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Abstract

We investigated the effectiveness of a heterogeneous Fenton-like reaction (ZVI-Fenton, i.e., ZVI + H_2O_2, where ZVI = zero-valent iron) towards the removal of potentially pathogenic bacteria in wastewater (WW). The effectiveness of the process was investigated towards live bacteria (measured by flow cytometry) as well as potentially pathogenic bacteria (identified by 16S rRNA amplicon sequencing), antibiotic resistance genes and class 1 integrons (assessed by qPCR). The ZVI-Fenton process resulted about as effective as H_2O_2 alone to decrease live bacteria ($p>0.05$), if compared with the blank controls (neither ZVI nor H_2O_2, $p=0.00005$), although it did not reduce the relative abundance of the tested antibiotic resistance genes or class 1 integrons ($p>0.05$). However, ZVI-Fenton was quite effective in lowering the total content of the potentially pathogenic bacteria in WW when compared to the controls with ($p=0.0186$) and without H_2O_2 ($p=0.0252$). These findings suggest that ZVI-Fenton has potential as an effective WW treatment technique that should be further investigated for future application in WWTPs.

Keywords: Zero Valent Iron; Fenton-like process; wastewater; pathogens; antibiotic resistance genes; Advanced Oxidation Processes.

1. Introduction

In wastewater treatment plants (WWTPs), disinfection is the last step devoted to minimize the pathogenic bacterial load (Di Cesare et al., 2020a). However, pathogenic bacteria can escape disinfection processes and can thus be found in treated wastewaters (WWs), from which they enter the aquatic environment (Shi et al., 2021). This is a serious concern for human health because treated WW can become a source for waterborne diseases, depending on its further use (Li et al., 2015). Furthermore, WWTPs are considered as hotspots of antibiotic resistance (Berendonk et al., 2015) and represent a direct source of antibiotic resistant bacteria and antibiotic resistance genes (ARGs) to the aquatic environment (Czekalski et al., 2014; Rizzo et al., 2013). Once in the aquatic environment, pathogenic and/or antibiotic resistant bacteria can infect humans through different ways that include skin contact, ingestion, as well as fish and shellfish contamination (Arnone and Perdek Walling, 2006; Leonard et al., 2018). Therefore, alternative and innovative disinfection processes should be investigated to improve the removal of the total potential human pathogenic bacteria from the WW bacterial communities, to prevent or reduce their release into the aquatic environment.

A possible future challenge for disinfection techniques is their coupling with depollution processes, that is, the elimination of chemical contaminants. In particular, several Advanced Oxidation Processes (AOPs) have been developed that rely on the production of strongly oxidizing transient species such as the hydroxyl ($^{\bullet}OH$) and sulfate ($SO_4^{\bullet-}$) radicals (Comninellis et al., 2008; Mirzaei et al., 2017). AOPs are involved in the degradation of pollutants including the contaminants of emerging concern (CECs), such as pharmaceuticals and personal care products (Huber et al., 2003; Wang and Zhuan, 2020). CECs are hydrophilic and often biorecalcitrant, and are usually not or incompletely removed by the traditional water treatment

methods (Richardson and Ternes, 2018). A common AOP is the Fenton reaction (1), which involves water-soluble ferrous salts and hydrogen peroxide to produce •OH (as well as additional oxidants such as ferryl, FeO^{2+}) (Pignatello et al., 2006, 1999):

$$Fe^{2+} + H_2O_2 \rightarrow FeOH^{2+} + {}^{\bullet}OH \quad (1)$$

Although it is cheap and easy to implement, the Fenton reaction has some important drawbacks: first of all, it is most efficient at pH 3 (Pignatello et al., 2006) that is hardly attainable in water treatment, due to both reagent costs and the salinity of treated water after the post-treatment neutralization step. Moreover, neutralization yields an iron-rich precipitate sludge that cannot be recycled in the process and has to be disposed of in some way (Huang et al., 2013). The addition of iron ligands can keep Fe dissolved and avoid formation of precipitates while often improving Fenton degradation, but it will cause issues with iron limits for wastewater discharge and will shift reactivity from •OH to less reactive oxidant species such as ferryl, thereby hampering degradation of some recalcitrant contaminants (Farinelli et al., 2020).

Several attempts have been made to improve the Fenton reaction, and one of the most promising is heterogeneous Fenton. It consists in the replacement of soluble Fe^{2+} species with solid Fe-based materials (Tang and Wang, 2020; Vorontsov, 2019), of which zero-valent iron (Fe^0 or ZVI) is one of the cheapest and most robust in terms of application (GilPavas et al., 2019; Joo et al., 2005; Rezaei and Vione, 2018). ZVI-Fenton also allows for water treatment to be carried out at higher pH compared to traditional Fenton (Minella et al., 2016). In particular, ZVI-Fenton has been shown to achieve effective degradation of pharmaceuticals (including hospital antibiotics) in real wastewater at pH 5 or even 6 depending on the contaminant, with competitive treatment costs (in the order of 0.04-0.1 \$ m^{-3} for the chemical reagents) (Furia et al., 2021; Minella et al.,

2019). Note that ZVI is a well-known reducing agent (Xu et al., 2019), but in the presence of dissolved oxygen it can trigger the Fenton reaction (Fu et al., 2014). However, the effectiveness of ZVI alone toward contaminant degradation (either reductive or oxidative) is only a very small fraction of what is observed in the presence of ZVI + H_2O_2 (Furia et al., 2021).

The production of •OH makes the Fenton reaction potentially suitable for disinfection, because •OH is also able to inactivate bacteria and viruses by damaging/destroying cell membranes or viral capsids (Giannakis et al., 2016; McGuigan et al., 2012; Ruales-Lonfat et al., 2014). For instance, Fenton-like techniques have been the object of considerable research in the framework of the improvement of Solar Water Disinfection - SODIS (Carratalà et al., 2016; García-Gil et al., 2021; Giannakis et al., 2018), which is a low-cost and solar-based disinfection of drinking water that is often the only feasible water treatment option in developing countries.

For the above issues, it is very interesting to investigate if the Fenton process (carried out in heterogeneous conditions through ZVI) can achieve both depollution from contaminants and disinfection of pathogens in a two-birds-with-one-stone approach. Therefore, this contribution considers ZVI-Fenton as an innovative and cheap AOP, which is able to effectively degrade pharmaceuticals (including hospital antibiotics) at very competitive cost compared to traditional Fenton or ozonation, to see if the same conditions that already gave promising results with pollutant degradation in real wastewater (Furia et al., 2021) are also effective towards disinfection.

2. Materials and Methods

2.1 Wastewater

The real WW sample used in this work was obtained from the secondary clarifier tank outflow of an urban WWTP from Verbania (Piedmont, NW Italy, population equivalents of 51k inhabitants, described in Di Cesare et al., 2016). The main chemical features of the studied WW sample are reported in **Supplementary Table S1** of the Supplementary Material (hereinafter, SM). The WW chemical characterization procedures are described in SM, **Paragraph S1**.

2.2 Choice of operational conditions

Although the Fenton reaction is most effective at pH 3 (Pignatello et al., 2006) and ZVI-Fenton makes no exception (Furia et al., 2021), and although such pH conditions would effectively kill practically all bacteria in wastewater, operation at pH 3 is not practical in an application scenario. Indeed, apart from the cost of the acid that would be a major expense voice at pH 3 (Minella et al., 2018), one has to neutralize acidic pH before wastewater discharge. That operation would *(i)* require a relatively high amount of a base with the associated costs, and *(ii)* produce a saline stream that might pose problems for discharge or reuse. Operation at pH 4 would pose similar problems, although to a lesser extent, while ZVI-Fenton suffers from a dramatic loss of effectiveness in wastewater between pH 6 and 7 (Minella et al., 2019). For these reasons we chose to operate at pH 5 and 6, where previous work has shown that suitable conditions can be found to achieve effective ZVI-Fenton degradation of antibiotics in

wastewater. Moreover, pH 5-6 allows for lower use of H_3PO_4 (*vide infra*) compared to pH 4, thereby decreasing costs and facilitating phosphate elimination in a following step.

2.3 Microbiological experiments

The experimental design consisted of two parts, referred to as "experiment 1" and "experiment 2", respectively (**Figure 1**). In the "experiment 1" we investigated the effect of the ZVI-Fenton process at different pH values on bacterial abundance and vitality of a partially treated WW microbial community. The aim was to select the best performing condition in terms of abatement of cell number and reduction of cell vitality. In this case, the collected WW sample (pH 7.5) was immediately transported to the laboratory. The sample was split in three equal aliquots and the pH adjusted as follows (time = T0): *(i)* pH 5 with H_2SO_4; *(ii)* pH 5 with H_3PO_4; *(iii)* pH 6 with H_3PO_4. H_2SO_4 and H_3PO_4 are the only two inorganic acids that proved effective in the Fenton process, considering that HCl is to be avoided because Cl^- scavenges $^{\bullet}OH$ in acidic solution and may thus inhibit Fenton degradation (Coha et al., 2021), while HNO_3 and $HClO_4$ are ruled out because of high toxicity of the conjugated anions.

From each sub-sample, three series of flasks (each one in triplicate) were prepared, each containing 50 mL of acidified WW. One set of flasks was untreated (control), the second one was amended with H_2O_2, and the third one with ZVI + H_2O_2. The values of H_2O_2 concentration, ZVI loading and their mode of addition, which may imply multiple additions during the course of the reaction, are described in the **Supplementary Table S2**. These conditions were chosen because of their effectiveness in degrading hospital antibiotics in WW (Furia et al., 2021; data of hospital antibiotic degradation in the same conditions with the same wastewater are also shown

in **Table S2**). The flasks were incubated at room temperature for 90 min in the dark. The pH value was monitored during treatment and, in case, corrected (**Supplementary Table S2**). At the end of the treatment, 1 mL of a catalase solution (0.1 g L^{-1}) was added to each flask, to degrade any potentially residual peroxide (the complete degradation of H_2O_2 was confirmed spectrophotometrically). Then, samples were neutralized with NaOH (0.1 mol L^{-1}) up to pH 6 if needed (time = T90S). Indeed, pH 6 is fully consistent with wastewater disposal into surface waters (Salgot et al., 2006). An aliquot (1 mL) from each sub-sample was fixed with formalin (final formaldehyde concentration 1.5%) and analyzed by flow cytometry.

The "experiment 2" had the aim of understanding how the selected ZVI-Fenton process affects the load of potential human pathogenic bacteria and the antibiotic resistome (total content of antibiotic resistance genes, ARGs) of the WW bacterial community. To do this "experiment 1" was replicated in the conditions that proved most promising/effective in the first experimental series, namely acidification at pH 5 with H_3PO_4, except that the acidified WW volume was increased to 350 mL and all the other reagents were added accordingly (**Supplementary Table S2**). At the end of the procedure, preliminary sample characterization was carried out by flow cytometry and, most notably, treated WWs and controls were processed for DNA extraction. An aliquot of DNA was twofold diluted and used for quantitative real-time polymerase chain reaction (qPCR) analysis. The other aliquot was processed for 16S rRNA gene amplicon sequencing, and shipped under controlled conditions to an external company for sequencing (IGA technologies, Padova, Italy). All the statistical analyses were conducted in the R environment version 3.6.0 (R Core Team, 2019).

165 *2.4 Flow cytometry*

166 The total bacterial number and the proportion of damaged cells in formalin fixed sub-samples
167 were obtained using previously described protocols for cytogram design (Corno et al., 2013) and
168 intact/damaged cells identification (after staining with SYBR green and propidium iodide 1:1)
169 (Amalfitano et al., 2015). For comparison, the initial WW sample was analyzed by flow
170 cytometry, too. An ANOVA (Analysis of Variance, Tukey post-hoc tested) was performed, after
171 a log-transformation of the data, to test for significant differences ($p < 0.05$) between sample
172 series.

173

174 *2.5 DNA extraction*

175 A volume of 100 mL of Fenton-treated WWs and controls derived from "experiment 2" was
176 filtered onto 0.22 µm polycarbonate filters (Millipore). The filters were then processed for DNA
177 extraction using the DNeasy UltraClean Microbial Kit (Qiagen), according to the manufacturer's
178 instructions. DNA was divided into two aliquots, and kept at -20°C before being treated as
179 described below.

180

181 *2.6 Antibiotic resistance genes (ARGs) and* intI*1 gene quantification*

182 As a general proxy for anthropogenic pollution in aquatic environments, we quantified the
183 integrase gene of the class 1 integrons (*intI*1) (Gillings et al., 2015). ARGs that are widespread in
184 wastewater and aquatic ecosystems were selected for analysis, namely: *sul*2, *tet*A and *qnr*S
185 (coding for the resistance against sulfonamides, tetracyclines and quinolones, respectively) (Di

186 Cesare et al., 2015, 2016a; Galafassi et al., 2021; Sabatino et al., 2020). qPCR was used to
187 quantify the gene abundances, with the methodological details as described elsewhere (Di Cesare
188 et al., 2015), on a RT-thermocycler CFX Connect (Bio-Rad). The primer pair sequences and
189 annealing temperatures are detailed in Supplementary Table S3. The limit of quantification
190 (LOQ) for each gene was determined according to Bustin et al. (2009) and was: 1548, 36, 122,
191 217, and 112 copy μL^{-1} for 16S rRNA, *sul*2, *tet*A, *qnr*S, and *intI*1 genes, respectively. Template
192 dilutions were used to ensure that there was no inhibition of the PCR reaction (Di Cesare et al.,
193 2013). Gene abundances were reported relative to the copy numbers of the 16S rRNA gene in the
194 same sample. In order to evaluate if the treatment affected the dynamics of resistance genes,
195 differences in their abundance were assessed by *(i)* Multivariate ANOVA (MANOVA),
196 analyzing the total tested ARG load (genes together), and then *(ii)* for each single gene by
197 ANOVA. For all the analyses, the normalized gene abundances were used as response variables.

198

199 *2.7 Bacterial community analyses*

200 The V3-V4 region of the 16S rRNA gene was sequenced to obtain a bacterial community profile
201 using the universal bacterial primer pair S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21
202 (Herlemann et al., 2011) on an Illumina platform (MiSeq) at IGA Technology Services Srl
203 (Udine, Italy). Raw reads were deposited at NCBI's sequence read archive (SRA) as project
204 PRJNA772528. Cutadapt 1.9.1 was used to remove adaptors and primers sequences before
205 analysis of the sequencing data (Martin, 2011). The Usearch pipeline was used to filter and
206 merge the sequences, following the instructions of the online tutorial (Edgar, 2010). Unique high
207 quality reads were identified and used as sequence variants to cluster the data into ZOTUs (zero
208 radius operational taxonomic units) with the *unoise* algorithm (Edgar, 2016). The *sintax*

command with the SILVA database (Quast et al., 2013) was used to identify taxonomically the sequences, which were confirmed if they were ≥80% similar to those in the database. In order to obtain a normalized dataset, all data were rarefied to the read number of the sample with the lowest count (namely 38,883). From the dataset, ZOTUs that were identified as chloroplasts or mitochondria and those not identified at least at a Phylum level were removed (reducing the original dataset from 4277 to 3839 ZOTUs). To investigate differences in bacterial communities between treatments, two datasets were used: 1) the total bacterial community and 2) a subset with only genera containing potentially pathogenic bacteria. The "potentially pathogenic bacteria" were defined as the bacterial genera that also included clinically relevant pathogenic species as reported in the pathogens database of the Bode Science Center (https://www.bode-science-center.com/center/relevant-pathogens-from-a-z.html) and previously published (Di Cesare et al., 2020b; Galafassi et al., 2021). For both, the whole community and potentially pathogenic bacteria only we calculated the richness, defined as the number of ZOTUs in each sample. Differences in richness between treated samples and controls were tested using a generalized linear model (GLM), considering a Poisson distribution of the data and a Tukey test as post-hoc analysis. Beta diversity was analyzed by calculating a matrix of all pairwise comparisons of the communities through abundance weighted Bray–Curtis dissimilarity index of the total bacterial community, using the *vegan* package (Oksanen et al., 2007). A dendrogram depicting the clustering of all samples was calculated using hierarchical cluster analysis (*hclust*) with average linkage (**Supplementary Figure S1**). To evaluate if the treatments could contribute to the variance of beta diversity, a PERMANOVA (Permutational ANOVA) was performed using the *vegan* package (Anderson, 2001; Oksanen et al., 2007). Furthermore, to better understand the efficacy of the treatments on the potentially pathogenic genera, their total

232 abundance and the abundance of each genus in treated and controls samples were compared
233 through a GLM (Tukey post-hoc tested). Only the results with p-value <0.05 were plotted,
234 meaning that they were considered significantly different between treatments.
235

236 # 3. Results

237 *3.1 Experiment 1*

238 Cell abundance and vitality in treated WW samples and controls significantly varied during the
239 experiment according to treatments and, most notably, pH values (ANOVA: $p \leq 1.0\times10^{-9}$;
240 **Supplementary Table S4** and **S5**) (**Figure 2**). In particular, at T0 the mere acidification of WW
241 to pH 5 significantly reduced cell numbers and vitality (**Figure 2**, **Supplementary Table S4** and
242 **S6**). Hereinafter, 'treatments' are referred to acidification followed by addition of H_2O_2 or ZVI +
243 H_2O_2 (ZVI-Fenton), 'controls' to acidification alone. At the end of the experiment, after
244 incubation and neutralization, no differences in terms of cell abundance were observed between
245 treatments in the same acidic condition (*e.g.*, control *vs*. sample treated with H_2O_2, both acidified
246 with H_3PO_4 at pH 5), nor when comparing similar treatments in slightly different conditions
247 (*e.g.*, sample treated with ZVI + H_2O_2, acidified with H_3PO_4 at pH 5, *vs*. sample treated with ZVI
248 + H_2O_2, acidified with H_2SO_4 at pH 5) (**Figure 2a**, **Supplementary Table S4**). On the contrary,
249 looking at cell vitality at T90S, the treatment with ZVI + H_2O_2 of the sample acidified with
250 H_3PO_4 at pH 5 (*i.e.*, 90 min contact time with ZVI + H_2O_2 in these conditions) significantly
251 increased the percentage of damaged bacteria both in respect with its control, and in respect with
252 the same treatment at different pH (H_3PO_4, pH 6) (**Figure 2b**, **Supplementary Table S5**).
253 Starting from these results, the ZVI-Fenton treatment with H_3PO_4 at pH 5 was selected for the

254 "experiment 2". All the pairwise comparisons that were significantly different are reported in
255 **Supplementary Table S4** (cell abundance) and **Supplementary Table S5** (percentage of
256 damaged cells).

257 Note that pH 5 was obtained by adding 1.4 mL of a 0.6 M H_3PO_4 stock solution to wastewater,
258 which produced a phosphate concentration that was higher than discharge limits. However, the
259 ZVI-Fenton treatment would be located after secondary WW sedimentation and before the
260 tertiary step of phosphate elimination (Furia et al., 2021), which would remove species with
261 eutrophication potential from the final stream and even favor the recovery of useful compounds
262 (fertilizers) such as struvite from WW.

263

264 *3.2 Experiment 2*

265 *3.2.1 Flow cytometry*

266 Flow cytometric analysis of samples from "experiment 2" confirmed the findings obtained for
267 "experiment 1". Indeed, significant differences between samples were observed both in terms of
268 cell abundance and vitality (ANOVA: $p \leq 6.36\times10^{-6}$; **Figure 3**, **Supplementary Table S6** and
269 **S8**). There was a significant drop of cell numbers and vitality after acidification of WW, and no
270 variation in cell abundance of the control and treated samples at T90S (**Figure 3**,
271 **Supplementary Table S6**). Again, at the end of the experiment the treatments with H_2O_2 and
272 ZVI + H_2O_2 caused significant increase in the percentage of damaged cells when compared to
273 the control (**Figure 3b**, **Supplementary Table S7**). All the pairwise comparisons that were
274 significantly different during the experiment are reported in **Supplementary Table S6** (cell
275 abundance) and **Supplementary Table S7** (percentage of damaged cells).

3.2.2 Bacterial community composition

When considering the whole bacterial community, richness was significantly higher in the control and H_2O_2-treated samples compared to samples treated with ZVI + H_2O_2 (GLM: $p = 2.2\times10^{-16}$; **Figure 4a**, **Supplementary Table S8**). In contrast, looking at the potentially pathogenic genera only, richness did not show any significant difference between samples (GLM: p= 0.413; **Figure 4b**, **Supplementary Table S8**). Regarding beta diversity, the treatment explained 48.4% of its variation in the case of the whole bacterial community (**Supplementary Figure S2a**, **Supplementary Table S9**), while the composition of the potential pathogenic bacteria was less influenced (30.8%) by treatment (**Supplementary Figure S2b**, **Supplementary Table S9**). Focusing on the dynamics of potentially pathogenic bacteria, the total abundance of reads associated to these genera was significantly lower in samples treated with H_2O_2 + ZVI if compared to controls (p= 0.0252) and to the H_2O_2 treatment (p= 0.0186) (**Figure 5**, **Supplementary Table S10**). When it comes to the single genus, *Acinetobacter*, *Legionella* and *Sphingomonas* were less abundant in the control and H_2O_2 + ZVI treated samples in respect with H_2O_2 treatment; *Bacteroides* had lower abundance in H_2O_2 + ZVI treated samples, whereas *Klebsiella* and *Micobacterium* in the control samples; *Clostridium_sensu_strictu_1* was less abundant in the control and H_2O_2 treated samples in respect with H_2O_2 + ZVI treatment; finally, *Prevotella_1* and *Prevotella_9* had higher abundance in the control samples (**Figure 6**). All the pairwise comparisons of potentially pathogenic genera that showed significant differences are reported in **Supplementary Table S11**.

3.2.3 ARGs and intI*1 gene quantification*

ARGs were quantifiable in all the samples, with concentrations ranging from 1.14×10^{-3} to 5.99×10^{-2} copies/16S rRNA gene copy. In particular, the abundance of *sul*2 ranged between 2.62×10^{-2} and 5.99×10^{-2} copies/16S rRNA gene copy; *tet*A between 1.14×10^{-3} and 2.29×10^{-3} copies/16S rRNA gene copy; *qnr*S between 1.75×10^{-2} and 4.67×10^{-2} copies/16S rRNA gene copy (**Supplementary Figure S2**). Also *intI*1 was always quantifiable, with concentrations ranging from 3.99×10^{-3} to 5.68×10^{-3} copies/16S rRNA gene copy. No significant differences in gene abundances between treatments were found when analyzing them either collectively (MANOVA: Pillai's trace = 1.1619, F = 1.3864, p = 0.3275) or as single gene (ANOVA: $p \geq 0.07464$; **Supplementary Table S12**).

4. Discussion

In this study we tested the efficacy of ZVI-Fenton as possible disinfection treatment to lower the load of potential human pathogenic bacteria and ARGs. In detail, we performed a first experiment testing the effect of acidification with different acids, *i.e.* H_2SO_4 and H_3PO_4 at different pH values (5 and 6), and of different treatments on a WW bacterial community, comparing ZVI-Fenton to the treatment with only H_2O_2 and to an untreated control. The best performing treatment in terms of cell vitality decrease was ZVI-Fenton, upon acidification with H_3PO_4 at pH 5. Therefore, this treatment was used in a second experiment to improve the analysis on bacterial community composition, as well as ARGs and class 1 integrons abundance.

Possible reason for the higher effectiveness of ZVI-Fenton (H_3PO_4) compared to ZVI-Fenton (H_2SO_4) is the fact that phosphate helps the dissolution of Fe species (Wilhelmy et al., 1985): in

particular, the ion HPO_4^{2-} binds Fe(III) to form the $Fe(HPO_4)^+$ complex, and dissolved Fe is thus more concentrated in ZVI-Fenton (H_3PO_4) compared to ZVI-Fenton (H_2SO_4) (Furia et al., 2021). On the one side, this would cause higher $^{\bullet}OH$ production (Keenan and Sedlak, 2008; Pan et al., 2020) that could play a role in bacterial inactivation. On the other hand, dissolved inorganic Fe species are toxic to bacteria, unless they are strongly bound by biocompatible organic ligands (Farinelli et al., 2021).

From the second experiment we found that, at the end of the process, cell abundance as measured by flow cytometry was not affected by ZVI-Fenton, nor by the treatment with H_2O_2 alone. This result is in agreement with what observed in "experiment 1" and in a previous study that tested another innovative AOP (UVC/H_2O_2/Cu-IDS) using H_2O_2 as comparative treatment, where neither treatment significantly affected the total bacterial abundances (Di Cesare et al., 2020b). Furthermore, similar results were also obtained when testing consolidated disinfection processes based on the use of oxidizing species, such as chlorination (Di Cesare et al., 2016b). Although it is extensively reported that $^{\bullet}OH$ can damage the cell membrane and the DNA (Zhang et al., 2021), extracellular $^{\bullet}OH$ would not reach DNA because of the high reaction rates with organic compounds, and it would be consumed by the cell membranes (Wu et al., 2021). The flow cytometer cell counting, which is based on the DNA staining with SYBR Green cannot discriminate between intact and damaged bacterial cells, and thus cannot be considered as the only measurement to evaluate whether a treatment is effective or not against bacteria. For this reason, we performed a flow cytometer cell counting based on double staining with SYBR Green and propidium iodide, which can discriminate between intact and damaged cells, i.e. live/dead stain (Amalfitano et al., 2015). ZVI-Fenton significantly decreased cell vitality compared to untreated samples; again, these results were similar to those previously obtained by testing other

342 AOPs (Di Cesare et al., 2020b) and consolidated disinfection treatments such as peracetic acid
343 (Di Cesare et al., 2016b).

344 Looking at the composition of the bacterial communities in treated and untreated samples, it was
345 evident that ZVI-Fenton strongly affected richness, which was significantly lower than that
346 determined for untreated samples and samples treated with H_2O_2 only. This shift is due to
347 different susceptibility of bacterial groups to the disinfection treatment (Di Cesare et al., 2020a),
348 and has previously been reported after treatment by several disinfection processes, *e.g.* ozonation
349 (Alexander et al., 2016; Becerra-Castro et al., 2016), chlorination (Pang et al., 2016), and UV
350 radiation (Becerra-Castro et al., 2016). Furthermore, the treatment explained approximatively
351 50% of the beta diversity. Samples treated with ZVI-Fenton were located in a distinct cluster in
352 the dendrogram obtained after the hierarchical cluster analysis (**Supplementary Figure S1a**),
353 which highlights once more that this AOP strongly affected the structure of the bacterial
354 community.

355 Focusing on the human potentially pathogenic bacteria only, ZVI-Fenton did not affect their
356 richness and was thus not able to carry out undesirable selection of pathogens. Furthermore, this
357 disinfection process was the best performing treatment in our experiment in terms of reduction of
358 the total pathogenic bacterial abundance. Therefore, the results shown here are a first but
359 promising evidence of the possible applicability of ZVI-Fenton as disinfection process, to
360 decrease the content of the total pathogenic bacteria in wastewaters.

361 Going deeper in the pathogenic bacterial community composition, ZVI-Fenton resulted
362 particularly effective against *Bacteroides,* which are relevant human pathogens. Indeed, once
363 outside the gut some *Bacteroides* species can cause bacteremia and abscess formation in multiple
364 body districts, and they are associated with lethality episodes for more than 19% of cases

(Wexler, 2007). ZVI-Fenton also significantly decreased the abundance of *Prevotella* compared to the untreated samples. *Prevotella* have frequently been detected in sputum samples of patients affected by cystic fibroses (CF) treated with antibiotics, and are defined as "emerging bacterial pathogens in the context of CF respiratory infection" (Mahenthiralingam, 2014). Furthermore, ZVI-Fenton did not determine the selection of other pathogenic bacteria, *i.e.*, *Legionella*, *Acinetobacter* and *Sphingomonas*, all associated to the formation of biofilm and/or capable to survive within amoebae (Pereira et al., 2017). Indeed, the latter were significantly more abundant in samples treated with only H_2O_2. However, further pathogenic bacteria (*Clostridium*, *Mycobacterium* and *Klebsiella*) were more abundant in samples treated by ZVI-Fenton compared to untreated samples and/or samples treated only with H_2O_2. This means that additional investigations targeting these specific bacterial genera by using ZVI-Fenton are needed.

Neither H_2O_2 nor ZVI-Fenton significantly affected the relative abundance of the tested ARGs and *intI*1. This result is in line with previous findings for other disinfection treatments including chlorination, peracetic acid, UV radiation and ozonation, where ARG abundances resulted unchanged or even increased after treatment (Di Cesare et al., 2016b; Galafassi et al., 2021). However, other studies showed that AOPs were effective in decreasing the load of antibiotic resistant bacteria or ARGs (Ferro et al., 2016; Zhou et al., 2020), suggesting that more efforts in setting the ZVI-Fenton process should be done to decrease ARGs in WWs.

A final comment is required about process costs and potential ZVI reuse. As shown in **Supplementary Table S13**, reagent costs for ZVI-Fenton with H_3PO_4 at pH 5 would amount to about 0.15 \$ m^{-3}, of which the majority (around 0.12 \$ m^{-3}) accounted for by H_3PO_4 itself. Because of this issue, operation with H_3PO_4 at pH < 5 would be problematic even if it could be effective in contaminant degradation. ZVI at pH 5 would account for only about 10% of total

388 reagent costs, which means that ZVI reuse is far less critical than H_3PO_4 saving in process
389 economics. Moreover, 0.02 g L^{-1} ZVI means that treatment of 1 m^3 water would require 20 g
390 ZVI, which is not a huge amount. Still, ZVI might be potentially reused for some times because
391 surface oxidation of a ZVI particle produces a layer of Fe(II) oxide that still retains Fenton
392 reactivity (Minella et al., 2016), although with some loss in performance compared to fresh ZVI
393 (Minella et al., 2019). Recovery of ZVI from wastewater at the end of the treatment would also
394 be made easier by its magnetic properties. However, the low ZVI cost should be compared with
395 the unavoidable costs of reuse, to see if the reuse route is economically viable compared to, e.g.,
396 direct employment of spent ZVI as secondary raw material.

397

398 ## 5. Conclusions

399 Overall, the results obtained from "experiment 1" and "experiment 2" showed a possible
400 applicability of ZVI-Fenton as promising treatment to achieve some disinfection of pathogens
401 along with decontamination of pollutants. ZVI-Fenton effectively decreased the total viable
402 bacterial numbers and caused a significant reduction in total human pathogenic bacterial
403 abundance. Unfortunately, it was ineffective in removing ARGs and *intI*1. However, it is here
404 necessary to underline that we used a target-based molecular approach (qPCR) that quantifies
405 only some ARGs. In contrast, other approaches such as shotgun metagenomics or High
406 Throughput qPCR could better characterize the bacterial community antibiotic resistome, and
407 provide a clearer overview of the effects of ZVI-Fenton on antibiotic resistance. Furthermore,
408 ZVI-Fenton resulted effective in the removal from WW of selected antibiotics reserved for
409 hospital settings (Furia et al., 2021). Therefore, coupling the results obtained by this

410 microbiological study with those previously obtained on antibiotic degradation, it is reasonable
411 to conclude that ZVI-Fenton is a promising process that deserves to be further investigated and
412 tested in order to be included in the processes applied in WWTPs.

413

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Figures

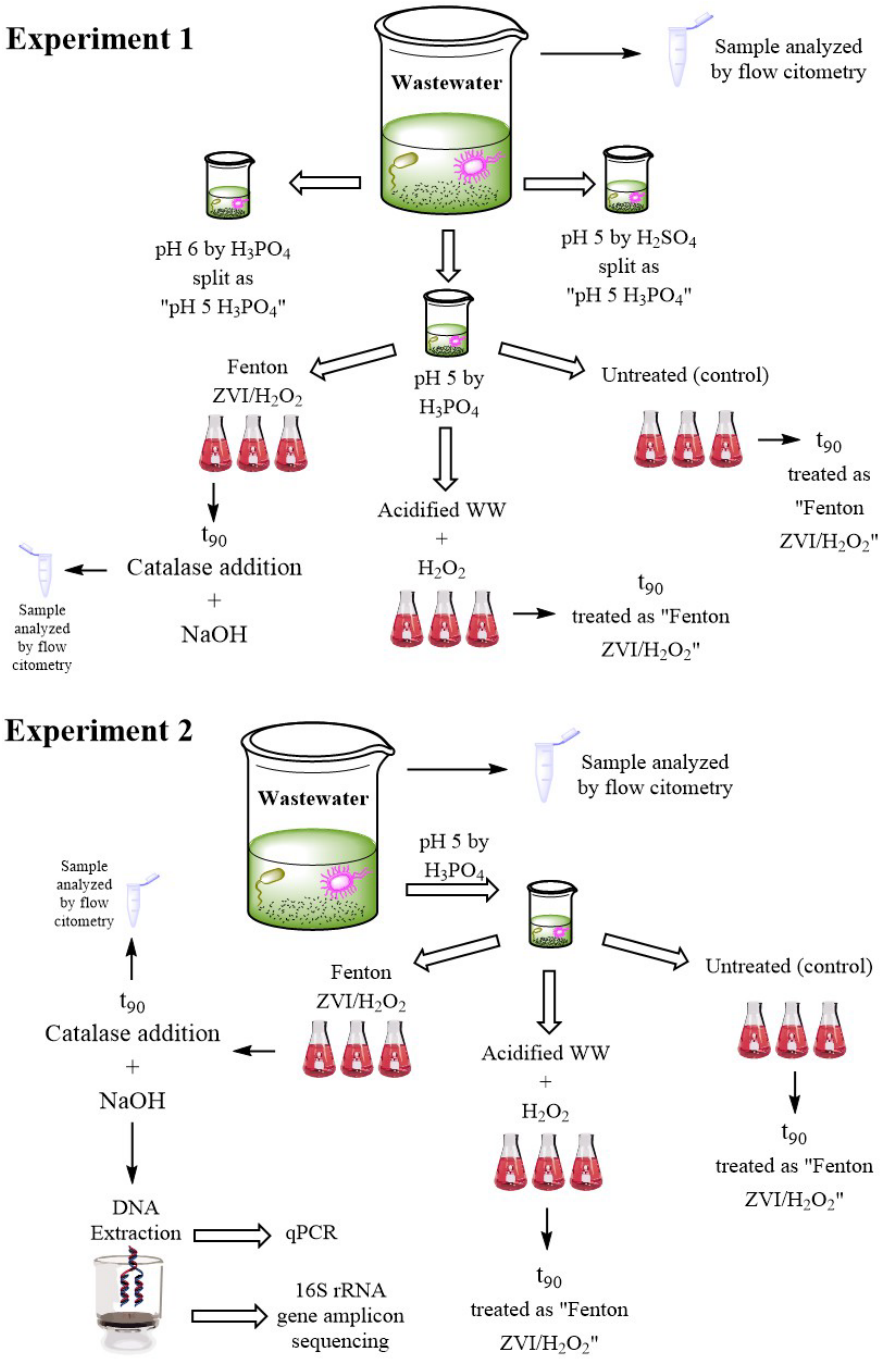
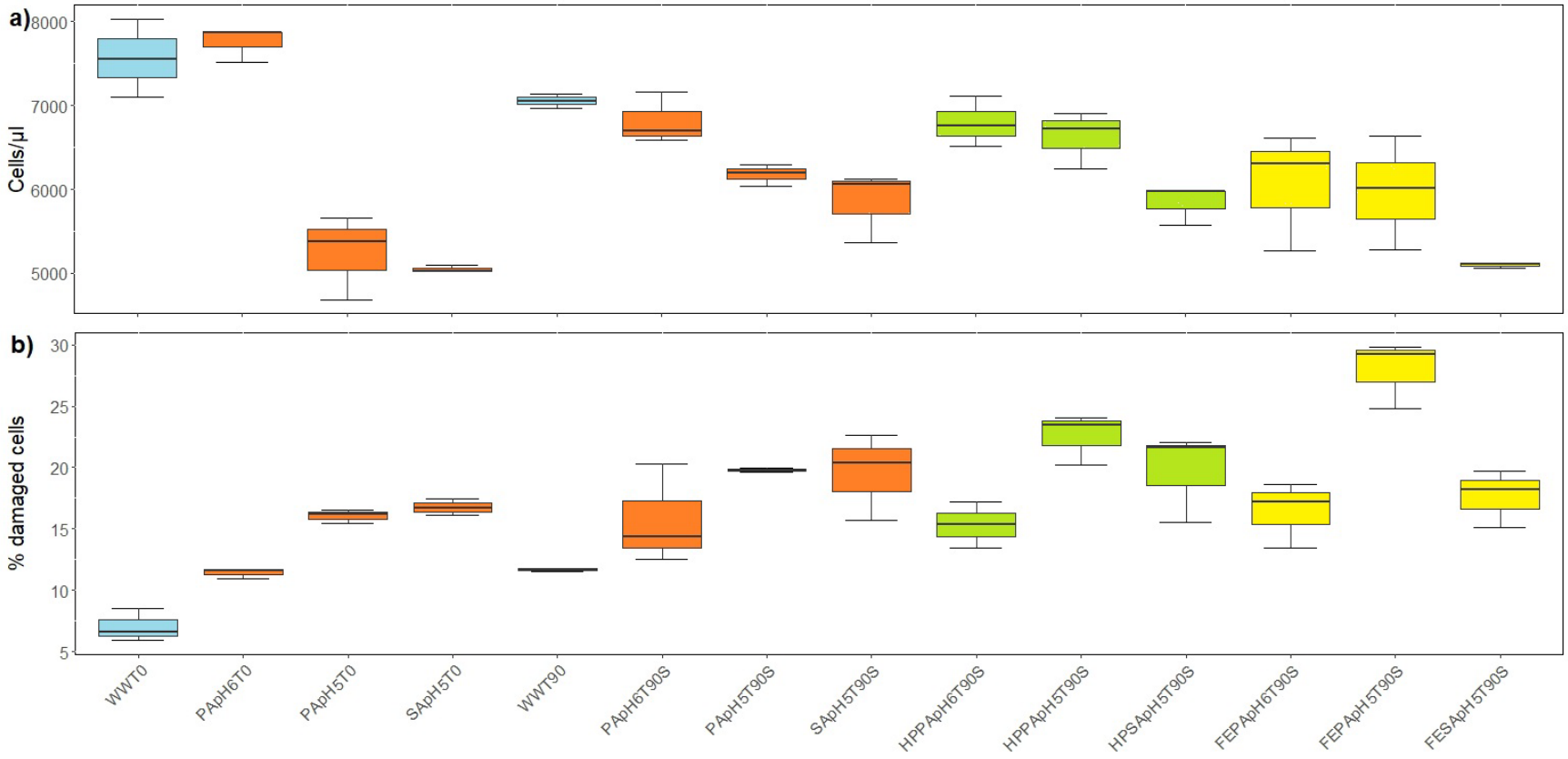


Figure 1. Illustration of the experimental design used in this work.



643

644 **Figure 2.** Flow cytometry results of "experiment 1". **a)** total cell abundance and **b)** percentage of damaged bacteria according to the
645 different treatments applied throughout the "experiment 1". Abbreviations: "PA"= phosphoric acid, "SA"= sulfuric acid, "HP"=
646 hydrogen peroxide, and "FE"= ZVI-Fenton Process. The thick horizontal line represents the median, the box represents 50% of the
647 values, the whiskers extend to the highest and lowest value within the 1.5 interquartile range.

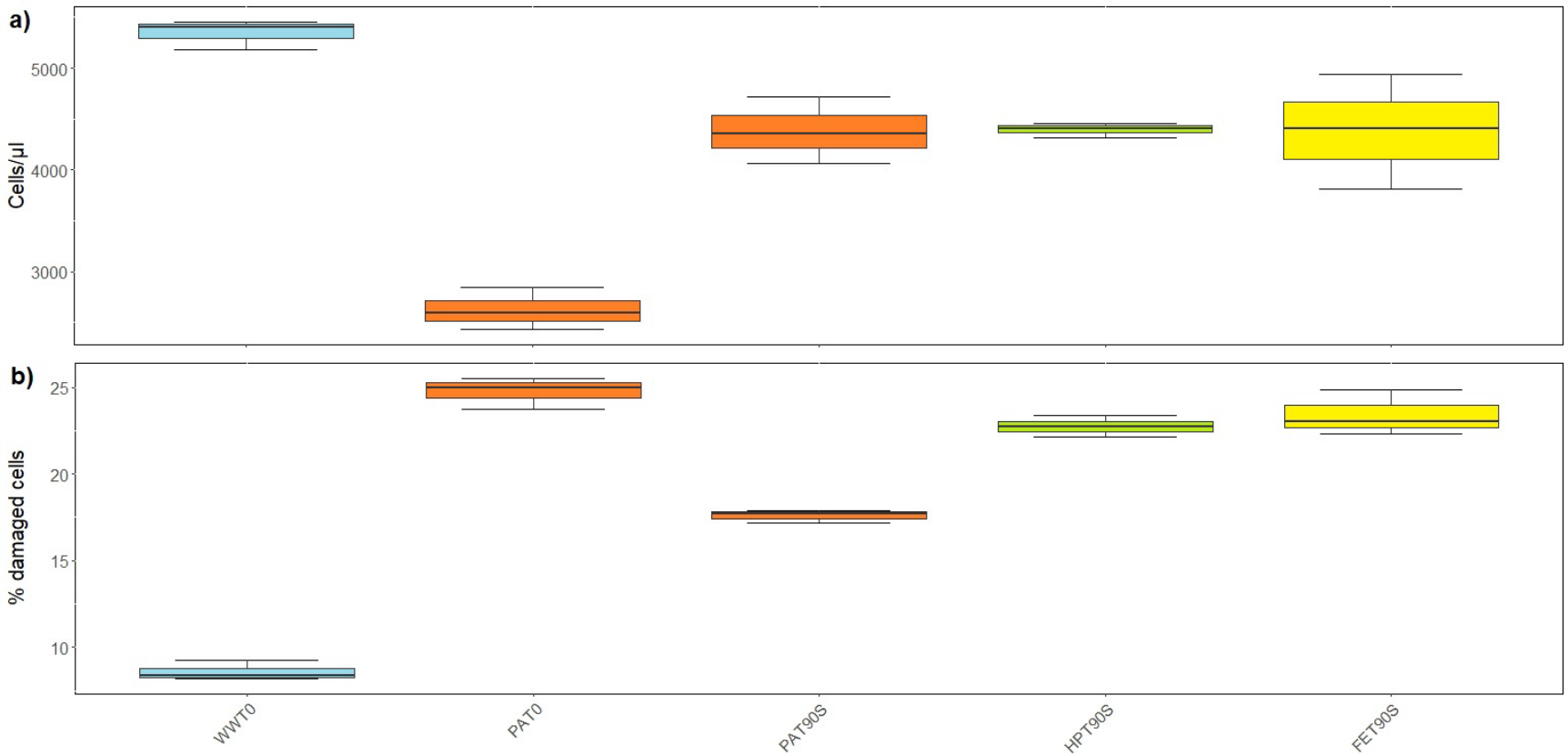


Figure 3. Flow cytometry results of "experiment 2". **a)** Total cell abundance and **b)** percentage of damaged bacteria according to the different treatments applied throughout the "experiment 2". Abbreviations: "PA"= phosphoric acid, "HP"= hydrogen peroxide, and "FE"= ZVI-Fenton Process. The thick horizontal line represents the median, the box represents 50% of the values, the whiskers extend to the highest and lowest value within the 1.5 interquartile range.

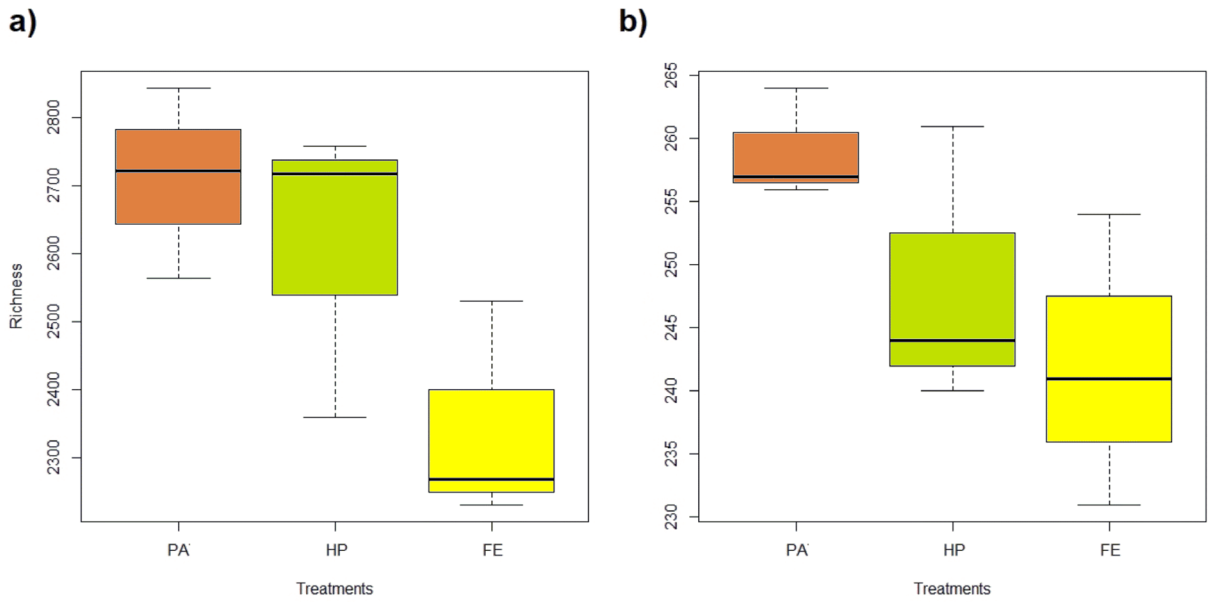
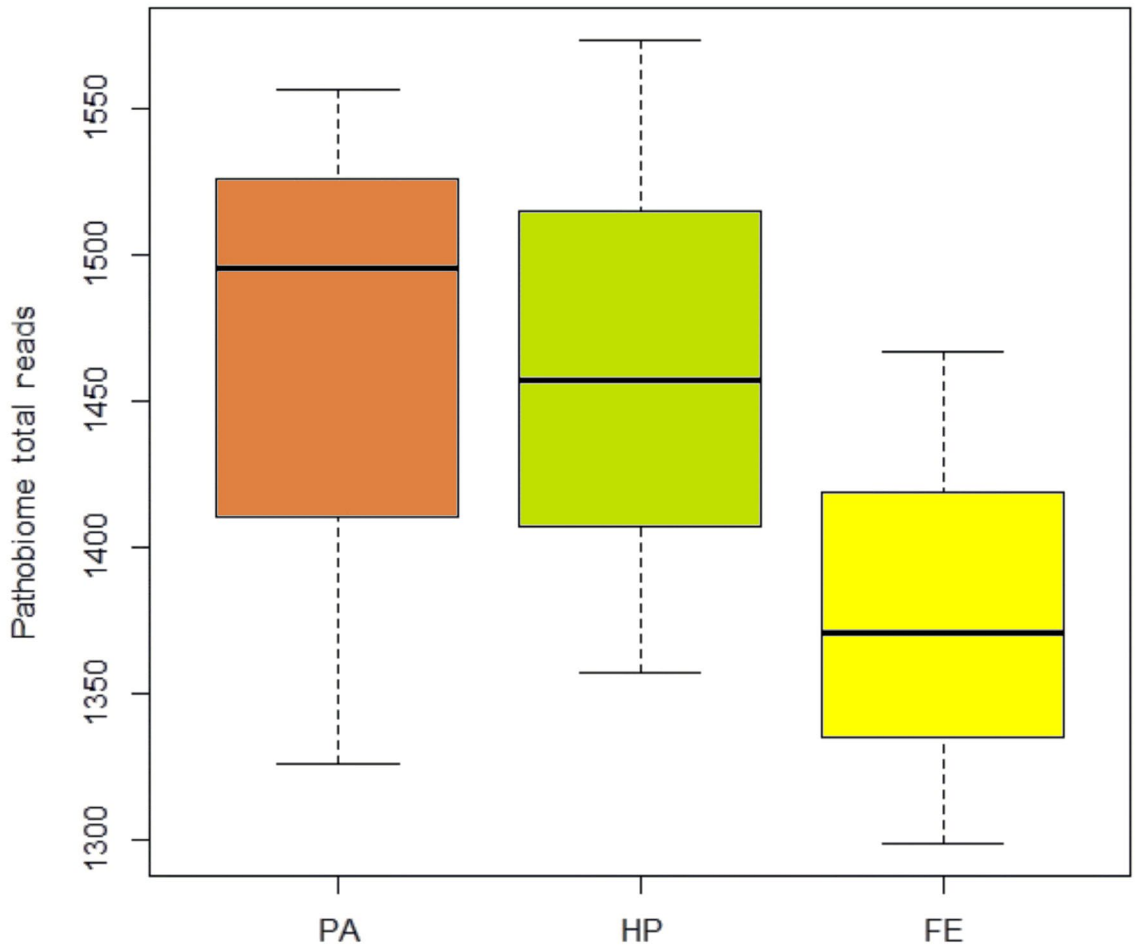


Figure 4. Richness of "experiment 2" samples. Boxplots of richness of **a)** total bacterial community and **b)** potentially pathogenic genera according to the different treatments. Abbreviations: "PA"= phosphoric acid, "HP"= hydrogen peroxide, and "FE"= ZVI-Fenton Process. The thick horizontal line represents the median, the box represents 50% of the values, the whiskers extend to the highest and lowest value within the 1.5 interquartile range.



661

662 **Figure 5.** Total reads abundance associated to the pathobiome. Boxplots of total reads
663 abundance of potentially pathogenic genera according to the different treatments. Abbreviations:
664 "PA"= phosphoric acid, "HP"= hydrogen peroxide, and "FE"= ZVI-Fenton Process. The thick
665 horizontal line represents the median, the box represents 50% of the values, the whiskers extend
666 to the highest and lowest value within the 1.5 interquartile range.

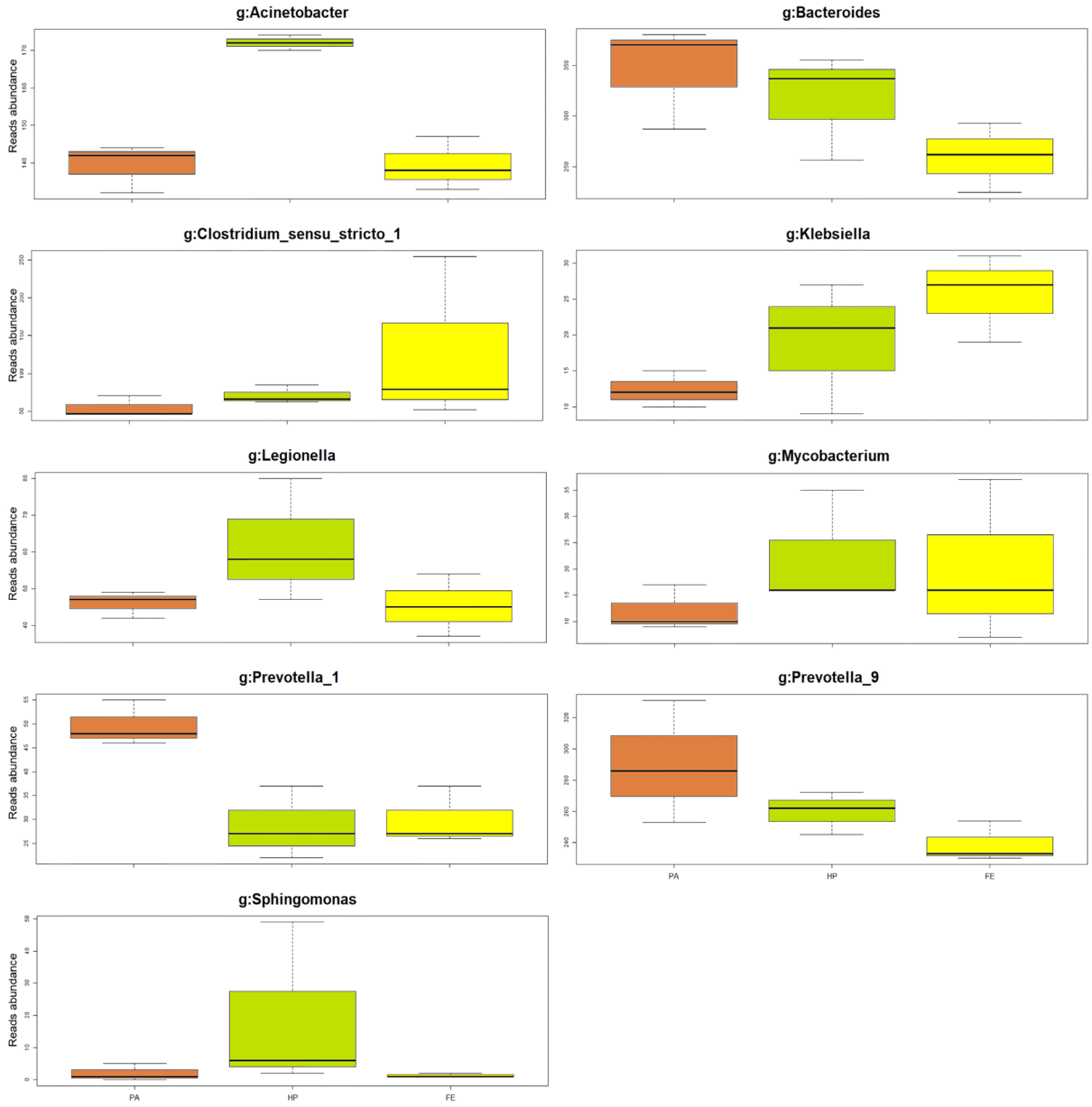


Figure 6. Reads abundance of potentially pathogenic genera as determined by amplicon sequencing. Boxplots of reads abundance associated to potentially pathogenic genera according to the different treatments. Only genera that were significantly different between treatments are depicted. Abbreviations: "PA"= phosphoric acid, "HP"= hydrogen peroxide, and "FE"= ZVI-Fenton Process. The thick horizontal line represents the median, the box represents 50% of the values, the whiskers extend to the highest and lowest value within the 1.5 interquartile range.