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DOI
10.2166/washdev.2025.094

Publication date
2025

Document Version
Final published version

Published in
Journal of Water, Sanitation and Hygiene for Development

Journal of
Water, Sanitation & Hygiene for Development

IWA PUBLISHING

 Journal of Water, Sanitation and Hygiene for Development Vol 00 No 0, 1 doi: 10.2166/washdev.2025.094

Research Paper

Antibiotic-resistant bacteria mirror the behaviour of faecal indicators during municipal wastewater treatment and discharge

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ABSTRACT

Classic faecal indicators, *Escherichia coli* and intestinal enterococci, were investigated as prospective proxies for presence of their resistant strains Extended Spectrum Betalactamase-producing (ESBL)-*E. coli* and Vancomycin Resistant Enterococci (VRE). These organisms are of global public health concern, and their tracing in water treatment systems is not yet standard practice. In this study, no significant difference was observed in the behaviour of the resistant bacteria and their sensitive counterparts during activated sludge treatment, chlorination, electrocoagulation and natural decay. Activated sludge treatment provided a 2.23 $\pm$ 0.13 log reduction value (LRV) for antibiotic resistant and sensitive bacteria alike. Disinfection by both free chlorine and electrocoagulation was slightly more effective against *E. coli* and ESBL-*E. coli* than against enterococci and VRE, though no significant difference was observed between the resistant bacteria and their sensitive counterparts. Decay experiments at 4, 13 and 24 °C showed a biphasic behaviour, with no relevant difference in decay between either of the indicators. It is therefore concluded that antibiotic-resistant ESBL-*E. coli* and VRE mirror the behaviour of faecal indicators *E. coli* and enterococci, experiencing the same rates of disinfection/decay, and maintaining similar ratios between sensitive and resistant populations before and after treatment.

Key words: antibiotic-resistant bacteria, disinfection, faecal indicators, wastewater treatment

HIGHLIGHTS

- Sensitive and resistant bacteria exist in stable ratios in sewage and treated effluents.
- Resistant bacteria have no competitive advantage during disinfection.
- Sensitive bacteria are a good proxy for antibiotic-resistant bacteria removal.
- Use of *E. coli* and enterococci, whose detection and quantification are simple, inexpensive, and low-tech, are valuable indicators for estimating disinfection of ESBL-*E. coli* and VRE.

1. INTRODUCTION

The World Health Organization highlighted in 2015 the dimension of the antibiotic resistance (AR) menace, which 'threatens the very core of modern medicine', as few viable replacement drugs are being developed (WHO 2015). A more recent global survey conducted by the World Health Organization in 2021 concluded that in 2019 alone, approximately 4.95 million people died due to AR-related complications, of which 1.27 million were a direct consequence of the antibiotic-resistant bacteria (ARB) infection, surpassing the 2019 death toll of HIV/AIDS and malaria combined. The survey indicates that the highest mortality rates occur in Sub-Saharan Africa and Southern Asia.

AR is the result of a process by which bacteria acquire resistance against specific antibiotics. Although commonly associated with clinical infections caused by pathogenic organisms, the term ARB does not limit itself to pathogens, as it can be observed in a wide range of bacteria, both human (or animal)-related and environmental. Several studies have traced the

origin of clinically relevant ARB strains and/or their resistance mechanisms to bacteria living in water or soil in natural environments (Finley *et al.* 2013; Singer *et al.* 2016).

AR exists since long before human developed antibiotics, as a part of a never-ending microbial warfare by which bacteria outcompete others by naturally producing toxic metabolites, some of which resemble the pharmaceuticals we know today (Larsson & Flach 2021). Antibiotics exert selective pressure over bacterial populations, killing those that lack the adequate defence mechanisms, thus allowing resistant ones to take over. Even at sub-lethal antibiotic concentrations, ARB tend to outcompete antibiotic-sensitive bacteria (ASB) (Gullberg *et al.* 2011; Liu *et al.* 2011; Hrenovic *et al.* 2017). The introduction of antibiotics has greatly accelerated the spread of resistance, promoting the acquisition of antibiotic resistance genes (ARGs) by horizontal gene transfer (HGT) and other processes that allow bacteria to acquire these genes from other bacteria, even if these belong to different species (Rizzo *et al.* 2013; Zhang *et al.* 2016; Lamba *et al.* 2017).

Waste water treatment plants (WWTPs) have been flagged as hotspots for AR dissemination (Hirsch *et al.* 1999; Díaz-Cruz *et al.* 2003; Brown *et al.* 2006; Kümmerer 2009; Czekalski *et al.* 2012; Rizzo *et al.* 2013) due to the simultaneous discharge of antibiotics, ARB and ARG into the environment. This is mainly facilitated by ARB and ARG in the incoming faecal matter, high cell densities associated with biological treatment, presence of nutrients and a steady selective pressure caused by low concentrations of incoming antibiotics and their metabolites in domestic sewage (Michael *et al.* 2013; Guo *et al.* 2017; Manaia *et al.* 2018). As a consequence, treated effluents usually carry high concentrations of human and animal bacteria, many of which harbour ARGs, thus becoming potential vectors for their dissemination into the environment (Pruden 2014; Berendonk *et al.* 2015; Manaia 2017).

Literature is divided on whether municipal WWTP selects for ARB during biological treatment or not. Some authors point to increases in the proportion of ARB in treated effluents (Łuczkiewicz *et al.* 2010; Biswal *et al.* 2014; Al-Jassim *et al.* 2015; Korzeniewska & Harnisz 2018), while others indicate a decrease of ARB relative abundance after treatment (Guardabassi *et al.* 2002; Varela *et al.* 2013; Nimonkar *et al.* 2019). The effect of the WWTP discharge in the receiving water bodies is also highly controversial, as some studies indicate ARB enrichment of water and sediment populations downstream (Akiyama & Savin 2010; Leclercq *et al.* 2013; Sidrach-Cardona *et al.* 2014; Osińska *et al.* 2016), others prove inconclusive or without significant variations (West *et al.* 2011; Czekalski *et al.* 2012; Schreiber & Kistemann 2013; Zhang *et al.* 2015), and a final group describes either simultaneous enrichment of certain ARB populations and decrease of others, or seasonal increase/decrease cycles (Koczura *et al.* 2012; Blaak *et al.* 2014; Marti *et al.* 2014; Zhang *et al.* 2015).

The efficacy of municipal wastewater treatment is evaluated based on a list of parameters, normally described in national/local guidelines, comprising diverse contaminant groups such as organic content, nutrients, metals and microbiological indicators. Regarding the latter, (sensitive) *Escherichia coli* and intestinal enterococci appear as the most commonly used microbial indicators, together with faecal coliforms (Scott *et al.* 2003; Lin & Ganesh 2013). Water quality screening designed for the evaluation of potential faecal contamination on other water uses such as recreational waters and reclaimed water for irrigation or potable reuse, also rely on these faecal indicators to assess suitability of use (Salgot *et al.* 2006; Rodrigues & Cunha 2017; Purnell *et al.* 2020). However, to date, no guideline limiting the presence of ARB and/or ARG in drinking water, wastewater, reuse water, or any other water of municipal concern, mainly due to a lack of consensus on which are adequate AR indicators to measure.

In this publication, we study the similarities between the classic microbial indicators *E. coli* and intestinal enterococci and specific resistant ESBL-*E. coli* and VRE in municipal effluents, in order to determine whether the former can be used as proxy for elimination of their resistant counterpart through conventional and novel water treatment processes, as well as their natural decay.

2. MATERIALS AND METHODS

2.1. Sewage and secondary effluent collection

Grab samples of raw sewage and secondary effluent were collected every 2 weeks from a large municipal activated sludge wastewater treatment plant (AS-WWTP) located in the southwest of the Netherlands. All samples were taken between the months of November 2020 and March 2021, with 12 sampling events in total. All samples were transported in coolers directly to the laboratory, with the initial microbial quantifications being conducted within 6 h of collection. In order to avoid debris, raw sewage samples were collected immediately after the screens. Samples of secondary effluent were collected from the discharge mains of the secondary settlers.

2.2. Disinfection and decay experiments

Experiments evaluating secondary effluent disinfection by Iron Electrocoagulation (Fe-EC) were performed by dosing continuous current into the liquid through two parallel and partially submerged ARMCO iron plates (maximum percentages: 0.14% carbon, 0.10% silicium, 0.80% manganese, 0.025% phosphorous, 0.015% sulphur, 0.010% nitrogen, 0.20% copper, and 0.080% aluminium). These were connected to a dual 30 V – 3 A TENMA 72-10500 bench DC supply by crocodile clip cables. Electrodes were square-shaped (40 mm × 40 mm), and provided with an thin elongation parallel to one of the sides (40 mm × 5 mm) to act as a dry contact for the clip cables (preventing the crocodile clips' dissolution). Plates were polished with coarse and fine sand paper and rinsed with demineralized water before each experiment. Beakers containing the effluent were fitted with a PTFE coated bars and placed on LABNICO L23 magnetic stirrers for mixing purposes. To maintain oxygen saturation, air was supplied continuously during the application of current using an OASE OxyMax200 air pump. A 30 L grab sample of secondary effluent was divided in two 15-L containers for duplicate purposes, and for each duplicate, samples were retrieved in 2-L beakers and exposed to a current of 287 mA during varying amounts of time in order to provide an increasing dosage of Fe in each sample. Once the desired dosages were achieved, samples were covered and let to settle during 2 h, after which the supernatant was collected for microbial and physical/chemical screening in triplicate.

Experiments evaluating disinfection by chlorine were performed using a 30-L grab sample of secondary effluent, divided in two for duplicate testing, and dosed with NaOCl (Sigma-Aldrich, Germany) for disinfection. The dosage of NaOCl (1.25%) was of 0.9 ml/L, which yielded an initial free chlorine value of approximately 0.50 mg/L as determined with the spectrophotometric US-EPA DPD method (HACH, USA), processed on a Spectroquant®NOVA60 spectrophotometer (Merck, Germany). Chlorine demand was measured in the same way throughout the experiment, simultaneously with the sample extractions once the desired exposure times were achieved, after which free chlorine was neutralized by the addition of 5 ml/L of 0.1 M $Na_2S_2O_3$ sodium thiosulphate solution (Sigma-Aldrich, USA). The disinfected effluent samples were then processed immediately for microbial screening in triplicate.

To simulate the microbial decay of sensitive and resistant bacteria in the secondary effluent, 30 L of effluent grab samples were divided into duplicate 15-L containers and stored in the dark atop orbital shakers, at different temperatures: 4, 13, and 24 °C. During the testing period, samples were extracted from each of the duplicated containers, and each screened for microbial concentrations in triplicate.

2.3. Microbial indicators and culture media

The screening of microbial indicators was based exclusively on culture methods. *E. coli* and enterococci are two of the most commonly used faecal indicators due to their presence in the human gut, and their ease of detection and quantification (Noble *et al.* 2004; Harwood *et al.* 2005; Petri *et al.* 2008; Rosenberg Goldstein *et al.* 2014; Al-Jassim *et al.* 2015; Anfruns-Estrada *et al.* 2017). For each of them, resistance against a specific type of antibiotic was selected, namely betalactams for *E. coli* and vancomycin for enterococci, as these ARB are listed under the category of 'serious threat' by the US CDC and the ECDC 2019 and 2022 Antibiotic Resistance reports respectively (US CDC 2019; ECDC 2022). The four indicator organisms and their respective growth media are indicated in Table 1.

2.4. Data analysis

Data series for inactivation of *E. coli*, ESBL-*E. coli*, enterococci and VRE were analyzed with the ANOVA (analysis of variance) statistical test in order to determine whether the different strains underwent statistically significant removal during

Table 1 | Selected indicators and growth media

Indicator	Growth medium
Escherichia coli (E. coli)	Chromocult® agar medium (ISO 9308-1), Merck Millipore.
Extended Spectrum β Lactamase (**ESBL**)-producing ***E. coli***	ChromID® ESBL agar medium. Biomerieux-Diagnostics (Marcy l'Etoile, France).
Enterococci	Slanetz-Bartley agar medium. Merck Millipore
Vancomycin Resistant Enterococci **(VRE)**	ChromID® VRE agar medium. Biomerieux-Diagnostics (Marcy l'Etoile, France).

activated sludge treatment (Section 3.1), spontaneous decay (Section 3.2), chlorination (Section 3.3), and iron electrocoagulation (Section 3.4). In all cases, the obtained data were comprised by triplicate microbial sampling in duplicate assays ($n = 6$).

3. RESULTS

3.1. Activated sludge wastewater treatment plant

During the 5-month sampling campaign on the AS-WWTP, samples of raw sewage and secondary effluent were collected every 2 weeks, and the concentrations of *E. coli*, ESBL-*E. coli*, enterococci and VRE were determined in triplicate in each sample. Within each sample, standard deviation was in almost all cases one order of magnitude lower than the concentration average for the triplicates, indicating the uncertainty in the observed concentrations was low (Figure 1). Microbial concentrations in the raw sewage and secondary effluent from the selected municipal WWTP were relatively stable during the sampling period for both the sensitive indicator bacteria as well as the ESBL-*E. coli* and VRE enterococci, with no clear temporal trends.

The activated sludge process and in particular its capability for removal of faecal indicator bacteria, have been extensively studied for decades, with most literature reporting log reduction values (LRVs) between 1 and 3 for diverse faecal indicators (Fu *et al.* 2010; De Luca *et al.* 2013; Hata *et al.* 2013). The selected WWTP performs as expected, with an average LRV of 2.1–2.4 $\log_{10}$, with standard deviations of 0.3–0.4 $\log_{10}$ for all indicators including ESBL-*E. coli* and VRE. This shows that the resistant ESBL-*E. coli* and VRE did not experience any better or worse removal than sensitive *E. coli* or enterococci during the activated sludge treatment, not being significantly better or worse suited to withstand the process (ANOVA p-value = 0.43), irrespective of their antimicrobial resistance condition.

For the raw sewage, average *E. coli* concentration was 5.5×10^7 cfu/L, while average ESBL-*E. coli* concentration was determined at 5.1×10^5 cfu/L, for which the *E. coli*/ESBL-*E. coli* ratio in the sewage was 124 ± 27 to 1. Enterococci average concentration in the sewage was 7.3×10^6 cfu/L, while that of VRE was 3.4×10^5, meaning that the enterococci/VRE ratio was in the 25 ± 13 to 1 range (Table 2). On the secondary effluent however, average *E. coli* concentration was 3.3×10^5 cfu/L, while average ESBL-*E. coli* concentration was determined at 2.2×10^3 cfu/L, for which the *E. coli*/ESBL-*E. coli* ratio in the sewage was in the 140 ± 36 to 1 order. Enterococci average concentration in the secondary effluent was 5.8×10^4 cfu/L, while that of VRE was 1.2×10^3, meaning that the enterococci/VRE ratio was in the 48 ± 28 to 1 order. This means that in the sewage, only 0.8% of *E. coli* cells were beta-lactam resistant and 4% of the enterococci colonies were Vancomycin resistant, while in the secondary effluent less than 0.7% of *E. coli* cells were beta-lactam resistant and 2% of the enterococci colonies were Vancomycin resistant. For both groups, results show that the resistant bacteria fractions did not increase as a consequence of the activated sludge treatment, whereas in fact a slight (not significant) decrease was observed.

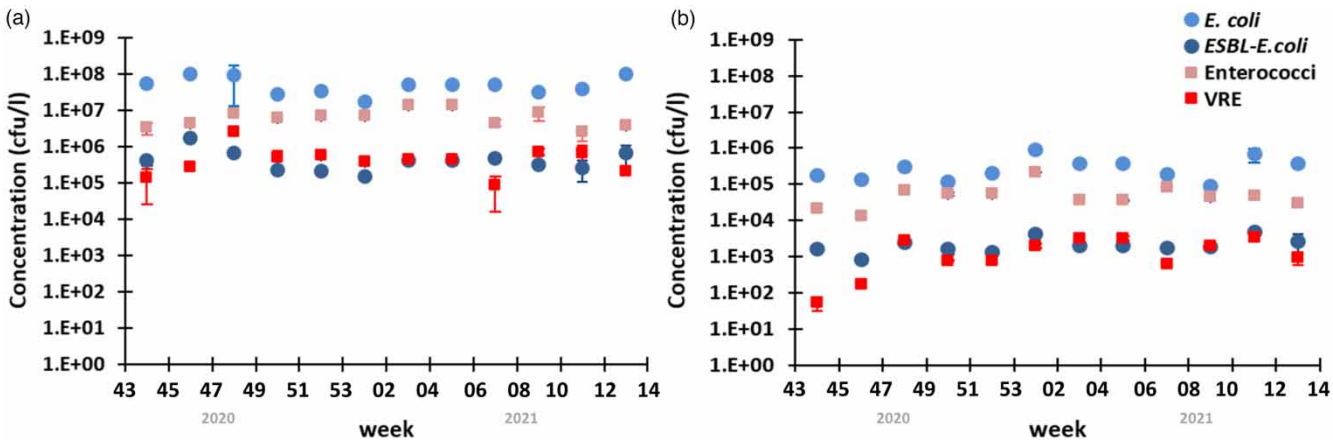

Figure 1 | Concentrations of *E. coli*, enterococci, ESBL-*E. coli* and VRE during November 2020–March 2021 sampling campaign in the WWTP's (a) raw sewage, and (b) secondary effluent. Microbial determination was performed in triplicate. Error bars represent standard deviation.

Table 2 | Average concentrations and standard deviation of *E. coli*, enterococci, ESBL-*E. coli* and VRE during the November 2020-March 2021 sampling campaign in the WWTP's raw sewage and secondary effluent, and mean Log removal value (LRV) for each indicator

	Influent (cfu/L)		Effluent (cfu/L)		LRV	
Indicator	**Average**	**stdev**	**Average**	**stdev**	**Average**	**stdev**
E. coli	5.5×10^7	3.1×10^7	3.3×10^5	2.5×10^5	2.2	0.4
ESBL-*E. coli*	5.1×10^5	4.5×10^5	2.2×10^3	1.2×10^3	2.4	0.4
Ratio	124	27	140	36		
Enterococci	7.3×10^6	3.7×10^6	5.8×10^4	5.3×10^4	2.1	0.3
VRE	3.4×10^5	3.1×10^5	1.7×10^3	1.2×10^3	2.3	0.4
Ratio	25	13	48	28		

3.2. Decay

In this publication, temperature decay kinetics were studied for real secondary effluent samples under controlled temperature conditions for all indicators, simulating a prospective discharge into a hypothetical receiving water body. Following effluent discharge into aquatic environments, faecal microorganisms generally progress towards non-viability, process usually termed as decay (Korajkic *et al.* 2019). Water temperature is a major factor in decay, as it has been show to influence first-order decay rate constants on a proportional basis, meaning that decay progresses faster in warmer waters and slows down when the water is colder (Medema *et al.* 1997; Easton *et al.* 2006; Hellweger *et al.* 2009). Though several other factors have also been identified in microbial decay on fresh and estuarine environments, such as the incidence of sunlight, salinity, presence of heavy metals, or predation (Gonzalez *et al.* 1990; Iriberri *et al.* 1994; Sinton *et al.* 2002; Noble *et al.* 2004; Deller *et al.* 2006), temperature is perhaps the most relevant. To determine whether sensitive and resistant *E. coli* and enterococci show similar survival in receiving water bodies in various climates, decay experiments were performed at different temperatures. Three temperature scenarios were assayed, namely; cold (4 °C), mild (13 °C), and warm (24 °C). For each assayed temperature, experiments were concluded once the concentrations of the resistant strains VRE/ESBL-*E. coli* were below levels that allowed accurate quantification. Results are displayed in Figure 2.

For the cold scenario (4 °C), microbial decay appeared to be biphasic, with a sharper decrease in concentration during the first 7 days. An inflexion can be observed in all the trendlines on day 7, after which a much slower decrease in microbial concentration is observed (Table 3). Experiments concluded at 21 days, with all indicators presenting a LRV of $\approx 2.15 \pm 0.35$ (Figure 2(a)). For the mild scenario (13 °C), similar observations were made, as a biphasic behaviour is displayed for all indicators, presenting an inflection point at day 3. Experiments concluded after 9 days, with all indicators presenting a LRV of $\approx 2.12 \pm 0.27$ (Figure 2(b)). For the warm scenario (24 °C), biphasic behaviour was also observed, presenting the highest rates of decay of all assayed conditions. The inflection point was determined at approximately 1.15 days, and experiments concluded at 4.15 days when all indicators presented a LRV of $\approx 2.05 \pm 0.28$ (Figure 2(c)).

Based on the biphasic behaviour of the decay process, and the good linearity observed in each of the phases, it can then be described as follows:

$$\log_{10}\left(\frac{C}{C_0}\right) = -k_1 \cdot t \quad \text{if } t < t_{\text{inflection}} \tag{1}$$

$$\log_{10}\left(\frac{C}{C_0}\right) = -k_1 \cdot t_{\text{inflection}} - k_2 \cdot (t - t_{\text{inflection}} \quad \text{if } t > t_{\text{inflection}}) \tag{2}$$

where C indicates bacteria concentration at time t (cfu/L); C_0 indicates bacteria concentration at time 0 (cfu/L); K_1 indicates first-order rate constant observed during the fast decay phase (d^{-1}); K_2 indicates first-order rate constant observed during the slow decay phase (d^{-1}); t = time since the beginning of the experiments (d); $t_{\text{inflection}}$ indicates timestamp in which a change in decay rate is observed (d).

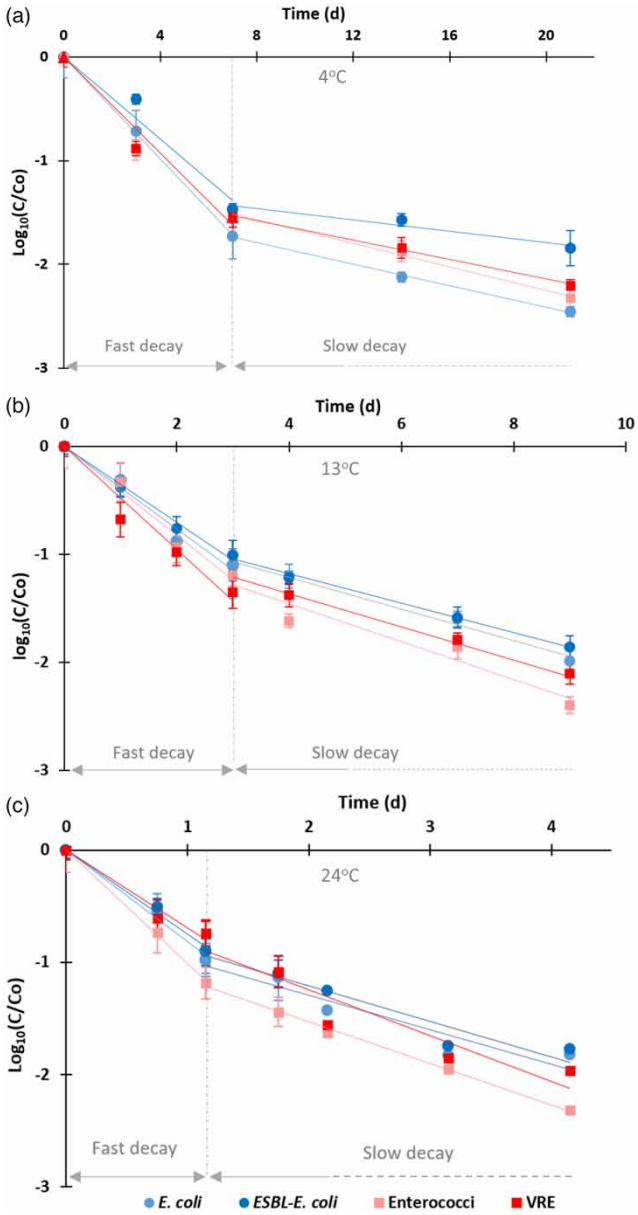

Figure 2 | Log_{10} data series of the relative microbial concentration (C/C_o) for *E. coli*, ESBL-*E. coli*, enterococci and VRE on secondary municipal effluent stored at (a) 4 °C; (b) 13 °C; and (c) 24 °C. Experiments were performed in duplicate. Microbial screening was performed in quadruplicate for T = 0, and triplicate for the rest of the samples. Error bars indicate standard deviation.

Table 3 | Slope values k_1 and k_2 for the linear trendlines of *E. coli*, ESBL-*E. coli*, enterococci and VRE during the temperature decay experiments.

	k_1 (d^{-1})			k_2 (d^{-1})		
	4 °C	**13 °C**	**24 °C**	**4 °C**	**13 °C**	**24 °C**
E. coli	0.25	0.38	0.82	0.05	0.15	0.31
ESBL- *E. coli*	0.20	0.35	0.75	0.04	0.14	0.32
Enterococci	0.23	0.41	1.02	0.06	0.18	0.37
VRE	0.23	0.48	0.70	0.05	0.15	0.41

3.3. Disinfection

Municipal effluent disinfection, usually termed tertiary treatment, is increasingly seen as a way of obtaining high quality polished effluents, with low organic and nutrient content, as well as reduced microbiological load (Henze *et al.* 2008). Chlorination, is the most popular disinfection technology currently applied in WWTPs (Manaia *et al.* 2018) due to its broad disinfection spectrum, high efficiency, and low operation and maintenance costs (Rizzo *et al.* 2013; How *et al.* 2017; Nihemaiti *et al.* 2020; Azuma & Hayashi 2021), hence selected for secondary effluent disinfection experiments. Secondary effluent samples were exposed to chlorine by applying 0.9 ml/L NaOCl [1.25%], which yielded an initial free chlorine concentration of ≈0.50 mg/L. This concentration remained relatively stable during the duration of the experiment, with a final average concentration of 0.44 mg/L after 16 min. A $Log_{10}(C/C_0)$ plot was constructed as a function of the product of free chlorine concentration (mg/L) and exposure time (min), commonly known as CT following the Chick–Watson equation for disinfection. Results are indicated in Figure 3.

Chlorine disinfection showed a good linear fit between the $\log_{10}(C/C_0)$ values and CT for all indicators ($R^2 \geq 0.95$), and disinfection data series were described by a first-order kinetic process, namely:

$$\log_{10}\left(\frac{C}{C_0}\right) = -k \cdot C_{FCl} \cdot t \tag{3}$$

where C indicates the bacteria concentration at time t (cfu/L); C_0 indicates the bacteria concentration at time 0 (cfu/L); K indicates first-order rate constant ($l \cdot mgCl^{-1} \cdot min^{-1}$); C_{FCl} indicates concentration of free chlorine (mgCl/L); T indicates exposure time to disinfectant (min).

The inactivation rate constant (k-value) of *E. coli* was very similar to that of ESBL-*E. coli* (0.370 vs. 0.359 $L \cdot mgCl^{-1} \cdot min^{-1}$). Similarly, the inactivation rate of enterococci was very similar to that of VRE (0.312 vs. 0.327 $L \cdot mgCl^{-1} \cdot min^{-1}$). The enterococci/VRE are somewhat more difficult to inactivate with chlorine than *E. coli*/ ESBL-*E. coli*. This distinct behaviour of gram positive and gram negative bacteria has been previously reported, and attributed to differences in bacterial membranes and cell wall structures, as chlorine reacts more aggressively with lipid-rich membranes (Mir *et al.* 1997).

3.4. Coagulation–sedimentation

Coagulation processes have seldom been reported as a mainstream disinfection mechanism, as more conventional technologies like chlorination, UV and ozonation usually take precedence. For this set of experiments, secondary effluent samples were subjected to a Fe coagulation process, conducted by electrolysis with high purity Fe-electrodes, with dosages up to 42.4 mgFe/L. Samples collected at regular intervals were left to settle for 2 h and then screened for the sensitive and resistant *E. coli* and enterococci. Linear models were used to fit the $Log_{10}(C/Co)$ plots versus Fe dosage (Figure 4), as this dose-response linearity for Fe-EC had been suggested in our previous research (Bicudo *et al.* 2022).

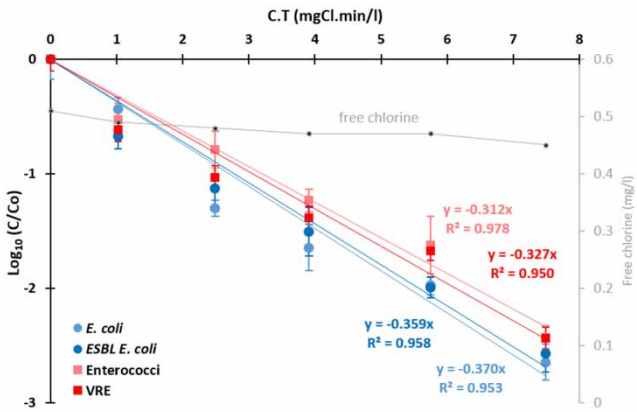

Figure 3 | Log_{10} data series of the relative microbial concentration (C/C_0) for *E. coli*, ESBL-*E. coli*, enterococci and VRE following chlorine disinfection in real municipal secondary effluent by the use of NaOCl during 15 min. Initial Free Chlorine values were ≈0.50 mg/L. Cumulative CT values were calculated based on the length of the time intervals and the measured Free chlorine value during said interval. Experiments were performed in duplicate. Microbial screening was performed triplicate. Error bars indicate standard deviation.

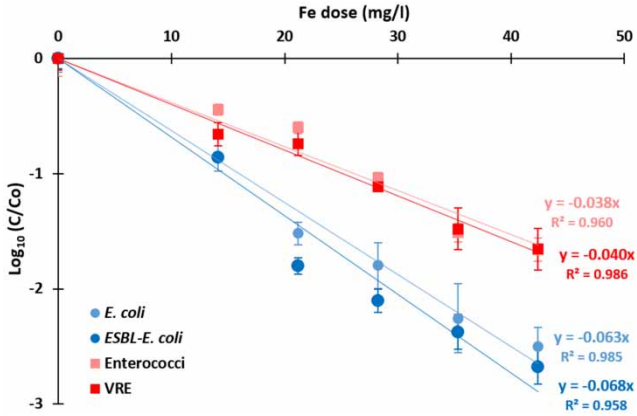

Figure 4 | Log_{10} data series of the relative microbial concentration (C/C_0) for *E. coli*, ESBL-*E. coli*, enterococci and VRE on secondary municipal effluent treated with Fe-EC. Fe dosage range was 0.0–42.4 mgFe/L. Experiments were performed in duplicate. Microbial screening was performed in quadruplicate for Fe = 0 mg/L, and triplicate for the rest of the samples. Error bars indicate standard deviation.

Results show that the LRVs for all indicators are directly proportional to the Fe dosage with removal reaching 2–3 log_{10} for Fe dosages of ≈42.4 mgFe/L, as previously observed by Bicudo *et al.* (2022). Other sources who had also investigated Fe-EC for secondary effluent disinfection arrived to similar results (2–3 log_{10} attenuation) using other microbial indicators, such as heterotrophic bacteria, somatic coliphages and *Clostridium perfringens* spores (Anfruns-Estrada *et al.* 2017; Bicudo *et al.* 2021). Because of the good linear fits obtained between the Log_{10}(C/Co) and the iron dosage for the selected bacteria ($R^2 > 0.95$), disinfection was described with first-order kinetics, namely:

$$\log_{10}\left(\frac{C}{C_0}\right) = -k \cdot [Fe] \tag{4}$$

where:

C = Bacteria concentration at time t (cfu/L)
C_0 = Bacteria concentration at time 0 (cfu/L)
k = First-order rate constant (l/mg Fe)
[Fe] = Iron dose in the bulk liquid (mg Fe/L)

Figure 4 indicates that the slopes (k-values) of all four indictors are clustered in pairs, with enterococci/VRE having k-values in the 0.039 ± 0.001 l/mg Fe range, and *E. coli*/ESBL-*E. coli* having k-values in the 0.066 ± 0.003 l/mg Fe. The slope analysis indicates that disinfection of enterococci was very similar to that of VRE (Δk ± 4.4%), while the same applies for *E. coli* and ESBL-*E. coli* (Δk ± 2.5%). A distinct response was observed between the enterococci/VRE cluster and the *E. coli*/ESBL-*E coli* cluster, indicating in this case that *E. coli* (sensitive and resistant) bacteria are better removed than enterococci (sensitive and resistant).

4. DISCUSSION

The present publication is a comprehensive evaluation of the similarities in behaviour between *E. coli* and ESBL-*E. coli* and between enterococci and VRE during conventional and non-conventional wastewater treatment/disinfection processes. Only real municipal sewage and secondary effluents were used for all experiments, as well as culture-based methods for quantification of all indicators. Discussion is geared towards understanding the value of classic microbial indicators as a proxy for both the presence and disinfection of resistant organisms using a simple, yet robust approach, currently lacking in AR literature.

Observations regarding the disinfection by activated sludge were in line with similar research including not only faecal indicators but also ARB (Yuan *et al.* 2016; Turolla *et al.* 2018;Wang *et al.* 2020), with most reported LRV in the 1–3 log_{10} range. The selected AS-WWTP does also not seem to affect the *E. coli*/ESBL-*E. coli* ratio significantly nor the enterococci/VRE ratio

between influent and effluent, further suggesting that removal of all four microbial indicators is proportional (ANOVA p-value > 0.05). Our results, which are exclusively culture-based, show that the studied ARB undergo the same removal process than that of ASB during activated sludge treatment. No enrichment of ARB was observed in the secondary effluent, as the fraction of resistant organisms in the effluent's microbial population was not larger than that of the influent, and as the total concentration of ARB decreased >99% when compared to the incoming sewage.

Following effluent discharge, this study also examined the decay of *E. coli*, ESBL-*E. coli*, enterococci and VRE in simulated receiving water bodies of different temperatures. In all scenarios a clear biphasic decline pattern was observed, consistent with previous research on faecal bacteria decay in fresh, estuarine and seawaters (Medema *et al.* 1997; Hijnen *et al.* 2007; Brouwer *et al.* 2017). For all assayed temperatures, die-off was faster at the beginning of the experiments and slowed down towards their end. Microbial decay plots indicated similar decay behaviour across *E. coli*, ESBL-*E. coli*, enterococci and VRE, both qualitatively and quantitatively, as indicated by similar k-values and inflection times in the decay curves. We observed no relevant differences between the temperature decay kinetics of *E. coli* and enterococci, and of their resistant strains ESBL-*E. coli* and VRE for any of the assayed temperature conditions. This suggests that classic microbial indicators such as *E. coli* and/or enterococci are good proxies for tracking the decay of ESBL-*E. coli,* VRE and possibly other ARB from municipal effluents in water bodies. Disinfection with both coagulation and chlorination showed a differential response for *E. coli* and enterococci.

For Fe-EC the first-order rate constant for *E. coli* and ESBL-*E. coli* (0.066 ± 0.003 l/mg Fe) was approximately 60% larger than that of enterococci and VRE (0.039 ± 0.001 l/mg Fe), indicating a higher sensitivity of *E. coli* towards the Fe-EC induced disinfection. Similar observations were obtained with secondary effluent chlorine disinfection (≈0.5 mg/L, room temperature and circumneutral pH), where no significant differences on inactivation first-order rate constants existed between *E. coli* and ESBL-*E. coli*, nor between enterococci and VRE. First-order rate constants obtained in this study for the selected resistant strains are not only similar to those obtained for the respective sensitive strains, but also to those obtained by other researchers involving the same sensitive strains in similar temperature and pH conditions (Tyrrell *et al.* 1995; Mwatondo & Silverman 2021). This indicates that the behaviour of VRE during Fe-EC and chlorination mirrored that of enterococci, in the same way that the behaviour of ESBL-*E. coli* mirrored that of *E. coli*, in both cases within a reasonable margin of error in their first-order rate constants (<5%). Hence, according to our observations, ESBL-*E. coli* and VRE deserve no further distinction in terms of disinfection than *E. coli* or enterococci, which are common faecal indicators, as the latter can be used to estimate inactivation of the resistant strains.

5. CONCLUSIONS

In this study, we subjected *E. coli*, ESBL-*E. coli*, enterococci and VRE obtained from municipal sewage and secondary effluents to diverse wastewater treatment processes. For all the studied microbial removal/decay processes, results conclusively demonstrated that no difference existed between the disinfection of *E. coli* and ESBL-*E. coli*, nor between enterococci and VRE, and that the ratios between the sensitive and resistant strain concentrations were not significantly affected by any of the processes. Activated sludge wastewater treatment offered 2.1–2.4 $\log_{10}$ average removal for all indicators, irrespective of them being antibiotic resistant or not. Fe-EC performed better for *E. coli*/ESBL-*E. coli* ($\log_{10}$ removal 0.066 ± 0.003 l/mgFe) than for enterococci/VRE ($\log_{10}$ removal 0.039 ± 0.001 l/mgFe), yet still removing sensitive and resistant bacteria in the same proportion. Disinfection by chlorine also proved enterococci/VRE to be hardier to inactivate than *E. coli*/ESBL-*E. coli* (LRVs of 0.320 ± 0.008 L·mgCl^{-1}·min^{-1} and 0.365 ± 0.006 L·mgCl^{-1}·min^{-1}, respectively), yet had no influence on the ratios between same-species sensitive and resistant organisms, also suggesting that neither ESBL-*E. coli* nor VRE fare better than their respective sensitive counterparts. Experiments by spontaneous decay under different temperatures showed that all four indicators present a biphasic behaviour, with decay progressing faster at the beginning and slowing down after a variable amount of time, with no significant difference in behaviour between resistant and sensitive organisms.

It may be concluded that for all the microbial disinfection/decay processes covered in this publication (activated sludge, chlorination, Fe-EC and spontaneous decay), our results demonstrated that the microbial reduction profiles, including those of the resistant strains ESBL-*E. coli* and VRE, are in line with sensitive faecal indicators. This means that the resistance status of these two organisms provided them with no competitive advantage over their sensitive counterparts *E. coli* and enterococci. Logically, these observations are method-specific and should not be lightly extrapolated to treatment operations not covered in this publication, nor to all ASB/ARB pairs. We do propose that the use of *E. coli* and enterococci, whose

detection and quantification are simple, inexpensive, and low-tech, remain very valuable indicators for estimating disinfection of ESBL-*E. coli* and VRE, and for inferring their presence in sewage, secondary effluents, disinfected effluents and possibly in the receiving water bodies of different temperatures.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to NWO TTW (grant no. 15424) for their sponsorship of the LOTUS^{HR} project (https://lotushr.org), which funded this research. We would like to acknowledge the efforts of Armand Middeldorp from the TU Delft Waterlab, and in particular the commitment and dedication of our intern, Ruby-Jane Crichlow.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 25 March 2024; accepted in revised form 7 June 2025. Available online 8 July 2025