Elimination from Wastewater of Antibiotics Reserved for Hospital Settings, with a Fenton Process Based on Zero-Valent Iron

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Abstract

The Fenton process activated by Zero Valent Iron (ZVI-Fenton) is shown here to effectively remove antibiotics reserved for hospital settings (specifically used to treat antibiotic-resistant infections) from wastewater, thereby helping in the fight against bacterial resistance. Effective degradation of cefazolin, imipenem and vancomycin in real urban wastewater was achieved at pH 5, which is quite near neutrality when compared with classic Fenton that works effectively at pH 3-4. The possibility to operate successfully at pH 5 has several advantages compared to operation at lower pH values: *(i)* lower reagent costs for pH adjustment; *(ii)* insignificant impact on wastewater conductivity, because lesser acid is required to acidify and lesser or no base for neutralization; *(iii)* production of undetectable dissolved Fe, which could otherwise be an issue for wastewater quality. The cost of reagents for the treatment ranges between 0.04 and 0.07 \$ m^{-3}, which looks very suitable for practical applications. The structures of the degradation intermediates of the studied antibiotics and

their likely abundance suggest that, once the primary compound is eliminated, most of the potential to trigger antibiotic action has been removed. Application of the ZVI-Fenton technique to wastewater treatment could considerably lower the possibility for antibiotics to trigger the development of resistance in bacteria.

Keywords: cefazolin; vancomycin; imipenem; wastewater remediation; advanced oxidation processes; degradation products.

1. INTRODUCTION

The spread of antibiotic resistance (ABR) among bacteria is a growing threat that stems from a combination of excessive prescriptions of antibiotics, incorrect disposal, use outside of the field of human medicine, and slowing-down of research into the development of new drugs (Levy, 1998). ABR could jeopardize the efficacy of antibiotics in future years, with the potential to produce many casualties because of diseases or medical procedures that are presently regarded as trivial (Wiles, 2016; Cox and Worthington, 2017). The spread of ABR takes place at various levels and involves many sectors of society (Cully, 2014; Cars, 2014; McCullough et al., 2016), including wastewater treatment (Amador et al., 2015).

Wastewater is a major contributor to the spreading of ABR because it contains: *(i)* antibiotic residues that are incompletely eliminated and end up in surface waters, where their concentration is too low to kill bacteria but can still be high enough (up to several hundred μg L^{-1} in the water matrix; Zhao et al., 2017) to favor the onset of resistance (Bengtsson-Palme and Larsson, 2016), and *(ii)* antibiotic-resistant bacteria and resistance genes (Rizzo et al., 2013). Unfortunately, the present techniques used in wastewater treatment plants (WWTPs) are poorly effective at removing biorecalcitrant compounds that include many contaminants of emerging concern (CECs), such as

51 the antibiotics, which are poorly compatible with biological treatment (Behera et al., 2011; Ahmed
52 et al., 2017; Richardson and Ternes, 2018). Moreover, the final step of wastewater disinfection is
53 often unable to totally eliminate resistant bacteria, and it could even select for antibiotic resistance
54 genes (Di Cesare et al., 2016).
55 A very important role in the fight against bacteria is played by the antibiotics reserved for hospital
56 settings. These antibiotics are used in hospitals to treat infections that are resistant to the other
57 "common" antibiotics. Clearly, the development of ABR toward hospital antibiotics is particularly
58 dangerous, because these antibiotics are our last line of defense against ABR bacterial infections
59 (Perl, 1999; Roche et al., 2016). Resistance against antibiotics reserved for hospital settings is
60 presently limited (although it is steadily growing), either because some of these antibiotics have
61 been discovered relatively recently (which means a couple of decades ago, as no new antibiotics
62 have been discovered afterwards), or because they have undergone little general use even if known
63 by more time, due to significant side effects or relatively difficult administration (Rattie and Ravin,
64 1975; Piddock, 2011). Because of restricted prescriptions, controlled use and focus on human
65 medicine, many routes to ABR development are currently minimized for antibiotics reserved for
66 hospital settings. The same is not true for wastewater, unfortunately, because of unavoidable
67 excretion by patients of these antibiotics and of the associated resistant bacteria (Das et al., 2019;
68 Hoelle et al., 2019; Ekwanzala et al., 2020).
69 Although at the moment antibiotics are not classified as contaminants and are not regulated by law,
70 there are already some initiatives aimed at tackling the ABR issue in wastewater. Therefore, it is
71 very likely that future technological developments in WWTPs will have to achieve the removal of
72 antibiotics and other CECs from wastewater (Sharma et al., 2016; Fiorentino et al., 2018; Oberoi et
73 al., 2019; Rizzo et al., 2020). Physical separation or degradation are some of the techniques that can
74 be used to eliminate chemicals from wastewater. Among such techniques, the Fenton reaction is a
75 notable example of a potentially cost-effective treatment (Wang and Zhuan, 2020; Brillas, 2020). It
76 is an Advanced Oxidation Process based on the production of $^{\bullet}OH$ and/or other oxidizing species

such as ferryl, FeO^{2+}. Classic Fenton uses soluble Fe(II) salts, and H_2O_2 usually in excess of the stoichiometric ratio (Neyens et al., 2003; Mirzaei et al., 2017):

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

$$FeOH^{2+} + H_2O_2 \rightarrow Fe^{2+} + H_2O + HO_2^{\bullet} \quad (2)$$

$$FeOH^{2+} + HO_2^{\bullet} \rightarrow Fe^{2+} + H_2O + O_2 \quad (3)$$

Classic Fenton has two major drawbacks: *(i)* It works best at pH 3, but its performance worsens quickly as pH increases, because of slower Fe(III)-Fe(II) recycling (Pignatello et al.,2006). It is next to impossible to carry out an effective treatment by classic $Fe^{2+} + H_2O_2$ under ~neutral conditions; *(ii)* The adjustment of pH before wastewater discharge causes Fe(III) precipitation, producing a sludge (Huang et al., 2013). To tackle these shortcomings, some modifications of the Fenton process have been developed: *(i)* Photo-Fenton, where radiation facilitates the conversion of Fe(III) into Fe(II) (Giannakis et al., 2018a; Giannakis et al., 2018b; Arzate et al., 2020). Unfortunately, natural sunlight may not be regularly available and artificial radiation is quite costly. *(ii)* Use of ligands to keep Fe(III) dissolved even at ~neutral pH (Wu et al., 2015; Qian et al., 2020). However, there are law limits for the total dissolved Fe in wastewater (Zhong et al., 2012), and the ligands may interfere with Fe elimination by precipitation. Moreover, ligands can change Fenton reactivity by inducing the generation of less-reactive ferryl over ${}^{\bullet}OH$, and may act as scavengers of reactive species (Pignatello et al., 1999; Farinelli et al., 2020). *(iii)* Heterogeneous Fenton, where Fe(II) salts are replaced by iron-based solid materials (Vorontsov, 2019; Tang and Wang, 2020). Research has focused on magnetic or magnetizable materials because they are very easy to remove after treatment (Jin et al., 2017; Gonçalves et al., 2020a; Gonçalves et al., 2020b). Among Fenton-active materials with magnetic properties, micro/nanoscopic zero-valent iron (Fe^o, or ZVI) offers several advantages such as availability, excellent reactivity, quite extended pH range and resistance to passivation (Joo et al., 2005; Rezaei and Vione, 2018; Gil Pavas et al., 2019). For instance, compared to other materials such as magnetite and titanomagnetites (Avetta et al., 2015; Bertinetti et al., 2019), ZVI

does not require particular storage attentions if used in the framework of the Fenton reaction, and even passivated ZVI still retains significant Fenton reactivity (Minella et al., 2016).

The ZVI-Fenton process, alone or in combination with other techniques (*e.g.*, ultrasound or radiation), has been studied for the degradation of some antibiotics, but the previously investigated compounds are also widely used outside of the field of human medicine (e.g., animal husbandry or aquaculture). Therefore, wastewater is definitely not the unique collector of the relevant residues (Perini et al., 2014; Lumbaque et al., 2019). Degradation of antibiotics bearing halogen or nitro groups has also been studied by exploiting the reductive (i.e., opposite to the oxidative ones used in ZVI-Fenton) capabilities of ZVI alone, without H_2O_2 (Liu et al., 2018; Xu et al., 2019; Xu et al., 2020). However, in such cases, the treatment requires ZVI (or sulfide-modified ZVI) doses of around 1 g L^{-1} (Cao et al., 2017), which would be very costly in wastewater treatment applications when compared with the ten-mg L^{-1} range of ZVI in ZVI-Fenton (Minella et al., 2019).

To the best of our knowledge, ZVI-Fenton has never been tested with antibiotics reserved for hospital settings, the elimination of which from wastewater is particularly critical. Therefore, in this work we have chosen three antibiotics reserved for hospital settings, belonging to different classes and having different structures (cefazolin, CFZ; imipenem, IMI, and vancomycin, VNM), to study their ZVI-Fenton degradation. While antibiotics could be partially excreted as glucuronate derivatives, ZVI-Fenton would be carried out after the activated sludge step, where bacteria detach the sugar moiety and set free the starting compound (deconjugation) (Polesel et al., 2016).

In this work, we carried out ZVI-Fenton degradation in the dark of the investigated antibiotics by focusing on: *(i)* optimization of ZVI and H_2O_2 doses; *(ii)* degradation efficiency at different pH values; *(iii)* impact on wastewater quality (conductivity, dissolved Fe, residual H_2O_2); *(iv)* formation of intermediates and, last but not least (von Gunten, 2018), *(v)* cost considerations.

2. MATERIALS AND METHODS

2.1. Reagents and materials

The used reagents, their manufacturers and purity grades are reported in the Supplementary Material (hereinafter, **SM**), **Paragraph S1**. Commercial ZVI particles (Sigma-Aldrich, Milan, Italy, purity ≥99.5%, product number 44890; *http://www.sigmaaldrich.com/catalog/product/aldrich/44890*) had diameter < 10 μm. The real wastewater (WW) samples used in this work (WWa, WWb) were obtained from the secondary clarifier tank outflow of two different urban WWTPs (population equivalents of 51,000 and 3.9 million inhabitants, respectively). The WW samples were used after rough pre-filtration with grade 1 qualitative filter paper (supplied by Whatman, Maidstone, UK), to remove large suspended solids. WW characterization is described in **Paragraph S2 (SM)**, and the main chemical features of the studied WW samples are reported in **Table S1 (SM)**. Note that, in Italy, hospital WWTPs have been largely dismissed and hospital wastewater is currently being treated together with the rest of urban wastewater, in centralized WWTPs. The disadvantage is that hospital antibiotics get diluted into the general urban wastewater flow. The advantage is that centralized WWTPs can afford the use of more advanced treatment technologies, and will do so in the future as well. Moreover, by so doing it is also possible to intercept the fraction of antibiotics that is excreted after the patients are dismissed from hospitals (prolonging hospitalization to wait for excretion kinetics would be totally unrealistic because of excessive costs, and even very dangerous due to increased risks of hospital infections) (Baietto et al., 2014).

To help quantify the amount of acid needed to fix WW pH in real wastewater treatment, WW aliquots (50 mL initial volume) were titrated with a standard H_2SO_4 solution (4.5×10^{-3} mol L^{-1}), using a potentiometric titrator (Titrino 702 SM by Metrohm, Herisau, Switzerland) equipped with a Metrohm combined pH glass electrode (code number 6.0233.100), and controlled by the Metrohm software "Tiamo" (titration and more).

2.2. Degradation experiments

Degradation experiments were carried out in beakers, where the reaction mixture (50 mL total volume) contained ZVI, H_2O_2, the substrate (CFZ, IMI, VNM, separately or in mixture), and a reagent for pH adjustment. Three experimental series were carried out: *(i)* separate substrates in ultra-pure water; *(ii)* mixture (CFZ+IMI+VNM) in ultra-pure water; *(iii)* mixture in wastewater (WWa, WWb). In the first two cases, a phosphate buffer at the desired pH value was added to the reagent mixture. Unfortunately, the amount of phosphate that had to be added to keep pH constant was higher than the law limits for phosphorous in WW. Therefore, in the third experimental series, the pH value was fixed with H_2SO_4, which was allowed by the WW buffer capacity and would be reasonable in WWTPs (Minella et al., 2019). HCl was avoided because Cl^- scavenges $^{\bullet}OH$ in acidic conditions (Jayson et al., 1973), and HNO_3 was also avoided because of toxicity and eutrophication issues connected with nitrate.

In analogy with other studies (Méndez-Arriaga et al., 2010; Minella et al., 2018), and considering that pharmaceuticals are typically found at trace levels in wastewater (CFZ and VNM have for instance been detected at μg L^{-1} level in effluents; Kümmerer and Henninger, 2003), substrate concentration was kept as low as possible, but still high enough to enable reliable monitoring by liquid chromatography (4-10 μmol L^{-1}, depending on the experiment).

At scheduled time intervals, a measured 0.9 mL sample aliquot was withdrawn from the reaction mixture and added with 0.1 mL methanol, to stop the Fenton reaction (Li et al., 2010; Minella et al., 2016). ZVI was then removed from the sample by filtration on Millipore Millex HV filters (0.45 μm pore diameter). CFZ, IMI and VNM were quantified by high-performance liquid chromatography – diode array detection (HPLC-DAD, VWR-Hitachi Elite series). Instrumental and operational details are reported in **Paragraph S3 (SM)**.

Repeatability experiments were carried out, to assess the degree to which differences in time trends can be considered as statistically significant. The results (see **Figure S1 (SM)** as an example)

suggest that the reproducibility of concentrations ranged between 5-20%. In these experiments, up to ten samplings were carried out in each beaker, for a total withdrawal of up to 9 mL. The results were compared with those of similar experiments, carried out in the same conditions but with just three samplings, to limit the reduction in the volume of the reacting system. The observed differences were well within the reproducibility of exactly replicated experiments.

The determination of dissolved (leached) iron and residual H_2O_2 was carried out with a Varian Cary 100 Scan double-beam UV-VIS spectrophotometer, using procedures (o-phenanthroline and peroxidase/4-aminoantipyrine, respectively; Sandell, 1950; Frew et al., 1983) that are described in detail in the **SM, Paragraph S3**. The spectrophotometric method used for Fe(II) and Fe(III) was very suitable in this context because its detection limit (0.05 mg_{Fe} L^{-1}) was much lower than the limit concentrations of Fe for wastewater discharge (1-2 mg_{Fe} L^{-1}, *vide infra*).

In dedicated runs, pH and conductivity of the WW samples under treatment were monitored over time by using, respectively, a 98100 Checker pH meter and a HI2030 Multi-parameter probe, both by Hanna Instruments (Rhode Island, USA).

2.3. Electron Paramagnetic Resonance (EPR) measurements

The reaction systems containing ZVI + H_2O_2 were added with the spin trap 5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO). Sample aliquots (50 μL) were taken at scheduled time intervals from the reaction mixtures using a capillary glass tube, which was sealed with plasticine on the one end and inserted into the EPR cavity. The instrument used was a Miniscope 100 EPR Spectrometer (Magnettech, Berlin, Germany). The operational parameters were as follows: middle of the range 3345 G, scan range 120 G, modulation amplitude 1000 mG, attenuation 7 dB, time 60 s, gain value 3, phase 180. The target was to identify the DMPO-OH adduct.

2.4. Identification of degradation intermediates

In this series of experiments the concentration of each antibiotic (2×10^{-4} mol L^{-1}) was higher than in the degradation runs, to make the identification of the intermediates easier. Wastewater was not used here, to avoid possible interaction or matrix effects that could complicate the interpretation of chemical structures. At scheduled reaction times, aliquots of 0.80 mL of the reacted solution were withdrawn and subjected to analysis by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS, Nexera Shimadzu, Kyoto, Japan), after the addition of 0.20 mL of methanol and filtration on 0.2 μm PTFE filters (VWR International, Darmstadt, Germany). See **SM, Paragraph S3** for details of the UHPLC-MS/MS measurements.

3. RESULTS AND DISCUSSION

3.1. Preliminary experiments of antibiotics degradation in ultra-pure water

A first series of experiments was carried out in ultra-pure water to check for the ability of ZVI-Fenton to degrade the three studied compounds, taken separately, under the most favorable conditions (absence of wastewater interfering agents). Coherently with previous findings (Minella et al., 2016; Minella et al., 2019) the ZVI-Fenton system worked best at pH 3, and performance gradually decreased (but was by no means totally lost) with increasing pH, in keeping with the fact that the $^{\bullet}OH$ yield of the Fenton reaction decreases with increasing pH (Farinelli et al., 2020). Actually, it was possible to offset the reactivity loss by increasing both H_2O_2 concentration and ZVI loading, obtaining complete or almost complete degradation also at pH 6 or 7. Compared to pH 3, it was necessary to increase the H_2O_2 concentration by 4-8 times, and the ZVI loading by 2-3 times to obtain effective degradation at pH 6-7 (see **Figures S2, S3 (SM)**). Note that the added costs linked with the dose increase would not hamper the economics of the process significantly (*vide infra*).

Most of the generation of $^{\bullet}OH$ (and/or other oxidizing species) in the ZVI-Fenton process stems from direct interaction between H_2O_2 and the ZVI surface, while the detected values of dissolved Fe(II, III) are very low and allow for excluding a significant role of the homogeneous-phase process (Minella et al., 2016 & 2019). The reasonable reactions involved in the heterogeneous process would take place between H_2O_2 and surface Fe^o or Fe(II) species. Fe(II) species are likely to already occur on the ZVI surface from the very start (*e.g.*, due to storage of ZVI in air), especially in the absence of acid-washing treatments to remove them. While decreasing the ZVI reactivity in general, surface Fe(II) can be involved in the Fenton reaction and would thus not be a problem in this context. Because the reaction involves ZVI + H_2O_2, by increasing the ZVI loading and/or the H_2O_2 concentration one could offset the decreasing production yield of $^{\bullet}OH$ as the pH increases, although only up to a certain degree. Actually, a further increase of ZVI and/or H_2O_2 beyond the optimum values would decrease the degradation efficiency, as both reactants can scavenge $^{\bullet}OH$ as well as contribute to its production (Minella et al., 2019):

$$Fe^{o}_{surface} + H_2O_2 + 2\ H^+ \rightarrow Fe^{2+}_{surface} + 2\ H_2O \quad (4)$$

$$Fe^{2+}_{surface} + H_2O_2 + H^+ \rightarrow Fe^{3+}_{surface} + {}^{\bullet}OH + H_2O \quad (5)$$

$$Fe^{2+}_{surface} + {}^{\bullet}OH + H^+ \rightarrow Fe^{3+}_{surface} + H_2O \quad (6)$$

$$H_2O_2 + {}^{\bullet}OH \rightarrow HO_2{}^{\bullet} + H_2O \quad (7)$$

Because satisfactory results are hardly achieved with the classic or modified Fenton reaction at ~neutral pH (in which case reactivity is often totally lost; Jung et al., 2009), the ability of ZVI-Fenton to still work well in ~neutral conditions makes it very attractive for water treatment.

IMI undergoes rather fast hydrolysis at pH 4 or lower. Therefore, the ZVI-Fenton degradation of IMI was only tested in the pH range 5-7 (**Figure S4 (SM)**), where hydrolysis is slow enough to be negligible at the experimental time scales. Significant hydrolysis of CFZ and VNM could be excluded at all the investigated pH values.

Some removal of the three studied compounds was also achieved in the presence of ZVI alone, without H_2O_2 (blank experiments; **Figures S2-S4 (SM)**). Part of the reason could be the adsorption of the substrates on ZVI, but this material is also known to trigger the Fenton reaction in the presence of dissolved O_2 (reactions 8-10) (Joo et al., 2005):

$$Fe^o + 2\ O_2 \rightarrow Fe^{2+} + 2\ O_2^{\bullet-} \quad (8)$$

$$2\ O_2^{\bullet-} + 2\ H^+\ (O_2^{\bullet-} + HO_2^{\bullet} + H^+) \rightarrow H_2O_2 + O_2 \quad (9)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (10)$$

However, antibiotic removal by ZVI alone was considerably less effective compared to ZVI + H_2O_2 (**Figures S2-S4 (SM)**), even when taking into account the experimental variability (**Figure S1 (SM)**). Indeed, the addition of H_2O_2 was strictly required to achieve complete removal of the contaminants in 1 h. The Fenton-like reaction ZVI + H_2O_2 is considerably more effective than ZVI adsorption and/or the process involving ZVI + O_2, where H_2O_2 is generated in rather small amounts by $O_2^{\bullet-}/HO_2^{\bullet}$ dismutation (Keenan and Sedlak, 2008). Moreover, the three antibiotics did not undergo degradation in the presence of H_2O_2 alone, without ZVI (data not shown).

To avoid IMI hydrolysis, the degradation of the three antibiotics in mixture was studied at pH 5-7. The three compounds taken separately underwent effective degradation under similar but not identical conditions. Actually, it was not possible to find conditions similar to those ensuring degradation of the separate compounds, under which the compounds in mixture could be degraded efficiently (see **Table S3 (SM)** for a summary of these preliminary experiments, where the doses of ZVI and H_2O_2 were varied). A possible solution to this problem is to make the reaction conditions more stringent, *e.g.*, by increasing the concentration of H_2O_2. However, ZVI and H_2O_2 behave as scavengers of ${}^{\bullet}OH$ and other oxidizing reactive species, in addition to producing them (Buxton et al., 1988). Therefore, both ZVI and H_2O_2 have optimal doses for degradation, and a further increase above a certain limit is actually detrimental (Minella et al., 2019). As an alternative, reagents can be added multiple times. In the case of H_2O_2, which is degraded over time under Fenton conditions (*vide infra*), multiple additions ensure sufficiently high but not excessive concentrations at any time

point, thereby limiting the role of H_2O_2 as scavenger (Minella et al., 2018). Very satisfactory performance was achieved by adding H_2O_2 both at the start of the reaction, and a second time after 30 min (see arrows in **Figure 1**). The addition at 30 min consisted in a very small amount of a concentrated H_2O_2 solution (5 or 10 μL depending on the pH value, 1 mol L^{-1}), not to significantly alter the overall volume of the reaction mixture. By so doing, complete or almost complete degradation of CFZ, IMI and VNM was achieved in one hour at pH 5-7 with ZVI + H_2O_2, while ZVI alone was much less effective (**Figure 1**). It was necessary to offset the decrease in Fenton reactivity with increasing pH by progressively increasing the initial/added H_2O_2 concentration and/or ZVI loading, at the same time avoiding the use of excessive amounts of either species.

A possible reason for the significant ZVI-Fenton reactivity under near-neutral conditions, which differentiates ZVI-Fenton from classic, homogeneous Fenton is that the latter relies on Fe(III)-Fe(II) recycling, which is hampered by Fe(III) precipitation at near-neutral pH. An additional reason could be a different reaction mechanism. Indeed, homogeneous Fenton yields significant amounts of $^{\bullet}OH$ at pH 2-3 ($^{\bullet}OH$ yield ~60%; Minero et al., 2013), but the production of $^{\bullet}OH$ decreases significantly as the pH increases. Ferryl (FeO^{2+}) replaces $^{\bullet}OH$ as the Fenton oxidant at near-neutral pH (Farinelli et al., 2020), and the lower reactivity of FeO^{2+} compared to $^{\bullet}OH$ might contribute to the decrease in Fenton degradation performance. Our EPR measurements showed that $^{\bullet}OH$ (identified as the DMPO-OH adduct) still occurred significantly with ZVI-Fenton at pH 6 (see **Figure S5a (SM)**). A $^{\bullet}OH$-based reactivity in near-neutral pH conditions would be consistent with the significant degradation of antibiotics observed with ZVI-Fenton at pH 5-7. Moreover, the generation of the Fenton oxidant would likely involve a reaction between H_2O_2 and the ZVI surface, as suggested by previous results (Minella et al., 2016), while aqueous Fe species played little to negligible role in $^{\bullet}OH$ generation (see **Figure S5b (SM)**). This finding agrees well with the negligible levels of leached Fe that were detected in the studied reaction systems (*vide infra*).

3.2. ZVI-Fenton degradation of antibiotics in partially treated wastewater

The ZVI-Fenton degradation was then carried out in wastewater matrix (the two wastewater samples WWa and WWb had different characteristics, see **Table S1 (SM)**), spiked with a mixture of CFZ, IMI and VNM, each at 4 μmol L^{-1} initial concentration.

Wastewater contains several organic and inorganic interfering compounds that inhibit the Fenton reaction (Salgota et al., 2006; Zhang et al., 2019; Ricceri et al., 2019). Therefore, compared to ultra-pure water, it is necessary to increase the doses of ZVI and H_2O_2 to still achieve effective degradation in wastewater. Multiple additions of reagents are often a compulsory choice to minimize the scavenging of reactive transient species by ZVI and H_2O_2 (Minella et al., 2018; Minella et al., 2019). Many optimization experiments were carried out to look for suitable degradation conditions, which returned the following key indications: *(i)* Almost negligible abatement of the studied antibiotics was observed with ZVI alone; therefore, significant adsorption on ZVI or degradation by ZVI + O_2 can be practically excluded in secondary wastewater. *(ii)* Effective degradation was achieved at pH 5 but not at pH 6 or 7 (see for instance **Figure S6** and **Table S4 (SM)** for a summary of the experiments carried out at pH 6, varying the H_2O_2 and ZVI doses within the framework of multiple additions); from this point of view, CFZ, IMI and VNM look more recalcitrant to ZVI-Fenton degradation compared to, *e.g.*, ibuprofen (Minella et al., 2019). *(iii)* Complete degradation in wastewater required 90 min reaction time, as compared to 60 min in ultra-pure water. *(iv)* The pH value tended to increase during the reaction (possibly due to H^+-consuming processes, such as (4)-(6)), and it was thus necessary to monitor and correct it by a small addition of acid as the reaction progressed. For instance, in both WWa and WWb at initial pH 5, it was necessary to adjust pH back to 5 at 30 min reaction time, to offset gradual pH increase and achieve satisfactory degradation. The pH of the two systems (WWa, WWb) continued increasing after 30 min to reach a final value of 5.7-5.8 at 90 min, which is suitable for wastewater discharge ($5.5 \leq pH \leq 9.5$) (Barbagallo et al., 2001). For this reason, and because effective degradation could be achieved anyway, after 30 min the pH was monitored but no longer corrected. The time trends of

CFZ, IMI and VNM at pH 5 are reported in **Figure 2**, for WWa (**2a**) and WWb (**2b**). The conditions reported in the figure are those allowing for the best degradation with the lowest consumption of reagents.

Conductivity is an important parameter for wastewater quality, because saline wastewaters are less suitable for discharge or, most notably, reuse in agriculture (Hussain et al., 1999). Treatments requiring pH adjustment may affect conductivity, thus the time trends of conductivity were monitored. The results observed in both wastewaters (WWa, WWb) are reported in **Figure S8 (SM)**. A very slight conductivity increase was observed, presumably because of SO_4^{2-} addition. However, conductivity changes were quite small and would not affect the wastewater requirements for discharge or reuse, even in the demanding field of agriculture (Salgota et al., 2006).

The ZVI-Fenton treatment relies on H_2O_2 addition, and in some contexts, the elimination of H_2O_2 after treatment might be an issue (in other cases, residual H_2O_2 may be welcome as a disinfectant) (Bownik and Stępniewska, 2015). For this reason, the time trend of H_2O_2 under ZVI-Fenton was studied (see **Figure S9 (SM)**). Interestingly, H_2O_2 underwent consistent degradation despite additions at 30 and 60 min, and H_2O_2 was negligible in solution at the end of the reaction (90 min). Therefore, the post-treatment elimination of H_2O_2 would not be necessary.

Dissolved Fe (either Fe(II) or Fe(III)) was monitored during the ZVI-Fenton reaction at pH 5 and 6, but it was always undetectable (< 0.05 $mg_{Fe}\ L^{-1}$; data not shown). This finding excludes significant Fe leaching from ZVI to the aqueous solution (as an alternative, there could be leaching followed by fast precipitation on the solid). Actually, dissolved Fe is not necessary for the ZVI-Fenton system to work, because the process can be triggered by the reaction of the iron sites at the ZVI surface with dissolved H_2O_2 (a genuine heterogeneous Fenton process) (Minella et al., 2016). The lack of dissolved Fe is very positive when considering the maximum Fe levels allowed by legislation in many countries (1 or 2 $mg_{Fe}\ L^{-1}$) (Barbagallo et al., 2001; Salgota et al., 2006; Zhong et al., 2012; National Environmental Agency, 2016).

To sum up, the optimized operational conditions for the effective degradation of CFZ, IMI and VNM (initial pH 5) are reported in **Table S5 (SM)**. They are the same for both WWa and WWb.

3.3. Identification of degradation intermediates

An MS/MS characterization study was first performed upon infusion into the mass spectrometer of methanolic standard solutions of each antibiotic at 100 μg L^{-1}, in order to find the most abundant product ions and the fragmentation pathways, which could be useful to identify the chemical structures of the intermediates. All the analytes were ionized in both positive and negative ion modes, but the first was the most sensitive and was selected for further runs. **Figures S10 - S12 (SM**) show the MS/MS spectra of CFZ, IMI and VNM, respectively.

Eighteen intermediates for CFZ, five for IMI and three for VNM were identified by UHPLC-MS/MS. An additional six compounds were detected for VNM, but they were likely synthesis impurities that already occurred at t = 0. Indeed, their abundance decreased as the reaction progressed. Chemical structures are proposed on the basis of MS spectra, isotopic cluster obtained by Enhanced Resolution experiments, and interpretation of MS/MS product ions. All the proposed structures gave 95-100% matching compared to *in silico* fragmentation. A tentative reaction scheme is reported in **Scheme 1** (more comprehensive structural data including the main fragments are reported in **Table S6 (SM)**).

Eighteen CFZ intermediates were identified, many of which are structural isomers (the relevant MS/MS spectra are reported in **Figures S13 – S30 (SM)**). Many chemical structures showed opening (CFZ-P5c, CFZ-P5d, CFZ-P6a, CFZ-P6b), loss (CFZ-P3 and CFZ-P7) or modification (CFZ-P1a, CFZ-P1b, CFZ-P1c, CFZ-P5a, CFZ-P5b) of the beta-lactam ring. CFZ-P4a and CFZ-P4b were the intermediates with the highest peak areas and were identified as sulfoxide compounds, in which thioether oxidation occurred at two different S atoms. The chemical structures of these two compounds have been already reported upon oxidation of CFZ by $KMnO_4$ (Li et al., 2016).

Sulfoxide groups also occur in the proposed structures of CFZ-P1a, CFZ-P1b and CFZ-P1c, where the formation of an aziridine ring would follow the loss of formaldehyde from the beta-lactam ring. CFZ-P5 showed two couples of sulfoxide-containing isomers: the first had an aziridine ring (CFZ-P5a and CFZ-P5b), the second was characterized by beta-lactam ring-opening (CFZ-P5c and CFZ-P5d). CFZ-P6a and CFZ-P6b were the hydroxylated forms of CFZ-P5c and CFZ-P5d, respectively. CFZ-P7 had the same chemical structure as CFZ-P1a, except for the formation of sulfoxide.

The proposed chemical structures of CFZ-P2a and CFZ-P2b are characterized by tetrazole loss and cleavage of the dihydrothiazine ring. The structure of CFZ-P3b (already present at trace level as CFZ impurity, and showing intensity increase during the degradation) corresponds to 5-methyl-4,5-dihydro-1,3,4-thiadiazole-2-thiol, as previously reported in the photocatalytic degradation of CFZ (Gurkan et al., 2012). In contrast, a linear structure compatible with the MS/MS fragmentation spectrum is proposed for CFZ-P3a. Finally, CFZ-P8a and CFZ-P8b were characterized by the loss of the thiadiazole group.

Five degradation intermediates were detected for IMI (see **Figures S31 – S35 (SM)** for their MS/MS spectra), among which IMI-P1 was already present at t = 0 and rapidly increased after 5 min reaction time, quickly becoming the most intense intermediate of IMI. The proposed structure is 3-(methylsulfanyl)-1H-pyrrole-2-carboxylic acid. IMI-P2, IMI-P3 and IMI-P4 were also present in traces at t = 0, probably due to IMI instability (De Souza Barbosa et al., 2019), and increased afterwards. IMI-P2 showed the opening of the beta-lactam ring, as already found in the metabolic degradation of IMI (Abdel-Moety et al., 2010), whereas IMI-P3, IMI-P4 and IMI-P5 were formed by reaction of the IMI diazene moiety to produce carboxylic and oxoacetic acids.

The chemical structure of VNM is very complex and includes an aminoglycoside and a polypeptide moiety. Direct infusion showed that the product ions at *m/z* 100, 118 and 144 were formed by fragmentation of the aminoglycoside moiety, which is important for the interpretation of the structures of the intermediates. Indeed, the latter preserved the aminoglycoside moiety, while degradation usually occurred on the polypeptide one.

The MS/MS spectra of the three detected VNM intermediates are shown in **Figures S36 – S38 (SM)**. VNM-P1 corresponds to $[M\text{-}OH+H]^{2+}$ at *m/z* 715.6, VNM-P2 (*m/z* 730.7) derives from CH_3N loss and N-acetylation, VNM-P3 (*m/z* 731.2) derives from water loss, oxidation and further methylation. The structures of VNM-P2 and VNM-P3 have been proposed as intermediates of VNM degradation in the environment (Cao et al., 2018). Other species were found at $t = 0$ (VNM-P4a, VNM-P4b, VNM-P5a, VNM-P5b, VNM-P6a, and VNM-P6b; **Figures S39 – S43 (SM)**), more likely as synthesis impurities rather than degradation intermediates (Hadwiger et al., 2012; Belissa et al., 2014; Cao et al., 2018). Indeed, their signal intensities decreased during the reaction.

The intermediates that potentially pose the highest concern are those with antibiotic properties, which could still promote the development of ABR and/or the selection of antibiotic-resistant bacteria. The antibiotic action of VNM stems from rather complex interactions (Ndieyira et al., 2017), the modification of which upon structural changes is poorly predictable. In contrast, the CFZ and IMI intermediates that might still act as antibiotics are those having an intact β-lactam ring (Takata et al., 1981; Wright et al., 2017). They are CFZ-P4a,b, as well as IMI-P3, IMI-P4 and IMI-P5.

The chromatographic peak areas give some tentative insight into the compounds abundance: the time trends of the peak areas of the primary antibiotic, the antibiotic plus the lactam ring-retaining intermediates, and the antibiotic plus all the intermediates are reported in **Figure S44a** (CFZ) and **S44b** (IMI) of the SM. The chromatographic peak areas of the primary antibiotics strongly prevail over those of the ring-retaining intermediates and, additionally, the latter would account for a small fraction of degraded CFZ and a negligible fraction of degraded IMI. Therefore, it can be assumed that when all the primary antibiotic has disappeared, as observed in the degradation experiments of **Section 3.2**, the anti-microbial action of the degraded mixture would be only a very small fraction of the initial one.

3.4. Approximate estimate of treatment costs

The optimized reaction conditions for wastewater reported in **Table S5 (SM)** were the basis for a preliminary cost assessment, scaling the total added amounts of ZVI, H_2O_2 and acid from the laboratory volume (50 mL) to 1 m^3 wastewater. Both the acid amount needed to fix the initial pH value and that used to adjust pH during the reaction were taken into account. Furthermore, there would be no need to add a base to adjust pH at the end of the treatment, because the final pH value was 5.7-5.8. Only the overall costs of the reactants are considered at this stage, thereby neglecting other costs such as investment costs, energy and human labor. This approach yields a lower limit for the actual costs, but chemicals are usually the main expenditure in Fenton treatments (Balabanič et al., 2012). Current commercial costs of bulk chemicals were thus considered (Made-in-china.com, 2020), and the results are reported in **Table 1**. These costs would be added to those of traditional wastewater treatment (around 0.38 \$ m^{-3}) (Minella et al., 2018), and they differ between WWa and WWb due to the different amounts of H_2SO_4 that were needed to fix pH. The reason is that WWa had lower alkalinity compared to WWb (**Table S1 (SM)**).

The addition of a tertiary treatment step based on ZVI-Fenton would increase the overall treatment costs by merely 9-17%, depending on the wastewater type, but it would ensure the degradation of compounds that the traditional treatment does not remove. These compounds are not limited to antibiotics reserved for hospital settings, because the same or very similar conditions are known to induce the degradation of other contaminants of emerging concern such as ibuprofen (Minella et al., 2019).

The ZVI-Fenton costs (**Table 1**) look very competitive, although successful operation at pH 5 requires higher doses of H_2O_2 and ZVI compared to, e.g., pH 3: costs are comparable with those of UV treatment intended for disinfection (which is much cheaper compared to UV for depolluting, as the latter needs higher irradiance and longer contact times), and they are quite lower than ozonation costs (Dore et al., 2020). As a further comparison, ZVI alone used as reductant at a dose of 1 g L^{-1}

for the dehalogenation of halogen-containing antibiotics (Cao et al., 2017) would cost about 0.8 $ m^{-3}, which could be an important economic burden in the field of wastewater treatment.

3.5. Implications for wastewater treatment

The ZVI-Fenton technique would not work at the natural pH of wastewater (7.5-7.8 in the present case), but the treatment methods that are based on pH adjustment (in the present case, acidification + neutralization) have the potential to adversely affect the conductivity of the water matrix. However, differently from classic Fenton that requires strongly acidic conditions, the present results show that the additions of H_2SO_4, needed to fix the initial pH to 5 and to maintain it during the reaction, would affect conductivity to an insignificant degree. Therefore, the ZVI-Fenton treatment step would not hamper even the demanding reuse of wastewater in agriculture, provided that initial wastewater conductivity meets the relevant guidelines. The reason is that pH 5 is still sufficiently near neutrality to limit the added amount of H_2SO_4, which benefits both process economics and treated effluent conductivity. This is a considerable advantage of ZVI-Fenton over traditional Fenton, which needs to be operated at pH 3 or near 3 to achieve effective degradation in the wastewater matrix (Minella et al., 2018).

Another important advantage of ZVI-Fenton over other Fenton-based techniques is that H_2O_2 reacts at the solid surface, with no need for dissolved Fe(II) species to trigger the reaction. Therefore, degradation is effective even in the presence of undetectable dissolved Fe, which cancels the need for a final step of dissolved Fe precipitation and sedimentation. There would still be the need to remove ZVI after treatment, but the procedure would be highly simplified by its magnetic properties. The lack of residual H_2O_2 after treatment might or might not be a positive issue in view of final disinfection (preliminary experiments suggest that such an additional step would be required, data not shown). However, unless H_2O_2 itself is used later to disinfect wastewater, the occurrence of residual H_2O_2 in solution would be more of a problem, because of possible unwanted interactions with other disinfectants such as HClO (Shams El Din and Mohammed, 1998).

Therefore, the lack of residual H_2O_2 after ZVI-Fenton would be a positive occurrence in most cases, and make a later step of H_2O_2 elimination unnecessary.

The scaling up of the ZVI-Fenton treatment from the lab size to an urban WWTP would certainly be challenging. Experiments were here made in batch mode, which in full-scale conditions would require a very large tank or even a series of large tanks, where stirring of the ZVI solid might be an issue. A flow-through system might be an interesting alternative, but studies on how such a system might be designed or conducted are currently lacking. Therefore, further research is still needed to understand which configuration is more likely to work under full-scale conditions.

The ZVI-Fenton step should ideally be located after secondary treatment (activated sludge process) and before the phosphorous elimination step. Such a step should also involve magnetic ZVI precipitation if the process is run in batch mode, which might aid the recovery by traditional precipitation of P-containing compounds such as struvite. In this way, the ZVI-Fenton step might positively interact with the recovery of added-value products from wastewater. In contrast, ZVI precipitation might not be needed in the case of a flow-through design, the detailed features of which should, however, still be defined.

4. CONCLUSIONS

This work shows that ZVI-Fenton can achieve effective and cheap elimination of antibiotics reserved for hospital settings, thereby giving a potentially important contribution to fight the onset of bacterial resistance against these life-saving and last-resort drugs. The identified transformation intermediates of the studied antibiotics (CFZ, IMI and VNM) indeed suggest that, once the primary contaminant is eliminated, most of the potential to trigger ABR is removed. In actual wastewater the process is effective at pH 5, with a very limited impact of acidification on treatment costs or wastewater conductivity, even in the case of wastewater reuse in agriculture. The absence of Fe

leaching from ZVI is positive for wastewater quality and, compared to other Fenton techniques, elimination after treatment of magnetic ZVI would be much easier than removal of dissolved Fe or Fe sludge. Finally, the same conditions would be effective towards other contaminants of emerging concern, including recalcitrant pharmaceuticals (Minella et al., 2019), thereby anticipating future wastewater legislation (Brack et al., 2015).

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Table 1. Assessment of treatment costs for chemical reagents, required to treat WWa and WWb with ZVI-Fenton at initial pH 5. Note that conventional treatment would cost around 0.4 \$ m^{-3}, to which these are added costs.

	Cost, \$ m^{-3}	
	WWa	**WWb**
ZVI	0.016	0.016
H_2O_2	0.012	0.012
H_2SO_4	0.008	0.038
Total cost	***0.036***	***0.066***

Captions to figures and schemes

Figure 1. Degradation of a mixture of 4 μmol L^{-1} CFZ + 4 μmol L^{-1} VNM + 4 μmol L^{-1} IMI in ultra-pure water, in the presence of ZVI + H_2O_2 (solid symbols) and of ZVI alone (open symbols). **(a)** pH 5 by PB, 100 μmol L^{-1} H_2O_2 per addition, 0.01 g L^{-1} ZVI; **(b)** pH 6 by PB, 200 μmol L^{-1} H_2O_2 per addition, 0.03 g L^{-1} ZVI; **(c)** pH 7 by PB, 200 μmol L^{-1} H_2O_2 per addition, 0.04 g L^{-1} ZVI. PB: phosphate buffer (H_3PO_4 + NaH_2PO_4) 0.001 mol L^{-1}. The vertical arrows highlight the time of the second addition of H_2O_2, in the case of the ZVI + H_2O_2 runs.

The pseudo-first order rate constants *k* of antibiotic degradation, obtained by fitting the curves with a mono-exponential equation, are as follows (error bounds represent ±σ, value in brackets refer to runs with ZVI alone).

pH	*k, min^{-1}*		
	CFZ	**IMI**	**VNM**
5	0.49±0.05 *(0.004±0.001)*	0.41±0.05 *(0.002±0.001)*	0.45±0.08 *(0.005±0.001)*
6	0.26±0.05 *(0.005±0.001)*	0.15±0.03 *(0.0015±0.0006)*	0.32±0.09 *(0.008±0.001)*
7	0.09±0.01 *(0.018±0.001)*	0.07±0.01 *(0.002±0.001)*	0.16±0.02 *(0.019±0.001)*

Figure 2. Degradation of a mixture of 4 μmol L^{-1} CFZ + 4 μmol L^{-1} VNM + 4 μmol L^{-1} IMI at pH 5, adjusted by H_2SO_4 before the beginning of the reaction and corrected again at 30 min. **(a)** WWa. **(b)** WWb. Conditions used in both cases: 0.02 g L^{-1} ZVI; 400 μmol L^{-1} H_2O_2 added in three aliquots (300 μmol L^{-1} at 0 min, 50 μmol L^{-1} at 30 min, and 50 μmol L^{-1} at 60 min). The solid vertical arrows highlight the times of further additions of H_2O_2. The dotted vertical arrow highlights the time of pH correction (H_2SO_4). Solid symbols: ZVI + H_2O_2; open symbols: ZVI alone. WWa had 270 μS cm^{-1} conductivity, pH 7.5 and 7.8 mg_C L^{-1} TOC. The corresponding parameters for WWb were 900 μS cm^{-1}, 7.8 and 38.9 mg_C L^{-1}.

WW	*k, min^{-1}*		
type	**CFZ**	**IMI**	**VNM**
WWa	0.049±0.006	0.048±0.007	0.030±0.005
	(0.003±0.001)	*(negligible)*	*(0.003±0.001)*
WWb	0.081±0.012	0.106±0.015	0.036±0.005
	(0.002±0.001)	*(negligible)*	*(0.002±0.001)*

Scheme 1. Tentative reaction schemes to account for the ZVI-Fenton transformation of the studied antibiotics (**a**: CFZ; **b**: IMI; **c**: VNM) into the detected reaction intermediates (P#). The dashed arrows indicate very tentative pathway proposals.

Figure 1

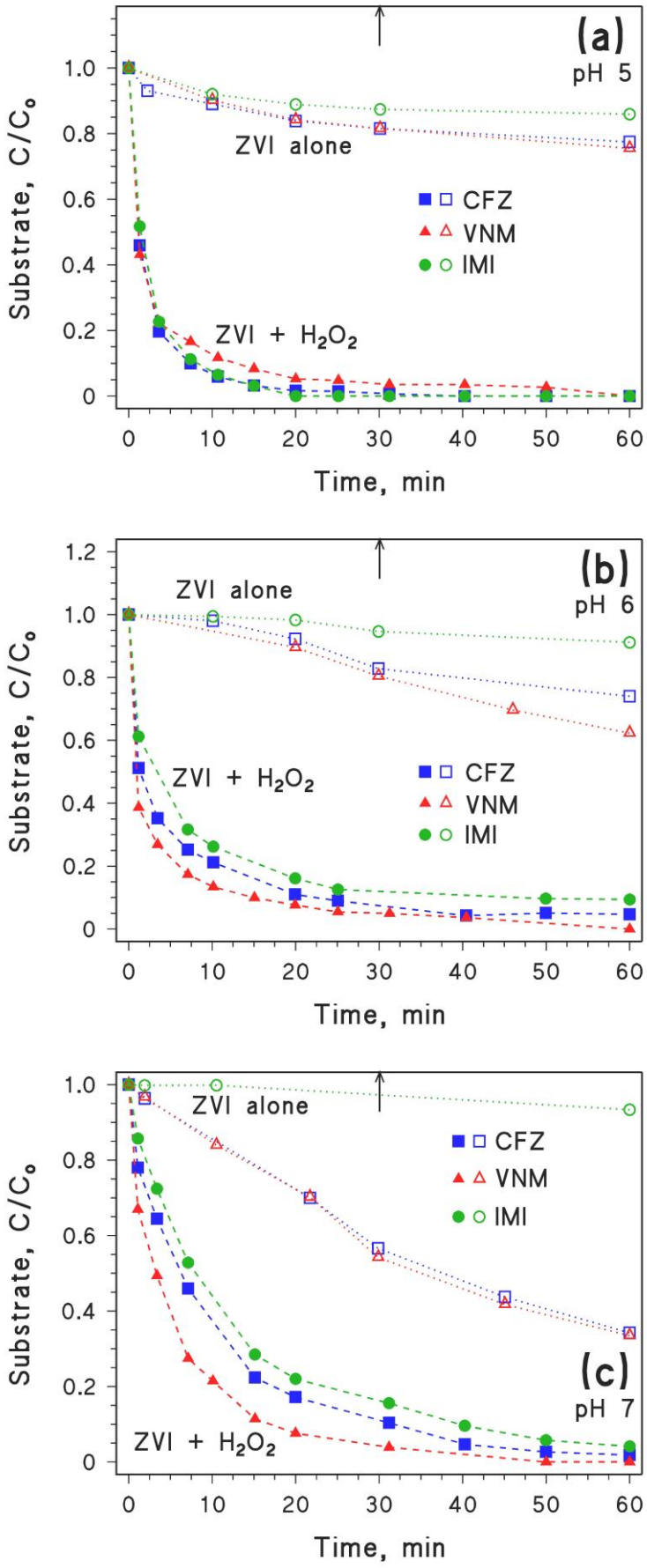
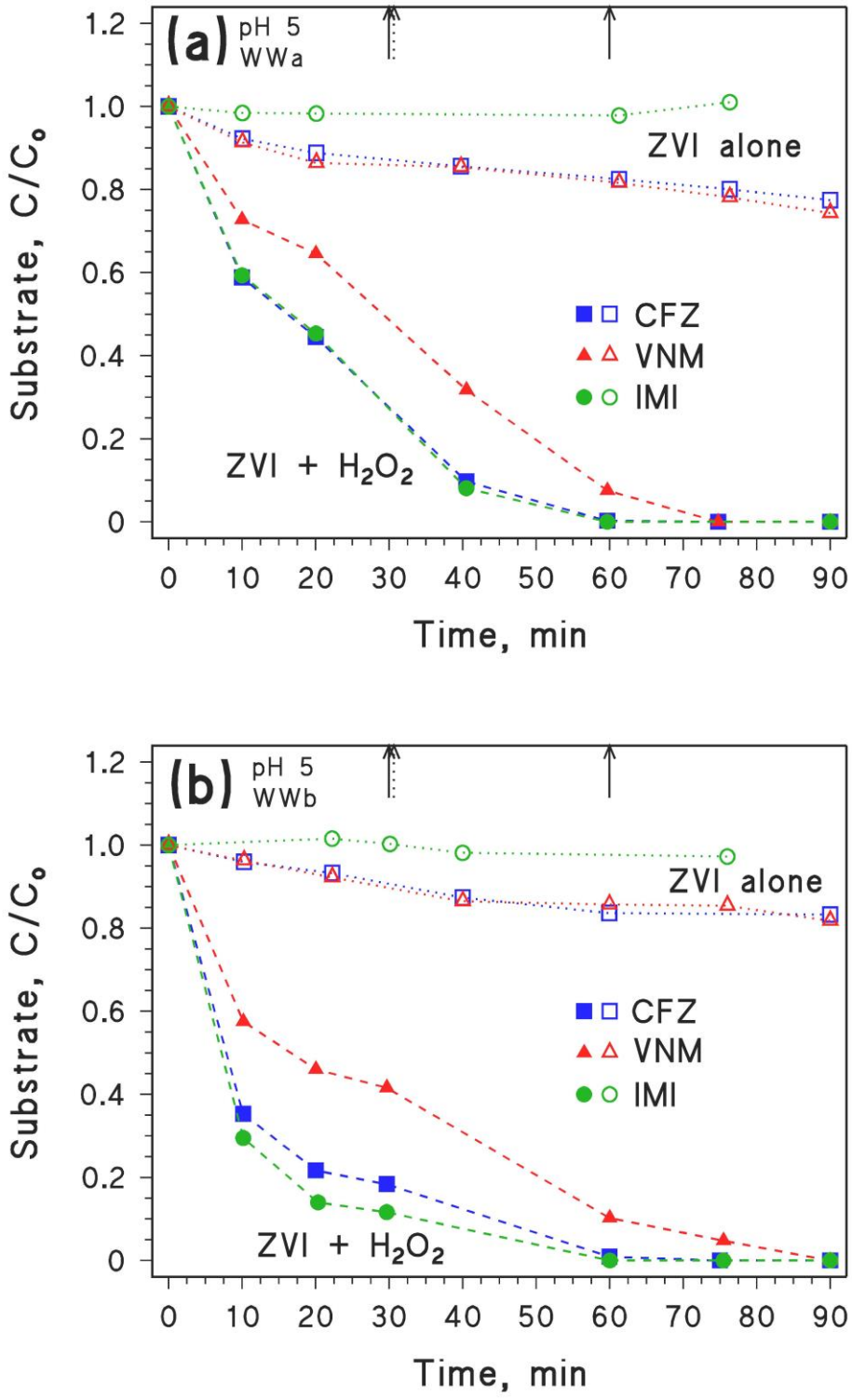
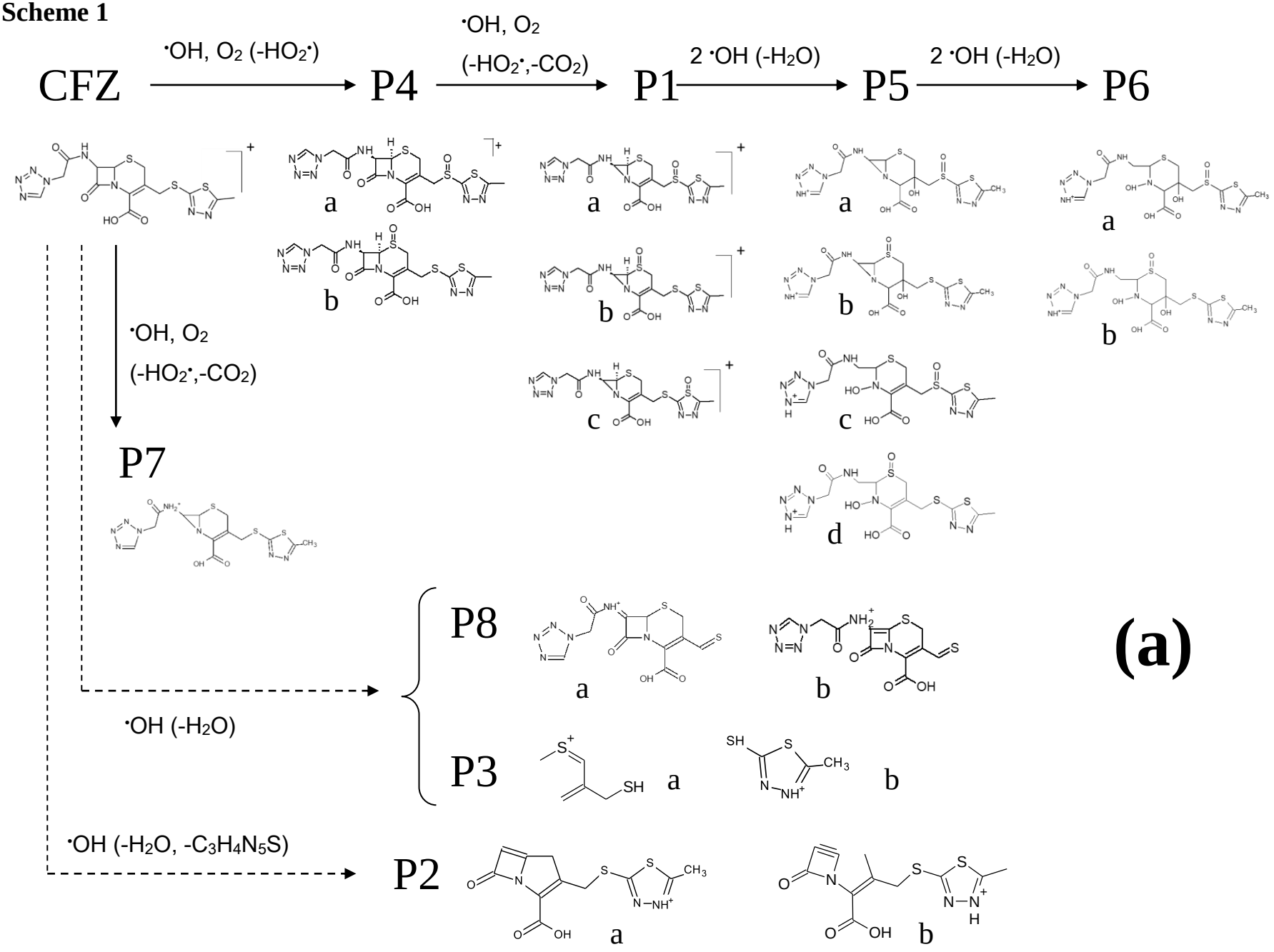
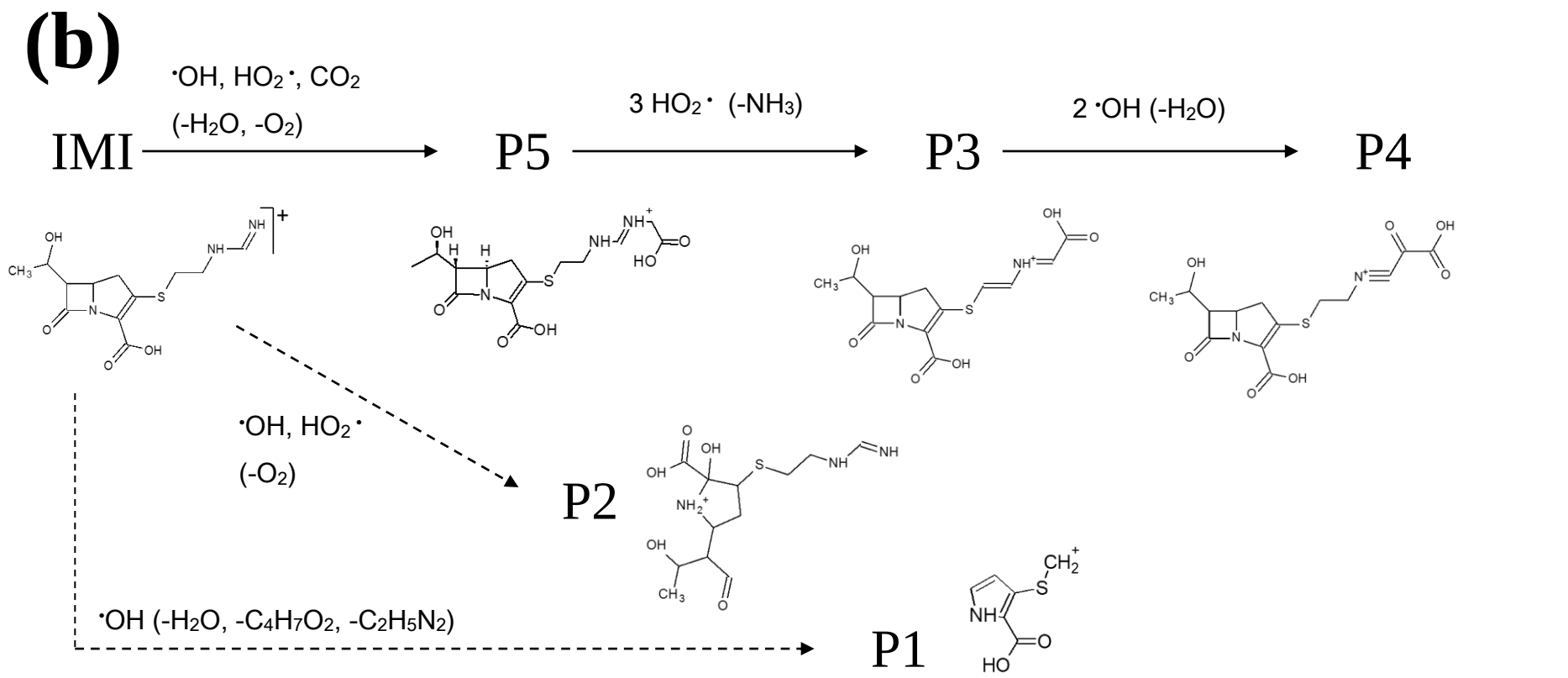

(a)
pH 5
ZVI alone
ZVI + H_2O_2
CFZ
VNM
IMI
Substrate, C/C$_o$
Time, min
(b)
pH 6
ZVI alone
ZVI + H_2O_2
CFZ
VNM
IMI
Substrate, C/C$_o$
Time, min
(c)
pH 7
ZVI alone
ZVI + H_2O_2
CFZ
VNM
IMI
Substrate, C/C$_o$
Time, min

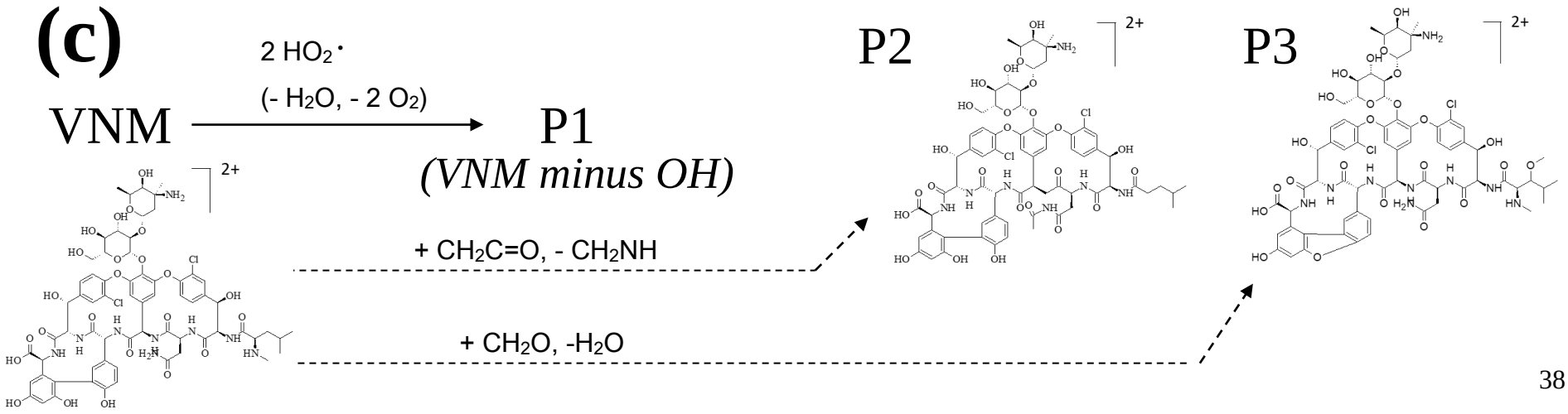
Figure 2


(a) pH 5
WWa
ZVI alone
CFZ
VNM
IMI
ZVI + H2O2
Substrate, C/Co
Time, min
(b) pH 5
WWb
ZVI alone
CFZ
VNM
IMI
ZVI + H2O2
Substrate, C/Co
Time, min

Scheme 1


CFZ
·OH, O2 (-HO2·)
P4
·OH, O2
(-HO2·,-CO2)
P1
2 ·OH (-H2O)
P5
2 ·OH (-H2O)
P6
a
b
c
d
·OH, O2
(-HO2·,-CO2)
P7
P8
P3
·OH (-H2O)
·OH (-H2O, -C3H4N5S)
P2
(a)


(b)
·OH, HO2·, CO2
(-H2O, -O2)
IMI
P5
3 HO2· (-NH3)
P3
2 ·OH (-H2O)
P4
·OH, HO2·
(-O2)
P2
·OH (-H2O, -C4H7O2, -C2H5N2)
P1


(c)
2 HO2·
(- H2O, - 2 O2)
VNM
P1
(VNM minus OH)
+ CH2C=O, - CH2NH
+ CH2O, -H2O
P2
P3
2+