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Urban wastewater disinfection by iron chelates mediated solar photo-Fenton: Effects on seven pathogens and antibiotic resistance transfer potential

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ABSTRACT

The effects of solar photo-Fenton (SPF) process mediated by the iron chelate Fe^{3+} imminodisuccinic acid (Fe:IDS) on both the inactivation of seven relevant pathogens and the potential for antibiotic resistance transfer (degradation of antibiotic resistance genes (ARGs) and after treatment regrowth), in real secondary treated urban wastewater, were investigated for the first time. A comparison with results obtained by sunlight/H_2O_2 process and Fe^{3+} ethylenediaminedisuccinic acid (Fe:EDDS) SPF was also carried out. ARGs were quantified by polymerase chain reaction (PCR) in samples before and after (3 h) the treatment. The persistence of the selected pathogens and ARGs was also evaluated in regrowth tests (72 h) under environmentally mimicking conditions. Fe:IDS SPF resulted to be more effective (from 1.4 log removal for *Staphylococcus spp*. to 4.3 log removal for *Escherichia coli*) than Fe:EDDS SPF (from 0.8 log removal for *Pseudomonas aeruginosa* to 2.0 log removal for Total coliphages) and sunlight/H_2O_2 (from 1.2 log removal for *Clostridium perfringens* to 3.3 log removal for *E. coli*) processes for the seven pathogens investigated. Potential pathogens regrowth was also severely affected, as no substantial regrowth was observed, both in presence and absence of catalase. A similar trend was observed for ARGs removal too (until 0.001 fold change expression for *qnrS* after 3 h). However, a poor effect and a slight increase in fold change was observed after treatment especially for *gyrA, mefA* and *intl1*. Overall, the effect of the investigated processes on ARGs was found to be ARG dependent. Noteworthy, coliphages can regrow after sunlight/H_2O_2 treatment unlike SPF processes, increasing the risk of antibiotic resistance transfer by transduction mechanism. In conclusion, Fe:IDS SPF is an attractive solution for tertiary treatment of urban wastewater in small wastewater treatment plants as it can provide effective disinfection and a higher protection against antibiotic resistance transfer than the other investigated processes.

1. Introduction

Recently, antibiotic resistance (AR) has emerged as a global threat to human health (Larsson and Flach, 2022). The World Health Organization (WHO) has defined this phenomenon as dangerously increasing across the world and able to pose a threat to our capabilities of dealing even with the most common diseases (Courvalin, 2016). The increase of AR also depends by anthropogenic activities (Darby et al., 2023; Tripathi and Cytryn, 2017). Inappropriate prescription and overuse of antibiotics contributes on a large scale to the spread of ARGs and antibiotic-resistant bacteria (ARB) (Llor and Bjerrum, 2014; Merlino and Siarakas, 2022). The release of partially or unaltered metabolized antibiotics and their availability at a sub-inhibitory concentration in the environment eventually induce an evolutionary pressure on pathogens, fostering mutations which make them more resistant to antibiotics (Davies and Davies, 2010). The acquisition of AR can occur through a vertical gene transfer (VGT) mechanism (Russell and Herwald, 2004), in which pre-existing ARGs are transmitted during the proliferation of bacteria, or through a horizontal gene transfer (HGT) mechanism (Li et al., 2021), in which genes are transmitted in a non-reproductive way (conjugation, natural transformation and transduction) (Li et al., 2021; Schneider, 2017). In the transduction mechanism, coliphages, and more

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generally, bacteriophages, play a key role (Balcazar, 2014). Noteworthy, coliphages are known to be more resistant than bacteria to the common disinfection treatments (Lamy et al., 2020).

Urban wastewater treatment plants (WTPs) are considered a hotspot for ARG and ARB spread (Cacace et al., 2019; Rizzo et al., 2013). Unfortunately, conventional wastewater disinfection processes (namely chlorination, ozonation, UV-C radiation and peracetic acid) are characterized by some drawbacks and/or are poor/not effective for the removal of ARGs (Di Cesare et al., 2020a). Photo-driven advanced oxidation processes (AOPs), generating highly reactive oxygen species, are experiencing growing interest as a tertiary and quaternary treatment of urban wastewater because they can effectively remove various pathogens and contaminants of emerging concern (CECs), being an attractive alternative to conventional processes (Rizzo, 2022), especially in relation to new regulations (in Europe, the proposed directive on wastewater treatment as well as wastewater reuse regulation (2020/741)). Therefore, the effect on AR of emerging processes such as AOPs deserves further investigation. The effect of UV driven AOPs (such as UV/H_2O_2) on AR has been investigated (Arslan-Alaton et al., 2021; Yoon et al., 2017), but the high energy demand makes the exploration of more sustainable solutions for wastewater treatment an increasingly pressing need. Sunlight/H_2O_2 and solar photo-Fenton (SPF) (coupling iron salts and H_2O_2) processes, are of particular interest for small WTPs because energy costs can be drastically reduced. However, only a few works have been focused on their effect on AR transfer with no conclusive and sometimes contrasting results (Ferro et al., 2016; Starling et al., 2021). The major limitation of SPF is the operation pH which should be acidic to avoid iron precipitation. In the last years, SPF implementing chelating agents to prevent iron precipitation and operate the process at neutral pH was successfully investigated (De la Obra et al., 2017; Gualda-Alonso et al., 2022) and different iron complexes have been tested for tertiary (disinfection) and quaternary (CECs removal) treatment of urban wastewater (Prete et al., 2021). Nevertheless, some drawbacks have been experienced. For instance, ethylenediaminotetraacetic acid (EDTA) while providing good performances in the photo-Fenton process (Sun and Pignatello, 1992) is poorly biodegradable (Hinck et al., 1997; Nörtemann, 2005). Nitrilotriacetic acid (NTA) exhibits high stability, good efficiency in CECs removal and bacteria inactivation, but it is potentially carcinogenic to humans (Rizzo, 2022); moreover Fe:NTA SPF was not effective in ARGs removal from urban wastewater (Fiorentino et al., 2022). Ethylenediaminedisuccinic acid (EDDS) is expensive and not always effective in bacteria inactivation under realistic conditions (Maniakova et al., 2021a; 2021b).

Imminodisuccinic acid (IDS) is a biodegradable (Cokesa et al., 2004) iron-chelating agent which has been recently investigated in the SPF process (Faggiano et al., 2022; Fiorentino et al., 2018; 2021b). SPF with Fe:IDS complex was compared to SPF with Fe:EDDS complex in urban wastewater disinfection and it was found more effective in the inactivation of some pathogens (La Manna et al., 2023).

Typically, the effects of advanced treatment methods on AR in urban wastewater were evaluated in terms of ARB inactivation and/or ARGs removal (Gmurek et al., 2022; Shi et al., 2022) but such an approach does not allow an exhaustive characterization of the effect of the investigated treatment process on the potential of AR transfer. Moreover, the contrasting and not exhaustive results available in the scientific literature about the effect of SPF on AR transfer as well as the promising results on urban wastewater disinfection by SPF with IDS make the evaluation of its effect on AR worthy of investigation. Therefore, in this work the effect on AR transfer of SPF with IDS in a pilot plant raceway pond reactor (RPR), using real secondary treated urban wastewater, was evaluated for the first time by measuring different relevant indicators. In particular, the inactivation rates of ARGs and relevant antibiotic resistant bacterial pathogens were investigated and subsequent regrowth tests (with and without catalase addition to discriminate possible H_2O_2 effect on regrowth) were carried out to evaluate the effect of treatment processes on VGT and HGT (in terms of conjugation and natural transformation mechanisms). Tests with and without catalase give also the possibility of verifying what would happen in real conditions if the wastewater is stored after treatment, before being reused for irrigation. Moreover, the inactivation rate of Total coliphages was also investigated to provide useful information concerning the potential of AR transfer through the transduction mechanism (HGT). Besides *Escherichia Coli* (*E. coli*), *Clostridium perfringens* (*C. perfringens*) and *Total coliphages*, chosen as indicators for effluent quality according to the EU regulation on wastewater reuse (Table 4, Annex 1, EU regulation 2020/741), *Enterococcus spp.*, *Staphylococcus spp.*, *Klebsiella* spp. and *Pseudomonas Aeruginosa* (*P. aeruginosa*) were also chosen as target antibiotic resistant bacterial pathogens, given their involvement in AR spread and serious or lethal infections according to the WHO global priority pathogen list of ARB (WHO, 2017). The following ARGs were selected and analyzed in this work, according to their abundance in wastewater and environment as well as their relevance from a clinical point of view (Hendriksen et al., 2019; Spänig et al., 2021): *cmIa* (chloramphenicol resistance), *sulII* (sulfonamide resistance), $bla_{OXA\text{-}48}$ and bla_{TEM} (carbapenems resistance), *qnrS* (quinolones resistance), *gyrA* (associated with fluoroquinolone resistance), *tetA* (tetracycline resistance), *mefA* (macrolide resistance). The process effect on integrase gene of the class 1 integrons *intl1*, which has been recommended for use as proxy for anthropogenic pollution (Gillings et al., 2015) and of ARGs in the environment (Ma et al., 2017), was also investigated. Finally, the effect of Fe:IDS SPF process was compared to Fe:EDDS SPF and sunlight/H_2O_2 processes for the first time.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in this work are summarized in paragraph 2.1, in the Supplementary Material (SM) file.

2.2. Wastewater characteristics

Real secondary treated wastewater samples were collected from a WTP located in the province of Salerno, Italy (650,000 p.e.) implementing a conventional activated sludge process. The chemical and microbiological characteristics of the samples are reported in the SM (Table SM1).

2.3. Solar-driven tests in RPR

2.3.1. Experimental set-up

Experiments were carried out in a RPR (90 cm × 45 cm, 5 cm liquid depth, working volume of 15 L and a surface area of 0.3 m^2), at the University of Salerno, Fisciano Campus, Southern Italy, in sunny days during summer, from 11:00 to 14:00 local time under natural solar irradiation. The outcomes were plotted as a function of the accumulated UV energy per unit of treated volume (Q_{UV}, kJ L^{-1}) (Malato et al., 2003).

A concentration of 50 mg L^{-1} (1.47 mM) of H_2O_2 and 5.5 mg of Fe^{3+} was used, according to previously set conditions (La Manna et al., 2023). Moreover, to reduce $HO^{\bullet}$ scavenging, the alkalinity of the wastewater samples was reduced from 335 mg L^{-1} to 75 mg L^{-1} by acidification and subsequent air stripping. Sunlight/H_2O_2 tests were carried out using the same concentration of H_2O_2. For more details, please refer to the SM file.

2.3.2. Preparation of Fe:L complexes and wastewater pre-treatment

Fe:L complexes were obtained by mixing the iron salt ($FeCl_3{\cdot}7H_2O$) and ligand solution in deionized water, according to the details provided in the SM file.

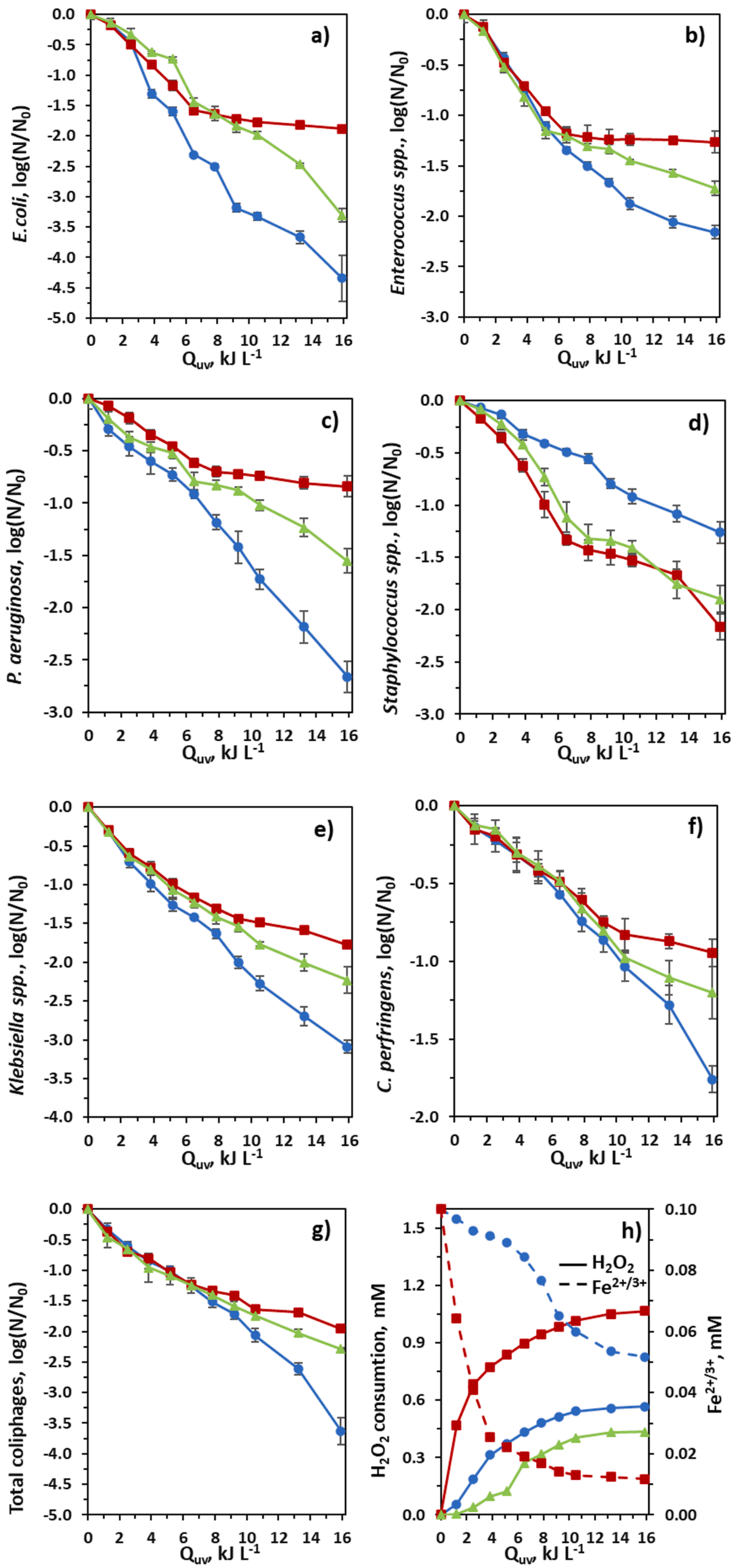

Fig. 1. Inactivation of several pathogens by Fe:IDS SPF (●), Fe:EDDS SPF (■) and sunlight/H_2O_2 (▲) processes: E. coli (a), Enterococcus spp. (b), P. aeruginosa (c), Staphylococcus spp. (d), Klebsiella spp. (e), C. pefringens (f), Total coliphages (g) and H_2O_2 consumption and $Fe^{2+/3+}$concentration (h). Total time= 180 min; H_2O_2 = 1.47 mM, Fe:L = 0.1mM:0.1 mM.

2.4. Analytical measurements

All the relevant parameters (Fe, H_2O_2, pH, turbidity, Biochemical Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), Alkalinity, Total Nitrogen, Nitrites and Nitrates) were analyzed according to the methods and instruments explained in the SM file.

2.5. DNA extraction

DNA was extracted from the wastewater samples (500 mL) taken just before, immediately after and 72 h (regrowth time) after SPF and sunlight/H_2O_2 experiments and analyzed according to the methods and instruments explained in the SM file.

2.6. Qualitative PCR

Specific primers (SM file, Table SM2) for the detection of ARGs were chosen on the basis of their relevance (**EU** Reference Laboratory for antimicrobial resistance) and were analyzed by qualitative and quantitative PCR. Details are provided in SM file.

2.7. Quantification of ARGs

ARGs were quantified by quantitative Real-Time PCR (qPCR) in triplicate according to the method and details explained in the SM file. The results were plotted evaluating the relative gene expression as fold change ($2^{-\Delta\Delta CT}$), where C_T (cycle threshold) is the number of amplification cycles required for the target gene to exceed a threshold level of fluorescence (Livak and Schmittgen, 2001), the higher the C_T the lower the genetic material. $\Delta\Delta C_T$ was calculated according to the formula: $\Delta\Delta C_T$ = unknown sample ΔC_T gene – control sample ΔC_T gene (at $t = 0$ h) where $\Delta C_T = C_T$ target gene – C_T housekeeping gene. ARGs results were statistically analyzed by ANOVA (one-way analysis of single variance) and Tukey post hoc test, using Past 4.03 statistical software (Hammer et al., 2001) with $p<0.05$ as the significance cut-off.

2.8. Microbiological analysis

The enumeration of bacteria was performed by standard plate counting method (Double Agar Layer, lysis plaque method for somatic coliphages) and filtration membrane method (all the experiments were carried out in triplicate), according to the details provided in the SM file.

3. Results and discussion

3.1. Effect of disinfection processes on pathogens inactivation and post-treatment regrowth

3.1.1. Effect of disinfection processes on pathogens inactivation

Preliminary control tests to evaluate the possible contribution of sunlight, Fe^{3+}/sunlight, ligand/sunlight and Fe-ligand/sunlight to *E. coli* inactivation were carried out and no significant effect was observed (Table SM3).

The effect of the Fe:IDS SPF process on the target pathogens in real secondary UWW treatment, was investigated under previously optimized conditions (Fe: 0.1 mM (5.5 mg L^{-1}), H_2O_2: 1.47 mM (50 mg L^{-1}), Fe:Ligand ratio 1:1) (La Manna et al., 2023), comparing Fe:IDS and Fe:EDDS SPF and sunlight/H_2O_2 processes (Fig. 1). The mechanisms by which these processes can inactivate bacterial cells are known as extracellular and intracellular photo-Fenton (García-Gil et al., 2022; Giannakis et al., 2016b, 2016a). In the case of sunlight/H_2O_2 process, the inactivation mechanism is mainly intracellular, due to the low natural iron content in the aqueous matrix and therefore the low production of hydroxyl radicals. Solar radiation and H_2O_2 can penetrate the cell wall, and the presence of intracellular iron can activate SPF process producing a sufficiently high amount of hydroxyl radicals to inactivate the bacterial cell. In the case of SPF, an extracellular mechanism can also take place because the external addition of iron will promote a higher formation of hydroxyl radicals compared to sunlight/H_2O_2 process. On the other hand, the intracellular photo-Fenton mechanism can be hindered by the increased turbidity compared to sunlight/H_2O_2 process, due to pH conditions and/or degradation of the chelating agent, which will increase iron precipitation, finally hampering or preventing the radiation from penetrating the cell wall.

The inactivation efficiency of the target pathogens (Table SM4-SM6) followed this order: Fe:IDS SPF> sunlight/ H_2O_2 > Fe:EDDS SPF, but for Staphylococcus (Fig. 1d). Fe:IDS SPF process resulted in 4.3 log, 3.6 log and 1.8 log reductions for *E. coli, Total coliphages* and *C. perfringens,* respectively after 180 min treatment (Fig. 1a, 1f and 1g, respectively). *C. perfringens* is able to resist to adverse/stress conditions generating endospores (Granger et al., 2011) and its ability to counter SPF has been described in the literature (Kokkinos et al., 2021). Mild inactivation performances (2.2 log) were observed for *Enterococcus spp*. (Fig. 1b). *Enterococcus spp*. is a genus of gram-positive bacteria whose cell wall has an outer layer composed of lipopolysaccharides (Gilmore, 2002). Compared to gram-negative bacteria, the cell wall is more resistant to the radicals attack, hampering the migration of iron and hydrogen peroxide, playing a crucial role in the intracellular photo-Fenton process (García-Fernández et al., 2019; G. Maniakova et al., 2021b). The resilience of *Enterococcus spp*. (and of *C. perfringens*) to the SPF processes was also confirmed by a recent work in which Fe:NTA SPF was investigated for wastewater disinfection in a RPR (Gualda-Alonso et al., 2023). *P. aeruginosa* was among the more resistant pathogens to the disinfection process (2.7 log removal, Fig. 1c) because of the low permeability of its outer membrane (1/100 compared to the outer membrane of *E. coli*) (Pachori et al., 2019). These results are comparable with those observed in a previous work by SPF (180 min treatment) with simulated and real wastewater in a compound parabolic collector under acidic conditions (Rodríguez-Chueca et al., 2013).

Staphylococcus spp. proved to be the most resistant pathogens to the Fe:IDS SPF process, with only a 1.4 log reduction (Fig. 1d). The possibility of an adaptive response to oxidative stress by the formation of H_2O_2 resistant colonies has been suggested in the literature (Painter et al., 2015). However, a complete inactivation of *Staphylococcus* spp. was achieved in a previous work but using significantly higher iron concentration (30 mg L^{-1}) (Vilela et al., 2021), which may require a post-treatment to make the effluent in compliance with iron limit for reuse. Noteworthy, Fe:IDS SPF process was less effective than the other disinfection processes in the inactivation of *Staphylococcus* spp., unlike of what observed for the other pathogens investigated. Such a different behavior could be due to several factors hard to investigate in real wastewater and complex systems, which include the diversity of staphylococci compared to other microorganisms and their tendency to form "clusters", also in response to stress actions (Haaber et al., 2012) as well as the different characteristics of the two chelating agents.

Klebsiella spp. was also severely affected, with 3.0 log reduction (Fig. 1e). In literature, a mild delay in inactivation kinetics during SPF was reported in ultrapure water (Giannakis et al., 2018) and a contribution of bacterial capsule composition has been suggested (Paczosa and Mecsas, 2016; Pan et al., 2015).

Fe:EDDS SPF was found to be less effective than Fe:IDS SPF, showing an efficiency (approximately 2 log unit inactivation) lower than Fe:IDS SPF for *E. coli*, according to a previous work (G. Maniakova et al., 2021b). This behavior can be explained by the higher and faster iron precipitation observed in the Fe:EDDS SPF tests (Fig. 1h), which resulted in higher turbidity (3 NTU for Fe:EDDS against 1.1 NTU for Fe:IDS), making sunlight penetration harder (Alkan et al., 2017) and extra- and intracellular photo-Fenton mechanisms less effective. Possibly, Fe:IDS complex resulted in a lower hydroxyl radicals production rate due to a lower reactivity compared to Fe:EDDS, with a consequent reduced degradation of the complexing agent, which finally delayed iron precipitation keeping the water clearer for longer time (La Manna et al.,

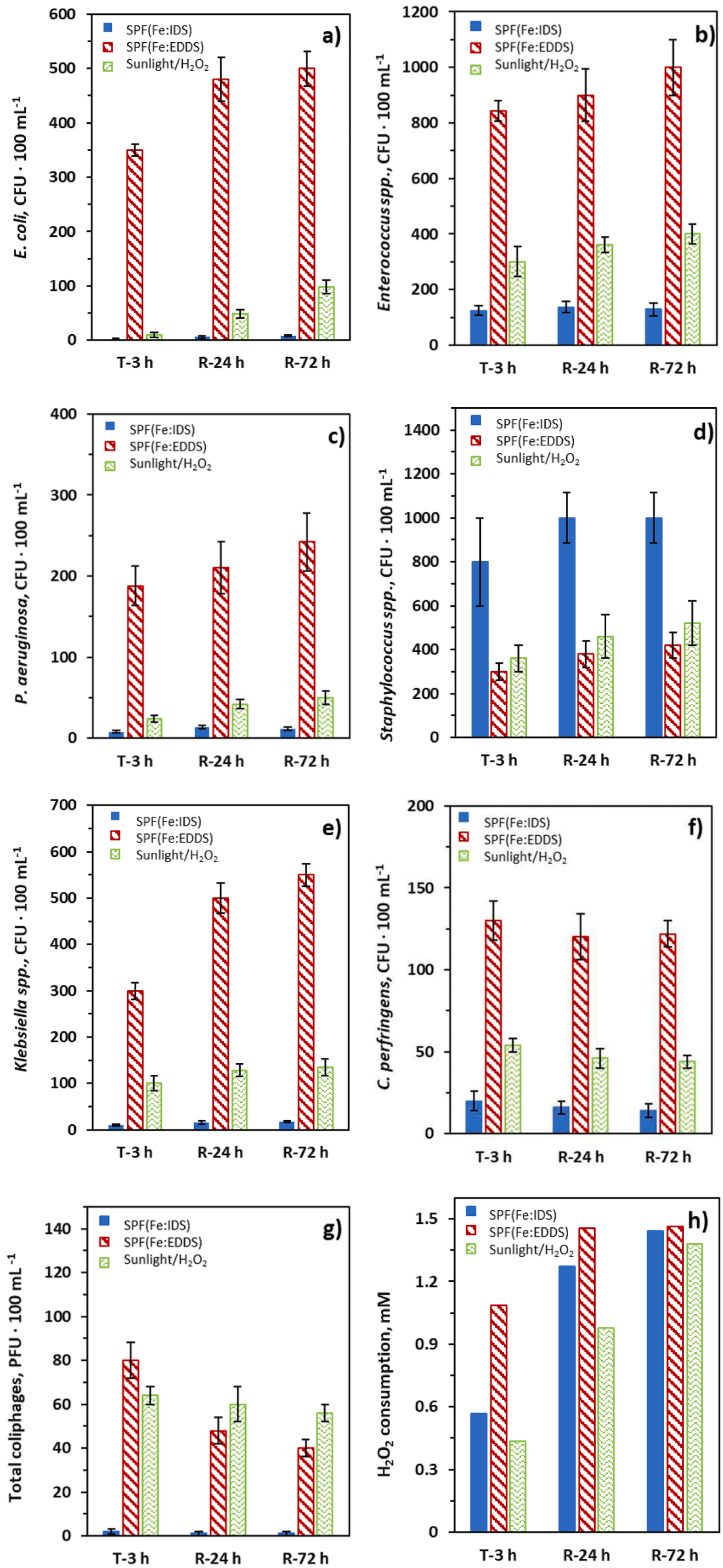

Fig. 2. Regrowth experiments without catalase and H_2O_2 consumption after Fe:IDS SPF, Fe:EDDS SPF and sunlight/H_2O_2 treatments (T- 3 h), after 24 h and 72 h of regrowth (R-24 h and R-72 h) for: E. coli (a), Enterococcus spp. (b), P. aeruginosa (c), Staphylococcus spp. (d), Klebsiella spp. (e), C. perfringens (f), Total coliphages (g), H_2O_2 consumption (h).

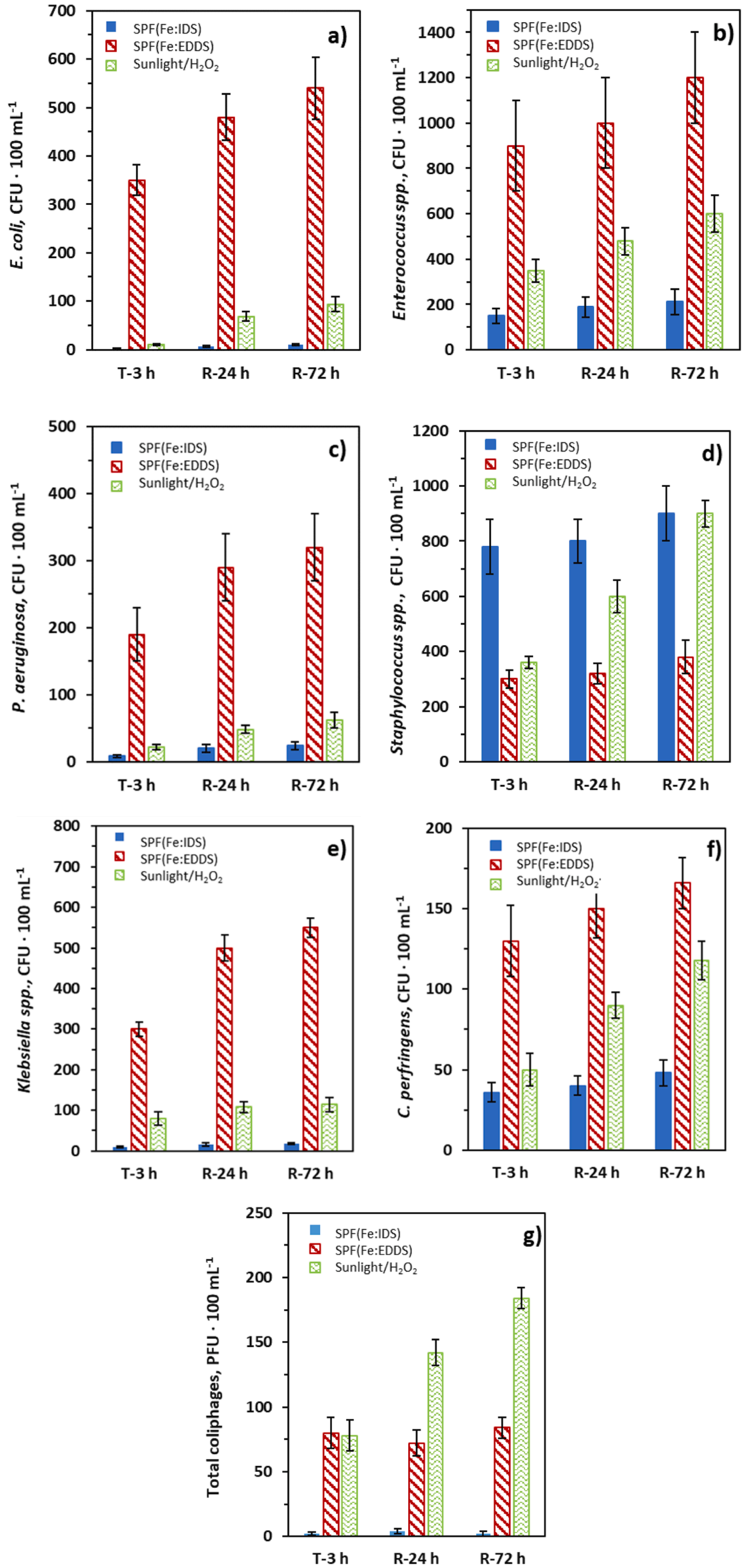

Fig. 3. Regrowth experiments with catalase after Fe:IDS SPF, Fe:EDDS SPF and sunlight/H_2O_2 treatments (T-3 h), after 24 h and 72 h of regrowth (R-24 h and R-72 h) for: E. coli (a), Enterococcus spp. (b), P. aeruginosa (c), Staphylococcus spp. (d), Klebsiella spp. (e), C. perfringens (f) and Total coliphages (g).

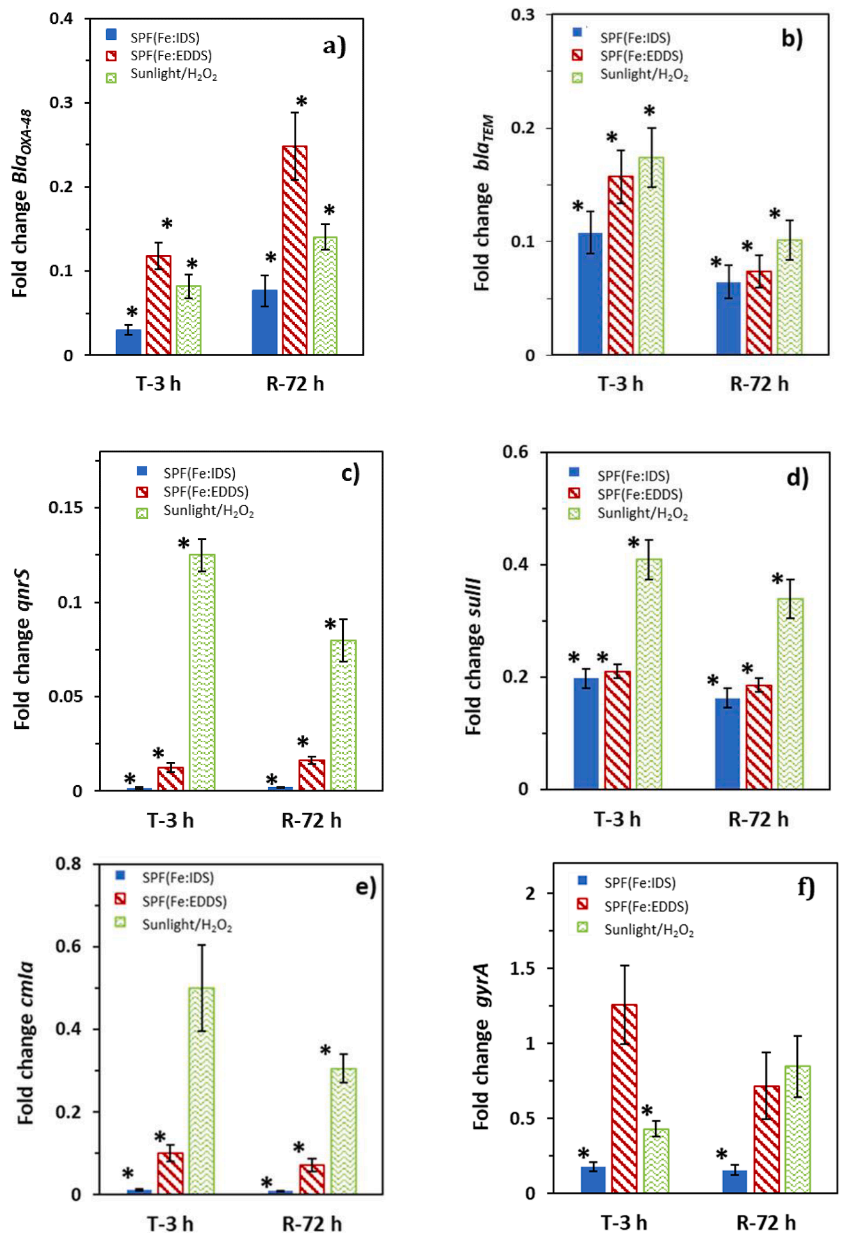

Fig. 4. ARGs removal for Fe:IDS SPF, Fe:EDDS SPF and sunlight/H_2O_2 process immediately after the treatments (T-3 h) and after regrowth tests under environmental mimicking conditions (R-72 h) in absence of catalase: bla_{OXA48} (a), bla_{TEM} (b), qnrS (c), sulII (d), cmIa (e), gyrA (f), tetA (g), mefA(h), intI1 (i). Fe:L = 0.1 mM: 0.1 mM, H_2O_2: 1.47 mM. The relative fold changes were analyzed and data were normalized with 16 s RNA COM. Normally distributed data were analyzed by one-way ANOVA, followed by Tukey's test for post-hoc analysis. $p<0.05$ was regarded as statistically significant. * = $p<0.05$.

2023). Moreover, the lower H_2O_2 consumption by Fe:IDS leads to an increased probability of H_2O_2 to pass through cell membrane and stimulate the intracellular SPF process. Coliphages also were less affected by Fe:EDDS SPF, as a consequence of the lower injury on *E. coli*, compared with Fe:IDS; the lower the number of host bacterial cells damaged, the higher the probability for coliphages to continue their replication cycle (Voumard et al., 2019).

Klebsiella spp inactivation (Fig. 1e) occurred at a lesser extent for the sunlight/H_2O_2 process (2.2 log reduction) and for Fe:EDDS SPF process (1.8 log reduction), (Fig. 1c). *P. aeruginosa* (Fig. 1b) also showed lower susceptibility to sunlight/H_2O_2 (1.6 log reduction) and Fe:EDDS SPF (0.8 log reduction) processes.

For the *Enterococcus spp*. (Fig. 1a) a decrease in the inactivation log was observed for sunlight/H_2O_2 (1.7 log) and Fe:EDDS SPF (1.3 log) processes compared with the 2.2 log of Fe:IDS SPF. Being a gram-positive bacteria, with a cell wall more resistant to the radicals attack than gram negative's one, the migration of iron and hydrogen peroxide to promote the intracellular photo-Fenton is hampered (García-Fernández et al., 2019; Maniakova et al., 2021a). Finally, *Staphylococcus spp*. proved to be the most resistant pathogens to SPF and sunlight/H_2O_2 processes (Fig. 1d), with only 1.4 log reduction for Fe:IDS SPF, while better results (1.7 and 2.1 log) were obtained with sunlight/H_2O_2 (Fig. 1d) and Fe:EDDS SPF processes (Fig. 1d).

3.1.2. Effect of disinfection processes on post-treatment pathogens regrowth

Post-treatment pathogens regrowth is a source of concern due to the release of the effluent into the environment (Xie et al., 2022) or its reuse for agricultural purposes (Rizzo et al., 2018). Two sets of experiments with and without catalase addition at the end of the treatment (180 min) were carried out to evaluate the effect of residual H_2O_2 on pathogens

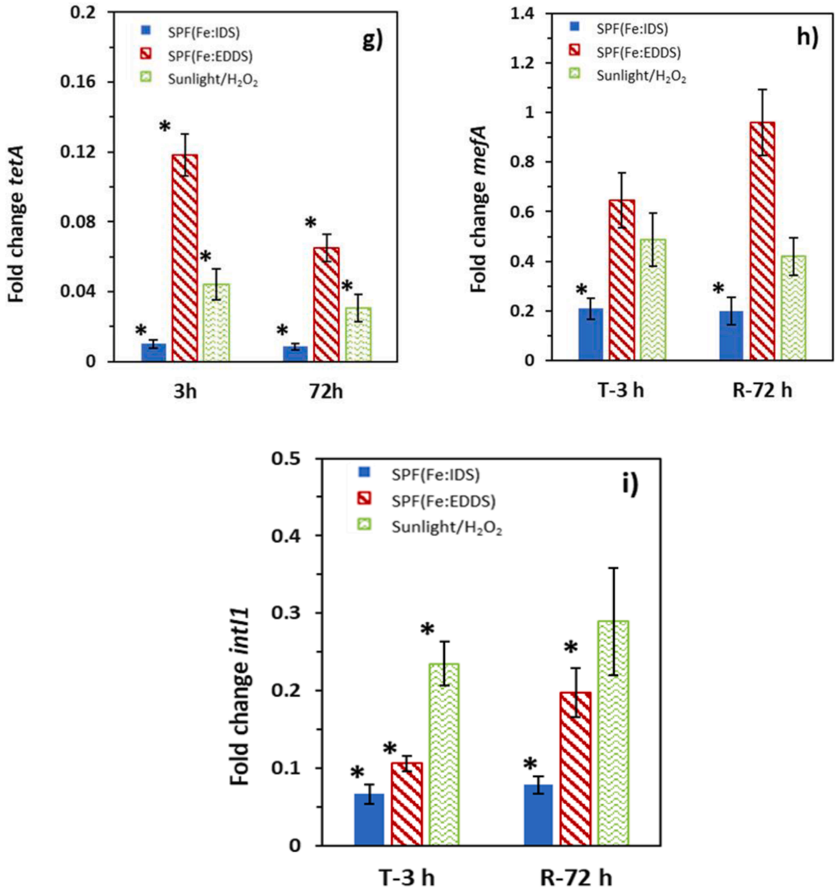

Fig. 4. (*continued*).

regrowth after 24 h and 72 h incubation time. Standard cultivation media are selective and may not detect injured cells, which can eventually regrow under favorable environmental conditions (Rizzo et al., 2004; Di Cesare et al., 2020b). No statistically significant regrowth (p<0.05) was observed for almost all the treatments in the samples without catalase addition (Fig. 2a-2g), but a slight regrowth after 24 h for *E. coli* and *Klebsiella spp*. when wastewater was treated with Fe:EDDS SPF process. Although the regrowth was not statistically significant, proving the processes to be effective in the control of pathogens regrowth under the investigated conditions, from a practical and operational point of view the risk that *E. coli* could exceed the very stringent limit for wastewater reuse in agriculture (10 CFU/100 mL for class A water) should not be overlooked.

The results available in the scientific literature on bacterial regrowth tests after disinfection treatment not only vary from process to process but also for the same disinfection process, as the operating conditions (e.g., dose of the disinfectant, treatment time), the characteristics of the aqueous matrix (e.g., real or simulated aqueous matrix) and type and number of organisms vary (Fiorentino et al., 2015; Iakovides et al., 2019; Li et al., 2013). A slight decreasing regrowth trend was observed for *C. perfringens*, which may be due to a higher sensitivity to residual hydrogen peroxide (Harmon and Kautter, 1976). Such a behavior is more evident for Total coliphages, possibly as a result of a combination of factors including the inactivation of the bacterial host cells (*E. coli*) and direct inactivation of the coliphages (Giannakis et al., 2016c; Naureen et al., 2020).

When catalase was added to the treated samples, the highest trend in the pathogens regrowth (statistically significant (p <0.05) after 24 h) was observed for sunlight/H_2O_2 (especially for *E. coli, Staphylococcus spp*., *C. perfringens* and coliphages, Fig. 3a, 3d, 3f and 3g, respectively). This result is in agreement with our previous work where the sunlight/H_2O_2 process, even if more effective than chlorination, could only delay bacterial regrowth (Fiorentino et al., 2021a). This behavior can be explained by the higher dependence of sunlight/H_2O_2 process from the residual H_2O_2 concentration (Fig. 2h). When H_2O_2 is quenched by catalase, the effect of sunlight/H_2O_2 process on pathogens stops. This is more evident for the most abundant pathogen (*E. coli*), for the *Staphylococcus spp*., which offers a certain resistance to disinfection (Fig. 2d) and for coliphages, whose life cycle relies upon *E. coli.* These outcomes confirmed the role of residual H_2O_2 to preserve the quality of the treated wastewater, especially for sunlight/H_2O_2, while a lower dependence was observed for SPF processes, possibly due to the different inactivation mechanisms explained above.

3.2. Effect of disinfection processes on AR transfer potential

Horizontal gene transfer of AR can encompass different DNA transfer mechanisms. Among these, conjugation is the mechanism by which an antibiotic resistant donor cell comes into contact with a non-resistant recipient cell by exchanging DNA through the pilus (Dodd, 2012). Natural transformation is as an AR transfer mechanism wherein cells take up DNA (ARGs) from the extracellular environment (Dodd, 2012). Although transformation has been found to result in the transfer of clinically relevant ARGs in a variety of human pathogens and it is generally considered to not be as important in the transfer of ARGs compared to the conjugation mechanism, it is likely that its contribution to HGT is underestimated (Winter et al., 2021). In our work, although the investigated processes proved to be effective in inactivating the target pathogens, the residual bacterial load can be still high (Fig. 1), especially for some species and in particular if the effluent should be in compliance with regulations for disposal into the environment and not those for reuse. Therefore, taking into account that the seven bacterial

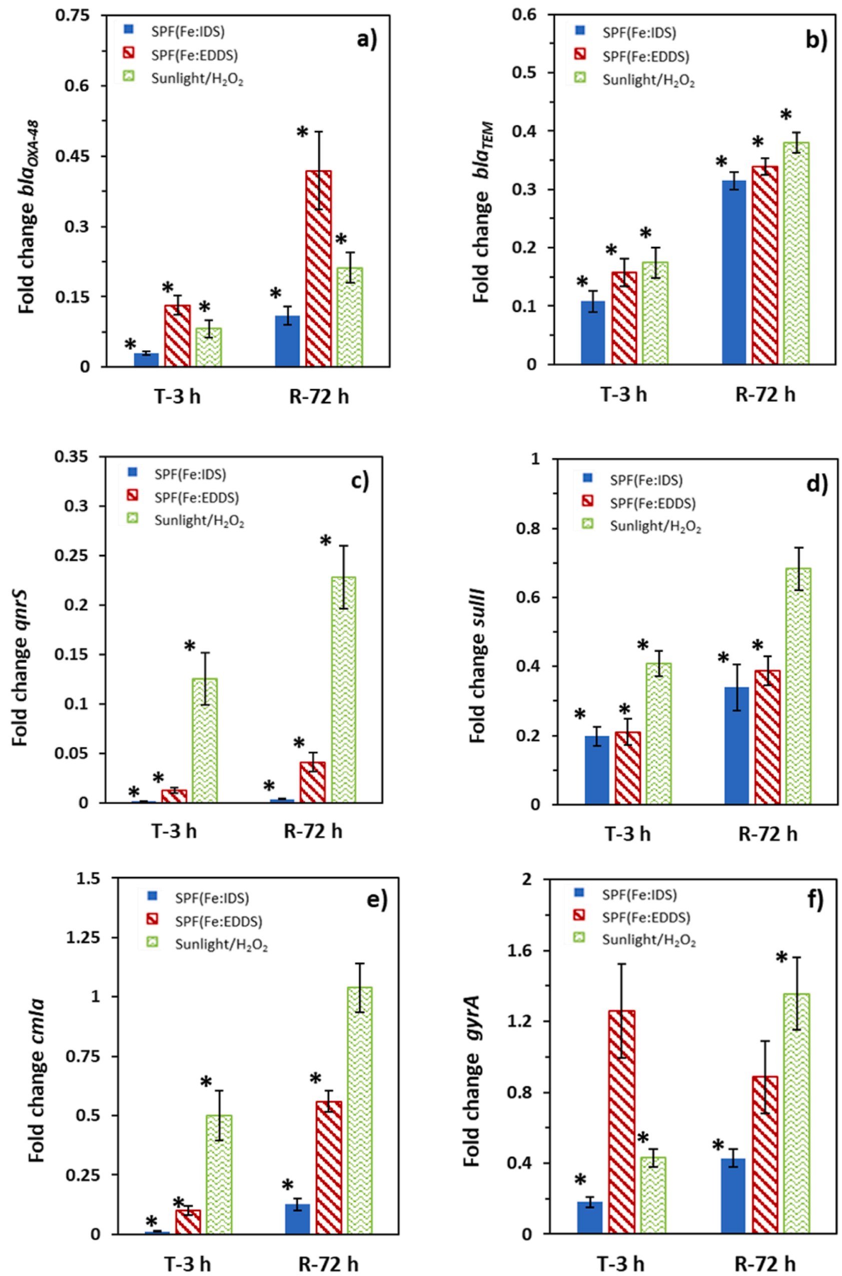

Fig. 5. ARGs removal for Fe:IDS, Fe:EDDS SPF processes and sunlight/H_2O_2 process immediately after the treatments (3 h) and after regrowth under environmental mimicking conditions (72 h) in presence of catalase: blaOXA48 (a), blaTEM (b), qnrS (c), sulII (d), cmIa (e), gyrA (f), tetA (g), mefA (h), intI1 (i). Fe:L = 0.1 mM: 0.1 mM, H_2O_2: 1.47 mM. The relative fold changes were analyzed and data were normalized with 16 s RNA COM. Normally distributed data were analyzed using one-way ANOVA, followed by Tukey's test for post-hoc analysis. $p<0.05$ was regarded as statistically significant. * = $p<0.05$.

families investigated in this work are a small part of the total bacterial population in the wastewater (Di Cesare et al., 2020b), a contribution from the residual bacterial population to the diffusion of the AR through the conjugation mechanism cannot be excluded, as also confirmed by the results on regrowth tests (Fig. 2).

The abundance of the target ARGs was analyzed before and after treatment (3 h) and the potential of AR transfer was evaluated through regrowth tests, with and without catalase addition (Fig. 4 and Fig. 5, respectively). Each gene was measured and normalized with respect to the 16S rRNA gene and the results were expressed as fold changes values (SM file, Table SM7-SM9). The quantification of ARGs takes into account both the intracellular and extracellular ones and therefore gives an estimate of the effect of the disinfection processes on the potential of AR transfer by conjugation and of natural transformation mechanisms.

The investigated processes affected ARGs abundance after 180 min in different extents depending on the target ARG (Fig. 4). Overall, Fe:IDS SPF showed better performances and the order of removal in terms of target ARGs was: *qnrS*> *tetA* >*cmIa*> bla_{OXA-48} > *intI1*-bla_{TEM}> *gyrA*-*sulII*-*mefA*. Fe:IDS SPF (fold change: 0.03) and sunlight/H_2O_2 processes (fold change: 0.08) were found to be more effective than Fe:EDDS SPF process (fold change: 0.12) in the removal of bla_{OXA48}. These two processes were also found more effective in controlling bla_{OXA48} in the regrowth experiments (72 h) after the end of the treatment ($p<0.05$), while for bla_{TEM}, a removal was observed 72 h after the end of treatment in the absence of catalase. Fe:IDS and Fe:EDDS (0.001 and 0.01 fold change respectively) SPF processes showed to be more efficient than sunlight/

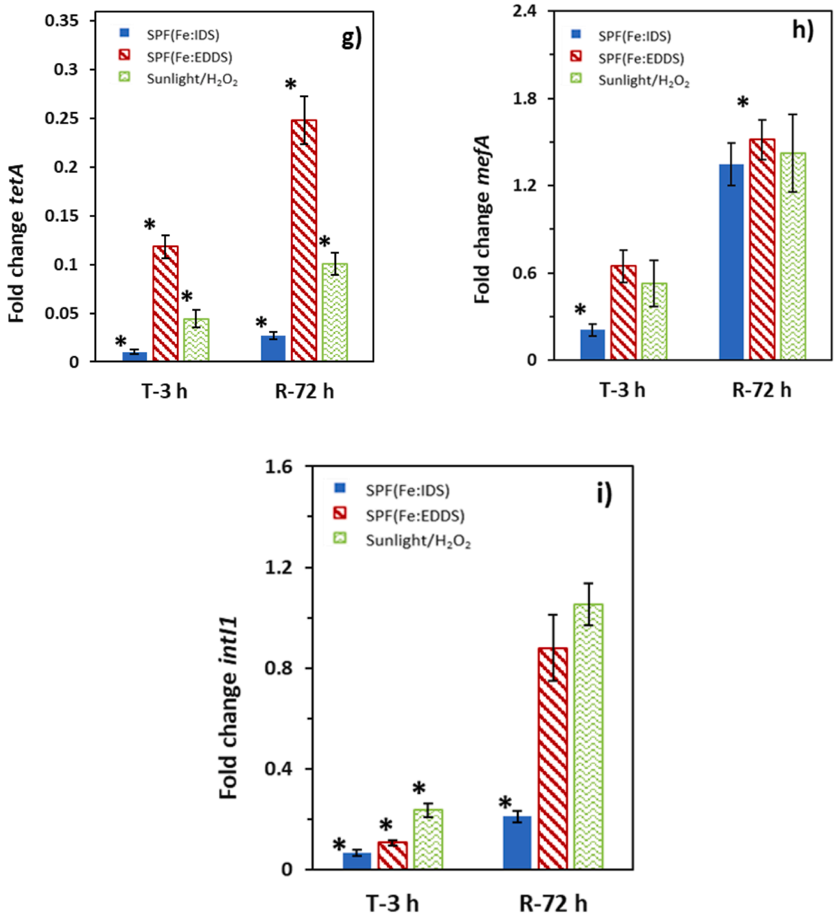

Fig. 5. (*continued*).

H_2O_2 (0.12 fold change) in the removal of the *qnrS* gene, and no increase in abundance was found 72 h after the treatment. No significant differences were observed between Fe:IDS and Fe:EDDS SPF processes in *sulII* removal, while for *cmIa, gyrA, tetA* and *mefA* Fe:IDS SPF process showed better performances than the other treatments (Fig. 4). In particular, the sunlight/H_2O_2 process resulted to be not very efficient to control the regrowth of *gyrA* (from 0.4 to 0.8-fold change). Noteworthy, the removal of *intI1*, a common indicator of anthropogenic environmental pollution (Gillings et al., 2015) and of potential HGT (Di Cesare et al., 2016), was higher for SPF processes than the sunlight/H_2O_2 process. The sunlight/H_2O_2 process has been showed to reduce ARGs with an average removal of 1 log (Moreira et al., 2018). In other studies, no significant effects on bla_{TEM} gene were observed after UV/H_2O_2 (Ferro et al., 2016) or Fe:NTA SPF (Fiorentino et al., 2022) treatment.

However, aqueous matrix is expected to drastically affect process efficiency in terms of ARGs removal (Ortega-Gómez et al., 2014). ARGs removal by Fe:EDDS SPF drastically decreased from ultrapure water (4 log) to real wastewater (1 log) (Ahmed et al., 2021).

Experiments made adding catalase to the treated wastewater samples at the end of each treatment and observing regrowth after 72 h, confirmed that the presence of H_2O_2 in solution plays a key role to keep under control the regrowth process, according to the results observed for the pathogens (Fig. 5). This is essentially true for Fe:IDS SPF process in the case of *sulII, cmIa, gyrA, tetA, mefA* and *intI1* removal (Fig. 5). Particularly, for *mefA*, catalase addition resulted in a value (fold change: 1.3) not significantly different from that occurring before the treatment, while without catalase a strict control of regrowth was observed. In the presence of catalase, sunlight/H_2O_2 process showed higher regrowth than in absence and than Fe:IDS SPF process, but for *mefA*. A regrowth of bla_{TEM} was observed for all the investigated processes when catalase was added (Fig. 5), even if the final expression of this gene remained below the starting one (fold change range: 0.3–0.4 (fold change <1), $p<0.05$).

Coliphages are bacteriophages that infect coliform bacteria such as *E. coli*. Bacteriophages have the potential to transfer ARGs among bacterial cells through a mechanism known as transduction (Dodd, 2012). Although total coliphages abundance is only one of possible indicators of transduction transfer mechanism, monitoring the effect of disinfection processes on bacteriophages, in addition to ARGs, has been recommended to better characterize the risk of AR spread (Kumlien et al., 2021). According to the results on pathogens inactivation discussed above, Fe:IDS was the best treatment for the inactivation of coliphages (3.6 log removal) as well as to control their regrowth in absence of catalase. When residual H_2O_2 concentration after treatment is roughly zero (regrowth tests with catalase), coliphages can regrow after sunlight/H_2O_2 treatment unlike of SPF processes (Fig. 3g).

4. Conclusions

The effects of SPF with Fe:IDS on the inactivation of seven relevant pathogens and ARGs in real urban wastewater were investigated and compared to SPF with Fe:EDDS and sunlight/H_2O_2 processes for the first time. Fe:IDS SPF resulted to be more effective in pathogens inactivation compared to the other processes due to a better combination of intra- and extra-cellular photo-Fenton mechanisms. Moreover, no relevant pathogens regrowth was observed, even in the absence of catalase, condition investigated to evaluate the effect of residual H_2O_2 as well as to simulate possible regrowth under a more realistic scenario. The effect of the investigated processes on ARGs was found to be gene dependent, which makes it difficult to draw conclusions on the effect of the investigated processes on the natural transformation and conjugation mechanisms. Noteworthy, coliphages can regrow after sunlight/H_2O_2 treatment unlike SPF processes; this factor leads us to consider that the risk of AR transfer by transduction mechanism is probably higher with sunlight/H_2O_2 process. Anyway, the presence of other factors contributing to the transduction process and requiring further deep investigations makes it difficult for us to define at what extent SPF process can prevent AR by transduction mechanism. In conclusion, Fe:IDS SPF is an attractive solution for tertiary treatment of urban wastewater in small WTPs as it can provide effective disinfection toward several pathogens and a higher protection against AR transfer than the other investigated processes.

CRediT authorship contribution statement

Pellegrino La Manna: Methodology, Investigation, Formal analysis, Writing – original draft. **Marco De Carluccio:** Methodology, Investigation, Formal analysis, Writing – original draft. **Gianmaria Oliva:** Formal analysis, Writing – review & editing. **Giovanni Vigliotta:** Methodology, Supervision, Writing – review & editing. **Luigi Rizzo:** Conceptualization, Methodology, Supervision, Writing – review & editing, 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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120966.

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