Metagenomic analysis of MWWTP effluent treated via solar photo-Fenton at neutral pH: Effects upon microbial community, priority pathogens, and antibiotic resistance genes

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HIGHLIGHTS

• Photo-Fenton had significant impact on secondary wastewater bacteria diversity.
• Proteobacteria enrichment in the presence of hydrogen peroxide must be investigated.
• Total removal of priority pathogens K. pneumoniae and P. aeruginosa by photo-Fenton
• Complete removal of resistance genes related to β-lactams and fluoroquinolones
• Sul1, tet(X), tet(C) are potential indicators of antibiotic resistance in wastewaters.

GRAPHICAL ABSTRACT

ABSTRACT

The effectiveness of advanced technologies on eliminating antibiotic resistant bacteria (ARB) and resistance genes (ARGs) from wastewaters have been recently investigated. Solar photo-Fenton has been proven effective in combating ARB and ARGs from Municipal Wastewater Treatment Plant effluent (MWWTP). However, most of these studies have relied solely on cultivable methods to assess ARB removal. This is the first study to investigate the effect of solar photo-Fenton upon ARB and ARGs in MWWTP by high throughput metagenomic analysis (16S rDNA sequencing and Whole Genome Sequencing). Treatment efficiency upon priority pathogens and resistome profile were also investigated. Solar photo-Fenton (30 mg L−1 of Fe2+ intermittent additions and 50 mg L−1 of H2O2) reached 76–86% removal of main phyla present in MWWTP. An increase in Proteobacteria abundance was observed after solar photo-Fenton and controls in which H2O2 was present as an oxidant (Fenton, H2O2 only, solar/H2O2). Hence, tolerance mechanisms presented by this group should be further assessed. Solar photo-Fenton achieved complete removal of high priority Staphylococcus and Enterococcus, as well as Klebsiella pneumoniae and Pseudomonas aeruginosa. Substantial reduction of intrinsically multi-drug resistant bacteria was detected. Solar photo-Fenton removed nearly 60% of ARGs associated with sulfonamides, macrolides, and tetracyclines, and complete removal of ARGs related to β-lactams and fluoroquinolones. These results indicate the potential of using solar-enhanced photo-Fenton to limit the spread of antimicrobial resistance, especially in developing tropical countries.

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1. Introduction

Antimicrobial resistance (AMR) challenges the treatability of infectious diseases as it decreases the performance of antibiotics used to treat infected patients. Treatment processes applied in Municipal Wastewater Treatment Plants (MWWTP) play a key role on the spread of ARB and ARGs to the environment. The collection of ARGs present in MWWTP (resistome) is influenced by the high density and rate of interaction between microbial communities aligned to subinhibitory concentrations of antibiotics in biological reactors. These factors favor ARG transfer to non-resistant strains resulting in ARB enrichment in the discharged effluent (Murray et al., 2018; Stanton et al., 2020). Therefore, reducing AMR in MWWTP remains a critical challenge (Fiorentino et al., 2019; Vikesland et al., 2019).

The World Health Organization (WHO) highlights that the surveillance of critical hotspots of AMR (i.e., MWWTP) is essential. In order to monitor the threat, it is necessary to take a “One Health” approach involving coherent and concerted multisectoral (human, animal, and environmental) actions to counter AMR at various levels. Hence, many countries adopt this perspective by tackling the spread of ARB promoted by MWWTP discharge (Collignon and McEwen, 2019). The WHO prioritized ARB on their list of ‘global priority pathogens’ which pose the greatest threat to human health, such as some bacterial species and their accompanying resistome (collection of ARGs carried by these species) (e.g., carbapenem-resistant Acinetobacter baumannii; cefalosporin-resistant Klebsiella pneumonia; vancomycin-resistant Enterococcus faecium, etc.) (WHO, 2017; Starling et al., 2021b). Consequently, investments in the development of advanced wastewater treatment strategies that promote the removal of ARB and ARGs from municipal wastewater (MWW) prior to discharge have increased.

Many studies indicate that tertiary treatment technologies such as chlorination, UV-C, and ozonation are ineffective to remove ARB and ARGs from MWWTP (Di Cesare et al., 2020; Lee et al., 2017; Narciso-da-Rocha et al., 2018). Chlorination may select ARB favoring their spread and affects intra and extracellular concentrations of ARGs (Guo et al., 2015; Hou et al., 2019; Liu et al., 2018). Recently, ozonation has been associated with the selection of Pseudomonas aeruginosa (Moreira et al., 2021), a priority pathogen according to the WHO (2017). In contrast, Advanced Oxidation Processes (AOP) are feasible methods for the inactivation of bacteria and elimination of ARGs as oxidative radicals damage cell membrane and DNA structure through free radical reactions (Guo et al., 2020; Li et al., 2021; Michael-Kordatou et al., 2018). Even though, regrowth was observed after treatment by some AOP treatments especially H2O2 + sunlight (Fiorentino et al., 2019; Michael et al., 2020; Wang et al., 2021).

Photo-Fenton (Fe2+ + H2O2 + UV–Vis) has been confirmed to promote effective removal of ARB and ARGs and to inactivate cell-free ARGs from MWWTP (Michael-Kordatou et al., 2018; Vilela et al., 2021; Starling et al., 2021a). Since photo-Fenton may be carried out under sunlight, its investigation for the improvement of MWWTP quality in areas of high solar irradiance (i.e., tropical developing countries) must be further stimulated. Yet, one of the main limitations of photo-Fenton treatment is the optimal pH of operation (2.8–3.0). Considering the natural pH of MWWTP (6.0–7.5), different strategies have been investigated to apply photo-Fenton at a neutral pH level (Clarizia et al., 2017). A feasible alternative for this purpose is the intermittent iron addition strategy which assures the presence of soluble and reactive Fe2+ species throughout treatment (Carra et al., 2014, 2013; Díaz-Angulo et al., 2021). This strategy has been proven effective for disinfection and ARB removal (Starling et al., 2021a).

Nevertheless, the quantification of ARB and ARGs in MWWTP samples and analysis of solar photo-Fenton impact upon resistome profile is still challenging. Most studies apply culture-dependent methods (Ioannou-Ttofa et al., 2019; Michael et al., 2020; Moreira et al., 2018; Rodríguez-Chueca et al., 2019; Starling et al., 2021a), which are relevant as they prove the viability of ARB and expression of ARGs after treatment. Yet, culture-dependent methods may be inadequate to analyze treatment effects upon uncultivable organisms, which represent public health risks (Manaa et al., 2018; Vaz-Moreira et al., 2011). In contrast, metagenomic analyses such as 16S rDNA sequencing show high specificity and sensitivity for all organisms, no matter their viability, and enable the analysis of treatment impact upon microbial community and resistome, which are fairly diverse in MWWTP (Rizzo et al., 2013). In addition, Whole Genome Sequencing (WGS) enables the identification of all genes present in a sample using high throughput screening (Ishii, 2020; Rice et al., 2020). So far, no previous studies have assessed WGS profile of MWWTP treated by solar photo-Fenton. Besides, only a few studies analyze treatment efficiency upon priority pathogens listed by the WHO (WHO, 2017) and present in MWWTP.

The goal of this study was to investigate the effects of the solar photo-Fenton process upon priority pathogens, bacterial community, and ARGs present in MWWTP by using metagenomic analyses (16S rDNA sequencing and WGS) with a deep examination of the effect of the proposed treatment upon WHO critical priority pathogens and resistome profiles.

2. Material and methods

2.1. Reagents

All reagents used in the experiments were of analytical grade. Hydrogen peroxide (H2O2, 35%) and sulfuric acid (H2SO4, 98%) were purchased from Neon. Hydrazine hydrate ferrous sulfate (Fe·SO4·7H2O) and ammonium metavanadate (NH4VO3) were provided by Nuclear. Bovine Serum Catalase (H2O2:H2O2 oxidoreductase) and acetic acid (CH3COOH, 96%) were purchased from Sigma-Aldrich. 1,10-Phenanthrolione (C12H8N2.H2O) were provided from Vetec. Synthetic supplied ammonium acetate (CH3COONH4), DNA ladder was purchased from Promega.

2.2. MWWTP sampling

MWWTP was sampled in the output of a secondary settling tank from a conventional activated sludge system throughout a whole year, comprising wet and dry seasons. Physicochemical characterization of MWWTP was performed for all samples, as presented in Table S1.

2.3. Solar photo-Fenton treatment

Solar photo-Fenton (Fe2+ + H2O2 + solar) treatment of MWWTP was conducted in a solar simulator chamber (SUNTEST CPS+, ATLAS) containing a Xenon lamp (300–800 nm) set at 268 W m−2 (30 W m−2), corresponding to the annual average irradiance in Belo Horizonte/MG. Temperature was kept constant at 35 °C. Experiments were performed at neutral pH in a glass recipient (400 mL) under continuous stirring. Preliminary Fenton and solar photo-Fenton experiments (30 mg L−1 of Fe2+ and 50 mg L−1 of H2O2) were conducted for 120 and 240 min to determine the most appropriate reaction length. Then, reactions were performed with 5 mg L−1 and 30 mg L−1 Fe2+ (intermittent additions: 0 min = 15 mg L−1; 5, 10 and 15 min = 5 mg L−1) in the presence of 50 mg L−1 of H2O2 (240 min) to determine the most appropriate iron concentration. These reagent concentrations were defined according to Vilela et al. (2021). Accumulated irradiation (Qsun) during treatments was calculated as according to Malato et al. (2009).

Solar photo-Fenton was performed using 30 mg L−1 Fe2+ (intermittent additions) and 50 mg L−1 of H2O2 for 240 min at neutral pH in all subsequent treatments. Controls consisted of Fenton (Fe2+ + H2O2), Fe2+ alone, solar + Fe2+, H2O2, solar + H2O2 and solar irradiation alone under the same operational conditions. Samples were withdrawn during reactions for residual H2O2 (Nogueira et al., 2005) and Fe2+ quantification (APHA, 2017). DNA extraction for ARB and ARG analysis


was carried out after 240 min of treatment ($Q_{in} = 22.28 \text{ kJ L}^{-1}$). Catalase enzyme (460 mg L$^{-1}$ in 0.04 M phosphate buffer) was added to consume residual H$_2$O$_2$ (Poole, 2004).

2.4. Culture-based analysis of antibiotic susceptibility for MIC

The determination of ARBs was performed by antibiotic susceptibility testing for minimum inhibitory concentrations (MIC) (CLSI, 2015). A non-selective agar medium was used to evaluate the growth of total heterotrophic bacteria (THB) present in MWWTPE before and after the proposed treatment. At the same time, ARB quantification was performed in agar medium enriched with sulfamethoxazole (SMX, 350 mg L$^{-1}$), trimethoprim (TMP, 350 mg L$^{-1}$), ciprofloxacin (CIP, 4 mg L$^{-1}$), tetracycline (TET, 16 mg L$^{-1}$), and amoxicillin (AMX, 32 mg L$^{-1}$). All plates were incubated at 37 $\pm$ 1 °C for 48 h, and colony-forming units were quantified in each plate.

2.5. DNA extraction, quality control, library preparation, and sequencing

Total DNA extraction was performed using FastDNA® Spin Kit for Soil (MP Biomedicals). DNA concentration and purity were measured by a NanoDrop UV–Vis spectrophotometer (Thermo Fisher Scientific), and structural integrity was determined by 1% agarose gel electrophoresis. Extracted DNA was shipped to Macrogen for library preparation and sequencing. Paired-end fragment libraries with a length of 450 nt from the 16S rDNA V3-V4 region were constructed using the primers 338F ACTCTACGGGAGGCAGCA and 806R GCAC TACHVGGGTWTCTAAT. 300 nt reads of each end were sequenced from fragments (Illumina MiSeq platform). Entire genomic DNA libraries were produced and sequenced (Illumina HiSeq) in 150 nt paired ends reads for WGS.

2.6. Bioinformatics analysis

2.6.1. Taxonomic assignment

All pre-processing was carried out using the Micca software (Albanese et al., 2015). Sequence read pairs were merged and quality filtering was performed by trimming primer adapters from the concatenated sequences and removing low-quality sequences (0.75% max. error and 400 nt min size). Operational Taxonomic Units (OTUs) were generated de novo by multiple and global alignments within each sample by grouping those which contained more than 97% identity. Next, resulting OTUs were classified taxonomically (Ribosomal Database Project) (Cole et al., 2014). The NAST algorithm globally aligned OTUs to generate phylogenetic profiles. Pre-processed data were used as input in R 3.6.3 (https://www.r-project.org/), Phyloseq (McMurdie and Holmes, 2013), and vegan (Oksanen et al., 2019) packages.

A total of 7,486,167 high-quality sequences (>465 bp) were retained by 16S sequencing analysis. Good’s coverage was higher than 99% (Good, 1953), indicating that the dataset was representative of the bacterial communities present in samples. Relative abundance in MWWTPE samples was compared by Kruskal-Wallis and Wilcoxon tests ($p < 0.05$) (Segata et al., 2011). Diversity and richness were calculated using R package pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html) and Circos (Krzywinski et al., 2009). Inhouse perl and R scripts were used to parse data.

Analysis of genes which confer resistance to H$_2$O$_2$ (KatA1, KatA2, KatMn, and KatE, AhpCF, Gpx1, Gpx2, and Gpx3) were carried out using filtered reads aligned against catalase (E1S7Y1_HELPL, G0L8N0_ZOBGA and CATE_ECOLI), hydroperoxidase (Q5RQT2_BACFG), and glutathione peroxidase (GPX1_SYNY3, GPX2_SYNY3 and A0A5P9CBT8_9PSED) peptide sequences through tblastn aligner (Camacho et al., 2009). Linhouse bash scripts were used to parse results and measure abundance.

3. Results and discussion

3.1. Bacterial community in MWWTPE

Phylogenetic analysis of MWWTPE bacterial community is summarized in Fig. S1. Proteobacteria was the dominant phylum in all samples (29.55% $\pm$ 10.87% of total sequences), followed by Bacteroidetes and Actinobacteria, which occurred less frequently. Actinobacteria (20.50 $\pm$ 10.78%) prevailed in samples from the dry season, whereas Bacteroidetes (19.70 $\pm$ 6.83%) were predominant in samples from the wet season. Phyla Chloroflexi (11.71 $\pm$ 9.68%), Firmicutes (3.08 $\pm$ 3.38%), and Acidobacteria (2.16 $\pm$ 1.88%) were also represented in samples. Proteobacteria along with divergent proportions of Bacteroidetes, Chloroflexi, Actinobacteria, Acidobacteria, and Firmicutes were also detected in MWWTPE from activated sludge reactors worldwide (de Celis et al., 2020; Numberger et al., 2019). No significant differences concerning bacterial composition were detected in the different samples ($p < 0.05$). This indicates the stability of MWWTPE microbial community and reflects the operational consistency of the activated sludge system applied in the MWWTP.

3.2. Effect of solar photo-Fenton on bacteria community

Solar photo-Fenton reaction applied for 240 min (22.28 kJ L$^{-1}$, 49.5 mg L$^{-1}$ of H$_2$O$_2$ consumption) was the most efficient condition for the reduction of microbial community diversity (Fig. S2c). In solar processes, treatment efficiency is highly associated with accumulated irradiation during treatment ($240 \text{ min} = 22.28 \text{ kJ L}^{-1}$; 120 min $= 11.14 \text{ kJ L}^{-1}$). The incident irradiation used in this study was equivalent to $30 \text{ W m}^{-2}$ which equals average incident irradiation in tropical locations. In this way, reaction time and reactor volume for the application of solar processes must be determined for each location and season after the conduction of scale-up experiments (Starling et al., 2021b). Enhanced disinfection rates are expected to occur after prolonged exposure to irradiation alone as it promotes cell membrane damage and leads to the formation of oxidative radicals from matrix components (Giannakis, 2018). Additionally, enhanced H$_2$O$_2$ consumption during photo-Fenton reactions is associated with a higher generation of oxidative radicals (Eq. (1)), thus resulting in exposure of bacteria to highly hostile conditions. Exposure of bacteria to these conditions initially causes reduced damage, yet prolonged treatment times lead to accumulated injury and eventual cell death (Serna-Galvis et al., 2019; Verbel-Olarte et al., 2021).
In contrast, recent literature points out to the spread and selection of ARB and ARGs after the application of traditional technologies such as Chlorine, ozonation and UV-C irradiation/oxidant (Jin et al., 2020; Kirchner et al., 2020; Lee et al., 2021; Moreira et al., 2021; Sharma et al., 2019). In addition, ozonation and UV-C irradiated processes are energy intensive and costly, which may hinder their application in developing countries. Meanwhile, solar photo-Fenton explores a natural and costless energy source which is abundant in tropical developing countries. Meanwhile, solar photo-Fenton explores a natural and costless energy source which is abundant in tropical developing countries.

Solar photo-Fenton using 30 mg L\(^{-1}\) of iron (intermittent additions) (Fig. S2b) had a higher impact upon microbial community diversity (640 OTUs) compared with 5 mg L\(^{-1}\) of iron (899 OTUs). Higher availability of Fe\(^{2+}\) in the system using 30 mg L\(^{-1}\) led to greater oxidant consumption (Fig. S2c) in the reaction between iron and H\(_2\)O\(_2\) (Eq. (1)) and consequently higher generation of hydroxyl radicals (HO\(^{•}\)) which react quickly with cell components such as DNA (10\(^{-2}\)–10\(^{-5}\) M s\(^{-1}\) (Neta et al., 1982), thus promoting disinfection and decreasing the diversity of microbial community present in MWWTPE.

The intermittent iron addition strategy ensured the continuous presence of Fe\(^{2+}\) during reactions at neutral pH even after 60 min (Fig. S2c), thus being shown to be effectively overcoming the limitation associated with optimal pH for the operation of photo-Fenton (Carra et al., 2014, 2013; Clarizia et al., 2017; Díaz-Angulo et al., 2021). Fe\(^{2+}\) cycling (Eq. (2)) is enhanced in the photo-Fenton system, and an extra route for the formation of HO\(^{•}\) (Eq. (3)) occurs under UV-A irradiation via light adsorption by iron hydroxides formed in the system (Tarr, 2003).

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{HO}^{•} + \text{OH}^{−} \quad (1) \\
\text{Fe}^{3+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{2+} + \text{HO}_2^{•} + \text{H}^{+} \quad (2) \\
\text{Fe(OH)}_{3}^{++} + \text{hv}(540-580 \text{ nm}) & \rightarrow \text{Fe}^{2+} + \text{HO}^{•} \quad (3)
\end{align*}
\]

PCA indicated significant (p < 0.05) differences in the taxonomic structure of bacteria community before and after treatment and controls (Fig. 1a). Solar photo-Fenton samples clustered on the bottom left side (Fig. 1a), showing significant differences in bacterial community diversity compared with MWWTPE samples (right upper side). The lowest average of microbiome diversity (Chao 1 diversity index) and abundance-based coverage estimator (ACE) (39% below MWWTPE sample) was detected in solar photo-Fenton samples, thus confirming the disinfection potential of this process (Fig. 1b). These results were confirmed by culture-based analysis of total heterotrophic bacteria and ARB. As shown in Fig. S2d, solar photo-Fenton treatment achieved nearly 70% removal of cultivable THB. The treatment was also efficient in eliminating ARBs (85% removal). ARB removal was enhanced under solar photo-Fenton (~1 log removal for ARB resistance to all tested antibiotics) compared to Fenton control for which removal of ARBs resistant to tetracycline (~1 log removal for ARB resistance (Galvis et al., 2019; Verbel-Olarte et al., 2021). These results were consistent with reports made in other studies (Serna-Galvis et al., 2019; Verbel-Olarte et al., 2021).

Solar photo-Fenton effectively removed most of the main phyla present in MWWTPE (Fig. 2a), achieving 86% removal of Acidobacteria, 80% removal of Chloroflexi, and around 79%, 76%, and 74% removal of Actinobacteria, Bacteroidetes, and Firmicutes, respectively. Unclassified taxa were reduced by 67%. These results are promising since priority ARB belong to Proteobacteria (e.g., P. aeruginosa, A. baumannii, N. gonorrhea, etc.), Firmicutes (e.g., E. faecium, S. pneumoniae, S. aureus), and Bacteroidetes (e.g., C. normannense, C. meningosepticum) phyla (Quintela-Baluja et al., 2019; Su et al., 2017). Besides, bacteria from Proteobacteria and Actinobacteria phyla are major hosts of ARGs carried by Methicillin-Resistant Staphylococcus aureus [MRSA]-related due to the addition of iron salts and H\(_2\)O\(_2\) to the system intensify outer cell damage (Giannakis, 2018); and (v) transport of H\(_2\)O\(_2\) to the inner cell compartment via porins (Feng et al., 2020) enhances internal photo-Fenton reactions.

As shown in Fig. 1c, pH ranged from 6.5 and 7.5 during solar photo-Fenton and control Fenton. The pH stability during photo-Fenton at neutral pH using the intermittent iron addition strategy was also observed by Stirling et al. (2021a). Recent studies indicate that solar photo-Fenton efficiency is hindered at neutral pH due to iron precipitation (Carra et al., 2013; Clarizia et al., 2017). However, the intermittent iron addition strategy mitigates this effect since dissolved iron is present in the system during the entire treatment. Iron precipitation occurs gradually, avoiding a turbidity peak usually associated with light scattering effects. Final Fe\(^{2+}\) concentration was under 5 mg L\(^{-1}\) after photo-Fenton and Fenton treatments, which is below discharge limits and presents no risks to aquatic environments (Silva et al., 2018; Stirling et al., 2021a).

A cluster containing control samples (H\(_2\)O\(_2\) + solar and H\(_2\)O\(_2\)) was formed on the upper left side (Fig. 1a). This is concurrent with results obtained in culture-based bacterial analyses of THB and ARB (Fig. S2d). These controls were less efficient in the removal of ARBs (except for ARBs resistant to tetracycline - removal ~1 log) compared to solar photo-Fenton treatment. Although H\(_2\)O\(_2\) + solar control showed ~75% removal of THB (Fig. S2d), the control containing only H\(_2\)O\(_2\) did not show a significant percentage of THB removal, being efficient only in removing tetracycline-resistant ARBs. The effect of these controls upon microbial community and cultivable bacteria may be associated with H\(_2\)O\(_2\) consumption (nearly 30%) (Fig. 1c), which is related to the oxidation of matrix and bacteria cell components. H\(_2\)O\(_2\) alone may disrupt the lipid bilayer of the bacteria cell membrane as it oxidizes lipids (Siddique et al., 2012). In addition, the transportation of H\(_2\)O\(_2\) to the inner cell compartment and the release of Fe\(^{2+}\) from enzymes by the action of irradiation alone (Feng et al., 2020) promotes the internal photo-Fenton during H\(_2\)O\(_2\) + solar (Giannakis, 2018), thus contributing to disinfection. Similar disinfection rates were also observed for solar photo-Fenton at neutral pH and H\(_2\)O\(_2\) + solar in previously published articles (Giannakis, 2018; Maniakova et al., 2021; Michael-Kordatou et al., 2018; Michael et al., 2020). Although not assessed in this study, ARB regrowth may be used as an additional indicator of disinfection efficiency. Bacteria regrowth was observed after 48 h of storage following H\(_2\)O\(_2\) + solar (Fiorentino et al., 2015; Michael et al., 2020) while no regrowth occurred after solar photo-Fenton treatment (Fiorentino et al., 2019).

The single cluster formed by MWWTPE and controls samples (Fe\(^{2+}\) + solar; Fe\(^{2+}\) + solar) (right center side) (Fig. 1a), shows that these controls had no significant effect upon the original bacterial community. This is confirmed in alpha diversity analysis (Fig. 1b) as no significant difference was detected between control samples and MWWTPE. Lack of impact upon MWWTPE microbial community diversity after solar irradiation alone is consistent with reports made in other studies (Serna-Galvis et al., 2019; Verbel-Olarte et al., 2021).

**3.3. Effect of solar photo-Fenton on bacterial phyla**

Solar photo-Fenton effectively removed most of the main phyla present in MWWTPE (Fig. 2a), achieving 86% removal of Acidobacteria, 80% removal of Chloroflexi, and around 79%, 76%, and 74% removal of Actinobacteria, Bacteroidetes, and Firmicutes, respectively. Unclassified taxa were reduced by 67%. These results are promising since priority ARB belong to Proteobacteria (e.g., P. aeruginosa, A. baumannii, N. gonorrhea, etc.), Firmicutes (e.g., E. faecium, S. pneumoniae, S. aureus), and Bacteroidetes (e.g., C. normannense, C. meningosepticum) phyla (Quintela-Baluja et al., 2019; Su et al., 2017). Besides, bacteria from Proteobacteria and Actinobacteria phyla are major hosts of ARGs carried by Methicillin-Resistant Staphylococcus aureus [MRSA]-related
(mecA, qacA, qacB, norA) and carbapenem-Resistant Enterobacteriaceae [CRE] (KPC, NDM, OXA-48) (Yin et al., 2019; Zhang et al., 2021, 2019). An almost four-fold enrichment was observed for Proteobacteria in all samples containing H₂O₂, thus indicating a positive selection of this group (Fig. 2a). Proteobacteria selection was also observed after ozonation of municipal wastewater (Moreira et al., 2021) and suggested higher resistance of this group to oxidative conditions. The bacterial community present in MWWTPE is sensitive to H₂O₂, which can function either as a disinfectant (Apel and Hirt, 2004) or as an oxygen source enhancing aerobic bacterial growth (Hinchee et al.,

**Fig. 1.** Beta diversity obtained by PCoA analysis (a), alpha diversity metrics obtained by non-parametric richness estimator (Chao 1 and ACE) (b), and pH, hydrogen peroxide consumption, and dissolved iron concentration after solar photo-Fenton treatment and controls carried out for 240 min \( Q_{av} = 22.28 \text{ kJ L}^{-1} \) (c).
Fig. 2. Phylogeny of bacterial communities (a); abundance of Proteobacteria classes (b); and classes and genera of Proteobacteria in samples treated by solar photo-Fenton (treatment time: 240 min, Q_{viso} = 22.28 kJ L^{-1}) (c). Taxa with abundance below 1% and unclassified taxa were designated as NA.
Six potential pathogens belonging to critical, high, and medium priority classes were detected in original MWWTP samples (WHO, 2017) (Fig. 3). Solar photo-Fenton and control Fenton completely removed high priority *Staphylococcus* and *Enterococcus* (Fig. 3). *Staphylococcus* infections carry high mortality levels when aggravated by antimicrobial resistance (Giulieri et al., 2020), and *Enterococcus* is an opportunistic pathogen associated with increased mortality rates (Leavis et al., 2006). In contrast, *Streptococcus* sp., inserted in the same group, was not entirely removed by proposed treatments. Failure to eliminate this priority group may be associated with its ability to adapt membrane composition in hostile oxidative environments (Pesakov et al., 2007).

Regarding *Acinetobacter* genera, phylogenetic analysis of solar photo-Fenton samples revealed an absence of *A. baumannii*, a critical priority pathogen (WHO, 2017). *A. johnsonii* predominated in all samples. Despite rarely causing human infections, this organism may actively acquire exogenous DNA becoming an ARG reservoir (Montaña et al., 2016). In the *Enterobacteriaceae* family, Fenton was not efficient at removing *Escherichia coli* or *Klebsiella pneumoniae* (*Enterobacteriaceae* family). Nevertheless, solar photo-Fenton completely removed *Klebsiella pneumoniae* and achieved 30% removal of *E. coli*. *Klebsiella pneumoniae* is known for having a negatively charged outer capsule (Podschun and Ullmann, 1998) which may have complexed with iron, thus lowering Fenton efficiency in the absence of light. Elimination of *Klebsiella pneumoniae* from MWWTP is critical to limit the spread of AMR as it is a major cause of hospital and community-acquired infections (Munoz-Price et al., 2013).

Growth of *Pseudomonas* and *Stenotrophomonas* was associated with their tolerance to H₂O₂. Regarding species within the *Pseudomonas* family, *P. yamanorum* predominated in treated samples. Within *Stenotrophomonas*, *S. maltophilia*, a harmful β-lactam resistant pathogen (Kumar et al., 2020), and *S. pavanii* ruled in MWWTP sample, yet were entirely removed by solar photo-Fenton.

Regarding bacteria known as ARG vectors, solar photo-Fenton achieved a substantial reduction of intrinsically multi-drug resistant *Chryseobacterium*. This is relevant as this genera is known for its resistance to chlorination applied in conventional MWWTP (Izaguirre-Anariba and Sivapalan, 2020). Solar photo-Fenton and Fenton achieved significant removal of *Legionella* and *Brevundimonas* (*Proteobacteria* cladon), commonly associated with nosocomial infections and considered pathogens of emerging concern in clinical locations (Li et al., 2018; Lytle et al., 2021; Ryan and Pembroke, 2018). In contrast, *Gordonia* sp., an opportunistic agent (Blaschke et al., 2007), and *Mycobacterium* sp. (*Corynebacteriales; Actinobacteria*), were not removed after Fenton and solar photo-Fenton. Extensively TB drug-resistant and multidrug-resistant organism *Mycobacterium tuberculosis* (Dua et al., 2018; Kumar et al., 2020) was not detected in any of the samples in this study (data not shown).
3.4. Effect of solar photo-Fenton on resistome profile: diversity and richness of ARGs

According to Fig. 4a, ARGs which confer resistance to different classes of antibiotics are abundant in the MWWTPE used in this study. Most current studies associated with ARG removal via solar photo-Fenton investigate a limited list of ARGs (blaCTX, blaTEM, blaOXA, Sul1, Sul2, emrB, tetQ, tetX, and tetM) via qPCR (Starling et al., 2021b). This is the first study to use WGS to investigate ARG removal from MWWTPE. A greater ARGs diversity (69 variations within 19 major types) (Fig. 4b) was detected, and their removal was analyzed in this study.

ARGs which confer resistance to broad-spectrum antibiotics, such as sulfonamides (sul1 and sul2), represented almost 74% of total ARGs present in MWWTPE, followed by ARGs associated to macrolides (mainly erm(F), mph(A), mph(E) and msr(E)) (~10%), tetracyclines...
(mainly tetA, tetB, tetO and tetW) (~9%) and aminoglycosides (mainly aadA, aph(3″), aph(6) and strA) (~6%). Overall, subtypes tet(X)_1, tet(X)_2, mph(E)_3 and msr(E)_4, and genes sul1 and sul2 (almost all subtypes) were the most abundant across MWWTPE samples (Fig. 4b). According to Nguyen et al. (2021) and Raza et al. (2021), ARGs conferring resistance to sulfonamides and tetracyclines are frequently detected in MWWTPE regardless of predominant bacteria taxa, season, and location. Gene sul1 usually prevails in MWW worldwide due to high sequence conservation and transfer among different species (Wei et al., 2018).

Solar photo-Fenton removed nearly 60% of ARGs associated with sulfonamides (55%), macrolides (61%), and tetracyclines (61%), and wholly removed ARGs associated with \(\beta\)-lactams and fluoroquinolones. Regarding subtypes, the treatment removed 66% of tet(X)_1 and almost 60% of tet(X)_2, mph(E)_3, and msr(E)_4 (Fig. 4b). Complete removal of ARGs associated with \(\beta\)-lactams is of remarkable relevance. Some of them are emerging threats to public health (i.e., carbapenem-resistant Enterobacteriaceae-related genes: BKC, GES, IMP, OXA, etc.) (Bush and Jacoby, 2010; Logan and Weinstein, 2017). Effect of control Fenton upon ARGs was limited to 24% and 30% for ARGs associated with sulfonamides and tetracyclines, respectively, and no substantial decay of ARGs associated to macrolide (~3%) and fluoroquinolone (~7%) classes were detected after treatment.

Results obtained by the most recent works published on the application of solar photo-Fenton have shown high efficiency of ARGs removal. Michael et al. (2019) reached complete removal of sul1, qnrS, blaOXA, blaCTX-M, and tetM, and 3 log units of blaTEM removal and Fiorentino et al. (2019) removed sul1 genes to levels below the detection limits. Although solar photo-Fenton treatment decreased the richness and diversity of the bacterial community in MWWTPE (Fig. 1a and b), significant enrichment in the abundance of Proteobacteria was observed after treatment (Fig. 2a). Proteobacteria have been reported to be potential hosts of genes that carry resistance to aminoglycosides (i.e., strA, aph, and aadA) and tetracyclines (i.e., tetQ, tetC, and tetX) (Luo et al., 2021).

3.5. Correlation between bacterial community and antibiotic resistance genes

As co-existing patterns between ARGs and bacterial communities indicate potential hosts of AMR (Jia et al., 2020) and bacterial community is considered one of the main drivers of ARGs spread, specific relationships between bacterial hosts and ARG subtypes were investigated for MWWTPE used in this study before and after solar photo-Fenton treatment. Taxa of contigs carrying ARGs were predicted at their specific phyla and family levels, respectively (Fig. 5). As shown in Fig. 5, different ARG-taxa relations were observed before and after solar photo-Fenton and control Fenton treatments. For example, tetracycline and macrolide resistance genes were correlated with almost all families in MWWTPE, while sulfonamide, \(\beta\)-Lactam, and aminoglycoside resistance genes correlated mostly with Proteobacteria (Pseudomonadaceae and Xanthomonadaceae).

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Therefore, the increased abundance of this phylum contributed to the persistence of these ARGs after treatment (Fig. 5). Within Proteobacteria, Pseudomonadaceae, Enterobacteriaceae, Campylobacteraceae, Comamonadaceae, and Xanthomonadaceae also showed a strong correlation with ARGs related to sulfonamides and macrolides in MWWTP samples. Increased abundance of Pseudomonadaceae and Xanthomonadaceae after treatment justifies the persistence of these ARGs after control Fenton and solar photo-Fenton.

The co-existence of ARGs associated with tetracycline, macrolides, and aminoglycosides (Fig. 5) was correlated with almost all families of bacteria present in MWWTP. These ARGs persisted even after high removal rates (>60%) since they were abundant in the untreated sample (Fig. 4b). Thus, relatively low removal of aph(3′), aph(6′), strA, mph(E), msr(E), and tetX (Fig. 4b) might be due to their occurrence in a varied spectrum of hosts.

Notably, families comprising multidrug-resistant bacteria (Mycobacteriaceae, Flavobacteriaceae, Xanthomonadaceae, Campylobacteraceae, Sphingomonadaceae, Pseudomonadaceae, and Enterobacteriaceae) were the leading carriers of beta-lactam resistance genes in MWWTP samples (Fig. 5). Nevertheless, these ARGs were removed after the proposed treatment (Fig. 5), confirming the results shown in Fig. 4.

Results obtained here confirm the elimination of priority pathogens (Fig. 3) and ARGs (Figs. 4 and 5) via solar photo-Fenton, thus ensuring the combat of AMR spread via MWWTPE discharge by this process. Nonetheless, some groups known as co-hosts of ARGs were selected during treatment. This fact requires further investigation and points out the use of these groups as potential AMR indicators. The establishment of global and regional indicators of AMR is critical for the control of priority pathogens and has been currently under discussion by the scientific community (Di Cesare et al., 2020).

4. Conclusions

The evaluation of the effects of solar photo-Fenton upon bacterial communities, priority pathogens, and ARGs using metagenomic analyses presented in this study appear to be novel in the scientific literature. The lowest species richness and diversity were achieved via solar photo-Fenton (30 mg L⁻¹ of Fe²⁺ and 50 mg L⁻¹ of H₂O₂; 240 min) compared to controls as the intermittent iron addition strategy was effective for treatment conduction at neutral pH. Solar photo-Fenton effectively removed the main phyta present in MWWTP (86% removal of Acidobacteria, 80% of Chloroflexi, 79% of Actinobacteria, 76% of Bacteroidetes, and 74% of Firmicutes). Solar + H₂O₂ and H₂O₂ alone showed a lower impact upon the microbial community when compared to solar photo-Fenton. Enrichment of Proteobacteria after the application of the solar oxidative treatment should be further investigated as it indicates positive selective pressure and led to the persistence of ARGs carried by this group in treated samples. Complete removal of high priority Staphylococcus and Enterococcus, critical priority K. pneumoniae and P. aeruginosa, and S. maltophilia, as well as substantial reduction of multi-drug resistant bacteria were observed. The proposed treatment also reached nearly 60% removal of ARGs associated with sulfonamides, macrolides, and tetracyclines, as well as complete removal of those related to β-lactams and fluoroquinolones. These results confirm the potential of applying solar photo-Fenton as an additional treatment stage in MWWTP to control the spread of AMR in tropical countries.

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CRediT authorship contribution statement

Study conception and design – P. B. Vilela, Rondon P. Mendonça Neto, A. S. Martins, M. C. V. M. Starling, and C. C. Amorim; experimental data – P. B. Vilela, Rondon P. Mendonça Neto, Felipe A. R. de Souza, Pires, G. F. F., A. S. Martins; manuscript writing and revision – P. B. Vilela, Rondon P. Mendonça Neto, M. C. V. M. Starling, and C. C. Amorim. All authors read and approved the final manuscript.

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.

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Appendix A. Supplementary material

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