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Chapter 8

# Molecular Biomonitoring of Microbial Communities in Tannery Wastewater Treatment Plant for the Removal of Retanning Chemicals

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Additional information is available at the end of the chapter

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**Abstract**

This chapter focuses on culture-independent characterization and monitoring of microbial communities in tannery wastewater treatment system, with special reference to the degradation of two xenobiotic chemicals used in retanning processes. Molecular survey of a tannery wastewater treatment system through metagenomic and metatranscriptomic approaches revealed a diverse microbial community in each component of the treatment system with high gene copies for enzymes involved in the degradation of cyclic aromatic compounds such as nitrotoluene. A combination of flow cytometry and molecular fingerprinting methods was used in a lab-scale reactor to monitor the dynamics of the microbes in the sludge and the fate of two retanning chemicals. The identified key microbial communities for the removal of the two xenobiotic chemicals belong to members of the group *Proteobacteria* and the phylum *Bacteroidetes*.

**Keywords:** tannery, retanning chemicals, bacteria, biomonitoring, wastewater, flow cytometry, fingerprinting

## 1. Introduction

### 1.1. The leather industry

The leather industry in developing nations is a sector in continuous growth but leaving behind the toxic pollutants in the environment. The economy of Eastern African countries is predominantly agricultural where the livestock subsector plays a substantial role. Livestock

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is an integral part of the national agricultural wealth of Eastern African countries serving as sources of power, meat, milk, egg, hides and skins, manure, and other products. Hides and skins, though by-products of animals, have been contributing greatly to the export earnings from the livestock sector since ancient times.

In most parts of sub-Saharan Africa and Asia, tanning is a family business, carried out in small- to medium-scale semi-mechanized units. Tanneries owned by different individuals are frequently grouped tightly in clusters which used to be nonresidential areas. Most of the tanning facilities are strategically located near to rivers and small streams so as to discharge their large amount of heavily polluted wastewater directly to these water bodies. Considering a case study in Ethiopia, the Awash River is used as inputs for small- and large-scale farms of fruits, vegetables, and sugarcane, yet experiencing a significant water quality deterioration. The discharge of properly untreated tannery effluent has caused severe pollution affecting surface and underground water resources, farms irrigated by such water, people working in the farms, and consumers of the farm products, not to mention the aquatic ecosystem.

### 1.2. The leather manufacturing process

The production of leather involves the whole process of converting raw hides or skins into useful commodities such as shoes and garments from the meat industry [1]. Hides and skins are processed to react with various chemical substances that prevent them from putrefaction to make them resistant to wetting and keep them supple and durable [2, 3]. It has been reported by Khan and colleagues [4] that about 130 chemicals are used in the entire process of leather production. The production process is generally divided into four main categories, namely, the beamhouse, tanning, retanning, and finishing processes. In this chapter, we will focus on pollutants of the retanning process and their fate during biological treatment processes.

### 1.3. The retanning process: a closer look

Retanning, also called post-tanning operation, involves neutralization and fat liquoring to improve the feel and handle of leather and provide frame retarding, water, and abrasion resistance properties [5, 6]. Retanning is carried out by employing various substances such as phenolic and naphthalene resins, melaminic resins, acrylic resins, and polymers [6].

#### *1.3.1. Melaminic resins as retanning agents*

Melaminic resins are condensation products from formaldehyde with amino and amido compounds, such as urea, melamine, and cyanamide (dicyandiamide). The amine resins are polymers synthesized by condensation of urea, formaldehyde, and melamine (2,4,6-triamino-1,3,5-triazine) [7]. The formaldehyde undergoes an addition reaction with amino group of urea or melamine with the formation of N-methylol groups. Urea-formaldehyde resins are synthesized and chemically modified by reaction with a sulfating agent to form a sulfonated soluble product. Regarding melamine, the methylol groups can react with amino or other methylol groups to form methylene or ether bridges based on the reaction scheme for melamine as depicted in **Figure 1**. These resins give light colored leathers with good resistance.

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Due to their availability, melaminic resins are among the widely used chemicals in industries processing leather to the retanning and finishing steps [7]. The trade name Retanal MD-80 refers to melamine-formaldehyde resin used in retanning of hides and skins.

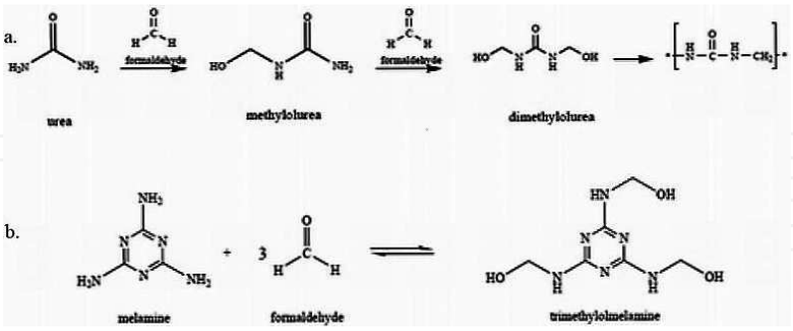



**Figure 1.** Condensation of urea (a) and melamine (b) using formaldehyde (after Ref. [7]).

### *1.3.2. Phenolic and naphthalene resins as retanning agents*

Phenolic and naphthalene resins are polymers synthesized using phenolic, naphthalene, and their derivatives condensed with urea and formaldehyde. The synthesis reaction which is patented by BASF in 1913 involves reaction of the basic phenolic and/or naphthalene constituents under acidic conditions which results in attachment of the aromatic compounds to one another with the aid of formaldehyde through methylene bridges. Then, they are adjusted to the optimum degree of condensation by making them binuclear or trinuclear and made water soluble by sulfonation or sulfomethylation which are finally adapted by buffering to meet the application requirements (**Figure 2**) [6, 8]. When used on chrome-tanned leather, they specifically impart it to a soft fullness and relaxed grain. These characteristics of mellowness and softness are very desirable in gloves, garment, and soft-type leathers [9].

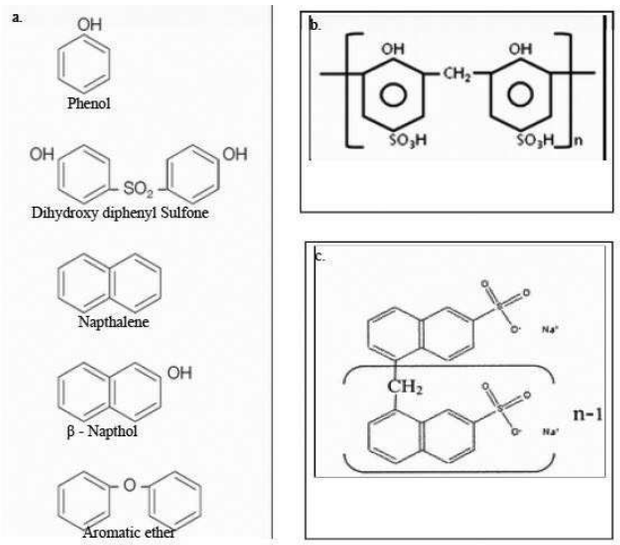



**Figure 2.** (a) Basic constituents of phenolic and naphthalene resins, (b) structure of phenol formaldehyde condensate, and (c) structure of naphthalene formaldehyde condensate (after Refs. [6, 10]).

| COD (mg/l) | BOD (mg/l) | $NH_4$(mg/l) | Cr (mg/l) | $S^{2-}$(mg/l) | TS (mg/l) | SS (mg/l) | VSS (mg/l) | pH | TDS (mg/l) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| 2250±565 | 1000±88 | – | 0.027±0.075 | – | – | 92±36 | – | 6.14±1.1 | – | [14] |
| 3700 | 1470 | 180 | – | 440 | – | 2690 | 1260 | 7.4 | – | [15] |
| 4800±350 | – | 225±18 | 95±55 | – | 10,266±1460 | 2820±140 | 1505±90 | 7.06 ± 0.26 | 18,800–19700 | [16] |
| 1320–54,000 | 840–18620 | – | 41–133 | 800–6480 | – | 220–1610 | – | – | – | [17] |
| 4100–6700 | 680–976 | 144–170 | 41,623 | – | – | 600–955 | – | 7.0–8.7 | – | [18] |
| 8000 | 930 | – | 11 | 228 | – | 2004 | 1660 | – | 15,152 | [19] |
| 2200 | – | – | – | – | – | 5300 | 1300 | 7.7 | 36,800 | [20] |
| 11123±563 | 2983±259 | 122±8 | 32±6 | 630±67 | – | – | – | 10.8-0.1 | 6646±557 | [21] |
| 2155 | – | 166 | 50.9 | 35.6 | – | 915 | 578 | 7.79 | – | [22] |
| 3114 | 1126 | 33 | 83 | 55 | 18,884 | 1147 | – | 10.5 | 17,737 | [23] |
| 2426 | – | 335 | 29.3 | 286 | – | – | – | 7.7 | – | [24] |
| 5650 | – | – | – | – | 19,755 | 5025 | – | 8.2–8.5 | 14,750 | [25] |

The first column lists the parameters used in the different studies. Parameters with "–" means data are not available.

**Table 1.** Characteristics of tannery wastewater based on studies from different countries and treatment systems.

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### 1.4. Characteristics of tannery wastewater

The tanning process consumes high amount of water, estimated to be 34–56 $m^3$ of water per ton of hide or skin processed [11]. Out of the total water consumed, 85% is discharged as a wastewater [12]. Interestingly, only 20% of the wet-salted hides/skins are converted into commercial leather, 25% becomes chromium-containing leather waste, and the remainder becomes non-tanned waste or is lost in wastewater as fat, soluble protein, and solid suspended pollutants [13]. Therefore, environmental pollution remains to be a serious problem in the leather sector.

The characteristics of the wastewater vary considerably from tannery to tannery depending on the size of the tannery, the chemicals used for the specific process, the amount of water used, and the type of final product produced by a tannery. The variations of effluent characteristics also occur through each working day in a tannery. According to Calheiros et al. [14], average COD and pH analyzed in 1 day were 2010 mg/l (±516) and 6.98 (±0.05), respectively, whereas 2068 mg/l (±446) and 7.93 (±0.08), respectively, in another day. **Table 1** summarizes the pollution load discharged from individual tannery processing operations.

Most of the studies on pollution load of tanneries do not include chemicals that are involved in the process after the tanning step. This is partly because the pollution load of the chemicals used in the retanning process is included in some parameters such as COD and TDS. The other reason is the absence of fast and cheap method to detect these specific chemicals. Reemtsma et al. [26] reported the presence of benzothiazoles in tannery wastewater in three forms, benzothiazole (BT), methylthiobenzothiazole (MTBT), and monobenzothiazole (MBT), with a dominance of MBT at a concentration of 3.3–6.9 μmol/L. These compounds have been detected in tannery wastewater samples by Fiehn et al. [27] in concentrations of 655 μg/L MBT, 10.5μg/L BT, and 39 μg/L of MTBT. A report by UNIDO [28] indicated that only 22% of all the chemicals used for post-tanning and finishing process is taken up and remained in/on the leather, whereas from the remaining waste chemicals (88%), 23% belongs to fat liquors and 20% to dyestuffs.

In this study, we explore the microbial community in the different components of a treatment plant and expressed genes for the target chemicals Basyntan and Retanal. In addition, we decipher the key microbial subcommunities responsible for the degradation of our target post-tanning chemicals.

## 2. Materials and methods

### 2.1. Reactor setup and sampling

The data shown in this chapter are from a study conducted on a pilot-scale biological wastewater treatment plant installed in the premises of a privately owned tannery in Modjo town, Ethiopia, 70 km south of the capital Addis Ababa. The system consists of two anaerobic reactors each with volume of 25 $m^3$: an aerobic reactor with a volume of 50 $m^3$ and subsurface-flow constructed wetland vegetated with the perennial grass *Phragmites australis* (Cav.) (**Figure 3**). Performance of the

treatment system was evaluated by taking samples of the influent and the treated effluent water and analyzing the different physicochemical parameters following the procedure in APHA [29].

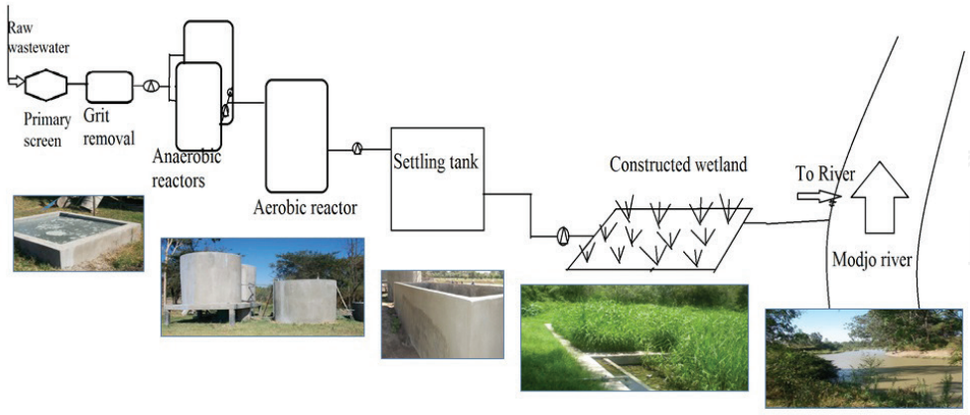



**Figure 3.** Schematic presentation of the pilot tannery effluent treatment site comprising anaerobic-aerobic reactors integrated with constructed wetland system.

### 2.2. Metagenomic and metatranscriptomic analyses

Sludge and sediment samples were taken from the anaerobic, aerobic, and different parts of the constructed wetland. The extraction of DNA and RNA was carried out using Zymo ZR® kit for DNA and Zymo ZR® kit for RNA (Zymo Research, CA, USA), respectively. Shotgun sequencing of the metagenome was conducted by means of Illumina Nextera XT® protocol. Total RNA was sequenced following the Illumina TruSeq® RNA preparation protocol.

The quality of the generated DNA and RNA reads was checked using FastQC toolkit [30]. FASTX-Toolkit was used to dereplicate, screen for ambiguous reads, and trim based on the cutoff value of Phred score >20 [31]. Assembly of the trimmed DNA and RNA reads was performed using Velvet (v 1.1) [32] and Trinity (v 2014) [33], respectively. Ribosomal RNA was removed using the riboPicker software (v 0.4.1) [34]. Binning and normalization were performed using an in-house Perl script. Taxonomic identification was done using BLASTN for the metagenome contigs and BLASTX for the metatranscriptome against a local download of NCBI nonredundant GenBank database. A set of contigs from the metatranscriptomic dataset were analyzed for the frequency of various identified genes, and Blast2GO (v 1) [35] was employed for the annotation of the genes.

### 2.3. Monitoring of microbial communities for the degradation of retanning chemicals

A bench-scale sequencing batch reactor (SBR) mimicking the treatment system was set up to analyze the dynamics of microbial community and its functional significance for the removal of the various pollutants in the wastewater. The SBR was operated continuously in cycles of around 72 hours with the fill, react, settle, and draw cycles as depicted in **Figure 4**. A number of abiotic parameters including liquid chromatography-based analysis of the two retanning chemicals (Basyntan and Retanal) were measured at each batch throughout the entire running period. Similarly, flow cytometry-based quantification and sorting of sludge microbial community stained with DAPI were carried out using the MoFlo cell sorter (DakoCytomation, Fort Collins, CO). The sorted cells

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were processed for taxonomic identification of the different subcommunities using T-RFLP and clone library-based 16S rRNA sequence analysis described in Koch et al. [36]. Correlation analyses between the abiotic parameters and the gated subcommunities were done by Spearman's rank-order correlation coefficient using the program R (http://CRAN.R-project.org) Version 2.14.0.

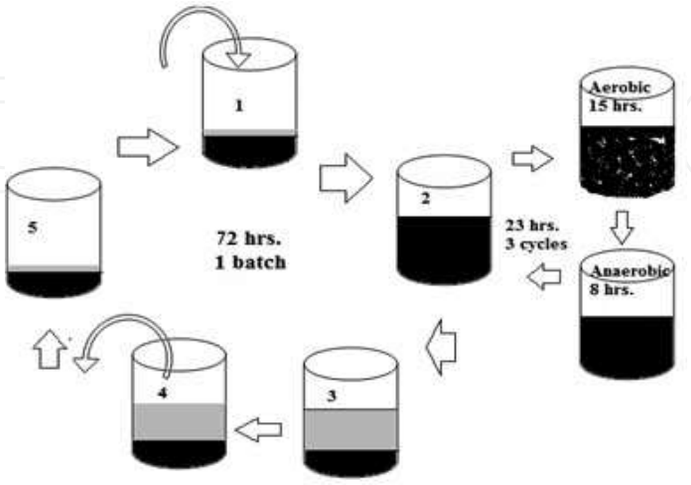



**Figure 4.** Schematic presentation of the different phases of the lab-scale sequencing batch reactor.

## 3. Results and discussion

### 3.1. Performance of the treatment system

Based on the physicochemical analysis, the untreated wastewater was characterized by its high concentration of sulfate, ammonia nitrogen, total suspended and dissolved solids, as well as high biological and chemical oxygen demands (BOD and COD). The high pH also indicated the alkalinity of the wastewater. Performance of the treatment system with regard to the removal of priority pollutants ranged between 70 and 99% (**Table 2**). The effluent parameters obtained for the COD, sulfate ($SO_4^{2-}$), sulfide ($S^{2-}$), nitrate ($NO_3$), and ammonia nitrogen ($NH_3$-N) were in line with the provisional emission limit values set for tannery effluents in Ethiopia which are 500 mg/l for COD, 1 g/l for $SO_4^{2-}$, 1 mg/l for $S^{2-}$, 20 mg/l for $NO_3$, and 30 mg/l for $NH_3$.

### 3.2. Metagenomic and metatranscriptomic analyses of tannery treatment system

Shotgun metagenomic analysis of the pilot reactors revealed the presence of seven phyla in the anaerobic reactor and eight phyla in the aerobic and the constructed wetland areas. The most abundant bacterial phyla in the anaerobic and aerobic reactors belonged to phylum *Firmicutes* and *Proteobacteria*, respectively. In the wetland, members of the phyla *Proteobacteria*, *Chlorobi*, and *Chloroflexi* were dominant (**Figure 5**).

A closer look into the dominant phylum *Firmicutes* showed that the genera *Bacillus*, *Clostridium*, and *Tissierella* were relatively the most abundant genera in the anaerobic system; these microorganisms have been implicated in the degradation of aromatic hydrocarbons in other tannery wastewater treatment systems [37].

| Parameter | Influent | Effluent | % Removal |
|---|---|---|---|
| TN | 245.25 ± 76 | 62.75 ± 14 | 74 |
| $SO_4$ | 800 ± 505 | 35 ± 61 | 96 |
| TP | 15.33 ± 1 | 4.23 ± 2 | 72 |
| $S^{2-}$ | 55.50 ± 6 | 4.91 ± 3 | 91 |
| $NO_3$ | 310 ± 203 | 40.25 ± 28 | 87 |
| $NO_2$ | 2.08 ± 3 | 0.03 | 99 |
| $NH_3$ | 287.70 ± 178 | 44.28 ± 26 | 85 |
| COD | 12,547.50 ± 3910 | 395 ± 139 | 97 |
| BOD | 4886.26 ± 266 | 308.91 ± 24 | 94 |
| TDS | 9470.50 ± 1335 | 2593.69 ± 344 | 73 |
| TSS | 1155 ± 203 | 92 ± 11 | 92 |
| VSS | 27,482.75 ± 197 | 2272.75 ± 724 | 92 |
| Total Cr | 27.25 ± 3 | 0.95 | 97 |
| pH | 10.40 ± 0.3 | 7.66 ± 0.1 | |

*Key*: TN, total nitrogen; TP, total phosphorous; TDS, total dissolved solids; TSS, total suspended solids; VSS, volatile suspended solids; total Cr, total chromium (Source: Desta et al. [37])

**Table 2.** Average characteristics of the influent and effluent wastewaters of the integrated treatment system at the time of sludge sampling (concentrations are in mg/l, except for pH).

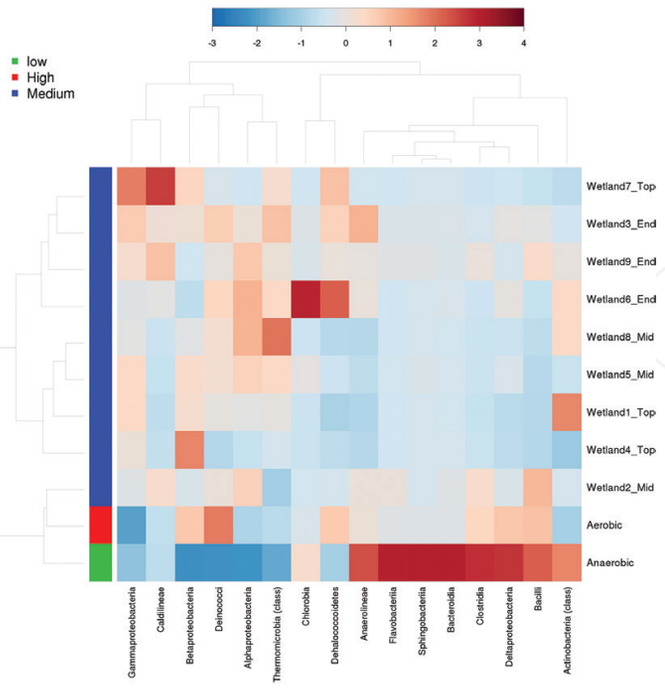



**Figure 5.** Relative abundance of bacteria as classified in phylum level. Sample sites were classified based on the concentration of salt (measured as TDS) and qualitatively designated as high, medium, and low levels.

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The phyla *Bacteroidetes*, *Cyanobacteria*, *Actinobacteria*, and *Chloroflexi* follow the next levels of abundance in the anaerobic reactor, with members identified in the degradation on both priority nutrients and synthetic aromatic compounds [36, 37].

In the aerobic reactor, members of the phyla *Cyanobacteria* and *Deinococcus-Thermus* were the most abundant bacterial groups. The genus *Deinococcus* was more abundant in the aerobic reactor than in any other part of the treatment system. Members of the class *Betaproteobacteria* such as the genera *Burkholderia*, *Rhodocyclus*, and *Nitrosomonas* were identified from the aerobic system and are inferred to be involved in ammonia oxidation and aromatic compound degradation [36, 37].

Metatranscriptomic analysis of biological samples from the anaerobic reactor of the treatment system revealed the presence of genes coding enzymes involved in the degradation of nitrotoluene, chlorocyclohexane, toluene, and benzoate, apart from the enzymes for common anabolic and catabolic pathways (**Figure 6**). Relatively higher number of expressed genes were detected for nitrotoluene degradation coded for the enzymes DNT dehydrogenase (EC 1.2.99.2) and DHAT reductase (EC 1.8.99.3). These enzymes are implicated in the degradation of compounds such as nitrotoluene and related aromatic compounds.

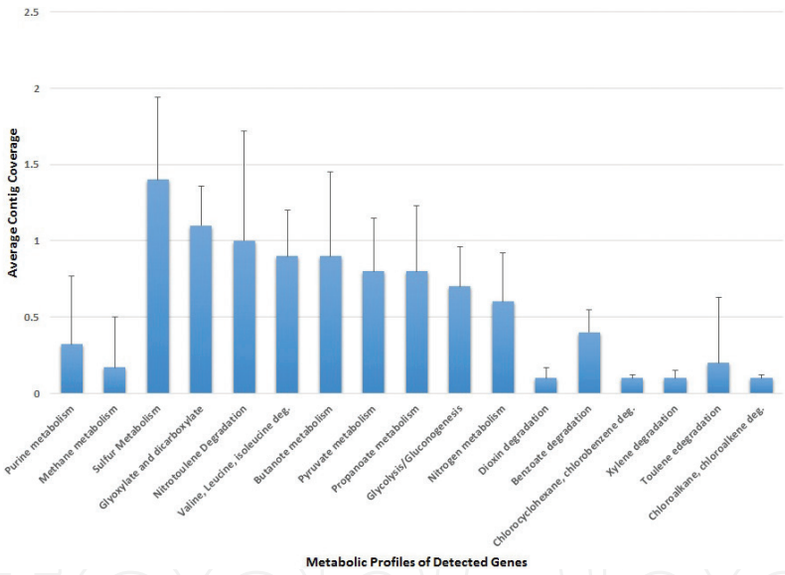



**Figure 6.** Average contig coverage for 17 common metabolic gene anaerobic reactors of tannery WWTP. X-axis, The genes involved in the metabolic pathways, and Y-axis, the average contig coverage. Error bars represent the standard error.

### 3.3. Dynamics and functional characterization of microbial communities

Flow cytometric characterization of bacterial community in the sludge of the reactor followed up at bench-scale sequencing batch reactor (SBR) revealed the dynamics, succession, and shift of the microbial subcommunities during the course of reaction, with typical patterns in each batch. Starting from the first batch of the operation of the SBR, changes expressed as shift of clusters in the x- and y-axes were observed in each batch of reaction of the SBR, indicating increase in cell size and proliferation activity of the microbial communities over the whole

running period of the SBR. Based on visual evaluation of the histograms of the dot plots, a gate template was created representing 30 clusters during the 14 batches of the reactor run (**Figure 7**). From each gate, cell abundance over the entire reaction period was evaluated.

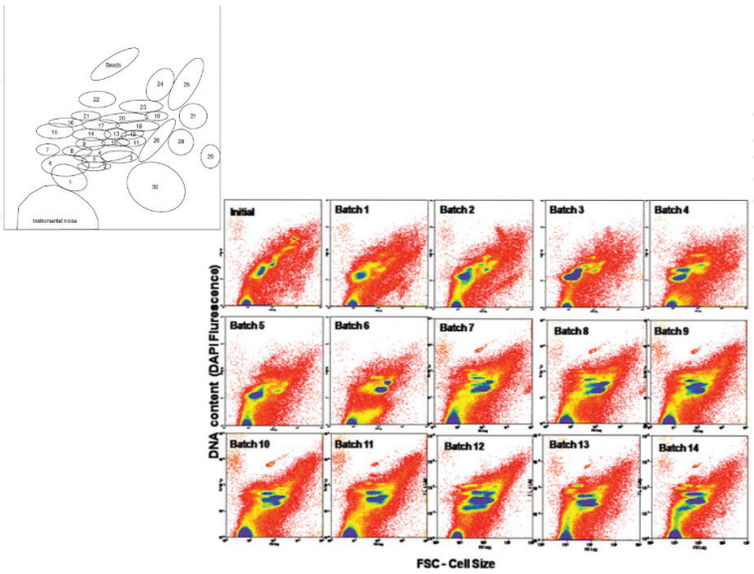



**Figure 7.** Bacterial community dynamics of tannery wastewater running in sequencing batch reactor (SBR) for 45 days. The first dot plot (initial) refers to the bacterial community in the acclimatized sample used to seed the reactor at the beginning of the SBR. The gate template (top-left box) which is used as the basis for fingerprinting of the different cell types.

Correlation analysis of bacterial cell abundance in each gate with the 13 measured abiotic parameters revealed positive correlations ($p<0.05$) between removal of the retanning agents and bacterial groups in gates G6, G12, and G20. Considering the different UPLC-based peaks of Basyntan, highly positive correlation was found specifically between peak 1 (ΔB1) and peak 3 (ΔB3) of Basyntan and the cells in G21 and G23. The correlations between the rest of the retanning agents (ΔB2 and ΔR) with the cells in G21 and G23 were still found to be positive ($p< 0.05$), suggesting the possible role of the cells in G21 and G23 for the biodegradation of Retanal and all the components of Basyntan. In order to have a closer look at the clusters and identify the consistent members in the flow cytometric pattern from each batch of the SBR, eight of the 30 gated subcommunities, namely, G1, G2, G6, G12, G14, G16, G20, and G21 were sorted to analyze the composition and abundance of bacteria in each sorted gate. From all the sorted gates, eight bacterial families and classes belonging to the phyla *Proteobacteria*, *Bacteroidales*, and *Bacteroidetes* were identified using terminal restriction fragments (T-RFs). Out of the eight gated clusters, gate 14 (G14) contained the smallest portion of the sorted bacterial community with predominant members belonging to *Proteobacteria* (6%) and showed strong positive correlation ($p< 0.01$) with the degradation of Basyntan and Retanal (**Figure 8**).

The gates 16, 20, and 21 (G16, G20, and G21) which showed positive correlations with retanning chemicals degradation were dominated by members of the phylum *Bacteroidetes* constituting 13,

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23, and 66%, respectively. *Rhodocyclaceae* (11%), *Brucellaceae* (10%), and unclassified *Proteobacteria* (8%) were the second, third, and fourth abundant groups identified in gate 20 (G20), respectively. The most abundant cells belonged to *Rhodocyclaceae* (48% and 22%). The second most abundant groups in this gate belonged to unclassified *Proteobacteria* (16 and 8%), followed by the family *Brucellaceae* (8%). The families *Caulobacteriaceae*, *Xanthomonadaceae*, and the phylum *Bacteroidetes* constituted a small proportion (15%) of the total community in the gate. The role of the identified bacterial groups in the degradation of the retanning agents is reflected by the positive correlation ($p < 0.05$) detected between cell abundances and removal of the retanning agents (**Figure 9**).

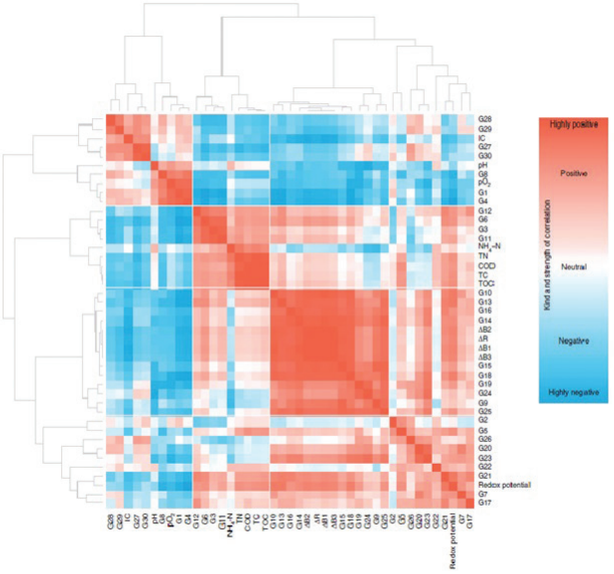



**Figure 8.** Correlation of 13 abiotic parameters with cell abundances in the 30 gates during the running period of the reactor (after Ref. [36]).

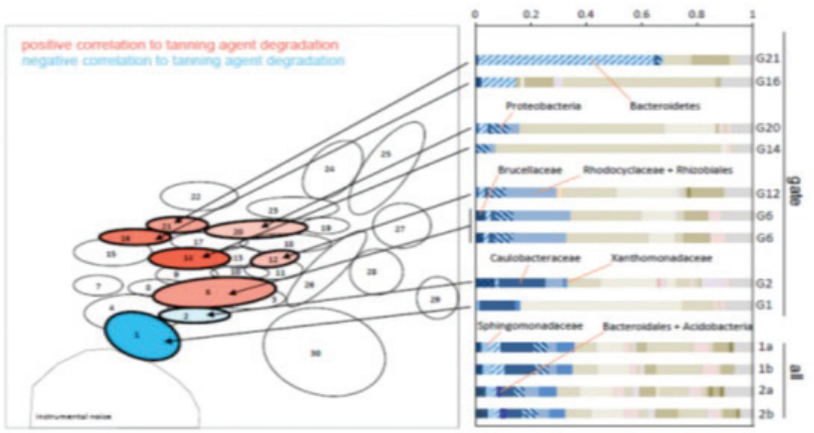



**Figure 9.** Taxonomic composition of the sorted gates associated with their role in the degradation of the two retanning agents Basyntan and Retanal (after Ref. [36]).

## 4. Conclusion

The findings of this study provided a preliminary investigation on the biodegradability of two of the several types of xenobiotic compounds used in the tanning industry. It was possible to single out bacterial groups such as *Bacteroidetes* and *Proteobacteria*, with strong correlation with the complete degradation of some of the compounds in retanning chemicals.

Management of wastewater treatment plants (WWTPs) primarily focuses on process parameters and physicochemical (abiotic) properties of the wastewater before and after treatment. Stable performance of any biological wastewater treatment system can be achieved by understanding and manipulating the microbial communities residing in the system besides the management of the conventional process parameters and abiotic properties. Investigations of microorganisms responsible for efficient reduction of pollutants in various biological wastewater treatment plants have been conducted for many years. This study was successful in identifying bacterial groups involved in different nutrient removal processes from tannery wastewater such as sulfur oxidation, denitrification, and cyclic aromatic compound degradation. Moreover, this study is one of the few studies conducted in field-scale reactors that integrate different approaches to interpret the functional property of a biological treatment system.

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