Sensitivity of antibiotic resistant and antibiotic susceptible *Escherichia coli*, *Enterococcus* and *Staphylococcus* strains against ozone

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ABSTRACT

Tolerance of antibiotic susceptible and antibiotic resistant *Escherichia coli*, *Enterococcus* and *Staphylococcus* strains from clinical and wastewater samples against ozone was tested to investigate if ozone, a strong oxidant applied for advanced wastewater treatment, will affect the release of antibiotic resistant bacteria into the aquatic environment. For this purpose, the resistance pattern against antibiotics of the mentioned isolates and their survival after exposure to 4 mg/L ozone was determined. Antibiotic resistance (AR) of the isolates was not correlating with higher tolerance against ozone. Except for ampicillin resistant *E. coli* strains, which showed a trend towards increased resistance, *E. coli* strains that were also resistant against cotrimoxazol, ciprofloxacin or a combination of the three antibiotics were similarly or less resistant against ozone than antibiotic sensitive strains. Pigment-producing *Enterococcus casseliflavus* and *Staphylococcus aureus* seemed to be more resistant against ozone than non-pigmented species of these genera. Furthermore, aggregation or biofilm formation apparently protected bacteria in subsurface layers from inactivation by ozone. The relatively large variance of tolerance against ozone may indicate that resistance to ozone inactivation most probably depends on several factors, where AR, if at all, does not play a major role.

Key words | advanced wastewater treatment, antibiotic resistance, extended-spectrum-ß-lactamase (ESBL), methicillin resistant *S. aureus* (MRSA), ozone, VRE

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INTRODUCTION

Surface water sources very often serve as drinking water reservoirs or as recreation facilities and therefore their water quality is of fundamental interest. This is manifested in WHO or EU guidelines, for instance in the European Union Water Framework Directive (EC 2000), which sets standards for acute and chronic toxicity in water for aquatic organisms to protect biological diversity and human health. To achieve these aims, improvements to sewage purification by ultrafiltration or ozonation as additional treatment stages in sewage treatment plants (STPs) are discussed and tested. The main goal of these additional wastewater treatment technologies is a reduction of the concentration of emerging contaminants in the effluent of STPs and ultimately in the receiving water bodies.

A positive side effect of, for example, ozonation is the inactivation of facultative pathogenic bacteria such as *Escherichia coli*, *Enterococcus* or *Staphylococcus* strains in effluents of STPs by several log units (Xu *et al.* 2002; Gehr *et al.* 2003; Lüddeke *et al.* 2015). The efficiency of ozone as a disinfectant depends on several parameters such as reaction rates of ozone or its decay products with compounds of sewage sludge, including bacteria (Gehr *et al.* 2003).

In batch assays Gehr *et al.* (2003) demonstrated that ozone decayed rapidly and formed hydroxyl radicals for non-selective oxidation, if the alkalinity was low and/or the organic concentration was high. Hunt & Mariñas (1999) reported that molecular ozone was the agent responsible for the inactivation of *E. coli*. To date, the exact mode

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of action and the specific target structures of ozone and of its decay products in microbial cells are not completely understood: ozone is expected to react rapidly with the unsaturated bonds of phospholipids and lipopolysaccharides in membranes and cell walls, but intracellular amino acids and proteins may also be affected (Dodd 2012). In addition DNA damages may also be caused by ozone (Gehr *et al.* 2003).

For advanced sewage treatment ozone is applied after secondary treatment either with a diffuser at the bottom of the tank or with a pump-injector system, injecting small gas bubbles into the influent water of the ozone reactor. Ozone concentrations between 2 and 10 mg/L, depending on the dissolved organic carbon (DOC) concentration, are used for final treatment of sewage. The minimally required contact time of ozone with sewage in STPs for a maximal oxidation is difficult to determine. Investigations in the STPs Bad Sassendorf, Duisburg-Vierlinden and Schwerte (Northrhine-Westphalia, Germany) indicated that the reactions triggered by ozone occurred within less than 30 minutes (Kommunalunternehmen der Stadt Warburg 2013).

In his recent review, Dodd (2012) raised the question whether antibiotic resistant bacteria have an advantage to cope with oxidative stressors such as ozone in comparison to bacteria that are not resistant against antibiotics. We addressed this problem and investigated if the treatment of conventionally purified sewage with ozone would favor a better survival of antibiotic resistant bacteria than of antibiotic sensitive bacteria. For this purpose, the sensitivity of antibiotic resistant and antibiotic susceptible *E. coli*, *Enterococcus* and *Staphylococcus* isolates from clinical samples, sewage and river water against ozone was determined for the first time in detail. Survival of antibiotic susceptible and antibiotic resistant bacterial strains was compared with data obtained from an ozonation unit in an STP.

MATERIALS AND METHODS

Laboratory assay simulating the ozonation of the STP in Eriskirch

In the pilot-scale ozonation unit (OCS system supplied by Wedeco, Herford, Germany) of the STP of Eriskirch (Federal State of Baden-Württemberg, Southern Germany), 34 m^3 secondary-treated sewage with a mean DOC of 5.5 mg/L were separated per day and dosed with 4 mg/L ozone that was injected by a venturi injector (Xylem Water Solutions GmbH, Großostheim, Germany). After the ozone dosage, the sewage was pumped into a closed tank (volume: 0.5 m^3). The contact time of ozone with sewage was 20 minutes, long enough to decompose organic material until ozone exhaustion (Tripathi *et al.* 2011).

Ozonation experiments in the laboratory were performed with 100 mL aqueous samples in 250 mL glass reactors, equipped with a diffuser and sterile 0.2 μm PTFE filters (Roth, Karlsruhe, Germany) at the inlet and outlet. Since the pH in the sewage-simulating, 20-fold diluted OECD-medium (Organization for Economic Cooperation and Development) (final DOC of 5.5 mg/L as in treated sewage) dropped below 5.5 after ozonation a phosphate-buffered medium (3.87 g/L K_2HPO_4, 2.42 g/L KH_2PO_4, 0.0138 g/L $C_6H_{12}O_6$, 9 g/L NaCl dissolved in deionized water, pH 7.0, autoclaved for 15 minutes) with 5.5 mg DOC per L and a pH of 7 after ozonation was used instead. Compared to typical wastewater, the phosphate-buffered medium used did not contain carbonates which might serve as radical scavengers. An ozone-generator (Laborozonisator 301.7, Sander, Uetze-Eltze, Germany) was connected via Norprene tubings with the inlet filter unit of the glass reactor. Ozone was generated from hydrocarbon-free compressed air for 3 minutes and introduced via the diffuser into the glass reactor. A concentration of 4 mg O_3/L was reached after 3 minutes. Then, 0.5 mL of an overnight culture of the respective strain, grown in DEV-nutrient broth, was inoculated. The used cell-concentrations were clearly higher than in secondary-treated sewage (e.g. Lüddeke *et al.* 2015) to be able to quantitatively detect surviving cells.

After 0.5, 1, 2, 5, 20 and 40 minutes of exposure to ozone, surviving cell numbers were determined. From *E. coli* cultures 10^{-2}, 10^{-3} and 10^{-4} fold dilutions and from *Enterococcus* and *Staphylococcus* cultures 10^{-1}, 10^{-2} and 10^{-3} fold dilutions were prepared and platted twice on DEV-nutrient agar (Roth, Karlsruhe, catalog number: CL02.1). To determine original cell densities in overnight cultures 100 μL of the dilution steps 10^{-6} and 10^{-7} were plated on DEV-nutrient agar (according to Deutsches Einheitsverfahren). After 20 hours of incubation at 37 °C

colonies were counted and inactivation with exposure time to ozone was calculated. The reproducibility of these assays was about +/− 0.3 log units.

Ozone decay in the phosphate-buffered medium was determined using potassium indigo trisulfonate according to DIN 38408-3 (DIN, Deutsche Industrie Norm 2011-04). Ozone concentrations were calculated based on decolorization of potassium indigo trisulfonate solution: an absorbance calibration curve at 600 nm for the phosphate-buffered medium plus different ozone concentrations was prepared. The half-life time of ozone in the medium was 6 minutes and 4 seconds (Figure 1). Preliminary experiments revealed that ozone inactivated bacteria within less than 30 seconds. Between 5 (mean ozone concentration: 2.96 mg/L) and 20 minutes after inoculation (mean ozone concentration: 1.54 mg/L) no further inactivation of bacteria was seen, although ozone was not yet depleted (Figure 1). In control assays with an *E. coli*, an *Enterococcus* and a *Staphylococcus* strain in the phosphate-buffered medium gassing with air instead of ozone did not change numbers of living cells during incubation (data not shown). For laboratory assays the contact time could thus be restricted to 5 minutes, compared to 20 minutes contact time of sewage with ozone in the pilot-scale ozonation plant in Eriskirch.

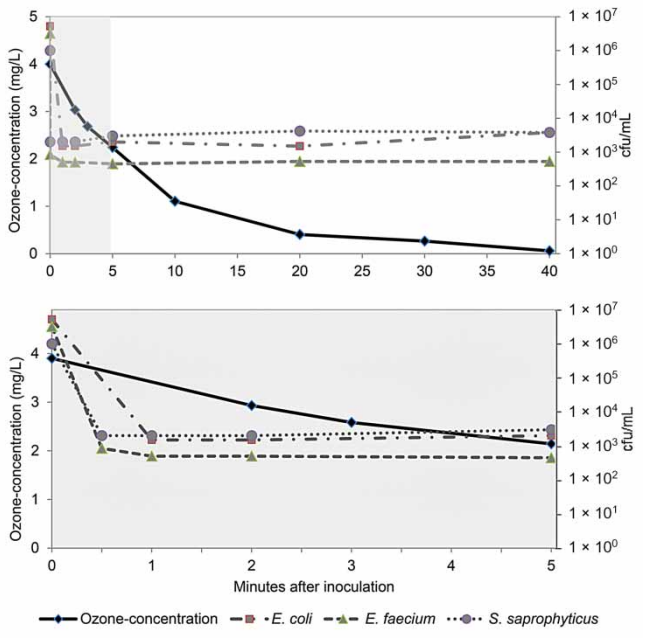

Figure 1 | Decrease of the ozone concentration and of colony forming units in an *E. coli*, *E. faecium* and *S. saprophyticus* culture in the presence of initially 4 mg/L ozone. Mean of four analyses of ozone and of two analyses of bacterial densities for each sample.

Identification and antibiotic susceptibility testing of isolated strains

The tested 52 *E. coli*, 89 *Enterococcus* and 80 *Staphylococcus* strains were isolated within the SchussenAktiv*plus* project during 2012 and 2014 from sewage and river water. This project, which was funded by the Federal Ministry for Education and Research (BMBF, Bonn), focused on the efficiency of advanced sewage treatment technologies to reduce the release of micropollutants and of facultative pathogenic and antibiotic resistant bacteria (for details see Triebskorn *et al.* 2013; Heß & Gallert 2014; www.schussenaktivplus.de). Strains that probably have already survived ozonation (e.g. isolated from the effluent of the STP of Eriskirch) were excluded from this study. Depending on the contamination level of the respective samples, direct plating or the filtration method was used to obtain *E. coli*, *Enterococcus* and *Staphylococcus* isolates.

For cultivation of *E. coli* isolates, ECD-agar (Merck Millipore, Darmstadt, Germany) and ESBL CHROMagar™ (MAST Diagnostica GmbH, Reinfeld, Germany) were used, following the manufacturer's instructions. On ECD-agar, blue fluorescent and indole positive colonies (red-colored after reaction with 10 µL Kovac's reagent) were identified by polymerase chain reaction (PCR) targeting of a specific *tuf*-gene fragment (Maheux *et al.* 2009). Briefly, the DNA-amplification was performed in a total volume of 25 µL containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA, USA), 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µL of template DNA (extraction with phenol/chloroform).

For cultivation of *Enterococcus* isolates bile-esculin-azide agar (Roth) and VRE CHROMagar™ (MAST Diagnostica GmbH), supplemented with 0.15 g/L sodium azide were used, following the manufacturer's instructions. Black colonies with a halo on bile-esculin-azide agar and pink colonies on VRE CHROMagar™ were identified on species level based on their biochemical properties with Micronaut-Strep2®-microtiter plates according to the manufacturer's instructions (MERLIN, Gesellschaft für Mikrobiologische Diagnostika mbH, Bornheim, Germany).

For cultivation of *Staphylococcus* isolates Chapman-Stone agar, containing 0.05 g/L sodium azide, was used. Colonies grown after incubation for 48 hours at 37 °C were streaked on Mannitol-Salt agar before they were identified by their physiological reactions on Micronaut-Staph®-microtiter plates (MERLIN). *S. aureus* and *S. saprophyticus* isolates from clinical specimen were obtained from Dr A. Becker, Städtisches Klinikum Karlsruhe.

Antibiotic resistance (AR) of the *E. coli* isolates against ampicillin (10 µg), ciprofloxacin (5 µg), cotrimoxazol (1.25 µg trimethoprim/23.75 µg sulfamethoxazol; SXT) was tested using the agar diffusion test according to Deutsche Industrie Norm (DIN 58940, 2011). For testing AR against cefotaxim (5 µg) clinical breakpoints according to EUCAST (2011) were applied. All ampicillin resistant isolates were additionally tested against ceftazidim (30 µg) and cefpodoxim (10 µg) according to Clinical and Laboratory Standards Institute (CLSI 2011); the inhibitory effect of clavulanic acid on ß-lactamase was checked as described by Bradford (2001). According to the definition of Robert-Koch-Institut (2007), extended-spectrum-ß-lactamase (ESBL) producers phenotypically were resistant against cefpodoxim as well as against ceftazidim and/or cefotaxim. All isolates that were resistant against the above-mentioned antibiotics were classified as ESBL producers.

AR of the *Enterococcus* isolates was tested against ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg) and erythromycin (15 µg) according to DIN 58940 (2011). The susceptibility of *E. faecium* and *E. faecalis* isolates against vancomycin (VRE) was tested according to CLSI (2011). Vancomycin resistance (VR) was confirmed by the presence of *van*A-E and *van*G genes using primers and PCR conditions previously described by Depardieu *et al.* (2004). The DNA-amplification was performed in a 25 µL assay containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µL of template DNA (extraction with phenol/chloroform). Intrinsic low-level vancomycin resistances of *E. gallinarum* and *E. casseliflavus* (*van*C1- and *van*C2-type) were not considered.

Susceptibility of the *Staphylococcus* isolates against oxacillin (5 µg), ciprofloxacin (5 µg) and erythromycin (15 µg) was tested by the disc-diffusion test with Mueller-Hinton agar according to DIN 58940 (2011) and against clindamycin (2 µg) according to CLSI (2011). Oxacillin resistance was confirmed by the presence of the *mec*A gene using previously described primers and PCR conditions (Predari *et al.* 1991). DNA-amplification was performed in a 25 µL assay containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 µM of both primers and 0.5 µL of template DNA (extraction with phenol/chloroform).

Pigment extraction and quantification

Cells of an overnight culture of the respective *S. aureus* strains, grown in DEV-nutrient broth, were pelleted by centrifugation. The cells were re-suspended in a 0.9% sodium chloride solution to obtain a turbidity of McFarland 2.0. Two mL of the suspension were used to extract staphyloxanthin and to quantify pigment production as described by Morikawa *et al.* (2001).

Statistical analysis

For descriptive analysis of the obtained data, box plots created with Microsoft Excel were used displaying variation within the respective 'clusters': the line dividing the rectangle built by the first and third quartile displays the median of the obtained data. Minimal and maximal values are indicated by the whiskers.

RESULTS AND DISCUSSION

Exposure time for inactivation of bacteria by ozone

A Gram-negative *E. coli* strain, a Gram-positive *Enterococcus* isolate and a Gram-positive *Staphylococcus* isolate were exposed to 4 mg/L ozone. Inactivation of cells by more than 3 log units occurred within the first 30 seconds contact time from $1–4 \times 10^6$ initially to $5 \times 10^2–5 \times 10^3$ bacteria per mL (Figure 1). No further inactivation occurred later on, although even after 5 minutes more than 2 mg/L ozone was still present, an ozone concentration that was higher than the initial concentration in some pilot-scale ozonation systems. A consistently higher ozone concentration and a longer exposition time might presumably result in a higher inactivation rate. The phenomenon of a rapid initial

inactivation of bacteria by ozone, however, seemed to be independent of the applied ozone concentration since Xu *et al.* (2002) also reported no difference in inactivation of fecal coliforms between 2 and 10 minutes hydraulic retention time for a given ozone dose. Differences in cell wall and membrane architecture between Gram-positive and Gram-negative bacteria seemed not to be the decisive structures that were responsible for survival in the presence of ozone.

Inactivation of *E. coli* by ozone

A relatively large variance of antibiotic susceptible and antibiotic resistant *E. coli* strains was observed for inactivation by ozone (Figure 2). Within the same species some strains were less susceptible against ozone than others (variation of the inactivation of living cells between 0.3 and 4.1 log units), independent of whether the strains carried AR genes or not (Figure 2): the median inactivation (2.8 log units) of the tested antibiotic resistant isolates was in the same order of that of antibiotic sensitive strains. Looking in more detail, the median of the cluster 'ampicillin resistant *E. coli*' was the lowest within all antibiotic resistant *E. coli* strains, indicating a lower susceptibility against ozone of ampicillin resistant isolates as compared to strains which were not at all resistant against the tested antibiotics or were resistant against at least one more antibiotic-class (Figure 2). The same trend was also observed in the pilot-scale ozonation plant in Eriskirch: the percentage of ampicillin resistant *E. coli* isolates increased during ozonation

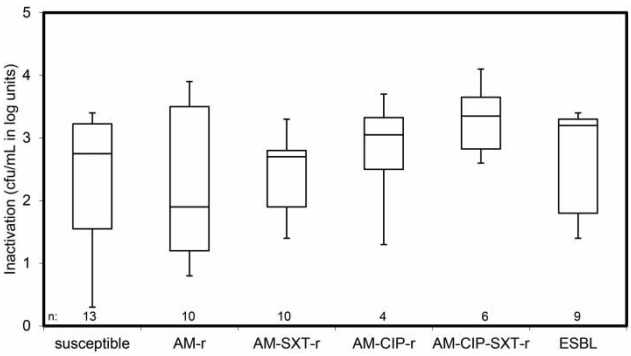

Figure 2 | Inactivation of 13 antibiotic susceptible and 39 antibiotic resistant *E. coli* strains that were isolated from sewage and river water samples after inactivation by 4 mg/L ozone for 5 minutes. Number of tested isolates, n; resistant strains against ampicillin (AM-r), cotrimoxazol (SXT-r), ciprofloxacin (CIP-r) and extended-spectrum-ß-lactamase producers (ESBL).

about 8.9% (influent: 10.7%, effluent: 19.6%, as noted by Lüddeke *et al.* 2015).

In the STP of Eriskirch, the percentage of ampicillin resistant isolates, which were additionally resistant against at least one more class of antibiotics, decreased from 8.3 to 3.8% after ozonation, as noted by Lüddeke *et al.* (2015). This was in line with the laboratory approach, where isolates that, in addition to ampicillin, were resistant against cotrimoxazol, ciprofloxacin or cefotaxim (ESBL) apparently were more sensitive against ozone, manifested in higher median values for inactivation of living cells (2.7–3.4 log units compared to 1.9 log units of only ampicillin resistant isolates; Figure 2). Händel *et al.* (2013) demonstrated that *E. coli* cells apparently compensated 'metabolic costs' for AR by physiological adaption. Comparing antibiotic susceptible and antibiotic resistant strains, changes in gene expression levels, mainly of genes for cell wall maintenance, DNA metabolic processes, cellular stress and respiration as well as for the electron transport, were observed. Overall the acquisition of AR primarily seemed not to require much extra energy, but seemed to cause a reduced ecological versatility (Händel *et al.* 2013). Nevertheless there were apparently differences between mechanisms for ARs: on average, the ampicillin-only resistant *E. coli* strains were less susceptible against ozone than *E. coli* strains that were additionally resistant against ciprofloxacin and cefotaxim, as judged from results of laboratory assays (Figure 2) and of pilot plant operation in Eriskirch. The tested ampicillin-only resistant *E. coli* isolates showed a very broad range of susceptibility against ozone. The inactivation of living cells varied between 0.8 and 3.9 log units (Figure 2). The reason could be that several mechanisms for resistance against ß-lactam-antibiotics are expressed, that are more or less energy intensive, e.g. overexpression of the intrinsic *amp*C-gene or the expression of one of the *bla*-genes (e.g. TEM, SHV, CTX-M; Robert Koch Institut 2007).

Inactivation of *Enterococcus* isolates by ozone

Inactivation of *Enterococcus* isolates by ozone ranged from 0.2 to 5 log units (Figure 3). Although there was a broad range of susceptibility within the respective 'clusters' of different species, the median of inactivation of *E. casseliflavus*

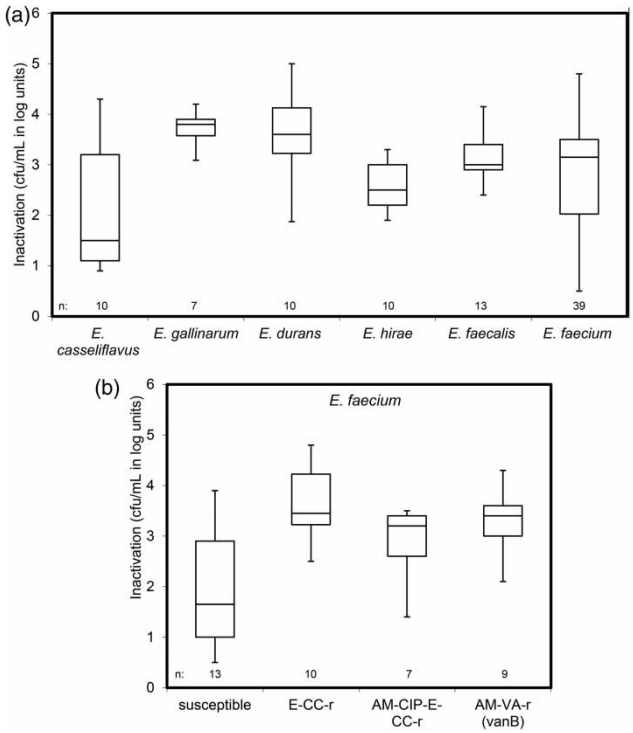

Figure 3 | Inactivation of 89 *Enterococcus* isolates belonging to (a) six *Enterococcus* species and (b) 39 *E. faecium* isolates (split into susceptible and resistant against the mentioned antibiotics) by 4 mg/L ozone. Number of tested isolates, n; strains resistant against: erythromycin (E-r), clindamycin (CC-r), ampicillin (AM-r), ciprofloxacin (CIP-r), vancomycin (VA-r). All *E. casseliflavus*, *E. gallinarum*, *E. durans*, *E. hirae* and *E. faecalis* strains were susceptible against the tested antibiotics.

(1.5 log units; Figure 3) by ozone was significantly lower than for *E. gallinarum* (3.8 log units), *E. durans* (3.6 log units), *E. hirae* (2.5 log units), *E. faecalis* (3.0 log units) or *E. faecium* (3.2 log units; Figure 3). A typical feature of the species *E. casseliflavus* is the production of a yellow pigment leading to intensively yellow colored colonies on agar plates. Pigment formation is a well-known strategy for protection against oxidative stress by many Gram-positive bacteria. Investigations of Clauditz *et al.* (2006) with *S. aureus* confirmed that staphyloxanthin, a carotenoid produced by this *Staphylococcus* species, plays a role for fitness and the ability to cope with oxidative stress. Possibly, the pigment itself or intracellular processes coupled with its synthesis are the factors that protect cells against damaging effects of ozone and its decay products.

Nevertheless, concerning the inactivation of *E. casseliflavus* strains by ozone, which varies over 0.9–4.3 log units, pigment synthesis itself could not explain the broad range of susceptibility against ozone within the strains of this species. In the effluent of the STP Eriskirch the percentage of isolates that were identified as *E. casseliflavus* was higher after ozonation (18.4%) than before ozonation (12.3%; as noted by Lüddeke *et al.* 2015), indicating a higher 'resistance' of the respective species against ozone. A notable reduction of 15.8% of *E. faecium* strains in the effluent of the STP Eriskirch after ozonation (as noted by Lüddeke *et al.* 2015) was in accordance with results of the laboratory experiments (Figure 3). Lüddeke *et al.* (2015) assumed that the recorded shift might explain the drastic decrease of antibiotic resistant enterococci of about 25.4% after ozonation.

The results of our laboratory experiments supported this hypothesis since the median for inactivation of active cells of antibiotic resistant *E. faecium* strains was higher (3.4–4.2 log units; Figure 3(b)) compared to that of susceptible strains (1.7 log units; Figure 3(b)). Thus, antibiotic resistant strains may reveal a higher sensitivity to ozone. Luczkiewicz *et al.* (2011) also reported an increased percentage of erythromycin, ciprofloxacin and chloramphenicol susceptible *Enterococcus* strains after ozonation. They also observed a decreased percentage of isolates that were identified as *E. faecium* and *E. faecalis* (10.2% and 25.2% decrease, respectively), mainly in favor of *E. hirae* (36.3% increase; Luczkiewicz *et al.* 2011). Händel *et al.* (2013) reported that *E. faecium* strains with ampicillin resistance invested the same maintenance energy as antibiotic sensitive *E. faecium* strains, whereas vancomycin resistant strains required extra energy. High-level vancomycin resistance (e.g. encoded by *van*B), associated with a metabolic burden, might result in a higher sensitivity against ozone compared to vancomycin susceptible strains in the absence of the antibiotic (Figure 3(b)). *E. casseliflavus* and *E. gallinarum* strains intrinsically are low-level vancomycin resistant (*van*C1- and *van*C2-type). Comparing ozone susceptibility of isolates of the two *Enterococcus* species in laboratory assays, *E. casseliflavus* strains (median inactivation of living cells 1.5 log units; Figure 3(a)) were more resistant against ozone than *E. gallinarum* strains (most sensitive *Enterococcus* species: median inactivation of living cells 3.8 log units; Figure 3). Therefore, low-level vancomycin resistance seemed not to be 'the' determining factor for ozone-susceptibility. Pigmentation of *E. casseliflavus* might be one possibility to explain this difference.

Inactivation of *Staphylococcus* isolates by ozone

S. aureus isolates were less susceptible against ozone (median inactivation of active cells 2.1 log units) than, for example, *S. saprophyticus* (3.2 log units), *S. sciuri* (3.1 log units) or *S. xylosus* (2.7 log units; Figure 4(a)). A possible explanation for this finding may be staphyloxanthin synthesis of *S. aureus* as a protecting substance. It is known that the orange-red triterpenoid carotenoid can function as an antioxidant and seems to play a role in the protection of *S. aureus* against oxidative stress (Clauditz *et al.* 2006). Clauditz *et al.* (2006) showed that staphyloxanthin, located in the cell membrane, scavenges free radicals with its conjugated double bonds and thereby probably protects primarily lipids. It might, however, also be involved in protecting proteins and DNA.

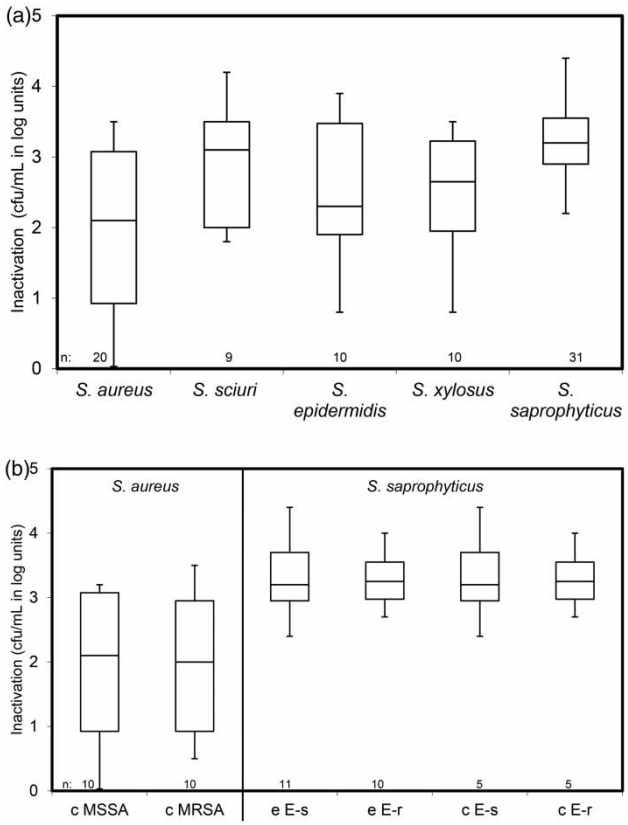

Figure 4 | Inactivation of 80 *Staphylococcus* isolates from environmental and clinical sources belonging to (a) five different species and (b) 20 *S. aureus* and 31 *S. saprophyticus* isolates by 4 mg/L ozone. The *S. aureus* strains were split in MSSA and MRSA, the *S. saprophyticus* strains in erythromycin sensitive (E-s) and erythromycin resistant (E-r). Number of tested isolates, n; isolates from the environmental samples, e; clinical strains. (c) All *S. sciuri*, *S. epidermidis* and *S. xylosus* strains were susceptible against the tested antibiotics.

Staphyloxanthin might also play a role in the defense against reactive oxygen species, whereas enzymes such as catalase and superoxide dismutase most likely contribute to a larger extent to the survival of cells during stress (Clauditz *et al.* 2006). Our results provide evidence for the assumption that pigments might confer an additional protection for *S. aureus*, as the tested non-pigmented isolates, identified as *S. sciuri*, *S. epidermidis*, *S. xylosus* and *S. saprophyticus*, were more sensitive against ozone (Figure 4(a)). Highest tolerance against ozone coincided with a high content of carotenoids in *S. aureus* colonies but the level of resistance against ozone and the amount of staphyloxanthin were not always in line (data not shown). This indicated that there must also be other factors responsible for resistance against ozone. The possession and expression of the *mecA* gene seemed to have no consequence for the susceptibility against ozone as the median inactivation of MRSA (methicillin resistant *S. aureus*) and MSSA (methicillin sensitive *S. aureus*) was identical (Figure 4(b)).

The median inactivation of active cells of *S. epidermidis* isolates (2.3 log units) was only slightly higher compared to that of *S. aureus* (2.1 log units, Figure 4(a)). *S. epidermidis* is a well-known biofilm-forming species (e.g. Cerca *et al.* 2005) and cells of an overnight culture already aggregated and formed visible flocs (data not shown).

Although cell flocs in the *S. epidermidis* culture could be disaggregated to single cells by high shear forces, extracellular polymeric substances (EPS) may have surrounded single cells and thus have protected cell walls or cell membranes from oxidation by ozone. The clearly higher resistance against ozone of cells embedded in biofilms has already been described and attributed to an EPS matrix (e.g. Hems *et al.* 2005). The same observation as for *S. epidermidis* was made with aggregating strains of *S. xylosus*, resulting in a median inactivation by ozone of 2.7 log units, whereas isolates of *S. sciuri* and *S. saprophyticus*, both of which neither produced pigment nor grew in aggregates were less resistant to ozone (Figure 4(a)).

Regarding the inactivation of *S. saprophyticus* isolates of clinical and environmental samples by 4 mg/L ozone, there were no significant differences with respect to their origin (Figure 4(b)). The median for inactivation, independently of origin or AR, e.g. against erythromycin was almost identical (Figure 4(b)).

Taking the results of *E. coli* strains, *Enterococcus* and *Staphylococcus* isolates from hospital sources, sewage and river water as a whole, then single isolates, that were resistant against at least one of the tested antibiotics, were not per se more resistant against oxidative stress by 4 mg/L ozone than antibiotic sensitive strains. Some species seemed to be a little more ozone-resistant than others and thus might be 'positively selected' by ozonation with the consequence that the percentage of such strains increases. Pigment and biofilm production may be factors affecting sensitivity against ozone but further tests particularly focusing on these aspects are necessary to estimate their influence on ozone sensitivity.

CONCLUSIONS

From this study we have established that AR per se did not lead to a reduced sensitivity of *E. coli*, *Enterococcus* and *Staphylococcus* strains against 4 mg/L ozone. Also, pigment-producing *E. casseliflavus* and *S. aureus* were a little less sensitive against ozone compared to non-pigmented species of the respective genera.

Within the same species, the susceptibility against ozone expressed as inactivation of active cells differed over a wide range up to 3.8 log units. And finally, cell wall and membrane architecture seemed not to be 'the' decisive structures that were responsible for survival in the presence of ozone. Several other factors may influence resistance against ozone within species or genera, including the ability to form aggregates or biofilms.

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