



## Article

# Diversity of Microbial Communities in Trade Wastes—Implications for Treatments and Operations

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**Abstract:** Industrial wastewaters display a complex and diverse range of physicochemical properties that are measured, studied, and treated by businesses and water service providers. Less frequently measured are the microbial communities in these wastes, despite possible implications for health, equipment maintenance, and the environment. This study aimed to assess the microbial communities of eighteen raw and discharge-ready wastewaters across eleven industrial sites to compare the microbial compositions of these wastewaters across different industry sectors, on-site treatment levels, and other wastewater components. The potential for variance in the biomethane yield, depending on microbial communities, was also measured. Using targeted sequencing, a unique taxonomy was identified, including genera linked to animals (*Acetivibrio*, *Lactobacillus*, *NK4A214*, *Prevotella*, and *Shuttleworthia*), cooling water (*Bosea*, *Legionella*, *Methylobacter*, and *Reyranella*), and extreme conditions (*Alkalibacillus*, *Geobacillus*, *Halorubrum*, and *Pyrobaculum*). However, the compositions of the microbial communities were not found to be directly correlated to industry sector or on-site treatment levels, nor were they found to have a direct effect on the biomethane potential. However, the presence of certain individual taxa is linked to the methane yield and treatment status and may be explained in the context of physicochemical properties while serving as potential markers for identifying, improving, or developing on-site processes.

**Keywords:** wastewater; microbial community; biomethane; amplicon sequencing; water treatment



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## 1. Introduction

Wastewaters from different industrial processes display a complex and diverse range of physicochemical characteristics [1], which can change because of the effects of the waste treatment processes undertaken at a particular site. These characteristics include pH, temperature, dissolved solids, and metals and can be affected by pumping, stagnation, exposure to light, and the combining of streams. The growth of individual microorganisms in wastewater is influenced by all these conditions and can be further influenced/varied directly through disinfection [2] or biological treatment [3].

The microbial community contained in these wastes is consequential. For example, within the bounds of a site and sewerage network, *Thiobacillus* spp. [4] and *Desulfobulbus* spp. [5] are examples of bacteria that biologically mediate the corrosion of concrete and steel. Furthermore, the health and safety of workers and others in the vicinity can be affected by pathogens, including *Mycobacterium* [6] and *Legionella* [7], while areas downstream of discharge points have been found to have increased abundances of potential pathogens, like *Arcobacter* and *Chlamydia* [8], as well as altered biofilms with increased antimicrobial resistances [9]. It is also possible that microbes in wastewater could influence or indicate the suitability of certain treatment processes, particularly resource recovery and biological methods. For example, the phosphorus removal of municipal wastewaters is improved in anoxic/aerobic membrane bioreactors by non-sterile substrates [10], and

nitrifiers in influents increase the stability and shorten the start-up times of activated sludge sequencing batch reactors that are treating municipal wastewaters [11]. Opportunities for exploiting such ecological knowledge are based on process optimisations without major capital expenditures for engineered solutions. However, research into the microbiome of industrial wastewater is limited, with large knowledge gaps remaining, preventing such process optimisations. Research usually focuses on individual biological treatment processes, such as the overloading of anaerobic digesters [12] or augmentation by selected bacteria [13]; municipal wastewater infrastructure, including temporal changes in sewer microbiota [14]; or end-of-pipe effects, like those on nitrogen-cycling bacteria in rivers near effluent discharge points [15]. The microbial diversity present in wastewater at the point of the source, as well as the effects that on-site treatment has on it, remain largely unknown, especially for industrial wastewater.

To improve our knowledge base, this study was undertaken to identify microbial communities of industrial wastewaters before and after on-site treatment, aiming to investigate any links between their microbiomes, levels of treatment, industries, and physicochemical properties. Hence, this work provides a novel inventory of microbial communities indigenous to selected treated and untreated industrial wastewaters, highlighting taxa of interest and links to undertaken treatments and resource recovery potential.

## 2. Materials and Methods

### 2.1. Sampling

Treated and untreated effluents were supplied from 12 de-identified industrial sites in Melbourne, Australia, as previously described [16]. Samples were stored at 4 °C before transport and physicochemical testing and frozen and stored at −18 °C for microbial analysis.

### 2.2. Physicochemical Analyses of Wastes

Standard gravimetric methods were used to calculate the total and volatile solids [17]. The pH was measured using a Hanna Instruments HI5221 pH meter (Hanna Instruments Australia, Keysborough, VIC, Australia), and the salinity was measured using a Horiba Scientific LAQUAtwin-Salt-11 salt meter (Horiba, Ltd., Kyoto, Japan). The chemical oxygen demand (COD), phosphate ( $\text{PO}_4^{3-}$  by acid–persulphate digestion), total nitrogen (TN by persulphate digestion), nitrates ( $\text{NO}_3\text{-N}$  by the chromotropic acid method), nitrites ( $\text{NO}_2\text{-N}$ , by the diazotisation method), and ammonia ( $\text{NH}_3\text{-N}$ , by the salicylate method) were measured using appropriate Hach Test 'N Tube methods with a Hach DR900 colorimeter and a DRB200-reactor heating block (Hach, Dandenong South, VIC, Australia) [18–23]. De-identified discharge data were supplied by Greater Western Water.

### 2.3. Biomethane Potential Assessment

The biomethane potential (BMP) was assessed, as previously described, using a custom lab-scale set-up [16] or “Nautilus BMP reactors” (Anaero Technology, cambridgeshire, UK) [24] as applicable. Briefly, digestion vessels were prepared with real industrial wastewater substrates and mature waste-activated sludge at an inoculum-to-substrate ratio (ISR) of 3.5 on a grams-per-VS basis. The digesters were sealed and held at 38 °C in a water bath. When the Nautilus was used, the gas flow volume was measured in near-real time through liquid displacement buckets and corrected for atmospheric temperature and pressure, followed by gas collection in 3 L Tedlar PLV gas-sampling bags with Thermogreen LB-2 septa (Sigma Aldrich, Saint Louis, MO, USA). Methane concentrations were measured using a calibrated portable multi-gas monitor with an infrared detector (MX6 iBrid,  $\text{CH}_4$  range 0–100%,  $\pm 1\%$ , Industrial Scientific, Pittsburgh, PA, USA) Tests were continued until the daily methane production had decreased to less than 1% of the cumulative total for a period of at least one week.

#### 2.4. Targeted 16S-rRNA Sequencing

Frozen genomics samples were thawed, and DNA was extracted using a DNeasy Powersoil Pro extraction kit (Qiagen, Hilden, Germany), as per the manufacturer's instructions [25], and stored at  $-20^{\circ}\text{C}$ . DNA concentrations were quantified using a Qubit 4.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The 16S Metagenomic Sequencing Library Preparation Guide (Illumina, San Diego, CA, USA) was followed [26], using primers targeting the V3V4 region of the 16S-rRNA gene with primers Pro341FB (CCTACGGGN-BGCWSCAG) and Pro805R (GACTACNVGGGTATCTAATCC) [27,28] prior to sequencing (Illumina MiSeq, 600 cycles) [26]. Blanks were included in the library preparation to assess contamination. A total of 20 libraries from this study were pooled together with 137 other 16S libraries from unrelated projects and then sequenced together at 8 pM on an Illumina MiSeq ( $2 \times 300$  bp), including 20% phiX, after library preparations using Nextera XT indices.

Processing of fastq files was conducted on the Research Cloud made available by the Australian Research Data Commons (ARDC). The combined processing of the sequences (all the sequenced libraries) included primer trimming with cutadapt [29] followed by pairing forward and reverse reads, denoising, filtering, and dereplicating the sequences using DADA2 [30] (error rates were trained on 277,158,882 bases in 1,015,234 reads from 53 samples) after truncating all the amplicon sequences to 273 and 220 nucleotides (forward and reverse reads, respectively) using the default settings of the QIIME2 [31] pipeline apart from relaxing the expected error from 2 to 6 for reverse reads.

This followed taxonomic classification (qiime feature-classifier classify-sklearn) [32] of amplicon sequence variants (ASVs) using the SILVA database after training the classifier (qiime feature-classifier fit-classifier-naïve-bayes) on taxonomy and sequence files [33,34]. The subset of samples for this study was then used for further analysis. Site 6 was removed from the analysis because of potential sample contamination.

#### 2.5. Data Analysis and Visualisation

A total of 175,557 quality reads at a median frequency of 9873 reads per sample and 6506 ASVs were obtained. All the analyses and charting were performed in R, version 4.2.2 [35]. ASVs with fewer than five reads were filtered for quality. Abundances were normalised through rarefaction to the minimum sample size prior to calculating the richness, Shannon diversity ( $H'$ ) and Pielou evenness ( $J$ ) using the phyloseq package [36]. Kruskal–Wallis tests were used to assess differences in alpha diversity between treated and untreated effluents. Prior to principal component analysis (PCA) analysis, relative abundances were transformed to centred log-ratios (clrs) using the microbiome package [37]. Phyloseq was then used for PCA ordinations of clr-transformed abundances to assess compositional differences between sites. Canonical correspondence analysis (CCA) was performed on phylum-level abundances (Bray–Curtis dissimilarities) using the vegan [38] package to assess associations between bacteria and effluent of different sites and their chemical properties. To detect any treatment or site-specific genera, differential abundance analysis was conducted using the ANCOM-BC package on clr-transformed abundances [39]. For treatment effects, sites were limited to those with both treated and untreated samples available.

### 3. Results and Discussion

#### 3.1. Wastewater Properties

Wastewater samples were obtained from businesses in a broad range of industries, including edible fats (Site 1), brewing (Site 2), waste management/logistics (Sites 3 and 9, respectively), chemical production (Sites 4, 8, 10, and 11), food (Site 5), and animal products (Sites 7 and 12), with both untreated and treated samples available for Sites 1, 2, 3, 5, 9, and 12 (Table 1).

**Table 1.** Measured characteristics of wastewater samples, including industries from which they were taken, whether before or after on-site treatment, chemical data, BMP yield, and microbial diversity.

Sample Name	Site	Treatment Level	Industry	COD (mg/L)	VS (mg/L)	PO <sub>4</sub> (mg/L)	pH	NO <sub>2</sub> (mg/L)	NO <sub>3</sub> (mg/L)	NH <sub>3</sub> (mg/L)	TotN (mg/L)	Na (mg/L)	Gas Yield	Observed Taxa	Shannon Diversity	Pielou's Evenness
1U	1	Untreated	Edible Fats	44,900	7430	272	6.02	0.7	5	0	11	2.1	Low	37	2.182	0.604
1T	1	Treated	Edible Fats	1086	614	170	6.33	0	10	0	8	2.1	Low	14	2.393	0.907
2U	2	Untreated	Brewery	13,800	3778	3.47	3.89	0.17	2.8	0	93	0.9	Low	37	2.896	0.802
2T	2	Treated	Brewery	5530	1864	63	4.02	0.4	7	0	49	0.9	Medium	46	3.243	0.847
3U	3	Untreated	Logistics	9710	5556	72	12.29	0.8	9	0	83	1.5	High	45	3.247	0.853
3T	3	Treated	Logistics	2880	874	53	6.72	0.6	7	20	65	1.7	Low	58	3.164	0.779
4U	4	Untreated	Chemicals	20	84	10	6.89	0.02	8	0	4	0.2	Low	60	3.57	0.872
5U	5	Untreated	Foods	22,200	6982	263	5.39	0	12	20	239	1.8	High	44	3.107	0.821
5CU *	5	Untreated *	Foods	1,466,300	279,271	1090	4.86	0.62	8	210	7500	3.7	High	19	1.984	0.674
5T	5	Treated	Foods	9210	3332	170	6.32	0.5	9	0	103	1.5	High	91	3.24	0.718
7T	7	Treated	Animal Products	>100,000	19,460	3.06	6.77	2.3	8	100	870	310	Low	50	2.571	0.657
8T	8	Treated	Chemicals	10,000	1326	10	6.50	0.15	11	0	0	0.4	Low	24	2.521	0.793
9U	9	Untreated	Logistics	17,700	9992	3.34	12.16	0	6.2	0	19	2.4	High	64	3.577	0.86
9T	9	Treated	Logistics	3650	1173	2.46	5.63	0.04	1.4	0	50	1.2	Medium	52	3.041	0.77
10T	10	Treated	Chemicals	2560	615	235	6.21	0.09	1	0	8	0.6	Low	47	2.981	0.774
11T	11	Treated	Chemicals	140	443	1.36	7.91	4.9	10.3	0	9	0.6	Low	153	4.465	0.888
12U	12	Untreated	Animal Products	2920	1109	95	6.72	0.03	2	270	254	1.4	High	128	4.175	0.86
12T	12	Treated	Animal Products	2000	211	16	6.96	0.04	0.7	120	96	1	Low	177	4.788	0.925

\* Sample 5CU is untreated source-separated wastewater from a single cooking process at Site 5, all the other untreated samples are the final combined effluents sampled immediately before treatment. COD: chemical oxygen demand; VS: volatile solids; PO<sub>4</sub>: phosphate; NO<sub>2</sub>: nitrite; NO<sub>3</sub>: nitrate; NH<sub>3</sub>: ammonia; TotN: total nitrogen; Na: sodium. Adapted with permission from [16]. 2023, Elsevier.

Treatments differed between sites. Water treatment unit operations undertaken included removing particles through straining, screening, and settling; separating hydrophobic liquids in oil interceptors; cooling the wastewater stream; adjusting pH through chemical addition; controlling sulphide levels; and more complex procedures for coagulating, flocculating, and removing solids, like polymer addition and dissolved-air flotation (DAF) (Table 2).

**Table 2.** Treatments undertaken at each site. Treatment processes are not in order. Treatments are general processes and are not performed identically at each site.

Site	Industry	Straining	Settling	Mixing	Oil Interception	Neutralisation	Cooling	Screening	Ferric Sulphate	Polymer	DAF *	Sludge Removal	Sulphide Control	Aeration	Separation	pH Adjustment
1	Edible Fats	✗	✓	✗	✓	✓	✓	✗	✗	✓	✗	✗	✓	✗	✗	✗
2	Foods	✓	✓	✓	✗	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗
3	Logistics	✓	✓	✓	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
4	Chemicals	✗	✓	✗	✗	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
5	Foods	✓	✓	✗	✗	✓	✓	✗	✗	✗	✓	✗	✗	✗	✗	✗
7	Animal Products	✓	✓	✗	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
8	Chemicals	✗	✗	✗	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✓
9	Logistics	✓	✓	✗	✓	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗
10	Chemicals	✓	✓	✓	✓	✓	✗	✗	✗	✗	✗	✗	✗	✓	✗	✗
11	Chemicals	✗	✓	✗	✓	✓	✗	✗	✗	✗	✗	✗	✓	✗	✗	✗
12	Animal Products	✓	✓	✗	✓	✓	✗	✓	✓	✓	✓	✓	✗	✗	✗	✗

\* Dissolved-air flotation.

Historical data (Supplementary Table S1) showed that the measured characteristics at discharge were unique to a site because of the interactions between the individual wastes produced and the treatment enacted. Although historical data should match the measured data of treated samples for a given site [40], this was not always the case. This may be affected by the long-term average composite nature of the data held by the water corporation. Consistent with the purpose of on-site treatment, the untreated samples were not reflected in the discharge data (Supplementary Table S1).

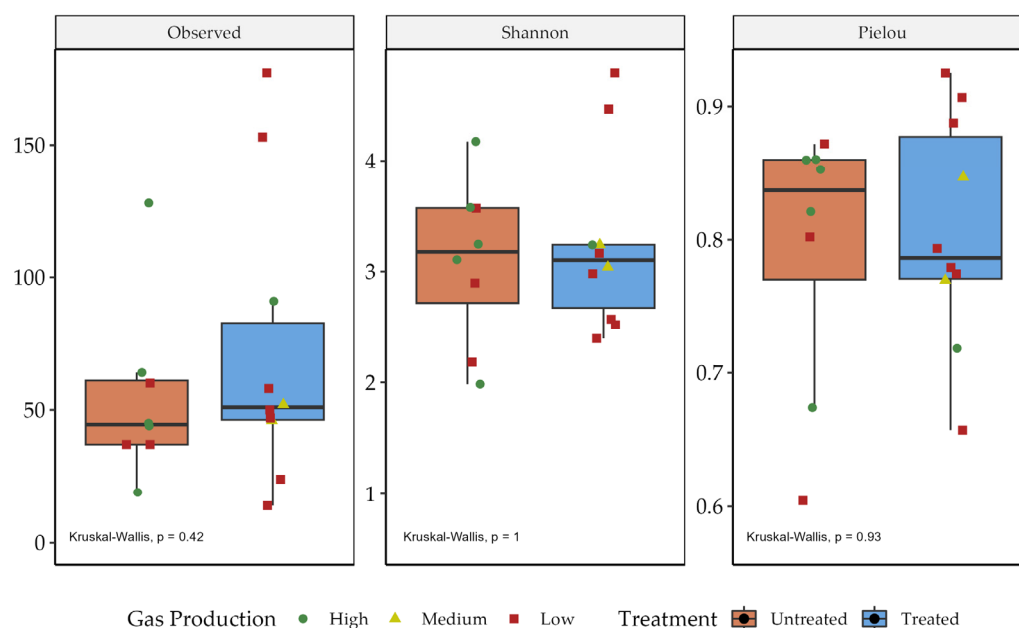
High COD (>9500 mg/L) and VS (>3500 mg/L) characterised the untreated streams of Sites 1, 2, 3, 5, and 9 [41] and the treated sample (7T), which also contained high measured nitrogen, sodium, and dissolved solid concentrations according to discharge data (Table 1). Otherwise, treated samples were lower in COD and VS, and Sample 4U was dilute in all the measured parameters. Sites 1, 5, and 10 contained high phosphate concentrations (>150 mg/L), consistent with other edible fat and food manufacturers [41–43] and certain chemical producers (Table 1). Most samples were in the pH range of 6–8; however, 3U and 9U had high pH values (>12), and 2U, 2T, 5U, and 9T had low pH values (<6). Low pH values in untreated food-manufacturing wastewater may be caused by microbial acidogenesis, where monomers, such as simple sugars, are converted to short-chain organic acids [44]. The pH in treated samples is predominantly the result of neutralisation treatments. The pH of the treated sample for Site 2 was much closer to that of the untreated sample than the historical data, indicating a potential treatment process upset. Confirmed by available discharge results, low total nitrogen concentrations were measured in samples from Sites 1, 4, 8, 10, and 11 and before treatment at Site 9 (Table 1). Discharge from Sites 3, 5, and

8 historically contained greater than 500 mg/L of oil and grease. Sites 4, 7, 8, and 10 generally discharged at a lower temperature (<20 °C) and 1, 2, and 9 at a higher temperature (>28 °C). High gas yields were measured from untreated streams at Sites 3, 5, 9, and 12 and the treated stream at Site 5. Medium yields were gained from post-treatment samples at Sites 2 and 9 (Table 1).

As expected, the chemical properties of the untreated effluents were diverse, reflecting the different production methods each site housed and the varying treatment processes undertaken. More surprisingly, despite general restrictions on the quality of the discharge [45], each of the treated samples also has unique chemical properties. Although in some instances, discrepancies between measured and historical data indicate a short-term fluctuation in treatment effectiveness, most differences between treated samples can be explained by the contents of the water before treatment and the relatively broad range of trade waste qualities allowed by guidelines or special agreements.

### 3.2. Richness and Diversity Assessments of Microbial Communities among Treated and Untreated Trade Wastes

To assess if the wastewater microbial diversity was different between treated and non-treated effluents or associated with high potential for biogas production, the richness (of observed taxa), Shannon diversity ( $H'$ ), and Pielou's evenness ( $J$ ) were calculated, representing the number of organisms, number of effective (or abundant) organisms, and their distributions within each sample, respectively (Figure 1).



**Figure 1.** Diversity measurements of microbial communities sequenced from wastewater samples. Colour of box-plot denotes whether sample was measured before or after on-site treatment; colour and shape of dots depict level of methane yield in BMP tests.

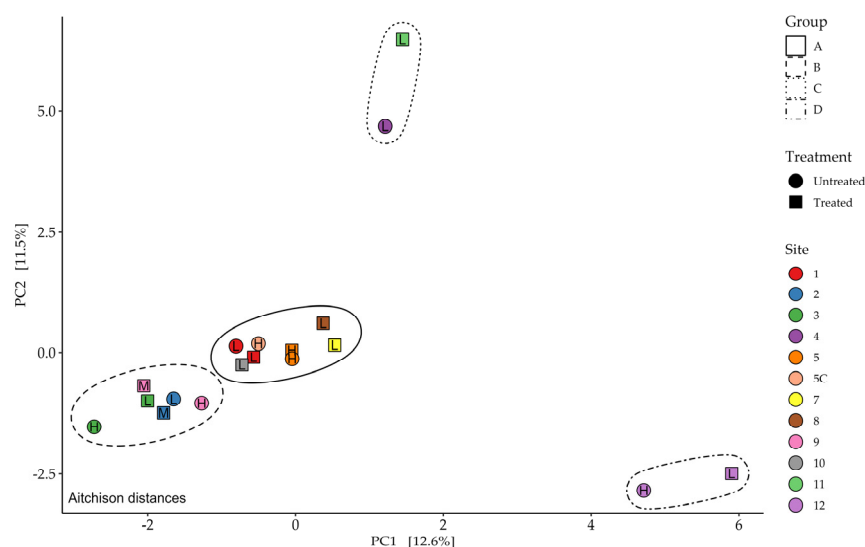
Across the 12 sites, both treatment and biogas yields were not associated with any of the diversity indices (Kruskal–Wallis,  $p > 0.05$ ). The mean richness and diversity ( $H'$ ) values appeared higher for treated samples ( $71.2 \pm 53.7$  and  $3.24 \pm 0.80$ , respectively) compared to untreated samples ( $52.3 \pm 32.9$  and  $3.09 \pm 0.73$ , respectively); however, the variability across the sites was too large (and sample sizes were too small) to confirm this observation. For sites with pre- and post-treatment samples available, no consistent trend was found. It was hypothesised that, generally, more concentrated and complex untreated wastewaters would support greater richness [46,47]. However, in some cases, inhibitors, like phenols or heavy metals, which cause the dominance of a limited number of species, are present in raw wastewater [48]. When these are removed by treatment, the growth of more



diverse microbes is possible. Sample diversity here may also be shaped by the stability of the wastewater over time, resulting in more predictable microbial communities, as shown in long-term studies of parallel anaerobic digesters [49], meaning that a complex but consistent substrate does not necessarily translate to higher diversity. It is also likely that individual treatment vessels are home to unique microbial communities [50] and seed the wastewater as it is processed. Diversity may be changed in ways, on-site, that have no effect on BMP tests, including exposure to high temperatures or pressures [51]. Consequently, in future studies on industrial wastewater sites, an appropriate number of sites for sampling may need to be determined through power analysis to ensure sample-level diversity can differentiate between treated and untreated effluents.

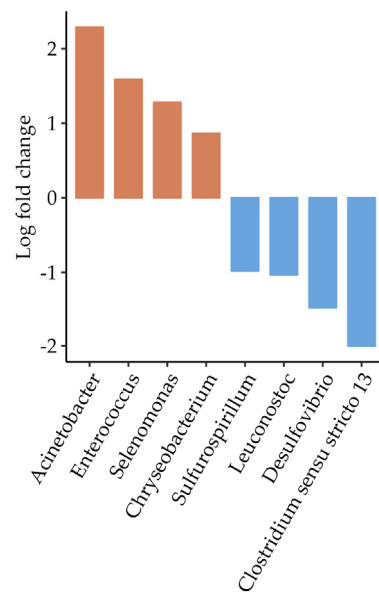
### 3.3. Microbial Community Analysis of Microbial Communities among Trade Wastes at Different Levels of Treatment or BMP Yields

Using PCA to visualise the dominance of the taxa identified in the samples, some groupings were evident. Considering the first two principal components, a general group of Sites 1, 5, 7, 8, and 10 can be seen (Group A). Of the other samples, those from Sites 2, 3, and 9 form a cluster (Group B); the communities at Sites 4 and 11 (Group C) were different from those at the other sites, and Site 12 communities were different again (Group D) (Figure 2).



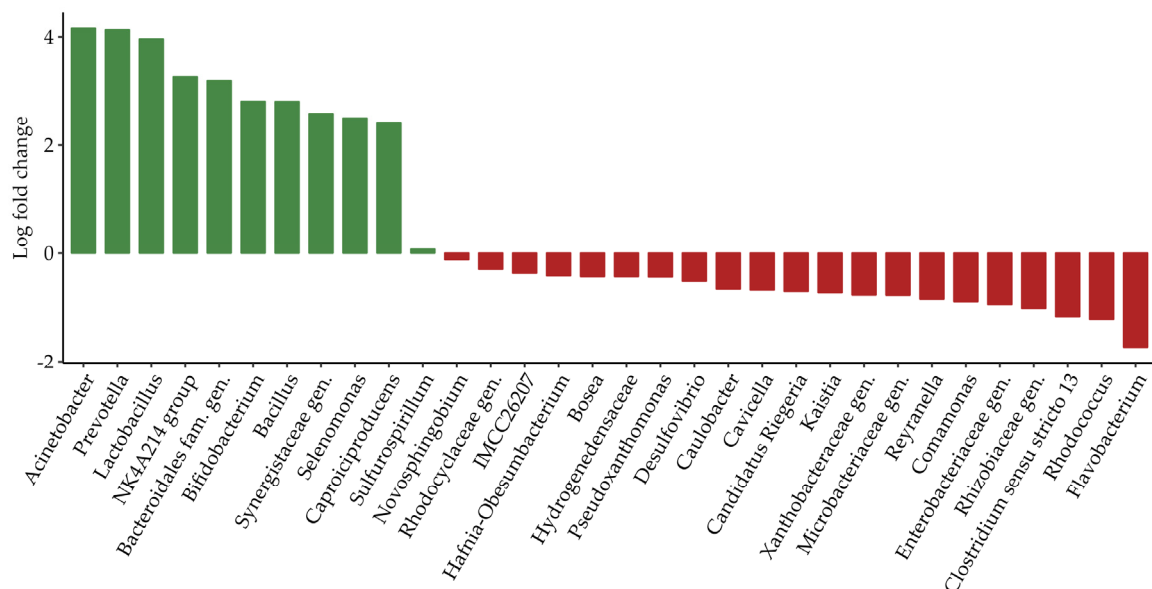
**Figure 2.** PCA of microbial communities in wastewater samples. Circles represent untreated streams, squares represent wastewater ready for discharge to sewers (treated), and colours denote sites. 5C represents the source-separated waste from Site 5. The letter at each point denotes a high, medium, or low (H, M, or L, respectively) gas yield in BMP tests. Groups shown by ellipses; 12.6% and 11.5% of the variance between samples are mapped by the x- and y-axes, respectively.

Group B consists of all the samples from the two logistics companies and the brewery. Both sites in Group C were chemical-manufacturing businesses, and Site 12 was in the animal product industry. Site 7, notable for high sodium concentrations, both measured and historically (Table 1, Supplementary Table S1), also shared very few taxa with other sites. None of treatment levels, measured water chemistry values, or BMP assay biogas yields (Table 1) predicted microbial community similarity between samples. Apart from logistics companies being confined to Group B, industry type also did not predict similarity. It was observed that some individual genera were more-or-less abundant, depending on the treatment level. Across all the samples with both treated and untreated samples available, *Sulfurospirillum*, *Leuconostoc*, *Desulfovibrio*, and *Clostridium sensu stricto* 13 were more abundant in the treated samples, while *Chryseobacterium*, *Enterococcus*, *Selenomonas*, and *Acinetobacter* were more abundant in the untreated samples ( $p < 0.05$ ) (Figure 3).



**Figure 3.** Genera with statistically significant ( $p < 0.05$ ) differences in relative abundance in untreated samples relative to treated samples. Samples limited to sites with both treated and untreated samples available. Y-axis shows log-fold change in relative abundance in untreated samples vs. treated samples. Positive values, in brown, denote genera more abundant in untreated samples; negative values, in blue, denote genera more abundant in treated samples;  $n = 12$ .

Consistent with untreated wastewaters generally producing higher BMP yields, the latter two taxa were also significantly more abundant in samples with high BMP yields ( $p < 0.05$ ), while *Desulfovibrio*, and *Clostridium sensu stricto* 13 were more abundant in lower-yielding samples (Figure 4).



**Figure 4.** Genera with statistically significant ( $p < 0.05$ ) differences in relative abundance. Y-axis shows log-fold change in relative abundance in high-yielding samples relative to low-yielding samples. Positive values, in green, denote genera more abundant in untreated samples; negative values, in red, denote genera more abundant in low-yielding samples;  $n = 16$ .

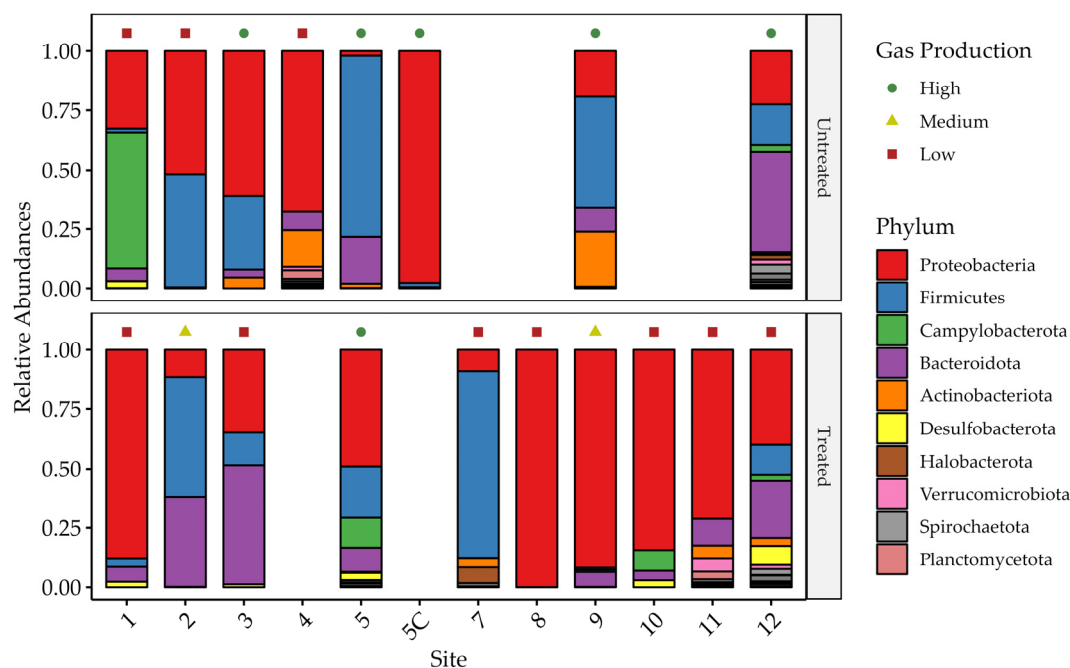
Conversely, *Sulfurospirillum* had a small but significant increase in higher-yielding samples. This genus was present in Sample 5T, the only treated sample with a high gas



yield. *Bosea* and *Reyranella* were also represented in low-yielding substrates, in Group C, the provenance of which is described further in Section 3.5 below. *Prevotella*, *Lactobacillus*, and NK4A214 are present in high-yielding samples from brewery, logistics, and food sites (Sites 2, 3, and 5, respectively). Members of the family *Synergistaceae* are implicated in higher biogas yields in anaerobic digestion [52], in agreement with an increase in high-yielding substrates in this study.

### 3.4. Taxonomic Abundances—Overall Trends

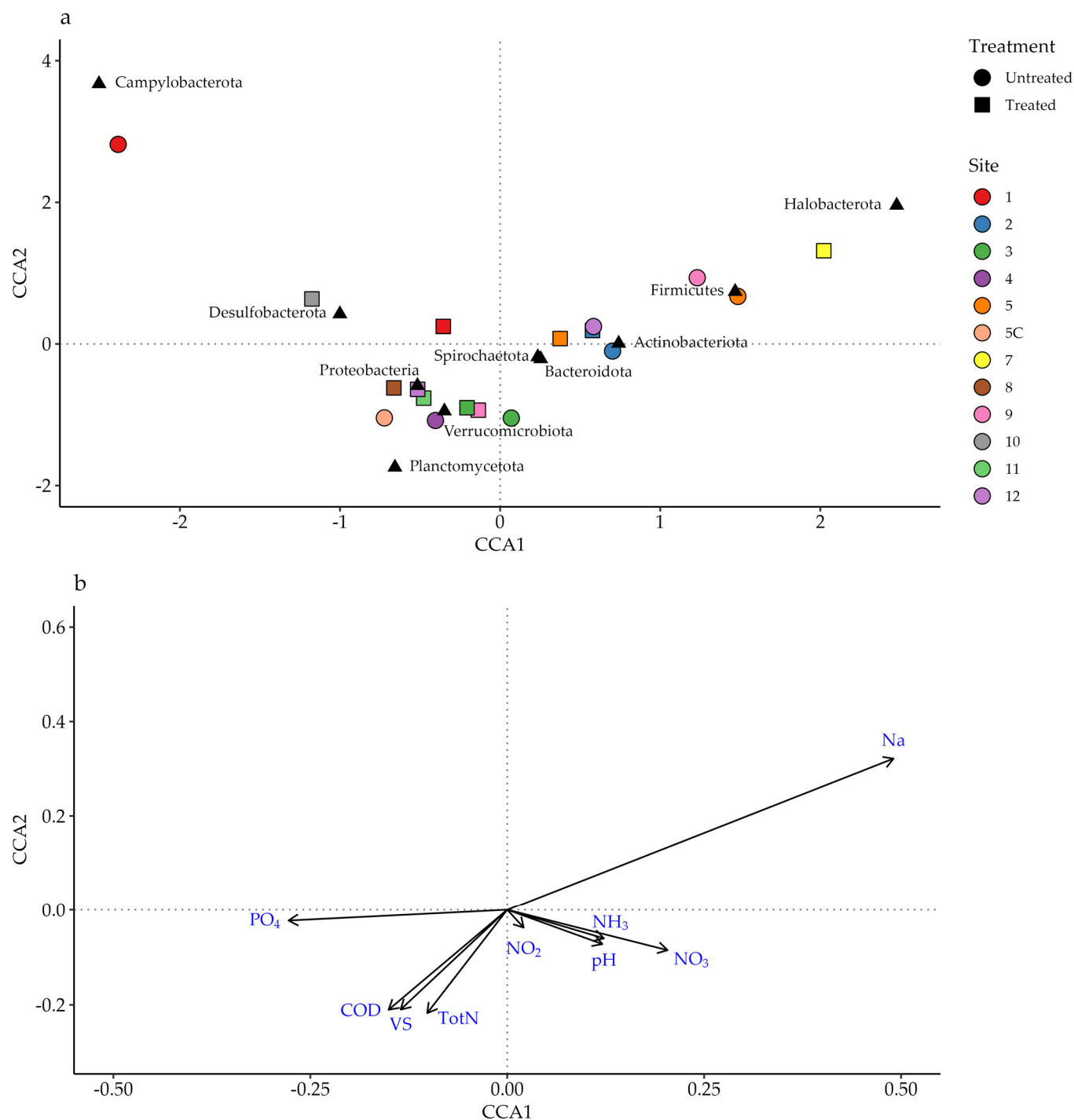
Comparisons were made to investigate which characteristics correlated to phylum abundance. At the phylum level, Proteobacteria were the most common across the samples (mean relative abundance = 0.519), followed by Firmicutes (0.224), Bacteroidota (0.133), and Campylobacterota (0.047) (Figure 5).



**Figure 5.** Abundance plot at phylum level, showing sites, treatment status, and level of gas yield from BMP tests. Proteobacteria were the most highly abundant across the samples. Phylum abundance had no significant difference between treatment levels.

Campylobacterota were classified within the phylum Proteobacteria until recently [53]. These phyla were three of the four most abundant in a combined domestic/industrial sewer [14], and Proteobacteria and Firmicutes were the two most common phyla in anaerobic digesters treating municipal waste-activated sludge [54]. Within individual sites, taxonomic abundances changed markedly after treatment, including the almost total disappearance of Campylobacterota at Site 1 or the large increase in Bacteroidota at Site 3. On-site treatment is undertaken to reach discharge specifications, so differences between the measured chemistries of untreated samples and discharge wastewater compositions are expected. Therefore, variation in the bacteria that are present in each medium is evident. There was no consistent phylum-level community between all the treated and all the non-treated samples or between the high-, medium-, and low-biogas-yielding samples. This is evident in Figure 2, as there is no grouping of samples with the same treatment status or yield level in the ordination.

Figure 6 shows the canonical correspondence analysis (CCA) of the samples, phyla, and measured chemical data.



**Figure 6.** Canonical correspondence analysis (CCA) plots of abundances (Bray–Curtis dissimilarities) of all the samples, showing the 10 most common phyla (a) and the influence of the measured chemical data on their abundance (b). Plot b axes are at a smaller scale for clarity. CCA1 represents 31.3% of the inertia; CCA2 represents 19.9%.

The most striking observation is the relationship between the sodium concentration at Site 7 and Halobacterota, a phylum containing many halophiles. The abundances of Firmicutes in this sample and the untreated wastewaters of Sites 5 and 9 are also represented along this axis. The samples containing larger abundances of Verrucomicrobiota, Planctomycetota, and Proteobacteria generally also contained lower concentrations of sodium. These plots also map the high abundance of Campylobacter and low concentrations of nitrite, nitrate, and ammonia in Samples 1U and 10T.

### 3.5. Microbial Community Phylogenetic Analysis—Individual Sites

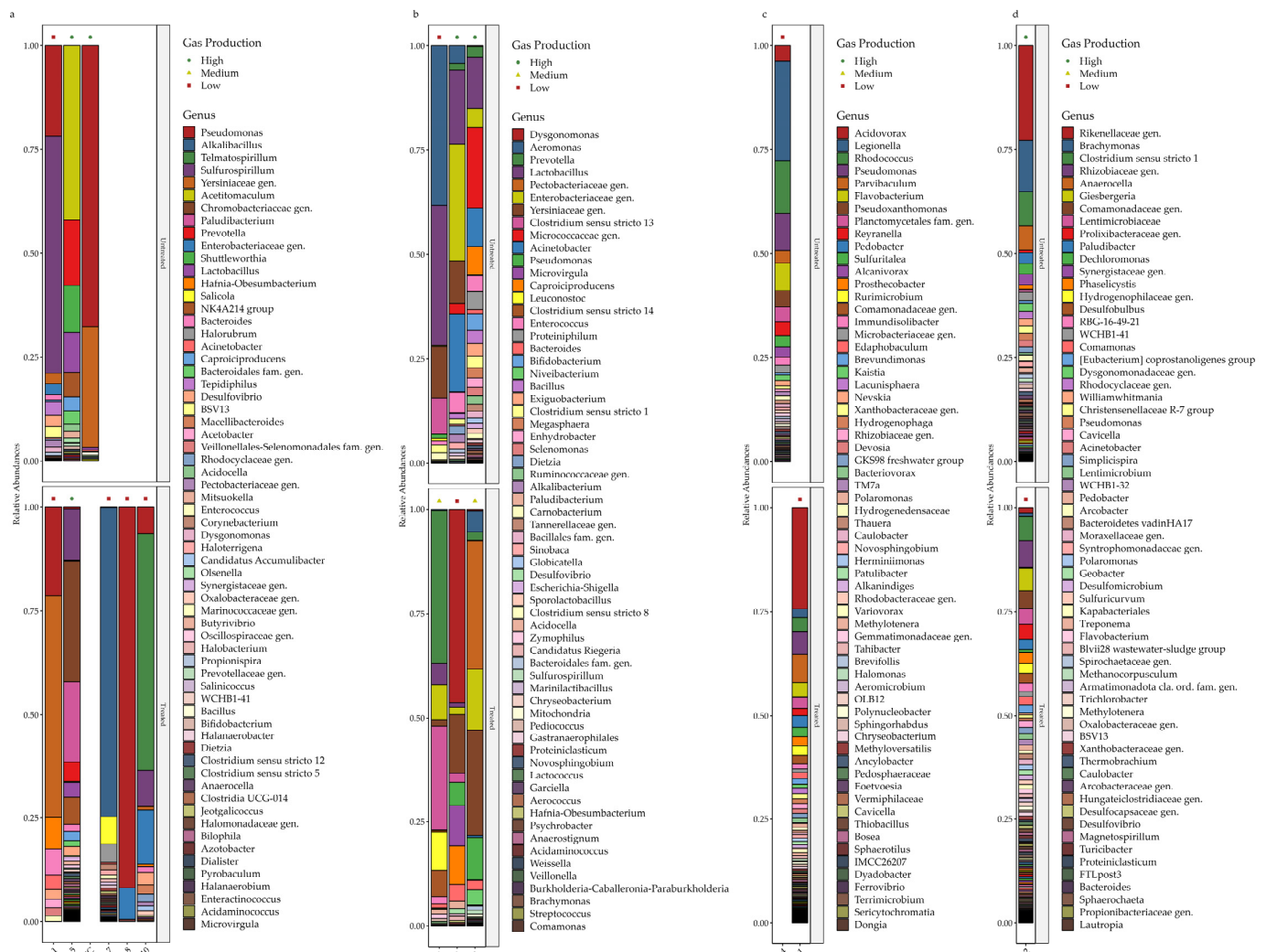
The microbes present in each sample are a function of conditions and wastewater composition, so links can be found between abundance and effluent properties. Genus-level investigation can provide more information about likely optimal conditions than the more general Phylum-level analysis [55]. Taxa described in this section were detected in the relevant samples, as per Figure 7. Sites have been ordered by groups observed in Section 3.3. Group A can be seen as a general grouping. Samples from brewery and logistics sites made up the entirety of Group B. Group C was characterised by the presence of genera found in cooling water, along with high diversity (observed taxa > 60,  $H' > 3.5$ , and  $J > 0.86$ ). Group D, consisting of treated and untreated samples from Site 12, was highly diverse.

#### 3.5.1. Group A, Site 1—Edible Fats

The untreated and treated samples at Site 1 were dominated by anaerobic or microaerobic microbes. *Sulfurospirillum* and *Desulfovibrio* both reduce sulphur compounds [56] and are found at very high abundances before on-site treatment, going against the overall trend for these genera described earlier. After treatment, which includes sulphide control, their numbers markedly decreased. *Desulfovibrio* is also potentially involved in the decomposition of fats, at this site, as a butyric acid oxidiser [57]. *Pseudomonas* maintains a consistent relative abundance before and after treatment. Members of the *Pseudomonas* genus are aerobic and strictly respiratory, but some species can grow under anaerobic conditions by reducing nitrates. Otherwise, they are widely distributed, with minimal nutrient requirements [58], as demonstrated by their position in the top ten most abundant ASVs in samples U1, T1, U2, 3T, 4U, 8T, 9T, 10T, and 11T. Treatment increases the relative abundance of *Yersiniaceae*, a family of spore-forming facultative anaerobes that include some pathogenic species [59], and of *Obesumbacterium proteus*, implicated in beer spoilage [60]. *Acetobacter*, found in fermented foods, is also present [56].

#### 3.5.2. Group A, Site 5—Foods

Site 5 is a food-manufacturing business, and the microbes present in the raw wastewater are associated with animals. *Shuttleworthia* is present in the digestive systems of healthy chickens [61] and weaned calves [62]; NK4A214 has been isolated from yaks [63]. *Acetivomaculum*, *Lactobacillus*, and *Prevotella* are present in increased numbers in the rumen of cattle suffering from acidosis [64]. The likely mechanism of this is *Acetivomaculum* producing acetate, and *Lactobacillus* and *Prevotella* thriving in the subsequent low-pH environment. This tolerance is reflected in the relatively low pH of the waste stream. After treatment, the proportions of all these ASVs decreased, while *Paludibacterium* and other unclassified members of the *Chromobacteriaceae* family increased. *Paludibacter* are facultative anaerobes [65], and the rest of the family members are either aerobes or facultative anaerobes [66]. Wastewater treatment at this site includes DAF, a process that clarifies water using air bubbles to remove suspended solids as a sludge [67], perhaps favouring aerobic species. DAF has been shown to separate algae and bacteria from water [67,68]. The effect of the treatment on the relative abundance correlates with neither typical cell sizes nor cell wall structures found in literature, despite DAF involving fluid dynamics and interactions between bubbles and solids. Surprisingly, despite these aerobic conditions, the methanogen *Methanobrevibacter* was only identified in the treated sample. This genus has been linked to stressed anaerobic digesters [12].



**Figure 7.** Abundance plots at genus level, showing sites, treatment status, and level of gas yield from BMP tests. Plot is separated into groups (a–d) as identified in PCA (Figure 2). Genera are ordered by the highest relative abundance in the group.

Site 5 was unique in this study in providing access to a source-separated wastewater stream: a relatively small volume discharged from cleaning a cooking vessel. Apart from *Pseudomonas*, *Yersiniaceae*, and other previously described taxa, thermophilic *Pyrobaculum* [69] and *Geobacillus* [70] were identified at low levels, reflecting the high temperatures involved in the individual unit's operation. This sample showed low diversity (observed taxa = 19;  $H' = 1.98$ ;  $J = 0.67$ ) but a high BMP yield. It is likely that the high-temperature conditions reduced the microbial diversity [71] in a way that was temporally limited and unmeasurable in the wastewater sample but may have served as a form of pre-treatment that increases the BMP yield [44].

### 3.5.3. Group A, Site 7—Animal Products

The extremely low gas yields of BMP assays for this substrate were linked to the high concentration of sodium found in discharge measurement data. High sodium levels are also reflected in the microbial community of the wastewater, which is dominated by halophiles.

*Alkalibacillus* and *Haloterrigena* require salt concentrations of 10% for optimal growth [55,72]. The optimal concentration is higher for *Salicola*, isolated from salterns and salt lakes, at 15% [73], and higher again for *Halorubrum* and *Halobacterium*, at over 20% [55,74]. *Marinococcaceae* inhabit high-salinity areas, such as the shores of salt lakes [75].

*Corynebacterium* has been identified in an anaerobic membrane bioreactor inoculated with intertidal wetland sediment for treating high-salinity wastewater [76]. *Jeotgalicoccus* is slightly halophilic and was first identified in a salted preserved seafood dish [77]. *Dietzia* has also been found in fish and fish-based food-processing water [78]. All the above-named bacteria are aerobes or facultative anaerobes. The genus *Corynebacteria* also contains facultative anaerobes and aerobes [78]. Conversely, *Halanaerobacter* is an obligate anaerobe [79]. The presence of obligate aerobes and anaerobes at this site and others indicates the presence of microniches of different conditions and potentially a temporal factor as operations progress. Another indicator of this is the presence, albeit at very low levels, of *Methanobrevibacter* and *Methanosphaerula*. Both genera are known to increase in relative abundance compared to other methanogens when anaerobic digesters are under chemical stress [12]. In this case, they may be the last surviving remnants of an individual process's low-sodium, low-oxygen wastewater before the combination of streams.

#### 3.5.4. Group A, Site 8—Chemicals

The wastewater at this site contained a very low concentration of extractable DNA. The majority of that which was extracted and sequenced was from the previously described Enterobacteriaceae family and *Pseudomonas* genus. This sample was shown to be a poor substrate for biomethane production, despite medium levels of COD and VS. Historical data for this site indicate testing for certain herbicides and other xenobiotic compounds, suggesting that wastewater at this site may contain chemicals that inhibit growth, both on-site and later if used as a digestion substrate. *Pseudomonas* have been found to be able to degrade herbicides [80], chlorinated aromatics [81], and coking wastewater [82], so they may be better suited to grow on this substrate.

#### 3.5.5. Group A, Site 10—Chemicals

*Telmatospirillum*, Enterobacteriaceae, *Pseudomonas*, *Desulfovibrio*, and *Macellibacteroides* have all been shown to increase in relative abundance in an anaerobic sludge after exposure to selenate [46], and some halorespiring *Sulfurospirillum* species can use selenate as an electron acceptor when chlorinated compounds are not available [83]. These bacteria were present at high relative abundances in treated wastewater at this site, suggesting the presence of selenate. Somewhat discounting the theory of high selenate levels is the alternate link between *Desulfovibrio* and *Sulfurospirillum* and the presence of sulphur compounds, as indicated in discharge data [84,85], and the presence of Bacteroides, which are negatively affected by selenate [46]. Selenium was found at low concentrations by the water service provider at this site. Another parameter that is in the discharge-testing data is phosphorus, a major elemental component of phosphates. *Candidatus accumulibacter*, the only identified genus of the *Rhodocyclaceae* family at this site, can accumulate phosphate and is often found in phosphate removal trains at wastewater treatment plants [86]. This is compatible with the high phosphate concentration measured in the wastewater sample.

#### 3.5.6. Group B, Site 2—Brewery

The predominant genera found in raw wastewater at Site 2 were *Aeromonas* and *Lactobacillus*. *Aeromonas* have been found in malting processes [87] and the treatment of brewing wastewater [88], along with *Zymophilus* [82]. *Lactobacillus backii* and *Lactobacillus coryniformis*, both present, are beer spoilage microorganisms [89,90] that show sensitivity to  $\alpha$ -acid compounds in hops. The previously mentioned beer-spoiling *Obesumbacteria* species were not found. Despite both samples being of low pH, the obligate acidophile *Acidocella* [56] was found in the treated wastewater. At this site, treatment was found to increase the abundances of *Prevotella oryzae* and *Prevotella paludivivens*, slightly acidophilic anaerobes first isolated from irrigated rice-fields [91,92], and *Clostridium* and *Leuconostoc*, both of which have been found in sludge granules of UASB reactors treating distillery and brewery wastewaters [93]. *Leuconostoc* requires a rich growth medium for culturing [72]. *Yersiniaceae*, selected by treatment at Site 1, conversely, decreased in abundance following



treatment at this site. Site 2 was also an outlier in previous BMP studies, showing a higher biogas yield for the treated substrate. The factors in *Leuconostoc* and *Yersiniaceae* abundances before and after treatments may also influence measured biogas yields.

### 3.5.7. Group B, Site 3—Waste Management/Logistics

The untreated effluent of Site 3, a logistics company, contained high proportions of *Acinetobacter*, *Lactobacillus*, *Enterococcus*, and members of the *Enterobacteriaceae* and *Yersiniaceae* families that could not be further classified. This is consistent with trends for untreated samples described earlier. Although precise species and strains could not be identified, members of each of these families and genera are known to be potentially pathogenic to humans, animals, and/or plants [58,59,94,95]. This is rare and opportunistic for *Lactobacillus* species, which are usually known as having probiotic effects [96,97]. After treatment, the proportions of all the above taxa were decreased, except for *Yersiniaceae*. The most common species identified in the treated stream was *Dysgonomonas alginatilytica*. *D. alginatilytica* is a facultative anaerobe that can metabolise alginate [98]. Alginate is a gelling agent for food products [99] and can be produced by *Pseudomonas* and *Azotobacter* [100]. As the increase in the abundance of *D. alginatilytica* occurs after treatment, it is unlikely that alginate is present from the main industrial process at this site. *Azotobacter* was not identified in these samples, and *Pseudomonas* were only identified at low levels, so *D. alginatilytica* is likely to be utilising a different substrate. *Microvirgula aerodenitrificans*, also found at higher levels post treatment, is an aerobic denitrifier shown to be able to remove nitrogen from wastewaters. Perhaps coincidentally, *M. aerodenitrificans* can be successfully seeded into activated sludge flocs, using alginate beads as a carrier [101]. *Caproiciproducens*, containing species capable for producing caproic acid from various substrates [102], including xylose [103], and *Bacteroides graminisolvens*, a xylanolytic species previously found in an anaerobic digester treating cattle waste [104], were also found at higher relative abundances in the treated stream.

### 3.5.8. Group B, Site 9—Waste Management/Logistics

*Pectobacteriaceae* are a family of facultatively anaerobic bacteria often implicated as plant pathogens [59]. Together with *Yersiniaceae* and *Enterobacteriaceae*, the order *Enterobacterales* make up a large proportion of the microbes identified in the treated effluent of Site 9, with *Pseudomonas* and *Aeromonas* also present. Before treatment, unidentified genera of the *Micrococcaceae* family were the most common. Like at Site 3, *Lactobacillus* and *Acinetobacter* were also present, confirming abundance trends. The presence of *Proteiniphilum*, found in methanogenic environments, to accelerate propionate degradation [105], suggests a link to the high biogas yield found for this sample. A higher number of unique taxa and greater Pielou evenness were observed before treatment (67 and 0.860) versus after treatment (52 and 0.770), respectively.

### 3.5.9. Group C, Site 4—Chemicals

The most common ASV found in the untreated wastewater of Site 4 was *Legionella*, a potential pathogen found in cooling water [7]. The presence of *Legionella*, *Reyranella* (which has also been isolated from cooling towers [106]) and *Bosea*, all of which can grow within amoebae [106], as well as *Methyloversatilis*, a prey organism of *Legionella* [107], indicates that cooling water can be a significant contribution to an enterprise's wastewater. Apart from the immediate influx of certain microbes, this is also a source of treatment chemicals, such as microbicides, phosphates, or heavy metals [108], which may have downstream effects. *Rhodococcus erythropolis* [109], identified from this sample, and *Pseudoxanthomonas* [110], *Acidivorax* [111], and *Immundisolibacter* [112] species have been reported to degrade hydrocarbons, and *Parvibaculum* has been shown to oxidise linear alkylbenzene sulphonate, a commercial surfactant [113]. The presence of these ASVs may reflect the products of this chemical industry site.



### 3.5.10. Group C, Site 11—Chemicals

The microbial community of this wastewater is similar to that of the untreated effluent at Site 4. The presence of *Parvibaculum*, *Rhodococcus*, *Immundisolibacter*, *Sulfuritalea* [114], and *Acidivorax*, as well as other unknown *Comamonadaceae* species, suggests the presence of hydrocarbons and surfactants, while *Reyranella*, *Legionella*, *Bosea*, and *Methyloversatilis* all indicate that cooling-tower water is being sent to treatment. *Rurimicrobium* has been shown to increase in growth and lead to fouling in membrane bioreactors exposed to sunlight [115], conditions that are also possible in cooling towers or open trade-waste pits.

### 3.5.11. Group D, Site 12—Animal Products

Both treated and untreated wastewaters at this site had high richness (177 and 128), Shannon index (4.79 and 4.17), and Pielou evenness (0.92 and 0.86) values, respectively. Taxa identified from the untreated wastewater indicate complex inputs. *Dechloromonas* species, of the family *Rhodocyclaceae*, have been isolated from wastewater treatment processes [56], where they reduce nitrate and accumulate polyphosphate [116]. *Brachymonas* is an aerobe, with species that have been isolated from soybean waste sludge [117] and waste stabilisation ponds [118], and have applications in nitrogen removal [13]. Reflecting the high methane yields obtained in BMP assays, this wastewater supported populations of methanogens *Methanocorpusculum* [119], *Methanospirillum* [55], and *Methanomicrobiales* [120], as well as Synergistaceae, which are correlated with higher methane contents from anaerobic digestion [52]. *Anaerocella* was found in a methanogenic reactor treating rice-straw residue from cattle farm waste [121]. Revealing the industry sector at this site, the wider *Rikenellaceae* family contains mostly anaerobic bacteria frequently found in the digestive tracts of animals [74]. *Clostridium sensu stricto* 1 is also present in the digestive systems of pigs and can increase in abundance when the animal has been infected with pathogens [122]. *Arcobacter* is an animal- and food-borne pathogen that can grow at low temperatures [123]. *Williamwhitmania*, a genus including psychrophiles [124], may degrade long-chain fatty acids at low temperatures [125], a characteristic shared with WCHB1-41. At the other end of the optimal-growth-temperature scale, *Thermobrachium* is a thermophile that can grow very quickly in certain microniches [126], revealing the range of conditions under which wastewater is produced at this site.

All the above-mentioned taxa decreased in relative abundance after treatment as the overall measured diversity increased. Members of *Rhizobiaceae*, a highly diverse family [127], were present in the post-treatment sample, but further classification was not possible. As a settling process takes place as a part of the treatment at this site, the decrease in *Arcobacter* contrasts with the effects of settlers found at wastewater treatment plants [128]. Of the taxa showing increased abundance post treatment, *Giesbergia* species have been found in wastewater aeration tanks [129], and *Lentimicrobium* have been found in UASB treating high-strength wastewater [130]. The presence of bacteriolytic *Phaselicystis*, which exhibit antibiotic resistance to aminoglycoside antibiotics [131], and *Desulfobulbus*, which have implications for steel corrosion due to sulphate reduction byproducts [5], may give early warnings to operators concerned about maintenance and discharge quality issues.

## 4. Conclusions

This study investigated the taxonomic compositions of eighteen treated and untreated wastewater samples across eleven industrial sites. The novel inventories that were produced show that microbial communities in industrial wastewaters are as many and varied as the wastewaters themselves and provide significant information for the classification of trade-waste streams. In general, *Proteobacteria* was the most common phylum, while *Pseudomonas* was the genus that was present in the most samples. The connection between the conditions and a small number of identified taxa was apparent in some circumstances, such as the high sodium concentration at Site 7 favouring halophiles; however, there were limited links between the overall microbiome and the basic measurements recorded in the historical water chemistry data. *Clostridium sensu stricto* 13, *Desulfovibrio*, *Leuconostoc*, and

*Sulfurospirillum* were positively correlated with treatments, and *Acinetobacter*, *Enterococcus*, *Selenomonas*, and *Chryseobacterium* were associated with untreated samples. Among others, *Acinetobacter*, *Prevotella*, and *Lactobacillus* were found to be more common in substrates with high BMPs, while *Clostridium sensu stricto* 13, *Rhodococcus*, and *Flavobacterium* were more abundant in low-yielding wastewaters. The presence of some organisms may allow for speculation on certain internal sources of wastewater or indicate certain parameters for closer inspection, like hydrocarbons.

The richness and diversity values of the treated wastewater group were not significantly different from those of the untreated group and do not predict a substrate's suitability for anaerobic digestion. Where the cause of the decreased diversity is temporary, such as exposure to high temperatures, the BMP is not affected. Where the cause of the decreased diversity is carried within the wastewater, e.g., microbicidal compounds, the BMP is affected.

This work provides an original and valuable reference point for understanding the microbial communities present in industrial wastewaters and the information that they can provide to operators and water service providers.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/applmicrobiol4020047/s1>: Table S1: Historical Data.

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