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Evaluating Tannery Wastewater Treatment Performance Based on Physicochemical and Microbiological Characteristics: an Ethiopian Case Study

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Abstract

Tanneries are an important industrial sector in Ethiopia; consequently, gaps in wastewater treatment process performance are needed as the country increases its emphasis on compliance. A case study was conducted ~~of~~ to evaluate physicochemical and microbial water quality at a tannery near Addis Ababa. The treatment process was designed for: sulfide oxidation; biological oxygen demand reduction; and chromium removal. While some of Ethiopia's standards for industrial wastewater treatment were met through treatment, effluent COD, sulfide, total nitrogen and total chromium guidelines were not. 16S rRNA gene analysis was used to evaluate the microbial community composition across the treatment train. The results show that common ruminant phyla were dominant throughout, with Firmicutes and Bacteroidetes comprising 77% to 82% relative abundance. The Firmicutes *Clostridium* increased consistently in relative abundance with treatment, comprising 39% to 61% of the total bacterial community in the effluent. Improved treatment is needed to meet environmental and public health goals.

Key phrases: leather manufacturing, chromium, bacterial diversity, Illumina, 16S rRNA, water quality indicators, industrial treatment

Introduction

Leather manufacturing is an industry with a long history in Ethiopia, which is the largest livestock producer in Africa and tenth largest in the world. The country's modern leather manufacturing method started in the 20th century. Today, more than 27 tanneries exist in the country that export semi-finished and finished leather (Coppeaux et al., 2016), and a few more are under construction. The processing of leather is an export industry for Ethiopia, making up almost 6% of annual exports per year (around $127 million USD) (OEC, 2017). Ethiopia is among the poorest but fastest growing economies in the world, and is evolving toward a market-based economy. These changes are motivating the leather industry to produce high quality exports while meeting environmental and public health needs (UNCTAD, 2018); nevertheless, progress toward this end is slow given the Country's economic constraints. Important to this end is: understanding how the country's current approaches to industrial wastewater management are affecting both the environment and public health; determining what methods can be implemented

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29 to create best treatment practices; and creating useful water quality guidelines that can inform
30 future regulations.

31 Although there is interest in moving toward "green", chromium-free leather manufacturing
32 (UNCTAD, 2018; ELIA, 2019), the process of manufacturing leather from raw skins and hides
33 at most tanneries in Ethiopia still uses a water-and chemical-intensive chromium-based tanning
34 method that is performed using the following steps: beam house, tanning, retanning and finishing
35 (Gutterres et al., 2015; Wosnie & Wondie, 2014). The beam house operation includes soaking
36 skins and hides in lime, which facilitates the removal of hair, and then removing the residual
37 lime by slowly reducing the pH with acid. Additionally, bating is conducted in the beam house
38 using proteolytic enzymes to softening the skins and hides. Ultimately, the beam house produces
39 the strongest COD wastewater with the greatest volume. The chromium tanning and retanning
40 steps convert the collagen from the skins and hides into leather; it is this step that produces the
41 most hazardous wastewater that contains a strong chromium residual (Goswami & Mazumder,
42 2014). Finishing includes stretching, buffing and/or drying the tanned product. The wastewater
43 treatment system that receives wastewater from all these steps aims to remove solid waste (e.g.,
44 grift, fibers), suspended and dissolved substances, and tannery-specific pollutants, such as
45 sulfides and chromium. It is also likely to receive factory wash waters that do not go through
46 tannery processing.

47 Wastewater from the tanning industry is complex and, consequently, tannery wastewater
48 treatment is difficult to afford in low-income countries. Ultimately, tanneries are an important
49 source of pollution in Ethiopian surface waters since most of the treated or semi-treated tannery
50 effluents are released to nearby rivers. Regulations were implemented for the effluent from
51 tannery treatment systems (EEPA, 2003) and will necessitate the use of appropriate technologies
52 to meet the standards. This is particularly important since most of the treated and semi-treated
53 tannery effluents are released to nearby rivers that serve as irrigation water for crops and animal
54 agriculture. The effluent discharge limits for tannery wastewater treatment set by the Ethiopian
55 Environmental Protection Authority (EEPA) target physicochemical parameters such as nutrients
56 (e.g., phosphates, total nitrogen, sulfides), chromium (total and VI), and phenol. Currently, the
57 treatment approach used at the tanneries around Ethiopia vary across locations, which is likely to
58 result in varying performance.

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Most of Ethiopia's tanneries are located in and around Addis Ababa and in the Oromia region, which is more water abundant than other parts of the country and can more easily support water-intensive tanneries (Gebre & Van Rooijen, 2009). Many of these tanneries only have primary treatment ponds, but some tanneries have started incorporating secondary treatment. For instance, four tanneries in Addis Ababa have completed construction of secondary wastewater plants, and three additional tanneries have secondary treatment systems under construction (Hailemariam, 2019). Past studies from Ethiopia, India and Brazil showed that a combination of biological and chemical treatment can produce improved effluent quality that meets environmental regulations based on chemical performance, although several studies point to high variability in effluent quality (Chowdhury & Mostafa, 2015; Gutterres et al., 2015; Sugasini & Rajagopal, 2015; Terfie & Asfaw, 2015; Tsegaye & Kaba, 2017). The EEPA regulations target chemical pollution but do not include microbiological indicators, perhaps reflecting an assumption that microbiological contaminants are not likely to survive the harsh chemical methods used during the tannery process. However, microbiological indicators such as rumen-originating pathogens like the Enterobacteriaceae, which includes *Clostridium*, or other pathogens that can be sources for zoonotic diseases (Kemunto et al., 2018; Knight & Riley, 2019), may be appropriate to include as their presence would suggest a failure by the factory to provide barriers to their environmental release. Both the microbial composition of tannery treatment processes and the microbial water quality of tannery effluents are poorly characterized. To address this gap, both culture-dependent and independent (i.e., DNA-based sequencing) methods can be used to advance our understanding of the microbial diversity in tannery treatment processes (Birtel et al., 2015; Rosselli et al., 2016) and the residual microorganisms left behind after treatment. One of the methods used to study water quality and the microbial ecology of wastewater treatment systems is based on Illumina sequencing of the 16S rRNA gene. This approach is enhanced by the growing size of reference databases and reduced sequencing costs (Barb et al., 2016; Derakhshani et al., 2016). Importantly, as we learn more about the microbial ecology of these systems, relevant microbial indicators will be identified that help to characterize the effectiveness of treatment performance in reducing the risk of zoonotic pathogens.

The objective of this case study was to couple an investigation of both physicochemical and bacterial composition across a full-scale tannery to better understand the treatment performance

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90 of the process and identify chemical and microbiological contaminants of concern. The outcomes
91 of this case study can guide future regulatory needs that will best protect environmental and
92 public health. We used an average-sized tannery located within the populated city of Addis
93 Ababa as our field site, and employed conventional physicochemical water quality testing with
94 Illumina sequencing of the 16S rRNA gene to quickly ascertain relevant microorganisms across
95 the treatment process and effluent. We characterized the influence that tannery processing and
96 wastewater treatment had on the effluent, its ability to meet existing regulated industrial
97 treatment guidelines, and reflect on the potential impact of microbial agents that survive
98 treatment.

99

100 **Materials and Methods**

101 ***Sample site and physicochemical analysis.*** This study was carried out at the tannery wastewater
102 treatment plant located in the Akaki-Kality Sub City of Addis Ababa, Ethiopia. The tannery is
103 located on the bank of the Little Akaki River (8°55′53″N, 38°45′29″E) to which tannery effluent
104 is released. The tannery is a medium-sized factory with an average processing capacity of 8,000
105 goat and sheep skins and 1,000 cattle hides per day (UNIDO, 2012). The tannery wastewater
106 treatment plant is designed to remove phosphate, suspended solids, dye stuffs used in the leather
107 finishing process, and chromium; nutrient removal was not fully evaluated in this study. The
108 wastewater treatment plant influent is comprised of segregated inputs from the beam house,
109 chrome and dying operations. The treatment plant includes: aerated equalization; an aerated
110 biological oxidation pond; and coagulation, flocculation and sedimentation (Figure 1). The
111 aerated equalization basin targets oxidation of sulfide. The biological treatment step targets
112 oxidation of COD. Finally, the aerated mixed liquor from the oxidation pond is pumped to a
113 small coagulation basin where chemical coagulants (alum and ionic polymers) are mixed in,
114 flocculated and settled in the sedimentation basin for organics, phosphate and chromium
115 removal. After sedimentation, the clarified effluent is discharged to the nearby Little Akaki River
116 without disinfection or further tertiary treatment.

117 Samples were collected in sterile bottles during two sampling events (designated 1 and 2) that
118 occurred ten days apart during the month of August (end of the wet season). Samples were

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119 collected at three distinct sites within each sample location and composited into one sample.
120 Influent wastewater samples (denoted G) were taken from the equalization sulfur oxidation pond;
121 the mixed liquor samples (denoted S) were taken from the aerated biological oxidation pond; and
122 the final effluent samples (denoted E) were taken from the tannery effluent that was released
123 from the sedimentation tank. Temperature, pH, and conductivity were measured on-site during
124 sampling with a digital portable pH meter that had separate probes for conductivity and
125 pH/temperature (Thermo AP85 meter, Fisher Scientific, Singapore). Samples were transported to
126 Addis Ababa University on ice in a cooler. Duplicate biomolecular samples were stored at -20°C,
127 while all physicochemical samples were immediately processed or stabilized via freezing,
128 according to established protocols. Analytes were measured by Standard Methods (Clesceri et
129 al., 1999) or designated protocols as follows (by the given method number, where available):
130 chemical oxygen demand (COD) by closed reflux (#5220); total dissolved solids (TDS) by a
131 thermogravimetric method (#2540C); ammonia-N by the Nessler method; sulfide by the
132 methylene blue method (#4500 S^{2-} D); sulfate by the turbidimetric method (#4500-SO_4^{2-} E); total
133 nitrogen by persulfate digestion (#4500 N); and total chromium by inductively coupled plasma
134 mass spectrometry (Agilent 7900). All analyses were conducted in triplicate, and reported
135 standard deviations reflect triplicate analyses.

136 ***DNA Extraction and Sequencing.*** All samples for biomolecular analysis were centrifuged at
137 2000xg and pellets were used to extract genomic bacteria DNA. DNA was extracted from
138 duplicate samples per location using the Fast DNA^{TM} SPIN Kit for Soil (MP Biomedicals, Solon,
139 OH, USA). Briefly, 300 mg of pellet and 978 μL of sodium phosphate buffer was added to a 2
140 ml microcentrifuge tube and vortexed for 15 seconds. A DNA stabilizing and solubilizing agent
141 (MT buffer 122 μL) was added and samples were homogenized by bead beating (BioSpec,
142 Bartlesville, OK, USA) for 40 second. The homogenate was centrifuged at 14,000xg for 10
143 minutes and the supernatant was transferred to a clean 2 mL microcentrifuge tube. Protein
144 precipitating solution (250 μL) was added, shaken ten times by hand, incubated at room
145 temperature for 10 minutes and centrifuged at 14,000xg for 5 minutes to remove cell debris.
146 Supernatant (800 μL) plus an equal volume of binding matrix was combined into a 2 mL tube
147 and shaken gently by inverting five times. Finally, the DNA was eluted using 100 μL DNA
148 elution solution and stored at -20°C until further use.

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149 The integrity of the extracted DNA was visually verified using gel electrophoresis with 1%
150 agarose and 1xTAE buffer. The quality and concentration of extracted DNA was verified with a
151 Nano Drop 1000 UV-Visible spectrophotometer (ND-1000 Thermo Fisher Technologies, USA).
152 Blank water was used during DNA extraction as a negative control and no detectable DNA was
153 recovered from the blank sample. DNA was submitted for sequencing at the University of
154 Michigan's sequencing core (Medical College, Ann Arbor, Michigan, USA), where Illumina
155 Miseq was performed with 2x250 paired-end chemistry. An amplicon library was generated from
156 the V4 region of the 16S rRNA gene after two step amplification of the DNA fragment using
157 universal dual index primers 515F/806R (Mwaikono et al., 2015; Saunders et al., 2016). The
158 amplicons were indexed by barcodes and adaptors, which allow sequencing on the same flow
159 cell and easier demultiplexing during sequence data analysis (Fouhy et al., 2015).

160 ***Data Analysis.*** The reads were analyzed using Mothur (version 1.36.1). Reads were filtered and
161 de-noised to remove low quality and ambiguous reads using the filter and screen codes. The two
162 sets of reads were overlapped and combined to form contigs using the function make.contigs.
163 Chimeric sequences were removed using the UCHIME algorithm embedded in Mothur by
164 checking against chimera free data bases of 16S rRNA gene sequences following the sequence
165 binning workflow. Sequence alignment was carried out using the Silva reference database
166 (www.arb-silva.de, version 123). Quality filtered sequences were assigned to taxonomic
167 identities by reference database project (RDP) classifiers (Fadrosh et al., 2014), and sequences
168 were clustered into OTUs at a 97% similarity threshold level using the UCLUST algorithm.
169 OTU identification was performed using BLASTn (www.ncbi.org). Bacterial community
170 richness was analyzed based on the number of OTUs obtained and using rarefaction analysis
171 (Wu et al., 2015) after sub-sampling (data not shown). Diversity indices (Shannon and inverse
172 Simpson) were calculated using summary.single command. The number of OTUs in each sample
173 was used to estimate diversity and evenness of bacteria community (Sinclair et al., 2015).
174 Principal coordinate analysis (PCoA) plots were generated from OTU data to assist with
175 visualizing changes in diversity between samples. The observed core bacterial genera in each
176 sample was presented in a heatmap using the clustvis package (Metsalu et al., 2015). All
177 sequences have been submitted to NCBI under submission numbers SAMN13738847 through
178 SAMN13738852. Additional information about sequence data analysis is provided in the
179 Supplemental Information.

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180

181 **Results and Discussion**

182 ***Tannery wastewater treatment underperformed.*** The physicochemical characterization of raw
183 (G1 and G2), activated sludge (S1 and S2), and treated effluent (E1 and E2) samples from the
184 tannery wastewater treatment plant is reported in **Table 1**. The water quality results from our
185 study are presented as a range from the duplicate sampling days. Our results show much lower
186 COD (2,100-4,000 mg/L) and sulfate (570-650 mg-S/L) in the raw influent than found in the
187 Modjo tannery (COD_{avg}=12,500 mg/L and $sulfate_{avg}$=800 mg-S/L; Desta et al., 2014), and lower
188 COD than the Dire tannery (COD_{avg}=12,900 mg/L; Birhanie et al., 2017). The total chromium
189 concentration (13-37 mg/L) was similar or lower than these other studies (27-68 mg/L). Tannery
190 wastewaters are highly variable (Gutterres et al., 2015), likely due to the type of leather tanning
191 activity (chrome or vegetable-based), the amount of hair, the cleanliness of hides and skins
192 collected from different regions, and the complexity of the leather making process itself (Desta et
193 al., 2017; Saxena, Chandra, & Bharagava, 2016). Indeed, in our study we saw raw COD and
194 ammonia-N concentrations that were higher and total chromium concentrations lower in the G2
195 sample than G1, possibly reflecting different input stocks being processed by the tannery at the
196 time of sampling.

197 Removal, defined as the change between influent (G) and effluent (E) for each sampling event at
198 the tannery treatment system, was: 32% and 47% for conductivity; 49% and 67% for COD; 48%
199 and 51% for TDS; 68% and 69% for sulfide; 79% and 96% for ammonia-N; 63% and 64% for
200 TN; and 54% and 87% for total chromium. These are moderate levels of removal in nearly all
201 cases. Indeed, as shown in **Table 1**, the effluent from the tannery treatment system exceeded
202 guidelines for COD, sulfide, total nitrogen and total chromium in both samples. COD and total
203 nitrogen loss occur mostly through chemically-enhanced clarification. Presumably, the total
204 nitrogen removed by coagulation reflects the organic fraction only. We do not have reliable
205 nitrate measurements and cannot conclude that nitrification occurred; nevertheless, we do not
206 find common aerobic ammonia or nitrite oxidizers in our sequencing data (Table SI-2), which
207 suggests the wastewater treatment plant did not nitrify to a detectable extent. Other studies show
208 that incomplete nitrification has occurred in Ethiopia's Modjo tannery (44% of effluent total N is
209 nitrate) or a tannery in India (13-18% of effluent total N is nitrate) (Sugasini & Rajagopal, 2015;

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210 Terfie & Asfaw, 2015). Nitrification is inhibited by both Cr(III) and Cr(VI) (Novotnik et al.,
211 2014), as well as sulfide (Delgado Vela et al., 2018); these chemicals are found in the tannery
212 effluent and could have contributed to limited nitrification. Furthermore, although TDS is not
213 regulated, it was routinely high in the effluent and could have influenced the microbial ecology
214 in the biological reactor and effluent. The chromium violation is of concern, given the potential
215 toxicity of the metal (Saxena et al., 2016) and propensity to activate antibiotic resistance gene
216 mobility between bacteria (Branco et al., 2005). Furthermore, alum is not a preferred coagulant
217 for chromium (Johnson et al., 2008), and alternative coagulants should be considered. Overall,
218 the water quality parameters exceeded the discharge limits from 1.5 to 63 times EEPA standards.
219 Furthermore, variability in raw wastewater composition can make treatment more challenging;
220 indeed, we see that the higher COD and ammonia-N loads brought by G2 resulted in higher
221 effluent COD and ammonia-N concentrations (E2). Despite the lack of compliance with
222 regulatory guidelines, most water quality parameters improved by the end of treatment during
223 both sampling events; the exception to this was sulfate during the second sampling, which
224 remained relatively constant. These results show that the treatment process used was either
225 unable to or was not operated in a way that resulted in generation of an effluent with
226 characteristics within EEPA guidelines. Furthermore, this confirms that modified treatment
227 approaches are needed to improve effluent quality, and to protect public and human health from
228 chemical pollutants of concern.

229 ***Dominant bacterial phyla reflected rumen origins.*** Illumina sequence data were successfully
230 annotated and characterized to produce bacterial community structure information from phylum
231 to genus. The distribution of bacteria across the treatment system was dominated by four phyla:
232 Firmicutes, Bacteroidetes, Proteobacteria and Synergistetes (**Figure 2** and Table SI-1). These
233 phyla constituted over 98% of the total sequence reads. Firmicutes was the most abundant
234 phylum in all sample points except G1, which was dominated by Bacteroidetes (38% versus
235 34%). The order of most to least average relative abundance among the influent and mixed liquor
236 samples was (G, S): Firmicutes (45.3%, 55.4%), Bacteroidetes (32.2%, 26.8%), and
237 Proteobacteria (19.5%, 14.5%) with Synergistetes a distant fourth (1.4%, 1.8%). The order of
238 average relative abundance in effluent samples was different: Firmicutes (44.8%), Bacteroidetes
239 (37.1%), Synergistetes (10.5%) and Proteobacteria (4.8%). In all sample locations, Firmicutes

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240 and Bacteroidetes are at least 77% of the total sequences, which shows their dominance and
241 persistence across the tannery wastewater treatment plant.

242 The dominance of Firmicutes and Bacteroidetes can be explained both because the tannery
243 wastewater is exposed to fluids from ruminant and non-ruminant animals, and possibly because
244 of the prevalence of chromium. Firmicutes are mostly Gram positive, low G+C content
245 anaerobic and facultative aerobic bacteria while Bacteroidetes are Gram negative, non-spore
246 forming aerobic and anaerobic bacteria. Both phyla are known to ferment undigested
247 carbohydrates and are often among the most dominant taxa in the rumen of bovine (Granja-
248 Salcedo et al., 2017; Jami & Mizrahi, 2012; Liu., 2016; Tapio et al., 2016), sheep (Tanca et al.,
249 2017), and goat (Han et al., 2015; Liu et al., 2017; Wang et al., 2016), as well as the
250 gastrointestinal tract of equine (Shepherd, Jensen, & Ponder, 2011), rabbit (Monteils et al.,
251 2008), and human (Smith et al., 2019). Another study reported the high relative abundance of
252 Firmicutes (46%) and Bacteroidetes (36%) in animal manure (Ozbayram et al., 2018). In
253 addition to their role in mammalian guts, Firmicutes (especially *Clostridium* genus) were found
254 by DNA-based methods to be a dominant taxa in a chromium-contaminated soil (Desai et al.,
255 2009) and soils treated with chromium-contaminated tannery sludges (Miranda et al., 2018),
256 which suggests that strains exist from this taxa that are tolerant of chromium-contaminated
257 environments.

258 The next most predominant phyla were Synergistetes and Proteobacteria. The distinct prevalence
259 of Synergistetes over Proteobacteria in the effluent was consistent across both sample dates, and
260 suggests that the treatment process at the tannery influenced this selective shift. The dominant
261 bacteria found in the tannery treatment system is different from what is typically found in
262 hundreds of domestic wastewater treatment plant activated sludge samples collected globally,
263 where Proteobacteria represent over ¾ of all bacterial taxa and would be expected to represent
264 the majority of effluent taxa (Wu et al., 2019). Interestingly, the dominant phyla identified in this
265 study were also reported in another study that used 16s rRNA gene clone libraries with samples
266 from a pilot plant at the Modjo tannery in Ethiopia. That tannery employed a distinctly different
267 treatment process (anaerobic/aerobic biological treatment followed by constructed wetlands; no
268 coagulation step was used). In the Modjo study (Desta et al., 2014), Firmicutes and
269 Proteobacteria dominated the last root zone samples from the wetlands, which are assumed to

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270 reflect the effluent, followed by Bacteroides and Cyanobacteria. *Synergistetes*, which are
271 anaerobic bacteria, were also detected in the Modjo study. This shows that the nature of
272 treatment can significantly influence the characteristics of the microbial communities present in
273 the effluent from a tannery treatment plant. It also shows that Firmicutes is consistently present
274 as an abundant phylum, independent of treatment method employed.

275 ***OTU diversity varied with sample location and treatment approach.*** Mixed liquor (S) and
276 effluent (E) sample OTUs reflected similar community structures within each sample type (i.e.,
277 S1 was similar to S2; E1 was similar to E2), while influent (G) sample OTUs were dissimilar
278 between G1 and G2, as depicted by principal coordinate analysis (PCoA, **Figure 3**). The
279 observed variability in the bacterial community structure in the influent samples may change due
280 to changes in animal sources and tannery manufacturing practices, and is a common feature
281 among tanneries (Amde, 2017; UNCTAD, 2018). The S and E samples each formed distinct
282 clusters. This indicates two things: (i) samples within each S and E sample location were similar
283 over time and (ii) there is a sustained difference in microbial community structure between the S
284 and E samples. The only treatment steps between S & E were coagulation, flocculation and
285 sedimentation. Therefore, the shift in microbial community composition was consistently
286 influenced by this treatment, which removes insoluble or flocculant particles. Microbial strains
287 detected in the effluent were either present in effluent suspended solids that originated in the
288 mixed liquor, which in tanneries are expected to be below 50 mg/L, are able to exist in
289 planktonic form, or are otherwise resistant to removal by coagulation and settling. The notion of
290 an abundant planktonic fraction being present in tannery wastewater is consistent with the fact
291 that rumen microbiomes include planktonic subpopulations that thrive in the rumen fluid (Cho et
292 al., 2006). Finally, we see that the large community structure differences between G1 and G2 are
293 not reflected in mixed liquor or effluent sample community structure changes. Therefore, at least
294 over the ten day period reflected by samples in this study, the treatment system's microbial
295 composition was resistant to influent changes. More sample points over time are needed to fully
296 capture the variation of influent samples and how they impact mixed liquor and effluent
297 community composition to fully evaluate the stability of the community.

298 A comparison of the relative abundance among genera present in samples is presented in **Figure**
299 **4**. Variation in G1 and G2 is apparent based on OTU. G2 was dominated by: the Firmicutes

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300 *Clostridium* (32.1%), the Gammaproteobacteria *Psychrobacter* (13.6%) and *Acinetobacter*
301 (11.9%), the Firmicutes *Anaerovorax* (7.9%), the Synergistetes *Synergistes* (7.6%), the
302 Bacteroidetes *Bacteroides* (4.0%), and the Firmicutes *Papillibacter* (3.1%). In contrast, the four
303 most dominant OTUs in G1 were similar in percent relative abundance and included:
304 *Bacteroides* (17.8%), the Gammaproteobacteria *Shewanella* (16.4%) and *Ignatzschineria*
305 (13.5%), and *Clostridium* (13.2%). Despite this variation between influent samples, the
306 dominance of genus *Clostridium* and the class Gammaproteobacteria were reestablished in the
307 mixed liquor, and was consistent across both sample dates. The tannery mixed liquor bacterial
308 structure is different from mixed liquors treating domestic wastewater, which are typically
309 predominated by bacteria from the class Betaproteobacteria (Nascimento et al., 2018; Wu et al.,
310 2019).

311 *Clostridium* was the most dominant genus in all sample locations (31.1%-61.2%) except for G1
312 (13.2%), where it had comparable relative abundance to the other most dominant taxa.
313 *Clostridium* was also dominant in mixed liquor samples from Modjo tannery (Desta et al., 2014).
314 Effluent samples in the current study were also consistently dominated by *Clostridium*.
315 Interestingly, although many strains within the phylum Firmicutes are biofilm formers, only a
316 few biofilm forming strains of *Clostridium* have been studied from the many strains known to
317 exist (Pantaléon et al., 2014). *Clostridium* that feed on solid cellulosic substrates can form
318 biofilms; however, they can be released as detached biomass or survive in the planktonic state in
319 the absence of adequate substrate (Desvaux, 2005; Gelhaye et al., 1993). Furthermore, they are
320 metabolically diverse (Xing et al., 2011) and can live in either a growth-supporting vegetative
321 state that requires anaerobic conditions since they are strict (but not obligate) anaerobes, or as a
322 non-growing endospore that is highly resistant to disinfection and other hostile forms of
323 treatment (Kiu & Hall, 2018; Mckew et al., 2013). It is unclear how *Clostridium* persisted
324 during the tannery process, across the tannery treatment plant, only to dominate in the system's
325 effluent (either through growth, or by out-surviving other strains that succumbed to coagulation-
326 flocculation-sedimentation). Given the harshness of the chemical tannery process, it is
327 reasonable to assume that surviving cells existed as endospores; however, whether they became
328 vegetative cells in anaerobic niches of the treatment plant could not be discerned by our study
329 and should be evaluated in a follow-up investigation. Our results are consistent with the prior
330 study by Desta et al. (2014), who used clone library analysis and showed that *Clostridium* was

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331 the most dominant genus in the last constructed wetland root zone sample collected from the
332 pilot plant at the Modjo tannery.

333 Besides *Clostridium*, other genera within the Firmicutes appeared in the current study, but at
334 much lower relative abundance across all samples. For example, the effluent samples showed
335 *Acetobacterium* to be the most abundant Firmicutes after *Clostridium* (1.9% and 2.9% RA in E1
336 and E2, respectively); however, this genus did not appear above 1% relative abundance in either
337 mixed liquor or influent samples. Instead, *Tissierella* was 4.4 and 2.2% RA in mixed liquor and
338 5.6 and 2.4% in influent samples. Similarly, *Anaerovorax* was 6.7 and 7.2% in mixed liquor, and
339 7.9% in G2 while it was 0.1% in G1. Finally, *Papillibacter* was 3.3 and 3.0% in mixed liquor,
340 and 3.1% in G2 while it was also 0.1% in G1. Beyond the Firmicutes, *Synergistes* (Synergistetes)
341 had the second largest relative abundance in effluent samples (15.8%, 33.9%), and Psychrobacter
342 (Proteobacteria) was third at 9.0% and 7.9% RA. Consequently, the effluent became dominated
343 by a few OTUs.

344 To evaluated changes in diversity across the treatment plant locations, alpha diversity was
345 estimated across all samples (Table SI-3). Community richness, defined by number of OTUs
346 characterized, was greatest in mixed liquor samples across both sample dates (1,059 and 1,008),
347 which is not surprising. Interestingly, influent sample G1 that was structurally quite different
348 from all other samples also had the next lowest richness (485 OTUs), and was quite different
349 from G2 (977 OTUs). Effluent samples had the lowest richness, with 874 and 873 OTUs.
350 Community diversity was evaluated by the Shannon diversity and inverse Shannon indices
351 (Table SI-3). Both show that effluent samples were least diverse, and influent samples were most
352 diverse although both influent and mixed liquor samples had comparable indices. The reduced
353 diversity of the effluent is consistent with what is shown in Figure 4, due mostly to the
354 dominance in the observed relative abundance of one (*Clostridium*) or two (*Clostridium* and
355 *Synergistes*) OTUs. Finally, all samples showed low evenness (value $\leq$ 0.1), indicating that the
356 communities are dominated by a relatively small number of OTUs. Indeed, only 20 OTUs had
357 relative abundance measurements at or above 1% among 254 total OTUs characterized, and
358 reflect 94% to 97% of the relative abundance among classified taxa. This outcome justifies
359 focusing community analysis on a subset of taxa, which we define as those present at a relative
360 abundance $\geq$1%.

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361 Importantly, most of the Illumina sequence reads at the genus level were not annotated and
362 presented as unclassified reads (Figure SI-1). A comparable high proportion of unclassified
363 bacteria at the genus level was reported in the gastrointestinal tract of cattle (Kim et al., 2014),
364 the feces of milk cows (Liu et al., 2016), and pond water samples (Qin et al., 2016). This reflects
365 a current limitation of applying 16S rRNA-based community analysis to understudied
366 environments, such as tanneries, despite knowing the source of wastewater entering the treatment
367 plant comes from processing of hides and skins. As more sequences from industrial treatment
368 systems are uploaded into public databases, these limitations will be overcome.

369 ***Tannery wastewater treatment enhances removal of most potential bacterial pathogens except***
370 ***Clostridium.*** An important consideration of the bacteria found in the tannery treatment system is
371 that six of the core (>1%) OTUs are of genera that include pathogens of importance to human
372 health. These six genera include: *Clostridium*, *Acinetobacter*, *Arcobacter*, *Shewanella*, *Vibrio*
373 and *Erysipelothrix*. While our methods cannot discern if the taxa present are, indeed,
374 pathogenic, we evaluated these genera to assess the effectiveness of the tannery treatment
375 process to reduce their relative abundance in effluent samples. Our assumption is that a lower
376 relative abundance translates into a lower risk of sending pathogenic strains into the receiving
377 stream. As shown in Figure 5, the tannery was effective at reducing or maintaining the percent
378 relative abundance between the influent and effluent for five of the six potential pathogenic
379 genera. The largest change from influent to effluent among these five in at least one sample per
380 location were *Acinetobacter* and *Shewanella*; we detected changes from 12% to 0.7% and 16%
381 to 0.01% respectively. Interestingly, in the case of *Acinetobacter*, the relative abundance peaked
382 in the mixed liquor. *Arcobacteria*, *Vibrio* and *Erysipelothrix* show continuous, albeit modest,
383 reductions in relative abundance from influent through the aeration basin into the effluent.
384 *Clostridium* was a notable exception relative to the other core potentially pathogenic bacterial
385 genera. We see that it consistently increased in relative abundance across the treatment system to
386 its highest levels in the effluent; next, we provide comments on the importance of this
387 observation.

388 *Clostridium*'s dominance in this study is relevant because several species within the genus (e.g.,
389 *C. difficile*, *C. perfringens*) are increasingly recognized as potential zoonotic pathogens of public
390 health concern (Freeman et al., 2010; Knight & Riley, 2019; Rood & Cole, 1991). Importantly,

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391 zoonotic pathogenic infections are garnering increasing attention in East Africa, albeit without
392 concomitant epidemiological studies (Kemunto et al., 2018). For this reason, it is important to
393 pay attention to tannery discharges as potential point-sources. While we cannot determine the
394 species or physiological form of *Clostridium* present in effluent samples from the methods used
395 in this study or if they were pathogenic, its ability to increase in relative abundance across
396 treatment designed to remove particles suggests that planktonic forms of *Clostridium* may have
397 been abundant in the effluent. *Clostridium* species can move between planktonic (the virulent
398 form, if pathogenic) and sessile (more antibiotic resistant) forms, as needed (Crowther et al.,
399 2014). Future studies that assess methods to enhance the treatment performance for tannery
400 wastewater treatment plants should use culture-dependent and culture independent methods to
401 monitor the fate of *Clostridium* across the treatment process to determine if the observations
402 made here and at the Modjo tannery (Desta et al., 2017) are consistent with tannery treatment
403 systems more generally. Notably, spore forming strains of *Clostridium* are known to be resistant
404 to conventional disinfection (e.g., Kenters et al., 2017; LeChevallier and Au, 2004), which
405 suggests that implementing such disinfection practices will not reduce their presence in the
406 tannery effluent. Indeed, use of *Clostridium* as a water quality monitoring agent is gaining
407 attention because, as a spore-forming microorganism that is purely of fecal origin, it is more
408 rubust in the environment and resists die-off that is typically seen with other, more common
409 indicators (e.g., Stelma Jr, 2018). The consequence of *Clostridium*'s presence in Ethiopian
410 tannery effluents and, presumably, receiving waters or their sediment deserves further attention,
411 especially since many of these water sources are used routinely to irrigate food crops.

412 **Conclusion**

413 Physicochemical analysis in the effluent to the Ethiopian tannery studied in this case study
414 showed that several water quality parameters (COD, sulfide, total nitrogen and total chromium)
415 violated the country's industrial treatment standards during the time samples were collected. 16S
416 rRNA gene-based sequencing using the Illumina platform showed that the dominant bacteria
417 found in the tannery influent wastewater were of the phyla typically found in the guts of
418 ruminant animals, and persisted during treatment. In contrast, the chemical composition of the
419 effluent varied more, and appeared to reflect changes in chemical composition in the influent.
420 Overall, the treatment approach used was insufficient to address expected variations in the

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421 influent, which can vary widely depending upon the type of animal skins being processed on any
422 given day. Microbial community analysis showed that less than 8% of the OTUs comprise over
423 94% of bacterial phylotypes, reflecting low community richness. Furthermore, while the
424 treatment process was able to reduce the relative abundance of most of the prominent genera that
425 include pathogens, coagulation-flocculation-sedimentation resulted in an increase in the relative
426 abundance of *Clostridium* in the effluent. An evaluation of which *Clostridium* species are
427 present, in what forms (planktonic versus sessile, vegetative versus endospore), and their
428 potential impact on public health is warranted.

429

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441

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Table1. Physicochemical characteristics of the tannery wastewater treatment plant samples (mean±STD) compared to the regulated limits or guidelines and other studies. Bolded E1 and E2 values exceeded EEPA guidelines during this study.

	Influent (G), mixed liquor (S) and effluent (E) water quality results from this study						EEPA Discharge Limit[a]	**Other Studies**	
Parameters	**G_1**	**S_1**	**E_1**	**G_2**	**S_2**	**E_2**		**Raw influent**	**Treated effluent**
pH	8.0±0.1	8±0.0	7.5±0.1	8.3±0.1	8.2±0.1	7.9±0.1	6.0-9.0	4.0-9.0[b], 6.5-12.5[c], 10.4±0.3[d], 8.2±2.4[e]	7.7±0.1[d], 8.0±0.1[e]
T (°C)	20.3±0.5	18.7±0.5	18.3±0.5	20.0	18.0	17.3±0.5	40	20.6±2.34[d], 20.3±1.9[h]	18.0±1.2[h]
Conductivity (mS/cm)	21.1±0.1	19.4±0.1	14.4±0.1	25.5±0.1	18.4±0.2	13.5±0.1	NL	15.5±2.0[e]	8.2±0.5[e]
COD (mg/l)	2,100±30	2,040±26	**1,070±58**	3,990±942	3,260±72	**1,330±67**	500	12,900±6,900[c] 12,500±3,900[d], 7,300±540[e], 1,760±945[h]	395±139[d], 1,143±262[e], 338±70[h]
TDS (mg/l)	4,310±5	3,820±9	2,220±72	4,650±11	4,190±31	2,250±133	NL	1,590±508[h]	2,960±223[h]
TSS (mg/l)	n/a	n/a	n/a	n/a	n/a	n/a	50	510-3,330[b], 2,430±515[c], 1,160±200[d] 1,040±438[h]	92±11[d], 90±9[h]
Sulfide-S (mg/l)	191±4	99±2	**58±2**	212±1	124±2	**68±3**	1	417±131[c], 56±6[d], 269±76[e], 92±54[h]	4.9±3.0[d], 6.6±3.8[e], 0.3±0.3[h]
Sulfate-S (mg/l)	571±13	464±3	98±1	646±4	677±3	669±5	NL	800±505[d], 489±71[e]	35±61[d], 433±162[e]
Nitrate-N **(mg/l)**	n/a	n/a	n/a	n/a	n/a	n/a	NL	124±13[c], 112±24[e], 11-18[f]	144±35[e], 9-11[f]
Ammonia-N (mg/l)	32±3	46±0.8	1.4±0.2	59±2	54±1	12±1	30	24-762[b], 36-127[f], 34+23[h]	41±22[e], 32-35[f], 79±26[h],
Total Nitrogen	289±3	236±2	**110±2**	321±3	294±4	**118±4**	60	545±12[e]	220±18[e]

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(mg/l)									
Total Kjeldahl Nitrogen (mg/l)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	265-12,900[g]	n/a
Total Cr (mg/l)	37±0.9	4.6±0.1	**4.8±0.2**	13±1	4.6±1	**5.8±0.3**	2	35.7±8.6[c], 27±3[d], 28±5[e], 15±14[h]	7.7±0.1[d], 8.7±7.2[e], 0.6±1.3[h]

[a]EEPA, 2003; [b]Gutterres et al., 2015; [c]Birhanie et al., 2017; [d]Desta et al., 2014; [e]Alemu et al., 2019; [f]Sugasini and Rajagopal, 2015; [g] Gutterres et al., 2015; [h]Tsegaye and Kaba, 2017; n/a = not available; NL = no limit set.

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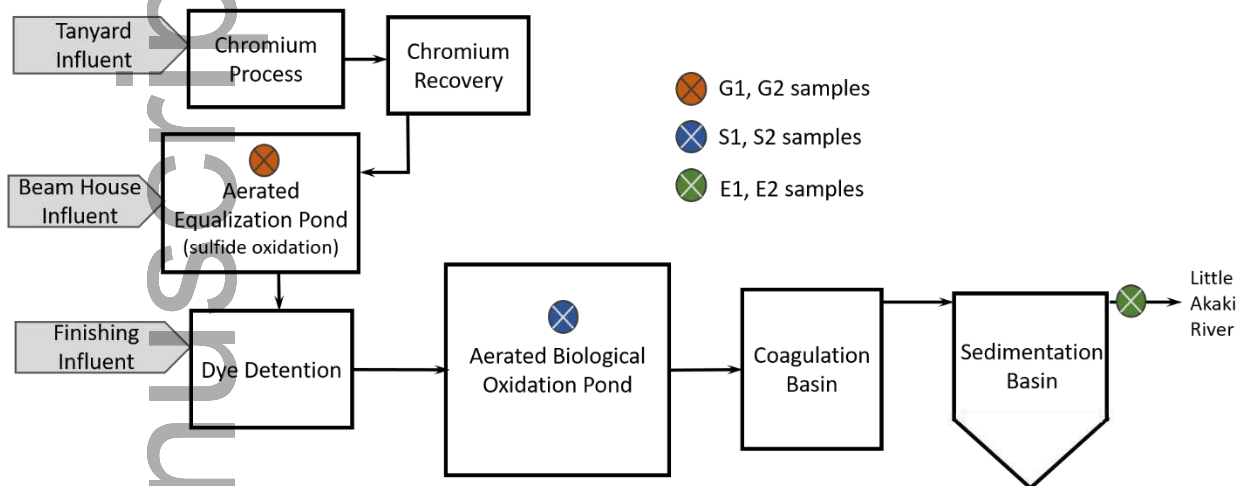

Figure 1. Schematic of full-scale tannery wastewater treatment plant and associated sample locations.

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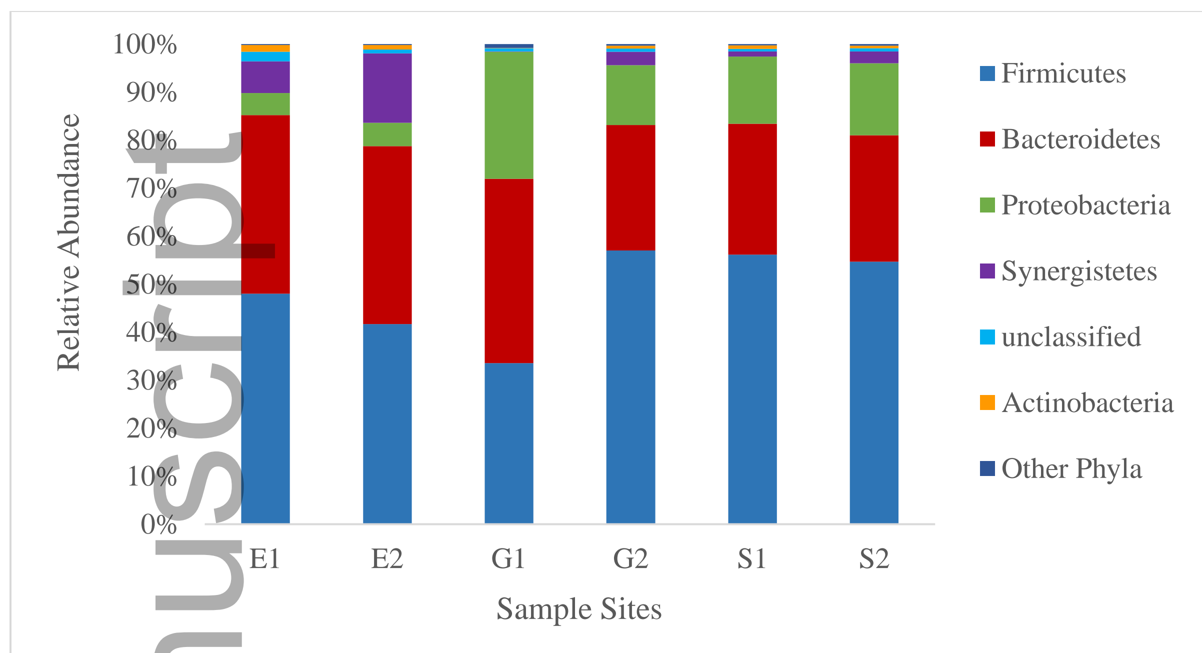

Figure 2. Composition and relative abundance of bacteria at the phylum level across all samples in the tannery wastewater treatment plant during the two sampling dates. Only phyla with relative abundance greater than or equal to 1% are shown.

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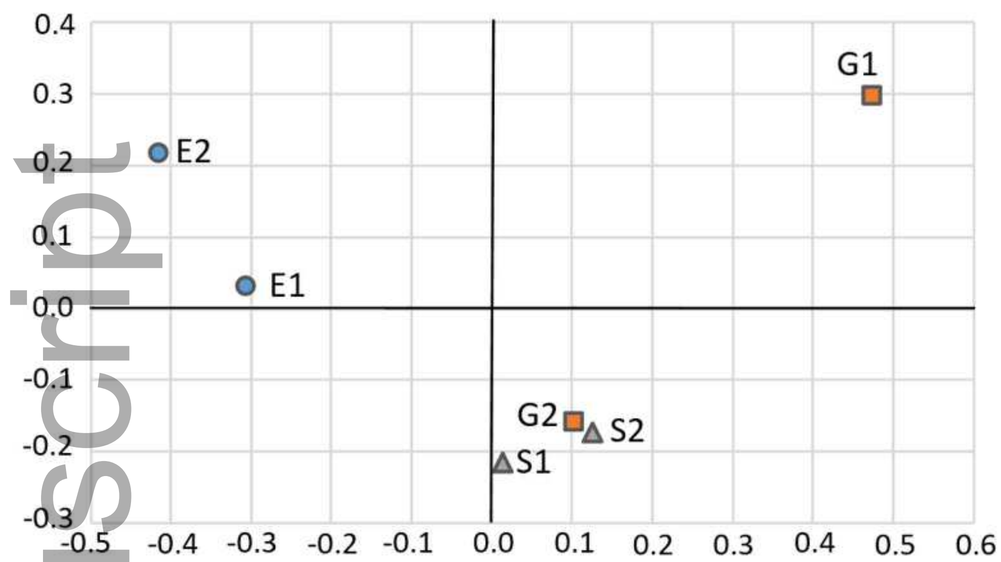

Figure 3. Principal Coordinate Analysis of the tannery wastewater treatment plant samples by location, using unweighted UniFrac distance as a measure of similarity between samples.

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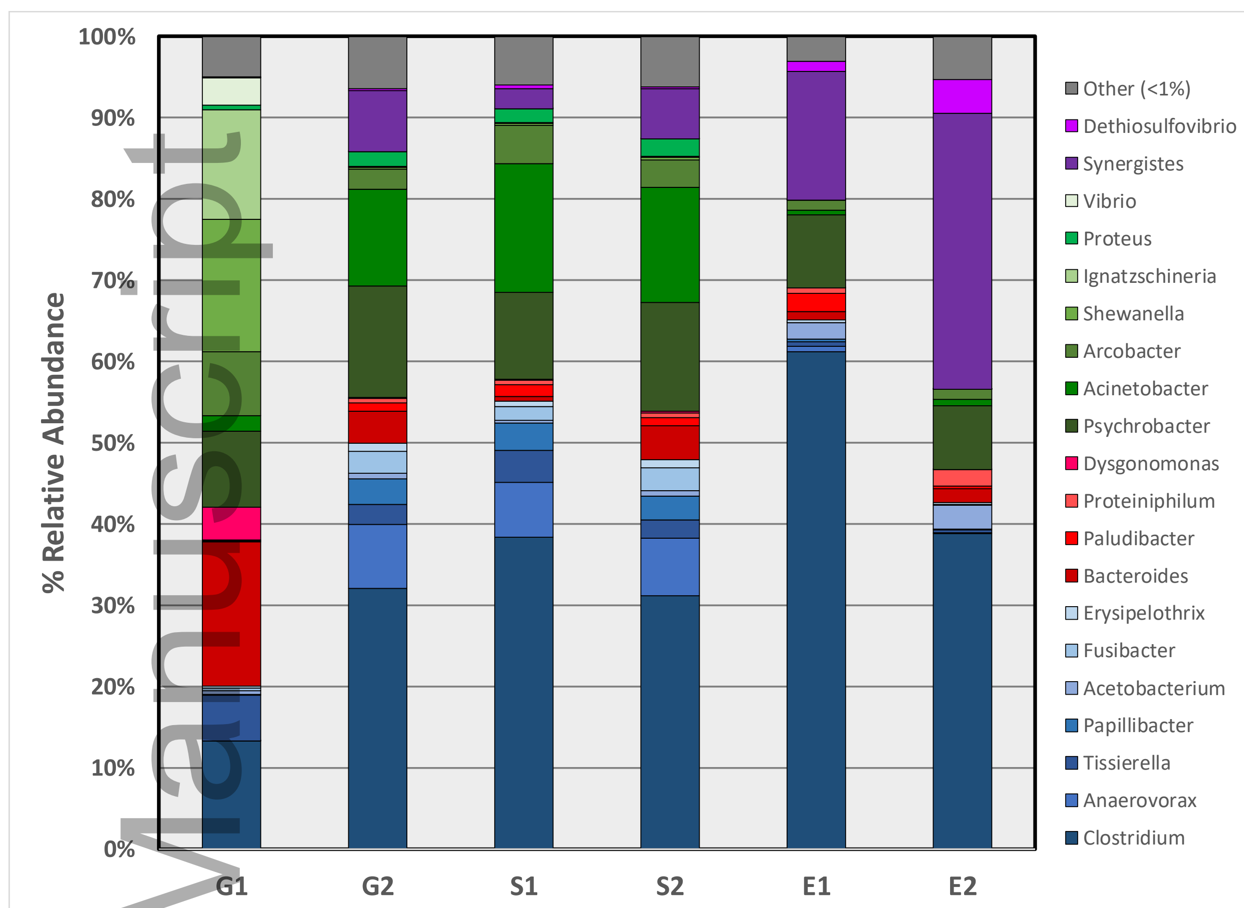

Figure 4. Relative abundance of classified Bacteria at the genus level at the three sampling locations across the tannery wastewater treatment plant during the two sampling events. Color coded bars are shaded in accordance with their Phyla from Figure 2: Firmicutes blue shades; Bacteroidetes red shades; Proteobacteria green shades; Synergistetes purple shades. Bacteria that were identified but exist at < 1% relative abundance are shaded gray and listed as Other.

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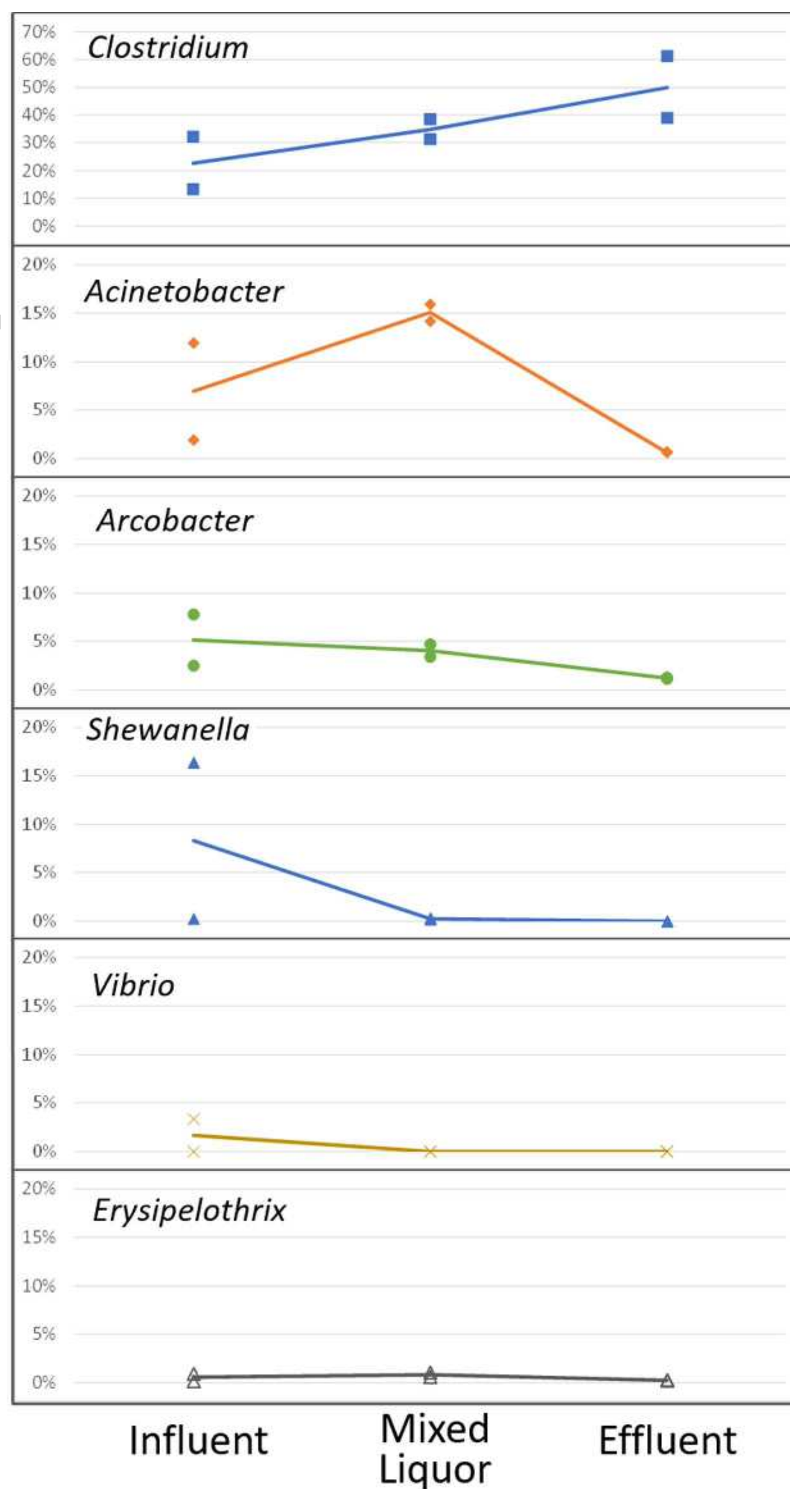

Figure 5. Changes in relative abundance of potential pathogenic bacterial genera across different sample points in the tannery. Symbols show the measured relative abundance in composited samples for each of two sampling dates, and the line graph shows the average values. Note that the scale for *Clostridium* is different from the others.

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