, and enterobacteria) together with their clinically relevant ARGs (*van*A, *bla*VIM, *erm*B, *amp*C) in extracted DNA from the whole communities before and after ozone treatment as well as subsequent aerated and non-aerated biofilters and granular active carbon filters. Molecular biology methods were used to quantify the target genes in total DNA from the whole bacterial community, which includes the detection of cultivable and also non-cultivable ARG carriers as well as possible horizontal gene transfer of ARGs via mobile genetic elements. To evaluate the impacts of ozone treatment on the whole bacterial wastewater communities methods were applied to discriminate between living and inactivated bacteria prior to PCR quantification. In addition to the quantification of taxonomic and antibiotic-resistance specific marker genes a whole community analyses (PCR-DGGE and Illumina 16S Amplicon Sequencing) were performed to study selective impacts of ozonation. As a consequence, this study is of far more comprehensive character, the objective being to evaluate the antibiotic resistance situation in natural wastewater populations after ozonation.

2. Experiments

Over two years, 48 24h-composite samples of wastewater were analyzed to determine the ARGs and opportunistic bacteria before and after ozone treatment. Samples were taken once every two weeks and were processed immediately for DNA extraction.

2.1. Sampling Locations

Different sampling points were chosen to investigate the impact of ozone treatment on the abundance of ARGs and opportunistic bacteria. An ozone treatment system was used to process the final wastewater of the local WWTP (population equivalents 43,000; average sewage quantity 6,400 m^3 per day; pH 7.25; COD 21 – 25 mg L^{-1}; NH_4—N 0.14 mg L^{-1}) (Fig. 1). A microstrainer (pore size 10 μm) was installed upstream of the ozone system to reduce the particulate matter for ozone application. The ozone system (WEDECO, type GSO) comprises two bubble columns operating in parallel flow. The ozone was injected in reverse flow by a ceramic-diffusor into the first bubble column. A bubble column is used as reaction chamber. The hydraulic retention time was 18 ± 2 min depending on the flow rate. Ozone concentration was adjusted to 0.9 ± 0.1 g ozone per 1 g DOC according to the dissolved organic carbon (DOC, average 10.0 ± 2.3 mg L^{-1}) present in the wastewater. This ozone concentration was specified by the operation company for further reduction of the organic load of treated wastewater. More than 90% of the contaminants that are marginally affected by conventional wastewater treatment are oxidized by ozonation doses between 0.8 and 1.5 g O_3 per g DOC (e.g., diclofenac, carbamazepine, metoprolol) (Prasse et al., 2015; Hollender et al., 2009; Huber et al., 2005; Ternes et al., 2015). Wastewater was sampled downstream of the microstrainer to determine the ARGs and opportunistic bacteria abundance before entering the ozone system, the ozone outlet, and the outlet of the connected four parallel filter systems (aerated and non-aerated biofilter/granulated active carbon (GAC) filter, filtration rate both 4-5 m h^{-1}, empty bed contact time of 25-30 min) (Fig. 1). In case of biofilters expanded clay was used. The filter unit sizes were 4 meters high with a diameter of 19 cm. The filling volumes were 0.113 m^3 each.

2.2. Sample Preparation for Molecular Biology Analyses

Wastewater samples were filtered using polycarbonate membranes with a diameter of 47 mm and a pore size of 0.2 μm (Whatman Nuclepore Track-Etched Membranes, Sigma-Aldrich, Munich, Germany). Up to 300 mL of conventional and ozone-treated wastewater were filtered for biomass separation. DNA extraction was performed using the Fast DNA spin kit for soil (MP Biomedical, Illkrich, France) utilizing the lysing matrix E and the manufacturer's protocol for wastewater. The quantities and purities of the DNA extracts were measured by means of the NanoDrop ND-1000 instrument (Peqlab Biotechnology, Erlangen, Germany).