

DRAFT: Tannery nanoremediation: Effect of Fenton-like process on microbial communities from tannery wastewater using nanoscale zero-valent iron.

Tentative author order

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ABSTRACT

The tanning industry is known for its severe impacts on the environment. Tanning effluents usually have high levels of hexavalent chromium, high chemical oxygen demand, and high levels of chlorides, among others. Here we explored two different methods of remediation for tanning effluents: traditional Fenton oxidation using ferrous sulfate as source of iron and the use of nanoscale zero-valent iron nanoparticles (nZVI) in a Fenton-like process. Results show significant effects in the microbial community structure along the remediation process. We found Fenton to be very effective at decreasing the chemical demand of oxygen (COD) affecting the relative abundance of several taxa. We also found differences in the effect of traditional Fenton vs Fenton-like processes. These findings are discussed in light of the biological and biochemical properties of the affected taxa.

INTRODUCTION

The tanning industry is known as an industry characterized by significant discharges of wastewater with a complex matrix, owing to its high composition of organic contaminants such as tannins, fats, and organic solvents, along with heavy metals such as chromium and other hazardous substances derived from the entire leather tanning process (Mannacharaju et al., 2020; Zhao & Chen, 2019). The discharge of these untreated effluents or those treated inadequately pose environmental risks to bodies of water, soil, and air, as well as public health issues (Nigam et al., 2023).

The microbial community inhabiting wastewater constitutes a dynamic and diverse ecosystem derived from environmental microorganisms, raw materials, and those introduced at each stage of tanning process, proliferating in response to available substrates (Fernandez et al., 2019; C. Li et al., 2021). This community exhibits various metabolic capacities involved in complex interactions, encompassing microorganisms capable of bioremediation and detoxification of actual contaminants, but also pathogens of significance to human health (Abate et al., 2021). Wastewater treatment aims to mitigate the environmental impact of contaminants generated during leather production. As the effluent undergoes treatment, physicochemical variables, nutrient availability, and contaminant concentrations experiment changes that influence the structure and composition of the microbial communities inhabiting this matrix (Fernandez et al., 2019; Sharma et al., 2021).

Advanced oxidation processes (AOPs) are technologies aimed at the degradation of persistent pollutants found in tannery wastewater, offering efficient and sustainable solutions for their remediation (Korpe et al., 2019; Korpe & Rao, 2021; Nigam et al., 2023). The Fenton reaction involves the generation of highly reactive hydroxyl radicals ($\bullet\text{OH}$) by the reaction of iron (Fe^{2+}) with hydrogen peroxide (H_2O_2) under low pH conditions, leading to the oxidative degradation of organic pollutants and thus reducing the chemical oxygen demand (COD) of the treated waters (Zhang et al., 2019). Conventional Fenton reaction

uses salts like FeSO₄ to provide iron to the reaction; however, in recent years, there has been interest in improving the performance of the Fenton reaction by using iron nanoparticles as catalysts instead of iron salts due to their unique properties, including high contact surface area, high reactivity, and catalytic efficiency, promoting better degradation of contaminants (Gil-Díaz et al., 2021; Xu et al., 2022).

Fenton bioremediation technologies have an impact in the bodies of water where it is utilized, affecting the microbial communities inhabiting these environments; Drastic changes in physicochemical conditions, exposure to oxidative stress, and the introduction of chemical agents can exert selective pressure on microbial populations, leading to changes in community composition, diversity, and functionality (Medina et al., 2018). The aim of this study is to elucidate the effects of conventional fenton (fenton-sulfate) and iron nanoparticle fenton (fenton-nano) on microbial communities in terms of their taxonomy, relative abundances, biological properties, as well as their relationship with physicochemical variables that may affect microbial composition.

MATERIALS AND METHODS

Sample Collection:

Samples were collected in the district of Villapinzón, a municipality in the north of Cundinamarca (5°11'46.86" N, 73°36'26.824" W; Colombia) at 2738 m.a.s.l, where most of the tanneries in this part of Colombia are located.

Water samples were collected before entering the tanning process (tap water) from the river that crosses the municipality (Bogotá river); 1,5 Liters of tap water were vacuum filtered using 0,025µm filter and were preserved in absolute ethanol until its processing. Wastewater was collected using a mix of beamhouse and tanning effluent in 20:1 proportion, respectively (tannery wastewater). Before tannery wastewater was collected, it was filtered using a common cotton filter to eliminate large solid particles. Tannery wastewater was used to evaluate the effect of the traditional Fenton process using ferrous sulfate as source of iron (fenton-sulfate), and the use of zero-valent iron nanoparticles (nZVI) in a Fenton-like process named (fenton-nano); At the end of each fenton treatment, 24 hours were allowed to pass before sample collection, waiting for the reduction of the foam generated during fenton process and pH neutralization. Replicates were taken from all these treatments at least 3 or 4 times each. Samples were taken to the lab and kept frozen in glycerol 20% (except tap water that was conserved in ethanol) until all of them were ready for DNA extraction and sequencing of the bacterial 16S gene.

Characteristics of sampled water

Physicochemical factors such as chemical demand of oxygen (COD), pH, oxidation - reduction potential (ORP), sulfates, chlorides, dissolved oxygen (DO) and conductivity were measured for all the treatments. To carry out these measurements a portable multi-parameter probe (HI 9828, Hanna Instruments, Woonsocket, RI) was used to test the pH, dissolved oxygen (DO), and oxidation reduction potential (ORP) for water samples in real-time. Using the closed reflux colorimetric method 5220, the chemical oxygen demand (COD) was determined (Water Environment Federation, 2012). Chlorides (Cl) were measured using Method 9253 (Water Environment Federation, 2012) and titrimetric silver nitrate. Lastly, Sulfate values were estimated using UV-VIS spectrophotometry according to the modified approach by (Viggi et al., 2010).

DNA extraction, library preparation and sequencing:

Samples were centrifuged at 4000 g for 15 minutes, the supernatant was discarded and the pellet was resuspended in 10 mL of NAP buffer and kept in agitation (120 rpm) overnight in order to release aggregated microorganisms. After that, samples were centrifuged at 4000 g for 15 minutes, the supernatant (buffer NAP) was discarded and the pellet was washed with TE buffer. Finally, the pellet was resuspended in 200uL of salt solution (0.89%). DNA extraction was performed with NucleoSpin® Soil Kit (Macherey Nagel, REF 740780), according to the manufacturer's protocol.

PCR amplicons were generated using the primers recommended by (Kozich et al., 2013) for the 16s rRNA gene V3V4 hypervariable region. The 3' ends of these primers were modified to accommodate the Illumina adapters. All PCR reactions were carried out in a 25uL final volume. PCR products were purified with magnetic beads. Illumina indexes (Nextera XT V2) were added in a second PCR reaction. PCR products were visualized by agarose electrophoresis in order to verify the expected amplicon size. Library was prepared by mixing PCR amplicons into a pool and quantified by fluorescence using Qubit 2.0 (Invitrogen). Library was normalized to 13ng/uL.

Illumina Miseq PE250 platform was used for sequencing. The library was diluted to 10 pM and mixed with 20% PhiX in order to increase sequence diversity.

Bioinformatics:

Sequencing files in FASTQ format were evaluated with FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016). Trimming was performed with Trimmomatic (Bolger et al., 2014), with the following parameters: SLIDINGWINDOW: 4:20 and a minimal length of 130nt. Trimmed sequences were imported into QIIME2 (Bolyen et al., 2019). Sequence denoising was performed with DADA2 as implemented in QIIME2. Sequences were deduplicated and ASVs were generated using QIIME's standard protocol.

Samples were rarified to 32000 sequences since sequence saturation was reached way below that point for all the samples. Samples were evaluated in terms of alpha and beta diversity. ASVs were classified using the classify-sklearn plugin, using the SILVA 138 database as source of 16S data (Quast et al., 2012)

Statistical Analyses:

Alpha diversity metrics, including Shannon index, Simpson diversity index and Chao1, were calculated as implemented in QIIME2.

Differences in treatments: tap water, tannery wastewater, fenton-nano and fenton-sulfate were subjected to statistical analysis such as Kruskal Wallis (differences among all samples) and Dunn's Test (paired samples). Mantel tests, Pearson correlation and canonical correspondence analysis were performed to explore the relationship between bacterial community structure and physicochemical variables. Variables associated with physicochemical properties and bacterial taxa were ranked using the randomForest package in R.

Relative bacterial abundance was determined and community structures were visualized using principal coordinate analysis (PCoA). Linear discriminant analysis and effect size (LEfSe) analysis were performed at phylum and genus level (Segata et al., 2011).

For all statistical analyses the chosen statistical significance was $P < 0.05$. All statistical analyses were performed using R 4.3.2 (R Core Team 2023).

Graphics were generated using a mixture of QIIME's python scripts, R packages: vegan (Dixon, 2003), phyloseq (McMurdie & Holmes, 2013), ggplot2, and in-house scripts.

RESULTS AND DISCUSSION

Alpha and Beta diversity Analysis of microbial communities

The α -diversity results indicate microbial diversity is high for all treatments (Fig 1). Shannon diversity index was higher for fenton-nano in comparison with tannery wastewater ($P < 0.05$), however, no significant difference was found between fenton-sulfate and tannery wastewater, despite the number of ASVs being higher for the former than for the latter (1271 vs 826 ASVs, respectively). This apparent increase in bacterial diversity after Fenton treatment might be attributed to chemical oxidation, given the fact that oxidation can produce less complex and more biodegradable molecules, which increases the productivity of certain taxa (Liao et al., 2019; Medina et al., 2018; Shi et al., 2024). Another explanation may be related to the inability of certain microorganisms to grow in the highly polluted environments existing before the Fenton treatment (Gou et al., 2019; W. Li et al., 2023).

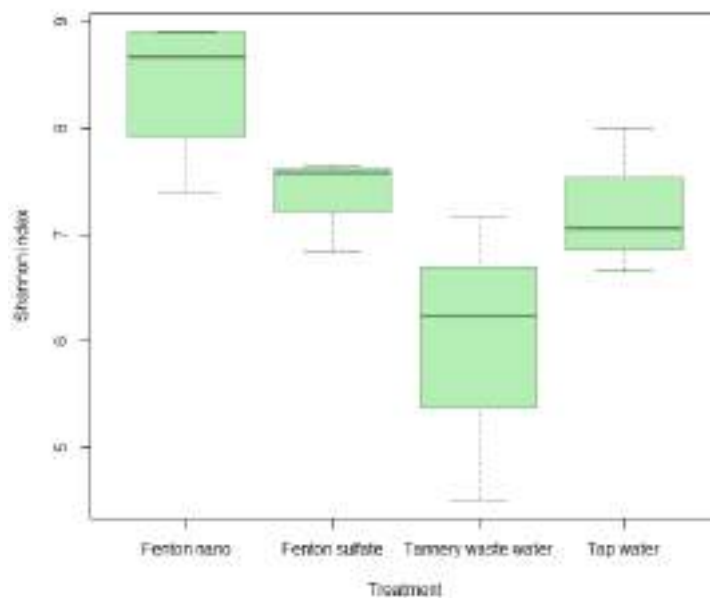


Figure 1. Boxplot showing Alpha diversity in Shannon entropy index for all treatments.

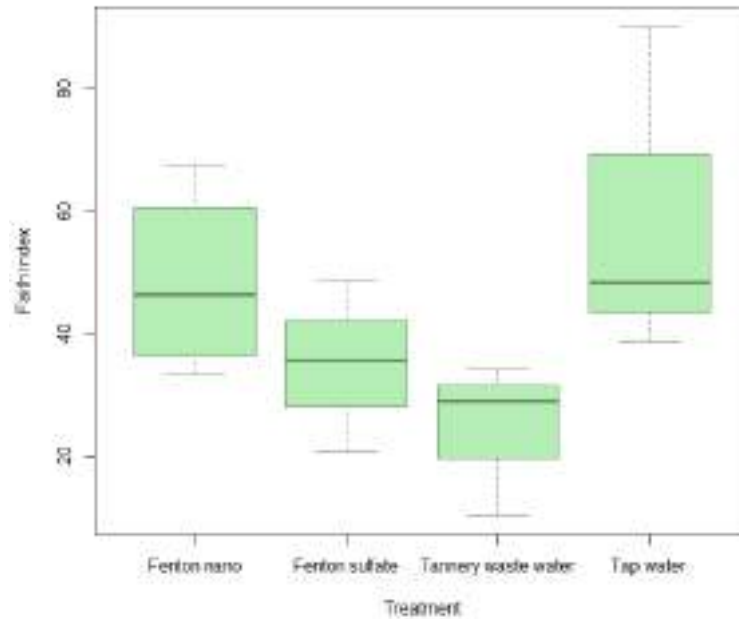


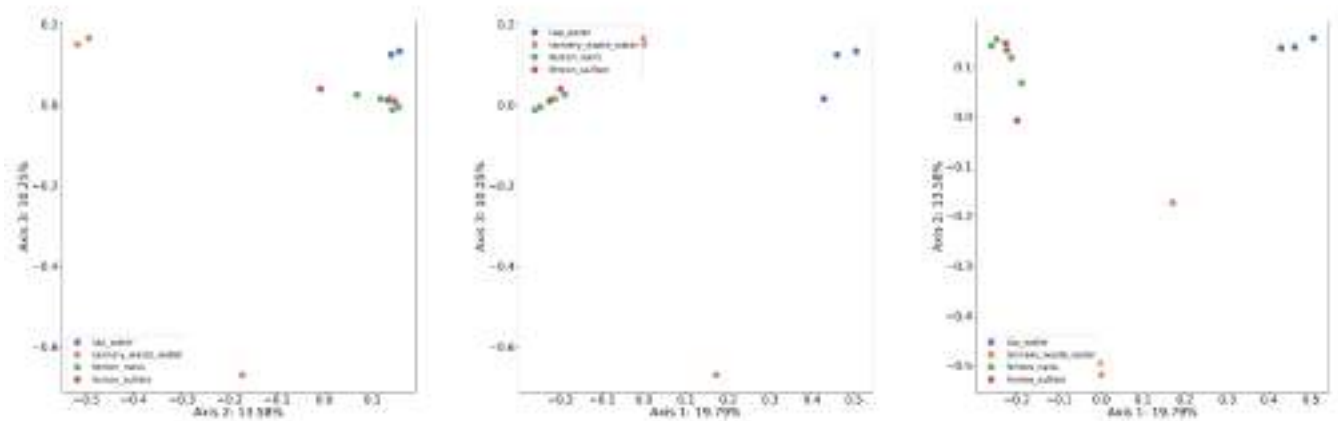
Figure 2. Boxplot showing Alpha diversity in Faith index for all treatments.

Furthermore, the statistically significant difference observed for fenton-nano treatment, but not for fenton-sulfate, may be attributed to a better oxidation rate of contaminants in the fenton-nano process. This is related to a higher reactivity of zerovalent iron compared to iron salts used in the conventional Fenton treatment. These properties allow more active sites where the Fenton reaction can occur, leading to a more efficient generation of hydroxyl radicals. Additionally, zerovalent iron can release Fe(II) ions that can further catalyze the fenton reaction, thereby increasing the oxidation of contaminants (Cuervo Lumbaque et al., 2019). Recently (W. Li et al., 2023) observed that the microbial community reveals more significant changes in soils contaminated with hydrocarbons when treated using Fenton reaction compared to only H₂O₂. This is because iron accelerates the production of free radicals, which intensifies the oxidation of contaminants. The application of fenton-nano treatment has demonstrated greater effectiveness in the oxidation of contaminants by significantly reducing the Chemical Oxygen Demand (COD) compared to conventional fenton, which have been reported before by (Vilardi et al., 2018). This could stimulate the growth of low abundance microorganisms and, consequently, significantly increase microbial diversity.

Phylogenetic diversity, as measured by the Faith index (Fig. 2), shows statistically significant differences between tap water and tannery wastewater ($P < 0.05$), being tap water the more diverse of the two, implying more divergence at the genus level than in tannery wastewater, perhaps because tap water originates from a metabolically more diverse environment: soil, surface water, and groundwater, contributing to the high phylogenetic diversity (K. Li et al., 2021). In contrast, tannery wastewater has more controlled microbiota primarily derived from animal skins processed during leather production (Abate et al., 2021). Moreover, the chemical treatments employed in the tannery process create selective pressures that limit the survival and growth of some microbial species (Lu & Lu, 2014), resulting in reduced microbial diversity compared to tap water.

For β -diversity Bray Curtis and Unweighted index analysis show a data grouping consistent for each treatment (Fig. 3), however, in PERMANOVA analysis, only fenton-nano treatment presented statistically significant differences (p -value < 0.05) against tap water and tannery wastewater (Table 1). This may be related with the previously discussed nutrient availability, which enriches some taxa on the community and could slightly change microbial composition. Moreover, it was observed that fenton-nano treatment has a minor effect, but not significant in the impact of gram negative bacteria compared to fenton-sulfato, which will be discussed later. This bias could influence the microbial divergence of this treatment.

A



B

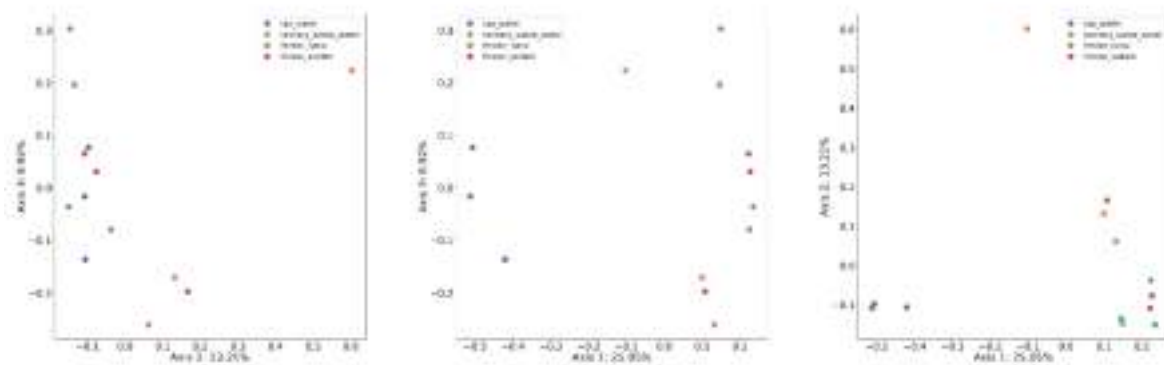


Figure 3. Beta Diversity. A) Bray Curtis Dissimilarity B) Unweighted index.

	Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Bray-Curtis Index	fenton_nano	fenton_sulfate	7	1,08047832	0,196	0,196
		tannery_waste_water	7	1,91403551	0,025	0,117
		tap_water	7	2,70739373	0,039	0,117
	fenton_sulfate	tannery_waste_water	6	1,65570236	0,096	0,1296
	fenton_sulfate	tap_water	6	2,38682862	0,108	0,1296
	tannery_waste_water	tap_water	6	2,06915806	0,104	0,1296

Unweighted Index	fenton_nano	fenton_sulfate	7	0,99356351	0,515	0,515
		tannery_waste_water	7	1,53649045	0,037	0,111
		tap_water	7	3,50244961	0,032	0,111
	fenton_sulfate	tannery_waste_water	6	1,32003581	0,096	0,1272
		tap_water	6	3,1006387	0,106	0,1272
		tannery_waste_water tap_water	6	2,48075366	0,094	0,1272

Table 1. PERMANOVA analysis of Beta-diversity for all treatments

Characteristics of microbial communities

The total microbial communities among all the samples consisted of 49 phyla, 125 classes, and 773 genera. The 5 most abundant phyla across all the samples were Proteobacteria (48.5% \pm 21), Firmicutes (20.8% \pm 18), Bacteroidota (12.2% \pm 9.5), Actinobacteriota (6% \pm 6) and Verrucomicrobiota (1.6% \pm 2.7).

For tap water samples, the most abundant phyla were Proteobacteria (68% \pm 19), Bacteroidota (20% \pm 15), Verrucomicrobiota (5.1% \pm 4), Actinobacteriota (1.8% \pm 1), and Bdellovibrionota (1.2% \pm 0.9), whereas for tannery wastewater the most abundant phyla were Proteobacteria (57% \pm 23), Firmicutes (29.5% \pm 29), Bacteroidota (4.4% \pm 4), Actinobacteriota (2.1 \pm 1.8), and Campilobacteriota (0.9% \pm 1.4). Most of the bacteria found in tap water correspond to gram negative environmental species, Proteobacteria in particular, which has been reported as being one of most prevalent phyla in freshwater and soils (de Oliveira et al., 2015; Zhang et al., 2021). These environmental bacteria are significantly reduced once this water is utilized in the tanning process and mixed with cattle hide residues, producing water that is rich in gram positive bacteria mostly related to cattle microbiota, with species related to reservoirs such as skin and intestines. Several of the Proteobacteria found in tannery wastewater have been shown as useful in remediation of contaminated matrices with high concentrations of chromium (Zhu et al., 2008; Thesai et al., 2020; Abate et al., 2021; Maurya et al., 2022).

The most abundant phyla for fenton-nano treatment were Proteobacteria (27.4% \pm 8), Firmicutes (27.2% \pm 11.7), Actinobacteriota (12.7% \pm 6.6), Bacteriodota (12.3% \pm 7), and Chloroflexi (3% \pm 2.8). The most abundant taxa for fenton-sulfate treatment were Proteobacteria (48.3% \pm 10), Firmicutes (24.3% \pm 8.7), Bacteroidota (12.3% \pm 6.1), Actinobacteriota (5.2% \pm 1.8), and Desulfobacteriota (2% \pm 1.4). It is important to note that Firmicutes appears markedly after Tannery wastewater and remains at high abundance in both fenton treatments. Firmicutes has been widely reported in tannery sludge and wastewater (Abate et al., 2021; Araujo et al., 2021; Liang et al., 2016; Verma & Sharma, 2020). The increase of Firmicutes in tannery wastewater may be related with the exposure to fluids of ruminant animals in early stages of hide processing; Bovine rumen is characterized by a high presence of Firmicutes (Pinnell et al., 2022; Weimer, 2015). Furthermore, several members of this group are biofilm formers (Abate et al., 2021; Peng et al., 2018) which could enable them to withstand chemical treatments, potentially explaining the prevalence of this phylum after both Fenton treatments.

Dominant genera for all the samples differed significantly. For tap water the most abundant genera were *Rhodoferrax* (found in stagnant waters), *Limnohabitans* (usually found in freshwater reservoirs), *Pseudarcicella* (freshwater species very susceptible to environmental changes (B. Wang et al., 2020), *Polaromonas* (a psychrophile), and *Sediminibacterium*, whereas for tannery wastewater the most abundant genera were: *Enterococcus*,

Pseudomonas, *Stenotrophomonas*, *Vibrio* and *Brevundimonas*, genera with at least some pathogenic or opportunistic species. This result shows that the environmental microbiota was completely replaced by the tanning process microbiota.

Effect of Fenton treatments on the microbial community of tannery wastewater

In relation to tannery wastewater, both Fenton processes had a significant effect on relative abundances of bacterial taxa (Fig. 4). We found a weak, but still significant bias of both Fenton processes towards the reduction of gram-negative bacterial populations (Kruskal-Wallis, $P=0.004$, Dunn test, $P=0.07$). This may be related to the fact that the peptidoglycan layer of gram-positive bacteria is thicker than for gram-negative bacteria, making it more difficult for iron ions to attack the cell, resulting in gram positives more resistant to the Fenton treatment. Our results show the abundance of the following taxa being reduced to minimum levels after both Fenton treatments: *Enterococcus*, *Acetoanaerobium*, *Shewanella*, *Achromobacter*, *Pedobacter*, *Variovorax*, *Aeromonas*, *Hydrogenophaga*, *Vagococcus*, *Tissierella* and a taxon belonging to the family *Alcaligenaceae*. The great majority of the aforementioned taxa are gram negatives. This bias may be related to the findings of (Huang et al., 2012), where they found gram-negative bacteria more susceptible to HO radicals than gram-positive bacteria. HO radicals are produced in traditional Fenton reactions and Fenton-like reactions like the one studied here, and could explain the bias in the abundances of gram positives and gram negatives. This bias appears to be slightly stronger for fenton-sulfate than for fenton-nano, although the difference is not statistically significant. This shows that fenton-nano is as effective in reducing bacterial populations as fenton-sulfate. Previous reports have shown a similar effect in bacterial populations (Manzoor et al., 2021).

The genus *Pseudomonas* is still abundant after both Fenton treatments. Other two genera that are present after both Fenton treatments are *Stenotrophomonas* and *Brevundimonas*, although their abundances are lower than in tannery wastewater. *Pseudomonas* lacks the OmpF and OmpC general trimeric porins that are usually present in other gram-negative bacteria like *Escherichia coli*, lowering *Pseudomonas* membrane permeability to only 1-8% of the permeability found for *E. coli* (Zgurskaya et al., 2015). Moreover, *Pseudomonas* is well-known as highly resistant to antibiotics, a phenomenon that is related to membrane permeability, but also to its ability to form biofilms which can restrict the diffusion of antibiotics (Pang et al., 2019). Likewise, *Stenotrophomonas* and *Brevundimonas* are considered as opportunistic pathogens with similar antibiotic resistance mechanisms in terms of membrane permeability and biofilm formation (Gil-Gil et al., 2020; Lepe & Martínez-Martínez, 2022; Soares et al., 2023). The presence of biofilm may restrict the contact of OH radicals with the cell membrane and the low membrane permeability confers some resistance for these microorganisms to the Fenton process, compared to other gram-negative bacteria.

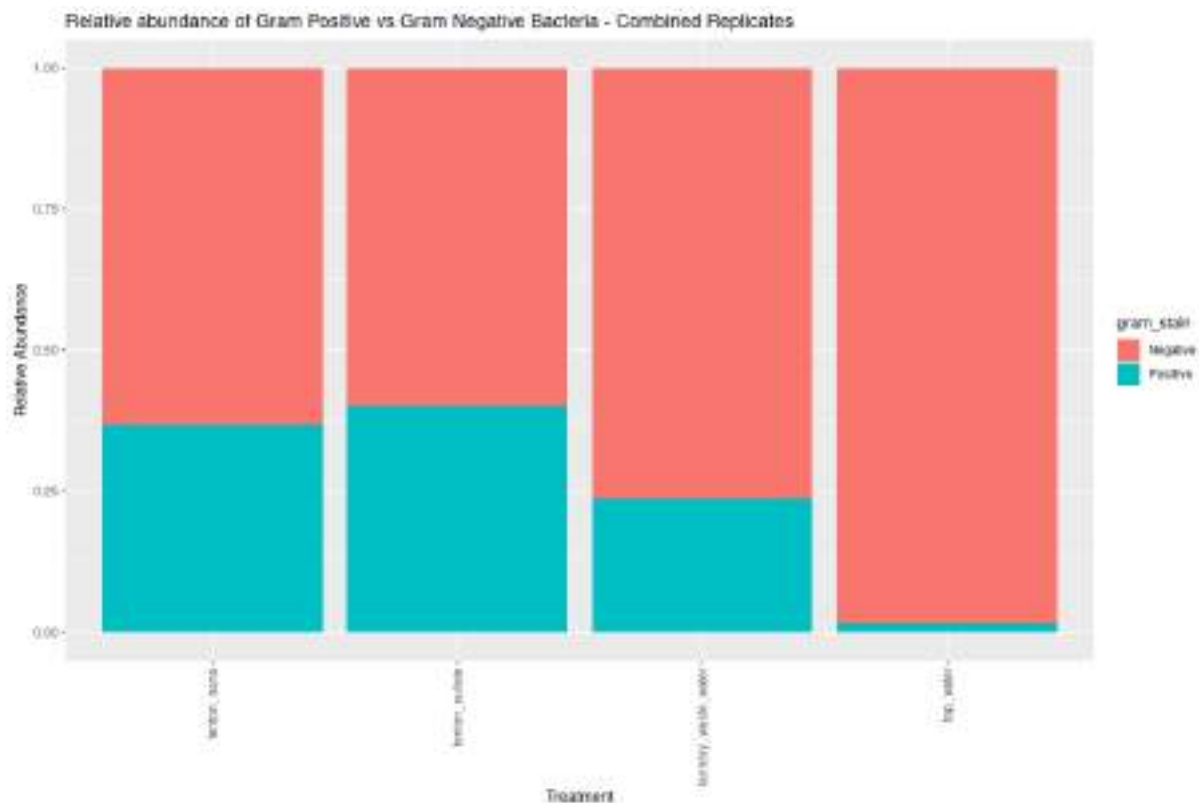


Figure 4. Relative abundances of gram-positive vs gram-negative bacteria among treatments.

In general the Fenton treatment, either fenton-nano or fenton-sulfate, seems to favor the abundances of some genera compared to their abundances in tannery wastewater. The genus *Acinetobacter* is the best representative of this observation, going from an abundance of 0.57% +/- 0.66 in tannery wastewater to abundances of 9.29% +/- 2.77 after fenton-nano treatment and 17.51% +/- 19.56 after fenton-sulfate treatment. Interestingly, there are other taxa whose relative abundances also increased with both treatments, and that taxa have new nomenclature, such as: Christensenellaceae R-7 group, Rikenellaceae RC9 gut group, NK4A214 group and UCG_005 group from the Oscillospiraceae family. As described above, the increase of relative abundances of certain taxa might be due to the availability of less complex molecules after fenton treatment, which allows the growth of low abundant taxa.

DIFFERENCES BETWEEN TREATMENTS

In order to identify differential taxa across treatments, we conducted a LefSe analysis which displays significant differences in abundances. The phylum Proteobacteria was significantly enriched in tap water, a result that is consistent with most of its taxa corresponding to gram negative environmental bacteria. Fibrobacterota and Actinobacterota were enriched for fenton-nano; the first is abundant in cattle microbiota as most of its members are able to degrade cellulose (Gharechahi et al., 2022). The role of Actinobacterota is still unknown as it only represents about 3% of the rumen microbiota (Sulák et al., 2012). No other phyla was found as significantly enriched for the other treatments. At the genus level 15 taxa were enriched for fenton-nano, five of them environmental species such as *Shingobium*, *Aminicentrales*, *Arthrobacter*, *Porticoccus*, and *Pseudoarthrobacter*. Two of these (*Arthrobacter* and *Pseudoarthrobacter*) have been associated with bioreactor and bioremediation

processes in the past (Guo et al., 2019; Naeimi et al., 2021). The remaining taxa has been associated with mammalian gut microbiota, some of them include taxa such as *Fibrobacter*, *Bacteroidales F082*, *Colidextribacter*, and bacteria of the group Ruminococcaceae. Several of these taxa organize themselves in biofilms in the rumen, the genus *Fibrobacter* and the family Ruminococcaceae among the best described (De Mulder et al., 2016; Huws et al., 2018). Biofilms and the exopolysaccharides (EPSs) associated with them may act as a protective barrier against oxidation processes such as Fenton. The protective properties of biofilms against oxidation have been reported in soils (W. Li et al., 2023).

For fenton-sulfate, 8 taxa were enriched at the genus level, two of them mostly environmental (*Pantotea* and *Delftia*) and 6 of them associated with mammalian microbiota (*Bacteroidales p251 o5*, *Prevotellaceae UCG 003*, *Acetitomaculum*, *Ruminococcus*, *Comamonas*, and *Acinetobacter*). The fact that more environmental taxa (gram negatives) appeared as significantly enriched for fenton-nano than for fenton-sulfate suggests fenton-nano is less aggressive with environmental gram-negative bacteria than fenton-sulfate.

ASSOCIATION OF BACTERIAL TAXA WITH PHYSICOCHEMICAL VARIABLES

Physicochemical variables, as well as the relative abundance of taxa between treatments were used to identify the variables that seem to affect the microbial composition the most.

It appears to be a strong correlation between the evaluated physicochemical variables. The most correlated variables were oxidation-reduction potential (ORP) and dissolved oxygen (DO) (Pearson correlation > 0.9), followed by pH, chlorides, chemical oxygen demand (COD) and conductivity (Pearson correlation > 0.7). Sulfate, on the other hand, doesn't appear highly correlated with any of the other physicochemical variables (Fig. 5). The high correlation existing between physicochemical variables implies that any association of bacterial abundance with physicochemical variables will also show association with other highly correlated variables.

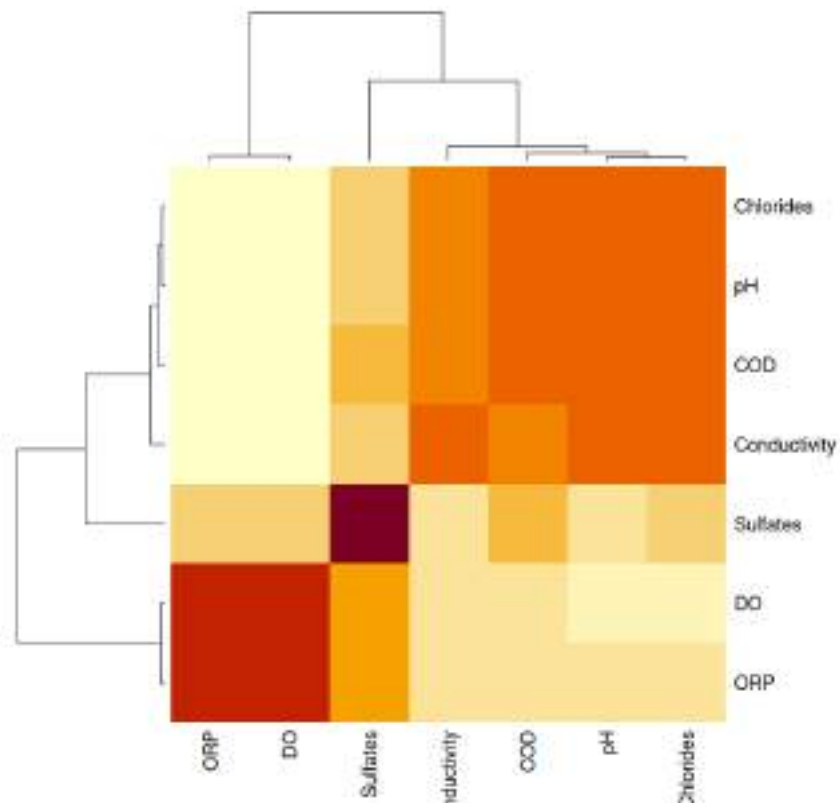


Figure 5. Heatmap showing Pearson correlation between physicochemical variables.

Physicochemical / Treatment	Tap water	Tannery wastewater	Fenton sulfate	Fenton nZVI
COD (mg/L)	128 +/- 10	16050 +/- 3281	4016 +/- 28	3271 +/- 118
pH	8.51 +/- 0.08	12.96 +/- 0.68	8.49 +/- 0.046	8.65 +/- 0.1
Chlorides (mg/L)	2307 +/- 18	26410	4176 +/- 227	2289 +/- 42
DO (mg/L)	3.1 +/- 0.61	0.08 +/- 0.017	3.5 +/- 0.36	3.4 +/- 0.23
Conductivity (mS/cm)	13.8 +/- 0.84	18.1 +/- 0.48	12.8 +/- 2.31	13.2 +/- 0.97
ORP (mV)	306 +/- 5.1	-518 +/- 11	310 +/- 0.17	298 +/- 1.96
Sulfate (mg/L)	59 +/- 1.2	667 +/- 24	1525 +/- 114	479 +/- 29

Table 2. Values of evaluated physicochemical variables. COD: chemical oxygen demand, ORP: oxidation-reduction potential, DO: dissolved oxygen

Water quality parameters showed significant variation. For tap water COD mean was 128 mg/L, whereas for tannery wastewater this value increased to a mean of 16050 mg/L, indicative of a high concentration of organic matter. However, after Fenton treatment, COD levels were reduced in at least 12000 units, with a COD removal of 74.9% for fenton-sulfate and 79.61% for fenton-nano, demonstrating the effectiveness of both Fenton processes. Interestingly, fenton-nano resulted in an overall better process, being almost 5% more efficient than fenton-sulfate at COD removal.

Sulfate concentrations showed a substantial increase in tannery wastewater (667 mg/L), and a further increase for fenton-sulfate (1525 mg/L). Since fenton-nano doesn't use ferrous sulfate to cause oxidation, sulfate levels remain similar to tannery wastewater.

phylum	COD	chlorides	conductivity	DO	ORP	pH	sulfates
p__Campilobacterota					0,041	0,017	
p__Deinococcota			0,066				
p__Firmicutes	0,071	0,053		0,063	0,075	0,062	
p__Fusobacteriota					0,084	0,076	
p__Nitrospirota							0,095
P-value < 0.1							

Table 3. P-values ($P < 0.1$) for Mantel test relating physicochemical variables and taxa at the phylum level.

At phyla level Firmicutes show a significant correlation with chemical oxygen demand (COD). This phylum was one of the most abundant for tannery wastewater as it originates from bovine rumen (Pinnell et al., 2022). This, along Proteobacteria and Bacteroidota are three phyla that have been shown as being the most abundant in the anaerobic phase of experiments related to municipal waste water decomposition (Yang & Song, 2019). In general, Firmicutes are high in all treatments, except tap water, which given the fact that it is a high oxygen level environment, doesn't favor the appearance of Firmicutes. Values for COD (16050 +/- 3281), and dissolved oxygen (OD) (0.08 +/- 0.01) are consistent with tannery wastewater being an anaerobic environment.

genus	COD	chlorides	conductivity	DO	ORP	pH	sulfates
g__Acetitomaculum							*
g__Acetoanaerobium		*			*	*	
g__Achromobacter	*	*	*	*	*	*	
g__Acinetobacter			*				
g__Aeromonas	*	*		*	*		
g__Alkalibacterium	*	*	*	*	*	*	
g__Brevundimonas	*	*	*	*	*	*	
g__Candidatus_Berkiella	*						
g__Caulobacter	*	*	*	*	*	*	
g__Comamonas							*
g__Denitromonas			*				
g__Enterococcus		*		*	*	*	

g_Eoetvoesia	*	*	*	*	*	*	
g_Erysipelothrix	*	*		*	*	*	
g_Halolactibacillus		*					
g_Hydrogenophaga		*	*	*	*	*	
g_Incertae_Sedis							*
g_Jeotgalibaca	*	*	*	*	*	*	
g_Leucobacter	*	*	*	*	*	*	
g_Macroccoccus				*			
g_Neisseria			*				
g_Ochrobactrum	*	*	*	*	*	*	
g_Pantoea							*
g_Prevotellaceae_UCG-001							*
g_Prevotellaceae_UCG-003							*
g_Proteocatella					*	*	
g_Pusillimonas	*	*	*	*	*	*	
g_Rheinheimera	*	*	*	*	*	*	
g_Ruminococcus							*
g_Shewanella	*	*		*	*	*	
g_Sphingobacterium		*					
g_Sphingopyxis		*	*	*	*	*	
g_Stenotrophomonas	*	*	*	*	*	*	
g_Tissierella		*		*	*	*	
g_Vagococcus		*	*	*	*	*	
g_Variovorax		*	*	*	*	*	
g_Verticiella		*	*	*	*	*	
*	P <0.01						
*	P <0.05						

Table 4. P-values for Mantel test relating physicochemical variables and taxa at the genus level.

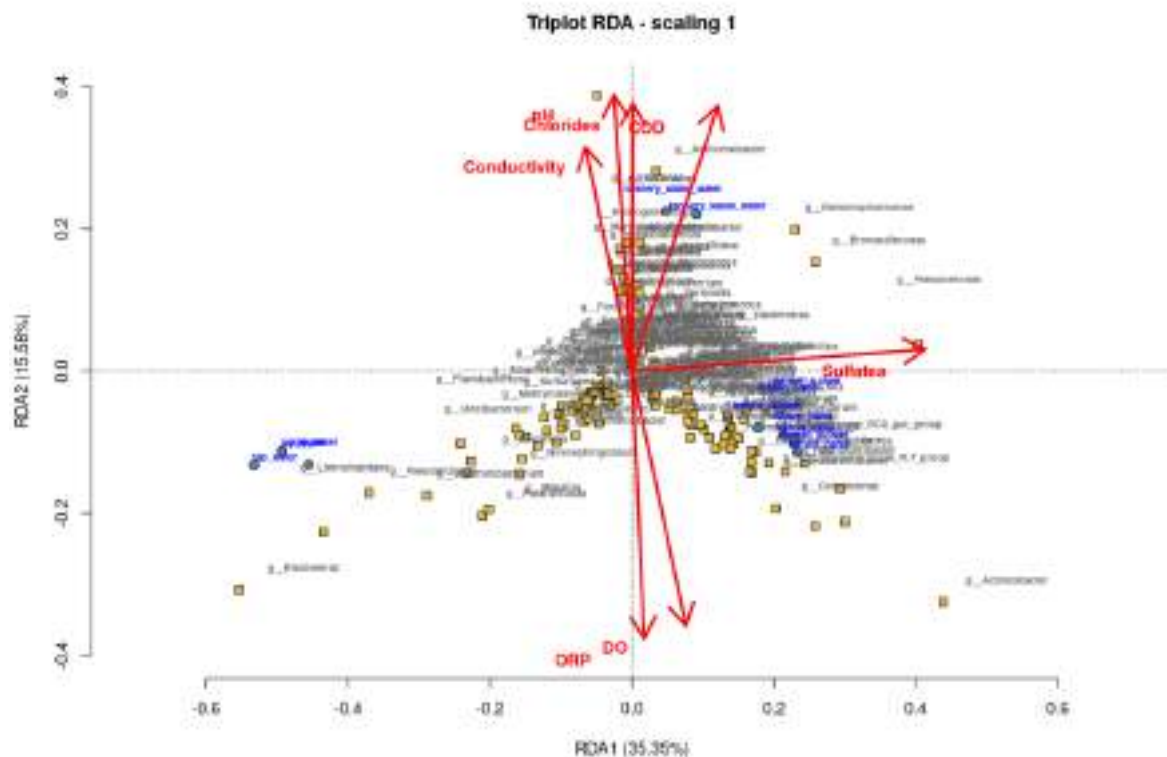


Figure 6. Effect of physicochemical variables on bacterial community composition at the genus level.

At genus level, 35% of the community structure is explained by axis1, and 15% of it explained by axis2. Overall, it appears that the variables with most influence (explanation power) in the community structure are chemical oxygen demand (COD) + Sulfates, which we will emphasize in this work.

At genus level, the Mantel test shows that all physicochemical variables affect the composition of bacterial communities ($P < 0.05$). It is worth noting that once a microorganism is affected by one of the highly correlated physicochemical variables (COD, pH, Chlorides, Conductivity), it appears that it is affected by the rest of them.

We found several taxa significantly associated with chemical oxygen demand (COD), and interestingly, most of these taxa have been somehow used for bioremediation processes. For instance, *Achromobacter* has been found to have a battery of at least 100 genes associated with the degradation of PHAs (Shi et al., 2024). *Aeromonas* shows bioaccumulation of metals such as Cu and Ni and is a promising candidate for heavy metal bioremediation of polluted areas (Qurbani et al., 2022). *Brevundimonas* has been isolated from oil contaminated areas and can use diesel as its sole source of carbon, with a fuel biodegradation percentage of up to 65% (Wang et al., 2016). *Eoetvoesia* and *Jeotgalibaca* species have been isolated from activated sludges of wastewater and pesticide plants (Felföldi et al., 2014; Geng et al., 2023). *Leucobacter chromiireducens* have been previously isolated from tannery contaminated soils and shows ability to reduce Cr(VI) both aerobically and anaerobically (Tahri Joutey et al., 2016). Same Cr(VI) bioremediation potential has been shown for *Ochrobactrum anthropi* (Cheng et al., 2010). *Pusillimonas* shows potential as a biodegrader of landfill leachates (Remmas et al., 2017). *Rheinheimera* shows increased

resistance to high mercury levels, shows bioaccumulation and potential for the bioremediation of mercury contaminated sites (Yadav et al., 2023). *Shewanella* has a long history in bioremediation, removing toxic elements from groundwater, given the versatility of its respiratory electron transport chain system (Tiedje, 2002). *Stenotrophomonas* can degrade many xenobiotic compounds, making it a good candidate for bioremediation as well (Ryan et al., 2009).

We also employed the Random Forest algorithm to investigate the genera correlated with physicochemical variables among treatments (tap water, tannery wastewater, and treated tannery wastewater with traditional fenton-sulfate and nanoparticle fenton (fenton-nano)). Several of our results show a significant correlation between COD levels and the presence of *Shewanella* and *Stenotrophomonas* (Mantel test, Random Forest), as also *Acetoanaerobium*, *Pseudoarthrobacter* and *Achromobacter* (Random Forest), which emphasizes the roles of these taxa in bioremediation and wastewater treatments.

Sulfate levels were correlated with *Ruminococcus*, *Acetitomaculum* (Mantel test, Random Forest), as well as *Comamonas*, *Jeotgalibaca*, and *Prevotella(ceae)* (Mantel test). *Comamonas* is a genus with a nonfermentative chemoorganotrophic metabolism, it is quite ubiquitous in the environment, has been isolated from soil and water and shows ability to degrade PHAs (Shah et al., 2008). The genus *Prevotella*, recently separated from *Bacteroides* has been shown to use chondroitin sulfate (a component of cartilage) as source of carbon (*Encyclopedia of Food Microbiology*, n.d.), and cause infection of cartilage in humans (Derouane et al., 2019).

CONCLUSIONS

This study shows the effect of Fenton oxidation on the microbial communities associated with the tanning process. Results show that almost all environmental bacteria is replaced by cattle microbiota and tannery-related microbiota. We also found a slight, but not statistically significant difference in the effect of traditional fenton-sulfate vs fenton-nano, being the latter less aggressive than the former over gram-negative environmental bacteria. Since all these bioremediated waters return to the environment eventually, fenton-nano appears to be superior to fenton-sulfate in this regard. Further studies will be necessary to corroborate this finding. Overall, these findings underscore the potential of Fenton-based treatments, especially with nanoparticles for mitigating the environmental impact of tannery wastewater.

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

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