Analysis of Microbial Community Dynamics during the Acclimatization Period of a Membrane Bioreactor Treating Table Olive Processing Wastewater

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Featured Application: The novel data and insights obtained in this study are expected to provide guidance in the start-up of large-scale applications of membrane bioreactor process for biological treatment of difficult to degrade table olive processing wastewater.

Abstract: Biological treatment of table olive processing wastewater (TOPW) may be problematic due to its high organic and polyphenolic compound content. Biomass acclimatization is a necessary, yet sensitive, stage for efficient TOPW biological treatment. Next-generation sequencing technologies can provide valuable insights into this critical process step. An aerobic membrane bioreactor (MBR) system, initially inoculated with municipal activated sludge, was acclimatized to treat TOPW. Operational stability and bioremediation efficiency were monitored for approx. three months, whereas microbial community dynamics and metabolic adaptation were assessed through metagenomic and metatranscriptomic analysis. A swift change was identified in both the prokaryotic and eukaryotic bio-community after introduction of TOPW in the MBR, and a new diverse bio-community was established. *Thauera* and *Paracoccus* spp. are dominant contributors to the metabolic activity of the stable bio-community, which resulted in over 90% and 85% removal efficiency of total organic carbon and total polyphenols, respectively. This is the first study assessing the microbial community dynamics in a well-defined MBR process treating TOPW, offering guidance in the start-up of large-scale applications.

Keywords: next-generation sequencing; membrane bioreactor; start-up; table olive processing wastewater; microbial community dynamics; biomass acclimatization

1. Introduction

Table olives are an important agro-industrial product, especially in Mediterranean countries. Table olive processing aims to eliminate the natural bitterness of the fruit, due to polyphenolic substances, and render it edible and marketable, through several process steps, namely: debittering, washing, fermentation, and packaging. Large quantities of freshwater are used, and 3–4 m^3 of table olive processing wastewater (TOPW) per ton of olives may be produced [1]. TOPW is a turbid effluent with a characteristic odor and high organic content, comprising sugars, acids, phenols, polyphenols, tannins, pectins, and oil residues [2,3]. Furthermore, TOPW possesses antimicrobial and phytotoxic properties [4], mainly due to its polyphenolic compounds content, wide pH variation (3.5–13.0), and high concentrations of sodium hydroxide (lye) and/or sodium chloride (brine) [2,3].

Biological wastewater treatment is likely the most widely used wastewater treatment technology [5]; nonetheless, the specific physicochemical characteristics of TOPW render its treatment uncertain through common biological processes [6,7]. Membrane bioreactor (MBR) technology, which combines the biological degradation process of activated sludge with a direct solid-liquid separation through membrane filtration [8], has inherent advantages, including the complete retention of suspended biomass, and the growth of a widely diversified bio-community that enhances the breakdown, and eventual assimilation and mineralization, of difficult to biodegrade organic components (xenobiotics, phenolic compounds, etc.) [8]. Patsios et al. [9] demonstrated in long-term experiments (six months), that MBR technology, after appropriate active biomass acclimatization, could successfully be employed for TOPW treatment, exhibiting stable performance with high percentages of total organic carbon (TOC) and total polyphenols (TPh) removal.

The inoculation and acclimatization of the suspended biomass during the start-up period of a MBR is an aspect that critically influences its performance, as in all such biological-based wastewater treatment processes [10,11]. Considering the specific characteristics of TOPW, a prolonged acclimatization period for the biomass community may be required to achieve satisfactory biological treatment [6]. TOPW may either introduce new microorganism species in the MBR or can exert a selective pressure on the bio-community leading to the supremacy of those species able to grow under the specific physicochemical conditions. Specific microorganism species have been identified as capable to metabolize the toxic polyphenolic compounds under such harsh conditions [12].

Studies on the acclimatization period of MBR systems have recognized the significance of advanced experimental techniques to gain useful insights into the phenomena involved [10] by analyzing the bio-community dynamics. In recent years, several studies have exploited next-generation, high-throughput, sequencing technologies (NGS) for phylogenetic and functional analysis of microbial bio-communities in environmental samples. Mainly, three NGS approaches are used to characterize microbial communities: (i) meta-barcoding or targeted amplicon sequencing, (ii) whole metagenome DNA sequencing, and (iii) whole metatranscriptome sequencing [13]. Activated sludge has been used as a specimen for developing novel analytical methods and data-processing techniques in metagenomic of complex samples [14–16]. Moreover, novel genes and pathways involved in the aerobic and anaerobic degradation of phenol, aromatic compounds, and phosphorus removal have been identified and evaluated in activated sludge samples [17,18]. Metagenomic analysis has been also suggested as a monitoring tool for activated sludge biodegradation capability and efficiency [19], for activated sludge foaming incidents [20], for the diversity of nitrogen-metabolizing microorganisms [21–23], and for assessing shifts in the microbial community during adaptation of anaerobic granular sludge from mesophilic to thermophilic conditions [24].

The present study addresses the acclimatization period of an aerobic pilot scale MBR treating TOPW, under well-defined operating conditions. The adaptation of the microbial community, over a period of three months, is interrelated to changes in the bio-community structure using meta-barcoding and metatranscriptomic analysis approaches which enable a thorough monitoring of microbial dynamics. Samples were collected and characterized through NGS analysis at four different points in time, namely at the beginning (Day0), the peak of acclimatization (Day16) and two later dates (Day58, Day87). Throughout the adaptation period, specific operating parameters were monitored to assess the overall MBR performance. The diversity trends in the microbial bio-community composition, the metabolic pathways of the expressed bio-community genes, and the specific bacterial organisms that dominantly contribute to successful adaptation and utilization of the TOPW nutrient resources are identified.

2. Materials and Methods

2.1. Experimental Setup

A laboratory scale aerobic, submerged type MBR system, which closely simulates the filtration operation of full-scale MBR through, constant flux operation with scheduled periodic backwashing,

was designed and constructed (Figure 1). The MBR tank has an effective volume of 20 L, in which a hollow fiber custom-made membrane module is submerged. The membranes, supplied by ZENON Environmental Inc., Oakville, Canada, are made of hydrophilized polyvinylidenefluoride. Their nominal pore size is 0.04 μm and the membrane module has an effective filtration area of 0.174 m^2. A coarse air bubble diffuser is installed under the membrane module to supply oxygen to biomass and to provide agitation hindering the deposition of bio-flocs on the membrane surface; the air flow rate is approx. 8 Ndm^3/min, and the mean dissolved oxygen concentration is significantly higher than 2.0 mg/L, to ensure aerobic conditions.
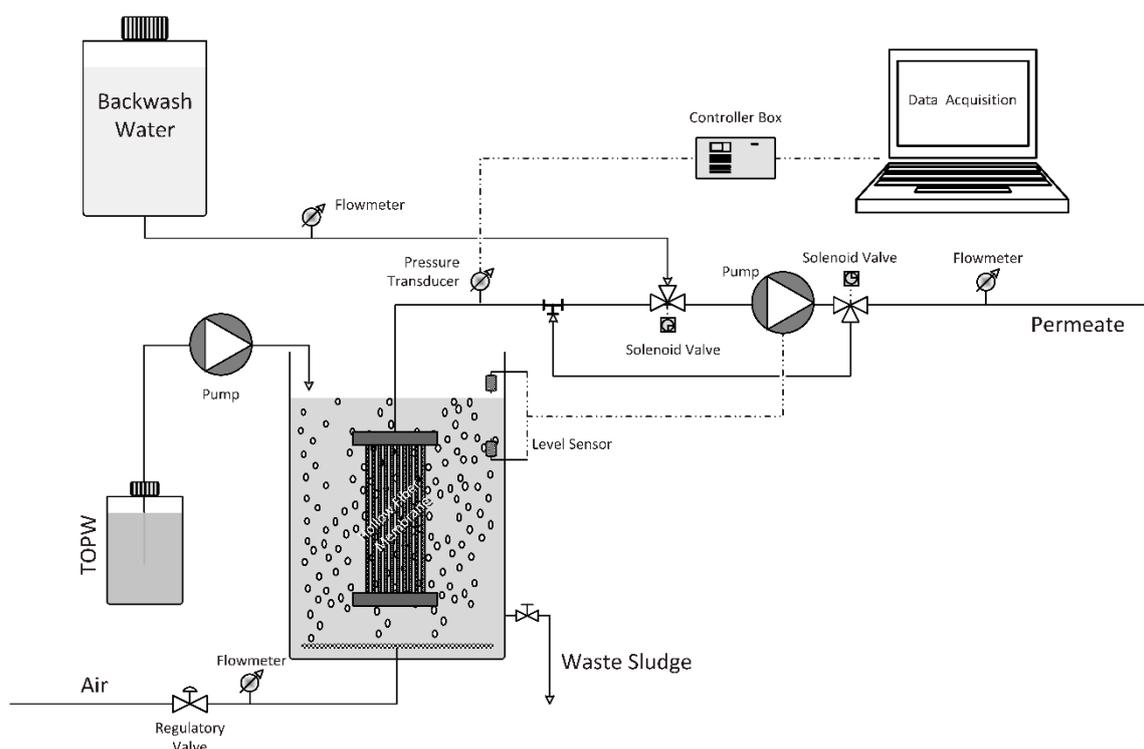

Figure 1. Schematic diagram of the laboratory scale submerged MBR system.

The wastewater is fed to the MBR through a piston pump (Fluid Metering Inc., Syosset, New York, USA). A pH-meter with integrated temperature sensor (713 type pH-meter, Metrohm Ltd., Herisau, Switzerland) is used to monitor the pH and temperature of the MBR. A piston pump (Fluid Metering Inc., Syosset, New York, USA) is employed for constant flux operation, whereas a pressure transducer (Tecsis GmbH, Offenbach, Germany), is connected at the outlet of the membrane module to monitor the Trans-Membrane Pressure (TMP) as an indicator of membrane fouling. A typical backwashing protocol of 3 min filtration followed by 1 min backwashing with tap water, at the same backwashing flux as the filtration mode, is applied throughout the start-up period. Computer-based data acquisition hardware (PCL-818L, Advantech Co., Taipei, Taiwan), and the software GeniDAQ 4.25 (Advantech Co., Taipei, Taiwan) have been used for monitoring the system. Two level sensors activate the permeate pump on and off in order to keep constant the level of the bioreactor at 10 ± 0.5 L.

2.2. Analytical Methods

The pH of the feed and the permeate were monitored on an almost daily basis. The TOC of the feed and permeate were measured every 2–3 days by a TOC analyzer (TOC-5000A, Shimadzu Co., Kyoto, Japan). Mixed liquor suspended solids (MLSS) and mixed liquor fixed suspended solids (MLFSS) were measured by filtration on a Whatman GF/A microfiber glass filter (1.6 μm pore size) according to APHA Standard Methods [25]. TPh were measured photometrically after the appropriate sample dilution, according to a modified Folin–Ciocalteu method at 750 nm [26]. The results were expressed as

mg/L equivalent of oleuropein, the main olive fruit polyphenol. The TMP was continuously monitored and recorded to assess membrane filtration performance.

2.3. DNA Extraction, Library Construction, and Amplicon Sequencing

Twenty-five milliliters of four activated sludge sample (Day0, Day16, Day58, and Day87) were mixed with an equal volume of 50% glycerol and conserved at $-80\,^{\circ}$C until analysis. Microbial DNA was extracted from each sample using 1 mL of mixed liquor with ZR Soil Microbe DNA MicroPrep (ZYMO RESEARCH; Irvine, CA, USA) according to the manufacturer's instructions. DNA concentration was measured on a Qubit 2.0 Fluorimeter using the Qubit® dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA) and its integrity was evaluated by electrophoresis on a 0.8% agarose gel. For the amplification of the 16S rRNA gene, libraries were constructed by amplifying the V3–V4 region (~460 bp), with gene-specific primers D-Bact-0341-b-S-17 and D-Bact-0008-a-S-16 selected from Klindworth et al. [27], whereas for the amplification of the SSU of the 18S rRNA gene, universal primers FR1 and FF390 were selected from Chemidlin Prevost-Boure et al. [28]. All primers were modified by adding an Illumina (Illumina Inc, San Diego, CA, USA) overhang adapter nucleotide sequence at the 5′ end. The sequences of the primers used were 16S_F: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′, 16S_R: 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′, 18S_F: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CGA TAA CGA ACG AGA CCT-3′ and 18S_R: 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GAN CCA TTC AAT CGG TAN T-3′. All libraries were constructed as described in the Illumina's 16S Metagenomic Sequencing Library Preparation (15044223 B) protocol. PCR products were purified to remove unincorporated primers and primer-dimer species using NucleoMag® NGS Bead Suspension (Macherey-Nagel, Düren Germany). PCR reactions were performed on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany). All libraries were quantified with fluorometric quantification using Qubit® dsDNA BR assay kit and their molarity was calculated in relation to the size of DNA amplicons after indexing. Quantitative PCR (qPCR) was performed with the KAPA Library Quantification kit for Illumina sequencing platforms (KAPA BIOSYSTEMS, Woburn, MA, USA). Libraries were sequenced in a MiSeq platform using MiSeq® reagent kit v3 (2 × 300 cycles) at 4 pM (Illumina Inc, San Diego, CA, USA). All sequences have been submitted to the European Nucleotide Archive database under study PRJEB27589 (sample accession ERR2681858, ERR2681859, ERR2681860, ERR2681861, ERR2681938, ERR2681939, ERR2681940, ERR2681941).

2.4. Sample Preparation, RNA Extraction, and Metatranscriptome Sequencing

One milliliter from activated sludge samples Day16, Day58, and Day87 was centrifuged at $13{,}000\times g$ for 2 min. The pellet was weighted, re-suspended in 1 mL of TriZol (Invitrogen, Carlsbad, California, USA) and total RNA was extracted from each sample according to manufacturer's instructions. RNA quantity was measured on a Qubit 2.0 Fluorimeter using the Qubit™ RNA BR assay kit (Invitrogen, Carlsbad, California, USA) and its integrity was evaluated by electrophoresis on a 0.8% agarose gel. RNA libraries were constructed using 1 μg total RNA as starting material from each time point. In brief, first strand cDNA was synthesized with random hexamers using SuperScript II-RT (Invitrogen, Carlsbad, California, USA) and second strand cDNA was accomplished with the NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs, Ipswich, Massachusetts, USA) following the manufacturer's instructions. Samples were further purified with NucleoSpin Gel and a PCR Clean-up Kit (Macherey-Nagel, Düren Germany). Purified second-stranded cDNAs were further used for metatranscriptome library construction using the Nextera DNA Library Preparation Kit (Illumina Inc, San Diego, CA, USA) with an average insert size of 250 bp. Library quantification was performed in the same manner as described above for amplicon meta-barcoding. Libraries were sequenced on a MiSeq platform (Illumina Inc, San Diego, CA, USA) using the MiSeq Reagent Kit v2 (2 × 150 cycles), according to the manufacturer's instructions. All sequences have been submitted to

the European Nucleotide Archive database under study PRJEB27589 (sample accession ERR2681943, ERR2681944, ERR2681945).

2.5. Data Processing and Bioinformatics Analysis

2.5.1. 16S and 18S Amplicon Meta-Barcoding

For prokaryotes, raw reads (*fastq* files) were quality-trimmed using Trim Galore with default settings [29]. Further analysis was conducted using Quantitative Insights into Microbial Ecology (QIIME) pipeline [30]. In brief, paired-end reads were joined using the fastq-join method (join_paired_ends.py) and demultiplexing of libraries was performed with split_libraries_fastq.py script. OTU picking was executed with pick_open_reference_otus.py script with the default clustering algorithm UCLUST [31]. Sequences were clustered into operational taxonomic units (OTUs) with 99% sequence similarity and aligned to SILVA 128 release for QIIME.

Eukaryotes were analyzed using the SOP provided by Kozich et al. [32] for microbiota processing in mothur [33]. Briefly, paired-end reads were joined and trimmed from adaptors and indices. The remaining sequences were checked for chimeric sequences using the chimera.uchime command. Sequences outside the range of 150–450 bp were removed and the remaining were aligned to SILVA database (release 123) for taxonomic assignment into OTUs, with 99% sequence identity.

2.5.2. Metatranscriptomics

The overall bioinformatics strategy included the following steps: (i) Trim and clean-up of the sequencing reads using the trim galore wrapper with default parameters, except for the –length 40 and the –fastqc options, so as to remove adaptors and low-quality sequences; (ii) stitching of the pair-end reads using the Fast Length Adjustment of SHort reads (FLASH) with default options except for -M 210; (iii) extraction of the open reading frames (ORFs). HMMER2GO [34] was used to extract ORFs and assign to Pfam (Pfam Version 31) using the default hmmer2go pipeline; (iv) search for similarities in the NCBI protein Non-Redundant (NR) database. From the NR database, downloaded on 17/11/2017, all the eukaryotic and bacteria protein sequences were extracted and used to create two distinct formatted databases for diamond blast [35]. Diamond blast (version 8.22) was used (parameters –outfmt 100 -p 20 -k 2 -e 10e-10) to find similarities of all the ORFs from the three samples (Day16, Day57, and Day84), in the entire NR, as well as separately in the bacteria and eukaryotic protein database. A total of 3,064,452 ORFs were used for diamond blastp queries from the three samples; (v) Gene Ontologies (GO) were assigned using the MEtaGenome Analyzer (MEGAN, community version 6.10.8) [36]. All analyses were implemented on a Linux-based HPC cluster assigning one node with 32 cores and 256 GB RAM.

2.5.3. Data Visualization

For amplicon meta-barcoding, OTU tables and *biom* files generated by QIIME and mothur were imported in R version 3.4.2 [37], to further process and visualize results. OTU counts and taxonomic assignments were merged to a phyloseq object with phyloseq R package [38] and analyzed using the ampvis2 R package [39]. All plots were visualized by combining functions provided by the ggplot2 R package [40]. Since the initial number of sequences obtained is unlikely to correspond to the true biomass fraction of each sample, all bar plots were normalized to 100% as abundance estimations within each sample. For metatranscriptomics, MEGAN was used to import diamond blast output and assign GO. Further analysis and plots were also performed using R.

2.6. MBR Acclimatization Procedure

The bioreactor was initially filled with activated sludge, sampled from the neighboring municipal wastewater treatment plant (WWTP) of Thermi/Thessaloniki, Greece. This WWTP is a conventional extended-aeration WWTP for secondary treatment of municipal wastewater and municipal sewage wastes. It has a capacity of approx. 13,000 equivalent population (e.p.) and the average feed organic

load is approx. 1000 Kg BOD_5/d. No industrial wastewaters flow into this WWTP. The activated sludge was withdrawn from the activated sludge recycle line after the secondary clarifier and was immediately transported and used as an inoculum for the MBR. The MLSS of the return activated sludge was 27,730 mg/L with pH = 7.7.

The MBR system operated for approx. three months in a semi-batch mode with 1 L of daily feed. A concentrated synthetic wastewater (CSW) recipe (Table 1) simulating domestic sewage [41] was slightly modified by including brown sugar, as a source of carbohydrates, and $NaHCO_3$ for pH regulation at slightly alkaline range. The measured TOC concentration of the CSW was 33,530 mg/L and its pH was 7.3. During the first six days, specific volumes (e.g., 30, 60, 120, etc.) of the CSW were diluted each day to a final volume of 1 L, which was used as the daily feed to the MBR system. Subsequently, a portion of the CSW was replaced by real TOPW, from a table olive processing plant located in Chalkidiki, Northern Greece; the final feed volume remained steady at 1 L per day. The ratio of the TOPW in the mixture of CSW and TOPW gradually increased from approx. 30% *v/v* at Day7 to approx. 95% *v/v* at Day44; thereafter, TOPW was the only daily feed to the MBR system.

Table 1. Composition of the CSW used in this study.

Component	Concentration (*g/L*)
Crude Sugar	75.0
Peptone	8.00
Meat Extract	5.50
Urea	1.50
$NaHCO_3$	10.5
K_2HPO_4	1.40
NaCl	0.35
$CaCl_2 \cdot 2H_2O$	0.20
$Mg_2SO_4 \cdot 7H_2O$	0.10

Permeate was withdrawn with a steady flux of 10 L/(m^2·h) when the level in the bioreactor exceeded approx. 10.5 L. The permeate pump operated for less than one hour per day until the level in the bioreactor dropped to less than 9.5 L. No excess activated sludge was withdrawn throughout the first 42 days of MBR operation; afterwards 100 mL of excess activated sludge was removed daily; this wasted sludge quantity corresponds to a solids retention time of 100 d.

3. Results

3.1. MBR Performance Efficiency during Acclimatization

The overall biomass concentration was estimated based on the mixed liquor volatile suspended solids (MLVSS) fraction, a safe indicator of the active biomass in wastewater treatment plants [5]. The initial MLVSS and MLFSS concentration of the MBR was approx. 6.0 g/L and 5.0 g/L, respectively. The temporal evolution of the MLVSS and MLFSS concentration during the operating period is shown in Figure 2. MLVSS varied between 5.2 g/L and 12.0 g/L when no excess sludge was removed (until the 42nd day); subsequently the MLVSS concentration stabilized at approx. 8.0 g/L.

MLFSS exhibited a more stable course as their concentration, quickly stabilized at 2.0–3.0 g/L (Figure 2). The increase of the MLVSS denotes that the bio-community shortly after the start-up started to biodegrade the wastewater organic compounds and multiply, producing new biomass. The pH values of the feed and the mixed liquor remained around neutral values (Figure 2). Interestingly, the mixed liquor, after a short drop of its pH value (around Day10) shifted towards mild alkaline conditions between 8.0 and 8.8, exhibiting a very stable and self-controlled behavior. Mixed liquor pH mean value after day 10 was 8.5, with a standard deviation of 0.22.
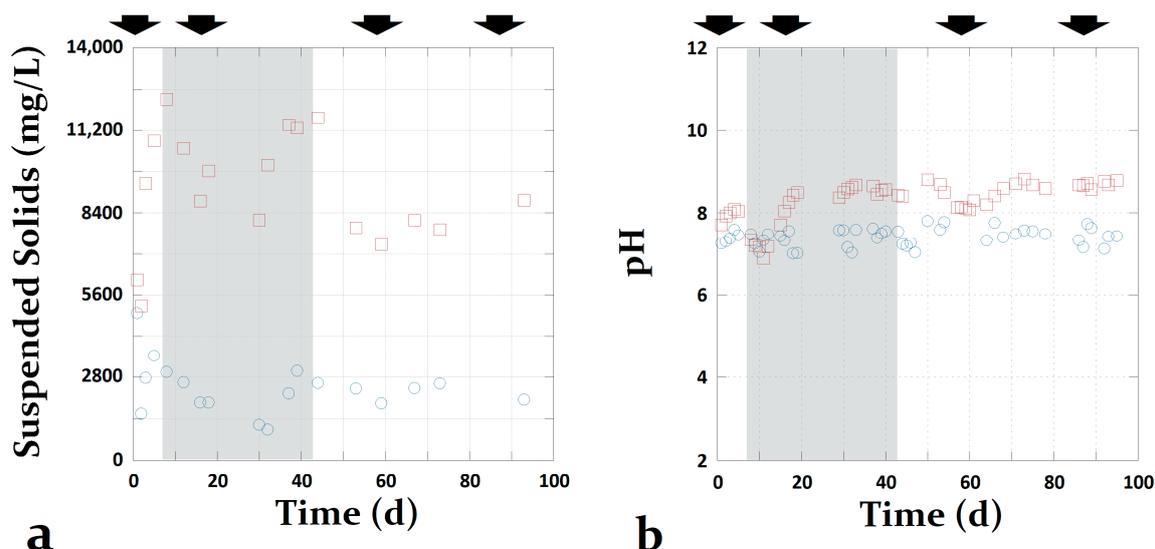
Figure 2. Temporal variation of mixed liquor characteristics. Plots of (**a**) □ MLVSS, and ○ MLFSS; and (**b**) pH values of the ○ feed, and □ mixed liquor during MBR acclimatization; the arrows indicate sample collection time points for metagenomic analysis; the shaded area indicates the time period when both CSW and TOPW were fed to the MBR, CSW, and TOPW were solely fed before and after this period, respectively.

The TOC concentration of the feed and permeate are shown in Figure 3. Upon inspection, it is obvious that the physicochemical characteristics of the TOPW are not stable; in fact, there are significant fluctuations regarding organic matter (Figure 3) and polyphenolic compounds concentrations (Figure 3). The TOC concentration of the feed fluctuates widely in a range between 500 to 9000 mg/L with a mean value of 3386 mg/L and a high standard deviation of 1897 mg/L, as expected from real wastewater samples. In contrast, the permeate TOC concentration is comparatively more stable. Lower TOC permeate concentration and high fluctuations are observed during the first 20 days of the acclimatization period (49.9 ± 35.1 mg/L), when CSW was initially used as a feed to the MBR (Days 0–8), whereas a somehow higher, but less variable, TOC concentration is observed between Days 21 and 100 (144.7 ± 38.2 mg/L). Polyphenolic compounds concentration (Figure 3), follows a similar pattern; TPh concentration in the MBR feed varies considerably between 120 and 901 mg/L, while TPh concentration in the MBR permeate has a mean value of 39.7 ± 9.6 mg/L. No polyphenolic compounds are measured during the first seven days when no TOPW was used as feed. Adsorption and bioaccumulation of the polyphenolic compounds in the suspended bio-flocs, as well as biodegradation by specific microorganisms are two possible mechanisms of polyphenolic compounds removal.

To quantitatively assess the MBR performance in TOC and TPh removal efficiency, the probability plot of the removal ratios of the TOC and TPh during the MBR operation is presented (Figure 4). The TOC removal ratio was greater than 0.85 in all the examined samples, 90% of the TOC removal ratios were over 0.90, and half of the calculated TOC ratios were greater than 0.96. TPh removal ratios were also quite high, though a little lower than those of TOC. The lowest TPh removal ratio was approx. 0.80, whereas 90% of TPh removal ratios were higher than 0.85, and half of TPh removal ratios were over 0.90. Therefore, it is evident that the MBR system exhibited excellent removal performance regarding TOC and polyphenolic compounds.
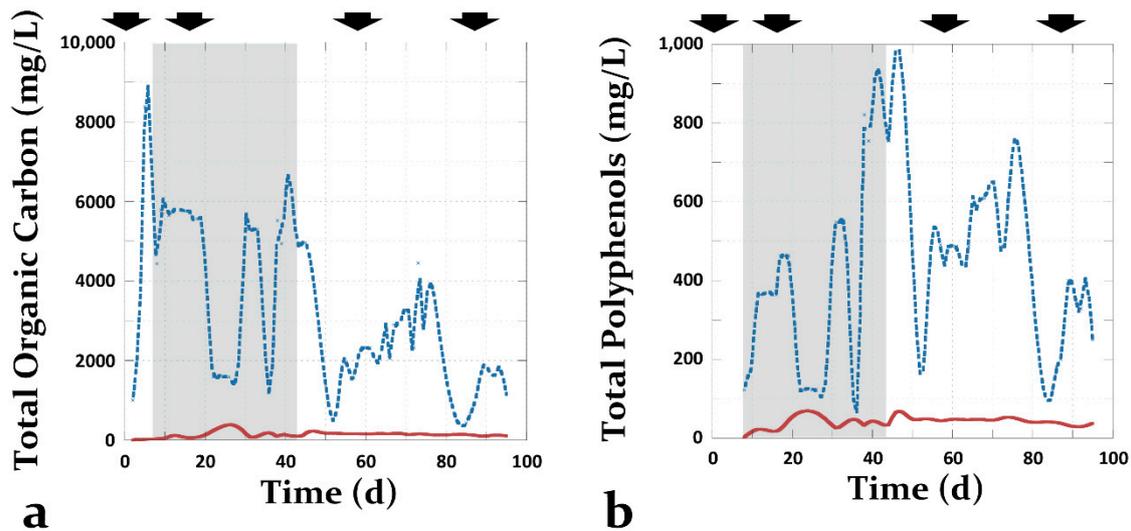
Figure 3. Temporal variation of feed and permeate characteristics. Plots of (**a**) TOC concentration; and (**b**) TPh concentration of the feed (discontinuous line), and the permeate (continuous line) during MBR acclimatization period; the arrows indicate sample collection time points for metagenomic analysis; the shaded area indicates the time period when both CSW and TOPW were fed to the MBR, CSW and TOPW were solely fed before and after this period, respectively.
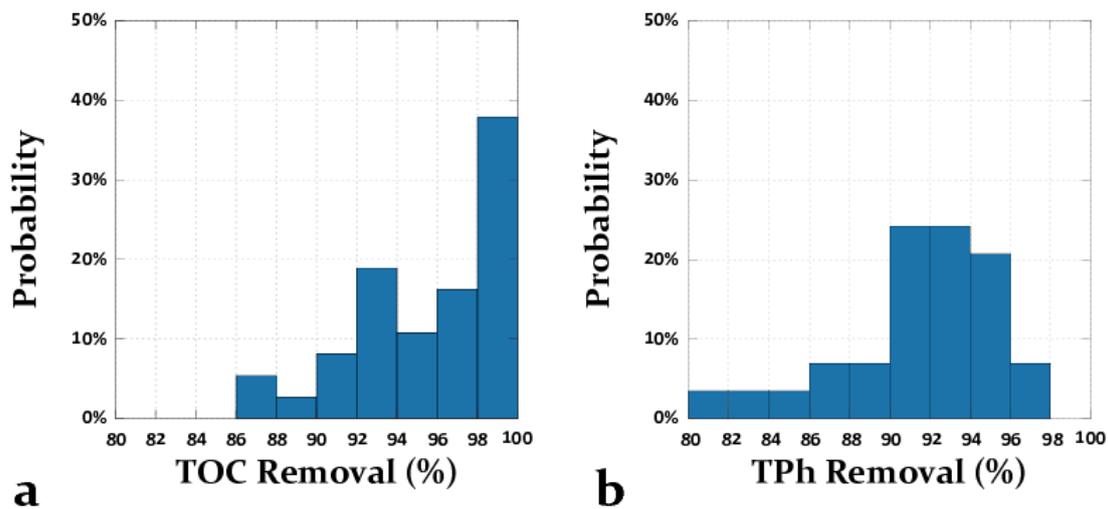

Figure 4. Probability plots of feed and permeate characteristics. Plots of (**a**) the TOC; and (**b**) the TPh removal efficiency during MBR acclimatization period.

To assess the long-term filtration operation during the acclimatization period, the temperature-corrected membrane permeability at the reference temperature of 20 °C was calculated based on the TMP data (Figure 5). A sharp drop of membrane permeability can be observed during the first 20 days of MBR operation. Afterwards, membrane permeability seems to stabilize around 200 L/(m²·h·bar) for approx. 20–25 days and it is followed by another drop at a level of approx. 175 L/(m²·h·bar) until day 80. In the last 20 days of operation the membrane permeability gradually increased to 200 L/(m²·h·bar). Overall, the membrane performance is satisfactory exhibiting long-term stable filtration operation despite some moderate fluctuations.
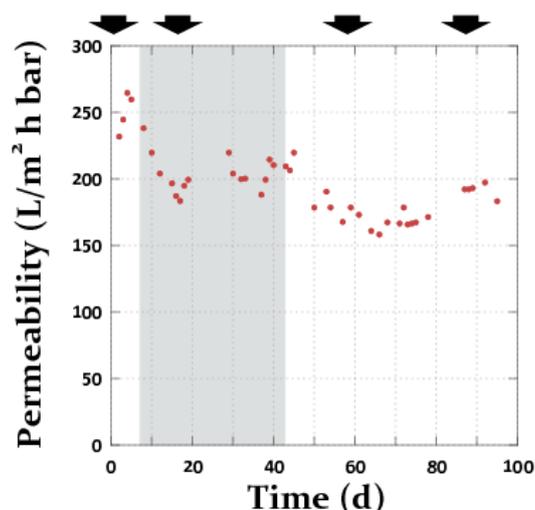
Figure 5. Membrane temperature corrected (20 °C) permeability. Data obtained during MBR acclimatization period; the arrows indicate sample collection time points for metagenomic analysis; the shaded area indicates the time period when both CSW and TOPW were fed to the MBR, CSW, and TOPW were solely fed before and after this period, respectively.

3.2. 16S and 18S Amplicon Meta-Barcoding

To assess the microbial community dynamics during acclimatization and operation, meta-barcoding analysis was performed for both prokaryotic and eukaryotic organisms, using the 16S rRNA and 18S rRNA genes, respectively, as taxonomic identification markers. Sequencing of the 16S rRNA gene resulted in 126,360, 140,229, 207,649 and 213,719 filtered reads for Day0, Day16, Day58 and Day87 time points, respectively (Table 2). For eukaryotes, the respective numbers were 147,664, 182,962, 169,154 and 151,733. In total, 39,108 and 3471 unique OTUs were obtained for bacteria and eukaryotes, respectively. The total number of identified bacterial OTUs per sample ranged from 12,597 (Day0) to 16,755 (Day87) (Table 2). However, the significant OTUs exceeding 0.1% in abundance in each sample time point were significantly less (Day0: 95 OTUs, Day16: 96 OTUs, Day58: 107 OTUs and Day87: 119 OTUs).

Table 2. Number of raw reads, sequences after operational taxonomic unit (OTU) picking and identified OTUs, for each timepoint, identified for 16S rRNA and 18S rRNA amplicon sequencing.

Amplicon	Sample	Raw Reads	Number of Sequencesafter OTU Picking	No OTUs
16S	Day0	221,672	126,360	12,597
	Day16	218,040	140,229	13,890
	Day58	290,839	207,649	15,357
	Day87	291,227	213,719	16,755
18S	Day0	221,664	147,664	1472
	Day16	269,715	182,962	876
	Day58	255,537	169,154	1038
	Day87	213,397	151,733	673

Comparisons of the shared organisms between samples identified a rather swift transition from the active sludge population at Day0 to an alternative core microbial community present at later stages of MBR function. The percentage of shared OTUs between Day0 and Day16 was 10.4%, which further diminished to 1% when compared to Day58 and Day87, whereas 44% of OTUs were common in the later samples. The active sludge inoculum (Day0) was dominated by *Proteobacteria* (68%) and *Firmicutes* (11%). *Actinobacteria* (6%), *Chloroflexi* (4%), *Bacteroidetes* (3%), and *Planctomycetes* were the minor phyla of the community (Figure 6). *Alphaproteobacteria* consisted of three OTUs of the order

Rhizobiales which amounted to 42% of the total OTUs of Day0. One of them, a *Methylocystis* spp., is a known methanotroph [42]; its existence is attributed to the fact that the initial inoculum comes from a WWTP that treats sewage wastes (sludge) from municipal septic tanks where anaerobic conditions and methane formation prevails. These three OTUs were effectively eliminated from the later samples. *Betaproteobacteria*, comprising 18% of the Day0 sample were dominated (>99%) by a single genus, *Dechloromonas* spp., a facultative aerobic, nitrate-reducing organism frequently encountered in WWT plants with a capacity to accumulate polyphosphate [43] (Figure 6). Its presence diminished in the MBR while another member of the *Rhodocyclaceae* family, *Thauera* spp. present initially in scarce numbers, increased in abundance displacing *Dechloromonas* spp. (Figure 6). *Firmicutes*, totaling 11% of Day0 active sludge, consisted of a single *Trichococcus* spp., a filamentous facultative anaerobe, fermenting readily metabolizable substrates. Its population also declined to zero in the MBR (Day58, Day87), where aerobic conditions prevail (Figure 6).
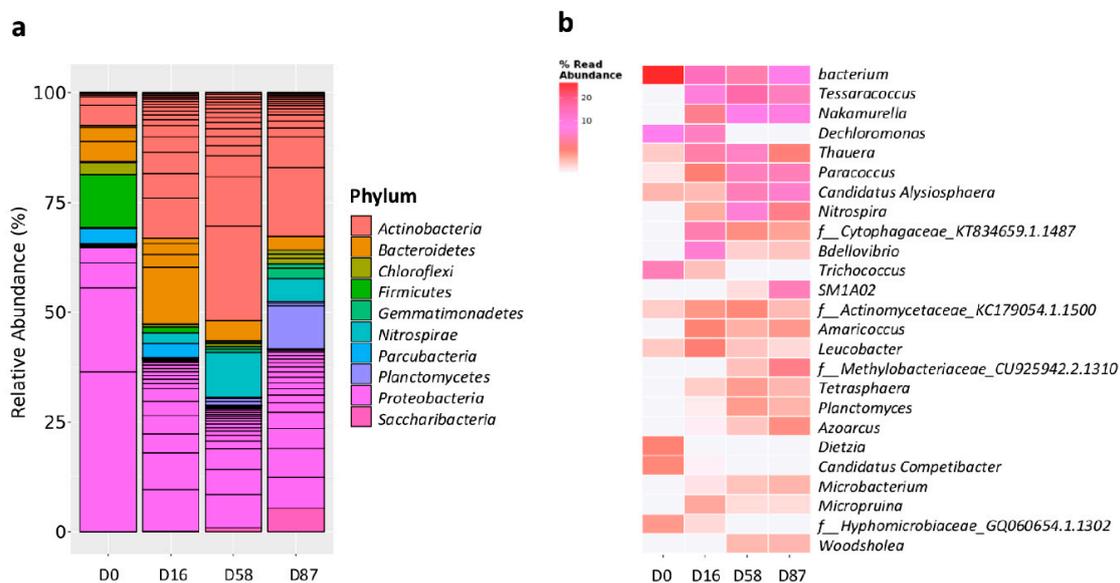

Figure 6. Metagenomic analysis of prokaryotic community. Distribution of (**a**) the major bacterial phyla, identified at four timepoints during MBR operation. The scale in the y axis reflects the normalized relative abundance percentages (%). Black lines within each bar separates each Phylum into lower taxonomic levels and (**b**) the relative abundances of the top 25 bacterial genera, identified in the four timepoints visualized through heatmap.

Actinobacteria increased in the MBR to a significant percentage and number of species. The main genus at Day0, *Dietzia*, was replaced by several OTUs. At Day16, seven major actinobacterial OTUs were identified collectively amounting to 28% of the microbial community. Two *Tessaracoccus* spp. accounted for one third of them. Members of the genus have been associated with enhanced biological phosphate removal and accumulate polyphosphate granules [44]. Another actinobacterial species which became dominant in the MBR was *Nakamurella* spp. (Figure 6). It can utilize numerous sugars and amino acids as carbon sources and accumulates glycogen possibly competing with phosphate accumulating bacteria.

Another introduced organism from the TOPW feed which likely affects the community balance was *Bdellovibrio* spp., a *Deltaproteobacteria* organism parasitizing on other Gram-negative bacteria, which could explain the decrease in their abundance in the MBR [45]. The nitrate reducing organisms *Paracoccus* spp. and *Thauera* spp. which increased in abundance were complemented with the nitrite oxidizing bacterium (NOB) *Nitrospira* spp. absent from Day0, but present at significant levels subsequently. *Nitrospira* is a globally distributed genus and represents the most diverse known group of NOB. Aside from the capacity to use nitrite as energy source, certain species of *Nitrospira* possess the capacity for complete ammonia oxidation [46].

Analysis of the eukaryotic load in the MBR community by 18S rRNA identified three major phyla, *Opisthokonta*, *SAR*, and *Archaeplastida*, accounting for most organisms and a pool of unclassified organisms ranging from 11% at Day0 to single digit percentages for the other time points (Figure 7). The main phylum at Day0, *Opisthokonta*, includes the true fungi and their protist relatives, as well as the animals and their protist relatives [47]. At Day0 sludge sample, 73% of the reads are *Opisthokonta*, and they remain substantial at later time points (Day16 = 45%, Day58 = 32%, Day87 = 57%) (Figure 7). True fungi were 47% of all reads at Day0, and their abundance decreases at later samples but remains at high levels (Day16 = 27%, Day58 = 11%, Day86 = 19%). *Ascomycota* (20%), *Basidiomycota* (15%), and *LKM11* (9%) a *Cryptomycota*, are the dominant genera (Figure 7). During MBR acclimatization *Ascomycota* and *Basidiomycota* diminish in abundance (OTUs 00008, 00013, 00015, 00016, 00019, 00021) and a single OTU from each, remains in significant numbers. In the *LKM11* clade, the single dominant OTU00009 at Day0 decreased dramatically, while a second OTU00004 absent at Day0 expanded to account for 18% of the total reads at Day16 and subsequently stabilizing at lower levels. The metazoan members of *Opisthokonta* belong to genus *Bilateria*, with a single OTU00003 accounting for 99% of the genus. It is present in all samples in stable abundance (12–19%).
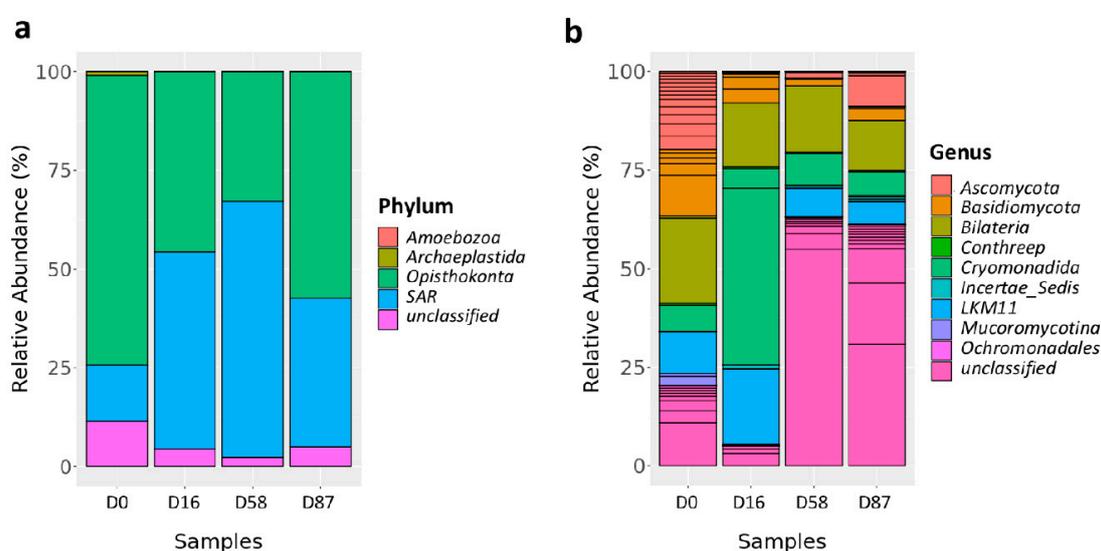

Figure 7. Metagenomic analysis of eukaryotic community. Distribution of the identified eukaryotes at the (**a**) phylum and (**b**) genus level, detected in four different timepoints. The scale in the y axis reflects the normalized relative abundance percentages (%). Black lines within each bar separates each Genus into lower taxonomic levels.

The phylum *SAR* clusters taxonomically three clades, *Stramenopiles*, *Alveolates*, and *Rhizaria* [47]. The *SAR* phylum expanded from 14% of the reads at Day0 to 50% at Day16, 64% at Day58 and 37% at Day87. *Rhizaria* account for 99% of *SAR* organisms. They are a group composed of numerous unicellular species mostly amoeboid in appearance. All OTUs were *Cercozoa*. Two OTUs dominate, a *Cryomonadida* genus present in all samples, with a peak at Day16 (Day0 = 6%, Day16 = 48%, Day58 = 8%, Day87 = 9%) and an unclassified genus absent at Day0 which expanded to 56% at Day58 and 31% at Day87.

3.3. Metatranscriptomics

To assess the dynamics of gene expression in the MBR microbial community, a metatranscriptomic analysis was performed for samples Day16, Day58, and Day87. Three metatranscriptomic libraries were prepared using total RNA extracted from the different time points, yielding 2,348,352, 3,786,095 and 2,835,755 paired-end sequences, for Day16, Day58, and Day87, respectively. A total of 3,064,452 ORFs, extracted from the stitched paired-end sequences, from the three samples were searched and

assigned to protein families, gene ontologies and metabolic processes using the PFAM, GO and SEED database, respectively. Proteins of prokaryotic and eukaryotic origin involved in amino acid derivatives, carbohydrate metabolism, and protein metabolism were in the top five most frequent families (Figure 8). The relative abundance in each of the metabolic categories remains constant between all three samples, indicating possibly a stable microbial community throughout the MBR operation period. A small increase is only noted for genes belonging to the category of fatty acids, lipids, and isoprenoids which increased from 3.34% at Day16, to 3.95% at Day58 and 4.33% at Day87, indicating increased metabolic activity towards TOPW lipids. The genes present in carbon metabolism and fatty acid degradation do not vary much in the three examined timepoints, though there are some indications that some enzymes may be differentially expressed. One such is alcohol dehydrogenase which is underrepresented at Day16. However, a great deal more sequencing depth is necessary to assess gene expression differences confidently.
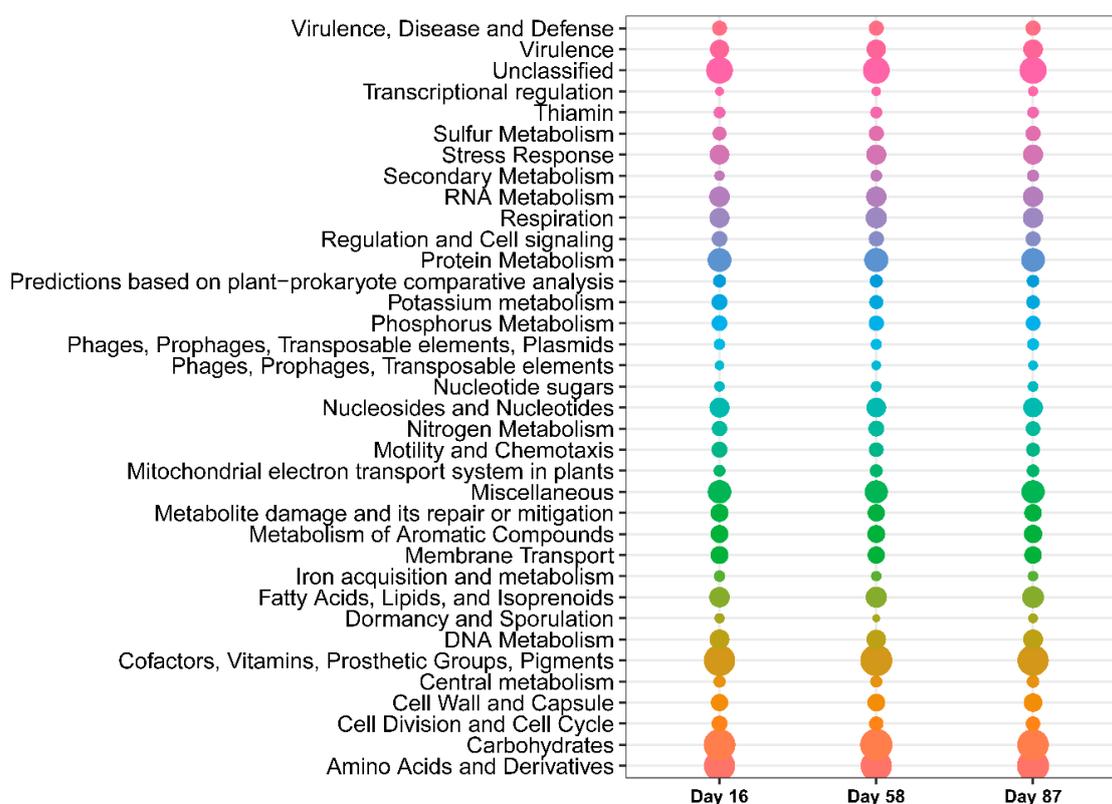

Figure 8. Dynamics of gene expression in the MBR microbial community. Annotation of the metabolic processes of the Day16, Day58, and Day87 samples based on the SEED database for both prokaryotes and eukaryotes.

3.4. Phylogenetic Composition of Metatranscriptomic Data

Community taxonomic analysis of the metatranscriptomic data was performed using MEGAN. Phylogenetic analysis of the expressed genes, which is an indicator of organismal metabolic activity, classified 82% of them in prokaryotes, as expected, and the rest of eukaryotic origin. Very few species dominated the expression frequency and numerous others maintained low levels of expression between 1% and 2% (Figure 9). *Thauera* sp. comprised almost half of expressed genes at Day16 and remained very high thereafter (33% at Day58 and 15% at Day87). The second most abundant expression was at 11% in a *Pseudoxanthomonas* sp. at Day16 which subsequently decreased to 1% and a mixed group of environmental samples-bacteria at 11% at Day58; both *Thauera* and *Pseudoxanthomonas* sp. have been identified as capable of aromatic degradation metabolism [48,49]. *Paracoccus* sp. is another versatile biodegrading organism increased in expression levels (3% at Day16, 5% at Day58 and 8% at Day87).

Nitrospira sp. is expressed at low but stable levels at all three time points, as well. *Nakamurella* sp. which was very abundant by 16S rRNA is expressed only at low levels (Figure 9).
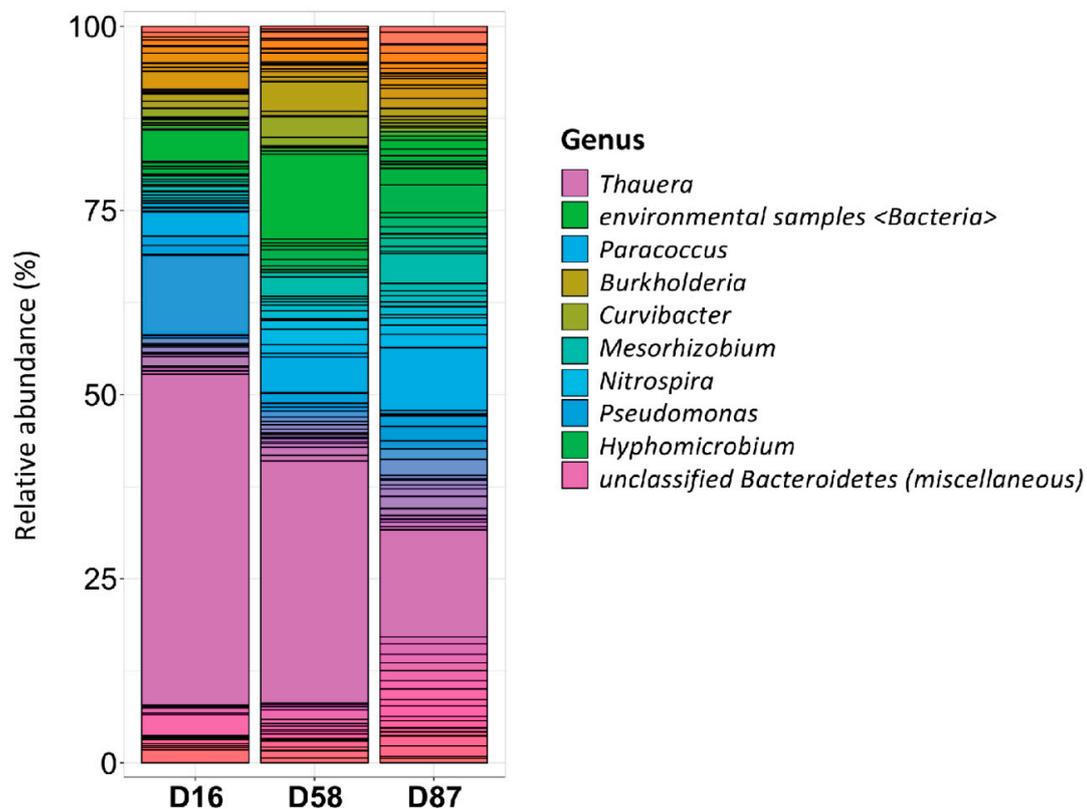

Figure 9. Prokaryotic community taxonomic analysis of the metatranscriptomic data. Distribution of the top identified bacterial organisms at genus level, identified in the three time points for the metatranscriptome analysis. The scale in the y axis reflects the normalized relative abundance percentages (%). Black lines within each bar separates each genus into different species.

4. Discussion

From the aforementioned results, it becomes evident that, upon introduction of TOPW, a drastic shift in the prokaryotes and eukaryotes present takes place; the organisms naturally present in TOPW and its specific chemical composition seem to exert a major pressure on the bio-community dynamics [50]. Such kind of shifts in wastewater treatment plants have already been described for other environmental factors, such as temperature [24] or salinity [51].

The number of OTUs identified in all samples is very large (39,108) and the significant bacterial OTUs present above 0.1% in abundance increase in the acclimatized MBR (Day0 = 95, Day87 = 119) indicating a strong diverse microbial biodegrading community. All major OTUs present in the samples from the latter part of the operating period are present in the Day16 sample, while the dominant OTUs present in active sludge disappear or drastically decrease. The acclimatization process of the MBR is significantly faster than it was hypothesized. The increase of the bioreactor pH value, together with the increase of the MLVSS, are indicators of a successful acclimatization of the microbial bio-community in the MBR and the biodegradation of organic matter present in the wastewater. The stable pH value (pH = 8.5), after Day20, has been reported in previous studies of biological treatment of wastewater originating from olive processing [52] and can be attributed to the efficient biodegradation of the organic acids in the bioreactor that results in rise of the pH value. Moreover, the operating efficiency in terms of TOC and polyphenols removal is quite high, while the acclimatization phase is on-going; the removal ratios in 90% of the analyzed samples were over 90% and 85% for TOC and TPh, respectively, denoting a rather stable biodegradation process.

From the prokaryote bio-community, *Thauera* and *Paracoccus* spp. remained present throughout MBR operation and appear to be important for the bioremediation process. Further studies on these two species may provide further insights into TOPW treatment. *Thauera* spp. have been recognized as specialists on lactate conversion, which is the main by-product of TOPW fermentation, as well as a wide variety of aromatic substrates under aerobic or denitrifying conditions [48]. The aromatic ring cleavage ability may be also employed for polyphenolic substances degradation given the relevance in the chemical structure of some aromatic substances (like benzoate and its hydroxylated derivatives) and simple polyphenols. *Paracoccus* sp. is capable of storing a variety of polyhydroxyalkanoates reserves of carbon and energy [53] as a response to feast/famine conditions. Microorganisms that possess such metabolic mechanisms (i.e., assimilation of internal carbon and energy pools, like glycogen and poly-P granules) take advantage of the feast/famine conditions of the MBR due to the periodic (once a day) feeding pattern; this is a characteristic indication of the significant effect that operating conditions have on the microbial community.

RNA based metatranscriptomics analysis is believed to provide more precise information on community functions by identifying only expressed gene transcripts, actively metabolizing microbes, and thereby more accurately reflect the pool of enzymes that are active within the community [54]. Surprisingly, the relative abundance of expressed prokaryotic genes differed significantly from the abundance of microbial organisms enumerated by 16S rRNA. A handful of species (*Thauera*, *Paracoccus* spp.) dominate the expression frequency, whereas numerous others maintain quite low levels of expression, usually lower than 1–2%. This diversity increases in later samples which is consistent with the increased OTUs identified by 16S rRNA.

Concerning technical aspects, it seems that municipal activated sludge could be successfully used as a starting inoculum for implementing a large scale industrial MBR for treatment of TOPW. It appears that minimal exogenous nutrient and/or starting culture additions may be required, since TOPW natural bio-community quickly dominates upon originally present microorganisms. Moreover, MBR offers the attribute of fully retaining the suspended microbial biomass, avoiding microbial community destabilization phenomena through abrupt wash-outs [55,56]. Hence, a high density and complexity bio-community is established facilitating the biodegradation of TOPW effluents under rather harsh operating conditions (high organic content, salinity, pH fluctuations etc.). Finally, specific species (e.g., *Thauera* spp.) were identified as novel pools of genes and enzymes potentially involved in bio-degradation of polyphenolic substances.

5. Conclusions

Advanced NGS-based techniques were used to analyze the microbial community dynamics and metabolic adaptation during the acclimatization of a well-controlled aerobic MBR pilot system treating TOPW. The MBR was inoculated with municipal activated sludge, whereas real TOPW was gradually introduced within a period of approx. three months. A swift change was identified in both the prokaryotic and eukaryotic bio-community after introduction of TOPW in the MBR, and a new diverse bio-community was established. *Thauera* and *Paracoccus* spp. are dominant contributors to the metabolic activity of the stable bio-community, which resulted in over 90% and 85% removal efficiency of total organic carbon and total polyphenols, respectively. The valuable insights obtained in this study are expected to provide guidance in currently planned start-up of large-scale applications of the MBR-based treatment of the difficult to degrade TOPW.

Author Contributions: Conceptualization: S.I.P., A.M.M., and A.A.; data curation: S.M. and A.A.; formal analysis: S.M. and A.A.; investigation: S.I.P. and K.P.; methodology: S.I.P., S.M., and A.A.; supervision: A.A. and A.J.K.; visualization: S.I.P., S.M., and A.A.; writing: S.I.P., S.M., and A.M.M.; reviewing and editing: A.A. and A.J.K.

Conflicts of Interest: The authors declare no conflict of interest.

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