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Research Letter

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16S rRNA gene based bacterial community structure of wastewater treatment plant effluents

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One sentence summary: A study of the microbiome in wastewater treatment plant effluent

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ABSTRACT

There is little known about the wastewater treatment plant (WWTP) effluent microbiome, its structure and dynamics. Here, we provide a study of the microbiome of effluent leaving conventional WWTPs and entering the water environment. DNA was extracted from WWTP effluent samples collected in 2015 and 2016. The bacterial communities were studied using Illumina MiSeq 16S rRNA gene amplicon sequencing and analysed using Calypso software. The *Proteobacteria*, *Bacteroides*, *Actinobacteria*, *Firmicutes*, *Tenericutes* and *Verrucomicrobia* phyla dominated the microbiomes. The bacterial community composition at high taxonomic levels is consistent between the tested WWTP effluents, and in agreement with previous studies of WWTP effluents in different global locations. The analysed microbiomes of the WWTPs shared high similarities with human faecal microbiome and contained potential human pathogens. The bacterial phyla/class composition of the bacterial communities varied greatly in both WWTP effluents in October 2015. The bacterial diversity was slightly different between studied WWTPs. Two main bacterial clusters were detected in all samples during all sampling periods. In conclusion, this work highlights the need for a better understanding of the bacterial communities in WWTP effluent. This data should be considered when analysing the risk posed by WWTP effluent to the environment and to human health.

Keywords: bacterial community; microbiome; bacterial diversity; wastewater; pathogens; effluent

INTRODUCTION

Wastewater treatment plants (WWTPs) receive wastewater from different sources including domestic waste released by urban residents, hospital wastewater and agricultural run-off. The ecology and dynamics of bacterial communities should be considered in the design and operation of wastewater treatment processes, as it allows for the prediction of possible variations in microbial community structure and its functioning under the environmental perturbation (Cydzik-Kwiatkowska and Zielinska 2016). A better understanding of bacterial communities can help to design wastewater treatment parameters and enrich the microbial ecological theory (Oerther *et al.* 2001; DeAngelis *et al.* 2011).

WWTPs can effectively reduce the bacterial load, including pathogens (Guo, Yuan and Yang 2013). The composition of bacterial communities in WWTP effluent can potentially alter the receiving ecosystem (Wakelin, Colloff and Kookana 2008; Drury, Rosi-Marshall and Kelly 2013; Garcia-Armisen *et al.* 2014; Lu and Lu 2014; Atashgahi *et al.* 2015; Price *et al.* 2018). Indeed, the WWTP effluent resulted in both an increase (Wakelin, Colloff and Kookana 2008; Garcia-Armisen *et al.* 2014; Price *et al.* 2018) and decrease (Drury, Rosi-Marshall and Kelly 2013; Lu and Lu 2014) in the diversity of bacterial communities in the receiving water environment. Monitoring the quality of WWTP effluent is essential when WWTP effluent is reused for irrigation in agriculture, as well as to prevent environmental contamination and the possible spread of human and animal pathogens.

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Our work aimed to characterize the structure and diversity of bacterial communities in the final effluent from two urban WWTPs with tertiary treatment over two years in Ireland. The bacterial community structures were identified and analysed based on targeted 16S rRNA gene amplicon sequencing. This work provides a comprehensive study of the bacterial community structure and potential pathogens present in urban WWTP effluents. We also present an analysis of bacterial diversity and clustering of the bacterial communities in WWTP effluent.

MATERIALS AND METHODS

Sample collection and WWTP characteristics

The 24 h composite samples of the final effluent were collected from two urban WWTPs (A and B) in Ireland. The characteristics of these WWTPs are presented in Table S1 (Supporting Information). The studied WWTPs were selected by Irish Water based on the following criteria: (1) they are representative of Irish Water medium-sized WWTPs and urban agglomerations, (2) they include tertiary treatment and (3) the distance between WWTPs was less than 100 km. The sampling was conducted over three consecutive days in March (10th, 11th and 12th) and October (6th, 7th and 8th) in 2015, and in March (2nd, 3rd and 4th) and September (28th, 29th and 30th) in 2016. Final effluent (2.5 L) was collected each day from each WWTP. They were transported to the laboratory in cooler boxes and analysed within 12 h.

DNA extraction, 16S rRNA gene amplification and sequencing

DNA was extracted from 250 mL of the WWTP effluent samples using the Mobio PowerWater DNA isolation kit according to the manufacturer's instructions. The DNA concentration and quality were evaluated using a DeNovix DS-11 spectrophotometer (A260/A280 ratio). The sequencing library was prepared following the 16S Metagenomic Sequencing Library guidelines (Illumina-a. 16s Metagenomic Sequencing Library Preparation). The library was pooled in the MiSeq v3 reagent cartridge, which has a standard flow cell (a single-lane) for the Illumina MiSeq platform. In each sampling period, DNA from 3 days (3 biological replicates per day) were sequenced. In September 2016, from WWTP A, the sequenced results from 3 days with eight DNA pools, and from WWTP B from 2 days with four DNA pools were analysed due to a poor DNA concentration in the other samples.

The sequenced data were filtered by Illumina chastity filter (Illumina-b. Miseq Reporter Software Guide (15042295)). The cluster of reads that had no more than 1 base call with a chastity value less than 0.6 in the first 25 cycles passed the filter. The analysis of 16S rRNA gene reads after the sequencing run were performed on BaseSpace-the Metagenomics workflow (16S Metagenomics app vesion 1.0.1.0 with Isis v2.5.35.6, Greengenes data base 13.5) (DeSantis *et al.* 2006; Illumina-c. 16s Metagenomics App). This demultiplexes reads, generates FASTQ files and then classifies reads. The 3' portion of non-index reads with low quality scores were trimmed by QualityScoreTrim in the FASTQ generation. The read classification was performed using the RDP Naïve Bayesian classifier (Wang *et al.* 2007), which provides taxonomic level classification for paired-end reads. In this process, the short sub-sequences of the reads (called words) are matched against the Greengenes databases of 16S rRNA reference sequences. The taxonomic levels are classified according to the accumulated word matches for each read. The main output is a classification of reads at kingdom, phylum, class, order, family and genus levels. Original data sets are available at the NCBI Sequence Read Archive under BioProject no. PRJNA43783, SRA accession: SRP135266.

Microbiome data analysis

Data analysis was conducted using Calypso software (http://cgenome.net/wiki/index.php/Calypso) (Zakrzewski *et al.* 2017). Sequenced data were normalized in Calypso to render the data suitable for statistical analysis. Data were filtered to remove samples with less than 1000 sequence reads. The taxa with less than 0.001% relative abundance and the rare taxa (having < 0.5 of sequences assigned in at least one sample) were removed. The relative abundance of phylum and class data were visualized by bar charts. Genera abundance were presented in a heat map with the colour code ranging from red (highly abundant) to blue (rare or absent). The relative abundances of Operational Taxonomic Units (OTUs) were compared across sampling dates and between WWTPs in all sampling periods using ANOVA. The bacterial alpha diversity and richness were estimated using Shannon and Chao 1 indices respectively, and compared in an ANOVA test. The calculated *P*-values (ANOVA) were adjusted for multiple testing by Bonferoni correction and false discovery rate. Principal component analysis (PCA) and rarefaction analyses were performed in Calypso with default parameters.

RESULTS AND DISCUSSION

General data analysis

From all samples, 11 418 560 raw reads with lengths of 301 bp were obtained. The number of reads per sample ranges from 12 096 to 357 709. The total number of passing filter reads is 10 965 998, which were used for further analysis. A total of 2013 OTUs were identified. Those found in WWTP A effluent were higher than in WWTP B.

After filtering sequenced data of WWTP A effluent samples, 30 phyla, 58 classes, 113 orders, 243 families and 635 genera were included; and 4 classes, 11 orders, 36 families and 207 genera were removed as they did not fulfil the selection criteria. From the sequencing data of WWTP B effluent samples 29 phyla, 57 classes, 114 orders, 250 families and 650 genera were included; and 1 phylum, 5 classes, 10 orders, 39 families and 192 genera were removed. The quality of sequenced data representing the diversity of the studied bacterial communities was assessed by rarefaction analysis. The rarefaction curves presented in Fig. S1 (Supporting Information) indicated sufficient sequencing depth.

Visualization of community composition

The PCA projects the relative abundance of OTUs of all WWTP effluent samples into a 2D plane (Fig. 1). Each subplot in Fig. 1 corresponds to data collected from one WWTP (A or B) at phylum or class level. Data collected in four different sampling periods (March 2015, October 2015, March 2016 and September 2016) formed four clusters, which are highlighted using four colours, respectively. The data were analysed in one cluster (based on the intra-cluster distance: the average distance between data in the same cluster to the cluster centre) and between clusters (based on the inter-cluster distance: the distance between two clusters represented by the Euclidean distances between two cluster centroids) (Wikipedia 2018).

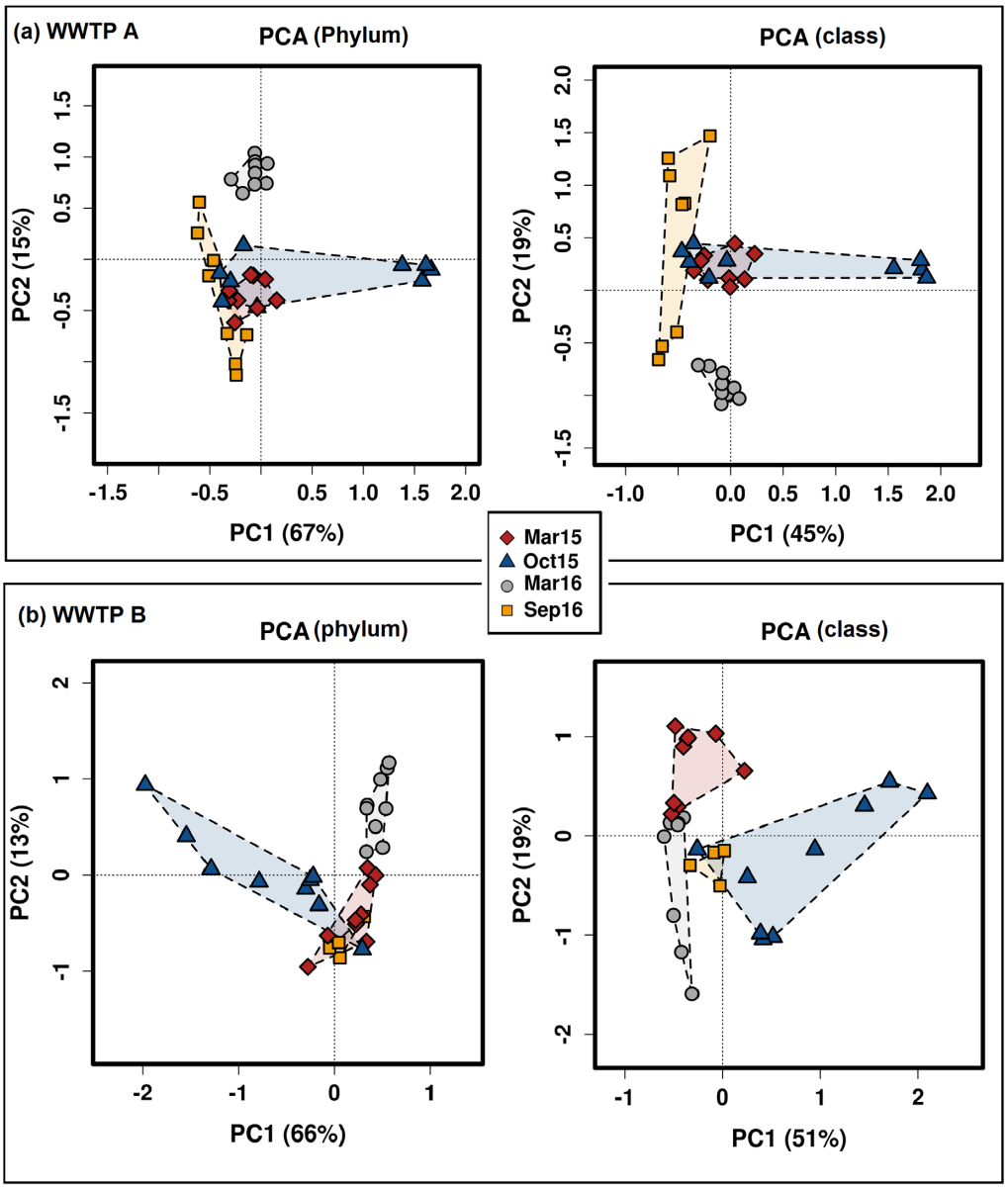

Figure 1. PCA profile of the microbial communities in WWTP effluent samples. (a) WWTP A effluent samples, (b) WWTP B effluent samples.

As can be seen in Fig. 1, data collected in March 2015–2016 and September 2016 have a small average intra-cluster distance. The data collected in October 2015 (the blue cluster) has a larger intra-cluster distance along the PC1 axis. That pattern was clearly observed in both WWTPs, and both at phylum and class level. This indicates the difference in the bacterial communities in all samples taken in October 2015. Moreover, data collected in March 2016 has the largest average inter-cluster distance to the other three clusters, which slightly overlap in all the subplots. This pattern seems to be consistent across the two WWTPs at the two taxonomic levels. This suggests the bacterial communities in March 2016 are the most different from the others.

The relative abundances of the top 20 phyla from both WWTP effluents are shown in Fig. 2a. The main phyla detected in the different sampling periods from both WWTP were similar. Among the top 20 phyla from all effluent samples, *Proteobacteria* was the most dominant with up to 67.34% of the classified reads in WWTP A effluent samples (WWTP A), and up to 64.84% in WWTP B effluent samples (WWTP B). The following dominant phyla were *Actinobacteria* (up to 32.07% in WWTP A and 49.83% in WWTP B), *Bacteroidetes* (up to 17.52% in WWTP A and 18.09% in WWTP B) and *Firmicutes* (up to 12.17% in WWTP A and 16.31% in WWTP B). The 20 most dominant classes are presented in Fig. 2b. The most abundant classes were *Betaproteobacteria* (up to 45.8% in WWTP A and 34.55% in WWTP B), *Gammaproteobacteria* (up to 38.89% in WWTP A and 35% in WWTP B) *Actinobacteria* (up to 29.33% in WWTP A and 47.67% in WWTP B) and *Alphaproteobacteria* (up to 11.95% in WWTP A and 10.07% in WWTP B).

The bacterial community composition in both WWTPs varied notably in October 2015 (Fig. 2). The relative abundance of phyla such as *Bacteroidetes* (from 1.36% in one sample to 16.05% in another), *Actinobacteria* (from 2.7% to 49.83%), *Spirochaetes* (from 0.47% to 5.07%), *Chloroflexi* (from 0.38% to 6.39%) and *Planctomycetes* (from 0.86% to 12.3%) changed remarkably between samples collected on different days. A big variation in relative abundance was also observed for the classes such as *Actinobacteria*

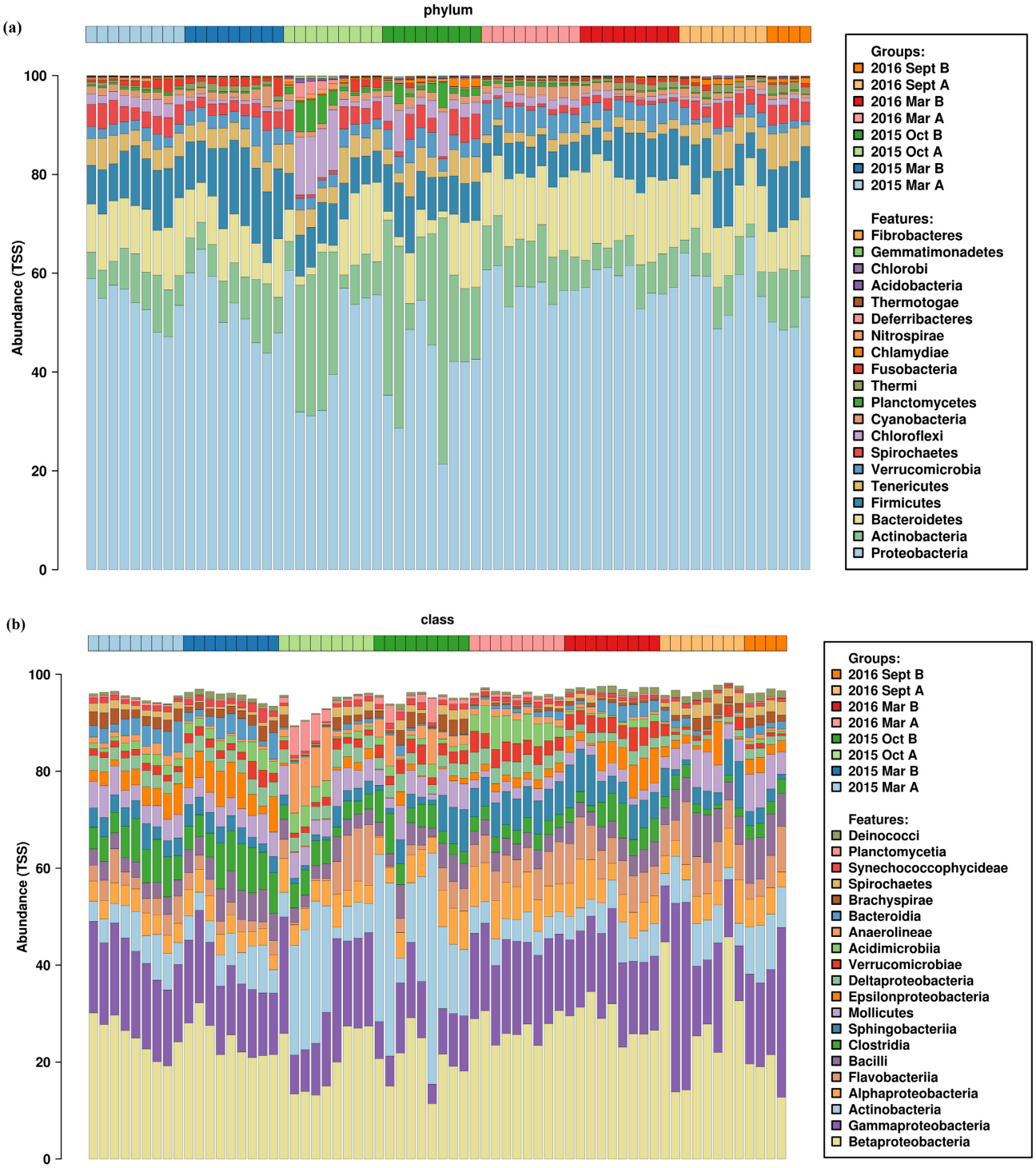

Figure 2. Taxonomic relative abundances: (a) Relative abundances of the top 20 dominant phyla in WWTP effluent samples. (b) Relative abundances at class levels for both WWTP A and B effluents.

(from 2.36% to 47.67%) and *Flavobacteriia* (from 0.23% to 12.53%). This confirms the cluster of bacterial communities in the PCA result. This variability was not seen in other studies, and the reasons for such variability are not yet known and have not previously been documented. The integration of rainfall data for Ireland did not show any significant difference over the sampling period. The temperature in October 2015 (10.7°C) was lower by about 1°C than in 2016 and 2017 (Met Éireann 2018). The strong relationship between temperature and variations in bacterial community composition was reported previously (L Liu *et al.* 2013; K Liu *et al.* 2017). Other studies demonstrated that temperature is one of the main parameters driving the change in bacterial comm0unity composition (Crump and Hobbie 2005; W Zhang *et al.* 2014).

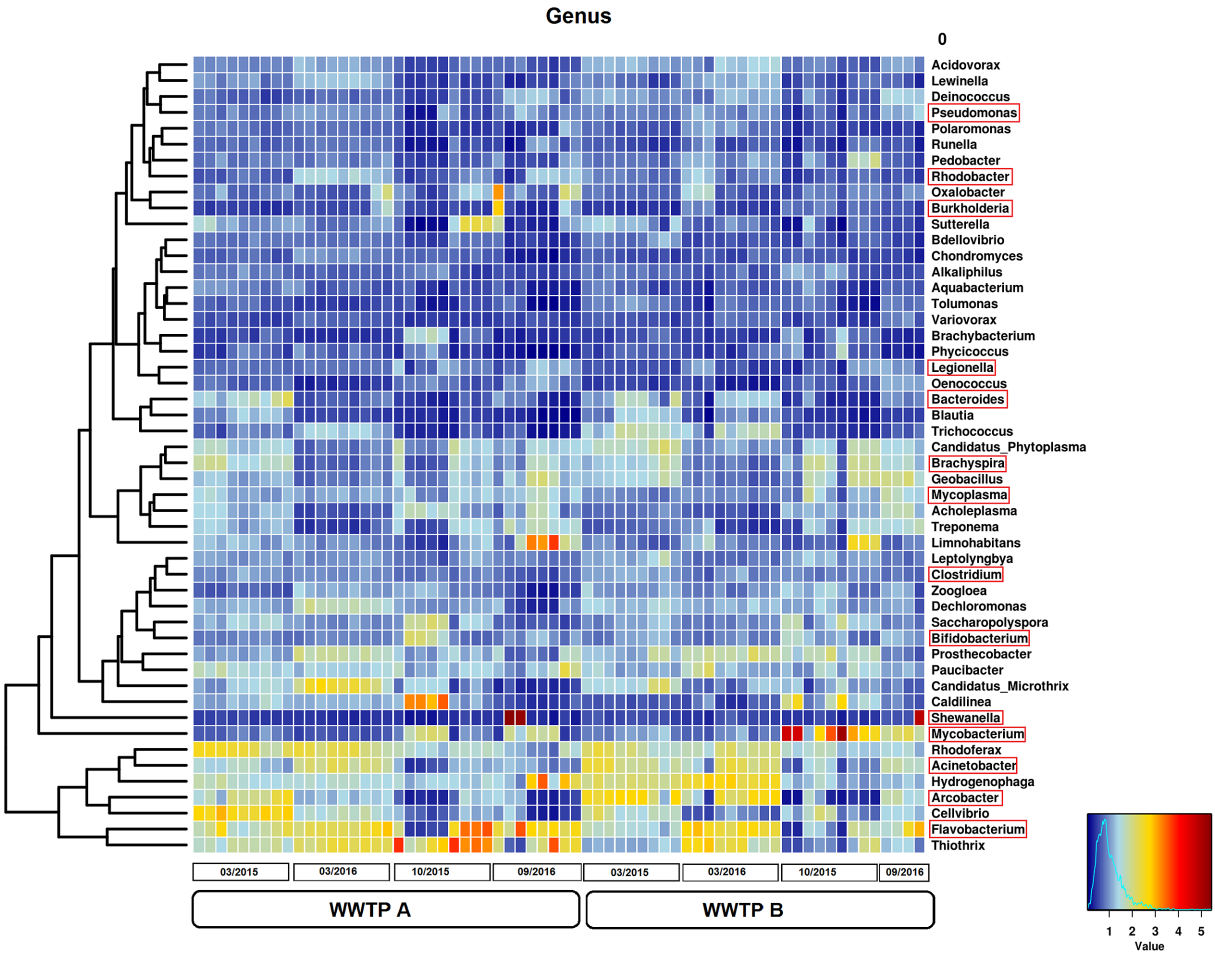

Figure 3. Relative abundances of top 50 genera and potential pathogens. The genera are listed from the highest relative abundance (*Thiothrix*) to the least relative abundance (*Acidovorax*). The pathogens are marked with a red box around their name.

The relative abundances of the top 20 most abundant phyla and classes were compared between two WWTPs in all sampling periods using ANOVA (Fig. S2, Supporting Information). The difference in the OTU relative abundance is considered significant when $P < 0.05$. The relative abundance of bacterial community composition showed a higher similarity in Autumn samples than in Spring samples. The number of phyla which have a significant difference in the relative abundance between the WWTPs were 3 (March 2015) and 5 (March 2016); the number of classes were 4 (March 2015) and 3 (March 2016) (Fig. S2, Supporting Information). In the Spring samples, 13 phyla and 10 classes showed a significant difference in their relative abundance between the WWTPs. This result matches the PCA profile (Fig. 1), where the bacterial communities in March 2016 are most different from March 2015 and from others.

The same treatment type in the studied WWTPs may have led to the similarities in the bacterial community structure in their final effluent. The bacterial community structure from tested WWTP effluents were similar to previous studies in Hong Kong, Denmark, Belgium and Colombia (Adrados *et al.* 2014; Cai, Ju and Zhang 2014; Garcia-Armisen *et al.* 2014; Silva-Bedoya *et al.* 2016). The most abundant phyla detected in the WWTP effluents were also found in influent in Denmark and Hong Kong, but the effluent bacterial communities are less affected by, or have no relation to, influent bacterial communities (Adrados *et al.* 2014; Cai, Ju and Zhang 2014). The most predominant taxa found here such as *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* were also found to be the most abundant in water surfaces (lakes, rivers); mineral, drinking and wastewater (Vaz-Moreira, Nunes and Manaia 2014). The presence of these core bacteria in different WWTP effluents might be the result of a similar composition of wastewater in different WWTPs (Tchobanoglous, Burton and Stensel 2003). The similarities in microbiome composition at high taxonomic levels between WWTP effluents in our work and those in other WWTPs from different locations suggest that the bacterial composition of effluent entering the environment is consistent between WWTPs (Adrados *et al.* 2014; Cai, Ju and Zhang 2014; Silva-Bedoya *et al.* 2016).

Potential bacterial pathogens in the WWTP effluent

The phyla *Bacteroidetes*, *Actinobacteria*, *Firmicutes* and *Proteobacteria* were the most predominant in all samples, which are reported as the most abundant in the human microbiome (Davenport *et al.* 2014; Fernandes *et al.* 2014; Thursby and Juge 2017). These phyla were also found in water surfaces, mineral and drinking water and soil (Janssen 2006; Vaz-Moreira, Nunes and Manaia 2014; Miyashita 2015). Other phyla such as *Verrucomicrobia*, *Spirochaetes*, *Cyanobacteria*, *Fusobacteria* and *Chloroflexi* were also detected in the top 20 most abundant phyla in our work. The same dominant phyla detected in WWTP effluent and other

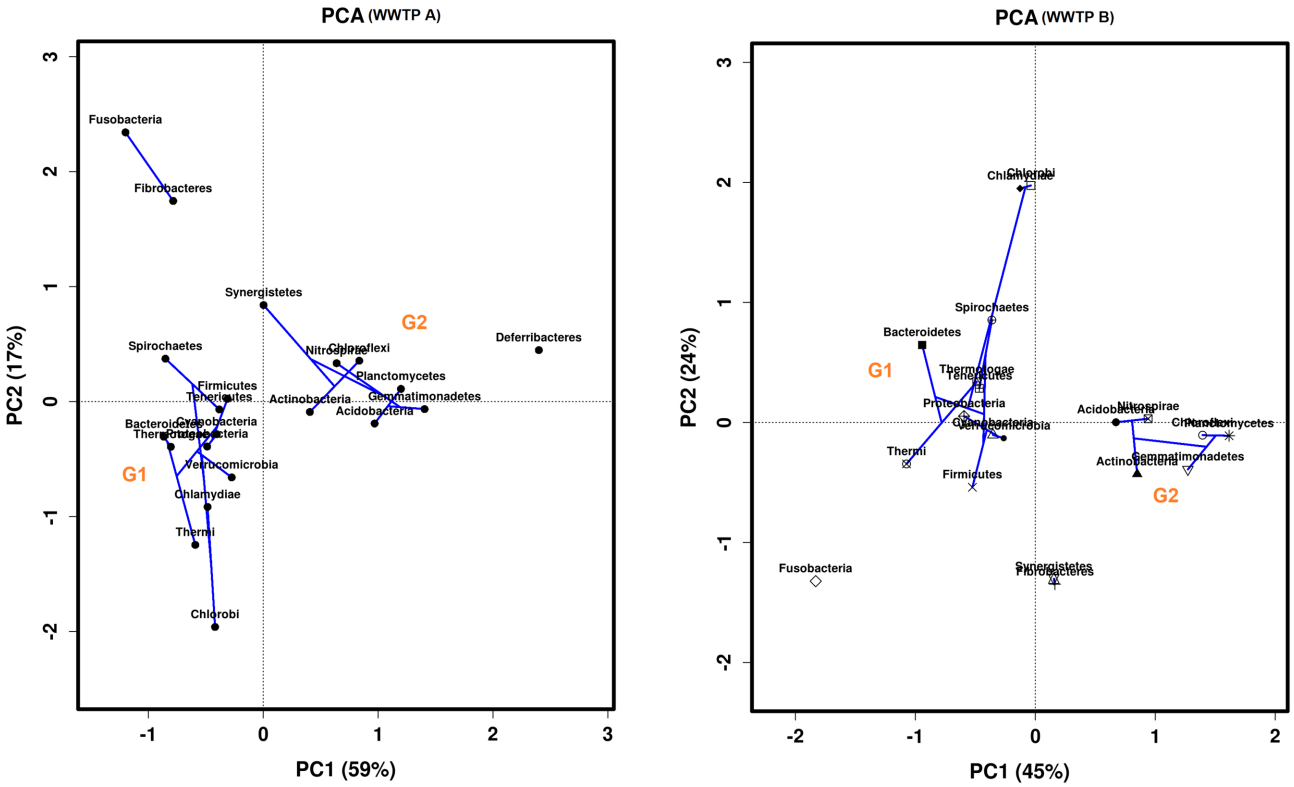

Figure 4. Clustering analysis of microbial phyla in WWTP effluent samples. Microbial phyla were ordinated by PCA, and the phyla sharing high similarities were grouped into clusters by hierarchical clustering.

sources indicate a high similarity between the microbiota of these environments at the analysed taxonomic rank.

The relative abundances of the top 50 genera detected in all WWTP effluent samples are shown in Fig. 3. Among them, many bacteria found in the human gut (*Flavobacterium*, *Acinetobacter*, *Bifidobacterium*, *Clostridium*, *Blautia*, *Bacteroides*, *Pseudomonas* and *Prevotella*) and sources including surface water (*Flavobacterium*) and soil (*Flavobacterium*, *Acinetobacter* and *Pseudomonas*) were detected. Other human gut bacteria such as *Ruminococcus*, *Enterococcus*, *Dorea* and *Faecalibacterium* were found < 0.3% in our study (Furet *et al.* 2009; Cai and Zhang 2013; Cai, Ju and Zhang 2014; Backhed *et al.* 2015). We also detected genera present in the human body: *Streptococcus*, *Lactobacillus*, *Corynebacterium*, *Moraxella*, at a low relative abundance (< 0.3%) (Ravel *et al.* 2011; Huttenhower *et al.* 2012). The total abundance of these genera (an average of 12.15% in WWTP A and 12.47% in WWTP B) show that these bacteria may have originated from human microbiome, besides other sources (soil) and they may contribute to shaping the bacterial profile in WWTP effluent. In the top 50 genera, 14 genera (28%) could be potential pathogens (Table 1). Most potential pathogens were found at a higher relative abundance in WWTP B effluent samples than in WWTP A effluent samples. The bacterial composition was found to vary with geographic location, and the differing wastewater characteristics between WWTPs can also lead to differences in bacterial composition (Ma *et al.* 2013; T Zhang, Shao and Ye 2012). The genera present in the top 50 such as *Flavobacterium*, *Arcobacter*, *Acinetobacter*, *Mycobacterium* and *Shewanella* (Table 1) are of most significance to human health. It suggests that WWTP effluent may be considered as a potential source of the release of bacterial pathogens into the environment. Bacterial species in the genus *Acinetobacter* are known as opportunistic human pathogens (Visca, Seifert and Towner 2011). *Acinetobacter baumannii* is of imperative clinical importance and a difficult pathogen to treat. The association of this bacterium with diseases such as pneumonia, bacteraemia, meningitis, wound infections, bloodstream infections and urinary tract infections have been well reported (Dijkshoorn, Nemec and Seifert 2007). *Mycobacterium tuberculosis*, a member of the genus *Mycobacterium*, is a medically important pathogen which causes tuberculosis (Smith 2003). The presence of pathogenic *Mycobacterium* was reported in previous studied in WWTP effluent (Cai and Zhang 2013; Cai, Ju and Zhang 2014). The species of the genus *Shewanella* implicated in human infections are *S. algae*, *S. putrefaciens*, *S. haliotis* and *S. xiamenensis* and incidences of infections are low (Janda and Abbott 2014; PY Liu *et al.* 2013). However, *Shewanella* spp. has been identified as a progenitor, reservoir and vehicle for the transmission of resistance genes and multi-drug resistance plasmids, and is now classified as an emerging cause of human infections (Yousfi *et al.* 2017). There are some reports of *Flavobacterium* infections in humans. An outbreak of respiratory infections caused by *Flavobacterium* spp. occurred in the 1980s (Flaherty *et al.* 1984; Liebert *et al.* 1984). More recently, a *F. lindanitolerans* strain was isolated from an ascites patient in China, who died of fatal pulmonary edema and haemorrhage (Tian *et al.* 2011). Three species in the genus *Arcobacter: A. butzleri*, *A. cryaerophilus* and *A. skirrowii*, have been shown to be involved in human diseases (Vandenberg *et al.* 2004; Ferreira *et al.* 2014; Figueras *et al.* 2014). *Arcobacter butzleri* is known as a causative agent of abdominal cramps and persistent, watery diarrhoea (Arguello *et al.* 2015). They can survive in aquatic environments and therefore may cause a threat to human health downstream from their site of release, or if used to irrigate vegetables (Rangel *et al.* 2005; Rubino, Cappuccinelli and Kelvin 2011; Søraas *et al.* 2013). The genera *Enterococcus* and

Table 1. Relative abundance of potential pathogens (within top 50 abundant genera) detected in WWTP effluent samples. The calculated P-values (Anova) were adjusted for multiple testing by Bonferoni correction and false discovery rate (FDR). The relative abundance of microbial genera was compared across sampling periods. There was a significant difference in microbial relative abundance when $P < 0.05$.

	Relative abundance (%) in WWTP A effluent samples							Relative abundance (%) in WWTP B effluent samples						
Genus	P-Anova	Adjusted P (Bonferroni)	FDR	Mar15 mean	Oct15 mean	Mar16 mean	Sep16 mean	P-Anova	Adjusted P (Bonferroni)	FDR	Mar15 mean	Oct15 mean	Mar16 mean	Sep16 mean
Mycobacterium	0.0043	0.077	0.0065	0.64	2.11	0.35	2.16	0.000015	0.00054	0.00005	1.04	12.73	0.71	4.18
Acinetobacter	2.2E-12	1E-10	2.8E-11	1.95	0.87	4.17	1.08	0.000067	0.0021	0.00018	4.25	1.13	3.73	2.93
Arcobacter	0.0000015	0.000052	0.0000047	3.77	0.8	1.56	0.76	0.0000052	0.00019	0.000019	6.17	0.49	4.73	2.6
Bacteroides	6.9E-09	0.00000027	0.000000029	2.63	0.27	0.5	0.47	0.00088	0.014	0.0013	1.92	0.27	1.35	0.87
Clostridium	0.0000022	0.000075	0.0000065	1	0.69	0.91	0.47	0.00011	0.0031	0.00024	1.23	0.82	0.84	0.59
Rhodobacter	0.0000007	0.000026	0.0000025	0.47	0.6	1.87	1.2	0.00098	0.015	0.0014	0.73	0.41	1.05	0.32
Brachyspira	0.0000086	0.00027	0.000022	2.77	1.35	0.53	1.71	0.00027	0.0062	0.00048	2.07	2.58	0.63	2.09
Pseudomonas	0.0096	0.15	0.014	0.72	0.47	1.02	0.95	0.00000069	0.000029	0.0000038	0.79	0.41	1.01	1.51
Mycoplasma	0.069	0.54	0.078	1.48	1.47	1.19	1.89	0.00052	0.0094	0.00079	0.82	1.39	0.83	2.48
Flavobacterium	0.28	0.96	0.29	3.82	5.2	5.56	6.9	0.00000017	0.0000076	0.0000014	2.35	1.62	6.61	5.13
Legionella	0.08	0.54	0.089	0.6	0.87	0.65	1.05	0.07	0.6	0.081	0.35	0.59	0.58	0.97
Bifidobacterium	0.015	0.21	0.02	0.76	2.05	0.78	1.06	0.096	0.67	0.11	1.04	1.33	0.74	1.33
Shewanella	0.068	0.54	0.078	0.24	0.15	0.16	7.25	0.064	0.6	0.076	0.33	0.2	0.27	6.11
Burkholderia	0.26	0.96	0.27	0.23	0.33	0.7	1.46	0.0013	0.049	0.002	0.18	0.61	0.44	0.34

Escherichia were found at an average of 0.03% and 0.02% in both WWTPs. Even though these bacteria are indicators of faecal contamination, they are not the most prevalent in wastewater (Vaz-Moreira, Nunes and Manaia 2014). The analysis of 16S rRNA gene sequences in our work determined the presence of the bacterial genera but not species. These genera may contain both pathogenic and non-pathogenic species. Therefore, the identification of pathogens requires further study.

Alpha diversity, richness and evenness

The alpha diversity of the bacterial community compositions of WWTP effluent samples were analysed with the Shannon's diversity index. The Shannon's diversity index is a measure that takes into account the species richness and evenness (Hollenbeck and Ripple 2007). These parameters were analysed to further understand the bacterial diversity in WWTP effluent. The OTU diversity in the bacterial community of WWTP A effluent samples are shown in Fig. S3(a) (Supporting Information) and Table 2. A significant difference in Shannon's diversity index was identified within the bacterial community in WWTP A effluent samples at all taxonomic ranks with $P < 0.05$. The median of Shannon's diversity indexes distinguished between microbiotas of effluent samples collected in Spring and samples collected in Autumn in WWTP A (Table 2). The diversity indexes were slightly higher in Spring than in Autumn. The alpha diversity of the bacterial community in WWTP B effluent samples is presented in Fig. S3(b) (Supporting Information) and Table 2. The significant differences in Shannon's diversity were identified in the microbiota at phylum and family levels with $P < 0.05$, but not at other taxonomic levels ($P > 0.05$). The Shannon's diversity indexes (at phylum and family level) were higher in effluent samples collected in Autumn than those in Spring. The difference in bacterial diversity between different sampling locations depends on the physical and chemical variables which likely select for different bacterial types (Jordaan and Bezuidenhout 2013; Silva-Bedoya *et al.* 2016).

The richness of bacterial communities was estimated using Chao 1 indexes (Fig. S4 and Table S2, Supporting Information). The median values of Chao 1 indexes were higher in Spring than those observed for Autumn from both WWTPs, indicating that the bacterial richness from both WWTPs was higher in Spring than in Autumn. It determined the seasonal change in the number of species in all samples. This number was higher in Spring (mean temperature was 6.3°C, in March 2015 and 6.6°C in March 2016) when the temperature was lower, compared to Autumn (10.7°C in October 2015 and 14.7°C in September 2016). The higher richness estimations at time-points with lower temperatures were reported previously in the water environment (Gilbert *et al.* 2010; Ghiglione and Murray 2012). The low bacterial activity in cold weather may cause the species distribution to be more even in cold seasons than in warm seasons. However, other environmental factors can also affect bacterial richness. The Shannon's diversity indices were significantly different for all taxonomic levels in WWTP A effluent samples, and for class and genus levels in WWTP B ($P < 0.05$).

The evenness of bacterial communities from all WWTP effluent samples are shown in Fig. S5 and Table S3 (Supporting Information). There was a significant difference in bacterial evenness in WWTP A effluent samples at all taxonomic ranks across sampling periods ($P < 0.05$). The evenness indexes were similar in 2015 for all sampling periods and slightly higher in 2016 in Spring than in Autumn. In WWTP B effluent samples, a significant difference in evenness was observed for phylum and family levels ($P < 0.05$). At phylum rank, the evenness indexes were lower in Spring than in Autumn. However, at family rank these values were similar in 2015, and were lower in Spring than in Autumn in 2016. Wittebolle *et al.* reported that the functioning of bacterial communities with a low evenness is less resistant to environmental stress (Wittebolle *et al.* 2009). Indeed, the community with low evenness is dominated by one or few species, and the resistance to environmental stress will only occur if these dominant species are tolerant to the stress. Another study by Mukherjee *et al.* showed that the evenness of the bacterial community was lower at higher pollution levels (Mukherjee *et al.* 2014). The discrepancy in the evenness may distribute the difference in the seasonal change of bacterial diversity between two WWTP effluents.

Table 2. Medians of Shannon's diversity indexes estimated in two WWTP effluent samples.

Median of Shannon's diversity indexes in WWTP A effluent samples										
Time	Phylum		Class		Order		Family		Genus	
	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index
Mar15	0.00113	1.63	1.65e-06	2.63	3.73e-09	3.28	6.55e-07	3.85	9.02e-08	4.62
Oct15		1.64		2.55		3.24		3.84		4.54
Mar16		1.52		2.54		3.27		3.80		4.67
Sep16		1.50		2.33		2.93		3.45		4.09
Median of Shannon's diversity indexes in WWTP B effluent samples										
Time	Phylum		Class		Order		Family		Genus	
	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index	P-value (Anova)	Shannon index
Mar15	2.75e-05	1.66	0.142	2.70	0.113	3.32	0.00733	3.88	0.724	4.66
Oct15		1.79		2.53		3.18		3.89		4.61
Mar16		1.48		2.46		3.12		3.69		4.58
Sep16		1.72		2.61		3.40		3.99		4.81

Clustering analysis of bacterial phyla across sampling periods

The cluster structure of the phyla across sampling dates was studied based on the relative abundances of the individual phylum in all WWTP effluent samples. Each identified phylum were ordinated by PCA, and the phyla sharing high similarities were grouped into clusters by hierarchical clustering. The phylum clusters of both WWTP effluents are presented in Fig. 4. The bacterial phyla clustered in two main groups (G1 and G2), which shared key members in both WWTP. Indeed, key phyla of group 1 from both WWTP effluent samples (G1) were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Tenericutes*, *Thermotogae*, *Thermi*, *Spirochaetes*, *Chlamydiae* and *Chlorobi*. The group 2 (G2) consisted of *Planctomycetes*, *Chloroflexi*, *Nitrospirae*, *Actinobacteria*, *Gemmatimonadetes* and *Acidobacteria*. All samples shared the same outliers including *Fusobacteria* and *Fibrobacteres*. As the clustering structure was similar between the two WWTPs, it suggests that the phyla grouped with each other independent of WWTP location.

The cluster structure of bacterial phyla in each WWTP effluent in each sampling year are shown in Fig. S6 (Supporting Information). Two main groups were also detected in both WWTPs with similar key cluster members and the same outlier phyla (*Fusobacteria*) for all sampling times. It is unlikely that the clustering structure originated from this temporal factor (years). The average relative abundances of all phyla in each group were plotted for each sampling year (Fig. S7, Supporting Information). It is clear that in both WWTPs that the relative abundance of all phyla in G1 was significantly higher than that observed in G2. It suggests that these groups of phyla in WWTP effluent might be formed due to the difference in their relative abundance. The microbiome data needs to be considered when analysing the risk of WWTP effluent to the environment and human health, as many of the bacteria identified are not analysed when assessing the risk of pollution from WWTPs globally.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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