

CRANFIELD UNIVERSITY

Cranfield Water Science Institute
School of Water, Energy and Environment

**Engineering Biological Wastewater Treatment for the Removal of
Hazardous Chemicals**

Lawson Mensah

PhD

Academic Year: 2014 - 2017

Supervisors:
Dr. Pablo Campo Moreno
Dr. Mark Scrimshaw

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THESIS ABSTRACT

The European Union's Environmental Quality Standards (EQS) continue to be stringent with regards to discharge of hazardous chemicals (HCs) (mainly organic micropollutants) from wastewater treatment plants (WWTPs). The strict limits are driven by the growing interest in wastewater reuse, evidence of feminisation of male aquatic species and bioaccumulation of HCs in water-based biota. WWTPs are the last barrier to these chemicals getting into the environment from homes, institutions and industries and therefore optimisation of existing WWTPs is critical. The operational conditions of the WWTPs such as solid retention time (SRT), hydraulic retention time (HRT), dissolved oxygen (DO) concentration and seasonal temperature variations have been proven to influence the removal of these HCs and also affect the microbial/bacterial diversity in WWTPs. This study therefore aimed to find the missing link in the literature, which is to seek the relationship between the microbial/bacterial diversity or abundance and HCs removal in WWTPs. A pilot-scale study was conducted to analyse the microbial and bacterial diversity using phospholipids fatty acid and 16S rRNA analysis respectively at 3, 10 and 27 d SRT (at constant 8 h HRT) and then repeated at a fixed 27 d SRT while HRT varied at 8, 16 and 24 h. The concentrations of nonylphenols and estrogens in the influent and effluent were also analysed using LC-MS/MS to determine the plant's chemical removal efficiency. The results showed that raising SRT (from 3 to 27 d) and HRT (from 8 to 24 h) increased bacterial diversity by 2.7 times and increased the removal of EE2 by 11%. The pilot-scale also revealed 32 novel positive correlations between bacterial genera abundance and HCs removal. The abundances of *Hyphomicrobium*, *Mesorhizobium*, *Planctomyces*, and *Rhizomicrobium* positively correlated with the removals of estrogens (E1, E2 and E3) and with nonylphenols (NP₂EC, NP₅EO) at *r*-values >0.7.

The experiment was repeated by surveying the bacterial diversity in 12 full-scale activated sludge plants (ASPs) operating at varying DO levels, SRTs (4.9 to 22.3 d), HRTs (6.2 to 26.6 h) and temperature (10.6 to 20.3 °C). The concentrations of 26 micropollutants (analgesics, antibiotics, anti-depressants, beta-blockers, estrogens, flame retardants etc.) were also analysed in the influent and effluent. The results showed that bacterial diversity in the full-scale reactor differed from that of the pilot-scale plant. Euclidean distance matrix shows that temperature (*p*<0.005) was the most influential parameter in chemical removal and

bacterial diversity. SRT and HRT were also important in bacterial diversity and HC removal but not in every ASP. Removal of HC followed a pseudo first order kinetics with metformin and ibuprofen achieving >98% removals. DO concentration did not show any effect due to lack of variation across the WWTPs. Once again, some novel correlations between bacterial genera abundance and chemical removal were found. The abundances of *Candidatus Accumulibacter* (a phosphate accumulating bacteria capable of denitrification) and *Nitrosomonas* (nitrite-oxidising bacteria) correlate positively with the removal of trixylenyl phosphate (flame retardant) at $R>0.7$. *Anaeromyxobacter dehalogenans* abundance also correlates positively with the removal of erythromycin. This work has shown that process parameters do influence both bacterial diversity and hazardous chemical removal, and there are correlations between bacteria taxa abundance and HCs removal. This knowledge will be vital in the discussion on improving existing activated sludge plants achieve better chemical removal and it will also be the foundation for future research into correlations of bacterial taxa abundance with chemical removal in the ASP reactor.

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ABBREVIATIONS

ACN	Acetonitrile
ACT	Actinomycete
AOB	Ammonium oxidising bacterium
AMO	Ammonia mono-oxygenase
APHA	American Public Health Association
ASP	Activated sludge plant
ASR	Activated sludge reactor
ATNL	Atenolol
ATOVo	o-Atorvastatin
ATOVp	p-Atorvastatin
β -PRO	Betaproteobacteria
BOD	Biochemical oxygen demand
BZT	Benzotriazole
CBAZ	10,11-epoxy carbamazepine
CBAZe	Carbamazepine
CIP	Chemical Investigation Programme
CIPR	Ciprofloxacin
CLMY	Clarithromycin
COD	Chemical oxygen demand
DCF	Diclofenac
DCM	Dichloromethane
DNA	Deoxy-ribonucleic acid
DO	Dissolved oxygen
E1	Estrone
E2	17 β -estradiol
E3	Estriol
EE2	17 α -ethynylestradiol
E1-3S	Estrone 1-3 sulphate
EPS	Extracellular polymeric substance
ERMY	Erythromycin
ERMYn	Nor-erythromycin
EPSRC	Engineering and Physical Science Research Council
EQS	Environmental Quality Standards
EtOAc	Ethyl acetate
FE	Final effluent
FLXT	Fluoxetine
GAC	Granular activated carbon
GC	Gas chromatography
GPC	Gel permeation chromatography
GRAM+	GRAM positive bacteria
GRAM-	GRAM negative bacteria
HCS	Hazardous chemicals
HPLC	High-performance liquid chromatography

HRT	Hydraulic retention time
IBPF	Ibuprofen
MeOH	Methanol
METF	Metformin
MLSS	Mixed liquor suspended solids
NOB	Nitrite oxidising bacterium
NP	Nonylphenols
NP _x EC	Nonylphenol carboxylate
NP _x EO	Nonylphenol ethoxylates
NSAID	Non-steroidal anti-inflammatory drug
PCA	Principal component analysis
PE	Population equivalent
PRPL	Propranolol
PNEC	Predicted no effect concentration
RAS	Return activated sludge
RNTD	Ranitidine
SERM	Selective estrogen receptor moderator
SERT	Sertraline
SERTn	Norsertraline
SPE	Solid phase extraction
SRT	Solid retention time
SS	Suspended solids
SSVI	Suspended solids volume index
STWs	Sewage treatment works
TMXF	Tamoxifen
TOC	Total organic carbon
TOXN	Total oxidizable nitrogen
TP	Total phosphorus
TS	Total solids
TXP	Trixylenyl phosphate
TZT	Tolyltriazole
UKWIR	UK Water Industry Research
UP	Ultra-pure
VS	Volatile solids
WAS	Wasted activated sludge
WWTP	Wastewater treatment plant

NOTATIONS

F:M	Food-to-microorganisms
K _{biol}	Biodegradability
K _{oc}	Octanol-carbon coefficient
K _{ow}	Octanol-water coefficient
K _d	Sorption coefficient
14:0i	12-methyltridecanoic acid
15:0i	13-methyltetradecanoic acid
15:0ai	12-methyltetradecanoic acid
15:1 ω 4	Pentadec-4-enoic acid
10Me16:0	10-methylhexadecanoic acid
16:0i	14-methyltetradecanoic acid
16:1 ω 9c	Cis-hexadec-9-enoic acid
16:1 ω 5c	Cis-hexadec-5-enoic acid
16:1 ω 7c	Cis-hexadec-7-enoic acid
17:0i	15-methylhexadecanoic acid
17:0ai	14-methylhexadecanoic acid
10Me17:0	10-methylhexadecanoic acid
cy17:0	Cyclo-heptadecanoic acid
17:1 ω 9c	Cis-heptadec-9-enoic acid
18:1 ω 7t	Trans-octadec-7-enoic acid
18:1 ω 9c	Cis-octadec-9-enoic acid
18:3 ω 3	Alpha-linolenic acid
18:2 ω 6	(9Z,12Z)-octadeca-9,12-dienoic acid
18:1 ω 9t	Trans-octadec-9-enoic acid
10Me18:0	10-methyloctadecanoic acid
cy19:0	Cyclo-nonadecanoic acid
20:5 ω 3	(5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-eicosapentaenoic acid

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 RATIONALE OF THE RESEARCH

This research aims to find out how the microbial diversity in activated sludge treatment processes (ASPs) is affected by the engineering parameters of a wastewater treatment plant (WWTP) and how the microbial diversity influences the removal of chemicals in the treatment of wastewater.

WWTPs are sources of ubiquitous chemicals into the environment. The chemicals are mainly synthetic organic compounds with different physical and chemical properties, and their uses range from pharmaceutical to industrial to domestic. They have acute toxicological effects ranging from behavioural abnormalities, genetic mutation potential, endocrine disrupting capabilities and physiological malfunctions on aquatic species. They also have varying degrees of degradability, with some recalcitrant chemicals seemingly non-degradable, leading to bioaccumulation in the environment. These chemicals are therefore collectively referred to as *hazardous chemicals* (HCs) in this study.

The European Union's Environmental Quality Standards (EQS) continue to be stringent (Vorkamp and Sanderson, 2016) with regards to the discharge of HCs from WWTPs. The strict limits are driven by the growing interest in wastewater reuse (Bixio et al., 2005; Gomez et al., 2012), evidence of feminisation of male aquatic species (Kidd et al., 2007; Blanchfield et al., 2015) and bioaccumulation of HCs in water-based biota. The increasing regulatory requirement by the EU implies that new WWTPs are built to be efficient in removing significant amounts of the HCs, and existing plants should be improved or optimised to meet future regulations, because existing WWTPs were built for the removal of nutrients and organic matter, so their serendipitous removal of HCs is a bonus.

Improved HCs removal at existing WWTPs may be achieved through the addition of advanced tertiary treatment systems such as microfiltration-reverse osmosis to remove dissolved inorganic solids or advanced oxidation processes (UV/O₃ or UV/H₂O₂) for the degradation of recalcitrant organic compounds, but these will result in about £45 billion (UKWIR, 2013) additional costs to wastewater companies,

arising from capital investment, increased energy and chemical usage. Alternatively, some existing WWTPs could be operated differently to improve their HC removal, as shown in several pilot-scale studies (Li et al., 2011; Petrie et al., 2014; Ito et al., 2015; Coello-Garcia et al., 2019) to reduce the need for tertiary treatment processes and potentially minimise carbon emission, chemical use and costs.

Typically, a full-scale WWTP would be operated between 10 and 12 d solids retention time (SRT), 8 – 16 hours hydraulic retention time (HRT) and 2 – 3 mg/L dissolved oxygen (DO), with reactor temperature varying seasonally. Bench-scale studies have shown that increasing the SRT and HRT beyond typical range increases the removal of HCs in the ASP. For instance, Akarsubasi et al., (2009), Clara et al., (2006) and Petrie et al., (2014) reported that increasing SRT subsequently increased the percentage of HCs removed, and the marginal gain in removal depends on the extent of SRT increase and other operational parameters. Petrie et al., (2014) also found that at a constant 27 d SRT, increasing HRT from 8 to 24 h resulted in further removal of steroid estrogens by 10%. Seasonal temperature rises in full-scale WWTPs, and the deliberate increase of temperature in laboratory-scale experiments in ASP has also led to improvements in the removal of HCs (Thompson et al., 2011).

Literature shows that the structural complexity of a WWTP affects the microbial community and the differences in engineering parameters resulted in diversity changes in microbial communities (Harris et al., 2012). For example, Sailkaly et al., (2005) found that at lower SRTs, the microbial diversity was higher, Akarsubasi et al., (2009) reported that between 2.6 and 10.4 d SRT the microbial diversity was statistically similar, whilst Lui et al., (2007) reported that there are significant differences in microbial diversity at 4, 8, 16 and 32 d SRTs, with more significant differences as the SRT increased. These findings are inconsistent and often reported in isolation from other process parameters. HRT increases caused low microbial density as it reduced organic loading rate and food-to-microorganisms ratio (Almasi et al., 2016). In a global study of ASPs, variation in temperature was found to be the main contributor to differences in microbial communities in WWTPs (Wu et al., 2019).

Even though the effects of process parameters on the removal of HCs and on microbial diversity are known, the influence of bacterial taxa abundance on HC removal remains unknown. Therefore, a gap in the literature where the combined effects of SRT, HRT, DO and temperature on the microbial diversity and taxa abundance in ASPs are under-reported; and the correlations between bacterial diversity taxa abundance and HCs removal is unknown.

Hence, the rationale for this work is to study the effects of process parameters (SRT, HRT, DO temperature) on microbial/bacterial diversity in a pilot and full-scale ASPs, and to characterise the influence of microbial diversity and abundance on the removal of HCs. The novelty of this project is to provide correlations between bacterial taxa abundance and the removal of various HCs in WWTPs.

The study is in two parts with three technical chapters. The first part (chapter 3) studies the correlations of microbial taxa with the removal of nonylphenols and estrogens at pilot-scale. The second part is the full-scale study to assess the influence of process parameters on microbial/bacterial diversity and the influence of diversity on the removal of hazardous chemicals, replicating the pilot-scale study. Due to the enormity of microbial/bacterial diversity data, chapter four is the first half of the full-scale study and it discusses the influence of process parameters on microbial and bacterial diversity. Chapter five is the second half of the full-scale study and presents the results on the chemical analysis and subsequently the correlations between bacterial taxa abundance and chemical removal.

1.2 AIMS AND OBJECTIVES

The thesis presents research funded by the UK Water Industry Research and the Engineering and Physical Sciences Research Council. The research aim is to understand the effects of engineering process parameters on microbial/bacterial diversity and to explore the influence of various bacteria taxa abundance on the removal of hazardous chemicals. The outcomes will inform the discussion on improving of existing ASPs towards compliance with future EQSs. The objectives are:

- 1.** Adapt the phospholipid fatty acid analysis for use in studying microbial diversity in activated sludge mixed liquor.
- 2.** Examine the effect of process parameters on microbial/bacterial diversity and chemical removal and then assess correlations between bacteria taxa and hazardous chemical removal in pilot-scale ASP.
- 3.** Survey of the influence of process parameters on microbial diversity and chemical removal in full-scale WWTPs and assess the correlations between bacterial taxa abundance and chemical removal in full-scale ASPs.

1.3 THESIS PLAN

This thesis is written in the format of papers for publication. The thesis was authored by Lawson Mensah and edited by Dr Pablo Campo-Moreno. All the work was done by the author except the following. The 16S rRNA analysis in chapter 3 was done by Gregg Icton of PROKARYA LTD, the running of the 16S rRNA analysis on the Ion Torrent PGM and the bioinformatic data processing was done by Tamara Coello-Garcia and the hazardous chemical analysis in the full-scale works was done by ALS Laboratories, Coventry. The flow chart of the thesis plan is shown in Figure 1.1.

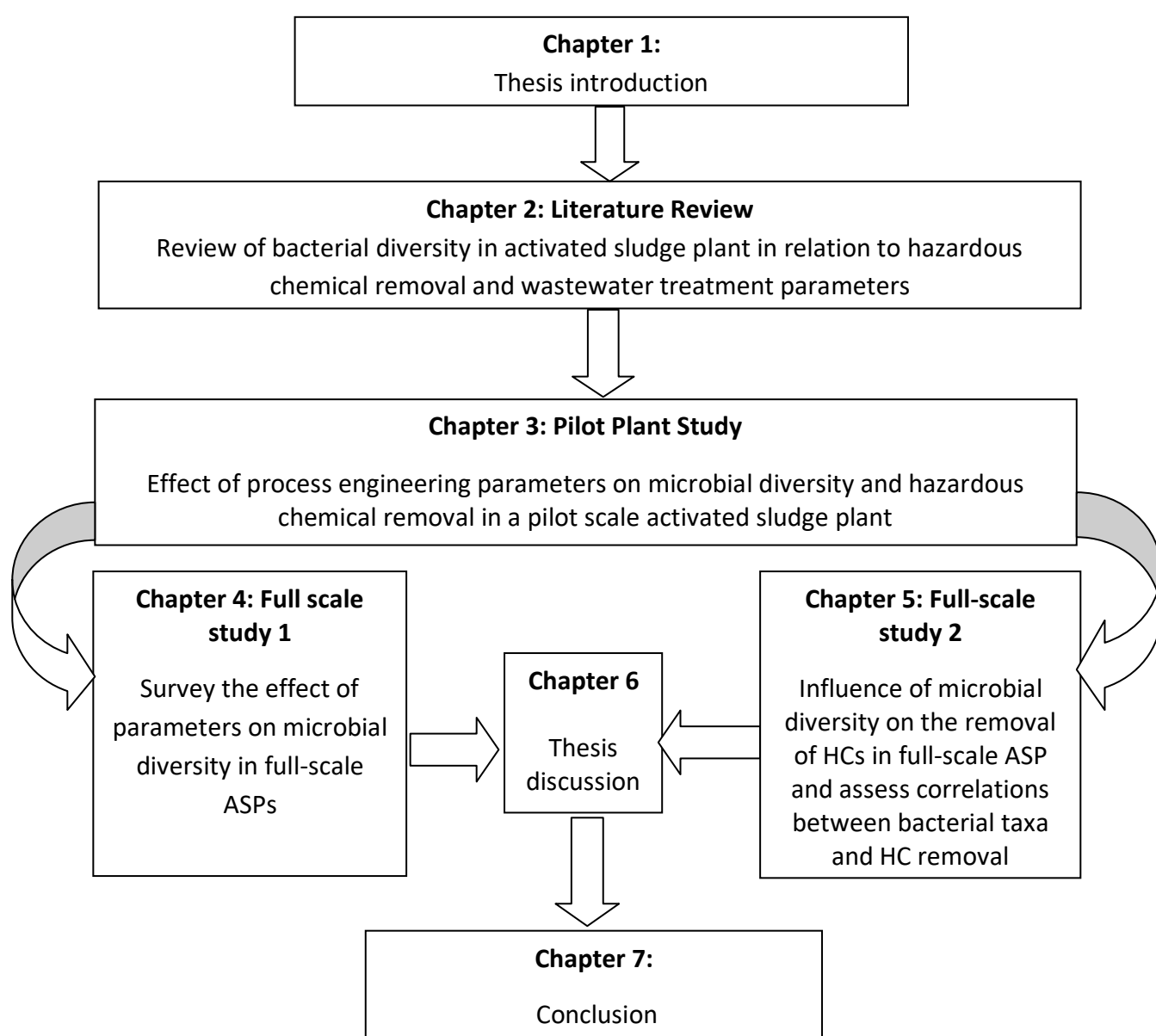


Figure 1.1. Structure of the thesis

CHAPTER TWO

BACTERIAL DIVERSITY IN ACTIVATED SLUDGE PLANTS IN RELATION TO PROCESS PARAMETERS AND THEIR INFLUENCE ON HAZARDOUS CHEMICAL REMOVAL

2. BACTERIAL DIVERSITY IN ACTIVATED SLUDGE PLANTS IN RELATION TO PROCESS PARAMETERS AND THEIR INFLUENCE ON HAZARDOUS CHEMICAL REMOVAL

ABSTRACT

Effluents from wastewater treatment plants (WWTPs) constitute a significant source of hazardous chemicals (HCs) in water bodies. The fate and behaviour of HCs in the environment is a concern worldwide due to their studied effects on aquatic biota. Sustainable removal of these HCs could be aided by enhancing activated sludge plant performance through the manipulation of process parameters to achieve an optimum composition of microbial communities inside the reactor. This review discusses the bacteria known to have the metabolic potential of a wide range of aromatic and other compounds, the process conditions under which different species become prevalent and the influence of the operational parameters on the bacterial diversity and the removal of hazardous chemicals. It also deliberates the theories underpinning microbial assemblage in engineered and environmental systems, explains the approaches to measuring diversity and the limitations and biases of each measurement. Finally, the pathways for removal of organic pollutants are discussed. The meaning of 'chemical removal' as used in this thesis is also elaborated.

Keywords: biodegradation; endocrine-disrupting compounds (EDCs); microbial analysis; pharmaceuticals and personal care products (PPCPs); wastewater treatment

2.1 INTRODUCTION

In both developing and industrialised countries, an increasing number of environmental contaminants are entering aquatic systems from anthropogenic discharges (Gardiner *et al.* 2013), these include effluent from wastewater treatment plants (WWTPs). Particularly, non-biodegradable chemicals, which are stable and remain hazardous in the environment even at low concentrations, are of increasing concern. Large-scale environmental studies, undertaken by Hope *et al.* (2012) in the United States and by Von der Ohe *et al.* (2011) in Europe confirm the global abundance of these hazardous chemicals.

The European Union's Water Framework Directive (WFD, 2000) identifies 20 priority substances and 13 priority hazardous substances which must be restricted from entering the aquatic environment due to their known effects on human health or ecosystems. Most of these chemicals are ubiquitous contaminants such as polycyclic aromatic hydrocarbons (PAHs) which are carcinogenic and immunotoxic (Abdel-Shaffy and Monsour, 2016); brominated diphenyl ethers (BDEs) capable of altering endocrine signalling in aquatic species (Thornton *et al.*, 2016) and other substances that can mimic natural hormones, commonly known as endocrine disruptors. For example, nonylphenols are in the list of priority hazardous substances because they mimic 17 α -ethynylestradiol (EE2) and has been reported to interfere with the sexual development of male fish leading to their feminisation (Aris *et al.*, 2014). A watch list of substances was also created to be continuously monitored for data gathering to aid in the decision to add them to the priority substance list. This watch list which is reviewed bi-annually, and the current watch list include: 17 α -ethynylestradiol (EE2), 17 β -estradiol (E2), estrone (E1), macrolide antibiotics (erythromycin, clarithromycin, azithromycin), methiocarb, neonicotinoids (imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid), metaflumizone, amoxicillin and ciprofloxacin (JRC Technical Report, 2018). WWTPs are facing a future of stricter regulations on discharge permit arrangements and hence are looking for solutions to improve the performance of existing treatment plants in removing hazardous chemicals (HCs).

Engineered systems such as WWTPs rely on the accumulation of microbial communities in its reactor to remove these chemicals wholly or partially from its influent, before returning it back to the water bodies (Pholchan et al, 2013). To improve the removal of hazardous chemicals, several studies have explored the impact of different process parameters on the removal of specific compounds or a group of compounds with similar characteristics. Some studies have been devoted to expanding the knowledge on how these process parameters affect microbial diversity, others on assessing the impact of process parameters on the removal of hazardous chemicals while a few have studied the impact of microbial diversity on chemical removal. This review discusses the existing knowledge on microbial diversity found in ASPs as a result of the operational conditions of the plant, and the influences of process parameters on hazardous chemical removal as well as the degradative abilities of bacterial species on individual or groups of the hazardous chemicals. It also discusses the theories underpinning microbial ecological systems as explored in the context of microbial diversity in WWTPs, and the relationship between α -diversity and rare functions in microbial reactors.

2.2 MICROBIAL DIVERSITY IN ACTIVATED SLUDGE PLANTS

Microbiologists have been interested in finding out the microbial communities in activated sludge tanks since their invention. The measurement of biodiversity over spatial scales has been describe as alpha, beta and gamma diversity by ecologists for nearly half a century (Whittaker et al., 1972) . Alpha-diversity refers to the types and abundance of individual species in a population or sample. However, beta-diversity is a measure of the changes in species diversity between different ecosystems whereas gamma diversity is a geographic-scale species diversity which measures the overall diversity for different ecosystems within a region. Diversity studies in ASPs started with the identification and quantification of protozoans (single-celled organisms) such as nematodes, amoeba, ciliates and flagellates (Curds et al., 1968) at different sludge ages with the aim of understanding and preventing failures, and later used as indicators for predicting plant performance (Zhou et al., 2006). It then progressed into bacterial phylogeny and taxonomy in ASPs but the progress in this area of study

was slow due to the limitations of traditional microbial analytical methods such as culturing and low-resolution microscopy. With technological advancements in instrumentation such as the invention of the DNA sequencing machines, and improvements in DNA extraction, purification and amplification techniques, an explosion in microbial research in recent years has led to a dramatic increase in knowledge of microbial diversity in WWTPs and to a deeper level of species identification and quantification of bacteria and archaea (Reyes et al., 2014).

Recent studies have identified more protozoa that are present in low abundance and were undetectable in earlier studies. For example, an abundance of *Litonosus obtusus* and *Opercularia coarctata* was found to correlates with high sludge volume index (SVI) and a high ratio of suspended solids (SS) to biochemical oxygen demand (BOD₅) respectively (Zhou et al., 2006) and hence should be minimised. The populations of *Arcella* and *Euglypha* were associated with nitrification, *Arcella hemisphaerica* with low total nitrogen (TN), BOD₅ and SS. *Vorticella convallaria*, *Epistylis plicatilis* and *Arcella hemisphaerica* were found in activated sludge plants (ASPs) all year round (Zhou et al., 2006). There are 94 species of ciliates, 40 species of amoeba and 13 species of large flagellates in ASP. *Aspidisca cicada* had the highest mean abundance in 98% of the analysed samples (Cheng et al., 2004). In ASP reactors, the formation of flocs and the inhomogeneity of the mixed liquor have resulted in variations in dissolved oxygen distribution and concentration in the reactor. Hence the ASP reactor has been found to contain aerobic, facultative and anaerobic microorganisms in varying abundance (Figure 2.1). Anaerobes such as phosphate-accumulating organisms (PAO), glycogen-accumulating organisms (GAO), sulphate-reducing bacteria (SRB), denitrifiers and methanogenic archaea are found in ASP because of the prevalence of pockets of anaerobic conditions and their ability to survive aerobically. SRB is present because of the high concentration of sulphate from recycled sludge, and methanogenic archaea were present due to recycling of digested sludge (Reyes et al., 2014). Figure 2.1 shows the structure of flocs in both conventional and granular activated reactors and the relative locations of PAO, denitrifiers, nitrifiers and GAO in the anoxic, aerobic and anaerobic zones (Winkler et al., 2013).

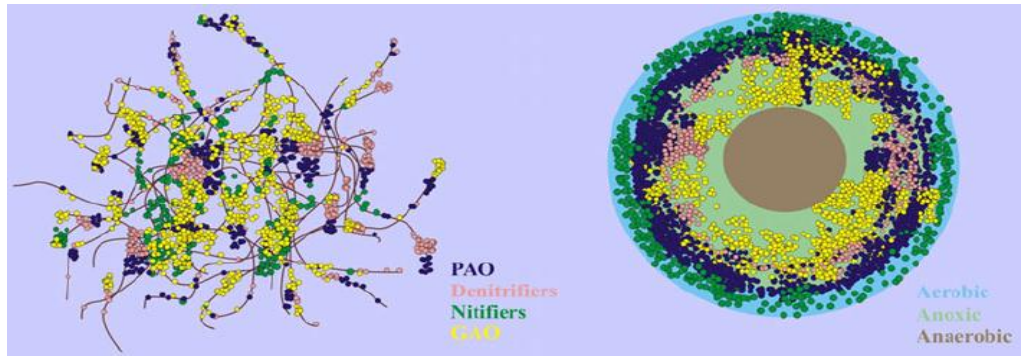


Figure 2.1. Structures of flocs in ASP (conventional and granular) reactor and the type of microbes in the floc (Winkler et al., 2013).

Although microorganisms already exist in sewage on route to the WWTP and may be expected to continue to thrive in the plant, comparative analysis of influent and mixed liquors found significant differences in their microbiota. Influent has a relatively higher abundance of *Epsilonproteobacteria*, *Firmicutes* and *Fusobacteria* but these are significantly reduced in the ASP reactor (Paiva et al., 2015). The influent microbial diversity has remarkable similarities with that of human faecal matter, comprising of *Bifidobacteriaceae*, *Coriobacteriaceae*, *Bacteroidaceae*, *Lachnospiraceae* and *Ruminococcaceae* (McLellan et al., 2010). Both influent and activated sludge contains *Flavobacteriaceae*, *Campylobactereceae* and *Comamonadaceae*. Neither *Nitrospiraceae* nor *Chitinophagaceae* is found in influent, but *Moraxellaceae* and *Neisseriaceae* are exclusively found in influent (Lee et al., 2015). The change in diversity from influent to mixed liquor is due to the difference in temperature and dissolved oxygen concentration (and other conditions) in sewers and aerated lanes in comparison with conditions in the human alimentary canal.

Microbial diversity in ASPs has been found to differ with process layout or changes in parameters or size of the reactor. Winkler et al, (2013) found that in an aerated granular ASP had a different bacterial diversity from that in a conventional flocculent ASP although they had similar hydraulic variations and fed from the same influent. Gast et al., (2006) also found that the size of the reactor correlates directly with the diversity and population in the reactor. Figure 2.2 shows the bacterial phyla diversity in different types of ASPs which may be operating at different parameters. The ASPs have differences in their *Proteobacteria*, *Nitrospira*, *Bacteroidetes* and *Chloroflexi* abundances.

It is also important to understand that these changes observed in diversity are only concerning the 1% of the true microbial diversity (Quince et al., 2008) which has been isolated and identified. That is the 30,000 microbial gene sequences that have been isolated through molecular techniques from environmental matrices and identified by comparison with sequences from clone libraries of cultured microbes.

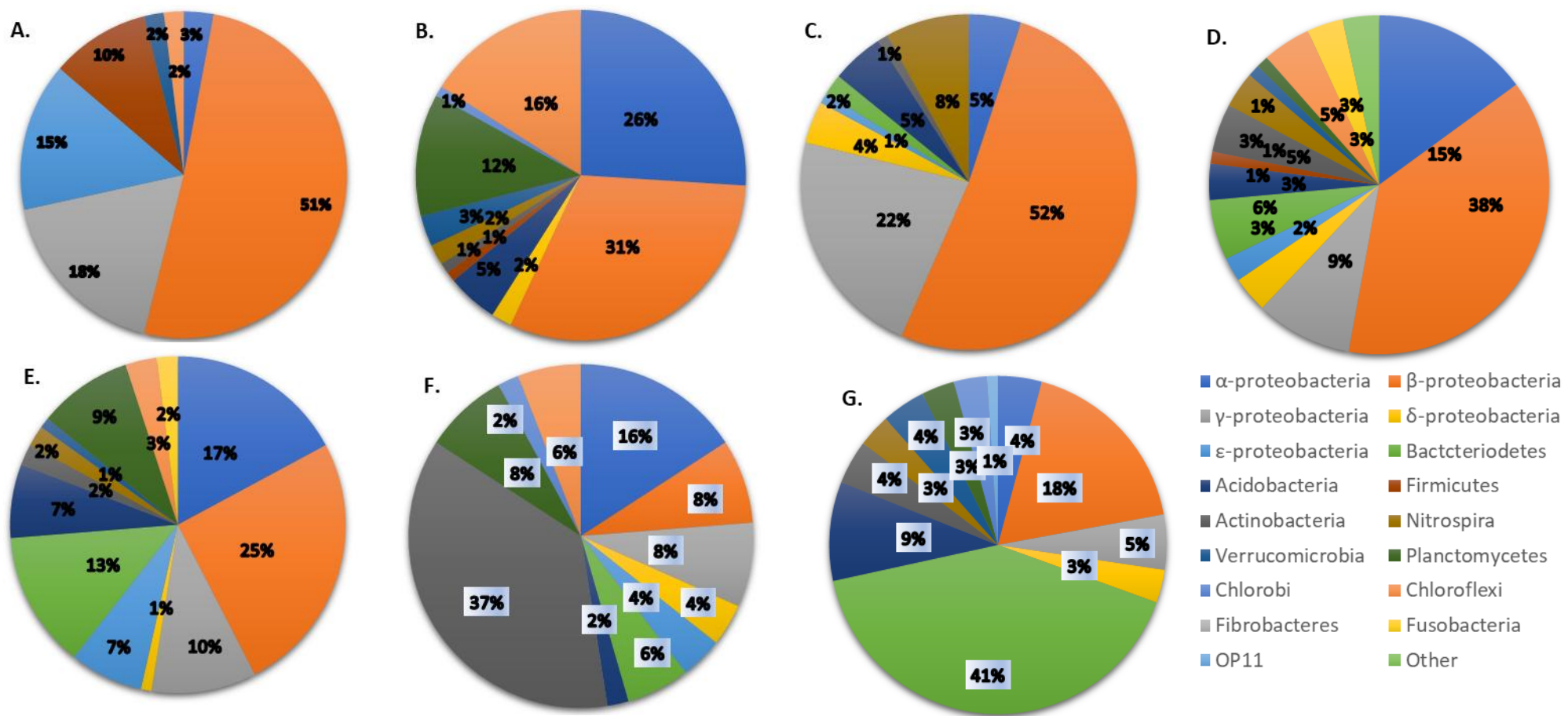


Figure 2.2. Bacteria phyla diversity and abundance in activated sludge plants. (A) high-load aeration basin of a full-scale municipal WWTP (Snaidir et al., 1999); (B) full-scale nitrifying/denitrifying plant (Juretschk et al., 2002); (C) pilot-scale nitrifying SBBR (Daims et al., 2001); (D) EBPR laboratory-scale SBBR + sodium acetate (Bond et al., 1995); (E) EBPR laboratory-scale SBBR unsupplemented (Christensson et al., 1998); (F) EBPR laboratory-scale continuous flows reactor (Dabert et al., 2001) and (G) EBPR laboratory-scale SBR (Lui et al., 2001).

2.3 LIMITATIONS OF MEASURING α -DIVERSITY

Studying the relationship between α -diversity and function in microbial communities is complicated because of the difficulties associated with the accurate quantification of richness and evenness (Coello-Garcia, 2018). With over 10^9 cells/mL in activated sludge (Curtis et al., 2006) and between 4×10^{23} to 6×10^{23} bacteria in ASP reactors worldwide (Wu et al., 2019), a grab sample to capture diversity is inherently problematic. However, technological advancements in microbial analysis and the development of next-generation sequencing technologies permit deeper community analysis, but it only sequences a few microlitres in metagenomic studies. The extraction and amplification of DNA also introduce biases and errors (Schloss et al., 2011) and noise from the data generated makes accurate assessment of microbial diversity difficult. Furthermore, sequencing platforms can add their own distinctive errors which may lead to overestimation of low occurring taxa (Bragg et al., 2013), despite continuing efforts to develop algorithms to de-noise the data. The bioinformatic computer systems used to improve the quality of data employ different methods to cluster sequence reads into operational taxonomic units (OTUs) and assume the most abundant sequenced OTU to be representative sequence of the cluster (Navas-Molina et al., 2013). All these limitations make the measurement of α -diversity in microbial communities difficult, especially for the rare taxa. To make the best of the sample data, parametric estimators have used the lognormal (Hong et al., 2007) and Bayesian distributions (Quince et al., 2008), assuming species abundance align with certain probability of distribution, to estimate the number of unaccounted species. Non-parametric estimators use sample coverage to predict the entire taxa abundance (Hughes et al., 2016). Sometimes, these estimates do not fit the shape of microbial ecological curves. Haegeman et al., (2013) challenged the reliability of the estimated microbial diversities acquired from these models because they are built on questionable assumptions about the shape of microbial-abundance distributions.

Diversity indices such as Shannon and Simpson have been used in microbial ecology for decades because of their ability to enumerate the species richness, and to some extent the evenness, although their interpretation is limited to the sample because it does not include sample coverage in its calculation (Mugurran, 1988).

Hill diversities derived from combining Shannon and Simpson indices and interpreted as effective number of species has also been used (Haegeman et al, 2013). An assessment of the various indices by Feranchuk et al., (2018) found Shannon index to be the most effective index in measuring statistical significance of differences in microbial communities. Notwithstanding all the limitations, meaningful studies on microbial diversity are still attainable through the use of estimators or diversity indices because they allow useful comparative analysis of the relationships between α -diversity and microbial ecosystem functions.

2.4 ECOLOGICAL THEORIES ON MICROBIAL ASSEMBLAGE

In order to design engineered systems which allow the natural assemblage of microbial communities to perform functions with benefit to human now and in the future, it is essential to understand factors that control microbial diversity, and the relationship between diversity and function (Pholchan et al., 2013). There are two opposing but inter-connected theories that govern the accumulation of microbes in natural or engineered system. Firstly, the deterministic theory which postulates that the presence of an organism signifies the presence or absence of that organism's niche (Chase and Leibod, 2003); and secondly, the stochastic (neutral) theory in which microbial communities assemble in the environment and change randomly in it (Bell 2001; Hubbell, 2001). Although microbial ecologists often interpret their work in the deterministic context, the neutral theory has been used to successfully predict microbial taxa volume curve (Woodcock et al, 2007). The inter-relatedness of the theories is: the neutral theory is based on the processes of birth, death and migration, and the rate of these phenomena is influenced by differences in species (deterministic). Ofiteru et al., (2010) created a dynamic neutral model by bringing together niche and neutral processes. This study takes the deterministic view to assess how changes to process parameters influence microbial/bacterial diversity in the activated sludge reactor at pilot and full-scale but recognise that some neutral processes are in play and therefore microbial diversity cannot fully be explained by changes in processes.

2.5 RELATIONSHIP BETWEEN OPERATIONAL CONDITIONS AND BACTERIAL DIVERSITY IN ACTIVATED SLUDGE PLANT

Statistically, 25 per cent of microbial diversity in ASP depends on the influent characteristics: COD, TN, ammonia, total phosphorus, pH and conductivity; and 23 per cent of the diversity can be explained by operational parameters such as SRT, HRT, temperature and DO concentration (Wang et al., 2014), while the remaining 52 per cent comes from a multiple factors. The phylotypic and phenotypic diversity of microbial species are also affected by the structural complexity, engineering parameters or both (Curtis et al., 2011). The operational parameters known to influence microbial diversity are discussed below.

2.5.1 Influent characteristics

Domestic and industrial sewage differ in their loading of nutrients, organic and inorganic chemical concentrations. This gives rise to differences in the microbial diversity because the chemicals in the wastewater which serves as the carbon source for the microbial species and affect influent pH. It also dictates which species will dominate the reactor based on their ability to utilise the most common chemical in the influent, with all other parameters kept the same. For example, the type and concentration of volatile fatty acid (propionate and acetate) in influent stimulated a substrate-metabolic competition in polyphosphate accumulating organisms; propionate was found to be the preferred substrate (Carvalho et al., 2014). Furthermore, *Clostridium sp.* and *Bifidobacterium sp.* were dominant in an ASP reactor fed with influent with high starch concentration, whereas *Halomonas spp.* dominated in a similar reactor operating at same condition but fed with hyper-saline wastewater (Lee et al., 2014; Cheng et al., 2008) shown in Table 2.1. Also, *Arthrobacter sp* W1 is an aromatic-degrading strain capable of directly or co-metabolically degrading naphthalene and phenols (Jiang et al., 2015) and it dominated the zeolite-biological aerated filters (Shi et al., 2015) fed with coking wastewater containing high levels of phenol and naphthalene. Also, pilot and full-scale WWTPs treating influent with high methanol and nitrate concentrations resulted in *Methylophilaceae* dominance (Isazadeh et al., 2016). Influent rich in nonylphenol polyethoxylates showed a significant change in diversity to

Betaproteobacteria (33%) dominance in the ASP reactor, but the carbon removal remained unchanged. This suggests that *Betaproteobacteria* is involved in the degradation of nonylphenols (Lozada et al., 2004). Moreover, when high levels of saline bath waters were received in the summer months at a WWTP, the *Nitrosomonas oligotropha* and *Nitrosomonas marina* dominance in the nitrifying ASP reactor changed to halophilic or halotolerant bacteria dominance and caused nitrification failure (Beneduce et al., 2014). Microbial diversity in WWTP reactor fed with an influent high in fluoroquinolones was dominated by *Alcaligenaceae* and *Pseudomonadaceae* bacteria; both have high antibiotic resistance (Marathe et al., 2016). Therefore, the microbial diversity in the ASP reactor is affected by the chemicals in the wastewater (Gannoun et al., 2013; Li et al., 2014; Cheng et al., 2008). The presence of sulfonamide antibiotics in wastewater produces conjugates that maintain their antibiotic capabilities after biotransformation. These conjugates can inhibit the synthesis of folic acid and stunt the growth of certain bacteria (Achermann et al., 2018).

2.5.2 Solids retention time (SRT)

The microbial diversity in WWTPs is influenced by SRT variations (Vuono et al., 2015; Akarsubasi et al., 2009), and the differences in diversity are more pronounced with increasing SRT difference (Lui et al., 2007). These recent findings concur with early studies using a light microscope, which showed the identity and population of protozoans in mixed liquor (Curds et al., 1968) and how their abundance changed with increasing SRT. Recent studies on microbial diversity have extended the depth of knowledge about diversity in ASPs and revealed the presence of several bacterial and archaeal species at different SRTs. For example, *Betaproteobacteria* and Bacteroidetes are the dominant taxa in membrane bioreactor systems, but the abundance of *Deltaproteobacteria* and *Epsilonproteobacteria* increased with longer SRT (Hu et al., 2012; Ahmed et al., 2007). Clara et al., (2005) suggest that lengthening the SRT allows the slow-growing microbes to increase in population. In addition, it is very likely these bacteria possess a suite of enzymes that metabolise aromatic ring-structures, e.g. oxygenases, coenzyme A ligases, dioxygenases and steroid dehydrogenases (Pérez-Pantoja et al., 2010). While one bacterial group increases,

others may reduce, remain unchanged or increase too, but their relative abundance may appear to be reducing. This may explain the observation by Roh and Chu (2011) that the ratio of estrogen-degrading bacteria (*Sphingomonas strain* KC8 and AOB) to the total bacteria in ASP reactor decreases as the SRT lengthens, while the evenness of diversity remained unaffected. A commonly accepted theory that a species with a high substrate affinity and low growth rate (k-strategist) will out-compete species with a lower substrate affinity and higher growth rate (r-strategist) if both species are subjected to low substrate concentrations (Winkler et al., 2017). Extending the SRT diminishes the food-to-microorganism ratio, and the ubiquitous 'K-strategists' heterotrophic bacteria will scavenge on the low concentrations of hazardous chemicals (Vuono et al., 2015). Therefore, decreasing SRT from 30 to 3 d caused a shift in microbial diversity from k-strategists (*Nitrospira*) to r-strategists (*Nitrosomonas*) and resulted in reactor failure to nitrify, denitrify and remove phosphorus. Hence, k-strategists bacteria disappear at low SRTs (Vuono et al., 2015, Whittaker et al., 2017).

2.5.3 Hydraulic retention time (HRT)

HRT is the ratio of the reactor volume of a reactor to the influent flow rate. Changing the HRT is manipulated by changing influent flow rate, but in doing so, it changes the organic loading rate, which has a significant impact on the substrate-to-microbe ratio, substrate availability and metabolic activity of the overall microbial biomass, which can have different effects at various SRTs (Winkler et al., 2017; Coello-Garcia et al., 2019). At constant SRT, increasing the HRT caused an increase in biomass concentration (Kargi and Eker, 2006) and changed the bacterial diversity (Zhang et al., 2014). Fewer bacterial species were found in an ASP operated at 25 h HRT compared to a similar plant operated at 42 h HRT (Han et al., 2010; Shin et al., 2010) – suggesting an increase in bacterial population and diversity when HRT is increased. The proportion of ammonia-oxidising bacteria (AOB) within the total bacteria population decreased from 33 to 15% as HRT decreased from 30 to 5 h. Conversely, the fast-growing nitrite-oxidising bacteria (NOB) increased from 4 to 15% under the same conditions (Li et al., 2014). In a conventional ASP operated at HRT

greater than 20 h, AOBs and NOBs were found to dominate but reducing the HRT caused a decrease in the AOBs and least affected the NOBs (Li et al., 2006).

2.5.4 Dissolved oxygen (DO) concentration

Microbial diversity is affected by DO concentration in the wastewater and microbes can be categorised based on their ability to function with or without oxygen. DO concentration is the most crucial selective parameter in the first phase of the nitrification process (Aleksandra et al., 2011), and the *Nitrosomonas oligotropha* lineage is found to be the dominant species in nitrifying ASP reactors operated at DO levels of 0.25, 0.5, 3.0 and 3.4 mg/L (Bellucci et al., 2011). At low DO concentrations (<0.3 mg/L), the conversion of ammonia to nitrates may not be necessarily a function of only ammonia oxidising bacteria, but other autotrophic and heterotrophic bacteria are involved (Fitzgerald et al., 2015).

2.5.5 Temperature

The effect of temperature on the ASP is multi-facet. Biologically, it affects the growth rate of microorganisms; chemically, it affects the rate of reactions, and physically, it influences the solubility and volatility of chemicals in water (Metcalf and Eddy, 2014). Although it may be operationally challenging to increase the temperature of a full-scale AS reactor, seasonal temperature changes showed that the dominant bacteria in winter was of *Nitrospira* lineages but in summer were *Nitrosomonas* lineages (Siripong and Rittman, 2007). The reduction in temperature impedes the growth of mesophilic *Nitrosomonas* and favours the growth of *Nitrospira* bacteria. Knowing the benefits of warmer temperature on the performance of WWTPs and the operational difficulties and financial constraints to increasing the temperature of WWTPs, research interest in the use of cultured psychrobacteria in aerobic and anaerobic processes is growing. Acclimatised dominant bacterial species from cold climate such as *Candidatus Brocadia fulgida* was used in a cold-adapted anammox reactor in treating wastewater at 10 °C (Hendrickx et al., 2014). Bowen et al., (2014) reported of methanogenic activity by psychrobacteria at 4 °C from the degradation of acetate, although it had a longer lag time due to acclimatisation, compared to degradation at 8 °C. Table 2.1 shows the bacterial genera dominating different WWTPs under various operational parameters and the resulting performance of the

plant. The influence of SRT, HRT, temperature, DO and influent on bacterial diversity is also presented.

Table 2.1. Summary of bacterial diversity in WWTPs and reactor performance at different process parameters

Dominant bacterial genera	Influent type	Operational conditions	Removal efficiency	Author(s)
Activated sludge reactor				
<i>Halomonas</i> spp. (Proteobacteria); <i>Paenibacillus</i> sp. (Firmicutes)*††	4% hypersaline wastewater	60 h HRT	84% COD	Lee et al., 2014
<i>Thiothrix</i> sp.*	Domestic wastewater		--	Xie et al., 2011
1 st : <i>Nitrospira</i> (Nitrospirae)	Municipal wastewater	12 d SRT;		Tian et al., 2015
2 nd : <i>Thauera</i> (Betaproteobacteria)				
3 rd : <i>Dechloromonas</i> (Betaproteobacteria)				
<i>Acinetobacter</i> (Moraxellaceae)			75% 4-MCHM	Yuan et al., 2016
Up-flow anaerobic reactor				
<i>Clostridium</i> spp. (Fermentative) and <i>Desulfovibrio</i> spp. (SRB) ‡~	--	24 h HRT, WLR of 0.34 – 0.85 g/L/d	--	Khelifi et al., 2009
<i>Methanosaeta</i> spp.*	Purified terephthalic acid (TA) Wastewater (from petroleum industry)	18.9 h HRT and 37 °C	93.7% COD; 93.3 TA	Li et al., 2014
<i>Methanosarcina</i> spp.*	--	55 – 60 °C; 4 d HRT	--	Ho et al., 2014
<i>Macellibacteroides</i> fermentans; *‡ ~	Abattoir wastewater containing blood, fats, faeces	37 °C; 0.75 – 5 h HRT & 55 °C	72 – 90% COD removal	Gannoun et al., 2013
<i>Desulfotomaculum peckii</i> & <i>Defluviitalea saccharophila</i> <i>Clostridiaceae</i> (Firmicutes)*	High concentration organic cassava wastewater	30 – 35 °C; pH 7;	98.5% sCOD; 71.4% TN; 62.1% TP; 100 % Acetic acid	Gao et al., 2012
Activated granular sludge bed				
<i>Methanoseata</i> and <i>Methanobacteria</i> †	--	18 h HRT, Temp 15 °C & 20 °C	--	Xing et al., 2009
<i>Clostridium</i> spp. and <i>Bifidobacterium</i> sp. †*	Synthetic wastewater containing starch 20g COD/L	37 °C, 0.5 h HRT, pH of 6.0 and 5.5	--	Cheng et al., 2008
<i>Pseudomonas</i> , <i>Shinella</i> and <i>Flavobacterium</i> †‡	--	10, 13 and 19 h HRT; COD/N ratio of 4.5 and 2.3	--	Cydzik-Kwiatkowska et al., 2014
MBR				
<i>Nitrosomonas</i> – like spp.†*	66 – 150mg/L NH4	50 d SRT; 3.3 h HRT	--	Aleksandra et al., 2011
Zeolite-biological aerated filters (Z-BAF)				
<i>Arthrobacter</i> sp. W1 strain *	Coking wastewater with high phenol and naphthalene content	--	94 – 98 % naphthalene	Shi et al., 2015
Integrated fixed-film Activated Sludge				
<i>Thauera</i> sp. R26885* (Betaproteobacteria)	Synthetic municipal wastewater	17 h HRT; 80 d SRT, 15 – 25 °C; 3000 mg/L MLSS & 6-7 mg/L DO	94 % COD; 99% NH4 ⁺ -N	Duan et al., 2015
Codes for analytical methods used: *16S rRNA †DGGE *FISH ‡PCR ~SSCP				

2.6 DIVERSITY AND RARE FUNCTIONS

In macro and micro ecological systems, the relationship between diversity and function is important for several reasons. Cardinale et al., (2012) state that a more diverse community can withstand external shocks and is more productive and a less diversity ecosystem easily succumbs to ecological stressors and lead to extinction of species. Not all loss of diversity will lead to loss of function. There are some basic functions performed by all or most (e.g. respiration and decomposition of organics), and continues despite loss of diversity, but there some rare functions (e.g. degradation of estrogens) that is performed by a few taxa, and hence vulnerable to diversity loss. It is worth noting that presence does not always translate into function (Liang et al., 2017). Therefore, understanding the principles by which microbial community and composition determine ecological functions in WWTPs is very important. It has been demonstrated in ASPs that loss of diversity through the reduction of SRT leads to loss of nitrification (Vuono et al., 2015). Several others have found better removal of chemicals corresponds with increased microbial diversity (Johnson et al., 2015; Torresi et al., 2016).

2.7 PATHWAYS FOR CHEMICAL REMOVAL IN ACTIVATED SLUDGE PLANTS

Percentage-chemical-removal is a phrase used to denote a collection of values from different mechanisms by which a chemical has been eliminated from the influent in WWTP. The value contributed from the different pathways can be derived from a comprehensive mass balance analysis of wastewater treatment processes (Racz and Goel, 2010). Chemicals can be removed through abiotic mechanisms such as sorption to sludge, volatilisation and photodegradation. Depending on the sorption coefficient (K_d) of the chemical, sorption to sludge can be a major mechanism by which removal of a chemical takes place in WWTP. Joss et al., (2006) found that chemicals with less than 300 L/Kg are less likely to be sorbed to sludge. Another mechanism by which chemicals are removed from WWTPs is biological transformation (Kagle et al., 2009). This could be direct metabolic use of a chemical as a carbon and energy source or a coincidental transformation of a compound without its use as a carbon and energy source, co-metabolism (Pauwel et al., 2008).

Biotransformation mechanisms of organic compounds may depend on the chemical structure of the compound and the environmental conditions within which it is found. For example, enzymatic *hydrolysis* tends to be prevalent in chemicals with a good leaving group attached to a saturated carbon structure (e.g. alkyl halide) or an unsaturated carbon (e.g. esters). Hydrolysis can happen with or without oxygen because the HC is neither *oxidised* nor *reduced*. Oxidation is another mechanism by which some HCs; mainly hydrocarbons without a leaving group, but has an electron-rich site (lone pair of electrons, pi of sigma bond) in its structure; may be biotransformed. In oxidation, the initiation step is under aerobic conditions and the microorganism spend energy to change oxygen from its less reactive form O₂, into a more reactive electrophilic oxidant and keep it in that oxidative state using a metal (Boer et al., 2014). For HCs with a structure containing a highly electronegative atom such as oxygen or chlorine, oxidation is less likely, especially under anoxic conditions. For such compound, biotransformation may happen through enzymatic *reduction* reaction. Enzymes such as nitroreductase are used to generate a hyride ion (H⁻) to start the reaction. Reductive dehalogenation is one of such biotransformations and it can be hampered by the presence of sulfur reducing bacteria because they are more efficient at stripping the environment of hydrogen (Berggren et al., 2013). Under anoxic conditions, a chemical compound that do not contain a 'good leaving group' and is already rich in electrons (hence cannot be reduced) may undergo enzymatic addition biotransformation where the microorganisms adds a carbon-containing group such as fumerate. For example, toluene has been biotransformed into benzoyl-Co-A by adding fumerate (Jarling et al., 2015). Biological transformation may either lead to complete mineralisation; that is the HCs turned to carbon dioxide or water; or may lead to degradation of the parent compound to lower molecular-weight compounds. Sometimes, it can also result in minor chemical alterations which may lead to the formation of another hazardous chemical. For example, in Figure 2.3 the degradation of nonylphenol poly-ethoxylates into nonylphenol or nonylphenol mono or diethoxylates (Petrie et al., 2014). Chemical analysis for only the parent compound in influent and effluent might not indicate the fate by which a chemical may have been removed (Joss et al., 2006). Biotransformation can be facilitated by a species of bacteria through catalytic enzymes and co-substrate, or it can be

accomplished by microorganisms working together (syntrophy) as consortia, with each species executing a particular sequence in the transformation of the HC (Schwarzenbach et al., 2016). For instance, 3-chlorobenzoate is transformed by a consortium of microorganisms, starting with a species that reductively removes the chlorine atom using hydrogen, followed by a species which convert the remaining benzoate to acetate, carbon dioxide and hydrogen. Finally, a third species also uses some of the hydrogen to convert acetate to methane and CO₂. Depending on which fate is employed for the removal, different factors in the WWTP impact differently and the physicochemical characteristic of the chemicals may play a role. Mass balance analysis of steroid estrogens in WWTPs showed that their fates of removal are sorption to sludge and biodegradation (Zhuo et al., 2012; Petrie et al., 2014; and Racz and Goel, 2010). Both mechanisms are improved by increasing SRT and HRT, because longer SRT increases microbial diversity (richness) to allow the K-strategist to accumulate, and longer HRT restricts the supply of substrate, hence improved biodegradation. Also, the increase in sorption is attributed to the decrease in floc size from 358 µm (at low SRT) to 164 µm (at high SRT) which increases the surface area of sludge for sorption (Petrie et al., 2014).

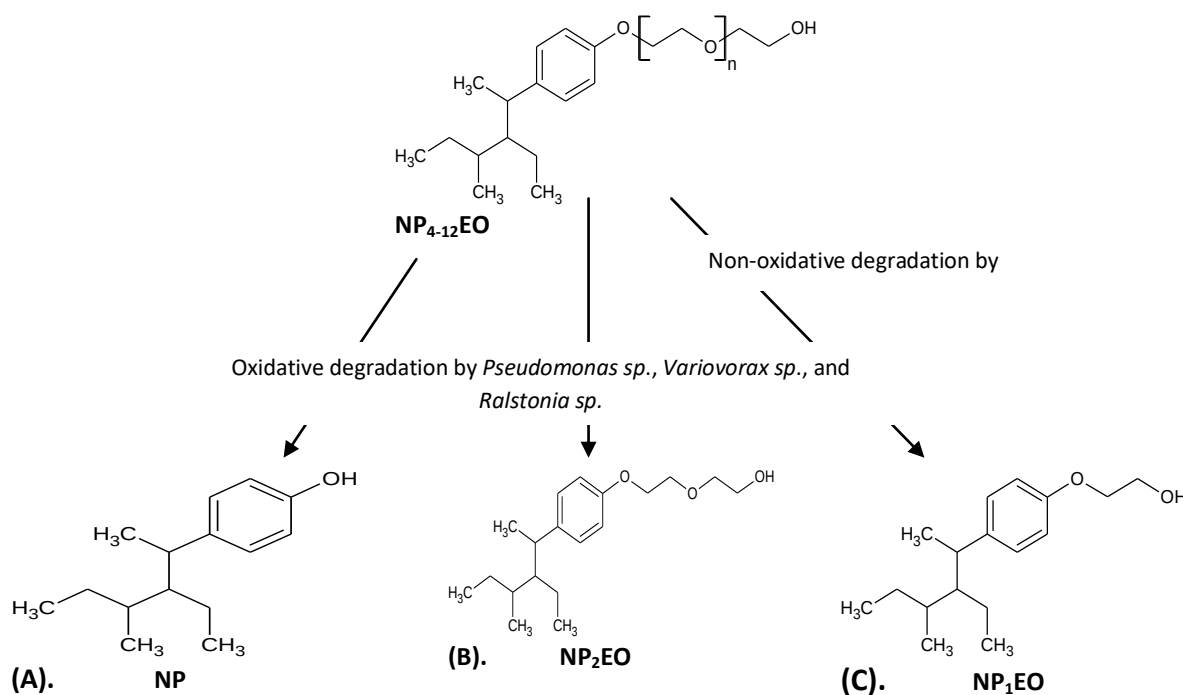


Figure 2.3 Pathways of biodegradation of nonylphenol poly-ethoxylate by *Pseudomonas* and *Variovorax* spp. into conjugates (A) nonylphenol, (B) nonylphenol di-ethoxylate, (C) nonylphenol mono-ethoxylate.

The use of only percentage-chemical-removed values to characterise the efficacy of an ASP at degrading harmful chemicals is misleading (Coello-Garcia, 2019) because an environmental risk of a chemical is based on exposure from final effluent concentration of the chemical. Thus, 90% chemical removal may be deemed as good performance, but if that chemical was metformin which has influent concentration $\sim 200 \mu\text{g/L}$ and PNEC of $10 \mu\text{g/L}$, then the effluent from that plant is exceeding the PNEC for metformin. Since the focus of this research was not to conduct an environmental risk assessment or to evaluate WWTP performance but rather to assess correlations between bacterial diversity and chemical removal, using the value of percentage chemical removal alone was adequate.

2.8 OPERATIONAL CONDITIONS FOR THE REMOVAL OF HAZARDOUS CHEMICALS IN ACTIVATED SLUDGE PLANTS

2.8.1 Solids retention time (SRT)

To some extent, it could be demonstrated that the diversity and abundance of specific microbial groups putatively involved in the removal of HCs can be controlled by altering the operational parameters. Several studies (Clara et al., 2005; Kreuzinger et al., 2004; McAdam et al., 2010) found enhanced HC removal with extended SRT. With a few exceptions such as a decrease in degradation of 4-methylcyclohexane methanol with increasing SRT (Yaun et al., 2016) most HCs removal correspond positively with increasing SRT, HRT and temperature (Armstrong et al., 2018). For instance, removal of estrone increased from 70 to 95% when SRT was extended from 3 to 10 d. As the SRT increases, the number of live cells per volume drops while the level of influent HCs such as estrogen stays the same (Petrie et al., 2014). Further to this, SRT has been found to influence the biotransformation rate constant of HCs, with higher rates found at longer SRT. SRT gradient was also observed to promote certain biotransformation mechanisms over other (Achermann et al., 2018).

2.8.2 Hydraulic retention time (HRT)

Extended HRT reduces the organic loading rate, increase the contact time between HCs and biomass and exposes HCs to prolonged biodegradative attacks after the readily degradable bulk organics have been removed by the active biomass (Petrie et al., 2014). Increasing HRT often results in the enhanced removal of HCs with a few exceptions. For instance, 94 and 87% removal of E2 and equivalent estrogenic quantity of E2 was observed at 12 h HRT, but complete removal was observed at 72 h HRT (Ma et al., 2017). Also, increasing the HRT from 5 to 40 h corresponded to an increase in the removal of dichlorophenol (Kargi and Eker, 2006) and lowering HRT from 25 to 5 h reduced the removal of trichloroethylene from 95 to 85% (Zhang et al., 2014). Removal of trihalomethane is increased by 62% when HRT is lengthened from 8 to 24 h, but no significant gain is observed with a further increase to 72 h (Galapate et al., 1999). These observations align with the theory that long HRTs promote the growth of K-strategists and lead to improved chemical removals. However, such high HRTs are unrealistic in ASPs due to high daily influent flows and limited tank sizes but it may be achievable constructed wetland (Akratos and Tsihrintzis, 2007). For chemicals which are mainly removed by sorption to sludge (e.g. tetracycline), HRT also affects their removal (and desorption) because it determines whether their sorption kinetic equilibrium will be reached or not (Kim et al., 2005).

2.8.3 Dissolved oxygen concentration

Increases in DO concentration has been observed to improve plants' performance in removal of ammonia, total nitrogen and chemical oxygen demand as well as phosphorus (Zhao et al., 2017), but sludge production is increased at high DO due to decrease in fermentative microbes (Niu et al., 2016). The effect of DO on the removal of pharmaceuticals varies widely between chemicals and under different DO concentration. Stadler et al., (2015) found that atenolol and trimethoprim removal was high under high DO but low when DO concentration was <0.3 mg/L while sulfamethoxazole removal was the opposite.

2.8.4 Temperature

Seasonal temperature increases in WWTPs have also been noticed to positively correlate with further removal of HCs (Thompson et al., 2011). For example, removal of progesterone, testosterone, estrogens (E1, E2 and EE2) was higher in spring than in winter (Manickum and John, 2014) while a decrease in temperature by 6 °C reduced the removal of estrone-1,3-sulfate by 20% (Koh et al., 2009).

2.9 BIODEGRADERS OF HAZARDOUS CHEMICALS

A variety of microorganisms that are capable of degrading different priority HC have been isolated (Zhang et al., 2014, Poursat et al., 2019). Some of them can metabolise a broad spectrum of chemical structures and some co-metabolise (Joss et al., 2006). However, most individual bacterial species appears to target a specific chemical group, e.g. *Arthrobacter* metabolises aromatic compounds (Jiang et al., 2015). Some biodegradation pathways identified in the literature include cleavage of bonds or the displacement of carbon from a compound to obtain energy. Tables 2.2 to 2.6 presents a list of priority compounds isolated and the identified degrading bacterial taxa, source, degradable quantity per day, mechanism of degradation and references. For example, *Sphingomonas* degrades nonylphenol poly-ethoxylates to nonylphenol mono-ethoxylate (Gabriel et al., 2005), and the *Sphingomonas* Ndbn-20 and *Sphingobium* Ndbn-10 degrade the herbicide Dicamba by O-demethylation mechanism into 2,5-dichlorosulfanilic acid. Also, *Stenotrophomonas maltophilia* KB2 degrades naproxen through the non-oxidative degradation mechanism (Wojcieszynska et al., 2014).

Sphingopyxis strain KCY1 degrades triclosan to chloride ion and unchlorinated end-products by dechlorination mechanism (Lee et al., 2012) whereas *Labrys portucalensis* degrades fluoroquinolones through a similar mechanism by cleavage of the piperazine ring and displacement of the fluorine substituent. *Pseudomonas sp.*, *Variovorax sp.* and *Ralstonia sp.* converts nonylphenol polyethoxylates to nonylphenols and nonylphenol di-ethoxylate through oxidative degradation mechanism (Zhang et al., 2014). The biodegradation of HCs may be driven by chemical-and-enzymatic structural compatibilities, half-saturation constant and

communal function. Bacterial species are genetically suited to acquire energy from a single or multiple chemical groups through redox reactions. Their genes determine the chemicals that they can metabolise or co-metabolise because the rate of degradation may be influenced by chemical-and-enzymatic structural compatibilities. For example, Fenner et al., (2013) found that N-substitution type biotransformation by ammonium monooxygenase (AMO) is faster than oxidative N- and O-dealkylation transformations.

The preference of substrate is also determined by the *half-saturation constant* (K_s) of the HC concerning the microbial species, which regulates the uptake of the HC. Hence, biodegradation of HCs by any bacteria occur to a different extent because microbial species have feeding preferences to certain chemicals. For example, the AMO bacterium degrades ten chemicals to different extents, ranging from one to four times in magnitude.

Biodegradation is also influenced by '*communal function*' of microorganisms. Degradation and removal of substrates may occur in stages, with each stage mastered by a different bacterial taxon, resulting in symbiotic relationships. For instance, the AOB and NOB degrade ammonia and nitrite respectively, but together, they convert ammonia to nitrate. Knapp and Graham (2007) described the relationship between *Nitrospira* and *Nitrobacter* with respect to the failure of nitrification in an activated sludge plant in four steps: *Nitrospira* dominates – nitrification; AOB community becomes unstable; *Nitrospira* crash; *Nitrobacter* left; nitrification is lost. Although there is no example in the literature of any hazardous chemical that is removed through the symbiotic effort of several species, the conversion of ammonia to nitrate described above provides the premises for the possibility of HCs being degraded in such a manner.

A summary of bacterial species that have been isolated and identified to degrade different types of HCs are presented in Tables 2.2 to 2.4. It shows that most HCs degraders in WWTPs are *Alphaproteobacteria* or *Betaproteobacteria* and various strains of *Sphingomonas* (*Alphaproteobacteria*) tend to be the common degraders of nonylphenols and estrogens.

Table 2.2. Bacteria with broad aromatic metabolic potential

Priority HC groups	affiliation	Species	Degradation conditions	Degradation mechanism	References
PBDEs, PCBs, phthalates, steroids	<i>Actinobacteria</i>	<i>Rhodococcus jostii</i> RHA1	Isolated from lindane-contaminated soil	A wide range of aromatics and steroids as sole carbon & energy source	McLeod et al., (2006)
PCBs, phthalates	<i>β-Proteobacteria</i>	<i>Burkholderia xenovorans</i> LB400	Isolated from PCB-containing landfill	Complete degradation, sole carbon & energy source	Ponce et al., (2011)
Alachlor, acetochlor and butachlor	<i>α-Proteobacteria</i>	<i>Roseomonas chloroacetimidivorans</i> sp. nov		63% of alachlor, 52% of acetochlor and 49% of butachlor were degraded after incubation for 4 d at 30 °C	Chu et al., 2016
4-methylcyclohexane methanol	<i>γ-Proteobacteria</i>	<i>Acinetobacter bouvetii</i> strain EU40		Degrades up to 80 mg/L of cis and trans-4-MCHM	Yuan et al., 2016
PAHs, phthalates		<i>Mycobacterium vanbaalenii</i> PYR-1	Aqueous media		Stingley et al., (2004)
E1, E2, E3; EE2	<i>γ-Proteobacteria</i>	<i>Acinetobacter</i> sp. BP10	Compost	Degradation of E1, E2, E3; co-metabolises EE2 in the presence of E1, E2, E3; Lowest reported degradation level: 1.8 ng/L	Pauwels et al., (2008)
E2	<i>Novosphingobium</i>	(E2)-degrading bacterium E2S	activated sludge	50 mg/L of E2, strain E2S exhibited 66.40% degradation efficiency after 7 days	Li, et al., (2017)
E1		<i>Sphingomonas</i> sp. AHC-F and strain <i>Sphingobium</i> sp. AX-B	activated sludge	Degrade 2 mg/L of E2 and its metabolite E1	Ma et al., (2016)
EE2, DEHP, PAHs	<i>Firmicutes</i>	<i>Bacillus subtilis</i> ssp. (Gram +)	Culture; Soil	EE2 percent removal 27% ± 2%; 81.6% DEHP degradation in 5 days (3,900 mg/L)	Larcher and Yargeau (2013)
17β-estradiol	<i>α-Proteobacteria</i>	<i>Sphingomonas</i> ssp. KC8			Roh and Chu, 2011

Table 2.3. Bacteria with nonylphenol degrading capabilities

Priority HC groups	Phylogenetic affiliation	Species	Degradation conditions	Degradation mechanism	References
Nonylphenols	<i>α-Proteobacteria</i>	<i>Sphingomonas sp.</i> TTNP3	Municipal WWTPs	Degradation capacity up to 100 mg/L/day; growth on NP as a sole carbon & energy source.	Tanghe et al., (1999) Corvini et al., (2006)
Nonylphenols	<i>α-Proteobacteria</i>	<i>Sphingomonas cloacae</i>	Municipal WWTPs	Degradation capacity up to 100 mg/L/day; growth on NP as a sole carbon & energy source.	Fujii et al., (2001) Corvini et al., (2006)
Nonylphenols	<i>α-Proteobacteria</i>	<i>Sphingomonas xenophaga</i> Bayram	Municipal WWTPs	Degradation capacity up to 100 mg/L/day; growth on NP as a sole carbon & energy source.	Gabriel et al., (2005) Corvini et al., (2006)
Nonylphenol diethoxylate (NP₂EO)	<i>α-Proteobacteria</i>	<i>Sphingomonas sp.</i> (NP42a)	Isolated from ASP	Non-oxidative degradation of NP ₂ EO with nonylphenol mono-ethoxylate as the main product	Zhang et al., 2014
Nonylphenol diethoxylate (NP₂EO)	<i>β-proteobacteria</i>	<i>Variovorax sp.</i> (NP427b)	Isolated from activated sludge		Zhang et al., 2014
Nonylphenol diethoxylate (NP₂EO)	<i>β-proteobacteria</i>	<i>Ralstonia sp.</i> (NP47a)	Isolated from activated sludge		Zhang et al., 2014

Table 2.4. Bacteria capable of degrading priority HC groups

Priority HC groups	Phylogenetic affiliation	Species	Degradation conditions	Degradation mechanism	References
Acetaminophen	γ-Proteobacteria	<i>Delftia tsuruhatensis</i> and <i>Pseudomonas aeruginosa</i>		Degrades up to 100 µg/L of acetaminophen as sole carbon and energy source	De Gusseme et al., 2011
Acetaminophen	γ-Proteobacteria	F1, F2 and FG-2; <i>Pseudomonas aeruginosa</i> strain HJ1012 (<i>Stenotrophomonas</i> & <i>Pseudomonas</i>)		Degrades up to 4000 mg/L and mineralise 87%	Zhang et al., (2013) Hu et al., (2012)
Aromatic amide, ester and thiophosphate	Proteobacteria (α, β, δ or γ)	<i>Ammonium monooxygenase bacteria</i>		N-substitutions type hydrolysis	Fenner et al., (2013); Helbling et al., (2012)
Carbamazepine	α-Proteobacteria γ-Proteobacteria	<i>Sphingopyxis</i> Strain KCY1 <i>Pseudomonas</i> sp CBZ-4		46.6% as sole carbon source after 144 h of incubation at 10 °C	Lee et al., (2012) Li et al., (2014)
Ibuprofen and naproxen	Firmicutes	<i>Bacillus thuringiensis</i> B1	chemical factory soil	Degrades up to 25 mg/L of ibuprofen and 6 mg/L of naproxen	Marchlewicz et al., (2016)
Triclosan	α-Proteobacteria	<i>Sphingomonas</i> sp. Rd1 <i>Sphingopyxis</i> Strain KCY1		Degrades ~35% Triclosan in complex media	Hays et al., (2001) Lee et al., (2012)
Naproxen	γ-Proteobacteria	<i>Stenotrophomonas maltophilia</i> KB2		28% as a sole carbon source, 78% and 40% as a co-metabolic substrate with glucose and phenol respectively.	Wojcieszynska et al., (2014)
Naproxen	Firmicutes	<i>Planococcus</i> sp. Strain S5	Culture	Degrades 30% of naproxen as a sole carbon source, 75% and 86% as a co-metabolic substrate with 1mg/L glucose and 282 mg/L phenol respectively.	Domaradzka et al., (2015)
Naproxen	γ-Proteobacteria	<i>Pseudomonas</i> sp. Strain CE21		Degrades 78% of 0.01 mg/L naproxen in 3 days	Wojcieszynska et al., (2014)
Fluoroquinolones (FQs)	α-Proteobacteria	<i>Labrys portucalensis</i> F11	Isolated and cultured from soil	Degrades ~91% of an individual or mixed FQs (Ofloxacin, norfloxacin, ciprofloxacin) with a readily available carbon source	Amorim et al., (2014)

2.10 FUTURE RESEARCH

As advancements in techniques for the identification and quantification of bacteria and archaeal continue and the quantitative analysis of HCs in effluent becomes more sensitive to detect even low concentrations, research into metagenomics should continue to collect information on bacterial diversity and function in the environment to drive improvement in existing WWTPs.

With environmental issues and concerns on the rise, a failure at a WWTP in the future will be a taboo. Hence, researchers should develop a cheap, less technical and fast microbial analytical method to be used by WWTP technicians for day-to-day monitoring of the diversity in wastewater treatment systems to provide warning signs to changes in diversity before works failure or to drive performance. Even better, the development of an on-line microbial-diversity-monitor should be researched to automate the processes from DNA extraction, through to sequencing and interpretation by artificial intelligence.

Collaborative studies on chemical mass balance through WWTP alongside microbial diversity and function should continue on large scale, across many WWTPs with the aim of finding the functions of bacterial communities and required operational conditions for their dominance. This will lead to the engineering of bespoke reactors connected in series, each with a unique set of operational parameters to enrich specific bacterial taxa for the degradation of targeted hazardous chemicals.

Future research should focus on identifying analytical methods suitable for studying different microbial species and their preferred substrate for degradation. For instance, it will be an excellent strategy to make use of fluorescence in situ hybridisation (FISH) to target exceptionally versatile HC degraders in samples from different sites whereas pyrosequencing could be applied to identify the abundance of bacterial genera of interest depending on the WWTPs operational conditions.

CHAPTER THREE

EFFECT OF PROCESS PARAMETERS ON MICROBIAL / BACTERIAL DIVERSITY AND HAZARDOUS CHEMICAL REMOVAL IN PILOT-SCALE ACTIVATED SLUDGE PLANT

3. EFFECT OF PROCESS PARAMETERS ON MICROBIAL / BACTERIAL DIVERSITY AND HAZARDOUS CHEMICAL REMOVAL IN PILOT-SCALE ACTIVATED SLUDGE PLANT

ABSTRACT

Recent bench-scale studies show that removal of estrogens and nonylphenols from wastewater can be enhanced through process parametric changes such as increasing solid and hydraulic retention times (SRT and HRT). It has been suggested in the literature that enhanced removal is due to corresponding changes in microbial diversity. In this study, the microbial diversity in an activated sludge pilot-plant fed with domestic sewage and operated at 3, 10 and 27 d SRT (constant HRT at 8 h) and at 8, 16 and 24 h HRT (constant SRT at 27 d) was monitored. The microbial identity and abundance were analysed using phospholipids fatty acid (PLFA) analysis, and the archaeal and bacterial diversities were assessed using genome sequencing of the 16S rRNA and compared at 97% similarity cut-off. The concentrations of estrogen and nonylphenols in the influent and effluent were quantified using UPLC MS/MS. The abundance ranking of microbial groups in all experiments fitted the log series of microbial diversity and the order was Gram-negative > fungi > Gram-positive > actinomycetes. Within the bacteria domain, the order of dominance was Proteobacteria followed by Bacteroidetes. At 3, 10 and 27 d SRTs, the dominant species were *Thiothrix eikelboomii* (11.9 %), *Nitrospira sp.* (6.6 %) and uncultured species from Comamonadaceae (5.9 %) respectively. The uncultured Comamonadaceae also dominated at HRTs of 8 and 24 h, while the *Nitrospira sp.* was dominant at 16 h HRT. The microbial diversity from PLFA changed and the bacterial diversity from DNA analysis increased as SRT and HRT were increased. Estrone (E1) and ethynylestradiol (EE2) removals were 19 and 30% (at 3 d SRT), 93 and 29% (at 10 d SRT) and 84 and 41% (at 27 d). Nonylphenol ethoxylates (NP₄EO and NP₅EO) removal rose from 44 and 49 % (at 3 d) to 73 and 85% (at 10 d) then to 84 and 89% (at 27 d). The effect of HRT was significant on α -diversity and on chemical removal. The abundances of several bacterial taxa showed correlation ($R > 0.7$) with the removal of various estrogens and nonylphenols. *Proteobacteria* negatively correlated with the removal of estrone, estradiol, estriol, nonylphenol ethoxylate (NP₃EO, NP₄EO, NP₅EO, NP₆EO, NP₇EO) while the *Chloroflexi* and *Verrucomicrobia* positively correlated with EE2 removal. The abundances of *Hyphomicrobium*, *Mesorhizobium*, *Planctomyces* and *Rhizomicrobium* positively correlated with the removals of E1, E2 and E3.

Leucobacter correlated negatively with all the estrogens except the readily degradable E1-3S. *Thiothrix*, which was dominant at 3 d SRT negatively correlates with the removal of NP₂EC and all nonylphenol ethoxylate with a chain length of three or more. Therefore, this study has shown for the first time, correlations between the abundance of bacterial taxa and the removal of estrogens and nonylphenols. These findings have the potential to explain the improvement in chemical removal associated with extending SRTs and HRTs which has been known for some time now. The research also confirmed the increase in bacterial diversity which occurs as a result of longer SRT and HRT.

Keywords: *microbial diversity, beta diversity, SRT, HRT, 16S rRNA, PLFA, estrogens, nonylphenols*

3.1 INTRODUCTION

The release of estrogens and nonylphenols into surface waters are of significant concerns due to their potential effects on human health, bioaccumulation in the environment (Barber et al. 2015) and the growing interests in wastewater reuse (Gomez-Lopez et al. 2009). It has been demonstrated that steroid estrogens, nonylphenol (NP) and nonylphenol ethoxylates (NPE) have the capability of feminising male aquatic organisms (Kidd et al., 2007; Blanchfield et al., 2015). Wastewater Treatment Plants (WWTPs) constitute a significant source of these chemicals and hence discharge from these outlets must be curtailed.

The removal efficiency of estrogenic compounds can be optimised by changing operational parameters such as solids retention time (SRT) and hydraulic retention time (HRT). For instance, the removal of estrogens in an activated sludge pilot-plant ASP increased from 70 to 95% when the SRT was changed from 3 to 10 d (Petrie et al., 2014). Amin et al., (2017) also showed improvement in estradiol and ethynylestradiol removal as SRT increased from 5 to 20 d, and increased removal of estrogens and attenuation of estrogenic activities with longer SRTs was observed by Kumar et al., (2015); Maeng et al., (2013) and Roh and Chu, (2011). Furthermore, removal efficiencies of nonylphenols and its ethoxylates and carboxylates derivatives increased as SRT lengthened (Koh et al., 2009; Gori et. al., 2010; Petrie et al., 2014). For instance, 37% of total alkylphenol removal was achieved at 8 d SRT while 59% was achieved at 20 d SRT (McAdam et al., 2011). Nonylphenol degradation rates were also reported to increase with longer SRT and it was suggested that the improved removal was due to greater species diversity (Langford et al., 2005). HRT increases leading to lower chemical loading rates also resulted in better removal of alkylphenols (McAdam et al., 2011).

Comparatively, the effect of HRT changes on the removal of chemicals is not as predictable as that of SRT. For example, Petrie et al. (2014) showed that increasing HRT from 8 to 24 h increased estrogen removal by 6% and Zhang et al., (2014) found that decreasing HRT from 25 to 5 h caused removal to drop by 10%. However, Kumar et al., (2015) observed no change in the removal of estrogens when HRT was altered. The improved removal during SRT and HRT increments has been attributed to increases in microbial activities, sorption to sludge or other chemical removal mechanisms. McAdam et al., (2011) explained that longer SRT allowed the slow-growing microbes to increase in population and degrade chemicals.

Roh and Chu (2011) suggested that longer SRTs led to a more diverse microbial community and facilitated the acclimatisation of the microbial community to the chemicals in the influent. HRT elongation leading to improved removal has also been credited to enhanced contact time between the chemicals and microorganism (Petrie et al., 2014) and reduced chemical loading rate which causes the microorganisms to compete and work harder for the limited resources available (Almasi et al., 2016). Other studies have observed the effects of SRT and HRT on microbial population and diversity in the WWTPs. For instance, the proportion of ammonia-oxidising-bacteria decreased from 33 to 15 % as the HRT decreased from 30 to 5 h; but nitrite-oxidising-bacteria increased from 4 to 15 % under the same conditions (Li et al., 2013). *Betaproteobacteria* and *Bacteroidetes* were the predominant phyla in a membrane bioreactor at low SRT but Delta and *Epsilonproteobacteria* abundance increased with longer SRT (Hu et al., 2012; Ahmed et al., 2007). Delta and *Gammaproteobacteria* abundances, as well as the removal of diclofenac, are favoured by longer SRTs.

Missing from literature is the link between bacterial taxa abundance and the removal of chemicals in WWTPs. This study explored the influence of SRT and HRT on microbial diversity and removal of estrogens and nonylphenols; and sought the correlations between chemical removal and bacterial genera abundance. For the purpose of this study, correlations with regression ($R > 0.7$) were considered as significant to report, to give future researchers a starting list of chemicals and bacteria taxa to investigate with a larger dataset.

The experiment involved setting up an activated sludge pilot-plant fed with real sewage and operated at a constant HRT while SRT is varied, and vice versa. Under each experimental condition, the impact of varying SRT and HRT on microbial diversity was assessed, by the wide-scoping phospholipids fatty acid analysis (PLFA), a method often used to study microbial diversity in soil by quantifying their phospholipid content arising from the cell wall/membrane of microbes living in the soil (Frostegard, 1993). Additionally, the effects of longer SRT and HRT on bacterial and archaeal diversity were analysed using 16S rRNA analysis. The influent and effluent concentrations of estrone (E1), estradiol (E2), estriol (E3), estrone-1,3-sulfate (E1-3S) and ethynylestradiol (EE2)] and nonylphenols (including ethoxylates and carboxylates) were measured by UPLC-MS/MS. The correlations between bacterial taxa abundance and chemical removal were investigated.

3.2 MATERIALS AND METHODS

3.2.1 Pilot-plant configuration and operation

A pilot activated sludge plant consisting of primary sedimentation tank (0.18 m^3), aerated basin (0.36 m^3) and a final clarifier (0.10 m^3) was fed with crude sewage from an area with population equivalent of 3,000. The raw sewage was pumped at $1.08 \text{ m}^3/\text{d}$, and the recycle ratio of the return activated sludge (RAS) to the flow rate was 0.60 of the settled sewage flows. Daily measurements of final effluent suspended solids and MLSS informed on the volume of sludge to be wasted to control the SRT.



Figure 3.1. Picture of pilot-plant showing the set-up of the primary and final sedimentation tanks (grey PVC tanks) and the mixed liquor tank (white PVC tank).

The pilot-plant was operated for a period no less than three times the length of the required SRT to establish steady-state. While HRT was maintained at 8 h, 3 d SRT was established and stabilised for 22 days after which, the plant was sampled for seven consecutive days from crude, settled and final effluents to analyse for removal of sanitary indicators (BOD, COD, TSS and ammonia) and estrogens and nonylphenols removals. Additionally, mixed liquor samples were collected for microbial diversity analysis. Three samples were taken each morning at an hour interval, 1L each for the crude, settled and final effluent and 2.5 L of mixed liquor sample. After the sampling campaign, the wastage regime was altered to achieve MLSS equivalent to 10 d SRT, followed by 35 days of stabilisation and then sampled.

The wastage of sludge was stopped until 27 d SRT was established, and wastage begun and continued for 85 days to achieve steady-state and then sampled thereafter. After the 27 d SRT and 8 h HRT sampling, the influent flow rate was adjusted to 22.5 L/h to increase the HRT to 16 h and operated at the 27 d SRT for thirty days and sampled for seven consecutive days thereafter. The flow rate was further reduced to 15 L/h to obtain an HRT of 24 h and further operated for thirty-two days before sampling. A total of 70 samples comprising of 7 mixed liquor samples, and 63 samples (21 each) of crude, settled and final effluent were taken.

3.2.2 Sanitary determinants analysis

Chemical oxygen demand (COD), ammonia ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$) concentrations were measured with cell test kits from VWR International (Leicestershire, UK) using a 5487 spectrophotometer (Cheshire, UK). Suspended and volatile solids and biochemical oxygen demand (BOD) were determined following standard methods (APHA, 2012).

3.2.3 Microbial analytical methods

3.2.3.1 Rationale and modification of the PLFA analytical method

Phospholipid fatty acid analysis is widely used in soil studies to characterise microbial community variations during changes in the environment (Frostegard et al., 1993). PLFA measures the abundance of individual fatty acids extracted from microbial cell membranes. The fatty acids can be used as indicators of microbial diversity as different microbial groups have been associated with specific fatty acids (Zornoza et al., 2009). Also, changes in a system are known to affect the type and abundance of lipids in the microbial cell membrane, for example, external stressors such as lack or abundance of nutrients (from variations in SRT and HRT) causes a shift in fatty acid types and abundance. The degradation of phospholipids in the cell membrane is rapid upon the death of the microorganism, hence this method is indicative of live microbial mass. The proportion of Gram-positive (GRAM+) bacteria in each sample was quantified as the sum of relative abundances of methyl esters of fatty acids: 14:0i, 15:0i, 15:0ai, 16:0i, 17:0i and 17:0ai; Gram-negative (GRAM-) bacteria by 16:1w7c, 15:1w4, 16:1w9c, cy19:0, cy17:0, 17:1w9c, and 18:1w9c, 18:1w7t; actinomycetes (ACTS) by 10Me16:0, 10Me17:0 and 10Me18:0; and fungi abundance by 16:1w5c, 18:1w9c, 18:2w6, 18:3w3 and 20:5w3 (Zornoza et al., 2009).

PLFA was chosen to see if the microbial diversity changes in ASPs as a result of changes in process parameters can be detected by a faster, inexpensive and lesser number of steps in the method than DNA analysis (Fornasier et al., 2014). Preliminary trial of the method led to a modification of the mass required for the analysis. In this study, ~0.5 g of freed dried sludge sample was used (instead of 5 g of soil sample) because >1.0 g mass of dried resulted in merged chromatograms from the Gas Chromatography and <0.5 g resulted in tiny peaks which made quantification difficult.

3.2.3.2 Phospholipids fatty acid analysis

Standard solutions and reagents

Reagent A [Toluene: methanol, 1:1], *reagent B* [0.56 g KOH in 50 mL CH₃OH]; *reagent C* [acetic acid 59 mL/L] and *reagent D* [Hexane: chloroform, 4:1]. Citrate buffer was prepared by dissolving 7.2 g of anhydrous citric acid and 11 g tri-sodium citrate in 250 mL ultrapure deionised water from 18.2 MΩ quality (Elga, Marlow, UK).

Bligh-and-Dyer solvent was made up of 21% citrate buffer, 26% chloroform, 53% methanol (v/v/v) and 30 mg of butylhydroxytoluene. Chloroform, toluene, ammonium hydroxide and ammonium acetate were acquired from Sigma Aldrich (Dorset, UK).

Sample preparation

Mixed liquor samples (250 mL) were transferred into Nalgene bottle and centrifuged at 8000 rpm for 20 minutes at 4 °C. The supernatant was decanted, and the remaining sludge was re-suspended in approximately 20 mL of supernatant and transferred into a 50-mL plastic sample bag and kept at -80 °C for 48 h followed by freeze-drying at -50 °C and then stored until analysis.

Extraction of phospholipids

Approximately 0.5 g of freeze-dried sludge was weighed into glass media bottle, topped with 15 mL of Bligh-and-Dyer solvent, sonicated for 30 minutes and then centrifuged at 700 relative centrifugal force (RCF) for 10 minutes. The upper layer was decanted into a clean glass media bottle. 4 mL chloroform and 4 mL citrate buffer were added before the solution was centrifuged again. The top (aqueous) layer was removed and discarded. The remaining solvent was then evaporated to dryness at 37 °C under nitrogen gas.

Solid-phase extraction (SPE)

An SPE cartridge topped with approximately 0.5 g sodium sulphate (Na_2SO_4) was washed twice with 2 mL chloroform. The phospholipid extract was then dissolved in 1 mL chloroform and transferred into the SPE cartridge and washed with 1 mL chloroform. The sterols and glycol lipids were eluted to waste with 5 mL chloroform and 12 mL acetone respectively. The phospholipids were eluted with 8 mL methanol into a clean 25-mL centrifuge bottle followed by drying at 37 °C and under N_2 .

Methyl esterification

The phospholipids were converted to fatty acids methyl esters (FAMES) by adding 1 mL each of reagents A and B followed by incubation at 37 °C for half an hour. A 0.25 mL aliquot of reagent C, 5 mL of reagent D and 3 mL of deionised water were added and then centrifuged at 700 RCF for 10 minutes. The organic phase was carefully pipetted and dispensed through a column (packed with glass-wool and Na_2SO_4) into a clean glass bottle and rinsed twice with 1 mL of hexane. The hexane was evaporated under N_2 at 20 – 25 °C and the residue was then reconstituted with 1 mL hexane, transferred into a vial. The FAMES were then analysed by GC–FID.

3.2.3.3 Rationale for 16S rRNA analytical method

The 16S ribosomal ribonucleic acid (16S rRNA) analysis is widely used for bacterial phylogeny and taxonomy (Johnson et al., 2019) because (i) it is present in almost all bacteria, often found as a multigene family; (ii) its function over time has not changed, which implies that random gene mutation in this sequence is an accurate measure of time of evolution; and (iii) the 16S rRNA gene (1500 bp) is large enough for informatics purposes (Janda & Abbott, 2007). The 16S rRNA gene sequencing was used to further investigate the bacterial diversity at various taxonomic levels of the prokaryotes. In this analysis, the V4 region of the 16S rRNA gene of bacteria and archaea is amplified in a pool of microbial DNA previously extracted from the mixed liquor sample. The conserved nature of the 16S rRNA gene lends itself to species identification as differences between sequences can be indicative of different taxa. With increasing literature on DNA sequences from pure bacterial and archaeal cultures, gene sequences from the V4 region of the 16S rRNA gene can be compared to clone libraries to identify the taxa at a specified operational taxonomic unit (OTU) which is a level of similarity between sequences for them to be related. In this study,

the sequences were compared at 97% similarity cut-off. The 16S rRNA gene sequencing method was chosen to assess how changes in SRT and HRT influence bacterial diversity at genus and species level and how bacterial taxa abundance correlate with hazardous chemical removal.

3.2.3.4 16S rRNA analysis

The 16S rRNA analysis was carried out by Gregg Icton of PROKARYA using the same method described in section 4.3.2.2 in chapter four; and the bioinformatic processing of the data was also done with the same processing pipeline used in chapter four by the Davenport Group at Newcastle. PROKARYA then provided the list of bacteria and archaea taxa identified in the samples as well as their abundances.

3.2.4 Hazardous Chemical Analysis

3.2.4.1 Estrogens

Chemicals, standard solutions and reagents

Analytical grade (99.9% purity) estrone (E1), 17 β -estradiol (E2), estriol (E3), 17 α -ethynylestradiol (EE2), estrone sulfate (E1-3S) from Sigma Aldrich (Dorset, UK). Hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH) from Rathburn Chemicals (Walkerburn, UK). Two ng/L mixed estrogens, 15 ng/L mixed estrogens, EtOAc:hexane (10:90), 3% NH₄OH in MeOH, DCM:MeOH (90:10), ultrapure (UP) water:MeOH (80:20) containing 0.1% NH₄OH and 0.1% acetic acid in MeOH. Deuterated internal standards estrone-2,4,16,16-d₄, 17 β -estradiol-2,4,16,16,17-d₅, estriol-2,4,17-d₃, 17 α -ethynylestradiol-2,4,16,16-d₄, sodium estrone-2,4,16,16-d₄ sulfate from QMX Laboratories (Thaxted, UK).

Extraction of estrogens

One-litre samples of influent or effluent were filtered through 1.2 μ m GF/C filter paper (VWR, Lutterworth, UK) and spiked with low (2 ng/L) and high (15 ng/L) deuterated estrogens to monitor recoveries of the extraction process and to correct for any variability in the response of the detector. These spike concentrations are typical of those found in

environmental samples and are approximately one order of magnitude greater than the detection limit of the method. Samples were passed through 500 mg: 6cc tC18 cartridge (Waters, Elstree, UK), pre-conditioned with 5 mL methanol followed by 5 mL UP water, at 5 mL/min. Cartridges were changed after passing 500 mL of sample to avoid saturation. Afterwards, the cartridge was washed with 5 mL UP water and left to dry for an hour under vacuum. The estrogens were eluted with 10 mL MeOH methanol followed by 10 mL DCM, and the extract was evaporated to 1 mL in a rotary evaporator and to complete dryness under nitrogen gas. The analyte was reconstituted with 0.2 mL DCM:MeOH (90:10) and cleaned through gel permeation chromatography (GPC) by injecting it onto a 5 μ m, 300 mm x 75 mm gel permeation size exclusion column (Varian, Oxford, UK), under isocratic conditions (DCM:MeOH, 90:10 v/v) at constant flow rate of 1 mL/min. The fraction between 5.5 and 11.5 minutes was collected and reduced to approximately 0.2 mL by evaporation. The eluent was re-constituted to 2 mL with hexane, passed through a pre-conditioned 500 mg: 6 cc NH₂ SPE cartridge (Varian, Oxford, UK) at a flow rate between 5 and 10 mL/min and washed with 4 mL EtOAc: hexane (10:90). The non-polar steroids estrogens (E1, E2 and EE2) were then eluted with 6 mL EtOAc and the polar estrogens (E1-3S and E3) with 6 mL of 3% NH₄OH in MeOH. Each aliquot was evaporated separately under a stream of nitrogen to dryness, reconstituted with 0.2 mL UP water:MeOH (80:20) containing 0.1% NH₄OH and transferred into autosampler vials for UPLC detection and quantification.

UPLC MS/MS detection

Ultra-performance liquid chromatography (UPLC) method was performed using a Waters Acquity UPLC (Waters, Manchester, UK). Acquity UPLC BEH C18 column (100 mm x 2.1 mm i.d., 1.7 μ m particle size; Waters, Manchester, UK) maintained at 45 °C was used for separation of the estrogens. A gradient separation of two mobile phases was used consisting of water containing 0.1 % NH₄OH (A) and methanol containing 0.1 % NH₄OH (B) passed at a constant flow rate of 0.2 mL/min. Initial conditions of 80 % A and 20 % B was increased gradually to 80 % B over 6 minutes and maintained for 1 minute. This was then returned to starting conditions for 2 minutes for equilibration. The total run time was 9 minutes. The maximum injection volume was 10 μ L to prevent band broadening and loss of efficiency (Petrie et al., 2014). Detection was achieved by a Waters Quattro Premier XE mass spectrometer with a Z-spray ESI interface (Micromass, Watford, UK). This was operated in

the negative electrospray ionisation mode using multiple reaction monitoring (MRM). Two MRM transitions were monitored for each estrogen compound; the most sensitive transition monitored for quantitation and the second for analyte confirmation. To maximise sensitivity the detection, estrogens was divided into two acquisition periods. E1-3S and E3 were monitored in the first period whilst the less polar estrogen (E1, E2 and EE2) were observed in the latter. The m/z values for the detection of the estrogens are listed in appended table AT29. The parameters for the mass spectrometry detection were 3.20 kV capillary voltage, 650 V multiplier voltage, 1000 L/h desolvation gas flow, -55 V cone voltage, 0.2 V RF lens, 49 L/h cone gas flow; 350 °C desolvation temperature and 120 °C source temperature (Koh et al., 2007).

3.2.4.2 Nonylphenols

Standard solutions and reagents

Technical 4-NP, 4-nonylphenol-mono-ethoxylate (NP₁EO), nonylphenol di-ethoxylate (NP₂EO), long chain NPEOs mixtures CO210, CO520 and CO720 were acquired from Sigma Aldrich (Dorset, UK). Nonyl-phenoxy acetic acid (NP₁EC) from QMX Laboratories (Thaxted, UK). HPLC grade (99.9% purity) acetone, dichloromethane, ethyl acetate, hexane, methanol (MeOH) and acetonitrile (ACN) from Rathburn Chemicals (Walkerburn, UK).

Extraction of nonylphenols

Final effluent (250 mL) and influent (100 mL) samples were filtered through 1.2 µm GF/C filter paper (VWR, Lutterworth, UK). The solids were discarded while the aqueous phase was loaded on a 500mg:3cc tC18 SPE cartridge (Waters, Elstree, UK) at 5 – 10 mL/min, pre-conditioned with 5 mL MeOH followed by 5 mL UP water. The cartridge was then washed with 5 mL UP water and left to dry under vacuum for an hour. The analytes were eluted with 5 mL EtOAc followed by 5 mL of 0.1% acetic acid in MeOH and 5 mL DCM, the eluent was then evaporated to 1 mL and dried under N₂. Subsequently, the extract was reconstituted with 0.25 mL of UP water: MeOH (80:20) and transferred into autosampler vials for detection. Prior to the extraction, samples were spiked low concentration (100 ng/L) and high concentration (1000 ng/L) of mixed compounds to assess recoveries and to ensure the reliability of the analytical method. McAdam et al., (2011) and Petrie et al., (2014) showed that these concentrations of the mixed compounds used to spike the samples are

representative because they are greater than the method's limit of detection by one order of magnitude as well as being in the range of concentrations expected to be found in the samples.

UPLC-MS/MS detection

Ultra-performance liquid chromatography was done using Waters Acquity UPLC (Waters, Manchester, UK). Chromatographic separations were achieved using an Acquity UPLC BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm particle size; Waters, Manchester, UK) controlled at 30 °C. The solutions used for gradient elution were 0.1 % NH₄OH in UP water (A) and 0.1 % NH₄OH in MeOH (B) at a flow rate of 0.4 ml per minute. Initially, 20 % B was maintained for 4 minutes and then slowly increased to 80 % over 5 minutes. This was held for 12 minutes and then returned to starting conditions over 3 minutes. The total run time was 26 minutes and an injection volume of 10 µL was used. Waters Quattro Premier XE mass spectrometer with a Z-spray ESI interface (Micromass, Watford, UK) was used for detection. The mass spectrometer was operated in the negative and positive electrospray ionisation mode utilising multiple reaction monitoring. The parameters for detection by the mass spectrometer were as follows: -2.3 kV (negative mode) and 3.20 kV (positive mode) capillary voltage; 1.0 V (negative mode) and 0.5 V (positive mode) RF lens, 3.0 V extractor lens, 650 V multiplier voltage, 1000 L/h desolvation gas flow, 50 L/h cone gas flow, 350 °C desolvation temperature and 120 °C source temperature (Petrie et al, 2014). The m/z values for the detection of the nonylphenols are presented in appended table AT29.

The limit of detection (LoD) and limit of quantification (LoQ) were derived based on the standard deviation of the response of blanks and the gradient approach. Seven blank samples were prepared by following the analytical procedure and analysed in the UPLC-MS/MS. The mean and standard deviation of the results were calculated using Excel 2016. Results were plotted and the concentration gradient was obtained. The LoD and LoQ were calculated as:

$$LoD = 3 \times SD / S \quad \text{(Equation 3.1)}$$

$$LoQ = 10 \times SD / S \quad \text{(Equation 3.2)}$$

where SD is standard deviation and S is the gradient.

3.2.5 Statistical analysis of the data

To compare richness and evenness of species in the pilot-scale reactor under each experimental condition, Simpson's index (D) of diversity was used. It was calculated in Microsoft Excel (2016) as:

$$\text{Simpson index (D)} = \frac{\sum_{i=1}^R n_i(n_i - 1)}{N(N - 1)} \quad (\text{Equation 3.3})$$

n_i = number of individuals in one type of species

N = total number of individuals in a system

Although it is known that this list of species obtained is not an exhaustive list all the species present in the reactor but the most common captured in the grab sample, it was treated as if it was an exhaustive list, hence the percentage abundance summed up to 100.

3.3 RESULTS

3.3.1 UPLC-MS/MS method performance

The mean recoveries of the extraction of estrogens ranged between 85 and 101% for settled sewage and 89 – 98% for the final effluent. The nonylphenols recoveries were 55 – 98% and 58 – 99% for settled sewage and final effluent respectively. The relative standard deviations (RSD) of the recoveries were 6.4% or less in the estrogens. The limits of detections (LoD) of the estrogens were 0.04 – 0.17 ng/L in settled sewage and 0.03 – 0.13 ng/L in the final effluent. Estrone-1,3-sulfate had the lowest LoD in all samples and the least recovery in settled sewage. Recoveries of nonylphenol, nonylphenol carboxylate and nonylphenol ethoxylates in settled sewage ranged from 55 – 99% with RSD between 4.2 and 13.1%, whereas recoveries in final effluent were 58 – 99% with RSD from 1.6 to 12.8%. NP₄EO had poor recovery in all samples and high RSD. Long-chained ethoxylates had mostly poorer recoveries than short-chained ethoxylates. The LoDs of the NP, NPEOs and NPECs ranged from 1.5 ng/L to 47.9 ng/L in both settled sewage and final effluent samples (Table 3.1).

Table 3.1. Limits of Quantification and Limits of Detection

Hazardous chemical	Settled sewage			Final effluent		
	LoD (ng/L)	Recovery (%)	RSD (%)	LoD (ng/L)	Recovery (%)	RSD (%)
E1	0.06	100.6	1.1	0.05	96.8	0.6
E2	0.17	92.1	6.4	0.13	96.7	6.8
E3	0.12	88.4	6.1	0.11	89.3	8.8
EE2	0.06	100.6	5.8	0.06	97.5	5.5
E1-3S	0.04	84.5	5.3	0.03	92.9	6.2
NP	23.8	99.2	4.2	9.3	98.9	3.3
NP ₁ EC	47.9	56.4	10.2	16.7	79.9	4.9
NP ₁ EO	30.7	97.5	4.4	14.2	84.0	6.3
NP ₂ EO	12.2	65.9	9.7	3.5	94.0	1.6
NP ₃ EO	3.7	61.5	13.1	1.5	62.2	5.5
NP ₄ EO	25.9	55.2	8.7	9.6	57.6	12.8
NP ₅ EO	11.1	58.5	11.8	2.4	75.8	8.1
NP ₆ EO	14.2	58.2	6.5	3.6	72.8	4.7
NP ₇ EO	8.0	68.4	5.2	3.1	75.2	3.9
NP ₈ EO	5.7	65.9	8.9	1.7	84.4	4.6
NP ₉ EO	9.1	62.5	5.5	2.8	75.9	3.3
NP ₁₀ EO	8.2	71.3	7.2	3.3	77.6	2.0
NP ₁₁ EO	11.4	55.9	12.8	4.1	63.8	7.9
NP ₁₂ EO	32.7	57.2	7.4	10.4	65.4	5.8

Limit of detection (LoD); relative standard deviation (RSD)

3.3.2 Pilot-plant performance

A summary of the loading and percentage removal of nutrients under each experimental setting during the pilot-plant sampling is presented in Table 3.2 and the full data in appended Tables AT1 and AT2. The percentage removal was calculated as follows:

$$\text{Percentage removal} = \frac{C_i - C_e}{C_i} \times 100 \quad (\text{Equation 3.2})$$

$$C_i = \text{influent concentration} \quad C_e = \text{effluent concentration}$$

The pilot-plant achieved removals of 74% BOD, 62% COD, 6% ammonia and 37% suspended solids and no nitrification at 3 d SRT. The removals increased significantly ($p < 0.001$, $n = 7$) when SRT was extended 10 d with 88% BOD, 82% COD, 99.5% ammonia and 76% suspended solids. In the HRT study, 94 – 96% of BOD and 82 – 84% of COD removals were observed, and all simulations achieved nitrification. T-test of the removals showed significant differences between all the SRTs studied except for the removal of COD ($p > 0.05$, $n = 7$) and ammonia ($p > 0.05$, $n = 7$) between 10 and 27 d SRT. In the HRT study, there was insignificant variation in the removals of ammonia or COD between 8, 16 and 24 h, as well as BOD ($p > 0.05$, $n = 7$) between 16 and 24 h.

Table 3.2. Performance of the pilot-plant in each experimental set-up

Pilot plant set-up		BOD ₅			COD			Ammonia			Suspended solids		
		Settled	Final effluent	% Mean removal	Settled	Final effluent	% Mean removal	Settled	Final effluent	% Mean removal	Settled	Final effluent	% Mean removal
S R T	3 d	127	32.7	74.3	458	172	62.4	51.5	48.4	5.9	140	88.3	37.0
		±20	±6.1	±2.4	±28	±13	±3.1	±2.9	±2.4	±1.0	±9	±4.7	±4.1
	10 d	120	14.6	87.8	510	91.1	81.9	50.8	0.3	99.5	127	30.1	76.3
		±7.3	±1.6	±2.4	±83	±13	±3.6	±2.8	±0.1	±0.2	±5	±5.3	±4.1
	27 d	123	4.8	96.1	442	74.9	82.9	50.9	0.2	99.5	142	18.0	87.4
		±14	±1.3	±0.7	±46	±9.6	±2.4	±1.7	±0.1	±0.1	±7	±4.2	±2.6
H R T	8 h	123	4.8	96.1	442	74.9	82.9	50.9	0.2 ±	99.5	142	18.0	87.4
		±14	±1.3	±0.7	±46	±10	±2.4	±1.7	0.07	±0.14	±7	±4.2	±2.6
	16 h	103	6.1	94.1	363	56.4 ±	84.4	37.0	0.2 ±	99.6	109	27.9	74.4
		±9	±2.1	±1.8	±19	3.9	±1.0	±3.7	0.03	±0.08	±21	±4.8	±3.1
	24 h	102	4.1	95.6	367	65.5	82.2	35.6	0.1 ±	99.6	89.0	29.6	67.0
		±31	±2.0	±2.8	±25	±8.9	±1.4	±5.5	0.03	±0.09	±7	±4.5	±3.5

BOD₅ = Biochemical oxygen demand with 5-day incubation period; COD = Chemical oxygen demand

3.3.3 Impact of high SRT on aeration demand and final effluent quality

For a plant operating at 3500 mg/L MLSS, F:M ratio of 0.12 and 10 d SRT, for every 1-day rise in SRT, the MLSS will increase by 10% (to 3,850 mg/L) without increasing the total volume of influent to the plant. This 10% increase in biomass would result in an endogenous demand for air and impact on the aeration. This will equate to 15% of total oxygen demand and to supply this extra oxygen, the airflow must increase by 1.5%. Hence, for every 1-day increase in SRT, the energy consumption of the blowers in the activated sludge tank will increase by about 1.5%.

Settlement in the final sedimentation tanks (FST) become poorer at higher SRT and make the FTS prone to sludge blanket blow-up. The plant (at MLSS of 3,500 mg/L; 10 days SRT) with suspended solids volume index (SSVI) of 120 mL/L requires that the maximum upflow velocity (U_{max}) of the mixed liquor in the FST should be approximately 0.87 m/h. This U_{max} will allow the solids to settle adequately before reaching the outlet weir of the FST. A one-day increase in the SRT implies the new MLSS of 3,850 mg/l at the same SSVI will reduce the U_{max} to 0.75 m/h. As the upflow in FST is reduced, and the inflow of the plant is expected to remain unchanged, an additional 14% surface area is needed in FST capacity to maintain the same settlement performance and final effluent quality.

3.3.4 Microbial diversity from PLFA at different SRT and HRT

Under all experimental conditions, the order of microbial abundance ranked as Gram-negative > fungi > Gram-positive > actinomycetes, also reported by Amir et al., (2010) in sludge cake studies. The high abundance of Gram-negative bacteria is due to the naturally high proportion of Gram-negative bacteria found in environmental samples, estimated between 40-86% of total bacteria count (Forster et al., 2002). The ranked abundance plot (Figure 3.2) fitted the log series diversity index when the percentage abundances are treated as the number of individual species.

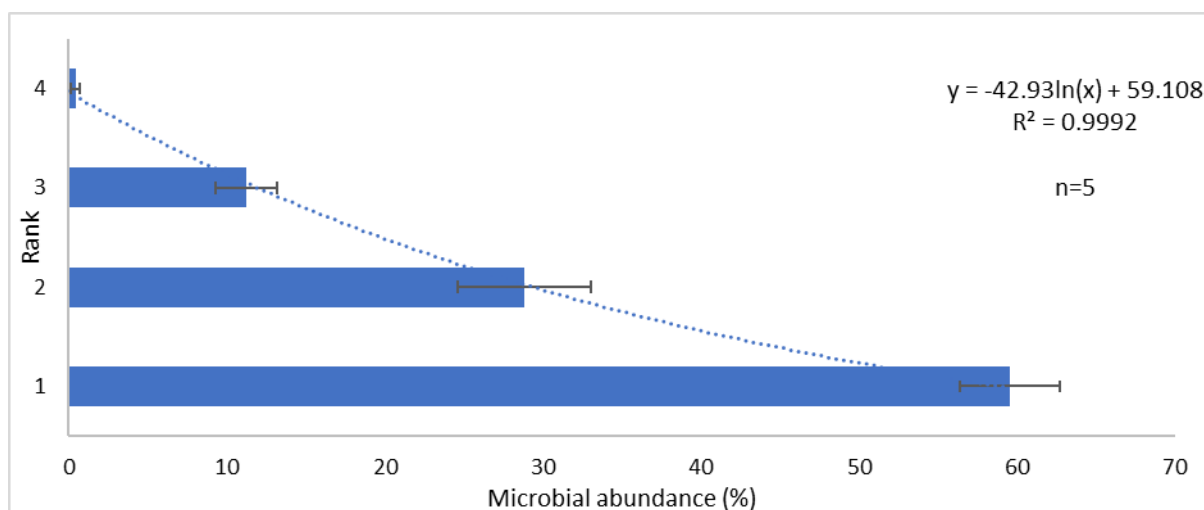


Figure 3.2. The rank plot of the mean abundance of Gram-, fungi, Gram+ and actinomycetes in the pilot-scale ASP samples

SRT increases results in changes in α -diversity in the pilot reactor as indicated by the differences in fatty acid profiles in Figures 3.3 and 3.4. The changes in fatty acid profiles as the process parameters changed were significant ($p < 0.01$, $n = 5$). SRT increase from 3 to 10 d coincided with a decrease in Gram-positive and Gram-negative bacteria abundance by 5 and 6% respectively, whereas fungi abundance increased by 10.7% and a significant ($p < 0.01$, $n = 7$) increment in the abundance of actinomycetes from 0.15% to 0.52%. Further elongation of SRT to 27 d had insignificant effect ($p > 0.05$, $n = 7$) on Gram-positive bacteria abundance, significant ($p < 0.01$, $n = 7$) reduction in actinomycetes, reduced fungal abundance by 8.7% and increased the gram-negative bacteria by 7.8%.

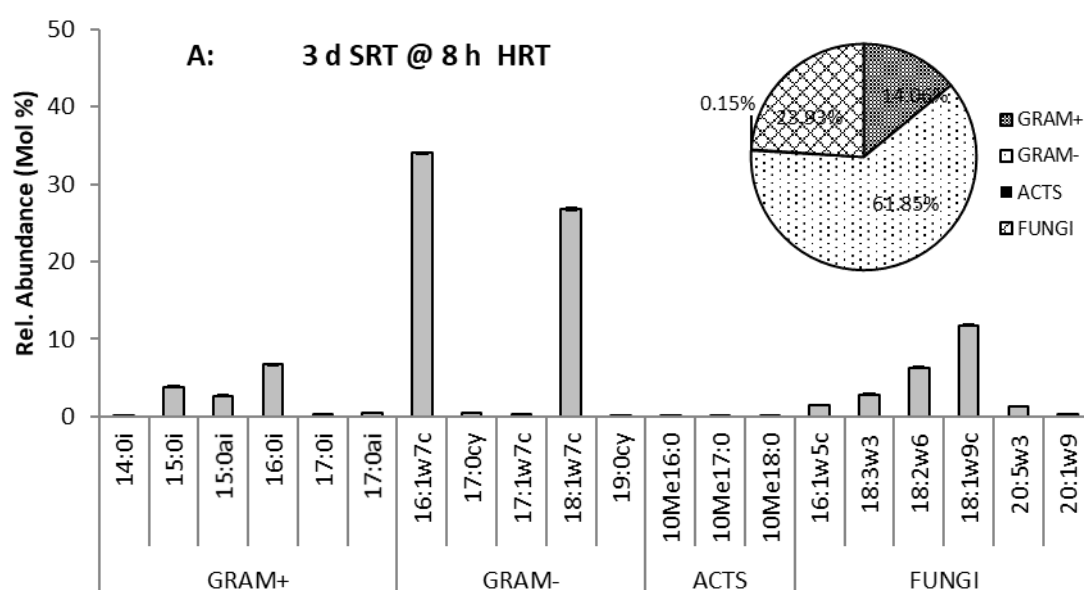


Figure 3.3A. Microbial diversity indicated by fatty acid methyl ester biomarkers at 3 d SRT

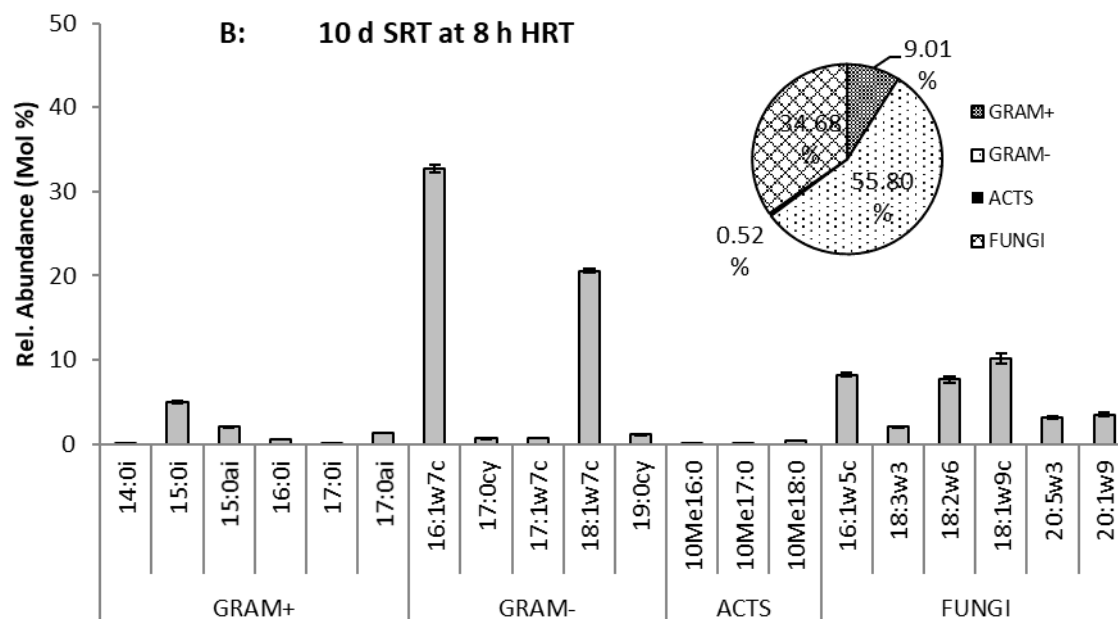


Figure 3.3B. Microbial diversity indicated by fatty acid methyl ester biomarkers at 10 d SRT

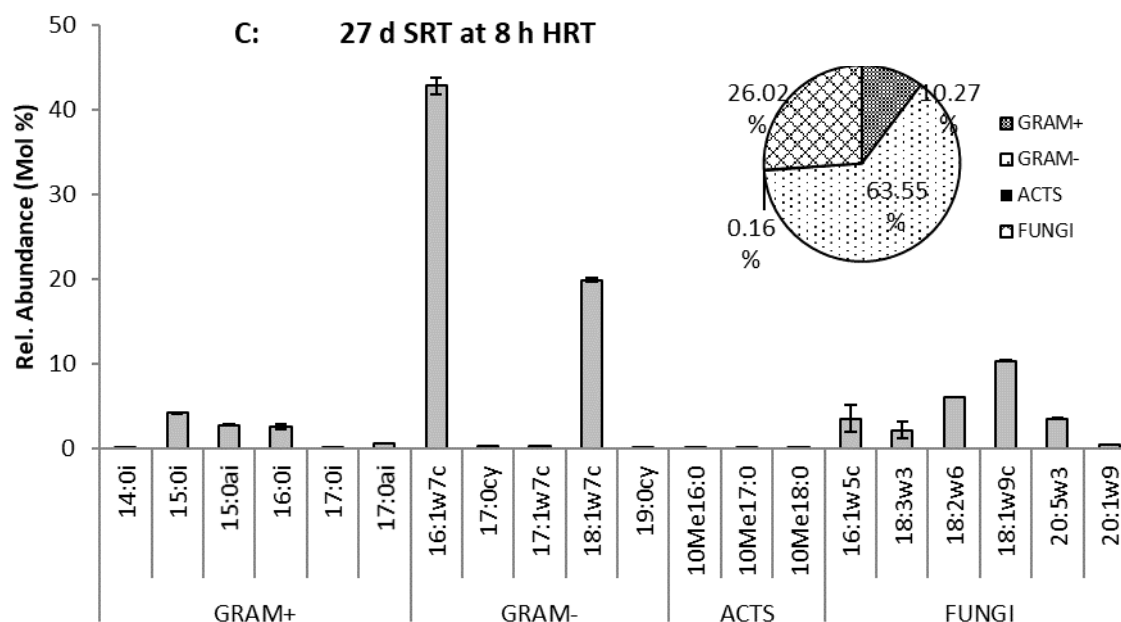


Figure 3.3C. Microbial diversity indicated by fatty acid methyl ester biomarkers at 27 d SRT.

A strong correlation between cy17:0 and 16:1w7c was identified ($R^2 = 0.86940$) as SRT increased from 3 to 27 d. The cy17:0 fatty acid is a modification of 16:1w7c fatty acid and is formed as a result of starvation or stress in the reactor (Frostegard et al., 2010). The fatty acids correlation affirms that increasing SRT reduces the food-to-microorganisms ratio and causes microbial diversity to change. Figures 3.4A and B are principal component analysis (PCA) and loading plot of the microbial diversities found at 3, 10 and 27 d SRT as indicated by the fatty acid profile, and it shows that at 3, 10 and 27 d SRT, the microbial communities were dissimilar.

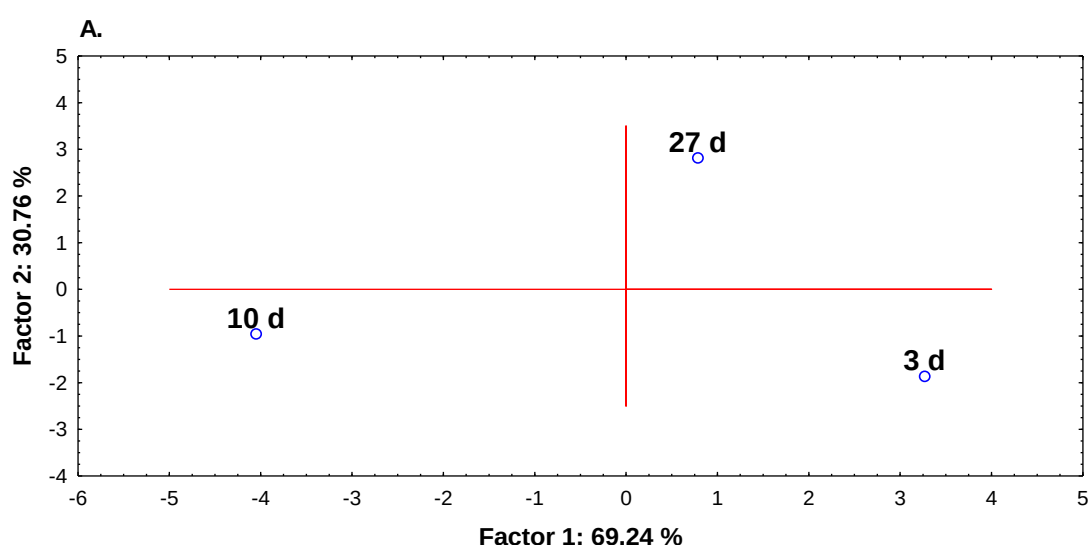


Figure. 3.4A: Microbial diversity from fatty acids profiles at 3, 10 and 27 d SRT (n=7).

The communities at 3 and 10 d differed from each other but both showed some similarities with 27 d SRT. That is, the community at 27 d had similar fungi profile to that at 3 d and similar Gram-positive profile to that at 10 d SRT.

The loading plot (Figure 3.4B) shows that at 3 d SRT, the fatty acids 16:0i, 17:0i, 18:3w3, 18:1w9c and 18:1w7c had high abundances. At 10 d SRT, the dominant fatty acids were 15:0i, 16:1w5c, 17:0ai, 10Me17:0, 19:0cy, 20:1w9, 17:1w7c, 10Me18:0 and 18:2w6. The 27 d SRT investigation had high percentages of 16:1w7c, 10Me16:0 and 20:5w3 fatty acids.

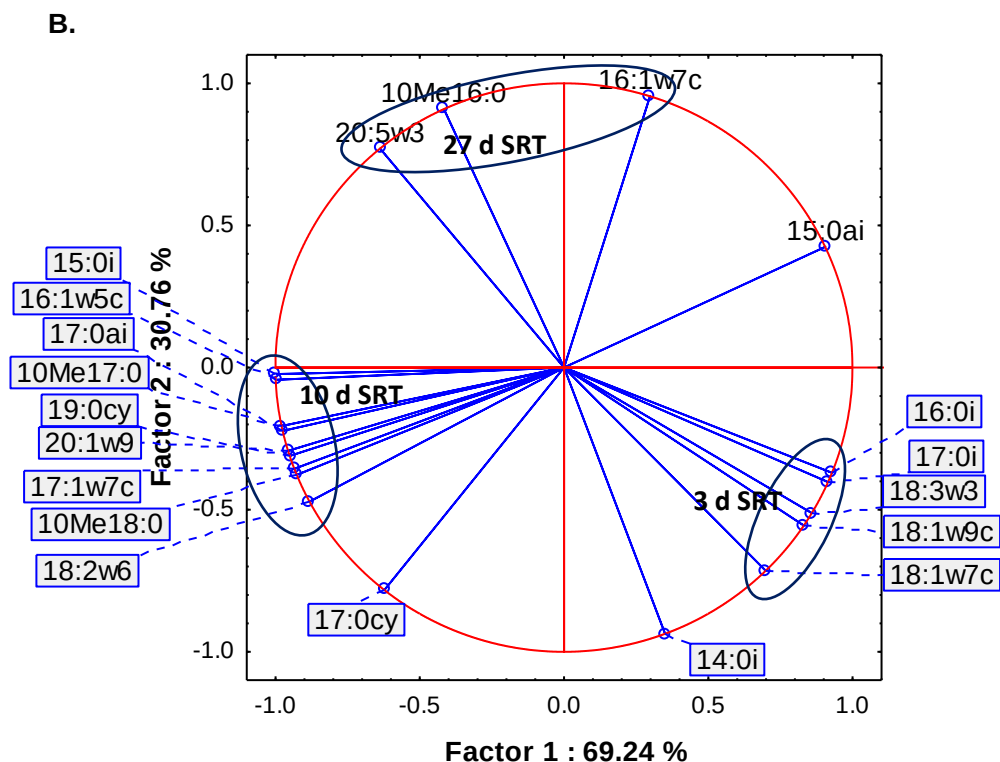


Figure. 3.4B: Loading plot of microbial diversity from fatty acids profiles at 3, 10 and 27 d SRT.

Figures 3.5(A, B and C) shows that HRT changes also caused shifts in microbial diversity. As HRT was lengthened from 8 to 16 h, the fungi proportion increased by 5.1% and Gram-negative bacteria showed a reduction of 6.0%. Further elongation of HRT to 24 h reduced fungi proportion and Gram-positive bacteria by 2.8% and 1.6% respectively, with a corresponding increase in Gram-negative bacteria by 1.41%. The order of abundance of the individual fatty acids was similar to that found at 8 h HRT, except for 18:3 ω 3 which became the second highest contributor by increasing from 1.2% at 16 h to 6.9% at 24 h HRT. These changes in fatty acid abundances imply that HRT increases caused shifts in microbial diversity.

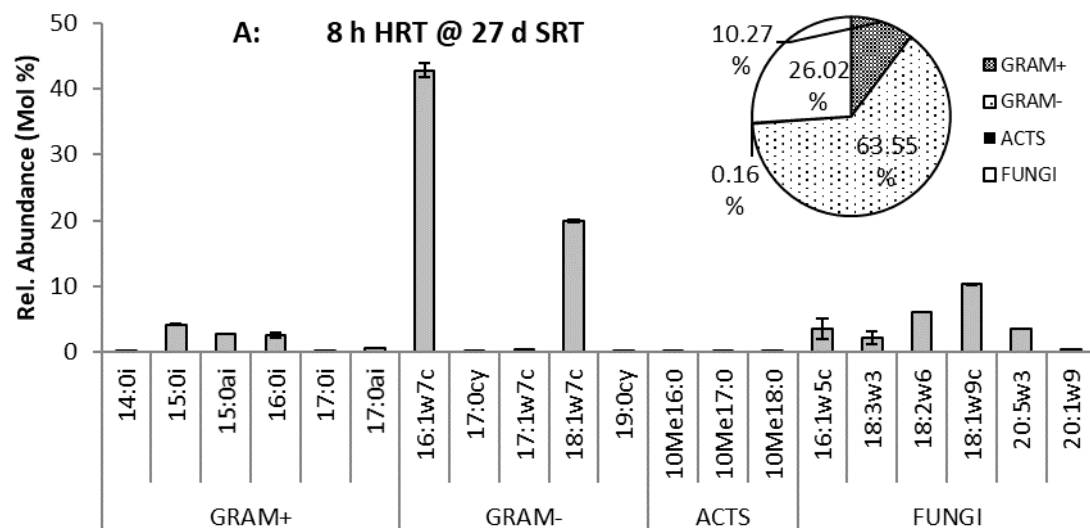


Figure 3.5A. Microbial diversity indicated by fatty acid methyl ester biomarkers at 8 h HRT.

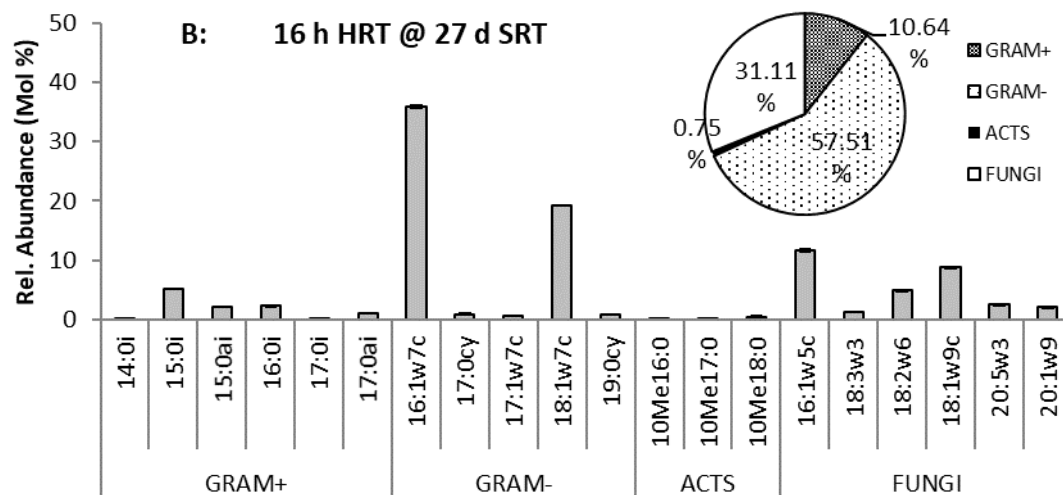


Figure 3.5B. Microbial diversity indicated by fatty acid methyl ester biomarkers at 16 h HRT.

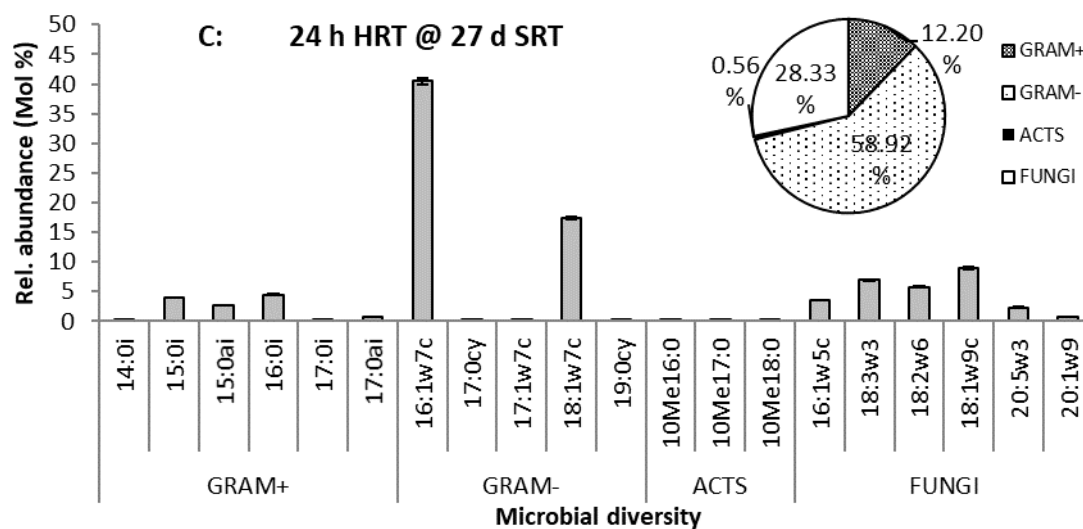


Figure 3.5C. Microbial diversity indicated by fatty acid methyl ester biomarkers at 24 h HRT.

Figures 3.6A and B shows the PCA and loading plot of microbial diversity as indicated by fatty acids at various HRTs. Figure 3.6A shows that at 8, 16 and 24 h, the microbial communities are unlike each other. At 8 h HRT, fatty acids 15:0ai, 17:0i, 16:1 ω 7c, 18:2 ω 6, 18:1 ω 9c and 20:5 ω 3 dominated in the microbial communities while fatty acids 15:0i, 16:1 ω 5c, 17:0ai, 17:0cy, 19:0cy, 10Me18:0, 17: ω 7c and 20:1 ω 9 were most abundant at 16 h HRT. At 24 h HRT, fatty acids 16:0i, 10Me16:0, 10Me17:0 and 18:3 ω 3 were higher in abundance (Figure 3.6B).

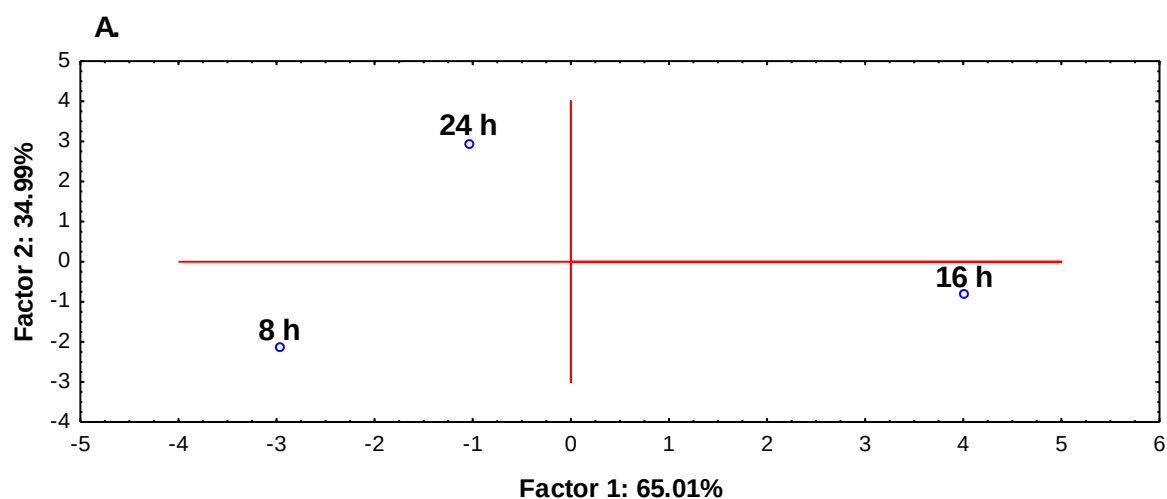


Figure. 3.6A: Microbial diversity from fatty acids profiles at 8, 16 and 24 h HRT (n=7).

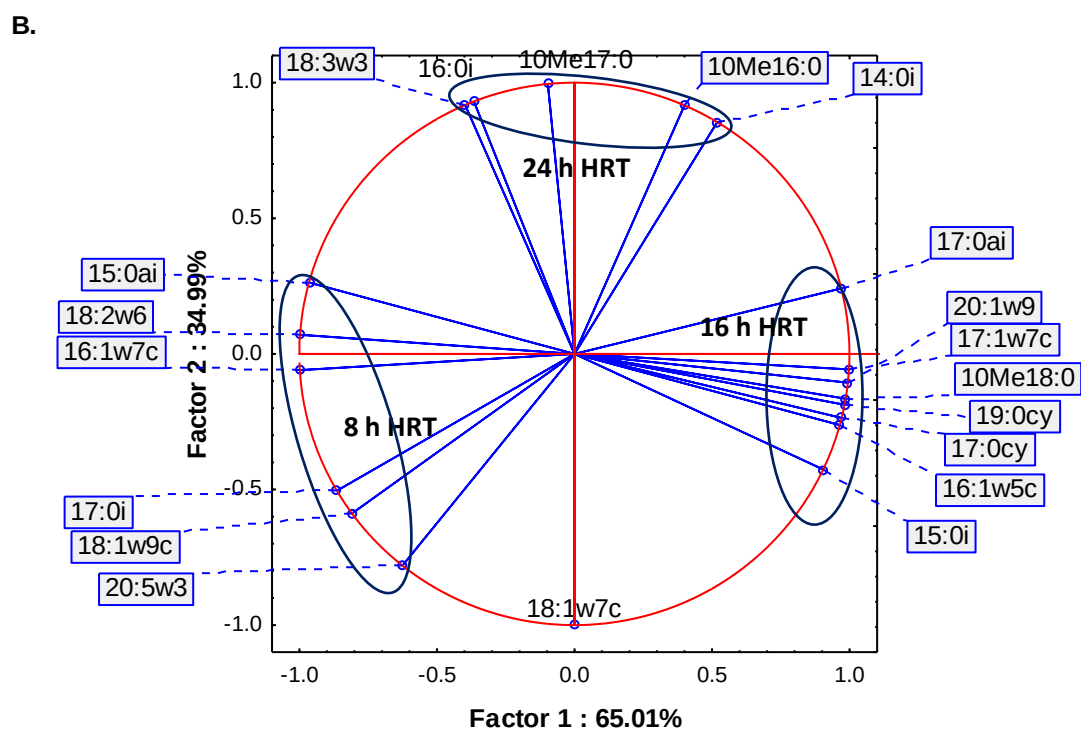


Figure. 3.6B: Loading plot of microbial diversity from fatty acids profiles at 8, 16 and 24 h HRT.

3.3.5 Bacterial and archaeal diversity from 16S rRNA analysis at different SRT and HRT

The Simpson index of microbial diversity measures the richness and evenness of species in a system. It ranks from zero to one, with zero being infinite diversity and one representing no diversity. The α -diversity assessed through the Simpson diversity index showed an increase as SRT and HRT are lengthened (Table 3.3). The Simpson diversity at 27 d SRT and 24 h HRT was 2.7 times higher than the diversity at 3d SRT and 8 h HRT. The samples analysed under each condition were not replicated, hence the significance of the variation could not be determined.

Table 3.3. Simpson diversity indices at various SRT and HRT

	SRT			HRT		
	3 d	10 d	27 d	8h	16 h	24 h
Simpson Index	0.0266	0.0115	0.0117	0.0117	0.0166	0.0099

3.3.5.1 Bacterial diversity in pilot-scale ASP

The bacterial diversity in activated sludge reactor, under all SRT and HRT conditions, is dominated by *Proteobacteria* followed by *Bacteroidetes* (Figures 3.7A, B and C), like observations made by Bagchi et al., (2015) and Jiang et al., (2008). The third most abundant phylum was different in each investigation. At 3 d SRT, the pilot-scale ASP contained 63% *Proteobacteria*, but this abundance was significantly reduced to 50% when SRT was extended to 10 d and decreased slightly to 48% when SRT was furthered to 27 d. The reduction in *Proteobacteria* abundance corresponded with increases in *Nitrospirae* and *Planctomycetes* at 10 d, and *Firmicutes* at 27 d SRT.

Figures 3.8A and B respectively show that the bacterial diversity at each SRT was distinct. The loading plot (Figure 3.8B) shows that *Proteobacteria*, *Hydrogenedentes* and WCHB1-60 had their highest abundance at 3 d SRT. In comparison with Figures 3.3A (PCA from PLFA analysis), it shows each SRT at a different location which implies the results from the two methods reveal a difference in microbial communities but with different emphases.

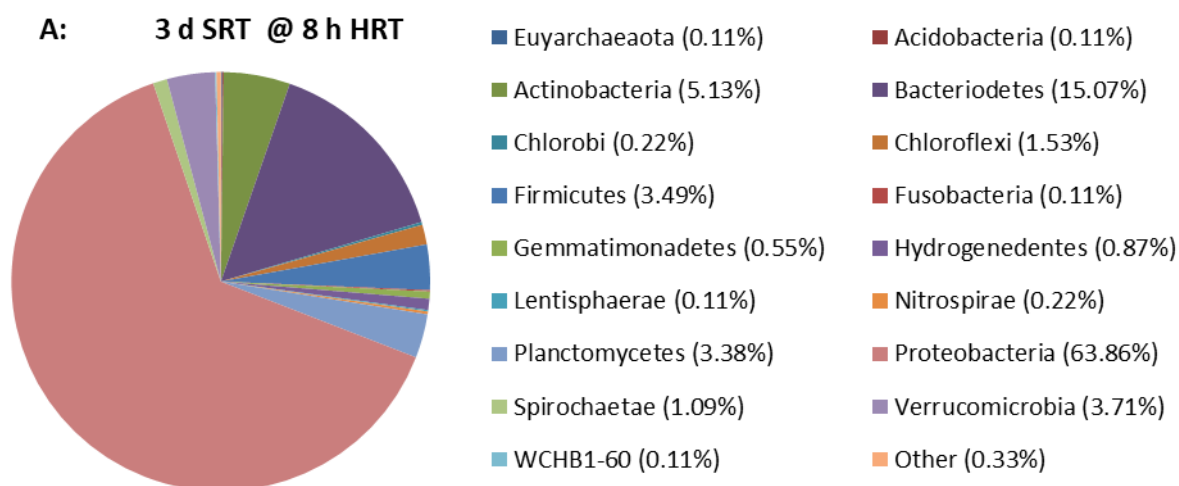


Figure 3.7A. Bacterial and archaeal diversity at phylum level at 3 d SRT.

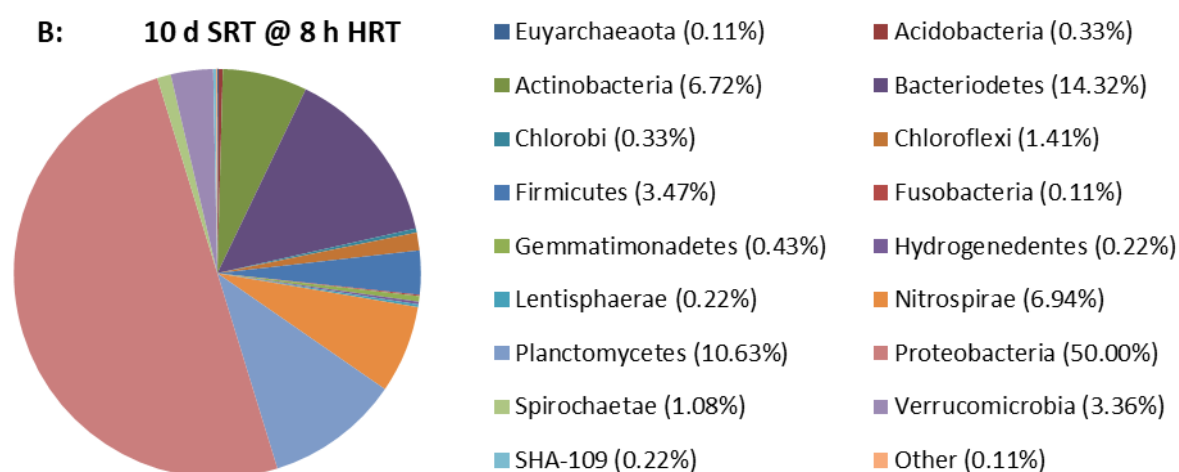


Figure 3.7B. Bacterial and archaeal diversity at phylum level at 10 d SRT.

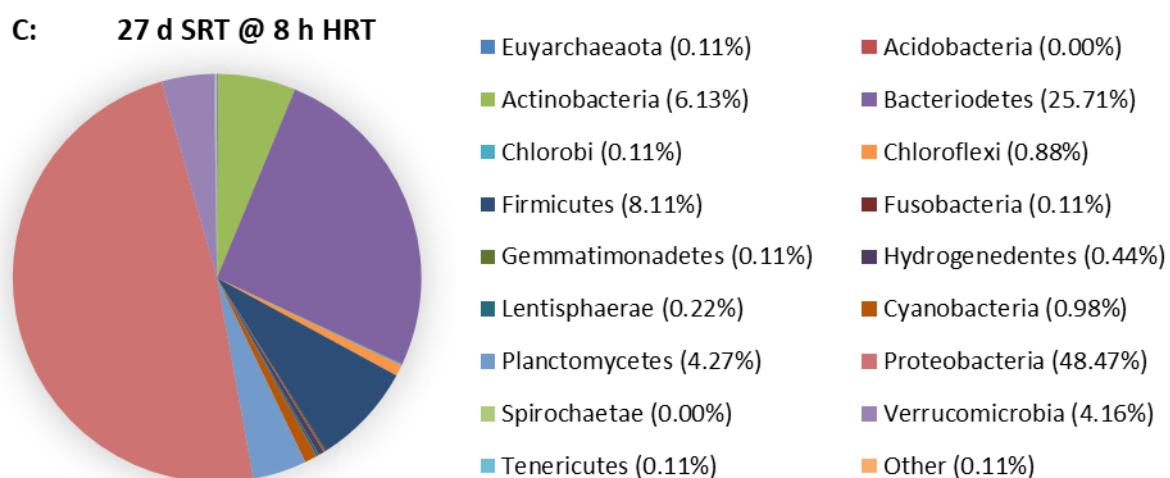


Figure 3.7C. Bacterial and archaeal diversity at phylum level at 27 d SRT.

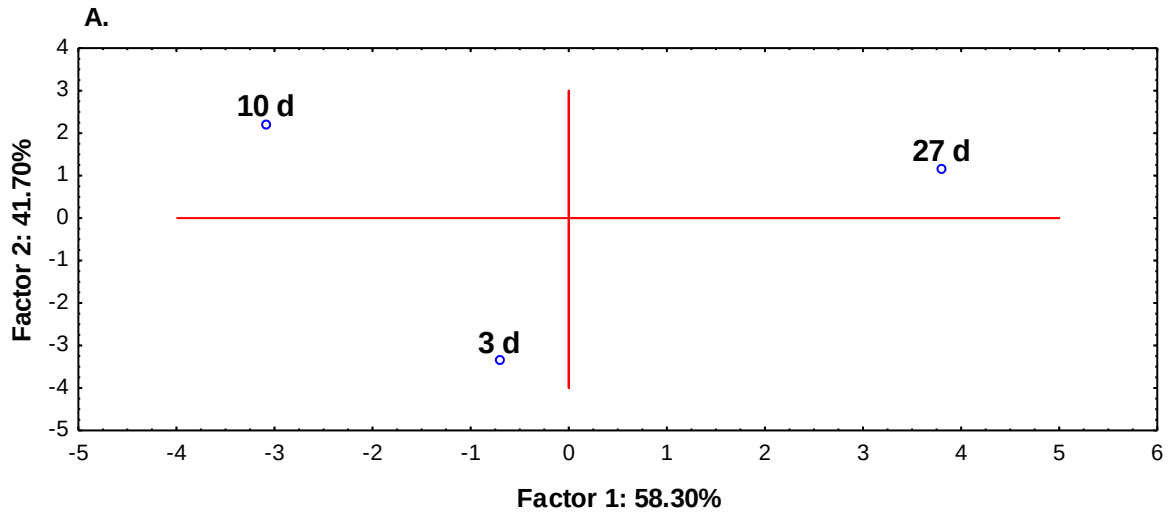


Figure 3.8A. PCA to compare the α -diversity of the bacteria communities at 3, 10 and 27 d SRT (n=7).

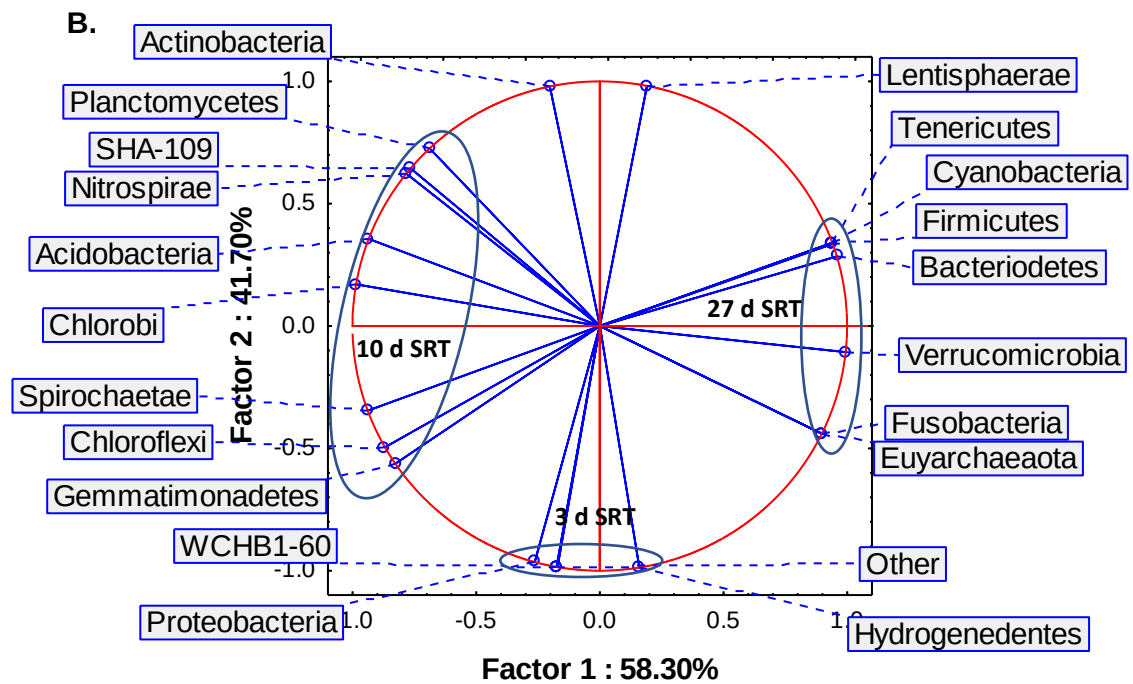


Figure 3.8B. Loading plots for the comparison of α -diversity in the bacteria communities of the pilot plant at 3, 10 and 27 d SRT.

HRT variation also induced changes in the bacterial diversity. Figures 3.9A, B and C showed that *Bacteroidetes* changed from 26% at 8 h to 14% at 16 h and then back to 24% at 24 h HRT. *Nitrospirae* and *Planctomycetes* increased from 0 and 4% to approximately 10% each as HRT increased from 8 to 16 h. The *Proteobacteria* decreased further to 42% at 16 h but recovered back to 48% at 24 h, as it was at 8 h HRT.

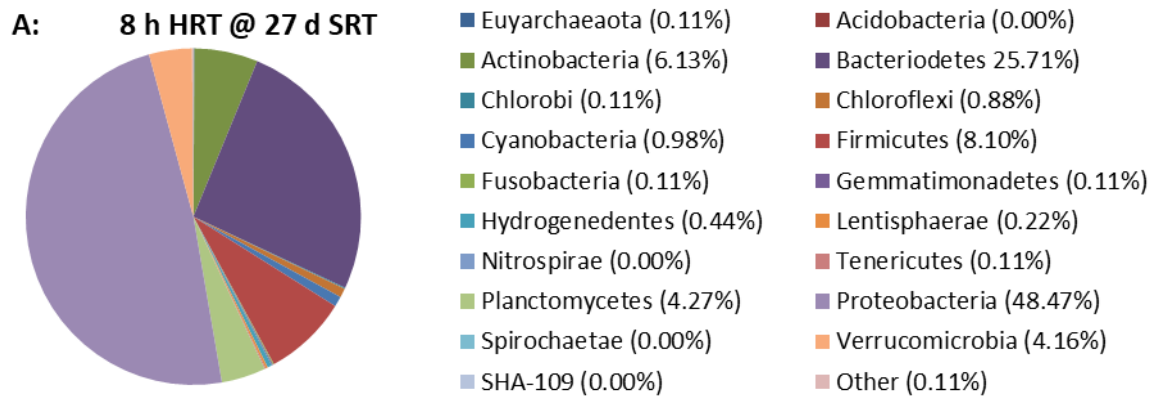


Figure 3.9A. Bacterial and archaeal diversity at phylum level at 8 h HRT.

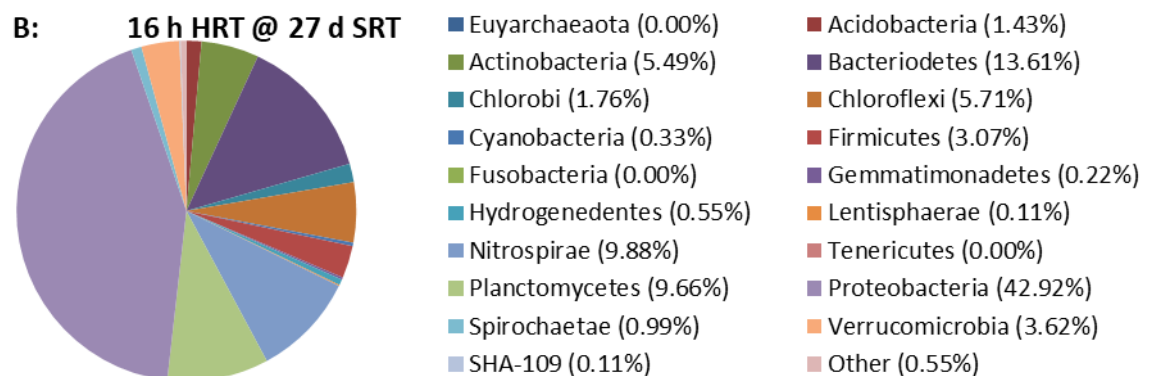


Figure 3.9B. Bacterial and archaeal diversity at phylum level at 16 h HRT.

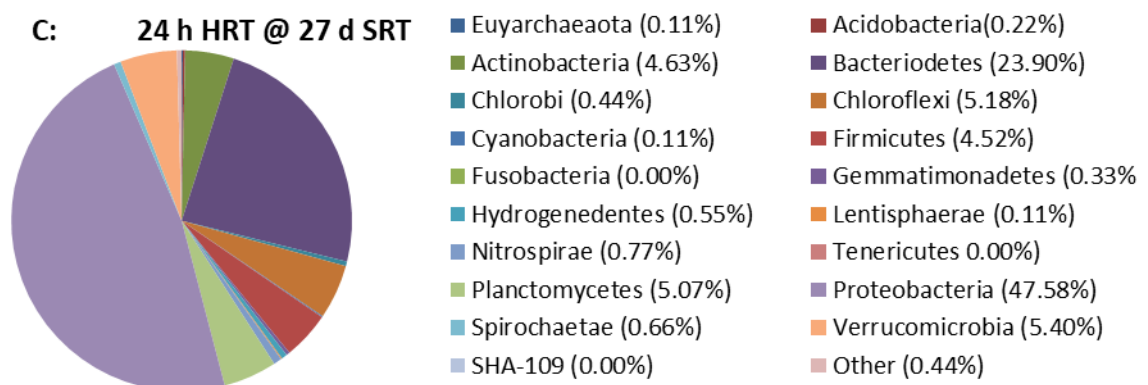


Figure 3.9C. Bacterial and archaeal diversity at phylum level at 24 h HRT.

Figure 3.10A shows that α -diversity at 8, 16 and 24 h HRT are dissimilar, with 8 and 16 h HRT been entirely different, while the diversity at 24 h has similarities with both. Figure 3.10B, the loading plot illustrates which bacterial phyla influenced the PCA in Figure 3.10A. The diversity at 8 h is like that of 24 h HRT due to their similar abundances of *Bacteroidetes*, *Proteobacteria*, and *Euyarchaeaota* while that of 16 h is similar to 24 h because *Chloroflexi*, *Hydrogenedentes* and *Spirochaetae* were similar.

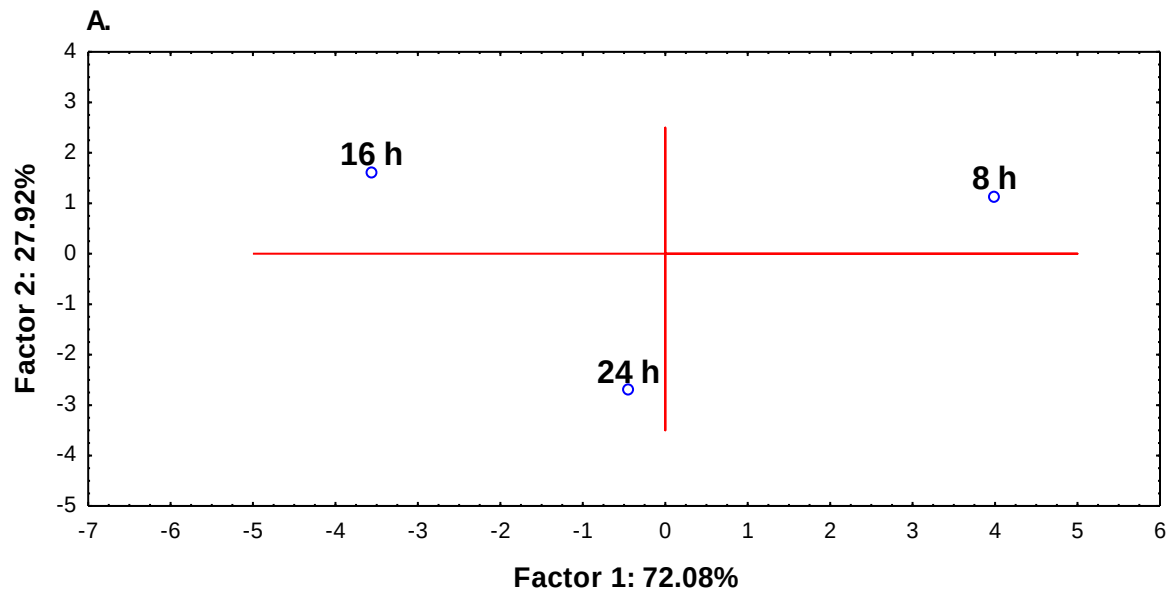


Figure 3.10A. PCA of phylum level bacterial and archaeal diversity at 8, 16 and 24 h HRT (n=7).

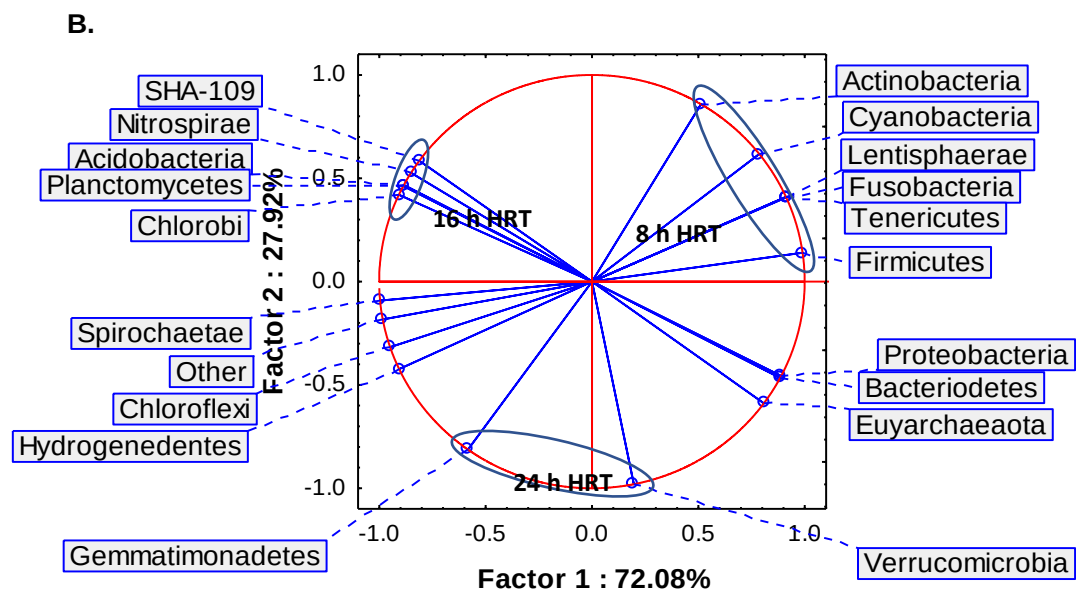


Figure 3.10B. Loading plots of bacterial and archaeal phyla diversity at 8, 16 and 24 h HRT.

3.3.5.2 Bacterial diversity at the class and species level

Comparing known species with taxa at higher levels introduces some degree of uncertainty because at phylum level, a DNA sequence may be adequately identifiable, but at genus or species it might be unknown. Under such circumstances, diversity measurements at higher levels can be significantly different from those at genus or species level (Bozzuto and Blanckenhorn, 2017). It is therefore imperative that this research examine diversity at lower taxonomic levels. The dominant species were *Thiothrix eikelboomii* (11.9%,) at 3 d; *Nitrospira sp.* (6.58%) at 10 d and an uncultured *Comamonadaceae* species (5.91%) at 27 d SRT. From 3 d to 10 d SRT, *Nitrospirae* abundance increased from 0.2 to 6.94%, and *Thiothrix eikelboomii* abundance reduced to 1.70% at 10 d and 0.66% at 27 d SRT (appended Tables AT3 to AT5). With *Proteobacteria* accounting for 42 – 63% of bacterial abundance in the reactor, the diversity within *Proteobacteria* was of interest. At 3 d SRT, Alpha, Beta and *Gammaproteobacteria* were 20, 16 and 23% respectively and the other most dominant genera were uncultured *Comamonadaceae*, *Flavobacterium*, uncultured *Chitiniphagaceae* and uncultured *Rhodobacteraceae*. At 10 d SRT the *Proteobacteria* abundance dropped to 50%, and the Alpha, Beta and *Gammaproteobacteria* abundances were 22, 12 and 7% (Figure 3.11). This increase in SRT had minimal affect the *Alphaproteobacteria*, slightly decreased the *Betaproteobacteria* and decimated the *Gammaproteobacteria*. Other dominant genera were uncultured *Comamonadaceae*, *Planctomyces*, *Rhodobacter* and *Haliscomenobacter*. At 27 d SRT, *Flavobacterium*, uncultured *Rhodobacteraceae*, *Hydrogenophaga* and *Leadbetterella* were prominent. Overall, *Planctomycetes* abundance fluctuated, but *Firmicutes* increased with increasing SRT.

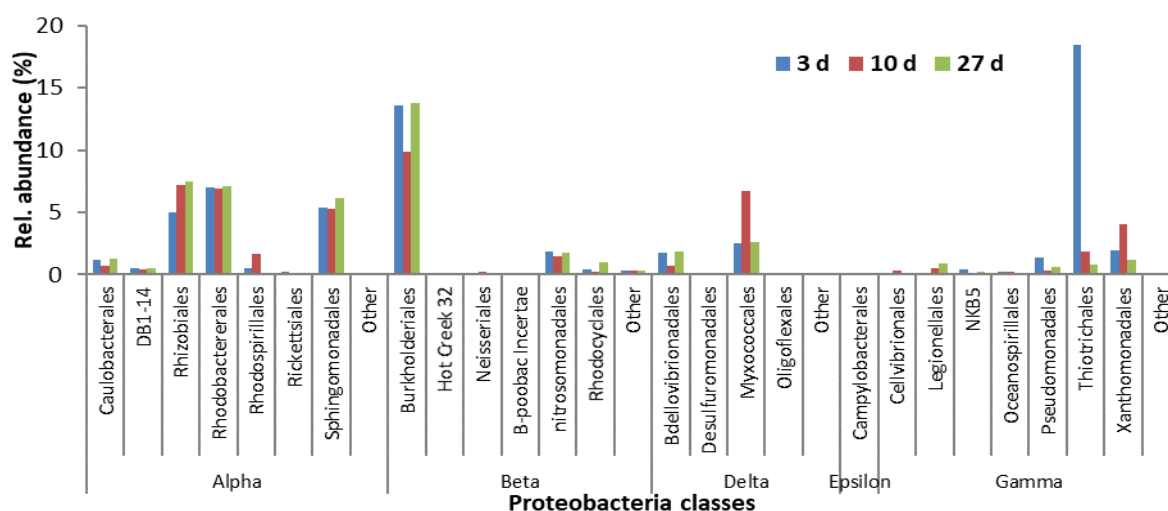


Figure 3.11. Diversity within the *Proteobacteria* phylum at varying SRT of 3, 10 and 27 d.

HRT increases caused the dominant species to change from the uncultured species from *Comamonadaceae* family (5.9%) at 8 h to *Nitrospira sp.* (9.4%) at 16 h and then to the uncultured *Comamonadaceae* species (5.6%) in Figure 3.12. Variation within *Proteobacteria* shows that HRT increase had no effect on the order of dominance but affected the percentage abundances (Tables AT6 to AT8 in Appendix). The dominant order, *Burkholderiales* was approximately 14 and 13% at 8 and 24 h. The abundance of *Thiothrichales* increased from 0.78% at 8 h to about 4% at 16 and 24 h HRT.

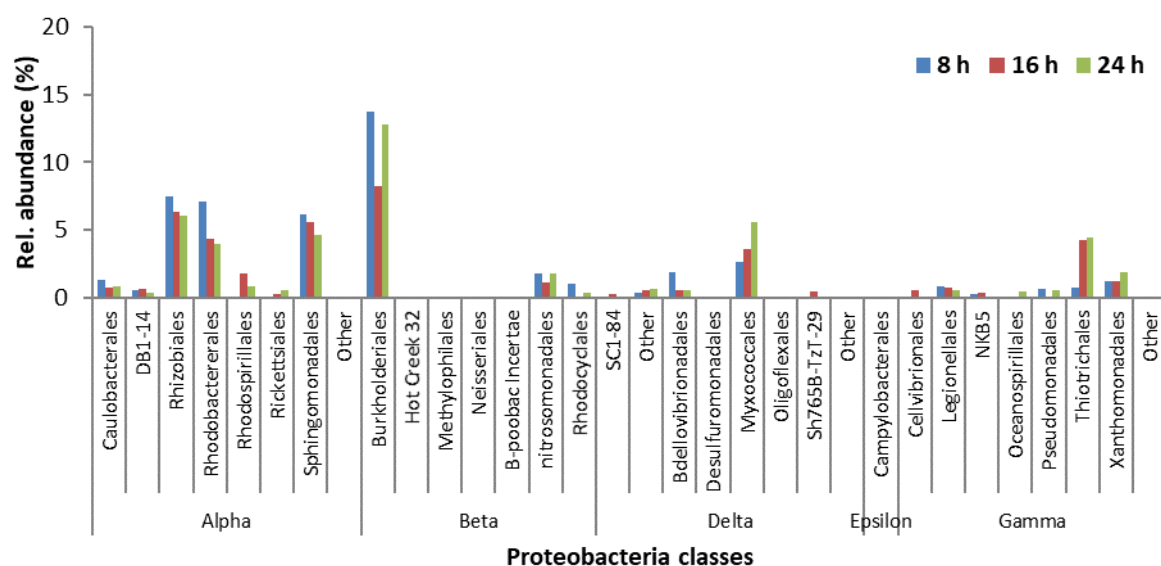


Figure 3.12. Diversity within the Proteobacteria phylum at varying HRT of 8, 16 and 24 h.

3.3.6 Removal of estrogens and nonylphenols

Figure 3.13A-F shows the influent and effluent concentrations of the estrogens as boxplots and their removals as scatterplots, at the various SRTs and HRTs studied. Appended table AT30 also shows the concentrations of nonylphenols in the settled sewage and final effluent at the SRTs and HRTs investigated. EE2 removals were 30, 29 and 41% at 3, 10 and 27 d respectively. Although the difference in removal at 3 and 10 d is insignificant ($p > 0.05$, $n = 7$), there was a significant ($p < 0.05$, $n = 7$) increase of 12% from 10 to 27 d SRT. E1, E2, E3 and E1-3S were better removed at 10 and 27 d SRT than at 3 d (Figures 3.13A-C). The removal of all the estrogens showed progressive increments at 8, 16 and 24 h HRTs, ranging from 62 to 97% (Figures 3.13D-F), as observed by Ma et al., (2016). At each SRTs, the removal of NP₆EO and longer nonylphenol ethoxylates exceeded 85% and improved as the SRT increased. The shorter NPEOs (< NP₆EO) showed significant increases in removal as the SRT lengthened.

For instance, 44, 73 and 84% of NP₄EO were removed at 3, 10 and 27 d SRT respectively (Table 3.4). At 3 d SRT, the concentration of short-chain nonylphenol (mono, di and tri) carboxylates were higher in final effluent than settled sewage because long-chain NPEOs degrade into short chains. This negative of removal was not observed at 10 and 27 d SRT.

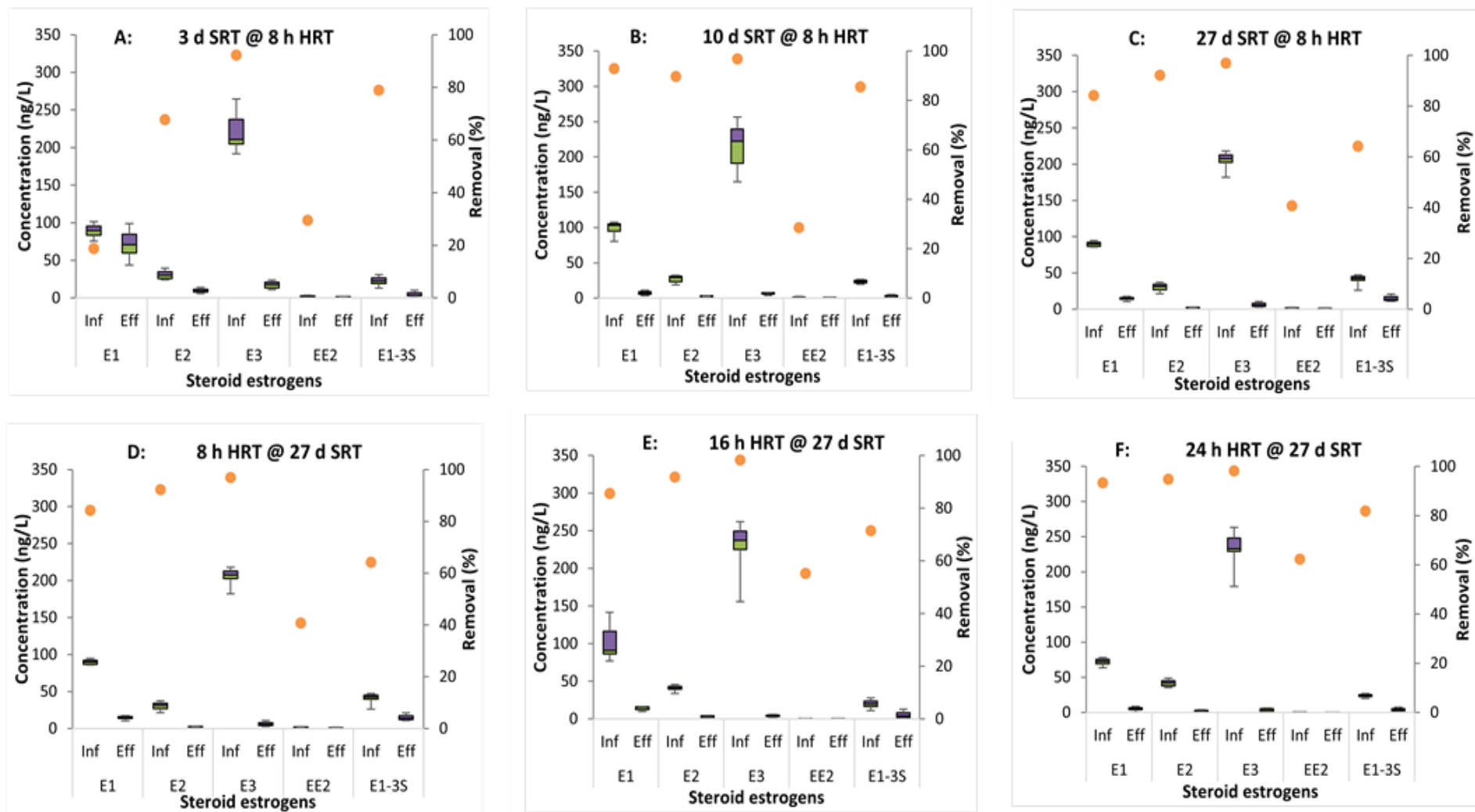


Figure 3.13. Boxplots of steroid estrogens concentrations in settled influent (Inf) and final effluent (Eff) in the pilot plant (n=7); (A) 3 d, (B) 10 d and (C) 27 d SRT; (D) 8 h, (E) 16 h and (F) 24 h HRT. Percentage removal is indicated by the orange dots. PNEC values for the estrogens are E1 = 3 ng/L; E2 = 1 ng/L; EE2 = 0.1 ng/L (Comber et al., 2019) and E3 = 60 ng/L (Cadwell et al., 2012).

3.3.7 Effect of SRT and HRT on the removal of estrogens and nonylphenols

The removal of estrogens and nonylphenols were similar at 10 and 27 d SRT (Figure 3.13A) but both differ from the removal at 3 d SRT. Figure 3.14B shows that the removal of all the chemicals apart from E1-3S, aligned with 10 d and 27 d SRT. E1-3S is readily degraded into estrone (Koh et al., 2011) and hence it has relatively high removal even at 3 d SRT.

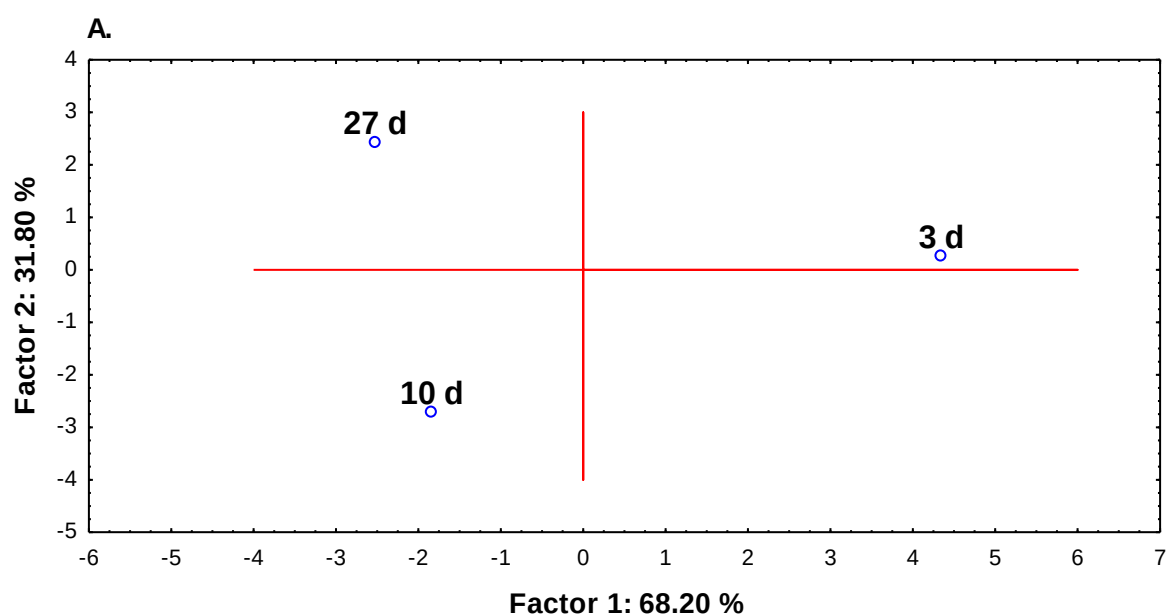


Figure 3.14A. PCA for comparing the removal of nonylphenols and estrogens at 3, 10 and 27 d SRT

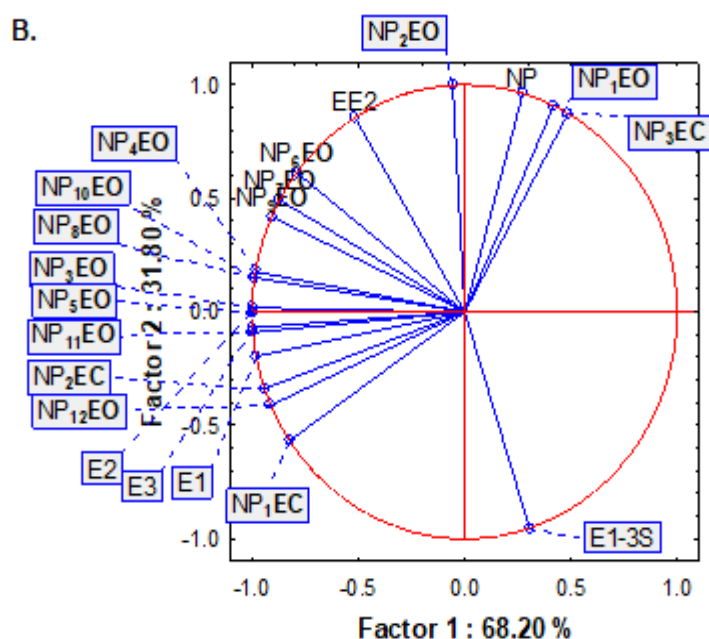
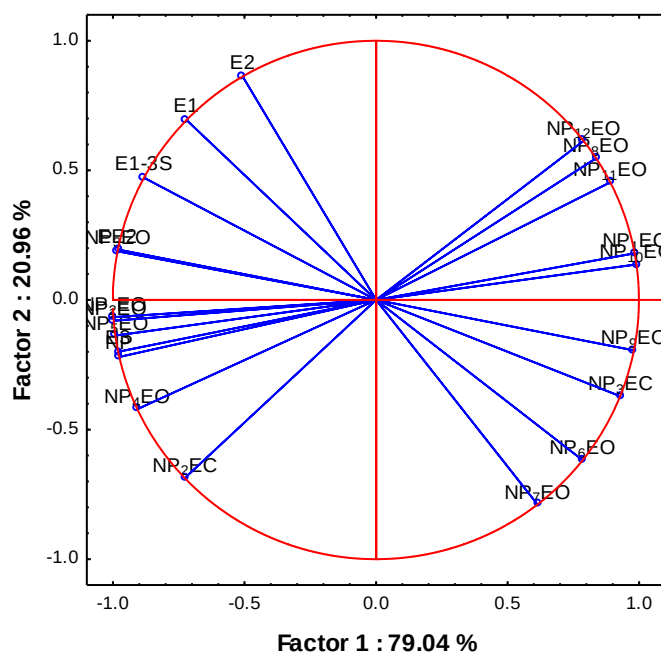
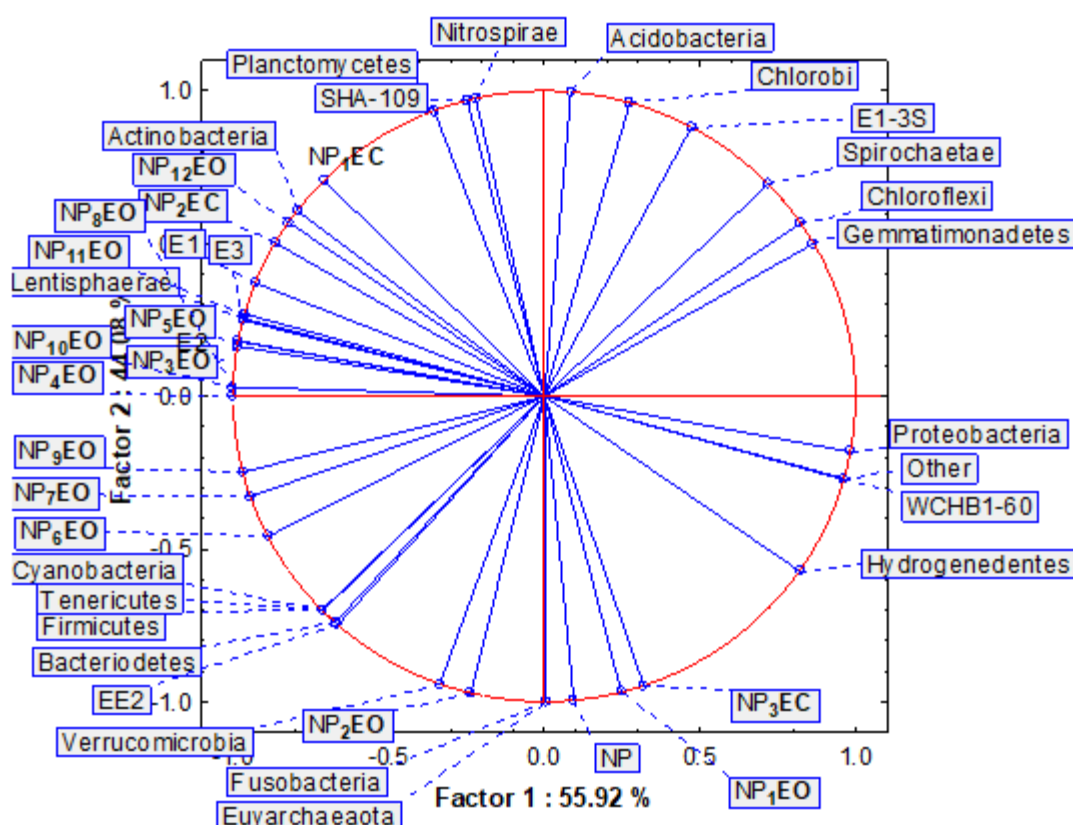


Figure 3.14B. Loading plots showing the similarities and differences in the chemical removal at 3, 10 and 27 d SRT

Figure 3.15B. Loading plots for the removal of nonylphenols and estrogens at 8, 16 and 24 h HRT



3.3.8 Correlation of bacterial phyla abundance with hazardous chemicals removal



In the HRT study (Figure 3.16B), the removal of NP₁EC and NP₁₀EO are high when *Fusobacteria*, *Tenericutes* and *Lentisphaerae* are at their highest abundances. *Actinobacteria* aligns with the removal of NP₆₋₇EO while *Cyanobacteria* abundance correlates with the removal of NP₉₋₁₀EO. The abundance of *Firmicutes* and removals of NP₈EO, NP₁₁EO and NP₁₂EO also correlated. *Spirochaeta* had its highest abundance coincide with the utmost removal of NP₄EO. The highest removal of EE2, NP₂EO and NP₃EO corresponded with a high proportion of *Gemmatimonadetes*. E1, E2 and E1-3S had their removals correlate with the abundance of *Verrucomicrobia* while the removal of E3, NP, NP₁EO and NP₃EO aligned with *Hydrogenedentes* and *Chloroflexi*.

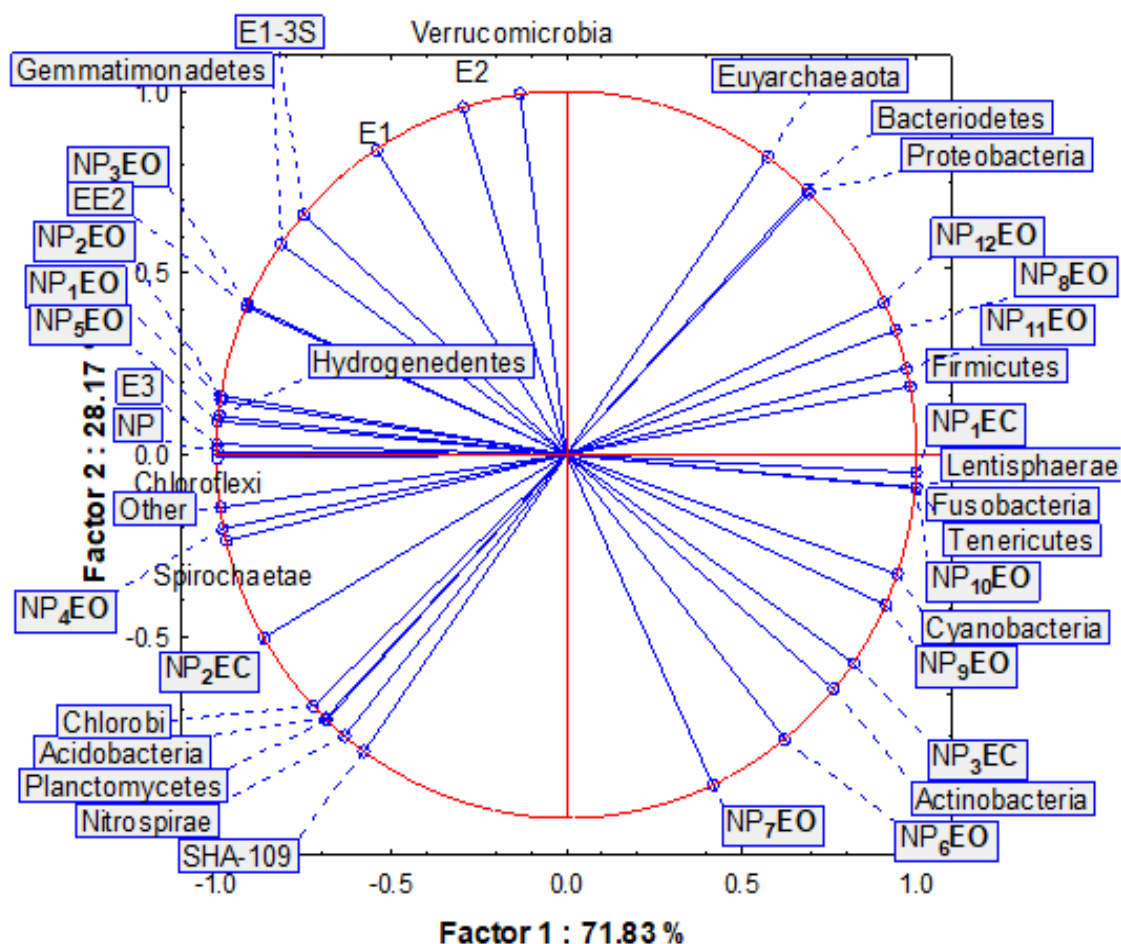


Figure 3.16B. PCA loading plots showing correlations between bacterial and archaeal abundances at phylum level and percentage removal of nonylphenols and estrogens at 8, 16 and 24 h HRT.

The different bacterial abundances from the SRT and HRT studies resulted in differences in chemical removal. The removal of estrogens and nonylphenols had remarkable correlations with bacterial abundance. Phyla abundance correlation analysis ($R > 0.7$) revealed that *Chloroflexi* and *Verrucomicrobia* positively correlate with EE2 removal while *Actinobacteria* negatively correlate with EE2. *Proteobacteria* negatively correlates with E1, E2, E3, NP₂EC, NP₃EO, NP₄EO, NP₅EO, NP₆EO and NP₇EO (Table 3.5).

Table 3.5. Correlations between bacterial phyla abundance and removal of estrogens and nonylphenols

Bacterial phyla	Chemical	R ² value	Linear equation
<i>Actinobacteria</i>	NP ₁ EC	0.91609	NP ₁ EC = -1408. + 211.07 * <i>Actinobacteria</i>
<i>Chloroflexi</i>	EE2	0.87986	EE2 = 26.297 + 5.7842 * <i>Chloroflexi</i>
	NP	0.96383	NP = -38.89 + 19.149 * <i>Chloroflexi</i>
<i>Cyanobacteria</i>	E1-3S	-0.9206	E1-3S = 81.804 - 19.03 * <i>Cyanobacteria</i>
	NP ₉ EO	0.87356	NP ₉ EO = 92.908 + 3.1321 * <i>Cyanobacteria</i>
<i>Gemmatimonadetes</i>	NP ₆ EO	-0.9876	NP ₆ EO = 99.433 - 23.90 * <i>Gemmatimonadetes</i>
	NP ₇ EO	-0.9587	NP ₇ EO = 97.823 - 16.54 * <i>Gemmatimonadetes</i>
	NP ₉ EO	-0.8584	NP ₉ EO = 96.228 - 7.406 * <i>Gemmatimonadetes</i>
	NP ₁₀ EO	-0.7519	NP ₁₀ EO = 95.327 - 6.487 * <i>Gemmatimonadetes</i>
<i>Hydrogenedentes</i>	NP ₁₁ EO	-0.8907	NP ₁₁ EO = 95.729 - 6.716 * <i>Hydrogenedentes</i>
	NP ₁₂ EO	-0.8698	NP ₁₂ EO = 95.424 - 9.561 * <i>Hydrogenedentes</i>
<i>Lentisphaerae</i>	NP ₁ EC	0.92529	NP ₁ EC = -675.6 + 2967.9 * <i>Lentisphaerae</i>
	NP ₁₁ EO	0.92075	NP ₁₁ EO = 87.948 + 27.796 * <i>Lentisphaerae</i>
	NP ₁₂ EO	0.90847	NP ₁₂ EO = 84.285 + 39.978 * <i>Lentisphaerae</i>
<i>Planctomycetes</i>	NP ₂ EC	0.73452	NP ₂ EC = -1279. + 131.64 * <i>Planctomycetes</i>
	NP ₃ EC	-0.7385	NP ₃ EC = 253.44 - 117.2 * <i>Planctomycetes</i>
<i>Proteobacteria</i>	E1	-0.9134	E1 = 260.52 - 3.669 * <i>Proteobacteria</i>
	E2	-0.9418	E2 = 154.03 - 1.320 * <i>Proteobacteria</i>
	E3	-0.9792	E3 = 112.06 - .3072 * <i>Proteobacteria</i>
	NP ₂ EC	-0.9296	NP ₂ EC = 3121.0 - 69.83 * <i>Proteobacteria</i>
	NP ₃ EO	-0.9599	NP ₃ EO = 443.95 - 8.016 * <i>Proteobacteria</i>
	NP ₄ EO	-0.9755	NP ₄ EO = 203.75 - 2.503 * <i>Proteobacteria</i>
	NP ₅ EO	-0.9704	NP ₅ EO = 195.46 - 2.256 * <i>Proteobacteria</i>
	NP ₆ EO	-0.9336	NP ₆ EO = 113.82 - .4395 * <i>Proteobacteria</i>
	NP ₇ EO	-0.9064	NP ₇ EO = 109.63 - .3408 * <i>Proteobacteria</i>
<i>Spirochaetae</i>	E1-3S	0.72023	E1-3S = 66.193 + 13.322 * <i>Spirochaetae</i>
<i>Verrucomicrobia</i>	EE2	0.71749	EE2 = -11.38 + 13.501 * <i>Verrucomicrobia</i>

3.3.9 Correlation of bacterial genera abundance with the removal of estrogens

Bacterial genera level abundance and estrogen-removal correlations (Table 3.6 to 3.10) showed that there were 82 bacteria genera which correlated with the removal of estrogens, with 32 positive correlations. Having more negative correlations than positives, suggest that the most abundant and perhaps easily detectable genera are fast-growing non-estrogen degraders, and therefore most estrogen-degraders are in low abundance in niche spaces, given that the removal of all estrogens increased with increasing SRT and bacterial diversity. The removal of E1-3S had the most correlation, with bacteria from all the phyla indicated in Table 3.8. *Leucobacter*, correlated negatively with all the estrogens except the readily degradable E1-3S. *Hyphomicrobium*, *Mesorhizobium*, *Rhizomicrobium* and *Planctomyces* positively correlated with the removals of E1, E2 and E3.

There were twenty-four bacteria genera that correlated with the removal of E1, thirty-five correlated with E1-3S, twenty-seven with E2, thirty-two with E3 and twenty-two correlated with EE2. None of the *Rhodococcus* and *Pseudomonas spp.* found in activated sludge (*B. subtilis*, *P. aeruginosa*, *P. putida*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, *R. zopfii*) to remove EE2 (Larcher and Yargeau, 2013) correlated with EE2 in this research.

Table 3.6. Bacteria genera abundance correlations with estrone (E1) removal

Estrogen	Bacterial genera	R value	Linear equation	
Estrone (E1)	<i>Leucobacter</i>	-0.8975	E1	= 96.921 – 13800 * <i>Leucobacter</i>
	<i>Microbacterium</i>	-0.9369	E1	= 98.953 – 7494 * <i>Microbacterium</i>
	<i>Clostridium sensu stricto 1</i>	0.87496	E1	= -5.480 + 18285 * <i>Clostridium sensu stricto 1</i>
	<i>Catenibacterium</i>	0.7306	E1	= 42.226 + 32746 * <i>Catenibacterium</i>
	<i>Planctomyces</i>	0.92328	E1	= 12.926 + 2846.2 * <i>Planctomyces</i>
	<i>Hyphomicrobium</i>	0.94017	E1	= 6.0640 + 18134 * <i>Hyphomicrobium</i>
	<i>Mesorhizobium</i>	0.79954	E1	= 38.628 + 30287 * <i>Mesorhizobium</i>
	<i>Rhizomicrobium</i>	0.99154	E1	= 18.758 + 70268 * <i>Rhizomicrobium</i>

Table 3.7. Correlations of bacteria genera abundance with estrone sulfate (E1-3S) removal

Estrogen	Bacteria genera	R-value	Linear correlation equation
Estrone sulfate (E1-3S)	<i>Iamia</i>	0.70166	$E1-3S = 70.463 + 1645 * Iamia$
	<i>Gordonia</i>	0.77843	$E1-3S = 71.524 + 12151 * Gordonia$
	<i>Filimonas</i>	0.77843	$E1-3S = 66.664 + 2430.2 * Filimonas$
	<i>Lachnoclostridium</i>	0.7965	$E1-3S = 79.430 - 15300 * Lachnoclostridium$
	<i>Pseudorhodobacter</i>	0.91518	$E1-3S = 67.813 + 14286 * Pseudorhodobacter$
	<i>Stella</i>	0.87989	$E1-3S = 65.746 + 10639 * Stella$
	<i>Polaromonas</i>	0.82166	$E1-3S = 55.202 + 3652.1 * Polaromonas$
	<i>Sphaerotilus</i>	0.89064	$E1-3S = 67.763 + 1724.4 * Sphaerotilus$
	<i>Byssovorax</i>	0.827	$E1-3S = 68.995 + 947.4 * Byssovorax$
	<i>Sorangium</i>	0.93902	$E1-3S = 68.237 + 3703.2 * Sorangium$
	<i>Arenimonas</i>	0.79181	$E1-3S = 67.214 + 2697.2 * Arenimonas$
	<i>Turneriella</i>	0.73182	$E1-3S = 65.973 + 1531.1 * Turneriella$

Table 3.8. Correlations of bacteria genera abundance with ethynylestradiol (EE2) removal

Estrogen	Bacteria genera	R-value	Linear correlation equation
Ethynylestradiol (EE2)	<i>Mycobacterium</i>	0.86643	$EE2 = 17.337 + 3166.2 * Mycobacterium$
	<i>Leucobacter</i>	-0.8016	$EE2 = 52.674 - 5859. * Leucobacter$
	<i>Hydrothalea</i>	0.75366	$EE2 = 33.686 + 1299.1 * Hydrothalea$
	<i>Aureispira</i>	0.7028	$EE2 = 38.536 + 23817 * Aureispira$
	<i>Candidatus Odysella</i>	0.7028	$EE2 = 38.536 + 4763.4 * Candidatus Odysella$
	<i>Acidovorax</i>	-0.9345	$EE2 = 58.814 - 25900 * Acidovorax$
	<i>Variovorax</i>	0.84237	$EE2 = 24.989 + 7629.5 * Variovorax$
	<i>Nannocystis</i>	0.73348	$EE2 = 33.377 + 2255.1 * Nannocystis$
	<i>Sandaracinus</i>	0.7028	$EE2 = 38.536 + 23817 * Sandaracinus$
	<i>Aquicella</i>	0.7028	$EE2 = 38.536 + 23817 * Aquicella$
	<i>Rickettsiella</i>	0.74764	$EE2 = 31.346 + 7471.3 * Rickettsiella$
	<i>Acidibacter</i>	0.78096	$EE2 = 31.464 + 11836 * Acidibacter$
	<i>Cerasicoccus</i>	0.7028	$EE2 = 38.536 + 23817 * Cerasicoccus$
	<i>Prostheobacter</i>	0.93517	$EE2 = 17.494 + 2688.1 * Prostheobacter$

Table 3.9. Correlations of bacteria genera abundance with estradiol (E2) removal

Estrogen	Bacteria genera	R-value	Linear correlation equation
Estradiol (E2)	<i>Prostheobacter</i>	0.71595	$E2 = 72.837 + 1502 * Prostheobacter$
	<i>Clostridium sensu stricto 1</i>	0.79748	$E2 = 61.663 + 5816.6 * Clostridium sensu stricto 1$
	<i>Catenibacterium</i>	0.7828	$E2 = 75.011 + 12246 * Catenibacterium$
	<i>Planctomyces</i>	0.84131	$E2 = 67.523 + 905.18 * Planctomyces$
	<i>Hyphomicrobium</i>	0.90798	$E2 = 64.029 + 6112.4 * Hyphomicrobium$
	<i>Mesorhizobium</i>	0.71712	$E2 = 75.879 + 9481.1 * Mesorhizobium$
	<i>Rhizomicrobium</i>	0.98669	$E2 = 67.732 + 24405 * Rhizomicrobium$
	<i>Pseudomonas</i>	-0.9292	$E2 = 101.45 - 7883 * Pseudomonas$
	<i>Thiothrix</i>	-0.9442	$E2 = 95.859 - 158.7 * Thiothrix$
	<i>Leucobacter</i>	-0.9559	$E2 = 95.415 - 5099 * Leucobacter$

Table 3.10. Correlations of bacteria genera abundance with estriol (E3) removal

Estrogen	Bacteria genera	R-value	Linear correlation equation	
Estriol (E3)	<i>Leucobacter</i>	-0.9834	E3	= 98.407 - 1174 * <i>Leucobacter</i>
	<i>Clostridium sensu stricto 1</i>	0.75835	E3	= 91.082 + 1237.8 * <i>Clostridium sensu stricto 1</i>
	<i>Planctomyces</i>	0.80692	E3	= 92.293 + 194.28 * <i>Planctomyces</i>
	<i>Hyphomicrobium</i>	0.83126	E3	= 91.770 + 1252.3 * <i>Hyphomicrobium</i>
	<i>Mesorhizobium</i>	0.76927	E3	= 93.797 + 2276 * <i>Mesorhizobium</i>
	<i>Rhizomicrobium</i>	0.96087	E3	= 92.274 + 5318.4 * <i>Rhizomicrobium</i>
	<i>Dechloromonas</i>	-0.7385	E3	= 98.357 - 1828 * <i>Dechloromonas</i>
	<i>Rickettsiella</i>	0.75517	E3	= 94.556 + 1232.6 * <i>Rickettsiella</i>
	<i>Pseudomonas</i>	-0.9549	E3	= 99.792 - 1813 * <i>Pseudomonas</i>
	<i>Thiothrix</i>	-0.8794	E3	= 98.322 - 33.08 * <i>Thiothrix</i>
	<i>Acidibacter</i>	0.7385	E3	= 94.701 + 1828.0 * <i>Acidibacter</i>
	<i>Prostheco bacter</i>	0.72873	E3	= 93.244 + 342.12 * <i>Prostheco bacter</i>

3.3.10 Correlation of bacterial genera with the removal of nonylphenols and derivatives

The correlation analysis of nonylphenol removals with bacteria genera abundance (Tables 3.11 to 3.22) at $R > 0.7$ ($n = 5$) revealed 122 bacteria genera that correlate with the removal of various nonylphenols carboxylates and ethoxylates, of which 88 genera correlated negatively. Several uncultured bacteria from various families were also found to correlate with removals of NPs but are not presented. *Thiothrix sp.* which was dominant at 3 d SRT but had its abundance reduced as SRT increased was found to negatively correlate with NP₂EC and NP₃₋₁₁EO. *Sphingomonas* had no correlation with any of the nonylphenols at $R > 0.7$ although several *Sphingomonas spp.* (*S. TTNP3*, *S. cloacae*, *S. NP42a*, and *S. xenophaga Bayram*) have been isolated from WWTPs and demonstrated to have the capacity to degrade up to 100mg/L/d nonylphenols as sole carbon and energy source (Tanghe et al., 1999, Corvini et al., 2006, Fiji et al., 2001, Gabriel et al., 2005 and Zhang et al., 2014). Further to that, neither the abundance of *Variovorax* nor the *Ralstonia* genera had correlations with any of the fourteen nonylphenols studied, although *Variovorax sp.* (NP427b) and *Ralstonia sp.* (NP47a) isolated by Zhang et al., (2014) from activated sludge and can degrade nonylphenol di-ethoxylate. However, the abundance of *Pseudomonas* and *Acidovorax*, members in a consortium of facultative microorganisms capable of degrading 76% of 1000 mg/L nonylphenol in 48 hours (Bai et al., 2017), correlated with the removal of nonylphenol and four nonylphenol ethoxylate (NP₂EO, NP₃EO, NP₄EO and NP₅EO), although all correlations were negative.

Table 3.11. Correlations of bacteria genera abundance with nonylphenol (NP) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol (NP)	<i>Roseiflexus</i>	0.77673	NP = -27.60 + 22500 * <i>Roseiflexus</i>
	<i>Turicibacter</i>	0.70555	NP = -136.0 + 59000 * <i>Turicibacter</i>
	Uncultured Delta proteobacterium	0.97861	NP = -15.33 + 81833 * uncultured <i>Deltaproteobacterium</i>
	<i>Polyangium</i>	0.80505	NP = -17.86 + 44071 * <i>Polyangium</i>
	<i>Rickettsiella</i>	0.83067	NP = -22.74 + 25087 * <i>Rickettsiella</i>
	<i>Acidibacter</i>	0.81329	NP = -19.85 + 37250 * <i>Acidibacter</i>
	<i>Prostheco bacter</i>	0.77621	NP = -47.33 + 6742.8 * <i>Prostheco bacter</i>

Table 3.12. Correlations of bacteria genera abundance with nonylphenol monocarboxylate (NP₁EC) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol monocarboxylate (NP₁EC)	<i>CL500-29 marine group</i>	0.85174	NP ₁ EC = -294.0 + 362000 * <i>CL500-29 marine group</i>
	<i>Candidatus Microthrix</i>	0.82626	NP ₁ EC = -526.9 + 27256 * <i>Candidatus Microthrix</i>
	<i>Iamia</i>	0.76345	NP ₁ EC = -364.8 + 39789 * <i>Iamia</i>
	<i>Flexibacter</i>	0.85174	NP ₁ EC = -294.0 + 362000 * <i>Flexibacter</i>
	<i>Ferruginibacter</i>	0.89464	NP ₁ EC = -396.4 + 41611 * <i>Ferruginibacter</i>
	<i>Sediminibacterium</i>	0.88388	NP ₁ EC = -557.6 + 168000 * <i>Sediminibacterium</i>
	<i>Pseudobutyrvibrio</i>	0.92982	NP ₁ EC = -350.7 + 322700 * <i>Pseudobutyrvibrio</i>
	<i>Isosphaera</i>	0.75938	NP ₁ EC = -319.0 + 69605 * <i>Isosphaera</i>
	uncultured	0.90301	NP ₁ EC = -590.9 + 205100 * uncultured
	<i>Novosphingobium sp.</i>		<i>Novosphingobium sp.</i>
	<i>Devosia</i>	0.84963	NP ₁ EC = -834.6 + 82842 * <i>Devosia</i>
	Uncultured	0.85174	NP ₁ EC = -294.0 + 362000 * uncultured
	<i>Alphaproteobacterium</i>		<i>Alphaproteobacterium</i>
	<i>Bauldia</i>	0.85174	NP ₁ EC = -294.0 + 362000 * <i>Bauldia</i>
	<i>Rhodobacter</i>	0.79487	NP ₁ EC = -646.6 + 27959 * <i>Rhodobacter</i>
	<i>Chitinivorax</i>	0.85174	NP ₁ EC = -294.0 + 362000 * <i>Chitinivorax</i>
	<i>Undibacterium</i>	0.92886	NP ₁ EC = -478.6 + 91784 * <i>Undibacterium</i>
	<i>Leeia</i>	0.85174	NP ₁ EC = -294.0 + 362000 * <i>Leeia</i>
	uncultured	0.91982	NP ₁ EC = -350.7 + 322700 * uncultured
	Desulfuromonadales bacterium		Desulfuromonadales bacterium
	<i>Phaselicystis</i>	0.86134	NP ₁ EC = -333.4 + 32875 * <i>Phaselicystis</i>
	<i>Chiayiivirga</i>	0.85174	NP ₁ EC = -294.0 + 362000 * <i>Chiayiivirga</i>
	<i>Dokdonella</i>	0.77797	NP ₁ EC = -316.8 + 29753 * <i>Dokdonella</i>
	<i>Pseudoxanthomonas</i>	0.8184	NP ₁ EC = -392.0 + 142000 * <i>Pseudoxanthomonas</i>

Table 3.13. Correlations of bacteria genera with nonylphenol dicarboxylate (NP₂EC) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol dicarboxylate (NP₂EC)	<i>Haliscomenobacter</i>	0.70678	NP ₂ EC = -1277. + 59352 * <i>Haliscomenobacter</i>
	<i>Clostridium sensu stricto 1</i>	0.92157	NP ₂ EC = -1994. + 360100 * <i>Clostridium sensu stricto 1</i>
	<i>Planctomyces</i>	0.955	NP ₂ EC = -1610. + 55047 * <i>Planctomyces</i>
	<i>Hyphomicrobium</i>	0.9091	NP ₂ EC = -1656. + 327900 * <i>Hyphomicrobium</i>
	<i>Mesorhizobium</i>	0.91869	NP ₂ EC = -1191. + 650700 * <i>Mesorhizobium</i>
	<i>Rhizomicrobium</i>	0.96689	NP ₂ EC = -1435. + 1281000 * <i>Rhizomicrobium</i>
	<i>Pseudomonas</i>	-0.9804	NP ₂ EC = 392.06 - 446000 * <i>Pseudomonas</i>
	<i>Thiothrix</i>	-0.922	NP ₂ EC = 40.073 - 8304 * <i>Thiothrix</i>
	<i>Aquimonas</i>	-0.9669	NP ₂ EC = -153.8 - 320000 * <i>Aquimonas</i>
	<i>Pseudoxanthomonas</i>	0.71899	NP ₂ EC = -876.8 + 389000 * <i>Pseudoxanthomonas</i>

Table 3.14. Correlations of bacteria genera with nonylphenol tricarboxylate (NP₃EC) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol tricarboxylate (NP₃EC)	<i>Fluviicola</i>	0.8086	NP ₃ EC = -830.3 + 774800 * <i>Fluviicola</i>
	<i>Phenylobacterium</i>	0.71845	NP ₃ EC = -751.8 + 144600 * <i>Phenylobacterium</i>
	<i>Chelatococcus</i>	0.77671	NP ₃ EC = -879.5 + 224400 * <i>Chelatococcus</i>
	<i>Bosea</i>	0.76392	NP ₃ EC = -849.1 + 149400 * <i>Bosea</i>
	<i>Kaistia</i>	0.82502	NP ₃ EC = -1295. + 968300 * <i>Kaistia</i>
	<i>Phreatobacter</i>	0.76917	NP ₃ EC = -927.1 + 84731 * <i>Phreatobacter</i>
	<i>Paracoccus</i>	0.77123	NP ₃ EC = -1697. + 452600 * <i>Paracoccus</i>
	<i>Novosphingobium</i>	0.84546	NP ₃ EC = -1610. + 89285 * <i>Novosphingobium</i>
	<i>Sphingomonas</i>	0.73496	NP ₃ EC = -988.5 + 260100 * <i>Sphingomonas</i>
	<i>Aquabacterium</i>	0.8086	NP ₃ EC = -1605. + 774800 * <i>Aquabacterium</i>
	<i>Leeia</i>	-0.825	NP ₃ EC = -326.8 - 969000 * <i>Leeia</i>
	<i>OM27 clade</i>	0.91388	NP ₃ EC = -1046. + 146000 * <i>OM27 clade</i>
	<i>Arcobacter</i>	0.8086	NP ₃ EC = -830.3 + 774800 * <i>Arcobacter</i>
	<i>Pseudomonas</i>	0.71845	NP ₃ EC = -1041. + 289200 * <i>Pseudomonas</i>

Table 3.15. Correlations of bacteria genera abundance with nonylphenol mono-ethoxylate (NP₁EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol mono-ethoxylate (NP₁EO)	<i>Kaistia</i>	0.99989	NP ₁ EO = -2391 + 2470000 * <i>Kaistia</i>

Table 3.16. Correlations of bacteria genera abundance with nonylphenol diethoxylate (NP₂EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol diethoxylate (NP₂EO)	<i>Kaistia</i>	0.81982	NP ₂ EO = -150.0 + 1935E2 * <i>Kaistia</i>
	<i>Prostheobacter</i>	0.84702	NP ₂ EO = -158.0 + 16957. * <i>Prostheobacter</i>

Table 3.17. Correlations of bacteria genera abundance with nonylphenol tri-ethoxylate (NP₃EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol tri-ethoxylate (NP₃EO)	<i>Mycobacterium</i>	0.75606	NP ₃ EO = -59.9 + 12012 * <i>Mycobacterium</i>
	<i>Clostridium sensu stricto 1</i>	0.70253	NP ₃ EO = -95.7 + 30522 * <i>Clostridium sensu stricto 1</i>
	<i>Planctomyces</i>	0.759	NP ₃ EO = -67.44 + 4864.2 * <i>Planctomyces</i>
	<i>Hyphomicrobium</i>	0.80958	NP ₃ EO = -84.76 + 32463 * <i>Hyphomicrobium</i>
	<i>Rhizomicrobium</i>	0.94685	NP ₃ EO = -73 + 139500 * <i>Rhizomicrobium</i>
	<i>Rickettsiella</i>	0.72605	NP ₃ EO = -11.87 + 31543 * <i>Rickettsiella</i>
	<i>Pseudomonas</i>	-0.9295	NP ₃ EO = 123.15 - 46900 * <i>Pseudomonas</i>
	<i>Thiothrix</i>	-0.8633	NP ₃ EO = 85.451 - 864.4 * <i>Thiothrix</i>
	<i>Prostheobacter</i>	0.79533	NP ₃ EO = -56.81 + 9938.8 * <i>Prostheobacter</i>

Table 3.18. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₄EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol polyethoxylate (NP₄EO)	<i>Mycobacterium</i>	0.7082	NP ₄ EO = 48.858 + 3456.4 * <i>Mycobacterium</i>
	<i>Hyphomicrobium</i>	0.7682	NP ₄ EO = 41.241 + 9463 * <i>Hyphomicrobium</i>
	<i>Rhizomicrobium</i>	0.91692	NP ₄ EO = 44 + 41500 * <i>Rhizomicrobium</i>
	<i>Pseudomonas</i>	-0.8791	NP ₄ EO = 101.76 - 13700 * <i>Pseudomonas</i>
	<i>Thiothrix</i>	-0.8323	NP ₄ EO = 91.077 - 256 * <i>Thiothrix</i>
	<i>Acidibacter</i>	0.70401	NP ₄ EO = 62.950 + 14250 * <i>Acidibacter</i>
	<i>Prostheobacter</i>	0.7974	NP ₄ EO = 47.813 + 3061.2 * <i>Prostheobacter</i>

Table 3.19. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₅EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol polyethoxylate (NP₅EO)	<i>Microbacterium</i>	0.9406	NP ₅ EO = 95.331 - 4354 * <i>Microbacterium</i>
	<i>Clostridium sensu stricto 1</i>	0.81081	NP ₅ EO = 38.261 + 9804.3 * <i>Clostridium sensu stricto 1</i>
	<i>Catenibacterium</i>	0.77117	NP ₅ EO = 61.4 + 20000 * <i>Catenibacterium</i>
	<i>Planctomyces</i>	0.84518	NP ₅ EO = 48.535 + 1507.6 * <i>Planctomyces</i>
	<i>Hyphomicrobium</i>	0.89934	NP ₅ EO = 43.259 + 10037 * <i>Hyphomicrobium</i>
	<i>Mesorhizobium</i>	0.7593	NP ₅ EO = 61.429 + 16643 * <i>Mesorhizobium</i>
	<i>Rhizomicrobium</i>	0.98766	NP ₅ EO = 49 + 40500 * <i>Rhizomicrobium</i>
	<i>Pseudomonas</i>	-0.9368	NP ₅ EO = 105.12 - 13200 * <i>Pseudomonas</i>
	<i>Thiothrix</i>	-0.9388	NP ₅ EO = 95.581 - 261.6 * <i>Thiothrix</i>
	<i>Aquimonas</i>	0.9877	NP ₅ EO = 89.5 - 10200 * <i>Aquimonas</i>

Table 3.20. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₆EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlations equation
Nonylphenol polyethoxylate (NP ₆ EO)	<i>Microbacterium</i>	0.7372	NP ₆ EO = 94.076 - 773.9 * <i>Microbacterium</i>
	<i>Catenibacterium</i>	0.85003	NP ₆ EO = 86.6 + 5000 * <i>Catenibacterium</i>
	<i>Pirellula</i>	0.80949	NP ₆ EO = 84.12 + 1168.7 * <i>Pirellula</i>
	<i>Rhizomicrobium</i>	0.75264	NP ₆ EO = 86 + 7000 * <i>Rhizomicrobium</i>
	<i>Methyloversatilis</i>	0.71762	NP ₆ EO = 89.055 + 1272.7 * <i>Methyloversatilis</i>
	<i>Thiothrix</i>	-0.745	NP ₆ EO = 94.152 - 47.09 * <i>Thiothrix</i>

Table 3.21. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₇EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlations equation
Nonylphenol polyethoxylate (NP ₇ EO)	<i>Catenibacterium</i>	0.83428	NP ₇ EO = 88.9 + 3500 * <i>Catenibacterium</i>
	<i>Pirellula</i>	0.76641	NP ₇ EO = 87.349 + 789.16 * <i>Pirellula</i>
	<i>Hyphomicrobium</i>	0.73855	NP ₇ EO = 87.333 + 1333.3 * <i>Hyphomicrobium</i>
	<i>Rhizomicrobium</i>	0.82916	NP ₇ EO = 88 + 5500 * <i>Rhizomicrobium</i>
	uncultured	0.7623	NP ₇ EO = 89.673 + 681.82 * uncultured
	<i>Deltaproteobacterium</i>		<i>Deltaproteobacterium</i>
	<i>Rickettsiella</i>	0.71129	NP ₇ EO = 90.174 + 1391.3 * <i>Rickettsiella</i>
	<i>Thiothrix</i>	-0.8023	NP ₇ EO = 94.36 - 36.17 * <i>Thiothrix</i>

Table 3.22. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₈EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol polyethoxylate (NP ₈ EO)	<i>Candidatus Microthrix</i>	0.75529	NP ₈ EO = 85.120 + 596.39 * <i>Candidatus Microthrix</i>
	<i>Dyadobacter</i>	0.72966	NP ₈ EO = 89.883 + 1916.7 * <i>Dyadobacter</i>
	uncultured prokaryote	0.75567	NP ₈ EO = 89.108 + 841.32 * uncultured prokaryote
	<i>Lactobacillus</i>	0.78744	NP ₈ EO = 89.28 + 787.44 * <i>Lactobacillus</i>
	<i>Christensenellaceae R-7 group</i>	0.71264	NP ₈ EO = 89.625 + 3625 * <i>Christensenellaceae R-7 group</i>
	<i>Clostridium sensu stricto 1</i>	0.77536	NP ₈ EO = 81.565 + 2326.1 * <i>Clostridium sensu stricto 1</i>
	<i>Pseudobutyrvibrio</i>	0.74238	NP ₈ EO = 89.333 + 6166.7 * <i>Pseudobutyrvibrio</i>
	<i>Catenibacterium</i>	0.9325	NP ₈ EO = 85.8 + 6000 * <i>Catenibacterium</i>
	<i>Pirellula</i>	0.88498	NP ₈ EO = 82.855 + 1397.6 * <i>Pirellula</i>
	<i>Planctomyces</i>	0.7739	NP ₈ EO = 84.334 + 342.48 * <i>Planctomyces</i>
	<i>Devosia</i>	0.77235	NP ₈ EO = 78.461 + 1802.6 * <i>Devosia</i>
	<i>Hyphomicrobium</i>	0.92965	NP ₈ EO = 82.019 + 2574.1 * <i>Hyphomicrobium</i>
	<i>Rhizomicrobium</i>	0.8355	NP ₈ EO = 85 + 8500 * <i>Rhizomicrobium</i>
	<i>Methyloversatilis</i>	0.79662	NP ₈ EO = 88.709 + 1545.5 * <i>Methyloversatilis</i>
	uncultured	0.74238	NP ₈ EO = 89.333 + 6166.7 * uncultured
	<i>Desulfuromonadales bacterium</i>		<i>Desulfuromonadales bacterium</i>
	<i>Legionella</i>	0.84493	NP ₈ EO = 86.123 + 1669.8 * <i>Legionella</i>
	<i>Thiothrix</i>	-0.9209	NP ₈ EO = 95.251 - 63.68 * <i>Thiothrix</i>

Table 3.23. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₉EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol polyethoxylate (NP₉EO)	<i>Candidatus Microthrix</i>	0.8417	NP ₉ EO = 91.337 + 219.88 * <i>Candidatus Microthrix</i>
	<i>Subtercola</i>	0.82916	NP ₉ EO = 93.250 + 2750 * <i>Subtercola</i>
	<i>Arthrobacter</i>	0.76405	NP ₉ EO = 93.035 + 546.51 * <i>Arthrobacter</i>
	<i>Dyadobacter</i>	0.87581	NP ₉ EO = 93.050 + 750 * <i>Dyadobacter</i>
	<i>Leadbetterella</i>	0.82916	NP ₉ EO = 93.250 + 98.214 * <i>Leadbetterella</i>
	<i>Chryseobacterium</i>	0.82916	NP ₉ EO = 93.250 + 229.17 * <i>Chryseobacterium</i>
	<i>Haliscomenobacter</i>	0.8264	NP ₉ EO = 91.264 + 173.69 * <i>Haliscomenobacter</i>
	<i>Lactobacillus</i>	0.82243	NP ₉ EO = 92.942 + 268.12 * <i>Lactobacillus</i>
	<i>Clostridium sensu stricto 1</i>	0.71129	NP ₉ EO = 90.739 + 695.65 * <i>Clostridium sensu stricto 1</i>
	<i>Coprococcus 3</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Coprococcus 3</i>
	<i>Lachnoclostridium</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Lachnoclostridium</i>
	<i>Pseudobutyrvibrio</i>	0.73855	NP ₉ EO = 93 + 2000 * <i>Pseudobutyrvibrio</i>
	possible genus Sk003-Sk004	0.82916	NP ₉ EO = 93.25 + 2750 * genus Sk003-Sk004
	<i>Ruminiclostridium 5</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Ruminiclostridium 5</i>
	<i>Ruminococcaceae UCG-013</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Ruminococcaceae UCG-013</i>
	<i>Subdoligranulum</i>	0.82916	NP ₉ EO = 93.25 + 1375 * <i>Subdoligranulum</i>
	<i>Catenibacterium</i>	0.95346	NP ₉ EO = 91.8 + 2000 * <i>Catenibacterium</i>
	<i>Holdemanella</i>	0.82916	NP ₉ EO = 93.25 + 1375 * <i>Holdemanella</i>
	<i>Pirellula</i>	0.95947	NP ₉ EO = 90.639 + 493.98 * <i>Pirellula</i>
	<i>Brevundimonas</i>	0.82916	NP ₉ EO = 93.25 + 916.67 * <i>Brevundimonas</i>
	uncultured	0.82916	NP ₉ EO = 93.25 + 2750 * uncultured
	<i>Gammaproteobacterium</i>		<i>Gammaproteobacterium</i>
	<i>Devosia</i>	0.81276	NP ₉ EO = 89.224 + 618.42 * <i>Devosia</i>
	<i>Hyphomicrobium</i>	0.80009	NP ₉ EO = 91.056 + 722.22 * <i>Hyphomicrobium</i>
	<i>Aminobacter</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Aminobacter</i>
	<i>Hydrogenophaga</i>	0.79167	NP ₉ EO = 92.898 + 102.52 * <i>Hydrogenophaga</i>
	<i>Methyloversatilis</i>	0.86244	NP ₉ EO = 92.709 + 545.45 * <i>Methyloversatilis</i>
	<i>Thauera</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Thauera</i>
	<i>Bacteriovorax</i>	0.82916	NP ₉ EO = 93.25 + 916.67 * <i>Bacteriovorax</i>
	<i>Peredibacter</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Peredibacter</i>
	uncultured	0.73855	NP ₉ EO = 93 + 2000 * uncultured
	<i>Desulfuromonadales</i>		<i>Desulfuromonadales</i> bacterium
	bacterium		
	Uncultured	0.86394	NP ₉ EO = 92.255 + 386.36 * uncultured
	<i>Deltaproteobacterium</i>		<i>Deltaproteobacterium</i>
	<i>Legionella</i>	0.98106	NP ₉ EO = 91.651 + 632.08 * <i>Legionella</i>
	<i>Thiothrix</i>	-0.7987	NP ₉ EO = 94.776 - 18 * <i>Thiothrix</i>
	<i>Luteolibacter</i>	0.82916	NP ₉ EO = 93.25 + 2750 * <i>Luteolibacter</i>

Table 3.24. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₁₀EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol polyethoxylate (NP₁₀EO)	<i>Candidatus Microthrix</i>	0.87172	NP ₁₀ EO = 90.687 + 224.4 * <i>Candidatus Microthrix</i>
	<i>Dyadobacter</i>	0.7785	NP ₁₀ EO = 92.533 + 666.67 * <i>Dyadobacter</i>
	<i>Haliscomenobacter</i>	0.82162	NP ₁₀ EO = 90.679 + 172.69 * <i>Haliscomenobacter</i>
	<i>Lactobacillus</i>	0.77056	NP ₁₀ EO = 92.396 + 251.21 * <i>Lactobacillus</i>
	<i>Clostridium sensu stricto 1</i>	0.84465	NP ₁₀ EO = 89.565 + 826.09 * <i>Clostridium sensu stricto 1</i>
	<i>Pseudobutyrvibrio</i>	0.80009	NP ₁₀ EO = 92.333 + 2166.7 * <i>Pseudobutyrvibrio</i>
	<i>Catenibacterium</i>	0.95346	NP ₁₀ EO = 91.2 + 2000 * <i>Catenibacterium</i>
	<i>Pirellula</i>	0.91267	NP ₁₀ EO = 90.193 + 469.88 * <i>Pirellula</i>
	<i>Planctomyces</i>	0.8066	NP ₁₀ EO = 90.663 + 116.37 * <i>Planctomyces</i>
	<i>Devosia</i>	0.83006	NP ₁₀ EO = 88.526 + 631.58 * <i>Devosia</i>
	<i>Hyphomicrobium</i>	0.9437	NP ₁₀ EO = 89.963 + 851.85 * <i>Hyphomicrobium</i>
	<i>Rhizomicrobium</i>	0.82916	NP ₁₀ EO = 91 + 2750 * <i>Rhizomicrobium</i>
	<i>Methyloversatilis</i>	0.79057	NP ₁₀ EO = 92.2 + 500 * <i>Methyloversatilis</i>
	Uncultured Desulfuromonadales bacterium	0.80009	NP ₁₀ EO = 92.333 + 2166.7 * uncultured Desulfuromonadales bacterium
	uncultured delta Proteobacterium	0.86394	NP ₁₀ EO = 91.655 + 386.36 * uncultured delta Proteobacterium
	<i>Legionella</i>	0.92249	NP ₁₀ EO = 91.179 + 594.34 * <i>Legionella</i>
	<i>Thiothrix</i>	-0.9303	NP ₁₀ EO = 94.337 - 20.97 * <i>Thiothrix</i>

Table 3.25. Correlations of bacteria genera abundance with nonylphenol polyethoxylate (NP₁₁EO) removal

Nonylphenol	Bacteria genera	R-value	Linear correlation equation
Nonylphenol polyethoxylate (NP₁₁EO)	<i>Candidatus Microthrix</i>	0.86832	NP ₁₁ EO = 89.181 + 269.58 * <i>Candidatus Microthrix</i>
	<i>Dyadobacter</i>	0.72618	NP ₁₁ EO = 91.45 + 750 * <i>Dyadobacter</i>
	<i>Ferruginibacter</i>	0.77973	NP ₁₁ EO = 90.766 + 341.32 * <i>Ferruginibacter</i>
	<i>Haliscomenobacter</i>	0.74067	NP ₁₁ EO = 89.459 + 187.75 * <i>Haliscomenobacter</i>
	<i>Lactobacillus</i>	0.70035	NP ₁₁ EO = 91.319 + 275.36 * <i>Lactobacillus</i>
	<i>Clostridium sensu stricto 1</i>	0.7925	NP ₁₁ EO = 88.087 + 934.78 * <i>Clostridium sensu stricto 1</i>
	<i>Pseudobutyrvibrio</i>	0.91856	NP ₁₁ EO = 91 + 3000 * <i>Pseudobutyrvibrio</i>
	<i>Catenibacterium</i>	0.79057	NP ₁₁ EO = 90.2 + 2000 * <i>Catenibacterium</i>
	<i>Pirellula</i>	0.75675	NP ₁₁ EO = 89.193 + 469.88 * <i>Pirellula</i>
	<i>Planctomyces</i>	0.77754	NP ₁₁ EO = 89.251 + 135.29 * <i>Planctomyces</i>
	<i>Devosia</i>	0.90332	NP ₁₁ EO = 86.066 + 828.95 * <i>Devosia</i>
	<i>Hyphomicrobium</i>	0.86753	NP ₁₁ EO = 88.611 + 944.44 * <i>Hyphomicrobium</i>
	<i>Undibacterium</i>	0.81373	NP ₁₁ EO = 90.081 + 756.76 * <i>Undibacterium</i>
	uncultured delta proteobacterium	0.71634	NP ₁₁ EO = 90.655 + 386.36 * uncultured delta proteobacterium
	<i>Legionella</i>	0.82559	NP ₁₁ EO = 90.019 + 641.51 * <i>Legionella</i>
	<i>Thiothrix</i>	-0.8181	NP ₁₁ EO = 93.406 - 22.24 * <i>Thiothrix</i>
	<i>Pseudoxanthomonas</i>	0.73995	NP ₁₁ EO = 90.75 + 1208.3 * <i>Pseudoxanthomonas</i>

3.4 DISCUSSION

SRT and HRT increases resulted in a better sanitary performance of the AS pilot-plant, in agreement with the findings by Petrie et al., (2014) and by Rehman et al., (2019) in pilot-plant studies. The low removal of BOD, COD, SS and ammonia at 3 d SRT and 8 h HRT, is due to the high chemical loading rate, and low biomass created under those conditions. This leads to a high F:M ratio and most of the nutrients to pass through the plant without degradation. The effluent from the reactor under these operational conditions (3 d / 8 h) breached the UK effluent discharge consent limits because it had BOD of 33 ± 6 mg/L and COD of 172 ± 13 mg/L as compared to the 25 mg/L O₂ BOD₅ and 125 mg/L O₂ COD. The 10 d and 27 d SRTs performance achieved adequate sanitary removal to meet the discharge limits. However, when operating at high SRT (27 d) removals of ammonia reached 99%, also found by Li and Wu et al., (2014), and lengthening HRT at 27 d SRT resulted in a marginal improvement in BOD, COD and ammonia removal. These are compatible with findings by Melidis et al., (2016) and Dialynas et al., (2009) that a preliminary nitrifying/denitrifying plant operated at relatively lower SRT, HRT and dissolved oxygen concentrations achieved 32% average COD and total nitrogen reduction, but an extended system operating a high HRT, complete solids retention and DO concentrations greater than 4 mg/L achieved 95 and 85% COD and TN reduction. Guven et al., (2017) demonstrated that high-energy-content-sludge to feed an anaerobic digester can be made from ASP by running at low HRT and removing <60% COD in a process termed high-rate activated sludge (HRAS).

The use of PLFA in tandem with 16S rRNA to study activated sludge mixed liquor was employed by Forney et al., (2001) to assess biomass levels and whole-community substrate utilisations and found that both methods showed temporal and geographical differences. Like Forney et al., (2001) microbial community differences were found as SRT and HRT varied. PLFA revealed the order of microbial abundance to be Gram-negative bacteria as dominant followed by fungi, Gram-positive and actinomycetes, like the findings of Amir et al., (2010) in a study of sludge cake from wasted activated sludge. The fatty acid 16:1 ω 7c dominated at each SRT and HRT as previously reported by Yi et al., (2011) and has been linked as a biomarker for *Pseudomonas* sp. (Coenye et al., 2001). The significant changes in fatty acid profiles were observed as the process parameters changed, and the sensitivity of the PLFA to variations in SRT and HRT is clear.

Therefore, PLFA analysis which is relatively cheap and a shorter protocol than DNA analysis (Fornasier et al., 2014) has the potential to be used to monitor microbial diversity in WWTPs, although it has several limitations: (i) it gives less detailed information on microbial community structure, (ii) it has challenges around linking individual PLFAs to specific microbial groups, (iii) it requires a larger amount of sample and (iv) there is uncertainty about the rate of PLFA turnover (Frostegard et al., 2011). Werker et al., (2003) held similar views in a study of microbial diversity in activated sludge using fatty acid isopropyl esters. Weekly measurements of fatty acid methyl esters in mixed liquor samples could be carried out so that significant changes in the fatty acid profile could be indicative of microbial health deterioration or improvement and enable treatment scientists to identify changes that may lead to treatment failure and avert it through appropriate interventions.

The dominance of *Proteobacteria* was observed from 16S rRNA analysis. Bagchi et al., (2015) and Jiang et al., (2008) reported the same findings with a similar average abundance. At 3 d SRT *Thiothrix eikelboomii*, a filamentous sulphur-oxidising bacterium responsible for bulking, loss of performance and sludge washout (Henriet et al., 2017) dominated, an observation also reported by Xie et al., (2011). The conditions in the ASP at 3 d SRT was ideal for *Thiothrix eikelboomii* because it is heterotrophic and capable of utilising a range of sugars and organics as sole carbon sources and a range of amino acids and ammonia as sole nitrogen sources (Howarth et al., 1999). Perhaps it was the high ammonia concentration in the mixed liquor due to insufficient nitrification that led to the dominance of *Thiothrix eikelboomii* at 3 d SRT (appended Table AT3) and consequently the poor plant performance (Henriet et al., 2017). At 10 d SRT, the ASP reactor was dominated by *Nitrospira sp.*, affirming the observation of nitrification at 10.4 d SRT by Clara et al., (2005), while at the same time, the *Thiothrix eikelboomii* abundance dropped by 85% which may be due to the efficient removal of ammonia by the *Nitrospira species*. The decrease of *Thiothrix sp.* along with an increase in plant performance agrees with findings of Henriet et al., (2017). The ammonia-oxidising bacteria (AOB), *Nitrosomonas europaea* known to co-metabolise E2 and convert EE2 to nitro-EE2, when ammonia concentration is high (Sabirova et al., 2008; Gaulke et al., 2008) was found to be decreasing as nitrification increased with SRT (appended Tables AT3 to AT5). This may be due to increase in other AOBs which are complete ammonia oxidisers (comammox) and therefore an SRT increase at high HRT (F:M decrease) caused

these slow-growing but highly energy-efficient comammox species to increase and maintain nitrification (Kessel et al., 2015).

Several diversity indices could be used in quantifying the α -diversity in the pilot-scale reactor, Shannon and Simpson are the widely used indices. As this was a single reactor under varying conditions, it was expected that the number of species detectable through the grab samples will be similar, but their abundances will vary.

That meant Simpson index which emphasizes on evenness of species (Nagendra, 2002) was the preferred index for quantifying α -diversity. The Simpson index showed increases in diversity as SRT and HRT were lengthened. The significance of these differences could not be determined due to insufficient data. The increase in number of identified taxa in the pilot reactor as SRT and HRT lengthened coincided with increases in the removal of nonylphenols and estrogens, a phenomenon also observed by Akarsubasi et al., (2009). Increasing HRT promote competition among degraders as the chemical loading rate reduces (Petrie et al., 2014) and changes the chemical degradation rate from first-order where it depends on chemical concentration to zero-order where microbial cell count becomes important (Dharmadasa et al., 2018). Therefore, a combination of long SRT and HRT enhance the removal of estrogens and nonylphenols because the chemical removal rate is zero-order and depends on microbial cell count which is high at long SRT, the chemical loading rate is low and there is an enhance contact time between biomass and hazardous chemicals.

Six known species capable of degrading estrogens and nonylphenols in the ASP were present at 3 and 10 d SRT, but nine were identified at 27d SRT (Yu et al., 2013; Zhang et al., 2015). *Novosphingobium* species which metabolises E1, E2 and E3 (Fujii et al., 2002) and the *Nitrosomonas sp.* which metabolises and facilitates the formation of nitro-EE2 (Gualke et al., 2008) were found in each experiment. *Pseudomonas sp* which metabolises E2 (Zeng et al., 2009) and converts E2 to E1 (Yu et al., 2007) was also found in the ASP reactor at each SRT and HRT studied. *Microbacterium sp* and *Flavobacterium sp.* which are capable of oxidising E2 to E1 (Yu et al., 2007) were also found, but at different populations. This may explain why the removal of estrogens occurred in every reactor, even at 3 d SRT. *Flavobacterium sp.* was the dominant estrogen-degrading species at all SRT and HRT, ranging from 0.80 – 4.10 % in abundance (appended Tables AT3 to AT8). Most E1 degrading bacteria are Beta and

Gammaproteobacteria (Zang et al., 2008) and increasing SRT caused an increase in Alpha and Beta but decreased Gammaproteobacteria. However, increasing HRT from 8 to 24 h caused the Gammaproteobacteria to increase while the Betaproteobacteria remained relatively unchanged and the Alphaproteobacteria reduced, which may have increased the removal of E1 from 84 to 93 %. The abundance of Bacteroidetes increased as the SRT increased and these estrogen-degrading Bacteroidetes were only found at 27 d SRT: *Sphingobacterium* sp JCR5 which degrades E1, E2, E3 and EE2 (Ren et al., 2007), *Sphingomonas* strain KC8 which metabolises E1 and E2 (Yu et al., 2007), *Aminobacter* strain which degrades E1 and E2 and *Brevundimonas diminuta* which converts E2 to E1 (Yu et al., 2007; Muller et al., 2010); and that might have contributed to the improved removal of estrogens at longer SRT. It must be cautioned that the presence of a taxa does not necessarily translate into activity. Liang et al., (2017) demonstrated that the active community in an activated sludge WWTP reactor differed significantly from the total community.

Ralstonia pickettii, *Rhodococcus zopfii*, *Rhodococcus equii*, *Escherichia coli*, *Denitratisoma oestradiolicum* and *Achromobacter xylosoxidans* are bacterial species that have been isolated from activated sludge and identified as estrogen-degraders (Weber et al., 2005; Kurisu et al., 2010; Yoshimoto et al., 2004) but were not found in this study. These taxa may be present but in small quantities, causing them to be missed in the sample which highlights the challenges in capturing true diversity in a reactor from grab sampling (Quince et al., 2008). Also, confounding factors such as temperature (Wu et al., 2019) and pH (Wei et al., 2017) known to influence bacterial diversity in ASP but were not controlled in this experiment (a drawback) may be contributing to the absence of these species. Another drawback of this research was that in the HRT experiments conducted at a constant SRT of 27 d, the exact contribution of longer HRT towards the removal of chemicals could have been masked by the high SRT atypical of full-scale WWTPs. For instance, removal of E1 was $84 \pm 4\%$ at 8 h, $86 \pm 6\%$ at 16 h and $93 \pm 3\%$ at 24 h HRT. This resulted in insignificant differences in removal of E1 when 8 h is compared with 16 h HRT ($p > 0.05$, $n=7$) but removals of E1 were significantly different between 8 and 24 h ($p < 0.01$, $n=7$) and 16 and 24 h ($p < 0.01$, $n=7$).

The novel correlations found between bacteria (phyla/genera) taxa abundance and the chemical removals with $R > 0.7$ include positive and negative relationships. *Proteobacteria* negatively correlates with E1, E2, E3, NP₂EC, NP₃EO, NP₄EO, NP₅EO, NP₆EO and NP₇EO; *Chloroflexi* and *Verrucomicrobia* positively correlate with EE2 removal while *Actinobacteria* negatively correlate with EE2. *Leucobacter* correlated negatively with all the estrogens except E1-3S. *Hyphomicrobium*, *Mesorhizobium*, *Rhizomicrobium* and *Planctomyces* positively correlated with the removals of E1, E2 and E3. Although this work is the first of its kind, the findings are comparable to recent research by Coello-Garcia (2018) correlations between bacteria some genera and biodegradation rates of E1 and EE2.

Coello-Garcia (2018) found that the degradation rate of E1 correlated with the abundance of *Gemmatimonas*, *Planctomyces*, *Mesorhizobium* and *Leptospira* at $R > 0.7$ whilst *Nannocystis* correlated with the degradation of EE2. Other studies focus on the role and correlations of functional bacteria and genes has used Network Analysis to find other correlations. Li et al., (2019) found that the abundance of *Denitratisoma* and *Dokdonella* positively correlated with nitrite reductase genes nirS and nirK ($p < 0.05$), while *Thermomonas* and *Pseudomonas* positive correlation with nitrate reductase gene narG ($p < 0.05$). Li et al., (2016) explored the correlation between influent antibiotic concentrations and antibiotic resistance genes and found that levels of several antibiotics correlated with some gene. These correlations set the stage for future works to develop. It is important to impress that these correlations are not being interpreted as causation in this research because that assertion is out of the scope of this work. These correlations might be driven by some confounding factor such as temperature changes which was not controlled in this study. The correlation of nonylphenol removals with bacteria genera abundance revealed 122 correlations of which 34 correlated positively. *Thiothrix* which was dominant at 3 d SRT but reduced as SRT increased was found to negatively correlate with NP₂EC and NP₃₋₁₁EO. Several genera and removal correlated but there is no comparable research available for these results.

This study has demonstrated that changes in microbial/bacterial diversity in activated sludge reactors are influenced by process parameter and can be monitored through fatty acid profile from PLFA analysis. Further evidence has been provided from this research to support the already established understanding that longer SRT gives improved chemical removal. Contrary to the finding that HRT did not affect the removal of estrogens (Kumar et

al., 2015) this work has shown that HRT increases can lead to about 20 % increase in the removal of EE2, as reported by Petrie et al., (2014) and Ma et al., (2016). Finally, this study has found that bacterial diversity influences chemical removal and has identified several genera that correlate either positively with chemical removal. However, future research to generate larger datasets may be required at pilot and full-scale to affirm these correlations.

3.5 CONCLUSION

- The use of PLFA analysis for microbial diversity in mixed liquors is viable and could be incorporated in routine WWTP health checks on a weekly basis.
- Extending the SRT and HRT of the activated sludge process increased diversity and improved the removal of estrogens and nonylphenols.
- The length of the extension depends on the WWTP's capacity to control variability in influent flow and the availability of spare final tanks capacity to meet the increase in demand of final settling surface area to ensure good settlement and good effluent quality.
- The abundances of *Hyphomicrobium*, *Mesorhizobium*, *Planctomyces*, and *Rhizomicrobium* positively correlated with the removals of E1, E2 and E3. *Leucobacter* however, correlated negatively with all the estrogens except the readily degradable E1-3S. 122 bacteria genera were found to correlate with the removal of various nonylphenols carboxylates and ethoxylates, of which 88 genera correlated negatively. *Thiothrix sp.* the dominant taxon at 3 d SRT negatively correlates with the removal of NP₂EC and all nonylphenol ethoxylate with a chain length of three or more.
- The improved removal may be attributed to the increase in abundance and diversity of known identified taxa capable of degrading HCs, although other removal routes such as sorption to sludge may have been enhanced. Further studies to generate a larger dataset are required to support the observed correlations.
- Therefore, this study should be carried out at several full-scale municipal WWTPs to ascertain the correlations between bacterial diversity and chemical removal.

CHAPTER FOUR

SURVEY OF MICROBIAL / BACTERIAL DIVERSITY IN 12 FULL-SCALE ACTIVATED SLUDGE PLANTS

4. SURVEY OF MICROBIAL / BACTERIAL DIVERSITY IN 12 FULL-SCALE ACTIVATED SLUDGE PLANTS

ABSTRACT

The era of treating a biological wastewater reactor like a '*black-box*' is gradually eroding. Microbial analytical techniques have advanced to collect detailed information from the natural and engineered system where culturing was impossible, including wastewater treatment plants (WWTPs). However, operational parameters of the WWTP such as solid retention time (SRT), hydraulic retention time (HRT) and dissolved oxygen (DO) concentration as well as other external factors like temperature, influent chemicals and pH do affect the microbial diversity in the reactor. In this study, the broad microbial diversity of 12 full-scale activated sludge plants (ASPs) was surveyed over a sixteen-month period through the fast and affordable phospholipids fatty acid (PLFA) analysis, followed by an in-depth bacterial and archaeal analysis using the 16S rRNA gene sequencing protocol. The operational parameters of the reactor: SRT, HRT, DO and temperature were also recorded during sampling. The influences of operational parameters on the microbial/bacterial diversity were assessed using principal component analysis and the Bray-Curtis assessment of dissimilarity. The PLFA revealed that microbial diversity in AS reactors had 59 ±5% Gram-negative bacteria, 31 ±4% fungi, 9 ±2% Gram-positive bacteria, 1 ±0.3% actinomycetes, and there is a strong negative correlation ($R^2 = 0.93$) between Gram-negative bacteria and fungi abundances. The 16S rRNA bacterial diversity showed that the dominant phyla were 48 ± 7% *Proteobacteria*, 22 ± 6% *Bacteroidetes* and 8 ± 4% *Actinobacteria*. *Albidiferax*, *Zoogloea*, *Dechloromonas*, *Flavobacterium* and *Gordonia* were the dominant genera in most reactors. About 98% of the dominant bacterial species are uncultured. The microbial / bacterial diversity was influenced by temperature, SRT, HRT and plants' configuration. Temperatures below 13 °C increased the abundance of *Firmicutes* and *Bacteroidetes* and subsequently reduced the abundance of *Proteobacteria*, often leading to an increase and dominance of *Flavobacterium* or uncultured *Clostridia* bacterium. Shannon index of the diversity in each reactor revealed that the changes in diversity are insignificant ($p>0.11$). This research has shown for the that variations in bacterial diversity as a result of changes in process parameters though significant, does not impact the reactor's ability to remove nutrients, which attest to the robustness of ASPs. It has also proven the potential use of PLFA to assess microbial diversity in WWTPs and identify changes that might lead to treatment failure.

4.1 INTRODUCTION

The microbial diversity in activated sludge reactor depends on several parameters, which in turn determine the performance of the bioreactor in removing chemicals from the sewage. Recent technological advancements have permitted researchers to study the effect of these process parameters on microbial diversity. However, due to the complexity of the interaction between the parameters and the microbial communities in the bioreactor, a lot remains unknown in our quest to understand the relationship. Good knowledge of the interaction will allow process scientist to manipulate these bioreactors to achieve optimum removal of chemicals.

Solids retention time (SRT), hydraulic retention time (HRT), temperature and dissolved oxygen (DO) concentrations are a few parameters known to impact on microbial diversity, and these parameters will be the focus of this study. These parameters collectively determine the structural complexity of a WWTP (Harris et al., 2012) and explain 23% of the microbial diversity in the bioreactor, [25% defined by influent characteristics, 5% from the interaction between the influent characteristics and process parameters, and the remaining 47% due to unknown factors] (Wang et al., 2014). SRT studies in the pilot-scale at 3, 10 and 27 d showed that different bacterial genera dominate the reactor at different SRTs. The dominant bacteria species were *Thiothrix eikelboomii*, *Nitrospira sp.*, and an uncultured species from the *Comamonadaceae* family respectively (Chapter 3). Increasing SRT causes subtle and slow changes in bacterial diversity, but the change is significant and more pronounced with larger SRT differences (Lui et al., 2007). Lengthening SRT allows the ‘slow-growing’ bacteria that can degrade the hazardous chemicals, to increase in population, leading to increased diversity (McAdam (2010).

Temperature affects richness and evenness of microbial species because of its direct influence on optimum growth of psychrophilic, mesophilic and thermophilic bacteria. At higher temperatures (> 20 °C), the community richness is decreased, because psychrophilic bacteria (optimum ~ 15 °C) populations are significantly reduced (Jo et al., 2016). In suspended media activated sludge, temperature decrease caused a reduction in biofilm density and thickness as well as extracellular polymerase substance concentration, all of which are indicative of an alteration in microbial community and abundance (He et al., 2016). A fall in temperature also resulted in the dominant genus change from *Alishewanella*

at 25 °C to *Pseudomonas* at 5 °C (Wang et al., 2015). Floc morphology is also observed to be influenced by temperature (Koivuranta et al., 2017).

HRT, the ratio of bioreactor volume to influent flow rate and the regulator of the availability of substrate in the reactor also affect microbial diversity. As the richness of microbial diversity in a bioreactor is a function of reactor volume (van der Gast, 2006), increase in HRT in upflow activated sludge bioreactor decreases the population of coliform bacteria (Almasi et al., 2016). Also, the relative abundance of AOB in an ASP decreased from 33 to 15% as the HRT dropped from 30 to 5 h while NOB increased from 4 to 15% under the same conditions (Li et al., 2013).

Dissolved oxygen (DO) concentration also impacts on microbial diversity. The degradation of chemicals with or without oxygen results in different products and varying amounts of energy to the degrader, hence different microbial communities function in aerobic and anaerobic reactors. Activated sludge reactors are aerated for two reasons, firstly to keep the flocculants thoroughly mixed in suspension and secondly to provide DO for the microbial communities. DO levels, therefore, affect the floc structure, size, strength and the number of zoogaea and filaments in the mixed liquor (Wilén et al., 2010). To reduce the cost of aeration, DO concentrations now range between 1 – 2 mg/L, as opposed to historical levels greater than 5 mg/L and this has resulted in greater microbial diversity including fermentative, predatory and slow-growing bacteria (Nui et al., 2015). Another cost-reducing approach to aeration is intermittent aeration which further alters the microbial diversity and decreases phosphorus removal (Bhatia et al., 2017).

Research on microbial diversity and process parameters has often been limited to one parameter, a couple of parameters, a few full-scale WWTPs or using one microbial analytical method (Coello-Garcia, 2019). This research surveys the microbial and bacterial diversity in twelve municipal ASPs to characterise the influence of four process parameters. The microbial diversity was analysed through the affordable and fast phospholipid fatty acids method to obtain a broad overview of microbial community changes and through the advanced 16S rRNA analysis to gain a detailed register of bacterial and archaeal species identity and quantity.

This chapter is the first part of the full-scale study to assess the influence of process parameters on microbial/bacterial diversity and the influence of diversity on the removal of hazardous chemicals, replicating the pilot-scale study. Due to the enormity of microbial/bacterial diversity data, chapter four discusses the influence of process parameters on microbial and bacterial diversity of the full-scale WWTPs and chapter 5 presents the results on the chemical analysis and the subsequently the correlations between diversity and chemical removal. The novelty of this chapter is the use of PLFA and 16S rRNA analysis to study diversity in activated sludge sample in several full-scale WWTPs to understand the influenced SRT, HRT, temperature and dissolved oxygen microbial/bacterial diversity.

4.2 RATIONALE FOR WWTP SELECTION AND SAMPLING FREQUENCY

In the first chemical investigation programme (CIP1) led by the UK Water Industry Research (UKWIR), the performance of activated sludge plants in removing different chemicals varied widely (Gardiner et al, 2013). This research aimed at finding the relationship between bacterial diversity and chemical removal and so it was expedient to choose the activated sludge process due to their known record of good and poor removal over a range of chemical. Twelve full-scale ASPs were chosen to statistically represent the proportion of activated sludge WWTPs previously studied in the CIP1. To understand the impact of catchment on the microbial diversity in the ASP reactor; and knowing that influent chemicals differs with plant location and population equivalent (P.E.); ASPs with different P.E. were chosen within 140 km proximity to the research centre, to allow same-day sample preparation and storage. The plants were categorised as small (P.E. < 100,000), medium (100,00 < P.E. < 300,000) and large works (P.E. > 300,000) based on their treatment capacity. Each WWTP was visited between four and seven times to ensure a minimum of two winter and two summer samples were taken (full sampling schedule in Table 4.1.) Samples were taken from raw sewage, settled sewage, mixed liquor and final effluent in triplicate. A description of each WWTP and the plant's operational parameters are also found in Table 4.2. Each WWTP comprised of primary sedimentation tanks, activated sludge lanes and then final settling tanks, except WWTP C which had an MBBR (Kaldness) reactor before its primary sedimentation and sand filtration beds after the final settling tanks. WWTP H also had sand filtration beds after final settling tanks.

Table 4.1. Sampling dates for the full-scale WWTPs

Sampling Year / Mon	WWTPs code and sampling dates											
	A	B	C	D	E	F	G	H	J	K	L	M
2015	Apr						29 th					
	May			26 th			29 th					
	Jun					16 th	2 nd 11 th			22 nd		25 th
	Jul	1 st 30 th				24 th			14 th		24 th	
	Aug											
	Sep			3 rd			3 rd					
	Oct				13 th			20 th	27 th		27 th	20 th
	Nov	24 th										
2016	Dec		18 th			10 th						
	Jan		28 th	21 st			19 th			28 th		
	Feb						2 nd			15 th		
	Mar	15 th	31 st		24 th	22 nd 30 th	29 th	29 th	15 th 22 nd	31 st	30 th	29 th
	Apr		8 th	6 th	15 th					8 th		
	May					19 th		31 st	19 th		19 th	
	Jun	9 th		1 st	1 st	9 th	7 th 10 th	7 th 10 th			9 th	7 th 10 th
	Aug		22 nd 26 th									
Total	5	6	5	4	5	5	7	5	5	5	5	5

Table 4.2. WWTPs code, population equivalent processes descriptions and operational conditions

WWTP (P.E.)	Key processes	Inlet flow (m ³)	RAS (mg/L)	SAS (m ³ /d)	DO (mg/L)	Temp (°C)	HRT (h)	SRT (d)
A 105,000	PST -> NASP -> SST	24409 ±4854	6154 ±721.5	544 ±38.85	1.74 ±0.59	16 ±3.17	10.82 ±1.99	9.76 ±1.46
B 71,100	PST -> NASP -> SST	35529 ±7675	5763 ±1084	974.3 ±403	1.46 ±0.47	15.88 ±3.38	13.53 ±3.05	11.48 ±4.88
C 147,975	MBBR -> PST -> NASP -> SST -> SBF	15534 ±6262	6988 ±956	549.6 ±292	0.942 ±0.83	17.86 ±2.23	11.36 ±3.08	9.64 ±2.99
D 25,000	PST -> NASP -> SST -> LGN	11044 ±2607	6220 ± 1195	437.5 ±47.87	1.2725 ±0.45	16.45 ±1.38	19.3 ±4.35	11.675 ±4.79
E 55,761	PST -> A/O -> SST	11924 ±4278	5684 ±1252	200.4 ±85.9	1.272 ±0.66	14 ±2.91	19.72 ±6.75	9.14 ±3.8
F 409,918	PST -> NASP -> SST	132848 ±15417	14020 ±4617	914.8 ±256	1.474 ±0.57	16.44 ±2.48	10.48 ±2.48	13.96 ±3.38
G 302,000	PST -> A/O -> SST	31029 ±9043	8234 ±1500	673 ±303	3.34 ±2.56	16.21 ±2.48	12.64 ±3.29	13.77 ±3.73
H 97,029	PST -> NASP -> SST	20397 ±3890	6124 ±477	308.6 ±63	2.598 ±2.3	15.7 ±2.88	9.1 ±2.3	10.84 ±2.59
J 122,127	PST -> A/O -> SST -> SBF	13459 ±3394	4540 ±564	479.2 ±72.3	1.996 ±1.8	14.08 ±3.03	14.92 ±3.11	8.08 ±1.92
K 101,000	PST -> NASP -> SST	25908 ±1613	7648 ±1213	438.8 ±97.1	1.65 ±0.13	13.32 ±2.27	17.64 ±1.02	17.56 ±3.87
L 66,205	PST -> BNR+P -> SST	24939 ±9648	4923 ±1386	587.4 ±72.4	2.598 ±2.02	15.78 ±3.02	10.42 ±4.3	9.3 ±2.8
M 1750000	PST -> NASP -> SST	93847 ±21593	5388 ±757	1607 ±131	1.082 ±0.22	16.7 ±2.94	10.22 ±2.15	10.98 ±0.65

PST = primary settling tank; NASP = nitrifying activated sludge plant; FST = final settling tank; MBBR = moving bed biofilm reactor (Kaldness); SBF = sandbed filtration; LGN = lagoon; BNR+P = biological nutrient removal with phosphorus removal

4.3 MATERIALS AND METHODS

4.3.1 Sanitary determinants analysis and operational parameters

Chemical oxygen demand (COD), ammonia ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$) concentrations were measured with cell test kits from VWR International (Leicestershire, UK) in a 5487 spectrophotometer (Cheshire, UK) on the raw, settled and final effluents. Suspended solids in the samples, as well as the mixed liquor and the return activated sludge (RAS), were determined following Standard Methods for the Examination of Water and Wastewater, 22nd edition (2012). The pH and temperature were measured in-situ with Hanna pH meter HI-991401 (Digitalmeters, UK) and the dissolved oxygen in the mixed liquor was measured using the Hach HQ40d meter. The amount of surplus activated sludge (SAS) removed for sludge treatment for the previous 24 hours was also copied from the daily site logbook, and the total influent treated, and effluent discharged over the preceding day were recorded from the respective flow meters. The size of the activated sludge lanes was obtained from the WWTP's construction manuals and used to compute the HRT and SRT. The means and standard deviations of the process parameters and the flows displayed in Table 4.2 and the full process parameters and results of the sanitary determinants are found in appended Tables AT9 to AT12.

4.3.2 Microbial analytical methods

4.3.2.1 Phospholipids fatty acid analysis

The chemicals and protocol for the phospholipids fatty acid analysis used for the microbial diversity in all the WWTPs have been described in chapter 3.2.

4.3.2.2 16S rRNA analysis

Sample preparation and storage

A 25 mL aliquot of mixed liquor was transferred into a 50-mL centrifuge tube and immediately preserved in analytical grade ethanol in the ratio 1:1 (v/v). The samples were then stored at 4 °C and transported to the laboratory and stored at -20 °C until further analysis.

Chemicals and reagents

MPBio FastDNA Spin Kit for soil (Q-Biogene, MP Biomedicals, UK) Lysing Matrix E Tube; Fastprep[®] Instrument (or Hybaid Ribolyser); Sodium Phosphate Buffer; MT buffer; sodium dodecyl sulphate; Eppendorf tube; PPS reagent; Binding Matrix Suspension; SPIN[™] Filter and catch tube; SEWS-M (ethanol/salt to wash DNA); DES (DNase / Pyrogen Free Water; to solubilise DNA); PCR mix provided (the PCR MIX contains reaction buffer, enzyme blend, dNTPs and reverse primer); Barcoded forward primer; Sterile molecular grade water (negative control); Thermal block cycler (PCR machine); Agencourt[®] AMPure[®] XP Reagent; DynaMag-2 magnet (magnetic rack); 70% ethanol; TE buffer or nuclease-free water; 1% agarose gels; 1 g of agarose; 1×TAE buffer (2 M Tris-Acetate, 0.05 M EDTA, pH 8.3); Nancy-520 DNA Gel Stain (Sigma Aldrich); 0.25% (w / v) bromophenol blue; 40% (w / v) sucrose in filtered sterilized water loading buffer; PCR marker; Hyperladder II (Bioline, UK); Bio-Rad Fluor-S[®] Multilimager (Bio-Rad, UK); Qubit dsDNA HS reagent 1:200; Qubit 2.0 Fluorometer.

DNA extraction from samples

About 250 µL of sludge sample was added to a Lysing Matrix E Tube and topped with 978 µL sodium phosphate buffer and 122 µL MT buffer. It was then processed in the Ribolyser at 6.5 speed for 30 seconds, followed by 10 minutes centrifugation at 13,000 g. The supernatant was transferred into a sterile 2-mL Eppendorf tube, along with 250 µL PPS reagent and mixed by shaking the tube by hand ten times. It was centrifuged at 14,000 g for 5 minutes to pellet the precipitate. Again, the supernatant was pipetted into another 2-mL tube, and 1 mL *Binding Matrix Suspension* was added, followed 2 minutes of mixing by hand inverting action after which it was rested for 3 minutes to settle the matrix. An initial 500 µL of the supernatant was removed and discarded. The remainder was resuspended, filtered through a SPIN[™] Filter and centrifuged at 14,000 g for 1 minute. 500 µL SEWS-M (ethanol/salt to wash DNA) was added to the SPIN[™] Filter and centrifuged again for a minute, emptied and re-centrifuged for 2 minutes to 'dry' the matrix of residual SEWS-M wash solution. The catch-tube on the SPIN[™] Filter was replaced, and the matrix was air-dried at room temperature for 5 minutes before adding 50 µL DES to resuspend the silica filter membrane for efficient elution and to solubilise the DNA. The eluent containing the DNA was transferred into the catch-tube by centrifuging for 1 minute at 14,000 g (MP Biomedicals, Santa Ana, USA).

Amplicon library preparation (fusion method)

A 24 µL of PCR mix containing reaction buffer, enzyme blend, dNTPs and the reverse primer 926r (5'-CCGGYCAATYYMMTTTTRAGTTT-3') was transferred into sterile 0.2-mL Eppendorf tubes. To each tube was also added 0.5 µL of unique a 12-base pair Golay barcoded-forward-primer 515F (5'-GTGNCAGMGCCGCGGTAA-3') (Hamady et al., 2009) which incorporated a sequencing adaptor and a GT spacers to allow multiplexed analyses of the samples. 0.5 µL of DNA extract was then added and mixed thoroughly by pipetting up and down. The DNA extract was then subjected to polymerase chain reaction under the following conditions: Initial denaturation at 95 °C for 2 minutes; 30 cycles of denaturation at 95 °C for 30 seconds each; 30 cycles of annealing at 55 °C for 30 seconds each; 30 cycles of extension at 72°C for 45 seconds each and a final extension at 72 °C for 7 minutes.

PCR product purification: removal of fragments < 300 bp

Directly into the PCR tube containing 25 µL PCR product, 27.5 µL of re-suspended Agencourt AMPure XP Reagent was added and pipetted up and down a few times to mix the bead suspension with the DNA thoroughly. The mixture was then incubated at room temperature for 5 minutes. The PCR tubes were placed in the DynaMag-2 magnetic rack until the solution cleared. The supernatant was then removed and discarded without disturbing the bead pellet. The PCR product was washed twice by adding 50 µL of freshly prepared 70% ethanol to the PCR tube while it remained in the magnetic rack, left for 30 seconds and all the supernatant removed and discarded. The sample was left in the magnetic rack for a further 4 minutes to air-dry the beads at room temperature. Afterwards, the PCR tube was removed from the magnet, and 20 µL of TE buffer was added, mixed and magnetised until a clear solution was observed. The supernatant containing amplicon DNA was transferred into a sterile 0.2 µL Eppendorf tube.

PCR products examined by gel electrophoresis

A gram of agarose was weighed into 100 mL of 1×TAE buffer, heated to dissolve, cooled to 60 °C and spiked with 20 µL of Nancy-520 DNA Gel Stain. The solution was poured into an agarose gel casting tray and comb assembly (to create sample wells), with ends of tray sealed with autoclave tape, and allowed to set for 30 minutes. The agarose gel tray was then placed into an electrophoresis tank filled with 1 × TAE solution after end-tapes and comb had been removed.

The PCR product (5 μ L) was mixed with 2 μ L of loading buffer before carefully loading 5 μ L of the mixture into the sample wells and 5 μ L of PCR marker in the flanking wells. The gel electrophoresis ran for 45 minutes at 100V, and the bands were visualised by UV illumination.

Amplicon Library quantification

From a single PCR library made by adding equimolar amounts of 500 pM and size selected from a 2% agarose-dye-free gel using the Pippin Prep System (Life Technologies, Warrington, UK) following the manufacture's protocol. The pooled PCR libraries were sequenced using Ion Torrent Personal Genome Machine (PGM) System, with an Ion PGM Hi-Q View Kit on a 318 chip (Life Technologies, Warrington, UK) in accordance with manufacturer's guidance. To maximize the number of reads per sample for better a-diversity studies (Bokulich et al., 2013), none of the samples was replicated.

Processing of sequencing reads

The bioinformatic processing of the data generated by the Ion Torrent PGM was done by Tamara Coello-Garcia using QIIME 1.9 to demultiplex the initial sequence reads, remove primers and barcodes, cluster the reads into Operational Taxonomic Units at 97% similarity cut-off and assign taxonomy.

4.3.3 Statistical treatment of data

The process parameters (SRT, HRT, DO and temperature) and the microbial diversity (Gram+, Gram-, ACTS and fungi) of the sampling events were compared by one-way analysis of variance (ANOVA) within each site to establish significant variations. The ANOVA was followed by Post Hoc Tukey HSD to identify which samples were different. Principal component analyses were also performed to compare the microbial diversity and the operational parameters to determine any clusters and the factors that drive it. These statistical analyses were repeated for the entire data to ascertain inter-site differences and similarities using Statistica₁₃.

The ANOVA for each process parameter showed that there were significant variations between most sampling events, appended Tables AT13 to AT16 shows the p-values as binary numbers, >0.05 as 1 and <0.05 as zero. There was significant variation across all WWTP with respect to temperature and SRT (except WWTP J).

Intra-site DO concentration variation was significant for all WWTP except A, F and M, but HRT variation within all WWTPs was insignificant apart from WWTP F. All WWTPs showed significant variation when compared with respect to SRT and temperature.

The microbial diversity emanating from the fatty acid profile: Gram-positive bacteria (G+: 14:0i, 15:0i, 15:0ai, 16:0i, 17:0i, 17:0ai), Gram-negative bacteria (G-: 16:1w7c, 17:0cy, 17:1w7c, 18:1w7c, 19:0cy), actinomycetes (ACTS: 10Me16:0, 10Me17:0, 10Me18:0) and fungi (16:1w5c, 18:3w3, 18:2w6, 18:1w9c, 20:5w3, 20:1w9) were also compared to ascertain variation. The fungal abundance showed significant differences within all WWTPs, but the ACTS abundances were similar in WWTPs H, J and L. The G- abundances were also similar in WWTPs A and J whilst G+ abundances was only similar within WWTP K.

The Post Hoc Tukey HSD showed significant variation at 95 % confidence limit and identified ten homogeneous groups when comparing the ACTS abundance; 17 in G+; 24 in fungi; 25 in G-; 7 in DO; 20 in HRT; 24 in SRT and 31 in temperature between all sampling event (appended Tables AT17 to AT24).

4.4 RESULTS

4.4.1 Comparison of sampling events based on their process parameters

Figure 4.1A is the distribution of sampling events based on their process parameters at the time of sampling. The large cluster in the middle confirms the observation that all WWTPs operate at similar process parameters with a few outliers due to occasional flow changes which may arise from rainfall and impact on HRT and SRT. Some extreme temperatures and DO measurements during sampling also contributed to outliers.

The seasonal changes in temperature across the WWTPs were also uniform. The outliers were sampling events C2, D1, E2, E5, H3, H5, H4, G2, L3 and B5 which scattered in isolation. Samples C2 and E5 are secluded because they had the shortest SRT of 5.2 d and 4.9 d respectively, sample B5 had the warmest temperature of 20.3 °C whereas sampling events E2 and D1 are remote because they had the longest HRT of 26.6 and 23.5 h respectively. The loading plot (Figure 4.1B) reveals that no individual parameter is responsible for the distribution of sampling events in Figure 4.1A.

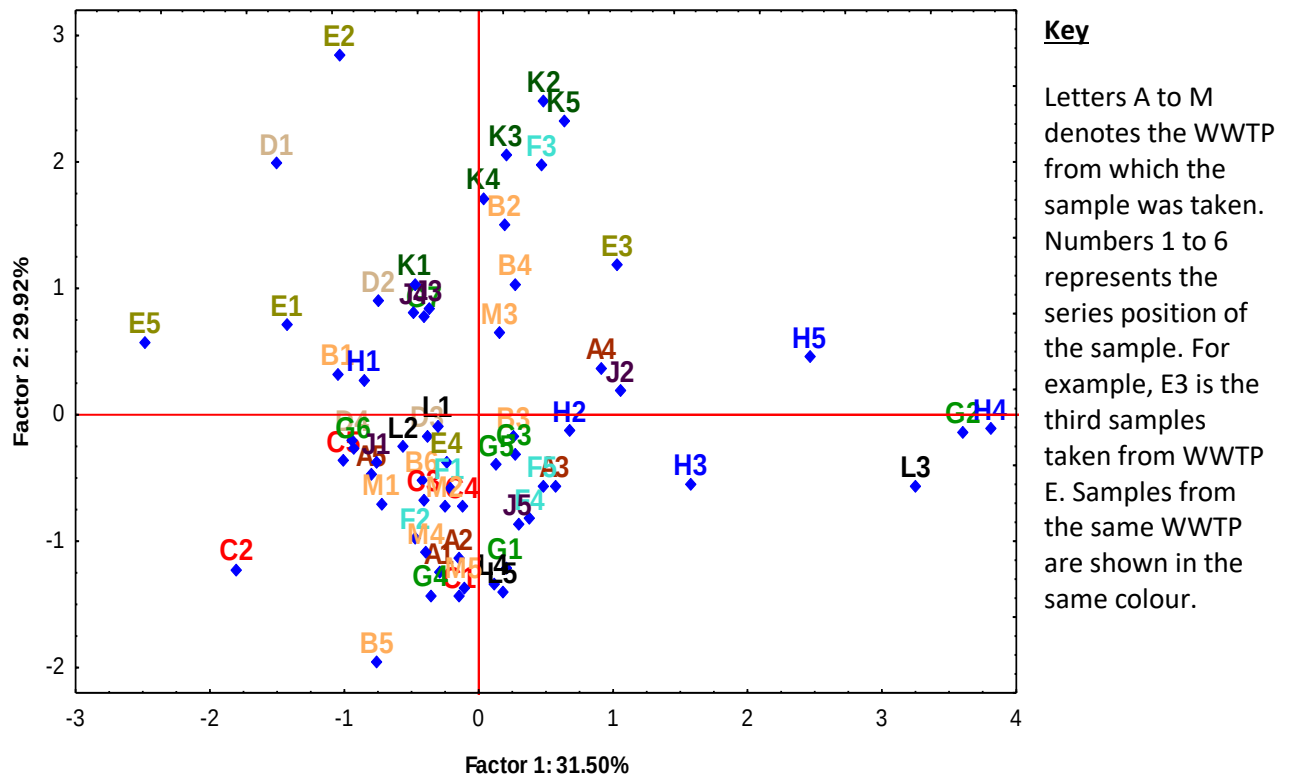


Figure 4.1A. Comparison of the sampling events based on their process parameters: SRT, HRT, temperature and DO.

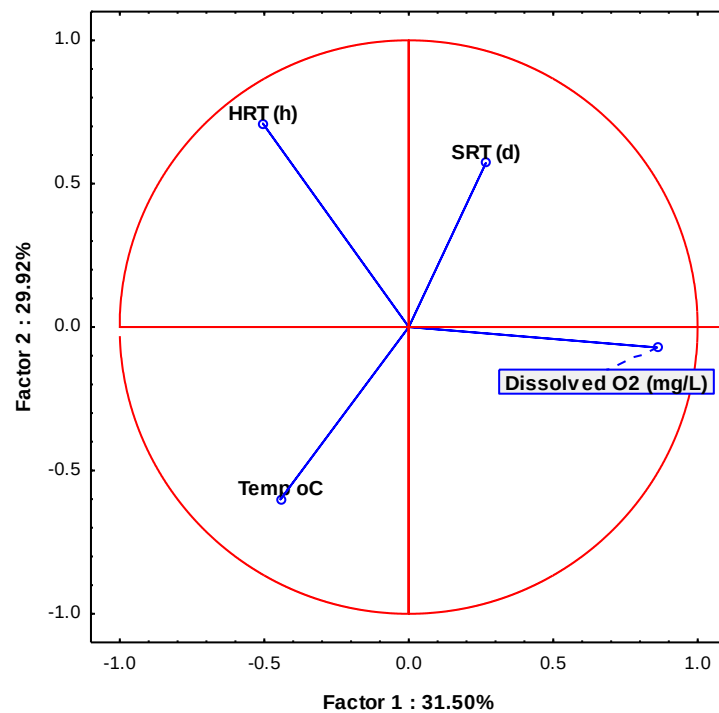


Figure 4.1B. Loading plot of the comparison of the sampling events based on their process parameters

4.4.2 Microbial Diversity from PLFA Analysis

The most occurring fatty acids in the microbial cell membranes from each sample were 16:1 ω 7c, 18:1 ω 7c; 18:1 ω 9c; 16:1 ω 5c; 18:2 ω 6 and 15:0i. A strong inverse correlation was found between Gram-negative bacteria and fungi abundances with $R^2=0.93$ (Figure 4.2).

The positive relationship observed between fatty acids cy17:0 and 16:1 ω 7c in the pilot-scale study is not found in the full-scale investigation.

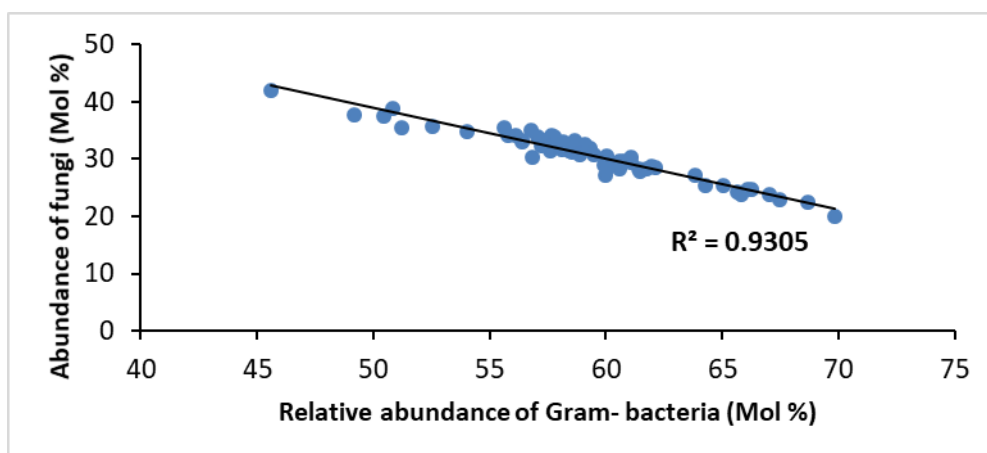


Figure 4.2. Correlation of abundances of Gram-negative bacteria with fungi

The individual fatty acids abundances and the relative abundances of the microbial communities in each sample (calculated as a sum of the fatty acids biomarkers) are in presented appended Figures AF1 to AF13. The overview of microbial diversity showed the dominance of Gram-negative bacteria in all sampling events with abundance ranging from 45.6 to 69.8%; seconded by fungi (20.0 to 42.0%), followed by Gram-positive bacteria (7.0 to 12.3%) and then actinomycetes (0.6 – 2.1%). WWTPs A and B had Gram-negative abundances of 57.7 – 61.5% and 50.8 – 57.7% respectively. The fatty acids profile was in the same order for both WWTPs. However, samples from WWTP A had relatively low percentages of the 16:1 ω 5c fatty acid (6%). The microbial groups found in WWTPs C and D had Gram-negative proportions between 45.6 – 59.9% and 54.0 – 61.1% respectively. Site C had the lowest Gram-negative percentages out of the twelve sites investigated. Samples from site C also had a more extended range of fungi abundance from 27.2 – 42%. WWTP E contained 52.5 - 61.4% Gram-negative bacteria, 28.2 – 35.6% fungi, 8.4 – 40.4% Gram-positive bacteria and between 0.72 and 1.02% actinomycetes. WWTP F had 0.8 – 1.4% actinomycetes, 7.4 – 9.0% Gram-positive bacteria, 29.7 – 32.7% fungi and 57.4 – 60.6% Gram-negative bacteria. WWTP G was sampled seven times due to its proximity to the research centre and an earlier access arrangement. Samples from G consistently contained

high proportions of Gram-negative bacteria (64.3 – 69.8%), and as such the lowest percentages of fungi (20.0 – 25.3%). Site H had 57.8 – 65.6% Gram-negative bacteria and 22.9 – 32.6% fungi. WWTP J had Gram-negative bacteria abundances between 55.8 and 58.6% while that of WWTP K were 56.4 and 63.9%. In sample K5, the Gram-negative bacteria proportion (56.49%) remained comparable to other samples from site K, but the abundance of fatty acid 16:1 ω 7c reduced from circa 40% to about 27% whereas fatty acid 18:1 ω 7c increased from its typical levels of 20% to approx. 26%. The percentage of Gram-negative bacteria in samples from WWTPs L and M were between 57.8 – 61.9% and 50.4 – 62.2% respectively. The fungi abundance for site L was 28.3 – 33.4% while those from site M were 28.6 – 37.6%.

4.4.3 Influence of process parameters on microbial diversity from PLFA.

The microbial diversity in the samples clustered into four groups (Figure 4.3A) when the influence of process parameters on it is assessed (Figure 4.3B). Group I samples contained high Gram-negative bacteria, low fungal abundances and had high dissolved oxygen concentrations.

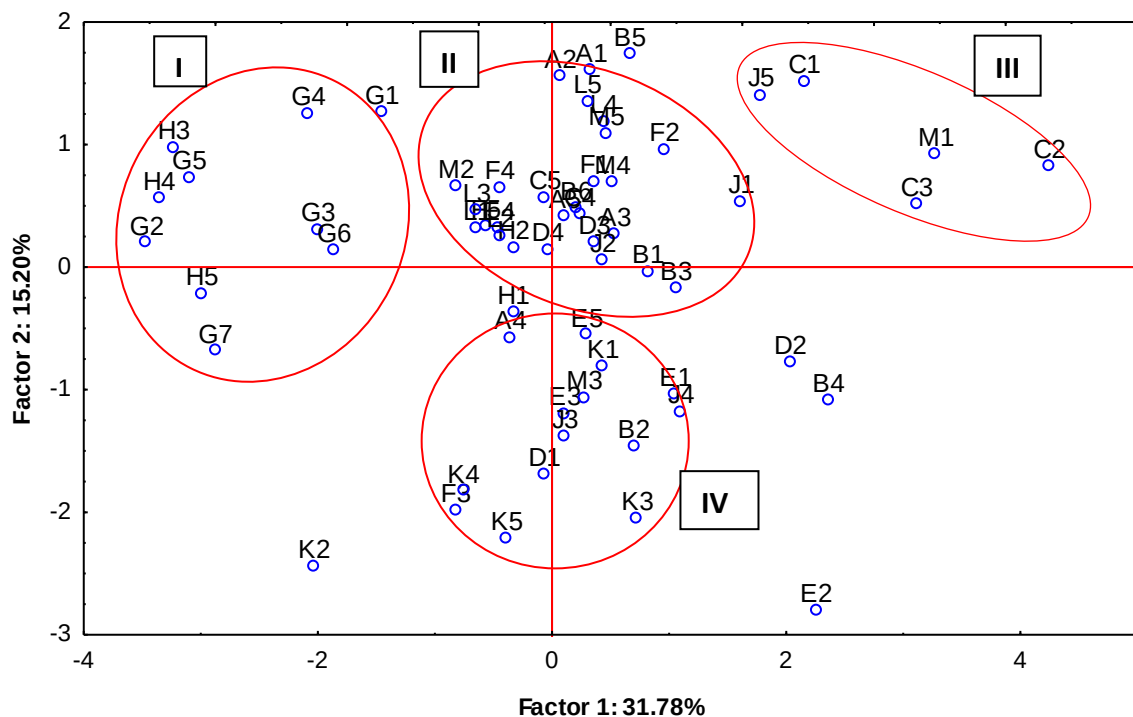


Figure 4.3A. Comparison of the microbial diversity in the full-scale WWTPs as influenced by SRT, HRT, DO and temperature

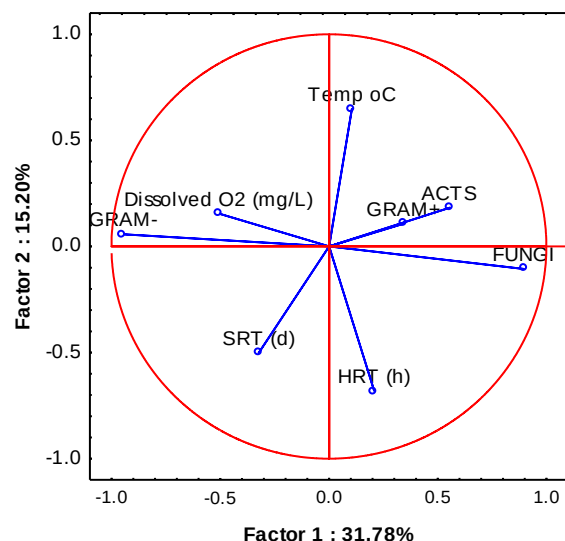


Figure 4.3B. Loading plot showing the influence of each process parameter and microbial diversity on the clustering of sampling events in Figure 4.3A.

Groups II and IV (which contained the most of sampling events) were driven by the low to mid ranges of SRT (6.5 to 12.5 d) and HRT (8 to 10 h), with group II being samples taken at warmer temperatures ($>14^{\circ}\text{C}$) while group IV corresponded to colder temperatures ($<14^{\circ}\text{C}$). Group III cluster arose from samples with relatively high Gram-positive bacteria, high actinomycetes and high fungi abundances which were taken at warmer temperatures and low DO concentration. Samples from WWTP C and G clustered in different quarters (Figure 4.3A), due to their different abundance of fungi, Gram-positive and Gram-negative bacteria. Samples C1, C2, C3, J5 and M1 cluster together in group III because they were taken at high temperatures ($\geq 14^{\circ}\text{C}$) and had a low DO concentration in their mixed liquors as well as low SRT. These process conditions lead to high abundances of Gram-positive bacteria and actinomycetes. Samples from sites G and H which taken were taken at relatively low temperatures, with high DO and they had high Gram-negative bacteria and low fungi abundances in their microbial diversity. The PLFA microbial diversity reveals that samples from sites C, G and M grouped sparingly well. However, the WWTPs groupings are merged into one broad cluster by the spread of all other sampling events. Across all WWTPs, it is observed that temperature and SRT were the most influential variables on microbial diversity, with temperature having the most significant effect (Figure 4.3B). The SRT increases did not have as significant an effect on the Gram-negative bacteria and fungi abundances as it did on the Gram-positive and actinomycetes. Also, the abundances of fungi are negatively impacted by high DO concentrations.

4.4.4 Bacterial diversity from 16S rRNA analysis

Figure 4.5 summarises the bacterial phyla with abundance greater than 1% in boxplots where the mean abundance is marked with x. The abundances were *Actinobacteria* (2.2 – 21%), *Bacteroidetes* (11.8 – 36.9%), *Chloroflexi* (0.6 – 12.1%), *Firmicutes* (2.1 – 25.8%), *Nitrospirae* (0.1 – 6.2%), *Planctomycetes* (0.5 – 9.9%), *Proteobacteria* (29.5 – 59.0%), *Spirochaetae* (0.1 – 4.9%) and *Verrucomicrobia* (0.4 – 2.2%). The bar charts in Figure 4.6 depict the entire phylum level abundance for each sample to demonstrate the changes that occur in the individual WWTPs with respect to time.

Notably, samples from WWTPs E and K had high abundances of *Bacteroidetes* with samples E2 and E3 being the only two where *Bacteroidetes* dominated followed by *Proteobacteria*. Sample H2 contained an unusually high proportion of *Firmicutes* (25.8%) and *Actinobacteria* (20.1%) than *Bacteroidetes* (13.7%). Samples from WWTP C had a significantly higher abundance of *Planctomycetes* compared to the others. The mean *Planctomycetes* abundance in reactor C was significantly higher (6.5 – 9.9%), and all other sites ranged between 0.5 – 4.4% and averaged around 2.47% (Figure 4.5). This unusual abundance correlates with an equally high abundance of the fatty acid 16:1 ω 5c in the WWTP C which ranges from 9.76 – 25.04% while all the other samples were within 5.1 – 10.9%. Sample C2 had high *Nitrospirae* abundance of 6.2%.

There was also some inter-phyla correlation such as Candidate division WS3 correlated with *Planctomycetes* ($R^2=0.7112$); *Euryarchaeota* and *Firmicutes* correlate ($R^2=0.6391$), *Chlorobi* correlates with *Chloroflexi* ($R^2=0.4996$); *Gemmatimonadetes* correlates with *Nitrospirae* ($R^2=0.4311$) and *Planctomycetes* ($R^2=0.5348$) and *Nitrospirae* correlates with *Planctomycetes* ($R^2=0.5574$).

4.4.4.1 Class and genus diversity

The bacterial class diversity (Figures 4.7A & B) and genus diversity (Figure 4.8 A & B and Table 4.4) shows changes in diversity occurring throughout the year, and the variations are more noticeable at the genus level. At the class level, the *Actinobacteria* abundance ranged from 1.0 – 15.5% in samples C2 and D2 respectively. Other samples with high *Actinobacteria* percentages were E2 (12.3%), E3 (14.8%), F3 (11.9%), H2 (12.7%) and M3 (10.4%). The *Sphingobacteriia* abundances were between 8.2 and 22.6% in L2 and A4 respectively.

The abundances within *Proteobacteria* were (5.6 – 18.0%) *Alphaproteobacteria*, *Betaproteobacteria* (11.1 – 42.8%), *Deltaproteobacteria* (3.3 – 10.1%), *Epsilonproteobacteria* (0 – 0.1%) and 2.3 – 11.1% *Gammaproteobacteria*. *Planctomycetacia* ranged from 0.4 – 7.1% while *Nitrospira* was between 0.1 and 6.2%. The abundance of *Clostridia* which was the most dominant class in sample H2 was 1.7 – 20.3%. *Anaerolineae* bacteria from *Chloroflexi* was high in many samples with the highest proportion found in D1 (6.7%). Samples E2 and E3 were dominated by *Flavobacteria*. The dominant genera in the *Actinobacteria* were *Ornithinibacter* (9.6% in E3 and 6.2% in E2), *Tetrasphaera* (6.1% in F3 and 3.3% in A5 and 3.7% in J3 and 3.0% in J2) and *Gordonia* (6.7% in D2 and 3.8% in D3). In the class of *Sphingobacteriia*, two of the three dominant genera were uncultured from *Saprospiraceae* and *Chitinophagaceae*, and the third dominant genus was *Terrimonas* (5.8% in sample H4). The genera *Rhodobacter* (4.0% in H2), *Amaricoccus* (2.8% in B3), *Sphingobium* (2.7% in K3), *Novosphingobium* (2.1% in K4) and *Defluviimonas* (1.1% in K3) dominated the *Alphaproteobacteria*. The *Betaproteobacteria* class was dominated by the genera *Dechloromonas* (20.1% in G1), *Albidiferax* (11.1% in sample J3) and an uncultured genus from *Comamonadaceae* (6.0% in L2). Other prominent *Betaproteobacteria* genera were *Lautropia*, *Zoogloea*, *Thauera*, *Leptothrix* and an unknown *Nitrosomonadaceae*. *Deltaproteobacteria* class was dominated by the genera *Haliangium* (4.7% in sample B1), *Nannocystis* (2.1% in C5), *Byssovorax* (1.6% in sample C5), *Sorangium* (1.0% in sample C1) and an unknown genus from *Myxococcales*. *Gammaproteobacteria* was dominated by an unknown genus from *Moraxellaceae* (4.6% in F3), *Dokdonella* (3.4% in L2), *Arenimonas* (3.2% in sample H4) and uncultured member of *Xanthomonadaceae* (1.5% in sample G6). Samples E2 and E3 were dominated by *Flavobacterium* genera. Other abundant genera were *Planctomyces* (2.6% in C2) and *Leptospira* (4.1% in sample B2).

Table 4.4 presents a list of the five most abundant genera in each sample which amounts to 245 places occupied by 31 different genera. *Albidiferax* and the uncultured *Saprospiraceae* bacteria appeared 38 times (15.5%) each, *Dechloromonas* counted 24 times while *Flavobacterium* appeared 22 times. These four bacterial genera accounted for nearly 50% of the dominance in the top five genera abundance in all samples.

4.4.4.2 Diversity measurements

Diversity within a sample or population is known as α -diversity. This intricate concept measures the number of species in a sample (richness) and the population of each species within the community (evenness). Shannon and Simpson indices are two popularly used metrics to measure diversity in a community, but each has its limitations. Simpson index emphasizes on the evenness component of the population while Shannon index, the richness (Nagendra, 2002). Since the focus of this study is to assess the effect of process parameters on diversity, Shannon index was used to emphasize differences in richness in different WWTPs.

$$\text{Shannon Index } (H) = - \sum_{i=1}^s P_i \ln P_i \quad (\text{Equation 5.2})$$

P_i = the number each species as a fraction of the total population

The Shannon indices presented in Table 4.4, where higher value equates to a more diverse population, showed that all the samples had diversity in the range of 4.25 to 5.25. Sample E2 which had the lowest diversity, contained 231 identified species with $\geq 0.1\%$ abundance while sample C2 was the most diverse with 337 taxa. Shannon index quantifies the visible changes in bacterial diversity between WWTPs in Figures 4.7A. The variation between WWTPs was insignificant with p-values ranging from 0.11 to 0.99.

Table 4.3. Shannon index (H) of the bacterial species diversity in each sample

Shannon Index (H)											
A1 (4.96)	B1 (4.92)	C1 (5.02)	D1 (5.00)	E1 (4.93)	F1 (4.76)	G1 (4.53)	H1 (4.88)	J1 (5.08)	K1 (4.96)	L1 (4.98)	M1 (4.94)
A3 (4.86)	B2 (4.94)	C2 (5.25)	D2 (5.00)	E2 (4.25)	F2 (4.96)	G4 (4.73)	H2 (4.64)	J2 (4.92)	K2 (4.73)	L2 (5.06)	M2 (4.99)
A4 (4.80)	B3 (4.92)	C3 (5.05)	D3 (4.79)	E3 (4.33)	F3 (4.78)	G5 (4.84)	H3 (4.58)	J3 (4.44)	K3 (4.98)	L3 (4.95)	M3 (4.84)
A5 (4.94)	B5 (5.06)	C5 (5.09)	D4 (4.83)	E5 (4.73)	F4 (4.73)	G6 (5.12)	H4 (4.57)	J5 (4.73)	K4 (4.69)	L4 (4.96)	M4 (4.94)
G7 (4.62)											

Beta diversity is the diversity between different samples, and it is often compared using distance matrices such as Euclidean, Chi-square, Bray-Curtis or Unifrac (weighted or unweighted). For this study, Bray-Curtis which is calculated as the sum of all the lower abundances in each sample divided by the total count of all species, was used because it does not include any notion of space from which species were counted but rather assumes that sampling area or volume are the same, which was the case in this study. Figure 4.4 displays the clustering of samples by Bray-Curtis distance (top dendrogram) and clustering of the genera by correlations between samples (left dendrogram) for the relative abundance of the top 25 genera. It shows that the uncultured *Saprospiraceae* was abundantly present in all samples as seen in Figure 4.4 and Table 4.3. *Dechloromonas*, *Albidiferax*, *Flavobacterium*, *Zoogloea*, *Nitrospira*, *Terrimonas* and *Hydrogenophaga* are found in most reactors. However, the phosphate accumulating bacteria, *Candidatus Accumulibacter*, is missing from WWTPs A, B, C and H. *Amaricoccus*, *Tetrasphaera*, *Gordonia*, *Leptospira* and *Ornithinibacter* are not prevalent in WWTP C (Figure 4.4) as it did in the others. Overall, two very different types of bacterial communities are present when the reactors are compared, reactors D and M are in one group whilst reactors A, B and K are in another, with reactors C and H sharing some similarity with both groups.

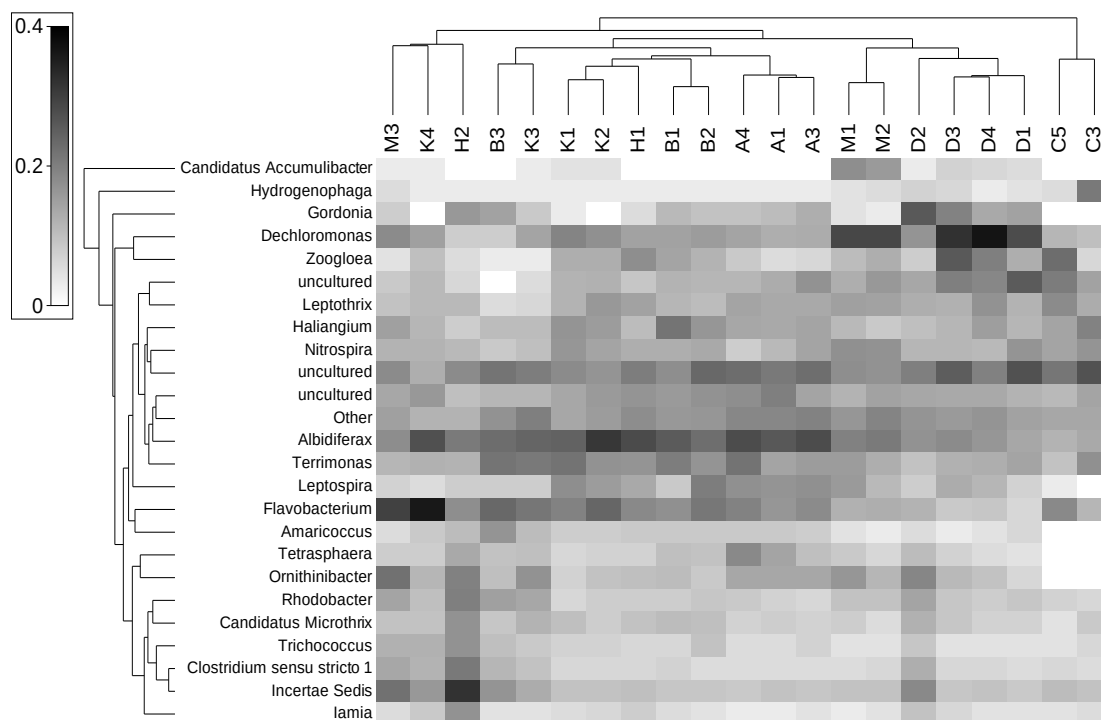


Figure 4.4. The normalised abundance of bacterial genera across the WWTPs displayed as a hierarchically-clustered heatmap, with the grey representing abundance.

Table 4.4. Top five bacteria genera and abundance, microbial diversity from PLFA and the operational conditions of the twelve WWTPs during sampling

	Process parameters				Microbial diversity (PLFA)				Top five dominant genera (16S rRNA) and their abundance (%)				
	DO	T °C	HRT	SRT	Gram +	Gram -	ACT	Fungi	First	Second	Third	Fourth	Fifth
A1	1.64	18.8	9.9	10.1	9.76	60.11	1.55	28.58	<i>Albidiferax</i> (6.8)	(4.4) uncultured Saprospiraceae	uncultured <i>Chitinophagaceae</i> (4.0)	oth. Comamonadaceae (3.5)	<i>Leptospira</i> (2.8)
A2	2.41	18.5	12.0	8.5	8.88	61.45	1.72	27.95	---	---	---	---	---
A3	1.48	12.7	8.0	8.2	6.95	57.66	1.31	34.08	<i>Albidiferax</i> (7.8)	Uncultured Saprospiraceae (5.1)	oth. <i>Comamonadaceae</i> (3.5)	<i>Flavobacterium</i> (3.3)	<i>Leptospira</i> (3.0)
A4	2.22	12.4	11.0	11.8	8.34	58.89	0.90	31.87	<i>Albidiferax</i> (7.8)	uncultured Saprospiraceae (5.2)	<i>Terrimonas</i> (4.8)	<i>Flavobacterium</i> (3.8)	oth. Comamonadaceae (3.5)
A5	0.93	17.5	13.2	10.2	8.19	60.03	1.14	30.64	Uncultured. Saprospiraceae (5.7)	<i>Albidiferax</i> (5.6)	<i>Flavobacterium</i> (3.3)	<i>Tetrasphaera</i> (3.3)	<i>Haliangium</i> (2.8)
B1	1.17	15.2	18.5	7.2	9.58	59.95	1.60	28.88	<i>Albidiferax</i> (6.6)	<i>Haliangium</i> (4.7)	<i>Terrimonas</i> (4.1)	uncultured Saprospiraceae (3.1)	<i>Flavobacterium</i> (3.0)
B2	1.07	13.2	14.5	17.4	9.18	56.41	1.21	33.20	Uncultured Saprospiraceae (5.6)	<i>Albidiferax</i> (5.1)	<i>Flavobacterium</i> (4.5)	<i>Leptospira</i> (4.1)	unc. Chitinophagaceae (2.9)
B3	1.35	12.7	10.5	8.4	7.63	55.63	1.17	35.57	<i>Flavobacterium</i> (5.6)	<i>Albidiferax</i> (5.1)	<i>Terrimonas</i> (4.7)	Unc. Saprospiraceae (4.7)	<i>Amaricoccus</i> (2.8)
B4	1.15	14.0	12.7	17.0	8.68	50.84	1.50	38.97	---	---	---	---	---
B5	1.67	20.3	10.4	6.5	7.74	57.71	1.10	33.46	Uncultured Saprospiraceae (4.0)	<i>Albidiferax</i> (3.8)	oth. Comamonadaceae (3.5)	<i>Terrimonas</i> (3.4)	<i>Nitrospira</i> (3.0)
B6	2.32	19.9	14.6	12.4	7.84	58.14	1.08	32.95	---	---	---	---	---
C1	2.40	19.9	15.8	9.8	12.26	51.22	1.00	35.52	<i>Leptothrix</i> (8.4)	uncultured Saprospiraceae (5.0)	<i>Nitrospira</i> (3.6)	Uncultured Anaerolineaceae (2.8)	oth. Comamonadaceae (2.4)
C2	0.46	19.4	21.3	5.2	11.51	45.61	0.89	41.99	<i>Nitrospira</i> (6.2)	uncultured Saprospiraceae (3.7)	<i>Terrimonas</i> (3.0)	<i>Planctomyces</i> (2.6)	oth. Comamonadaceae (2.4)
C3	0.44	14.8	9.5	8.5	12.04	49.18	1.11	37.68	uncultured Saprospiraceae (7.3)	Hydrogenophaga (4.4)	<i>Haliangium</i> (3.8)	<i>Terrimonas</i> (3.0)	<i>Nitrospira</i> (2.8)
C4	0.6	16.2	7.7	12.2	9.76	57.21	0.64	32.38	---	---	---	---	---
C5	0.81	19	13.9	12.5	11.99	59.96	0.78	27.26	<i>Zoogloea</i> (5.2)	uncultured Saprospiraceae (4.6)	uncultured Anaerolineaceae (4.2)	<i>Flavobacterium</i> (3.4)	<i>Leptothrix</i> (3.3)
D1	0.74	18.2	23.5	18.7	8.14	61.08	1.34	29.43	<i>Dechloromonas</i> (7.8)	uncultured Saprospiraceae (7.4)	Uncultured Anaerolineaceae (6.6)	<i>Nitrospira</i> (2.8)	uncultured Ardentacatenia (2.5)
D2	1.55	15.2	19.5	10.6	9.56	54.03	1.49	34.91	<i>Gordonia</i> (6.7)	uncultured Saprospiraceae (4.0)	<i>Ornithinibacter</i> (3.6)	(3.4) unknown Peptostreptococcaceae	<i>Albidiferax</i> (2.9)
D3	1.73	15.5	14.8	8.1	8.50	59.48	1.32	30.70	<i>Dechloromonas</i> (10.4)	<i>Zoogloea</i> (6.7)	uncultured Saprospiraceae (6.4)	Uncultured Anaerolineaceae (4.0)	<i>Gordonia</i> (3.8)
D4	1.07	16.9	15.5	9.0	8.42	60.74	1.08	29.75	<i>Dechloromonas</i> (13.5)	<i>Zoogloea</i> (4.0)	uncultured Saprospiraceae (3.9)	Uncultured Anaerolineaceae (3.5)	<i>Leptothrix</i> (2.9)

E1	0.46	13.7	20.0	6.0	8.38	57.37	0.95	33.30	<i>Flavobacterium</i> (8.9)	<i>Albidiferax</i> (4.90)	uncultured Saprospiraceae (3.4)	<i>Terrimonas</i> (3.3)	oth. Comamonadaceae (3.2)
E2	1.23	12.1	26.6	12.9	10.71	52.52	1.16	35.62	<i>Flavobacterium</i> (23.7)	<i>Ornithinibacter</i> 6.20	<i>Albidiferax</i> (3.5)	(3.4) unknown Peptostreptococcaceae	uncultured Saprospiraceae (2.6)
E3	2.19	10.6	13.0	13.1	9.87	57.62	1.02	31.48	<i>Flavobacterium</i> (18.1)	<i>Ornithinibacter</i> 9.60	uncultured Saprospiraceae (5.3)	<i>Albidiferax</i> (4.8)	uncultured Chitinophagaceae (2.8)
E4	1.57	15.6	12.8	8.8	10.36	60.60	0.72	28.32	---	---	---	---	---
E5	0.91	18.0	26.2	4.9	9.39	61.41	0.96	28.24	uncultured Saprospiraceae (6.6)	<i>Flavobacterium</i> (5.7)	<i>Albidiferax</i> (5.3)	<i>Dechloromonas</i> (5.1)	<i>Terrimonas</i> (3.5)
F1	1.05	17.4	9.7	12.7	9.07	58.86	1.33	30.74	<i>Dechloromonas</i> (8.5)	<i>Albidiferax</i> (6.5)	<i>Zoogloea</i> (4.3)	<i>Leptospira</i> (3.3)	oth. Comamonadaceae (3.1)
F2	1.05	17.6	10.2	9.9	8.40	57.36	1.40	32.84	<i>Dechloromonas</i> (6.8)	<i>Albidiferax</i> (4.7)	<i>Leptospira</i> (3.9)	<i>Zoogloea</i> (3.1)	uncultured Saprospiraceae (3.0)
F3	1.13	12.0	14.7	19.0	8.80	60.61	0.90	29.69	<i>Flavobacterium</i> (6.3)	<i>Tetrasphaera</i> (6.1)	(4.9) unknown Peptostreptococcaceae	uncultured Moraxellaceae (4.6)	Uncultured Anaerolineaceae (4.4)
F4	2.29	17.6	9.5	12.2	7.77	59.33	0.91	31.98	<i>Dechloromonas</i> (7.7)	<i>Albidiferax</i> (5.7)	<i>Zoogloea</i> (5.2)	uncultured Saprospiraceae (4.5)	<i>Flavobacterium</i> (4.2)
F5	1.85	17.6	8.2	15.4	7.41	59.08	0.84	32.67	---	---	---	---	---
G1	0.81	15.1	15.0	6.3	9.53	64.29	0.88	25.31	<i>Dechloromonas</i> (20.1)	<i>Albidiferax</i> (4.2)	<i>Zoogloea</i> (4.1)	uncultured Saprospiraceae (3.3)	<i>Candidatus Accumulibacter</i> (2.3)
G2	1.25	16.4	15.7	15.5	8.43	66.11	0.63	24.83	---	---	---	---	---
G3	0.60	17.0	14.7	13.4	8.97	65.02	0.72	25.29	---	---	---	---	---
G4	4.27	19.0	15.7	13.5	8.42	66.24	0.60	24.73	<i>Dechloromonas</i> (17.5)	uncultured Saprospiraceae (4.9)	<i>Zoogloea</i> (2.8)	<i>Nitrospira</i> (2.6)	<i>Albidiferax</i> (2.5) <i>Dokdonella</i> (2.5)
G5	4.66	19.4	10.2	17.0	9.31	69.82	0.84	20.04	<i>Dechloromonas</i> (11.8)	<i>Zoogloea</i> (5.4)	Uncultured Anaerolineaceae 5.20	uncultured Saprospiraceae 3.80	<i>Thauera</i> 2.50
G6	7.53	13.3	9.2	13.2	8.32	67.01	0.73	23.94	<i>Dechloromonas</i> (7.5)	<i>Albidiferax</i> (4.5)	oth. Comamonadaceae 3.50	<i>Terrimonas</i> (3.1)	uncultured Saprospiraceae 2.50
G7	4.25	13.3	8.2	17.5	8.25	68.69	0.67	22.39	<i>Dechloromonas</i> 17.8	<i>Albidiferax</i> (4.8)	<i>Zoogloea</i> (4.1)	uncultured Saprospiraceae (3.3)	<i>Ferribacterium</i> (2.5)
H1	1.77	16.4	8.2	8.7	7.59	61.05	1.06	30.30	<i>Albidiferax</i> (7.9)	uncultured Saprospiraceae (4.1)	<i>Flavobacterium</i> (3.4)	oth. Comamonadaceae (3.2)	<i>Zoogloea</i> (3.1)
H2	6.53	10.7	7.9	10.2	8.69	57.77	0.86	32.68	(10.3) unknown Peptostreptococcaceae	<i>Clostridium sensu stricto</i> 1 (4.4)	<i>Albidiferax</i> (4.3)	<i>Rhodobacter</i> (4.0)	<i>Ornithinibacter</i> (3.9)
H3	1.08	16.1	8.1	14.9	8.68	67.48	0.90	22.94	<i>Zoogloea</i> (10.0)	<i>Albidiferax</i> (6.0)	<i>Dechloromonas</i> (5.6)	uncultured Saprospiraceae (4.3)	<i>Leptothrix</i> (2.5)
H4	1.01	17.6	8.1	8.7	9.46	65.82	0.90	23.82	<i>Zoogloea</i> (11.3)	<i>Terrimonas</i> (5.8)	<i>Albidiferax</i> (5.7)	<i>Dechloromonas</i> (4.8)	<i>Arenimonas</i> (2.5)
H5	2.60	17.7	13.2	11.7	9.26	65.64	0.76	24.34	---	---	---	---	uncultured Saprospiraceae (4.0)

J1	1.23	18.2	14.0	11.2	7.91	56.11	1.77	34.22	<i>Albidiferax</i> (6.1)	uncultured Saprospiraceae (3.3)	oth. Comamonadaceae (3.1)	<i>Gordonia</i> (2.7)	<i>Leptothrix</i> (2.6)
J2	5.1	15.6	18.1	8.5	7.23	57.74	1.68	33.35	<i>Albidiferax</i> (9.1)	Haliangian (3.4)	oth. Comamonadaceae (3.2)	<i>Terrimonas</i> (3.1) & uncultured Chitinophagaceae (3.1)	<i>Nitrospira</i> (2.6) <i>Leptothrix</i> (2.6) Uncultured Moraxellaceae 2.60
J3	0.91	10.9	16.3	6.9	7.55	58.63	0.68	33.15	<i>Albidiferax</i> (11.1)	<i>Flavobacterium</i> (8.7)	uncultured Chitinophagaceae (4.4)	<i>Terrimonas</i> (3.8)	<i>Tetrasphaera</i> 3.70
J4	0.75	11.4	16.2	7.5	9.17	55.80	0.85	34.18	---	---	---	---	---
J5	1.99	14.3	10.0	6.3	11.08	56.81	2.07	30.25	<i>Albidiferax</i> (8.3)	<i>Flavobacterium</i> (6.4)	uncultured Chitinophagaceae (5.7)	<i>Leptospira</i> (4.0)	<i>Tetrasphaera</i> (3.0)
K1	1.54	17.1	17.6	16.5	8.41	58.53	1.43	31.63	<i>Albidiferax</i> (6.0)	<i>Terrimonas</i> (4.8)	<i>Flavobacterium</i> (3.8)	<i>Dechloromonas</i> (3.7)	uncultured Saprospiraceae (3.3)
K2	1.86	13	18.1	20.9	8.39	63.85	0.67	27.09	<i>Albidiferax</i> (9.8)	<i>Flavobacterium</i> (5.7)	<i>Dechloromonas</i> (3.0)	<i>Terrimonas</i> (2.9)	uncultured Saprospiraceae (2.8)
K3	1.54	11.1	18.2	14.6	9.53	56.42	1.12	32.93	<i>Albidiferax</i> (5.8)	<i>Flavobacterium</i> (4.6)	<i>Terrimonas</i> (4.4)	uncultured Saprospiraceae (4.1)	oth. Comamonadaceae (4.0)
K4	1.69	12.2	18.4	13.5	8.48	61.02	0.81	29.69	<i>Flavobacterium</i> (13.0)	<i>Albidiferax</i> (7.5)	uncultured Chitinophagaceae (2.6)	<i>Sphingobium</i> (2.4)	<i>Dechloromonas</i> (2.2)
K5	1.62	13.2	15.9	22.3	9.14	58.54	1.00	31.32	---	---	---	---	---
L1	2.3	18.7	15.4	13.0	8.79	61.75	1.16	28.29	<i>Dechloromonas</i> (7.8)	Uncultured Saprospiraceae (4.9)	<i>Roseiflexus</i> (3.8)	oth. Comamonadaceae (3.2)	<i>Dokdonella</i> (3.0)
L2	1.57	16.5	14.8	8.9	8.09	61.97	1.15	28.79	oth. Comamonadaceae (6.0)	<i>Dechloromonas</i> (4.3)	<i>Albidiferax</i> (4.0)	<i>Dokdonella</i> (3.4)	<i>Rhodobacter</i> (2.4)
L3	6.16	10.9	7.6	7.5	7.73	57.76	1.13	33.37	<i>Ornithinibacter</i> (5.2)	<i>Flavobacterium</i> (4.6)	<i>Dechloromonas</i> (4.3)	<i>Rhodobacter</i> (3.4)	<i>Albidiferax</i> (3.3)
L4	1.56	15.2	8.1	6.0	8.88	58.44	1.15	31.52	<i>Dechloromonas</i> (9.0)	uncultured Saprospiraceae (5.6)	<i>Albidiferax</i> (5.3)	oth. Comamonadaceae (3.7)	<i>Leptospira</i> (2.8)
L5	1.40	17.6	6.2	11.1	9.01	58.11	1.14	31.74	---	---	---	---	---
M1	0.74	18.4	11.2	11.9	10.17	50.44	1.85	37.55	<i>Dechloromonas</i> (8.3)	<i>Albidiferax</i> (3.9)	<i>Candidatus Accumulibacter</i> (3.1)	<i>Nitrospira</i> (3.0)	<i>Ornithinibacter</i> (2.7)
M2	1.34	17.3	10.6	11.0	8.24	62.15	0.96	28.64	<i>Dechloromonas</i> (8.3)	<i>Albidiferax</i> (4.3)	oth. Comamonadaceae (3.7)	<i>Nitrospira</i> (2.9) uncultured Saprospiraceae (2.9)	uncultured <i>Anaerolineaceae</i> (2.6)
M3	1.08	11.5	12.9	10.1	7.28	57.77	0.93	34.02	<i>Flavobacterium</i> (8.8)	<i>Ornithinibacter</i> (4.9) unknown (4.9) Peptostreptococcaceae	<i>Dechloromonas</i> (3.3) uncultured Saprospiraceae (3.3)	<i>Albidiferax</i> (3.2)	<i>Haliangium</i> (2.2) oth. Comamonadaceae (2.2)
M4	1.05	18.1	9.2	10.8	7.30	56.77	0.89	35.04	<i>Dechloromonas</i> (8.6)	<i>Albidiferax</i> (4.5)	oth. Comamonadaceae (3.6)	<i>Leptospira</i> (2.8) <i>Nitrospira</i> (2.8)	uncultured Saprospiraceae (2.7)
M5	1.2	18.2	7.2	11.1	8.01	57.04	0.98	33.97	---	---	---	---	---

oth = other

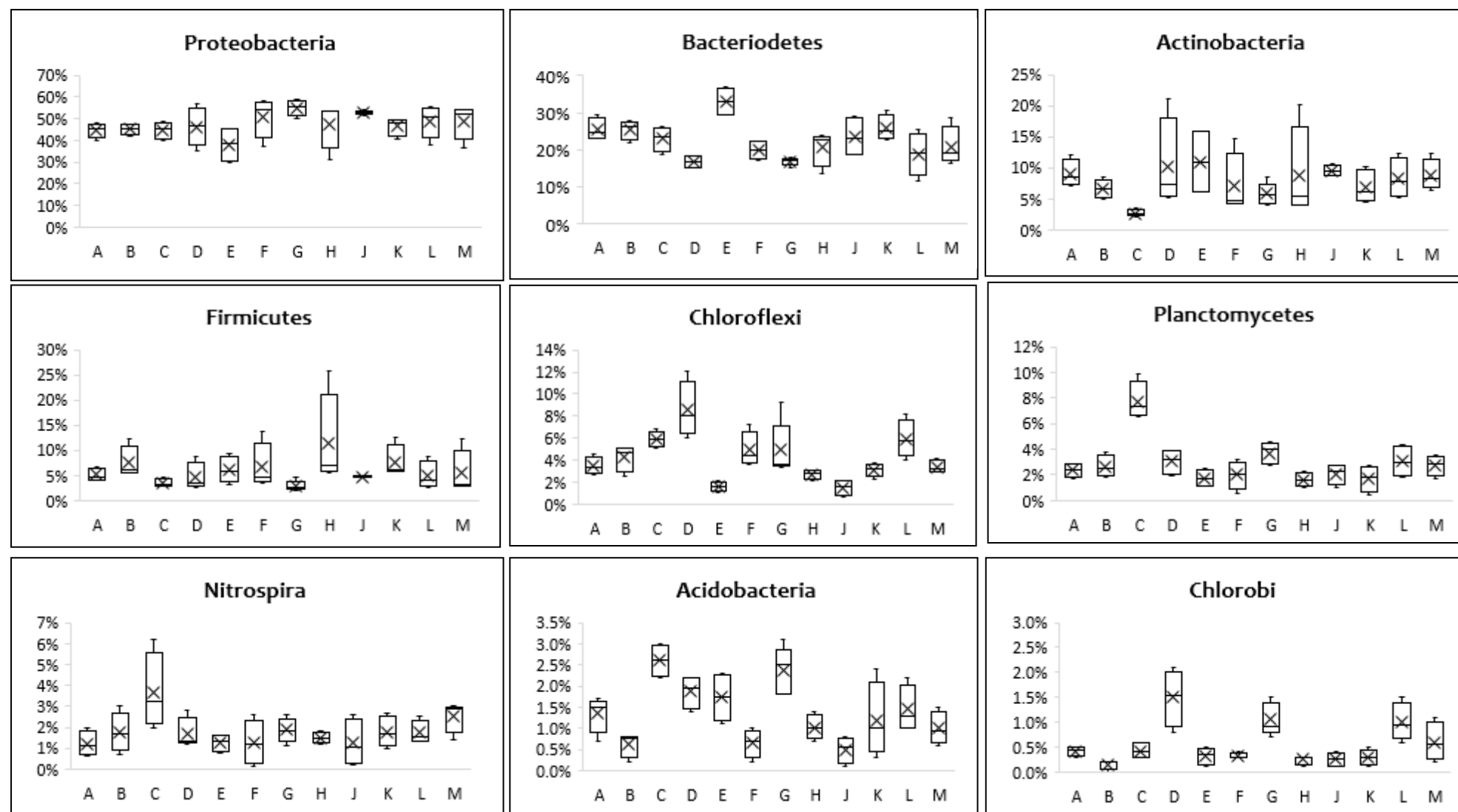


Figure 4.5. Boxplots of the nine most abundant bacterial phyla from 12 full-scale ASPs, the mean abundance is marked (x) in each boxplot (starting from the top, it shows the maximum, upper quartile, median, lower quartiles and minimum).

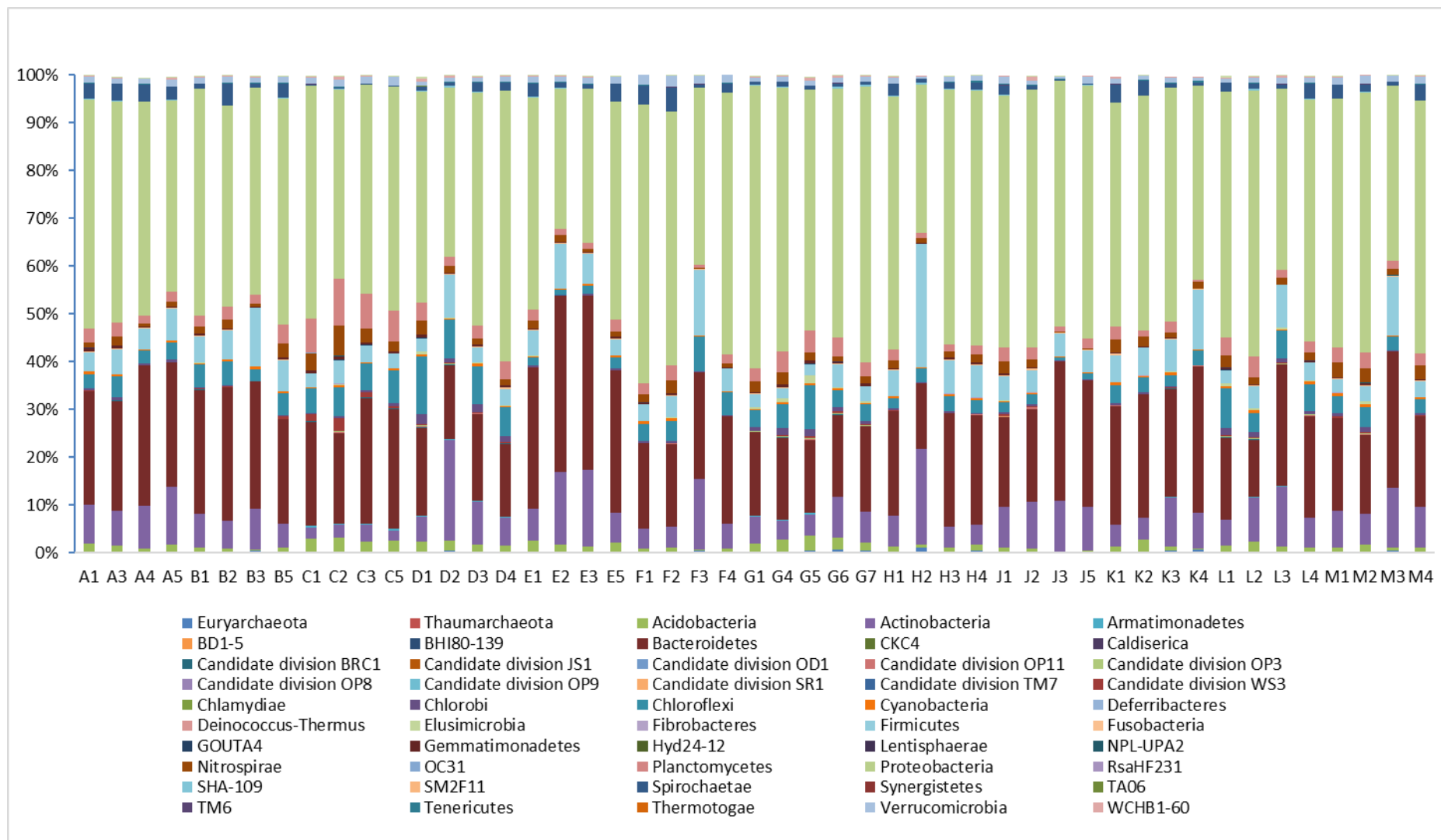


Figure 4.6. Bacterial and archaeal phyla diversity and abundance from twelve full-scale ASPs.

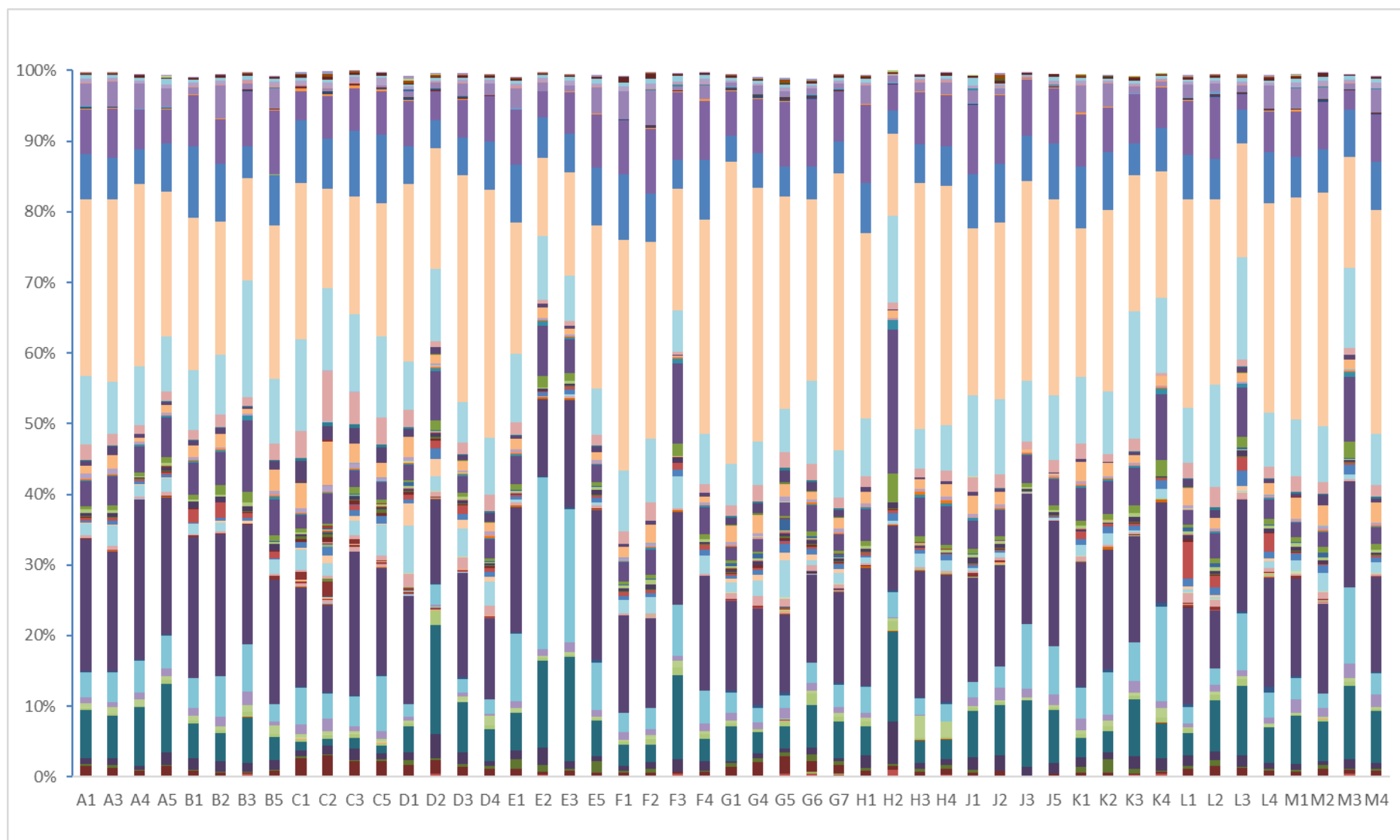


Figure 4.7A. Bacterial and archaeal class diversity and abundance from twelve full-scale ASPs.



Figure 4.7B. Legend for the class level bacterial and archaeal diversity and abundance from twelve full-scale ASPs

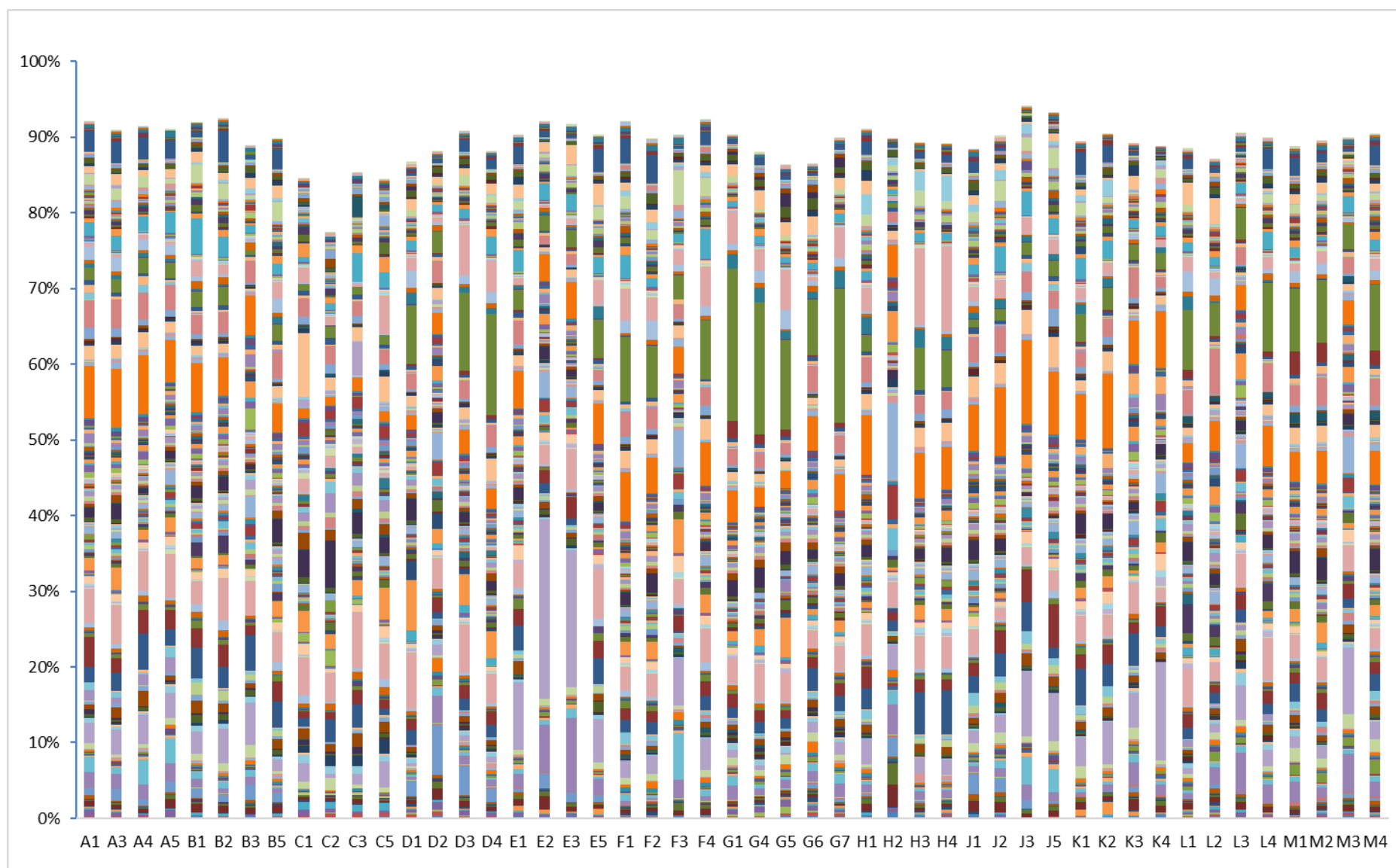


Figure 4.8A. Bacterial and archaeal genera diversity and abundance ($\geq 0.1\%$) from twelve ASPs.

4.4.5 Influence of process parameters on bacterial diversity

The phylum level bacterial diversity arising from 16S rRNA analysis showed less distinct groupings (Figure 4.9A) as to the effect of SRT, HRT, DO and temperature. Although the distribution of sampling events did not show strong grouping as the kind seen from the PCA of microbial diversity from the PLFA analysis, the loading plot (Figure 4.9B) shows that SRT and HRT are influential to the bacterial community structure but not in every WWTP. Temperature was the most influential parameter in determining bacterial diversity. The high abundances of *Verrucomicrobia*, *Planctomycetes*, *Proteobacteria* and Nitrospirae and the low abundances of *Firmicutes* and *Actinobacteria* aligned with warmer temperatures (Figure 4.9B).

Comparison at genus level (Table 4.4) reveals that, irrespective of location, samples from warmer seasons (with in-situ mixed liquor temperatures ≥ 14 °C) were dominated by either *Dechloromonas* or *Albidiferax* both from the *Betaproteobacteria*; except in C2 and D2 which were dominated by *Nitrospira* and *Gordonia* from *Nitrospirae* and *Actinobacteria* respectively. These discrepancies may be explained by the observations of foaming/bulking in the mixed liquor at WWTP D when sample D2 was taken, and the Kaldness reactor preceding treatment at WWTP C. Eight out of the ten sampling events dominated by a non-*Proteobacteria* species were taken in cold weather with temperature <14 °C. The influence of temperature on microbial diversity is further displayed in site E where all three winter samples were dominated by *Flavobacterium* species from phylum *Bacteroidetes* and the summer sample by *Albidiferax* from *Proteobacteria*. Also, the dominance of an unknown affiliation from *Firmicutes* in F3 and H2 shows by comparison of their process parameters, that temperature is the only common denominator according to Table 4.4. This observation is consistent with samples taken at temperatures below 13 °C. The proportion of *Firmicutes* decreased as temperature increased ($R^2=0.3153$).

A Distance-based Redundancy Analysis (dbRDA) was performed using S17 Bray-Curtis Resemblance matrix with the inclusion of MLSS because it is a direct measurement of the treatment process, unlike the calculated SRT which did not show any influence in Figure 4.9. The dbRDA determined that two out of the five parameters measured, temperature ($p<0.001$) and MLSS ($p<0.001$), were important drivers of the bacterial community structure,



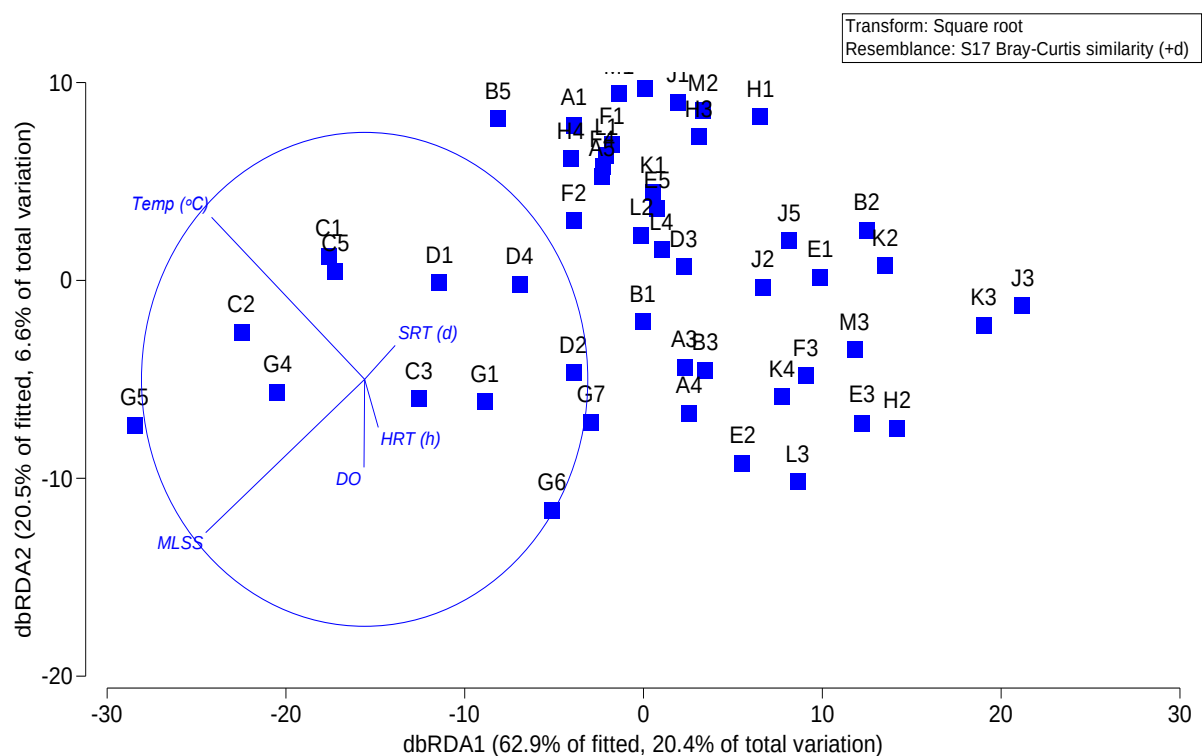


Figure 4.10. Distance-based Redundancy Analysis showing the influence of temperature, MLSS, SRT, HRT and dissolved oxygen (DO) levels on the bacterial community structure in twelve activated sludge plants; $n = 49$; p -values < 0.001 for temperature and MLSS.

4.5 DISCUSSION

The microbial diversity from phospholipids fatty acid analysis where the absolute dominance of Gram-negative bacteria was observed has similarities with Amir et al (2010) in sludge, Yi et al., (2012) in activated sludge. The PLFA profile was effective in identifying shifts in microbial diversity as process parameters changed, although these changes were subtle. Frostegard et al., (2011) cautioned against the use of fatty acids as indicative of different microbial groups because it is not as straightforward, recommend that comparison to be based on whole fatty acid profiles to identify changes as a result of environmental stresses. PLFA was good at identifying a microbial community that is different from the rest, that is, the PLFA profile of WWTP C was different from the other WWTPs. The added benefit of using PLFA is that it is fast and affordable, making it plausible for use in the wastewater industry for routine monitoring of microbial health in ASP reactors also suggested Werker et al., (2003). Recently, Veum et al., (2019) proved that fatty acid profiles from PLFA are influenced by sampling handling and storage condition. The length of storage time may be a drawback of this study, the sample preparation from wet mixed liquor to freeze-dried sludge was short and identical, but the storage time prior to PLFA analysis varied from one to three months. This length of storage time for some sample could have impacted on the fatty acid composition of some samples.

The bacteria diversity across the 12 WWTPs was closely related, irrespective of time, location or size of the WWTP. The abundance of *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, *Chlorobi*, *Chloroflexi*, *Nitrospirae* and *Firmicutes* is in the typical range often report in ASPs (Xu et al., 2018; Zhang et al., 2018). At the class taxonomic level, the dominance of *Betaproteobacteria* in most reactors agrees with findings of Bagchi et al., (2015) and Jiang et al., (2008). *Sphingobacteriia*, *Actinobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Flavobacteria*, *Anaerolineae*, *Planctomycetacia*, *Clostridia*, *Bacilli* and *Spirochaetes* (Zhang et al., 2011) also followed closely. Bacterial diversity studied using Illumina MiSeq (Zhang et al., 2018; Xu et al., 2018) or through 454 pyrosequencing (Zhang et al., (2011) have all found the prominent genera in different ASPs to be *Albidiferax*, *Dechloromonas*, uncultured *Saprospiraceae* and uncultured *Comamonadaceae*, *Zoogloea*, *Flavobacterium*, *Terrimonas*, *Tetraspharae*, *Leptospira*, *Nitrospira* and uncultured *Chitinophagaceae*, as found in this research.

Comparison of the bacterial diversity in the full and pilot-scale reactors (appended Figures AF20 and AF21) showed that the two communities are different. The 10 d and 16 h pilot plant samples had some similarities to the full-scale samples. This dissimilarity is due to differences in influent chemical composition and process parameters. The pilot plant's influent comes from residential accommodation, office and an airfield with P.E of ~3000. The full-scale WWTP's P.E varied in P.E (25,000 to 1.75 million) and sources of influent.

All the process parameters, temperature, SRT, HRT and DO, had an impact on microbial/bacterial diversity (Xu et al., 2018) but to different extents and in some or all WWTPs. The influence of temperature on microbial diversity (PLFA analysis) and bacterial diversity (DNA analysis) was found in all WWTPs. A worldwide study of bacterial communities in ASPs by Wu et al, (2019) also found temperature as a common driver of diversity. At high temperatures, the reactor is dominated by *Albidiferax*, *Dechloromonas* and *Zoogloea* but at lower temperatures (≤ 14 °C), the abundance of *Bacteroidetes*, *Firmicutes* and *Actinobacteria* increased. This led to the dominance of *Flavobacterium*, unknown *Peptostreptococcaceae*, (belongs to *Clostridia* in *Firmicutes*) and *Ornithinibacter*, also reflecting published work (Zhang et al., 2012). Temperature can influence bacterial growth rate, biodegradation rates (whether internally by bacteria or extracellular polymerase substances) and the solubility of chemicals. These biological, chemical and physical impacts of temperature make it a dominant factor in WWTP. The growth of filamentous bacteria is enhanced at lower temperatures and hence floc morphology has been found to correlate with temperature (Koivuranta et al., 2017). DO concentration did not show any impact on bacterial community structure (16S rRNA) but did impact on microbial diversity (PLFA). Nui et al., (2016) states that high DO concentrations were synonymous with lower microbial diversity, higher extracellular polymerase substance and high sludge production. Under high aeration, a higher abundance of *Firmicutes* was seen. This observation is at odds with existing knowledge that *Firmicutes* are weak and their abundance diminishes under high shear force (Larsen et al., 2008). Further studies may be required to substantiate this as it was only observed in one sample, and other confounding factors such as temperature may have contributed. The sequence of processes in treatment was also observed to influence bacterial diversity. WWTP C had a distinct community from the other WWTPs whether viewed through PLFA analysis or 16S rRNA analysis.

This distinction can be attributed to the pre-treatment MBBR (Kaldness) reactor before the primary settling tank. The Kaldness reactor removes most of the BOD and COD in the raw sewage resulting in low organic loading in the influent, low food-to-microorganisms ratio and consequently higher diversity as indicated in the Shannon index calculation, however insignificant, and an increase in the abundances of *Nitrospirae*, *Planctomycetes* and *Acidobacteria* (Li et al., (2008). The high abundance of autotrophic bacteria like *Planctomycetes* and *Acidobacteria* in WWTP C with pre-treat Kaldness reactor may be because slow-growing bacteria capable of generating energy for the synthesis of cell carbon may be promoted in the low-organic influents or at colder temperatures as the conditions inhibit the proliferation of fast-growing heterotrophs. The of dominance of *Nitrospira* in only one sample or the lack of *Nitrosomonas* dominance in any reactor, although each reactor was nitrifying at the time of sampling demonstrates that, the conversion of ammonia to nitrite to nitrate is a niche function performed by a small population of AOBs and NOBs in a delicate balance (Yao and Peng, 2017; Knapp and Graham, 2007). The average abundance of the dominant AOB and NOB genera were *Nitrosomonas* 0.39% and *Nitrospira* 1.78% respectively, which are low in comparison with the mean abundances of *Albidiferax* (4.92%) and *Dechloromonas* (5.12%). The *Nitrosomonas* and *Nitrospira* average abundances are also lower than abundances in other studies (Yao and Peng, 2017). The low abundance is due the low temperatures of the reactors in this research (10.6 to 20.3 °C) as opposed to the 20 – 25 °C in Yao and Peng (2017), because complete growth of nitrifying bacteria occur between 20 and 30 °C (Hao et al, 2002; Yao and Peng; 2017).

There was no observable effect of population equivalent on bacterial diversity, but van der Gast et al., (2006) found that larger reactors have more diverse taxa in membrane bioreactors. This uniformity in bacterial diversity of small and large WWTPs is because the construction of activated sludge lanes has similar dimensions across most WWTPs, with large plants having more lanes to accommodate the high volume of influent received; hence population equivalent of the WWTP did not influence bacterial diversity. The MLVSS which is a confounding factor for SRT was not measured or controlled because the focus was on the day-to-day process parameters related to the bacterial diversity in the reactor, and not the intrinsic meaning of the parameters. Likewise, organic loading rates is a confounding factor of HRT but was not measured for the same reason.

This research did not assess the effect of other known factors on bacterial diversity, because parameters such as elevation (Nui et al., 2015, Wu et al, 2019), pH and conductivity (salinity) of the influent (Wang et al, 2012) are not manipulatable to increase hazardous chemical removal in existing WWTPs (to be discussed later). A drawback of this research is the lack of control over the sampling timetable to ensure WWTPs are visited periodically as opposed to the sporadic manner dictated by the chemical analysis sampling timetable. This study has shown for the first time that PLFA analysis can be used in assessing the effects of SRT, DO, HRT and temperature on microbial diversity in full-scale ASPs in a way that may be useful in the wastewater industry.

4.6 CONCLUSION

Microbial diversity in ASPs comprises 59% Gram-negative bacteria, 9% Gram-positive bacteria, 1% actinomycetes and 31% fungi, whereas bacterial diversity was about 8% *Actinobacteria*, 22% *Bacteroidetes*, 4% *Chloroflexi*, 6% *Firmicutes*, 3% *Planctomycetes* and 48% *Proteobacteria*. The dominant genera averaged at 4.92% *Albidiferax*, 5.12 % *Dechloromonas*, 4% uncultured *Saprospiraceae*, 3.98% *Flavobacterium*, 2.28% *Zoogloea*, 2.42% *Terrimonas*, 2.03% uncultured *Chitinophagaceae* and 2.67% unknown Comamonadaceae. The microbial and bacterial diversities are influenced by WWTP configuration such as the inclusion of a pre-treatment as well as process parameters mainly SRT and temperature. Cold temperatures (<13 °C) increases the abundance of *Firmicutes* and *Bacteroidetes* and subsequently reduces the abundance of *Proteobacteria*. Occasionally, there were significant changes in SRT and HRT over a short period, but the microbial community structure remained relatively stable, with slight variations in abundances. Thus, depicting the operational resilience of the activated process to influent loading fluctuations. Both phospholipids fatty acid analysis and 16S rRNA analysis revealed that changes occurred in microbial diversity when the process parameters changed. The sensitivity of PLFA at detecting subtle changes in the microbial communities was observable but difficult to quantify its significance. This method can be used for routine microbial health in WWTP reactor.

CHAPTER FIVE

INFLUENCE OF BACTERIAL DIVERSITY ON REMOVAL OF HAZARDOUS CHEMICALS FROM WASTEWATER IN FULL-SCALE ACTIVATED SLUDGE PLANT

5. INFLUENCE OF BACTERIAL DIVERSITY ON REMOVAL OF HAZARDOUS CHEMICALS FROM WASTEWATER IN FULL-SCALE ACTIVATED SLUDGE PLANT

ABSTRACT

The removal of hazardous chemicals (HCs) from wastewater in activated sludge plants (ASPs) is influenced by the physicochemical characteristics ($\log K_{ow}$, $\log K_{oc}$, solubility) of the HCs and the process parameters (sludge age, HRT, DO and temperature) of the plant. The microbial diversity in a WWTP is influenced by the operational conditions of the plant as well as influent chemicals. This study explores the relationship the microbial diversity in the mixed liquor of the municipal ASP and the removal of HCs. We further explored the correlations between bacterial genera abundance and the removal of individual chemicals, although chemicals removal is not exclusive to biodegradation.

The results showed that several bacterial genera correlate positive and negatively with the removal of different HCs. *Candidatus Accumulibacter* (a phosphate accumulating bacteria capable of denitrification) and *Nitrosomonas* correlated positively with the removal of trixylenyl phosphate at $R > 0.7$. *Amaricoccus*, *BD1-7 clade* and *Subdoligranulum* correlated negatively with the removal of atorvastatin (including ortho and para) and the antidepressant sertraline. *Crocinitomix*, *Dongia*, *Cloacibacterium*, *Byssovorax* and an uncultured Gammaproteobacterium positively correlated with the removal of tamoxifen. *Crocinitomix* and *Anaeromyxobacter* also correlated positively with the removal of the antibiotic norerythromycin. Overall microbial diversity of the WWTP did not show a link to its performance in chemical removal, and neither did the physicochemical characteristics of chemical groups showed a bearing on the removal. Instead, chemicals with high influent concentrations were often removed efficiently ($> 70\%$) which reinforce the knowledge of chemical removal in WWTPs following a pseudo first-order degradation rate equation. As changes in process parameters result in shifts in microbial diversity but the diversity variations did not influence the performance of the ASP, it can be concluded that the activated sludge process is robust in its effectiveness in removing HCs despite changes in influent chemical concentrations, seasonal temperature fluctuations, SRT and HRT differences and the subsequent variations in microbial diversity; and it can remove most HCs at efficiencies above 70%. The novel correlations found will provided the basis for future research in microbial taxa correlations with degradation of chemicals.

5.1 INTRODUCTION

The removal of chemicals from wastewater is a multi-dimensional process. It can be sorbed to sludge, degraded by extracellular polymerase enzymes or microorganisms (Jesic et al., 2011), volatilised (Verlicchi et al., 2012) or photo-transformed. The pathway by which each chemical is removed hinges on their physicochemical characteristics (Ternes et al., 2004), the operational condition (Petrie et al., 2014), WWTP configuration (Harris et al., 2012) and influent flow patterns (Coello-Garcia, 2019). For example, the measured biodegradability (K_{biol}) for diclofenac under anoxic conditions was 0.04 L/g/ss/d (<0.1 L/g/ss/d) which indicates that it is non-biodegradable (Joss et al., 2006; Suarez et al., 2012), but when analysed in aerobic nitrifying conditions, the K_{biol} was 1.21 L/g/ss/d ($1 < K_{biol} < 5$) which suggest that it is highly biodegradable (Suarez et al., 2008). Further to this, the coefficients of organic-carbon distribution ($\log K_{oc}$) and octanol-water distribution ($\log K_{ow}$) may be used to predict whether a micropollutant may sorb to sludge or remain in solution, both of which impact on its removal rate and pathway. Chemicals that are highly sorbed to sludge; either by the absorptive interaction between the hydrophobic straight or cyclic chains of the compound and the phospholipids in the microbial cell membrane or by the adsorptive electrostatic interaction between the oppositely charged surfaces of the chemical and the microorganism; are likely to be influenced by solids retention time (SRT). Because increasing the mixed liquor suspended solids (MLSS) to extend SRT as a result of wasting less sludge will lead to more microbes/solid retained in the reactor.

Operational conditions such as SRT, HRT, DO concentration and temperature also influence the removal of micropollutants. Extending SRT improved chemical removal (Petrie et al., 2014; Roh and Chu, 2011; Kim et al., 2005) and has been suggested to be due to increases in the proportion of bacteria capable of reproducing in the reactor (Vieno and Sillanpää, 2014), and allowing the 'slow-growing' bacteria that are capable of degrading micropollutants to be retained and enriched (McAdam et al., 2010). For example, Simpson diversity index showed increases in diversity and estrogen removals with longer SRT as seen in chapter three and Petrie et al., (2014) respectively, although there may be other confounding factors such as temperature and HRT.

Furthermore, removal of the non-volatile and recalcitrant ethynylestradiol (EE2) is further enhanced with extended SRT and is mostly removed by sorption to sludge (Petrie et al., 2014). However, by increasing the SRT, the bacterial population capable of EE2 degradation increases, and sorption is no longer the only mechanism of removal of EE2, but also by biodegradation (Lust et al., 2015). Also, Santos et al. (2010) show that the removal of heavy metals (Cu and Zn) is enhanced as the SRT is increased, because most metals remain insoluble as the chelators in the settled sewage are degraded. Thus, a high proportion of the heavy metals are removed through sorption to sludge.

Chemicals that have the lowest tendency to undergo sorption to sludge and are highly biodegradable are the most impacted by increasing hydraulic retention time (HRT) (Gros et al., 2010); in fact, chemical with half-life shorter or equal to the HRT are most likely to be removed in the treatment process (Verlicchi et al., 2012). Although it may not always be that long HRT equates to better removal for all chemicals, it understandably so as explained by the half-life theory. Hence, increasing HRT from 8 to 24 h improves the removal of estrogens from 90% to 96% (Petrie et al., 2014), but the change in the removal of antibiotic tetracycline is insignificant when HRT is increased from 7.4 to 24 h (Kim et al., 2005). HRT also influences the order of decay rate of a chemical in the influent. Dharmadasa et al., (2018) revealed that at shorter HRTs the decay rate is first-order and the degradation is independent of microbial cell count but at longer HRTs the decay rate was zero-order and depends on the microbial cell numbers. Furthermore, HRT dictates the loading rate of the chemicals in the influent and therefore impacts on the food-to-micro-organisms ratio which results in organisms working hard for the limited resources (Coello-Garcia, 2019).

Temperature is another influential factor in chemical removal and microbial diversity in WWTPs. Although it may be operationally challenging to alter the temperature in a full-scale plant, it does increase seasonally and in so doing improve the removal of hazardous chemicals. For example, a 6 °C reduction in temperature resulted in a 20% decrease in estrone-1,3-sulfate (E1-3S) degradation (Thompson et al., 2011; Koh et al., 2009). This reduction in temperature could significantly lower microbial growth and inhibit enzymatic activities which will halt the biodegradation of E1-3S, or change the physicochemical characteristics (i.e. solubility, $\log K_{oc}$, $\log K_{ow}$, vapour pressure) of the E1-3S which will alter its interaction with the sludge or microbial cells.

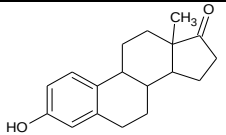
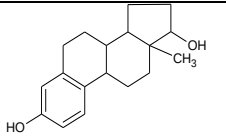
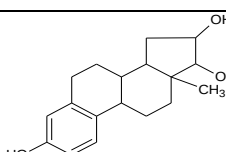
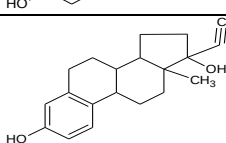
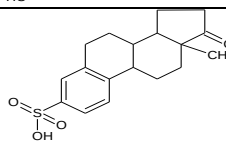
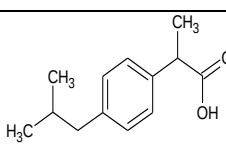
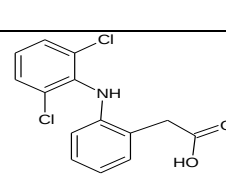
All these process parameters (SRT, HRT, DO concentration and temperature) also give rise to differences in microbial diversity in the reactor (Harris et al., 2012) and have been discussed in the introduction of chapter 4.

Missing from the literature is the link between microbial/bacterial abundance (diversity) and the removal of chemicals in WWTPs. In this study, the correlations between percentage removal of hazardous chemicals (estrogens, pharmaceuticals, flame retardants, corrosion inhibitors) and the abundance of bacterial genera are established. The study also discusses the influences of process parameters on chemical removal. From seven ASPs, a comprehensive data consisting of α -diversity, influent and effluent chemical concentrations and operational parameters was collected from twenty-one sampling events and analysed. The findings will provide valuable information for the discussion on improving existing ASPs to optimise removal of hazardous chemicals and minimise the addition of tertiary treatment processes, where possible. It will also provide the foundation for future research on bacterial taxa correlations with degradation of chemicals in ASPs.

5.2 PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE HAZARDOUS CHEMICALS

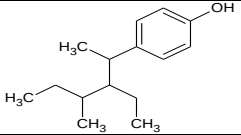
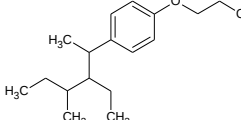
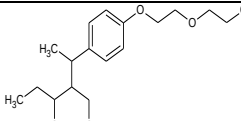
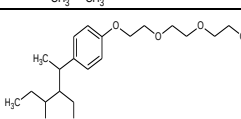
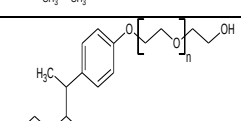
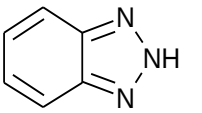
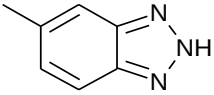
The hazardous chemicals studied were chosen from the list of priority substances to encompass differences in a range of uses and physicochemical properties such as hydrophobicity, degradability, solubility, etc. Tables 5.1 to 5.5 displays the names of the HCs, their code as used in this study (in parenthesis), chemical structure and physical characteristics such as solubility, molecular weight (MW) and vapour pressure (VP), octanol-water coefficient ($\log K_{ow}$), octanol carbon coefficient ($\log K_{oc}$) and the dissociation constants (pKa) of the chemicals.

Table 5.1. List of the hazardous chemicals and their physical and chemical characteristics, half-life in humans, uses and their mode of action

Micropollutant	Chemical structure	Molecular formula	MW (g/mol)	Solubility (mg/L)	Log K _{ow}	Log K _{oc}	pKa	VP (mmHg)	Use	Half-life in mammals	Mode of action
Estrone (E1)		C ₁₈ H ₂₂ O ₂	270.37	30	3.13	3.02	10.77	2.49 x 10 ⁻¹⁰	Hormone replacement	19 h (Human)	Binds to estrogen receptors
17β-estradiol (E2)		C ₁₈ H ₂₄ O ₂	272.39	3.6	3.94 – 4.01	3.97	10.71	6.38 x 10 ⁻⁹	Hormone replacement therapy and contraceptives	13 – 20 h (Human)	Estrogen receptor agonist
Estriol (E3)		C ₁₈ H ₂₄ O ₃	288.38	441	2.45 – 2.81	1.62 – 3.08	-	9.93 x 10 ⁻¹²	Hormone replacement therapy for menopausal symptoms	5 h (Human)	Estrogen receptor agonist
17α-ethinyl estradiol (EE2)		C ₂₀ H ₂₄ O ₂	296.40	11.3	3.67 – 4.15	2.71 – 4.65	10.40	1.95 x 10 ⁻⁹	Oral contraceptive	7 – 36 h (Human)	Estrogen receptor agonist
Estrone 1-3 sulfate (E1-3S)		C ₁₈ H ₂₂ O ₄ S	350.43	960	0.95	1.81	-	-	Steady source of estrone in hormone therapy	12 h (Human)	Estrogen receptor agonist
Ibuprofen (IBPF)		C ₁₃ H ₁₈ O ₂	206.29	21	3.97	2.35	4.91	4.74x10 ⁻⁵	Non-steroidal anti-inflammatory drug (NSAID)	2 h (Human)	Decreases cyclooxygenase
Diclofenac (DCF)		C ₁₄ H ₁₁ Cl ₂ NO ₂	296.15	2.37	0.7 – 4.51	4.20	4.15	2.18x10 ⁻¹²	NSAID with antipyretic and analgesic activity	1.2 – 2 h (Human)	Inhibition of prostaglandin synthesis by inhibition of cyclooxygenase

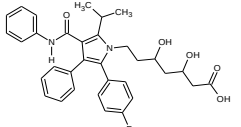
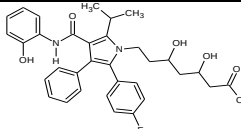
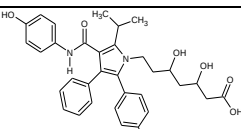
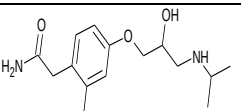
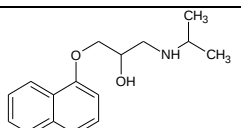
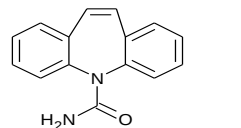
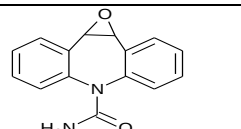
Sources: Astrazeneca Environmental Risk Assessment Data Sheets, SigmaAldrich Materials Safety Data Sheets, Cayman Chemicals Safety Data Sheets

Table 5.2. List of the hazardous chemicals and their physical and chemical characteristics, half-life in humans, uses and their mode of action

Micropollutant (CODE)	Chemical structure	Molecular formula	MW (g/mol)	Solubility (mg/L)	Log K _{ow}	Log K _{oc}	pKa	Vapour Pressure (mmHg @ 25°C)	Use	Half-life in mammals	Mode of action
Nonylphenol (NP)		C ₁₅ H ₂₄ O	220.35	7.6	5.77	4.28	10.28	4.39x10 ⁻⁵ (0.109 Pa)	Non-ionic surfactant	2 – 3 h (Human)	Allows water and oil to mix due to the presence of hydrophilic and hydrophobic ends
Nonylphenol monoethoxylate (NP ₁ EO)		C ₁₇ H ₂₈ O ₂	264.40	0.5 – 1.8	5.03 – 5.58	4.28	-	2.38x10 ⁻⁸ – 5.24x10 ⁻¹¹	Non-ionic surfactant	-	Allows water and oil to mix due to the presence of hydrophilic and hydrophobic ends
Nonylphenol diethoxylate (NP ₂ EO)		C ₁₉ H ₃₂ O ₃	308.46	0.5 – 1.8	5.03 – 5.58	4.28	-	2.38x10 ⁻⁸ – 5.24x10 ⁻¹¹	Non-ionic surfactant	-	Allows water and oil to mix due to the presence of hydrophilic and hydrophobic ends
Nonylphenol triethoxylate (NP ₃ EO)		C ₂₁ H ₃₆ O ₄	352.52	0.5 – 1.8	5.03 – 5.58	4.28	-	2.38x10 ⁻⁸ – 5.24x10 ⁻¹¹	Non-ionic surfactant	-	Allows water and oil to mix due to the presence of hydrophilic and hydrophobic ends
Nonylphenol polyethoxylate (NP ₄₋₁₂ EO)		C ₃₅ H ₆₄ O ₁₁	660.89	3.3 – 248	2.56 – 4.75	1.35 – 3.17	-	3.04x10 ⁻¹² – 5.43x10 ⁻²⁰	Non-ionic surfactant	-	Allows water and oil to mix due to the presence of hydrophilic and hydrophobic ends
Benzotriazole (BZT)		C ₆ H ₅ N ₃	119.13	19800	1.44	-	8.37	4.0 x 10 ⁻²	Corrosion inhibitor and anti-scaling agent	-	Forms an insoluble passive layer on the surface of the metal to stop corrosion
Tolyltriazole (TZT)		C ₇ H ₇ N ₃	133.15	6000	1.89	-	-	-	Corrosion inhibitor and anti-scaling agent	-	Forms an insoluble passive layer on the surface of the metal to stop corrosion

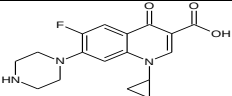
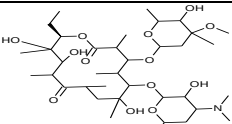
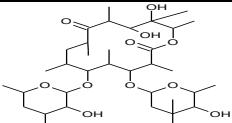
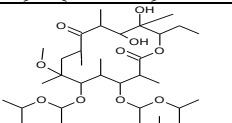
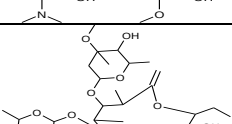
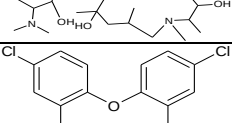
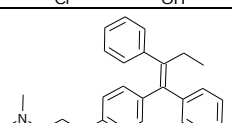
Sources: Astrazeneca Environmental Risk Assessment Data Sheets, SigmaAldrich Materials Safety Data Sheets, Cayman Chemicals Safety Data Sheets

Table 5.3. List of the hazardous chemicals and their physical and chemical characteristics, half-life in humans, uses and their mode of action

Micropollutant (CODE)	Chemical structure	Molecular formula	MW (g/mol)	Solubility (mg/L)	Log K _{ow}	Log K _{oc}	pKa	Vap. Pres (mmHg)	Use	Half-life in mammals	Mode of action
Atorvastatin (ATOV)		C ₃₃ H ₄₇ NO ₇	149.19	1.12X10 ⁻³	6.36	2.91	4.3	6.95X10 ⁻²⁵	To lower cholesterol	20 h (Human)	Decreases hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase
o-hydroxy atorvastatin (ATOVo)		C ₃₃ H ₃₈ FN ₂ O ₆	574.65	-	4.13		14.9	6.95X10 ⁻²⁵	Able to lower cholesterol	20 – 30 h (Human)	A metabolite of atorvastatin, a selective, competitive HMG-CoA reductase inhibitor
p-hydroxy atorvastatin (ATOVp)		C ₃₃ H ₃₅ FN ₂ O ₆	574.65	-	3.39		14.9	6.95X10 ⁻²⁵	Able to lower cholesterol	20 – 30 h (Human)	A metabolite of atorvastatin, a selective, competitive HMG-CoA reductase inhibitor
Atenolol (ATNL)		C ₁₄ H ₂₂ N ₂ O ₃	266.34	13300	0.16	4.38			Anti-arrhythmic	7 h (Human)	Beta-selective adrenoreceptor blocker
Propranolol (PRPL)		C ₁₆ H ₂₁ NO ₂	259.35	61.7	3.48	2.45	9.42	2.52x10 ⁻⁵	Angina, arrhythmia, hypertension	4 h (Human)	Beta- adrenergic antagonist
Carbamazepine (CBAZ)		C ₁₅ H ₁₂ N ₂ O	236.27	18	2.45	2.23	13.9	1.84 x 10 ⁻⁷	Anti-convulsant and mood stabilising drug for treating epilepsy and bipolar disorder	36 h (Human)	Gamma-aminobutyric acid (GABA) receptors agonist
10,11-epoxy carbamazepine (CBAZe)		C ₁₅ H ₁₂ N ₂ O ₂	252.27	18	2.45	2.23	13.9	1.84 x 10 ⁻⁷	Anti-epileptic metabolite from carbamazepine	8 h (Human)	Gamma-aminobutyric acid (GABA) receptors agonist

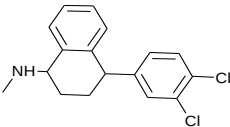
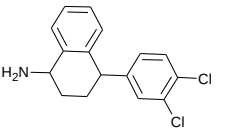
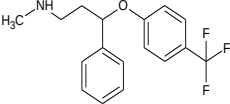
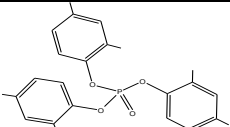
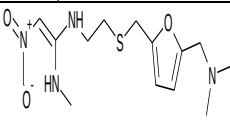
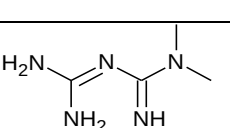
Sources: Astrazeneca Environmental Risk Assessment Data Sheets, SigmaAldrich Materials Safety Data Sheets, Cayman Chemicals Safety Data Sheets

Table 5.4. List of the hazardous chemicals and their physical and chemical characteristics, half-life in humans, uses and their mode of action

<i>Micropollutant</i>	<i>Chemical structure</i>	<i>Molecular formula</i>	<i>MW (g/mol)</i>	<i>Solubility (mg/L)</i>	<i>LogK_{ow}</i>	<i>Log K_{oc}</i>	<i>pKa</i>	<i>Vap. Pres (mmHg)</i>	<i>Use</i>	<i>Half-life in mammals</i>	<i>Mode of action</i>
Ciprofloxacin (CIPR)		C ₁₇ H ₁₈ FN ₃ O ₃	331.35	30000	-0.28	0.99	6.09	2.85x10 ⁻¹³	Antibiotic	3 – 7 h (Human)	Decrease DNA gyrase, a type II topoisomerase
Erythromycin (ERMY)		C ₃₇ H ₆₇ NO ₁₃	733.94	4.2	3.06	2.75	8.88	2.12 x 10 ⁻²⁵	Antibiotic	1.6 h (Human)	Decrease protein synthesis, binds 23S rRNA part of 50S ribosomal subunit
Norerythromycin (ERMYn)		C ₃₆ H ₆₅ NO ₁₂	703.91	4.2	3.06	2.75	-	-	Antibiotic	-	Decrease protein synthesis, binds 23S rRNA part of 50S ribosomal subunit
Clarithromycin (CLMY)		C ₃₈ H ₇₁ NO ₁₃	761.98	1.693	3.16	-	8.99	2.32 x 10 ⁻²⁵	Antibiotic	4 – 11 h (Human)	Decrease protein synthesis, binds 23S rRNA part of 50S ribosomal subunit
Azithromycin (AZMY)		C ₃₈ H ₇₂ N ₂ O ₁₂	748.99	2.37	4.02	3.49	8.74	2.65 x 10 ⁻²⁴	Antibiotic	68 h (Human)	Decrease protein synthesis, binds 23S rRNA part of 50S ribosomal subunit
Triclosan		C ₁₂ H ₇ Cl ₃ O ₂	289.53	10	4.76	4.3	7.9	4.6 x 10 ⁻⁶	Antiseptic	21 h (Human)	Decreases fatty acid synthesis
Tamoxifen (TMXF)		C ₂₆ H ₂₉ NO	371.52	16.7	6.30	-	8.87	3.46 x 10 ⁻⁸	Selective estrogen receptor moderator	5 – 7 d (Human)	Anti-estrogen inhibiting agent in the mammary gland

Sources: Astrazeneca Environmental Risk Assessment Data Sheets, SigmaAldrich Materials Safety Data Sheets, Cayman Chemicals Safety Data Sheets

Table 5.5. List of the hazardous chemicals and their physical and chemical characteristics, half-life in humans, uses and their mode of action

<i>Micropollutant</i>	<i>Chemical structure</i>	<i>Molecular formula</i>	<i>MW (g/mol)</i>	<i>Solubility (mg/L)</i>	<i>LogK_{ow}</i>	<i>Log K_{oc}</i>	<i>pKa</i>	<i>Vap. Pres (mmHg)</i>	<i>Use</i>	<i>Half-life in mammals</i>	<i>Mode of action</i>
Sertraline (SERT)		C ₁₇ H ₁₇ Cl ₂ N	306.23	3.5	5.1	4.17	-	-	Anti-depressant	25 – 26 h (Human)	Selective serotonin reuptake inhibitor
Norsertaline (SERTn)		C ₁₆ H ₁₅ Cl ₂ N	292.20	10.61	4.92		5.37	-	Anti-depressant	60 – 70 h (Human)	Selective serotonin reuptake inhibitor
Fluoxetine (FLXT)		C ₁₇ H ₁₈ F ₃ NO	309.33	38.35	4.05	4.72	-	-	Anti-depressant	4 – 5 d (Human)	Selective serotonin re-uptake inhibitor (SSRI)
trixylenyl phosphate (TXP)		C ₂₄ H ₂₇ O ₄ P	410.45	0.89	5.63		-	5.15 x 10 ⁻⁸	Flame retardant and plasticisers	-	Forms a charred phosphoric acid barrier on the surface which prevents further burning of the unburned part
Ranitidine (RNTD)		C ₁₄ H ₂₄ N ₄ O ₃ S	328.43	24.7	0.27	-	-	-	To treat stomach and intestinal ulcers	2 – 3 h (Human)	Histamine H2 receptor antagonist
Metformin (METF)		C ₄ H ₁₁ N ₅ ·HCl	165.62		1.43		12.4	7.58 x 10 ⁻⁵	Hypoglycaemic agent	8 – 12 h (Human)	Increases adenosine monophosphate (AMP)-activated protein kinase

Sources: Astrazeneca Environmental Risk Assessment Data Sheets, SigmaAldrich Materials Safety Data Sheets, Cayman Chemicals Safety Data Sheets

5.3 MATERIALS AND METHODS

5.3.1 Sampling and analysis of hazardous chemicals

The settled sewage and final effluent sampling at the full-scale WWTPs and the analyses for the hazardous chemical were carried out by ALS Environmental Wastewater Laboratory in Coventry for Atkins as part of the Chemical Investigation Programme 2, steered by UKWIR. The sampling was done using stainless samplers, transferred into glass containers and stored at 4 °C before transportation to the ISO 17025 accredited ALS Laboratories for analysis. To ensure that less than 20% change in the concentration of the hazardous chemicals occurs, the samples were analysed within 5 days of sampling. Influent and effluent samples earmarked for steroid estrogen analysis were preserved by the addition of 30% hydrochloric acid and copper nitrate (Gardner, (2012)). HPLC-MS and GC-MS were used to determine the concentrations of the HCs. A detailed description of the analytical method and the accompanying quality assurance, quality control and data handling protocols used by ALS Laboratories has been published in Comber et al., (2018) and Comber et al., (2019). To ensure parity between chemical and bacteria data, the ALS monthly sampling schedule was used for mixed liquor sampling for microbial analysis. The average concentrations of the chemicals in the settled sewage and final effluent for the required sampling dates were provided to the Cranfield research team for the purpose of this study.

5.3.2 16S rRNA analysis

The protocol for the 16S rRNA analysis is described in section 4.3.2 of chapter four.

5.3.3 Statistical treatment of the results

The physicochemical characteristics and the percentage removal of the chemicals were compared using principal component analysis (PCA) to identify similarities. The removals of each chemical were compared in a boxplot to identify outliers. The influence of operational parameters on microbial diversity and the effect of bacterial communities on chemical removal were assessed using Distance-based Redundancy Analysis and Bray-Curtis Similarity plots respectively. The correlations between chemical removal and microbial taxa were established using multiple regression analysis. The mean concentration of each HC was computed from the replicates using Microsoft Excel (2016) or IBM SPSS Statistics software (version 20) by the ALS Laboratories and disseminated to the research team.

5.4 RESULTS

5.4.1 Comparison of physicochemical characteristics of the hazardous chemicals

The chemicals can be grouped based on their pharmacological effects, functional group chemistry, physical properties or biodegradability. The physicochemical properties of some hazardous chemicals play a role in their fate and removal pathways.

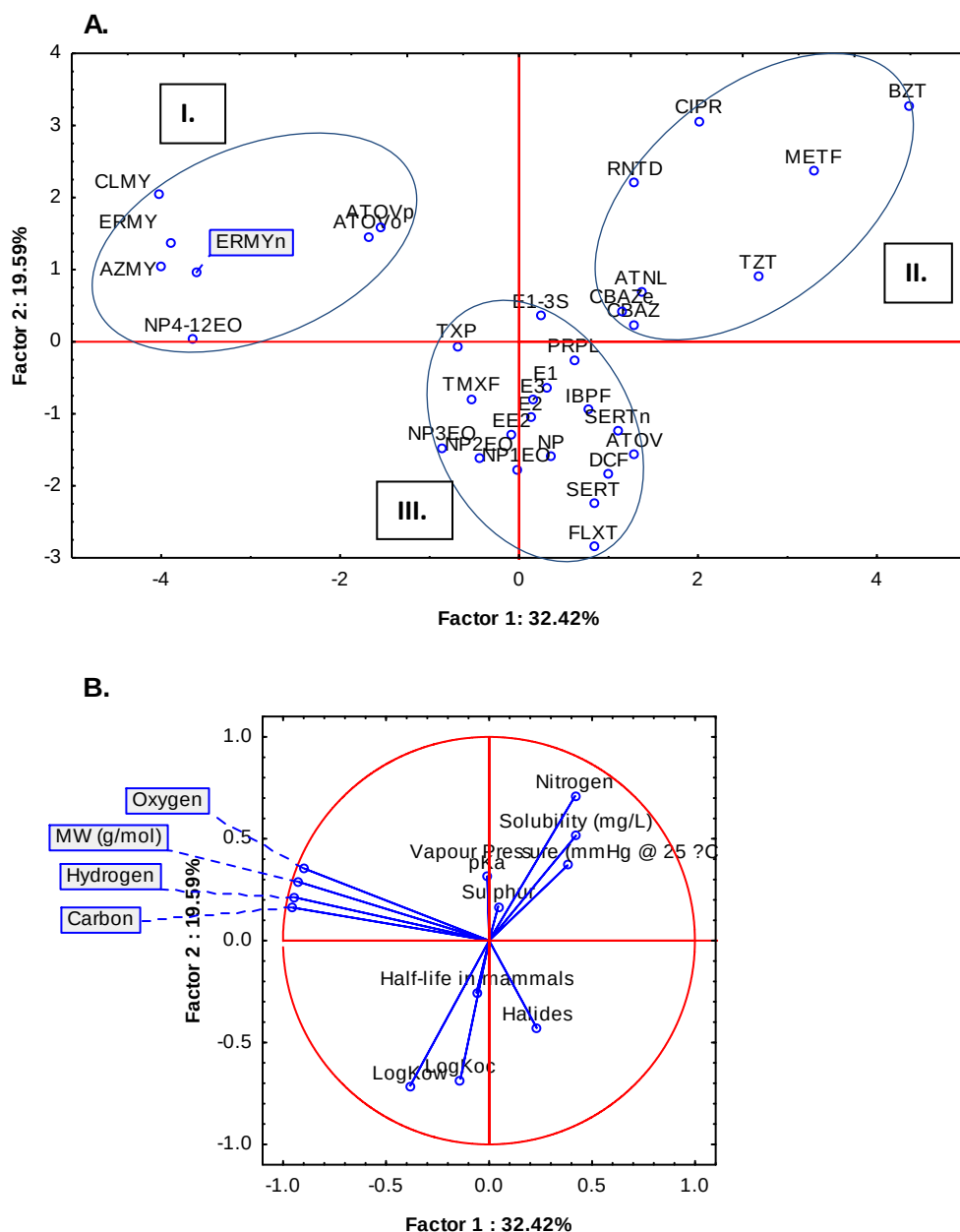


Figure 5.1. (A) PCA of the hazardous chemicals to show how they compare based on their physicochemical characteristics (i.e. molecular weight, vapour pressure, solubility, LogKow, LogKoc and pKa (as listed in Table 5.1) and the number of carbon, nitrogen, oxygen, sulfur and halogen atoms in each chemical). (B) Loading plot of the physicochemical characteristics of the HCs showing factors influencing the clustering

Figure 5.1 compares the chemicals based on their physical and chemical properties, to reveal the similarities between them and to assess the link between removal and their physicochemical properties. Comparison of the physicochemical characteristics of the hazardous chemicals in Figure 5.1 revealed three clusters: Group I were chemicals with comparable molecular weights, a similar number of carbon, hydrogen and oxygen atoms, and were predominantly antibiotics and beta-blockers. Group II comprised of highly soluble compounds which contain sulfur and nitrogen, and group III was made up of compounds that had high octanol-water coefficient or octanol-carbon coefficient or high values in both.

5.4.2 Removal of hazardous chemicals in full-scale activated sludge plants

The average concentration of each chemical in the settled sewage and final effluent for individual sampling events are presented in appended Tables AT13 to AT16. Box and whiskers plots (appended Figure AF14) show the removal of each chemical across the WWTPs. Most chemicals had a few data points which may be classed as outliers if all the results came from the same WWTP operating under the same parameters and the quest was to find a homogenous set of data. However, as each result was the recorded average for the sampling event by ALS laboratory, and this study seeks to find differences within each WWTP performance as the process parameters changed, or between plants operating differently, it was decided to include all the data in the analysis. The percentage removal of hazardous chemicals was calculated as:

$$\text{Percentage removal} = \frac{C_i - C_e}{C_i} \times 100 \quad (\text{Equation 5.1})$$

C_i = influent concentration and C_e = effluent concentration

The mean removal of hazardous chemicals ranged from –947.5 to 99.9 %. Figures 5.2 to 5.6 present the percentage removals of hazardous chemical from each sampling event to allow comparison within and between WWTPs. All the WWTPs removed metformin and ibuprofen efficiently throughout the year irrespective of operating conditions, with percentage removals ranging from 97 – 100% and 99 – 100% respectively. The high loading of metformin (76 – 331 µg/L) and ibuprofen (8 – 39 µg/L) in the influent compared to a typical influent concentration below 1 µg/L for most chemicals show that degradation of these chemicals follows a pseudo-first-order rate model (Lust et al., 2015), thus rate of removal

depends of the concentration of the reactant. Such a consistently high influent concentration of metformin and ibuprofen may also have caused the microbial communities to be adapted to metabolizing ibuprofen and metformin efficiently (Figure 5.2D and 5.3B). Effluent concentrations of ibuprofen ≤ 0.048 $\mu\text{g/L}$ exceeded its PNEC in some samples but metformin in final effluent was always below its PNEC of 10 $\mu\text{g/L}$. Conversely, diclofenac had low removals averaging to 27.8%, like the $<25\%$ removals reported by Salgado et al, (2012).

The removal of all other hazardous chemicals varied widely, and no individual WWTP achieved consistently high removal for all chemicals, although WWTPs C and D achieved the uppermost removal of chemicals (Table 5.8). High removals were also achieved in all sampling event (except sample H2) for estrogens E1 and E2. Sample H2 had more estrone, diclofenac and propranolol in the effluent than in the influent, resulting in negative removal, this is possible in activated sludge when the recycled sludge contains an adsorbed chemical which is then competitively desorbed by another chemical in the in-coming sewage, leading to higher effluent concentration than influent. This phenomenon has been observed in sludge-based activated carbon for phenolic compounds (Mohamed et al., 2011) and in activated sludge for heavy metals (Petrie et al., 2014). EE2 however, was difficult to remove in a few sampling events (Figure 5.2) with removals ranging between 20 and 93%. The removal of atorvastatin and its ortho and para derivatives were significantly high across 86% of sampling events with some removals reaching 97%.

The removal efficiencies of the beta-blockers, atenolol and propranolol showed a stark contrast. Atenolol achieved high removal, with an average of 81.5% across all samples whereas the removal of propranolol was low with mean removal of 7.5%, including their negative removals (without the negative removals, propranolol still achieved less than half the removal of atenolol, 40.6 and 88.4% respectively). This observation is similar to findings of Gallardo-Altamirano et al., (2019) which reported -90 and -100% propranolol removal but 41 and 88% atenolol removal at low and high MLSS respectively. On the contrary Miege et al., (2009) found a higher removal of propranolol than atenolol in conventional activated sludge processes. These two chemicals have contrasting solubility and $\log k_{ow}$ (Table 5.3) which led to their different groups (Figure 5.1) although they have similar pharmacological effects. Atenolol has high solubility and low octanol-water partition coefficient of 0.16 ($\log k_{ow} < 2.5$ has low sorption potential, Ternes et al., 2000) which make it less likely to sorb to

sludge and therefore likely to be removed by biodegradation which may be happening in this study. Propranolol is sparingly soluble and has a high octanol-water coefficient of 3.48 ($2.5 < \log k_{ow} < 4.0$, has medium sorption potential), therefore most likely removal pathway was sorption to sludge. The evidence suggests that propranolol could be effectively removed as seen in WWTPs C and D if the sludge characteristics are compatible. As sorption to sludge is the most probable mechanism for propranolol removal, samples A1, A3, H2, K2, K3, K4 and M1 which had increases in effluent concentration than influent may be due to competitive adsorption in recycled sludge as previously explained.

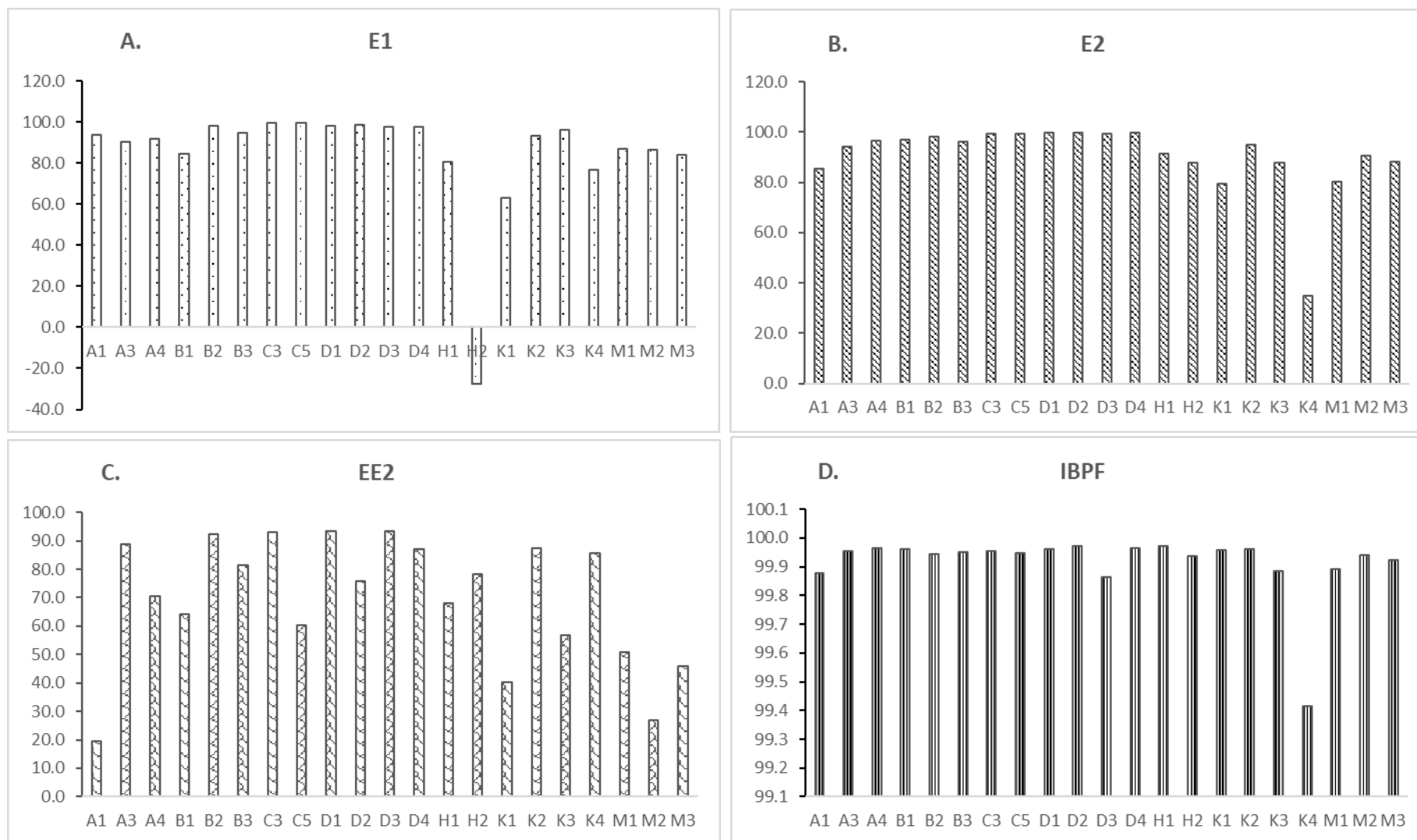


Figure 5.2. Average chemical removals from 21 sampling events at seven full-scale activated sludge WWTPs over a one-year period. E1 = estrone, E2 = 17 β estradiol, EE2 = 17 α -ethynylestradiol, IBPF = ibuprofen.

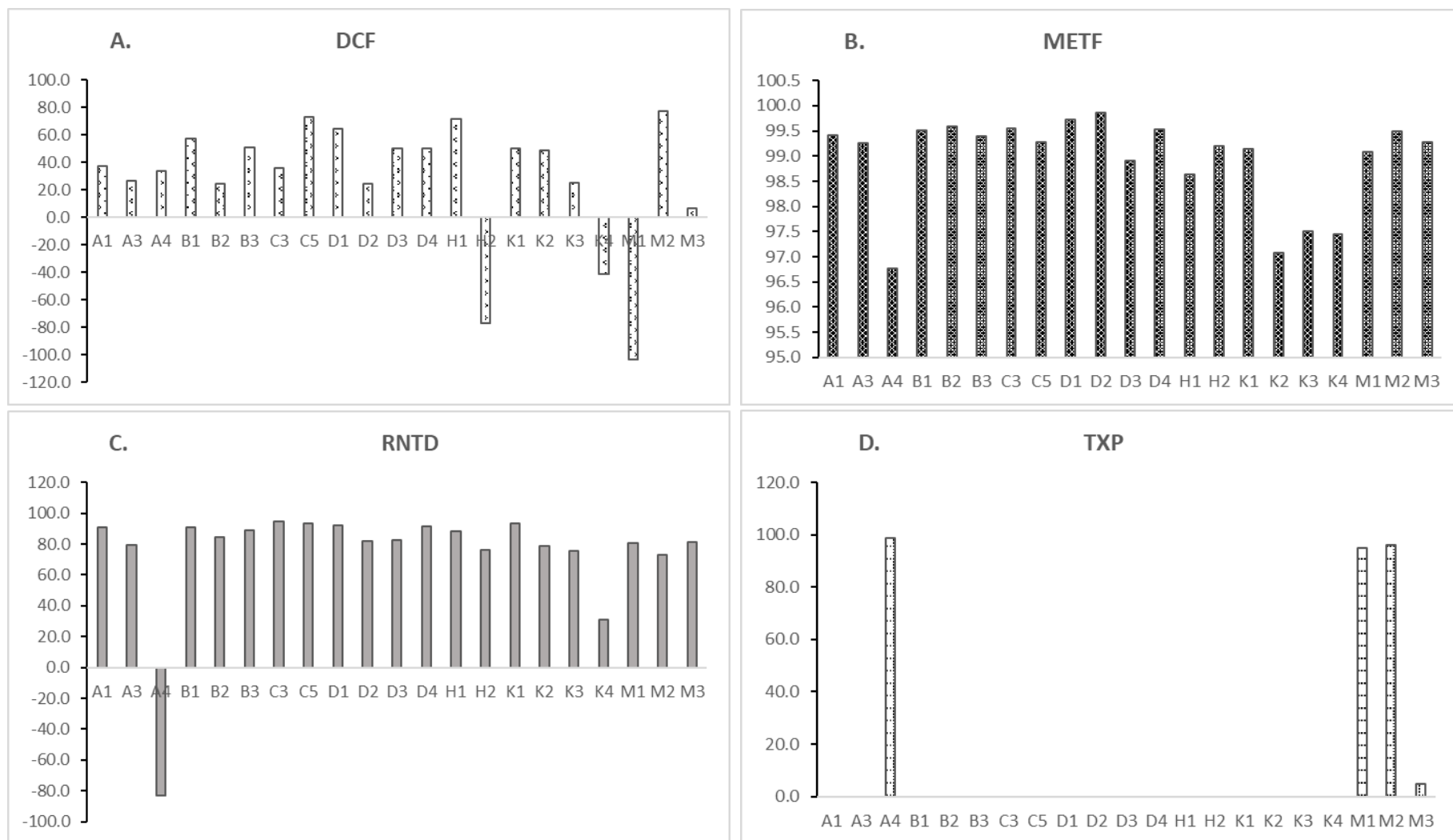


Figure 5.3. Average removals of chemicals from 21 sampling events at seven full-scale activated sludge WWTPs from April 2015 to August 2016. DCF = diclofenac, METF = metformin, RNTD = ranitidine, TXP = trixylenyl phosphate.

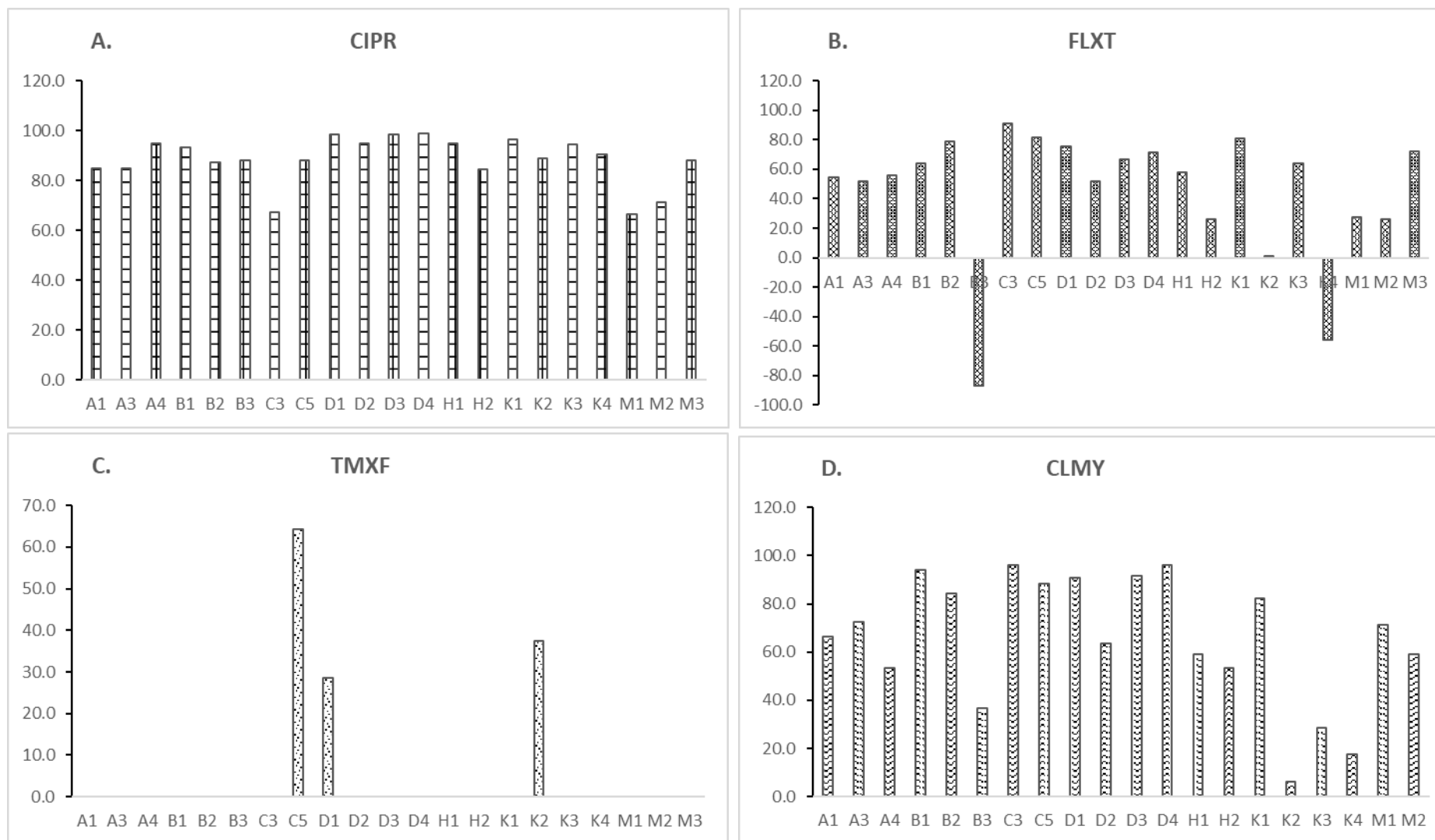


Figure 5.4. Average chemical removals from 21 sampling events at seven full-scale activated sludge WWTPs from April 2015 to August 2016. CIPR = ciprofloxacin, FLXT = fluoxetine, TMXF = tamoxifen, CLMY = clarithromycin.

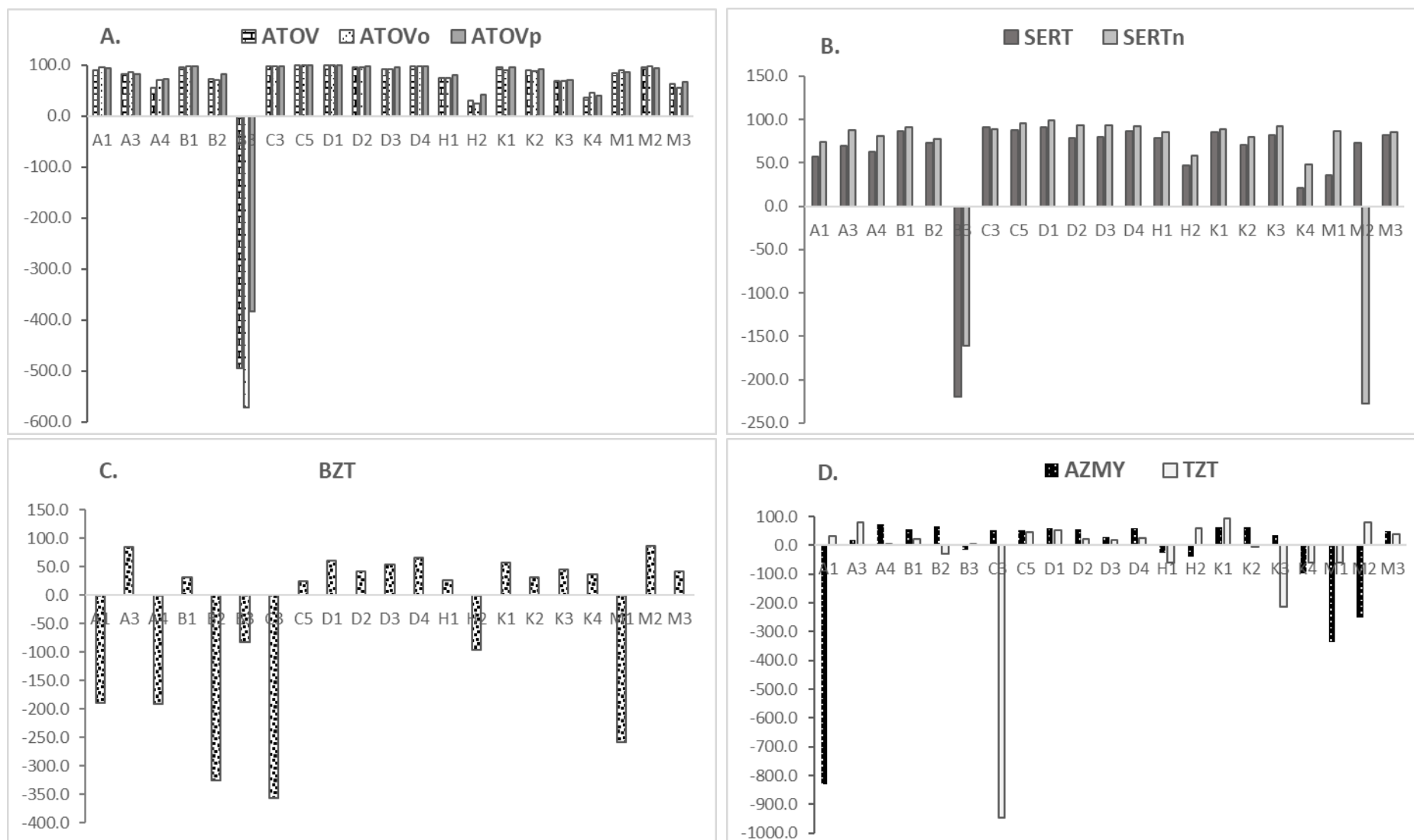


Figure 5.5. Average chemical removals from 21 sampling events at seven full-scale activated sludge WWTPs from April 2015 to August 2016. ATOR = Atorvastatin, ATOVo and ATOVp are ortho and para atorvastatin, BZT = benzotriazole, AZMY = azithromycin and TZT=tolyttriazole.

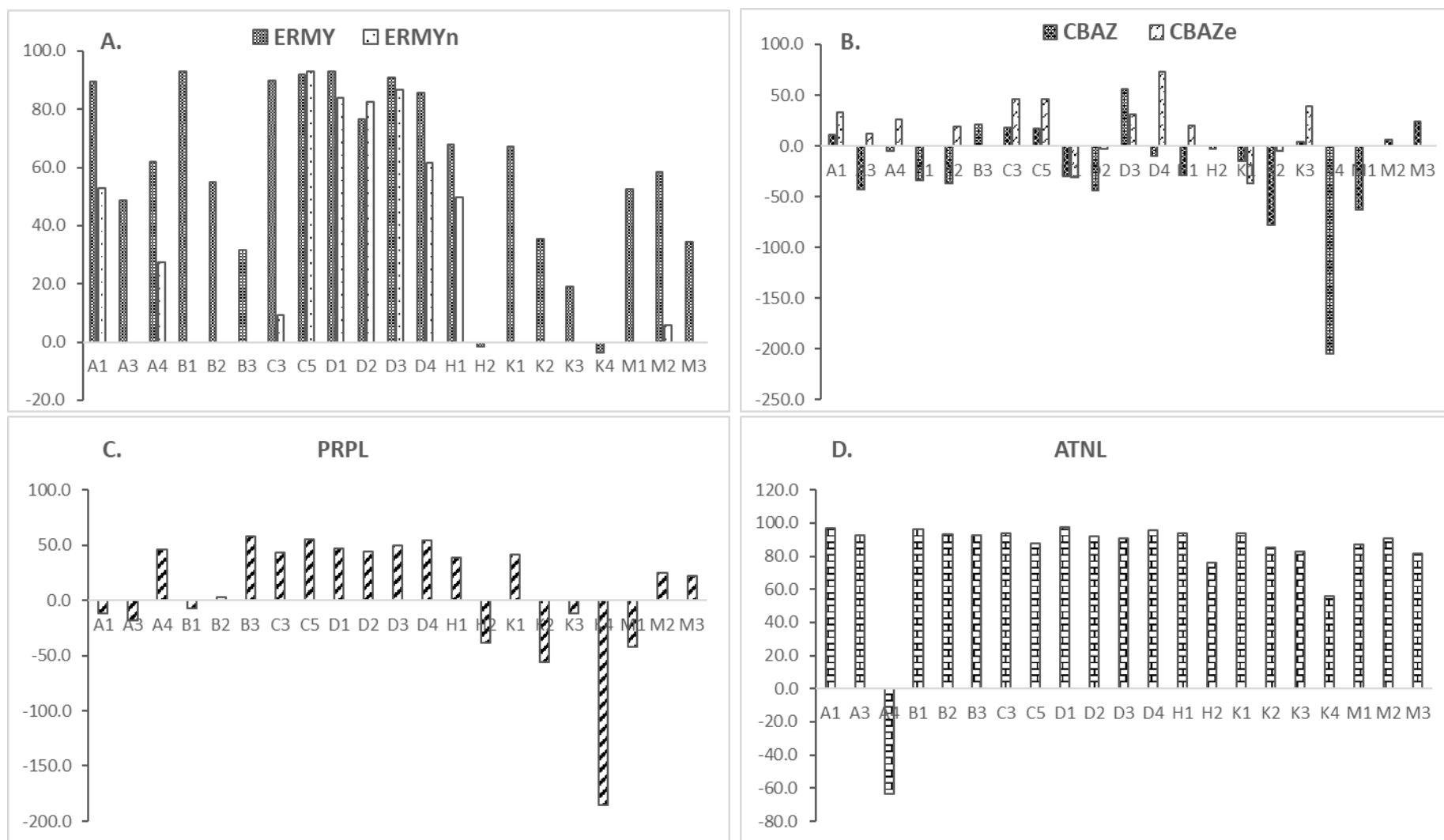


Figure 5.6. Average chemical removals from 21 sampling events at seven full-scale activated sludge WWTPs from April 2015 to August 2016. ERMY = erythromycin, ERMyn = norerythromycin, CABZ = carbamazepine, CABZe = epoxy-carbamazepine, PRPL = propranolol and ATNL = atenolol.

The anti-depressants, sertraline, nortriptyline and fluoxetine, had high average removals of 73, 85 and 58% respectively across all WWTPs but the extent of removal varied widely from sample to sample as was found in the removal of the antibiotics. Clarithromycin removal ranged from 6 – 96%, erythromycin from -4 – 93% and azithromycin from -829 – 74%. However, ciprofloxacin was consistently removed at high levels, between 67 – 99 %. Zero or negative removals were often seen in benzotriazole, carbamazepine, epoxycarbamazepine, tamoxifen, tolyltriazole and trixylenyl phosphate (TXP), although TXP had high removals in sampling events A4, M1 and M2 at 98.7, 95.1 and 96.3% respectively. Carbamazepine was unanimously recalcitrant in its removal across all WWTPs as previously reported by Zhang et al., (2008).

The removal of atenolol positively correlated with the removal of ranitidine with $R^2 = 0.97$ (appended Figure AF15), and these two chemicals (both in Group II) have similar physicochemical characteristics, and this may be the reason for the observed removal patterns. Correlations were not observed between any other chemicals.

5.4.3 Comparison of hazardous chemicals removal from different ASPs

PCA was chosen to compare the removal of hazardous chemicals across the WWTPs because each sampling event had a multiple of process parameters that made that event unique across space and time. The comparison revealed a cluster of chemicals (antibiotics, apart from azithromycin, estrogens, anti-depressants, metformin and ibuprofen) that were removed at 50% or higher in 95% of sampling events (Figure 5.7A). The cluster shows that the removal of the chemical does not favour any medicinal or chemical group, and suggests that, the influence of physicochemical characteristics is not as significant, but chemical loading rate is a more influential parameter for removal in full-scale ASPs. Figure 5.7B compares the sampling events in terms of their overall performance in chemical removal. Except for B3, the PCA shows that across space and time, sampling events had similar removal efficiencies for the HCs. Sample B3 was different because it had very high levels of statins and anti-depressants in its effluent than influent. No other sampling event showed this inability to remove all statins and anti-depressants.

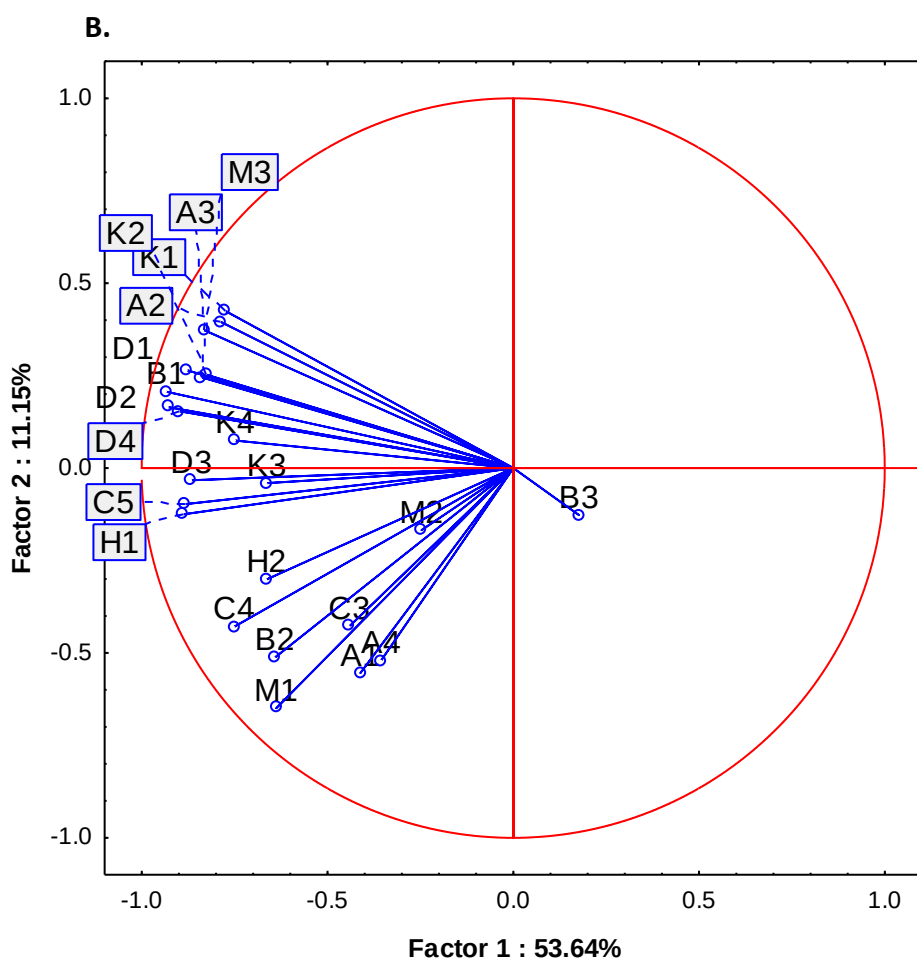
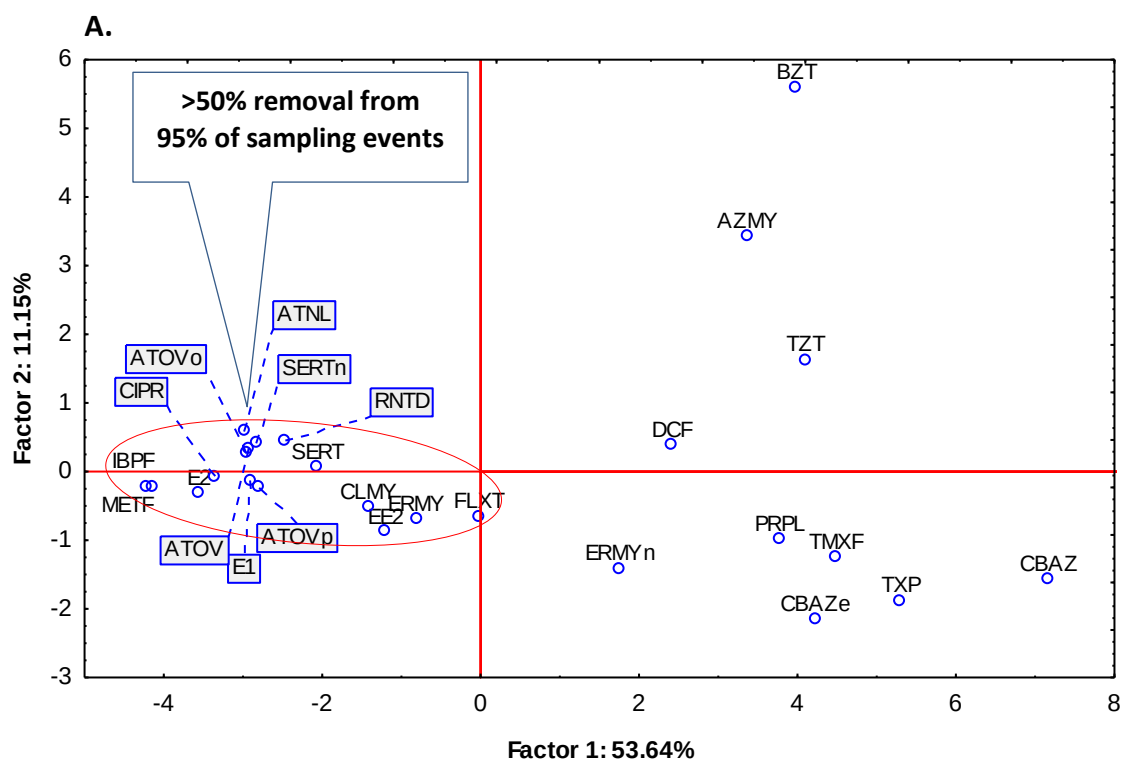


Figure 5.7 Comparison of (A) hazardous chemicals removal from all the sampling events (B) sampling events in terms of their chemical removals.

5.4.4 Bacterial diversity and process parameters

The microbial diversity and the process parameters have been fully analysed and discussed in chapter 4 but a summary of the relative abundances of PLFA microbial diversity and the dominant bacteria in each sample as well as the parameters are presented in Tables 5.6 and 5.7. The twelve WWTPs operated similar ranges of SRT, HRT and DO because they are all operating to achieve the same regulatory requirements of WWTPs in the UK to discharge into the environment, which are 25 mg/L O₂ BOD₅ (at 20 °C) and 125 mg/L O₂ COD. For a WWTP that discharges into a 'sensitive area' where eutrophication is prevalent, their phosphorus and nitrogen discharge limits are 2 mg/L and 15 mg/L for plants serving less than 100,000 populations equivalent. None of the chosen sites discharged into sensitive areas of the environment.

SRT ranged from 4.9 d to 22.3 d with an average of 11.4 d. The average HRT was 13.2 h, and it ranged from 6.2 to 26.6 h. On average, the DO concentration in the sampling events was 1.84 mg/L, but it ranged from 0.44 mg/L to 7.53 mg/L. The temperature ranged from 10.6°C to 20.3 °C, with an average temperature of 15.7 °C.

The alpha diversity in 80% of samples is dominated by an uncultured bacterium species from the genera *Albidiferax*, *Dechloromonas* or *Zoogloea*, all of which are *Betaproteobacteria*. The Gram-positive bacteria abundance ranged from 6.95% to 12.26%, the Gram-negative bacteria ranged from 45.6 to 69.8%, the actinomycetes abundance ranged from 0.60 to 2.1%, and the fungal abundance fluctuated between 20 and 42%.

Table 5.6. Dominant bacteria species and percentage abundance samples from sites A, B, C, D, E and F and their operating parameters

Site	Process parameters				Microbial diversity (PLFA)				Dominant bacterial taxa (16S rRNA)				
	DO	T °C	HRT	SRT	Gram +	Gram -	ACTS	Fungi	Phylum	Class	Genus	Species	[%]
A1	1.64	18.8	9.9	10.1	9.76	60.11	1.55	28.58	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	6.80
A2	2.41	18.5	12.0	8.5	8.88	61.45	1.72	27.95					
A3	1.48	12.7	8.0	8.2	6.95	57.66	1.31	34.08	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	7.80
A4	2.22	12.4	11.0	11.8	8.34	58.89	0.90	31.87	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	7.80
A5	0.93	17.5	13.2	10.2	8.19	60.03	1.14	30.64	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	5.50
B1	1.17	15.2	18.5	7.2	9.58	59.95	1.60	28.88	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	6.60
B2	1.07	13.2	14.5	17.4	9.18	56.41	1.21	33.20	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	5.10
B3	1.35	12.7	10.5	8.4	7.63	55.63	1.17	35.57	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	5.10
B4	1.15	14.0	12.7	17.0	8.68	50.84	1.50	38.97					
B5	1.67	20.3	10.4	6.5	7.74	57.71	1.10	33.46	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	3.80
B6	2.32	19.9	14.6	12.4	7.84	58.14	1.08	32.95					
C1	2.40	19.9	15.8	9.8	12.26	51.22	1.00	35.52	Proteobacteria	Betaproteobacteria	<i>Leptothrix</i>	Other	4.30
C2	0.46	19.4	21.3	5.2	11.51	45.61	0.89	41.99	Nitrospirae	Nitrospira	<i>Nitrospira</i>	uncultured organism	5.00
C3	0.44	14.8	9.5	8.5	12.04	49.18	1.11	37.68	Proteobacteria	Betaproteobacteria	<i>Hydrogenophaga</i>	Uncultured bacterium	4.40
C4	0.6	16.2	7.7	12.2	9.76	57.21	0.64	32.38					
C5	0.81	19	13.9	12.5	11.99	59.96	0.78	27.26	Proteobacteria	Betaproteobacteria	<i>Zoogloea</i>	Uncultured bacterium	5.10
D1	0.74	18.2	23.5	18.7	8.14	61.08	1.34	29.43	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Uncultured bacterium	3.70
D2	1.55	15.2	19.5	10.6	9.56	54.03	1.49	34.91	Actinobacteria	Actinobacteria	<i>Gordonia</i>	Uncultured bacterium	6.70
D3	1.73	15.5	14.8	8.1	8.50	59.48	1.32	30.70	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	7.50
D4	1.07	16.9	15.5	9.0	8.42	60.74	1.08	29.75	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	9.90
E1	0.46	13.7	20.0	6.0	8.38	57.37	0.95	33.30	Bacteroidetes	Flavobacteriia	<i>Flavobacterium</i>	Other	6.60
E2	1.23	12.1	26.6	12.9	10.71	52.52	1.16	35.62	Bacteroidetes	Flavobacteriia	<i>Flavobacterium</i>	Other	20.40
E3	2.19	10.6	13.0	13.1	9.87	57.62	1.02	31.48	Bacteroidetes	Flavobacteriia	<i>Flavobacterium</i>	Other	14.20
E4	1.57	15.6	12.8	8.8	10.36	60.60	0.72	28.32					
E5	0.91	18.0	26.2	4.9	9.39	61.41	0.96	28.24	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	5.30
F1	1.05	17.4	9.7	12.7	9.07	58.86	1.33	30.74	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	6.50
F2	1.05	17.6	10.2	9.9	8.40	57.36	1.40	32.84	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	4.60
F3	1.13	12.0	14.7	19.0	8.80	60.61	0.90	29.69	Firmicutes	Clostridia	<i>Incertae Sedis*</i>	uncultured microorganism	4.20
F4	2.29	17.6	9.5	12.2	7.77	59.33	0.91	31.98	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	5.70
F5	1.85	17.6	8.2	15.4	7.41	59.08	0.84	32.67					

**Incertae Sedis* means unknown taxa

Table 5.7. Dominant bacteria species and percentage abundance samples from sites G, H, J, K, L and M and their operating parameters

WWTP	Process parameters				Microbial diversity (PLFA)				Dominant bacterial taxa(16S rRNA)				
	DO	T °C	HRT	SRT	Gram +	Gram -	ACTS	Fungi	Phylum	Class	Genus	Species	[%]
G1	0.81	15.1	15.0	6.3	9.53	64.29	0.88	25.31	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	15.60
G2	1.25	16.4	15.7	15.5	8.43	66.11	0.63	24.83					
G3	0.60	17.0	14.7	13.4	8.97	65.02	0.72	25.29					
G4	4.27	19.0	15.7	13.5	8.42	66.24	0.60	24.73	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	12.80
G5	4.66	19.4	10.2	17.0	9.31	69.82	0.84	20.04	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	9.30
G6	7.53	13.3	9.2	13.2	8.32	67.01	0.73	23.94	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	5.6
G7	4.25	13.3	8.2	17.5	8.25	68.69	0.67	22.39	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	13.50
H1	1.77	16.4	8.2	8.7	7.59	61.05	1.06	30.30	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	7.90
H2	6.53	10.7	7.9	10.2	8.69	57.77	0.86	32.68	Firmicutes	Clostridia	<i>Incertae Sedis*</i>	uncultured microorganism	8.50
H3	1.08	16.1	8.1	14.9	8.68	67.48	0.90	22.94	Proteobacteria	Betaproteobacteria	<i>Zoogloea</i>	Uncultured bacterium	9.90
H4	1.01	17.6	8.1	8.7	9.46	65.82	0.90	23.82	Proteobacteria	Betaproteobacteria	<i>Zoogloea</i>	Uncultured bacterium	11.30
H5	2.60	17.7	13.2	11.7	9.26	65.64	0.76	24.34					
J1	1.23	18.2	14.0	11.2	7.91	56.11	1.77	34.22	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	6.10
J2	5.1	15.6	18.1	8.5	7.23	57.74	1.68	33.35	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	9.10
J3	0.91	10.9	16.3	6.9	7.55	58.63	0.68	33.15	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	11.00
J4	0.75	11.4	16.2	7.5	9.17	55.80	0.85	34.18					
J5	1.99	14.3	10.0	6.3	11.08	56.81	2.07	30.25	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	8.30
K1	1.54	17.1	17.6	16.5	8.41	58.53	1.43	31.63	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	5.90
K2	1.86	13	18.1	20.9	8.39	63.85	0.67	27.09	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	9.80
K3	1.54	11.1	18.2	14.6	9.53	56.42	1.12	32.93	Proteobacteria	Betaproteobacteria	<i>Albidiferax</i>	Uncultured bacterium	5.80
K4	1.69	12.2	18.4	13.5	8.48	61.02	0.81	29.69	Bacteroidetes	Flavobacteriia	<i>Flavobacterium</i>	Other	8.30
K5	1.62	13.2	15.9	22.3	9.14	58.54	1.00	31.32					
L1	2.3	18.7	15.4	13.0	8.79	61.75	1.16	28.29	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	5.00
L2	1.57	16.5	14.8	8.9	8.09	61.97	1.15	28.79	Proteobacteria	Betaproteobacteria	Other	Other	6.00
L3	6.16	10.9	7.6	7.5	7.73	57.76	1.13	33.37	Actinobacteria	Actinobacteria	<i>Ornithinibacter</i>	<i>Ornithinibacter aureus</i>	5.20
L4	1.56	15.2	8.1	6.0	8.88	58.44	1.15	31.52	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	6.10
L5	1.40	17.6	6.2	11.1	9.01	58.11	1.14	31.74					
M1	0.74	18.4	11.2	11.9	10.17	50.44	1.85	37.55	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	5.90
M2	1.34	17.3	10.6	11.0	8.24	62.15	0.96	28.64	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	6.30
M3	1.08	11.5	12.9	10.1	7.28	57.77	0.93	34.02	Bacteroidetes	Flavobacteriia	<i>Flavobacterium</i>	Other	5.90
M4	1.05	18.1	9.2	10.8	7.30	56.77	0.89	35.04	Proteobacteria	Betaproteobacteria	<i>Dechloromonas</i>	Other	6.30
M5	1.2	18.2	7.2	11.1	8.01	57.04	0.98	33.97					

5.4.5 Influence of process parameter on hazardous chemical removal

No two sampling events are identical when compared in terms of SRT, HRT, DO and temperature (appended Figures AF16 and AF17), but there is less variation between them, hence most sampling events (apart from D1 and H2) cluster together. Sample D1 lies to the far left due to its low DO and high temperature, while H2 lies to the far-right due to its high DO concentration and low temperature. WWTPs C and D had relatively better removals in most chemicals and hence cluster to the left, while H2 is shifted to the right due to its poor removal efficiency. The Euclidean distance matrix of the effect of process parameters on the removal of hazardous chemicals (Figure 5.8), shows that temperature ($p < 0.005$) is an essential driver in the variation in chemical removal in the ASPs. SRT played a more substantial role in chemical removal from WWTP K and HRT was an influential parameter in WWTP D as it seems necessary for the removal of chemicals.

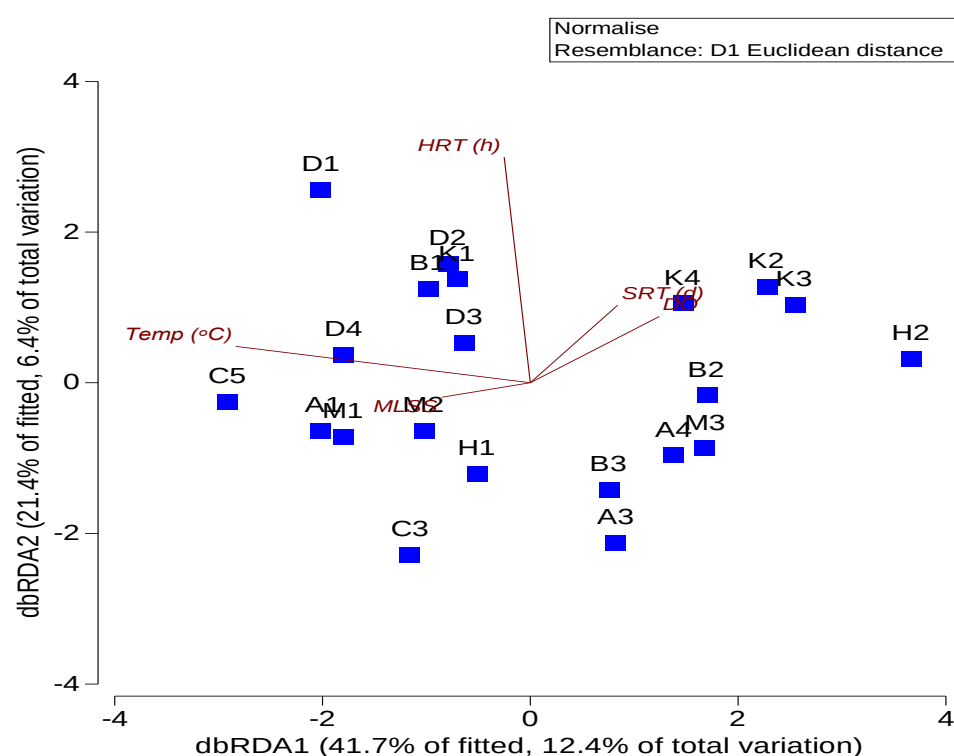


Figure 5.8. Euclidean distance matrix to show the influence of temperature, HRT, SRT, MLSS and DO on the performance of ASP reactors in removing hazardous chemicals.

From Table 5.8, the highest performance of any reactor was captured in the sampling event D1 with 80.2% average chemical removal. This sample had the second-highest SRT (23 d), longest HRT (23.5 h) and a high temperature of 18.2 °C. Its removal performance demonstrates the synergistic effect of optimum process parameters on chemical removal as

found in the pilot-scale study in chapter 3. Comparatively, sampling event K2, although had a high SRT (20.9 d) and high HRT (18.1 h), its cooler temperature of 13 °C might have impeded the reactor performance, hence it achieved 62.3% average removal. Sample B3 which had relatively low operational conditions (8.4 d, 10.5 h and 12.7 °C) failed to remove statins and anti-depressants from the influent. Furthermore, the one-off failure to remove estrone, diclofenac and propranolol in H2, coincided with a low sampling temperature of 10.7 °C, which is comparable to the findings of Thompson et al., (2011) that a temperature drop of 6 °C resulted in 20% reduction in E1-3S removal. The removal of atorvastatin was also affected by temperature, with lower removals observed in colder samples. Therefore, warmer temperature (Qarin et al, 2016), longer SRT (Petrie et al., 2014) and longer HRT (Coello-Garcia et al., 2019) are the ideal set of parameters for optimum chemical removal (Koh et al, 2009, Petrie et al, 2014).

Figure 5.9 shows that higher temperatures aligned with the removal of erythromycin, clarithromycin, estrone (Thompson et al., 2011) and carbamazepine. Longer HRT corresponded with higher removal of atorvastatin and sertraline. The removal of no group of chemicals aligned with any of the process parameters. For example, chemicals with low solubility and higher log K_{ow} (>4.0) may be predicted to be removed at higher SRT because removal may be restricted to sorption to sludge. Thus, trixylenyl phosphate (log k_{ow} 5.63, solubility 0.89 mg/L), azithromycin (log k_{ow} = 4.02, solubility = 2.37 mg/L), tamoxifen (log k_{ow} 6.30, solubility 16.70 mg/L) and ethynylestradiol (log k_{ow} 4.15, solubility 11.3 mg/L) are predicted to have high removal at extended SRT, but that was not the observed trend. Also, ciprofloxacin (log k_{ow} -0.28, solubility 30000 mg/L), tolyltriazole (log k_{ow} 1.89, solubility 6000 mg/L) and benzotriazole (log k_{ow} 1.44, solubility 19800 mg/L) have high solubility and lower log k_{ow} < 2.5, which makes their removal restricted to biodegradation, and therefore their removal should correlate with higher SRT and HRT, but was also unfounded. Since WWTPs operate similar processes, it is plausible that the removal efficiencies of these two groups of chemicals were not influenced by the process parameters as observed by Jesic et al., (2011).

B.

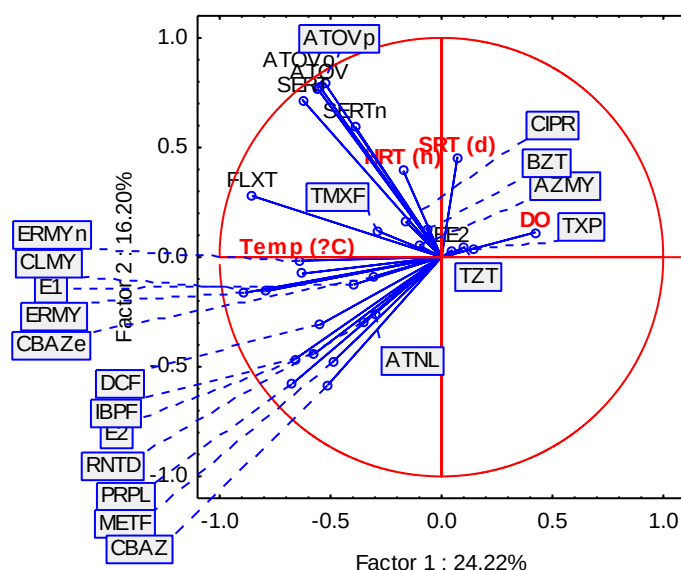


Figure 5.9. PCA showing the influence of process parameters on chemical removal in full-scale ASP

5.4.6 Influence of bacterial and archaeal diversity on hazardous chemical removal

The bacterial and archaeal phyla abundance (>1%), plants' operational conditions and the average chemical removal are compared in Table 5.9. It shows that the total dominance of *Proteobacteria* (>45%) often results in good removal, and when this dominance is reduced due to an increase in *Actinobacteria* or *Planctomycetes* abundances, the removal of HCs remains good or enhanced. However, when the dominance of *Proteobacteria* is reduced because of an increase in *Firmicutes* or *Bacteroidetes*, the average HC removal also decreased. To test these observations, correlation analysis between microbial phyla and chemical removal was performed. The results (Table 5.8) show eight correlations with $R > 0.7$ and confirmed that *Firmicutes* abundance correlate negatively with the removal of estrone and erythromycin. *Proteobacteria* did not strongly correlate with any chemical removal.

Table 5.8. Microbial phyla correlations with hazardous chemical removal

Chemical	Bacterial & archaeal phyla	Correlation equation	R-value
E1	Firmicutes	$E1 = 113.49 - 418.9 * \text{Firmicutes}$	-0.8196
	Euryarchaeota	$E1 = 113.10 - 9515. * \text{Euryarchaeota}$	-0.7488
ERMyn	Euryarchaeota	$ERMyn = 88.627 - 101E2 * \text{Euryarchaeota}$	-0.7087
	Gemmatimonadetes	$ERMyn = 13.492 + 14672. * \text{Gemmatimonadetes}$	0.72509
	Firmicutes	$ERMyn = 87.395 - 419.0 * \text{Firmicutes}$	-0.7333
TZT	Candidate division WS3	$TZT = 104.40 - 828E2 * \text{Candidate division WS3}$	-0.776
ERMyn	Chloroflexi	$ERMyn = -20.39 + 1007.8 * \text{Chloroflexi}$	0.70103
FLXT	Tenericutes	$FLXT = 97.468 - 436E2 * \text{Tenericutes}$	-0.727

The dominance of *Dechloromonas sp.* was often found to coincide with higher average chemical removal in the range of 63 – 80%. It was also noticeable that the dominance of species from *Proteobacteria* or *Actinobacteria* lineage resulted in above-average removal but increases in abundances of species from *Bacteroidetes* or *Firmicutes* lineages correspondingly decreased the average chemical removal. For example, sample H2 achieved low average removal of 49.7% with an unknown *Peptostreptococcaceae* species been dominant. Furthermore, samples K4 and M3 also had lower average removals of 42.9 and 53 %, and both were dominated by *Flavobacterium sp* from *Bacteroidetes*. However, samples C3 and C5 were dominated by *Hydrogenophaga sp.* and *Zoogloea sp.* respectively and had high average removals (> 70%), along with high *Planctomycetes* abundance of 7.4 and 6.5% respectively.

Anecdotally, excellent and poor average chemical removals were achieved at both high and low *Proteobacteria* abundance. Sample D4 contained the highest proportion of *Proteobacteria* (56.7%) and achieved 75.5% average removal whereas D2 had 35.5% *Proteobacteria* and reached 69.4% average chemical removal. The one-off E1-removal-failure in sample H2 also coincided with the emergence of *Firmicutes* as the dominant phylum instead of the *Proteobacteria*. The reduced removal may be due to the decrease in temperature which consequently increased the *Firmicutes* in the bacteria community. Total oxidizable nitrogen (TOXN) had higher removal when *Firmicutes* were high in abundance and temperature was lower.

Table 5.9. Process parameters, bacterial phyla abundance and average chemical removal in each sampling events

		Wastewater Treatment Plants sampling events																				
		A1	A3	A4	B1	B2	B3	C3	C5	D1	D2	D3	D4	H1	H2	K1	K2	K3	K4	M1	M2	M3
Parameters	DO (mg/L)	1.64	1.48	2.22	1.17	1.07	1.35	0.44	0.81	0.74	1.55	1.73	1.07	1.77	6.53	1.54	1.86	1.54	1.69	0.74	1.34	1.08
	Temperature (°C)	18.8	12.7	12.4	15.2	13.2	12.7	14.8	19	18.2	15.2	15.5	16.9	16.4	10.7	17.1	13	11.1	12.2	18.4	17.3	11.5
	HRT (h)	9.9	8	11	18.5	14.5	10.5	9.5	13.9	23.5	19.5	14.8	15.5	8.2	7.9	17.6	18.1	18.2	18.4	11.2	10.6	12.9
	SRT (d)	10.1	8.2	11.8	7.2	17.4	8.4	8.5	12.5	18.7	10.6	8.1	9	8.7	10.2	16.5	20.9	14.6	13.5	11.9	11	10.1
Abundance of bacterial phylum (%)	Acidobacteria	1.7	1.5	0.7	0.8	0.7	0.2	2.2	2.3	2.2	2.2	1.7	1.4	1.1	0.7	1.1	2.4	0.9	0.3	0.9	1.5	0.6
	Actinobacteria	8.1	7.2	9	7.1	6	8.5	3.5	2.2	5.2	21	8.9	5.8	6.4	20.1	4.6	4.7	10.3	7.5	7.8	6.4	12.4
	Bacteroidetes	23.8	23	29.4	25.9	27.8	26.5	26.2	25.1	18.4	15.5	18.2	15.3	22	13.7	24.7	25.7	22.6	30.7	19.2	16.4	28.5
	Chlorobi	0.4	0.5	0.3	0.2	0.2	0	0.5	0.6	2.1	0.8	1.8	1.3	0.3	0.1	0.5	0.3	0.3	0.1	0.7	1.1	0.2
	Chloroflexi	3	4.5	2.7	4.8	5.1	2.5	5.6	6.8	12.1	8.3	7.9	6	2.1	3	3.7	3.2	2.3	3.1	3.5	4.2	2.9
	Cyanobacteria	0.5	0.4	0.2	0.2	0.3	0.6	0.2	0.4	0.4	0.2	0.6	0.3	0.4	0.2	0.6	0.3	0.6	0.1	0.6	0.5	0.2
	Firmicutes	4.1	5.1	4.5	5.5	6.2	12.3	3.4	3.1	2.7	8.9	3.2	3.5	5.5	25.8	5.7	5.8	6.7	12.5	2.9	3.1	12.3
	Nitrospirae	1.2	2	0.6	1.6	1.8	0.7	2.8	2	2.8	1.3	1.3	1.2	1.6	1.2	2.7	2	1	1.4	3	2.9	1.4
	Planctomycetes	2.9	2.9	1.7	2.3	2.8	1.8	7.4	6.5	3.9	1.9	2.7	3.8	2.3	1	2.7	1.3	2.4	0.5	3.1	3.5	1.7
	Proteobacteria	47.8	46.2	44.8	47.5	42	43.3	43.7	46.8	44.2	35.5	48.8	56.7	52.9	31	46.9	49.2	48.9	40.6	52.1	54.3	36.5
	Spirochaetae	3.1	3.5	3.5	1	4.6	0.9	0.1	0.2	0.8	0.8	1.9	1.6	2.5	0.8	3.7	3.2	0.9	0.7	2.8	1.5	0.8
	Verrucomicrobia	1.2	1.1	1.1	1.2	1.3	1	1.4	1.9	0.9	0.8	0.9	1.1	1.2	0.4	1.1	0.7	1.1	0.6	1.5	1.7	1.1
*Average chemical removal (%)		63.4 ±34	64.0 ±38	62.6 ±27	67.2 ±37	63.6 ±36	59.0 ±41	70.9 ±35	74.6 ±29	80.2 ±26	69.4 ±32	70.7 ±32	75.5 ±30	65.2 ±30	49.7 ±34	69.1 ±32	62.3 ±36	55.6 ±35	42.9 ±33	64.0 ±34	63.3 ±36	53.0 ±33

*Average chemical removal and standard deviations were calculated without the negative removals

Further correlation analysis was conducted at the genus level with chemical removal. The results with $R > 0.7$ in Tables 5.10 and 5.11 revealed that the abundance of *Crocinitomix* positively correlates with the removal of norerythromycin and tamoxifen. The population of *Anaeromyxobacter dehalogenans* also aligns with the removal of erythromycin.

Amaricoccus, *BD1-7 clade* and *Subdoligranulum* correlated negatively with the statins and the anti-depressant sertraline. Several bacteria taxa correlated negatively with the removals of atenolol, clarithromycin, estrone and estradiol. The abundance of *Flavobacterium* correlated negatively with the removal of E2. *Hydrogenophaga* which dominated sample C3 where average removal was $>70\%$ correlated negatively with tamoxifen, which may suggest that abundance does not always translate into activity (Liang et al., 2017). *Perlucidibaca* abundance correlates negatively with E2 in both pilot and full scale. However, the correlation between *Candidatus odysella* and the removal of ethynylestradiol which was positive in the pilot-scale ($R = 0.7028$) is negative ($R = -0.7031$) in the full-scale set-up. Furthermore, the abundance of *Clostridium sensu stricto 1* and removal of E1 correlates positively in the pilot-scale ($R = 0.87496$) but negatively ($R = -0.8308$) in the full-scale study.

As these results are novel and there is no other literature to compare with, it is difficult to discern which one is valid as both studies had their limitations. The pilot-scale had only five data points as opposed to twenty-one in the full-scale, but the pilot plant's operational conditions were much more controlled than the full-scale study. The known degraders of EE2 such as *Rhodococcus* and *Pseudomonas* (Larcher and Yargeau, 2013) did not correlate with the removal of EE2 in this research nor the pilot-scale study.

Table 5.10 Correlations between bacterial genera abundance and chemical removal

Hazardous Chemical	Bacteria genera	R-values	Linear correlation
ATNL	<i>Tetrasphaera</i>	-0.7427	ATNL = 109.63 - 3319 * <i>Tetrasphaera</i>
	<i>Micropruina</i>	-0.7115	ATNL = 95.888 - 30300 * <i>Micropruina</i>
ATOV	<i>Amaricoccus</i>	-0.8858	ATOV = 166.53 - 18900 * <i>Amaricoccus</i>
	<i>BD1-7 clade</i>	-0.7253	ATOV = 169.05 - 52700 * <i>BD1-7 clade</i>
	<i>Subdoligranulum</i>	-0.8777	ATOV = 182.30 - 41000 * <i>Subdoligranulum</i>
ATOV _o	<i>Amaricoccus</i>	-0.8810	ATOV _o = 178.10 - 21200 * <i>Amaricoccus</i>
	<i>BD1-7 clade</i>	-0.7288	ATOV _o = 182.27 - 59900 * <i>BD1-7 clade</i>
	<i>Subdoligranulum</i>	-0.8724	ATOV _o = 195.75 - 46000 * <i>Subdoligranulum</i>
ATOV _p	<i>Amaricoccus</i>	-0.8852	ATOV _p = 153.85 - 15200 * <i>Amaricoccus</i>
	<i>BD1-7 clade</i>	-0.7259	ATOV _p = 156.02 - 42800 * <i>BD1-7 clade</i>
	<i>Subdoligranulum</i>	-0.8810	ATOV _p = 167.11 - 33500 * <i>Subdoligranulum</i>
AZMY	<i>Rickettsia</i>	-0.7967	AZMY = 146.29 - 187000 * <i>Rickettsia</i>
CBAZ	uncultured	-0.7174	CBAZ = -1.587 - 57800 * Uncultured
	Bacteroidetes bacterium		Bacteroidetes bacterium
CLMY	<i>Sphingobium</i>	-0.7625	CLMY = 82.580 - 2612 * <i>Sphingobium</i>
	uncultured	-0.7203	CLMY = 89.616 - 9240 * Uncultured
	Bacteroidetes bacterium		Bacteroidetes bacterium
E1	<i>Iamia</i>	-0.9073	E1 = 102.71 - 4130 * <i>Iamia</i>
	<i>Clostridium sensu stricto 1</i>	-0.8308	E1 = 104.11 - 2374 * <i>Clostridium sensu stricto 1</i>
	<i>Turicibacter</i>	-0.8300	E1 = 104.98 - 9532 * <i>Turicibacter</i>
	<i>Methanobrevibacter</i>	-0.7393	E1 = 107.15 - 9894 * <i>Methanobrevibacter</i>
	<i>Trichococcus</i>	-0.7996	E1 = 107.18 - 3373 * <i>Trichococcus</i>
	<i>Bifidobacterium</i>	-0.7326	E1 = 113.61 - 22200 * <i>Bifidobacterium</i>
	<i>Candidatus Microthrix</i>	-0.8017	E1 = 118.48 - 3862 * <i>Candidatus Microthrix</i>
	<i>Aestuariimicrobium</i>	-0.8540	E1 = 92.963 - 33400 * <i>Aestuariimicrobium</i>
E2	<i>Flavobacterium</i>	-0.7325	E2 = 103.48 - 354.9 * <i>Flavobacterium</i>
	<i>Proteiniclasticum</i>	-0.7417	E2 = 96.901 - 12000 * <i>Proteiniclasticum</i>
	VadinBC27 wastewater-sludge group	-0.7896	E2 = 98.320 - 11600 * vadinBC27 wastewater-sludge group
	<i>Perlucidibaca</i>	-0.7754	E2 = 98.359 - 6057 * <i>Perlucidibaca</i>
EE2	<i>Candidatus Odysella</i>	-0.7031	EE2 = 79.241 - 34000 * <i>Candidatus Odysella</i>
ERMY	<i>Microbacterium</i>	-0.8161	ERMY = 78.779 - 15500 * <i>Microbacterium</i>
	<i>Leptotrichia</i>	-0.7023	ERMY = 80.921 - 42100 * <i>Leptotrichia</i>
	<i>Trichococcus</i>	-0.7550	ERMY = 82.308 - 3561 * <i>Trichococcus</i>
	<i>Shinella</i>	-0.8050	ERMY = 83.559 - 24600 * <i>Shinella</i>
	<i>Bifidobacterium</i>	-0.7331	ERMY = 90.893 - 24900 * <i>Bifidobacterium</i>

Table 5.11 Correlations between bacterial genera abundance and chemical removal

Hazardous chemical	Bacteria genera	R-values	Linear correlation
ERMYn	<i>Anaeromyxobacter dehalogenans</i>	0.79104	ERMYn = 10.033 + 48819. * <i>Anaeromyxobacter dehalogenans</i>
	Uncultured bacterium	0.70442	ERMYn = 15.165 + 38995 * Uncultured bacterium
	<i>Crocinitomix</i>	0.72545	ERMYn = 15.610 + 28076 * <i>Crocinitomix</i>
FLXT	<i>Subdoligranulum</i>	-0.7281	FLXT = 83.286 - 12000 * <i>Subdoligranulum</i>
IBPF	vadinBC27 wastewater-sludge group	-0.7479	IBPF = 99.976 - 91.9 * vadinBC27 wastewater-sludge group
	<i>Perlucidibaca</i>	-0.8287	IBPF = 99.984 - 54.04 * <i>Perlucidibaca</i>
PRPL	Uncultured Bacteroidetes bacterium	-0.7672	PRPL = 29.569 - 66200 * Uncultured Bacteroidetes bacterium
	Uncultured Bacteroidetes bacterium	-0.7360	PRPL = 61.271 - 20500 * Uncultured Bacteroidetes bacterium
	Uncultured Bacteroidetes bacterium	-0.7360	PRPL = 61.271 - 20500 * Uncultured Bacteroidetes bacterium
RNTD	<i>Tetrasphaera</i>	-0.7341	RNTD = 105.52 - 3656 * <i>Tetrasphaera</i>
	<i>Micropruina</i>	-0.7410	RNTD = 91.231 - 35000 * <i>Micropruina</i>
SERT	<i>Amaricoccus</i>	-0.8571	SERT = 114.92 - 9494 * <i>Amaricoccus</i>
	<i>BD1-7 clade</i>	-0.7661	SERT = 121.52 - 29100 * <i>BD1-7 clade</i>
	<i>Subdoligranulum</i>	-0.8512	SERT = 123.01 - 20700 * <i>Subdoligranulum</i>
TMXF	Uncultured Gammaproteobacterium	0.70647	TMXF = .37374 + 3951.9 * Uncultured Gammaproteobacterium
	<i>Crocinitomix</i>	0.76985	TMXF = .90289 + 13925 * <i>Crocinitomix</i>
	<i>Dongia</i>	0.73981	TMXF = 1.7165 + 15718 * <i>Dongia</i>
	<i>Cloacibacterium</i>	0.70286	TMXF = 1.9337 + 12821 * <i>Cloacibacterium</i>
	<i>Byssovorax</i>	0.74538	TMXF = -9.558 + 3559.9 * <i>Byssovorax</i>
	<i>Byssovorax</i>	0.74538	TMXF = -9.558 + 3559.9 * <i>Byssovorax</i>
TXP	<i>Nitrosomonas</i>	0.73253	TXP = -18.20 + 8158.9 * <i>Nitrosomonas</i>
	<i>Candidatus Accumulibacter</i>	0.74858	TXP = 2.7382 + 3125.7 * <i>Candidatus Accumulibacter</i>
	<i>Candidatus Accumulibacter</i>	0.74858	TXP = 2.7382 + 3125.7 * <i>Candidatus Accumulibacter</i>
TZT	<i>Tepidicella</i>	-0.9318	TZT = 14.085 - 155000 * <i>Tepidicella</i>
	<i>Steroidobacter</i>	-0.9198	TZT = 15.427 - 33800 * <i>Steroidobacter</i>
	<i>Hydrogenophaga</i>	-0.9352	TZT = 45.331 - 22100 * <i>Hydrogenophaga</i>

5.5 DISCUSSIONS

The removal of chemicals did not conform to their broad groupings based on physicochemical characteristics, medicinal uses or chemical structure. Gardiner et al., (2013) is a study of 16 WWTPs removal of trace contaminants made a similar observation. Neither did the removal of all chemicals follow the model proposed by Byrns (2001) based on hydrophobicity where soluble chemicals with $\log K_{ow} \leq 1.5$ remain dissolved and might pass through the works with little or no removal, while those with $\log K_{ow} \geq 4.0$ which adsorb to the sludge as well as those with $\log K_{ow}$ between 1.5 and 4.0 which are biodegraded (Byrns, 2001) may be highly removed. However, metformin and ibuprofen were highly removed (Gardiner et al., 2013, Radjenovic et al., 2007) at >98% but benzotriazole and propranolol which had similar $\log K_{ow}$ were less removed. Differences in the removal of chemicals with similar hydrophobicity are attributed to the high loading rates of metformin and ibuprofen where their degradation followed a pseudo-first-order kinetic rate with respect to their influent concentration (Lust et al., 2015). Other factors like ionic interaction between charged solid surfaces and the chemicals (Gardiner et al., 2013) may have also contributed.

The variety of pharmaceuticals detected in all influent samples depicts their extensive usage and the range concentrations of detected demonstrate the enormity of the challenge facing the wastewater industry in removing these HCs, as well as the severity of the risk to the environment if the cleaning up process does not improve. Ibuprofen (8 – 37 $\mu\text{g/L}$) and diclofenac (0.17 – 1.59 $\mu\text{g/L}$) influent concentration were comparable to those found by Gardiner et al., (2013) and affirms the higher usage of over the counter medication. Influent concentrations of metformin (76 – 332 $\mu\text{g/L}$) were higher than levels found in a comparable study in Germany (Tisler and Zweiner, 2018) which reports 14 – 95 $\mu\text{g/L}$. The influent and effluent concentrations of antibiotics (ciprofloxacin, erythromycin) and the steroid estrogens are comparable to similar studies by Gardiner et al., (2013) in the UK and Kostich et al., (2014) in the USA.

A positive influence of temperature on chemical removal was observed in all WWTPs and the results agreed with Ho et al., (2014) and Razvi et al., (2014). The enhanced of chemical removals at longer SRT and HRT (Ma et al, 2017; Petrie et al, 2014) were observable in some WWTPs but not all. A synergistic effect of warmer temperature (>14 °C) and longer SRTs

(>15 d) was seen in a few samples to further improve the chemical removal. Dissolved oxygen concentration did not show any significant influence of chemical removal as revealed by Stadler et al., (2015). This is due to the lack of significant variation between sampling events with respect to DO concentrations.

The overall microbial diversity in the ASP reactor at full-scale did not seem to vary as much from each other, and hence no influence of microbial diversity on chemical removal was observed, as many reactors' performance were similar for some chemicals. However, there were several genera abundances that correlated with the removal of individual chemicals. The genera *Amaricoccus*, *BD1-7 clade* and *Subdoligranulum* correlate with the removal of atorvastatin and its ortho and para derivatives. There is no commonality in the literature about these three genera in terms of their known roles in ASPs. Therefore, it is unclear why these different genera correlate with the statins.

The removal of trixylenyl phosphate (TXP) positively correlates with the abundance of *Candidatus Accumulibacter*. Species from this bacterial genus are capable of accumulating large amounts of intracellular phosphate (Welles et al., 2016) and are often found in enhanced biological phosphorus removal WWTPs. *Nitrosomonas* (AOB) also correlated positively with the flame retardant TXP. It is therefore likely for the abundance of *Nitrosomonas* to correlate with TXP removal because phosphate accumulating organisms (PAO) has been suggested to have the capacity to denitrify under anoxic conditions (Oehman, 2010) although that is limiting in terms of energy outcome (Saad et al., 2016). This creates a symbiotic (syntrophic) relationship where the *Nitrosomonas* convert ammonia to nitrite and NOBs turn nitrite to nitrate and the PAO uses the nitrate as oxygen source while accumulating P from the TXP. Alternatively, the *Nitrosomonas* may be directly responsible for the biotransformation of TXP. Recent studies have shown that some ammonia-oxidising-archaea and bacteria has the ability to biotransform micropollutants. For instance, *Nitrososphaera gargensis* has been found to biotransform ranitidine and mianserin (Men et al., 2016). *Tetrasphaera*, a PAO, correlated with the removal of atenolol and ranitidine, but not TXP as might be expected. Unlike the other PAOs who rely on the availability of volatile fatty acids (VFA) created by other bacteria in anaerobic reactors, *Tetrasphaera* has a different metabolism where it can ferment glucose and use glycogen as a polymer storage but its correlation with atenolol and ranitidine or its non-correlation with

TXP cannot be explained. Perhaps, finding is novel and should be researched further. The EE2 degraders *Pseudomonas* and *Rhodococcus* (Larcher and Yargeau, 2013) did not correlate at $R > 0.7$ as was found by Coello-Garcia (2018) which emphasises the knowledge that degradation of EE2 may be a function carried out by several taxa, not a few (Helbling et al., 2015). There are several correlations of genus abundance with chemical removal that cannot be explained, and several others from literature on bacterial degradation of chemicals that were expected but unfounded. This research provides the basis for future work at pilot-scale to collect more data to substantiate or perhaps, eliminate some of the correlations established in this work.

5.6 CONCLUSION

- The physical and chemical characteristics of chemical groups in the influent did not affect their removal. The removal of chemicals followed a pseudo-first-order degradation rate with respect to influent concentration.
- The influent concentrations of chemicals show the magnitude of the risk to the environment from WWTPs and the challenge facing the industry. Most effluent chemical levels were below their PNEC values.
- *Candidatus Accumulibacter* and *Nitrosomonas* abundances correlate with the removal of trixylenyl phosphate. Several other bacterial genera abundance correlates with the removal of different chemicals.
- Removal of hazardous chemicals in ASPs across space and time is independent of overall microbial/bacterial diversity because the shifts in diversity are insignificant and do not translate into improvement or deterioration in plant performance.
- Full-scale ASPs operate under similar process parameters (temperature, DO, SRT, HRT) throughout the year. Therefore, the sampling events with exceptionally high removal or, failure to remove hazardous chemicals occasionally, coincide with a change in process parameter.
- Temperature increases corresponded with improved hazardous chemical removal. The improved removal at longer SRT and HRTs were observed in some ASPs but not in others because other factors may have contributed as the processes in full-scale plants are not finely controlled.

THESIS DISCUSSION

6. THESIS DISCUSSION

6.1 INTRODUCTION

Pilot-scale studies have shown that increasing the solids retention time (SRT) and hydraulic retention time (HRT) improves the removal of micropollutants in the activated sludge plant (Akarsubasi et al., 2009; Clara et al., 2006; Petrie et al., 2014), the marginal gains in removal has been suggested to be a result of increases in abundance of slow-growing microbial communities that are capable of degrading micropollutants. This research was aimed firstly, to determine the influence of SRT and HRT on microbial diversity at pilot-scale ASP fed with real sewage containing typical concentrations of hazardous chemicals. Secondly, assess the influence of the resulting microbial diversity on the removal of endocrine-disrupting chemicals (estrogens and nonylphenols). The revelation that various bacterial genera abundance correlates with the removal of estrogens and nonylphenols and that SRT and HRT affected microbial/bacterial diversity fuelled a full-scale study to learn if these findings are present in municipal WWTPs. Twelve full-scale activated sludge plants were chosen to study the influence of SRT, HRT, temperature and dissolved oxygen concentration on microbial diversity and on the removal of hazardous chemicals (*atenolol*, *atorvastatin*, *benzotriazole*, *carbamazepine*, *ciprofloxacin*, *clarithromycin*, *diclofenac*, *17 β -estradiol*, *17 α -ethynylestradiol*, *estriol*, *estrone*, *estrone-1,3-sulfate*, *erythromycin*, *fluoxetine*, *ibuprofen*, *metformin*, *nonylphenol (ethoxylates)*, *propranolol*, *ranitidine*, *sertraline*, *tamoxifen*, *trixylenyl phosphate*, *tolyltriazole*). Finally, the relationships between bacterial genera abundance and chemical removals were also assessed. The findings will inform the discussion on improving existing ASPs to optimise their removal of hazardous chemicals through alterations to operational parameter where possible, in order to meet prospective EU or UK compliance on the hazardous chemicals. The outcome will also improve our knowledge and understanding of microbial community structure in WWTPs in relation to process parameters and help the design WWTPs of the future in a deterministic manner to increase the richness of bacterial genera capable of chemical degradation in the reactor.

6.2 EFFECT OF PROCESS PARAMETERS ON MICROBIAL DIVERSITY AND HAZARDOUS CHEMICAL REMOVAL IN PILOT-SCALE ACTIVATED SLUDGE PLANT

The PLFA analysis demonstrates that SRT and HRT increase resulted in changes in microbial groups but the order of abundance (most to least) always remained Gram-negative bacteria, fungi, Gram-positive bacteria and actinomycetes. Findings by Amir et al., (2010) revealed the same order of microbial diversity in sludge cake from ASP. Lengthening HRT from 8 to 16 h increased the fungal proportion by 5.1% while Gram-negative bacteria reduced by 6.0%. Furthering HRT to 24 h reduced fungal and Gram-positive bacteria abundance by 2.8 and 1.6% respectively, with a corresponding increase in Gram-negative bacteria by 1.41%. SRT increase from 3 to 10 d coincided with a decrease in Gram-negative and Gram-positive bacteria abundance by 6 and 5% respectively, whereas fungal abundance increased by 10.7% with an insignificant increment in the percentage of actinomycetes. Further elongation of SRT to 27 d caused no change to the Gram-positive bacteria, insignificant reduction in actinomycetes, reduced fungal abundance by 8.7% and increased the Gram-negative bacteria by 7.8% because longer SRT reduces the food-to-microorganisms ratio and causes microbial diversity to change. PLFA has been routinely used in soil analysis to evaluate changes in microbial diversity due to environmental stressors (Zornoza et al., 2009; Lores et al., 2010), in wastewater treatment system (Quezada et al, 2007) and in sludge (Amir et al., 2010; Zhao et al., 2014). This is the first research to use PLFA analysis in assessing microbial diversity in mixed liquors from ASPs, as the reactor is stressed via changes in SRT and HRT. The observed microbial group changes as SRT and HRT are increased encouraged a deeper dive into the microbial diversity. Though PLFA is a fast and cheaper analysis (in comparison with DNA analysis), with ability to show broad changes in microbial diversity, it has limited use in identifying bacterial taxa (Frostegard et al., 2011) to enable the assessment of the correlation of bacterial taxa abundance with chemical removal.

The 16S rRNA analysis widely used in studying bacterial and archaeal diversity (Wang et al., 2014) in engineered (Coello-Garcia et al., 2019) and environmental (Edwards et al., 2018) systems was employed. The 16S rRNA analysis revealed changes in bacterial diversity at each taxonomic level of the prokaryotic nomenclature as SRT and HRT were varied. The

dominant phyla in each investigation were Proteobacteria followed by Bacteroidetes as reported by Sun et al., (2016) using Illumina MiSeq sequencing.

At low SRT (3 d), *Thiothrix eikelboomii* was dominant with 11.9%, as found by Xie et al., (2011) in ASP using fluorescence in-situ hybridisation (FISH) analysis that 10% of the bacteria population was due to *Thiothrix spp.* The *Thiothrix* is found in reactors that have significant foaming or bulking (Rubio-Rincon et al., 2017) or at short SRT (3.5 d) Perez et al., (2016). At 10 d SRT the dominance of *Nitrospira sp.* agrees with findings from Noophan et al., (2017) that at SRT ≥ 6 days, the dominant NOB in BNR plant was *Nitrospira sp.* through DGGE analysis. The uncultured *Comamonadaceae* which dominated at 27 d SRT was the only genus that remained in the top two most abundant in every experimental set-up (Damien et al., 2015). Some cultivars from *Comamonadaceae* are potential aerobic denitrifiers that directly convert ammonia into nitrogen under oxic conditions and use phenol and their derivatives as energy sources (Ni et al., 2013). Their predominance may be due to the high ammonia in the influent. The principal component analysis (PCA) and loading plots were used to show that the microbial/bacterial communities were dissimilar at each SRT and HRT. The use of PCA in comparing microbial diversity is a well-established statistical method for the evaluation of microbial ecology to find factors that drive the differences (Sun et al., 2016; Lee et al., 2015; Zhang et al., 2018).

The study of hazardous-chemical-removal found that estrone (E1) and ethynylestradiol (EE2) removals were 19 and 30 % (at 3d SRT), 93 and 29 % (at 10 d SRT) and 84 and 41 % (at 27 d). Nonylphenol ethoxylates (NP₄EO and NP₅EO) removal rose from 44 and 49 % (at 3 d) to 73 and 85% (at 10 d) then to 84 and 89 % at 27 d (Koh et al., 2009). The effect of HRT on chemical removal was not as high in comparison with Ho and Watanabe (2017) due to the high constant SRT, a shortcoming of this research. The correlation analysis of bacterial abundances with chemical removal revealed some novel relationships. The abundance of Proteobacteria negatively correlated with the removal of estrone, estradiol, estriol and nonylphenol ethoxylate (NP₃EO, NP₄EO, NP₅EO, NP₆EO and NP₇EO) while Chloroflexi and Verrucomicrobia positively correlated with EE2 removal. 82 bacteria genera correlated with estrogens, with 32 positive correlations while 122 genera correlated with nonylphenols of which 34 correlated positively at $R > 0.7$. *Leucobacter* correlated negatively with E1, E2, E3 and EE2 but positively with E1-3S. *Hyphomicrobium*, *Mesorhizobium*, *Rhizomicrobium* and *Planctomyces* positively correlated with the removals of E1, E2 and E3. Recently, Coello-

Garcia (2018) found the removal of E1 correlated with *Gemmatimonas*, *Planctomyces*, *Mesorhizobium* and *Leptospira* at $R > 0.7$ and *Nannocystis* correlation with the degradation of EE2. *Thiothrix* which was dominant at low SRT negatively correlate with NP_2EC and $NP_{3-11}EO$ removals. These new findings required further research at full-scale WWTPs to establish whether similar correlations exist in large reactors where there is much variability in flow that causes changes in SRT and HRT. To ensure the study is thorough, the range of hazardous chemicals was expanded to include antibiotic, anti-depressants, analgesics etc. and temperature and dissolved oxygen concentration were included in the operational conditions.

6.3 SURVEY OF MICROBIAL / BACTERIAL DIVERSITY IN 12 FULL-SCALE ACTIVATED SLUDGE PLANTS UNDER DIFFERENT PROCESS CONDITIONS

The PLFA analysis detected changes in the full-scale microbial diversity as process conditions changed but to a limited depth. It showed similar trends in microbial abundance ranking but varied in abundance in each WWTP. Similar to the pilot-scale, uncultured *Comamonadaceae* ranked in the top five most abundant genera in each WWTP at least once during the period of study. As the settled sewage entering the ASP reactor has high ammonia and phenolic compounds, coupled with the aeration needed, some *Comamonadaceae* strains did dominate because they are aerobic denitrifier capable of ammonia to nitrogen conversion (Ni et al., 2013). However, comparison of the bacterial diversity in the full and the pilot-scale reactors revealed differences. The disparity is due to differences in the abundance of *Proteobacteria*, *Planctomycetes*, *Lentisphaerae*, *Verrucomicrobia*, *Hydrogenedetes* and *Cyanobacteria*. This bacteria diversity difference between pilot and full-scale may be because of their different influent chemical composition (Gannoun et al., 2013; Li et al., 2014). However, Isazadeh et al., (2016) found similar diversity in pilot and full-scale plants. The full-scale ASPs were often dominated by *Betaproteobacteria* (Bagchi et al., 2015; Jiang et al., 2008) at class level and *Albidiferax*, *Dechloromonas*, *Flavobacterium* and uncultured *Saprospiraceae* (Xu et al., 2018; Wang et al., 2014) at genus level. The dominance of *Nitrospira* was observed in only one sample, and none was dominated by *Nitrosomonas* even though each reactor was nitrifying at the time of sampling.

This is indicative that nitrification is a niche function performed by a minority of species (Yao and Peng, 2017); hence a stable ASP reactor is vital for nitrification.

Temperature was the most influential parameter in determining bacterial diversity (Li et al., 2015; Wang et al., 2014; Siripong and Rittman, 2007). The temperature in ASP reactors directly impacts bacterial growth and floc morphology (Koivuranta et al., 2017), chemical degradation rates (Soares et al., 2006) and on the solubility of chemicals in the influent. At high temperatures the reactors were dominated by *Albidiferax*, *Dechloromonas* and *Zoogloea* but at lower temperatures (≤ 14 °C), the abundance of *Bacteroidetes*, *Firmicutes* and *Actinobacteria* increased, leading to the dominance of *Flavobacterium*, unknown *Peptostreptococcaceae* and *Clostridium sensu stricto 1* and *Ornithinibacter* (Zhang et al., 2012). Mielczarek et al., (2012) identified temperature as crucial for the dynamics of some species of filamentous bacteria in Danish WWTPs. Some strains of *Clostridium sensu stricto 1* and *Flavobacterium* are filamentous (MiDAS Field guide, 2019). WWTPs in locations with higher temperatures throughout the year e.g. Puerto Rico (Valentín-Vargas et al., 2012) and California (Wells et al. 2011) does not exhibit seasonal patterns in bacterial community composition.

The impact of DO in full-scale ASPs was difficult to assess due to the lack of significant variance in DO concentration between sampling events, as shown in the ANOVA. A high abundance of *Firmicutes* coincided with high DO concentrations. This observation is opposite to findings of Larsen et al., (2008) that *Firmicutes* are weak and their abundance diminishes under high shear force from aeration.

WWTP's configuration also affected the bacterial and microbial diversity found serendipitously in WWTP C. The MBBR (Kaldness) reactor which preceded the primary settling tank in WWTP C is attributed as the cause of the distinctiveness of the bacterial community (Harris et al., (2011). At the Rya WWTP in Sweden, bacteria community changed during maintenance works which by-passed the primary settlers for several weeks (Fredriksson, 2013). The Kaldness pre-treatment or by-passing primary settlement changes the influent chemicals concentrations and nutrient levels entering the AS reactor, hence the change in bacterial diversity. The low organic loading in WWTP C resulted in higher Shannon indices and increased diversity. Albeit, Siavash et al., (2016) investigated the impact of

manipulated environmental variables (pilot or full-scale, SRT, ASP configuration, fully oxic or anoxic/aerobic, chemical-stress induced by ozonation of the RAS) on bacterial diversity and found insignificant differences.

WWTP C had high abundances of *Planctomycetes* and *Acidobacteria* which maybe because the slow-growing bacteria capable of acquiring carbon from low-organic-influent are favoured in its reactor and fast-growing heterotrophs are inhibited. The Kaldness reactor is also well-known for its improved nitrification performance, and therefore a Kaldness pre-treat reactor will reduce the influent ammonia (Rusten et al., 2006) entering the AS reactor and stifle the abundance of *Thiothrix* and uncultured *Comamonadaceae* (Damien et al., 2015), both of which had low abundance in WWTP C.

Attempts at quantifying the influence of factor on bacterial diversity in WWTP show that diversity is neither fully deterministic nor stochastic. Isazadeh et al., (2016) states that 13% of bacterial diversity can be explained by process parameters (conventional, oxidation ditch or sequence batch reactor), Wang et al., (2014) found that 23% of diversity is due to process configuration, 5% due to influent interaction with process parameters and 25% can be explained by influent characteristic. This work found that 27% of bacterial diversity can be explained by SRT, HRT, temperature and DO concentration.

6.4. CORRELATIONS OF BACTERIAL ABUNDANCE WITH HAZARDOUS CHEMICAL REMOVAL IN ACTIVATED SLUDGE PLANT

Novel correlations were identified between the removals of chemicals and bacteria taxa at $R > 0.7$ for positive and negative relationships. Bacterial diversity increased with SRT or HRT (in the pilot plant) and improved the removal of estrogens and nonylphenols. The abundance of *Thiothrix*, which reduced as SRT increased, correlated negatively with the removal of nonylphenol dicarboxylate (NP₂EC) and nonylphenol ethoxylates (NP₃EO to NP₁₁EO). *Arenimonas* generally had low abundance and correlated negatively with the removal of NP₁EO and NP₃EC but positively with E1-3S in pilot scale. *Thiothrix* and *Arenimonas* are believed to play a role in degradation of sulfur-based and penicillin antibiotics (Xia et al., 2015) but never reported on nonylphenols or estrogens. The

association of *Thiothrix* and *Arenimonas* to degradation of sulphonamide antibiotics was undeterminable because macrolide and quinolone antibiotics were studied.

Ibuprofen removal correlated inversely with the abundance of *Perlucidibaca* and vanBC27 wastewater-sludge bacterium, not positively with *Sphingobium* as might be expected because *Shingobium* strain *Ibu-2* is an ibuprofen degrader (Langenhoff et al., 2013).

Amaricoccus, *BD1-7 clade* and *Subdoligranulum* correlate with the removal of atorvastatin and its ortho and para derivatives. Research on the link between *Amaricoccus*, *Subdoligranulum* or *BD1-7 clade* and statins is non-existence. Removal of trixlylenyl phosphate (TXP) positively correlated with the phosphate-accumulating bacteria *Candidatus Accumulibacter* abundance (Welles et al., 2016) as would be expected. Another correlation that could not be explained from the literature was that between phosphate-accumulating bacteria, *Tetrasphaera* with the removal of atenolol and ranitidine, but not with trixlylenyl phosphate. With such a vast number of unexplainable correlations, research at pilot and full-scale plants are needed to collect evidence to investigate these correlations and begin to elucidate the mechanisms of degradation. There were several known degraders of estrogens, nonylphenols and pharmaceuticals that were either unfounded in the bacterial abundance studies or did not correlate with their chemicals. For example, EE2 degraders *Pseudomonas* and *Rhodococcus* (Larcher and Yargeau, 2013) did not correlate with it (Coello-Garcia (2018)). Future bacterial analytical studies using targeted DNA primers for these bacterial genera should be conducted to understand their role in ASPs in relation to the removal of chemicals that they are known to degrade. *Planctomyces* and *Mesorhizobium* positively correlate with the removal of E1 and EE2 as reported by Coello-Garcia, (2018). *Hyphomicrobium* and *Rhizomicrobium* also positively correlated with the removals of E1, E2 and E3, but have not been reported in the literature. The degradation rate of E1 has been reported to correlate with *Gemmatimonas* and *Leptospira* whilst EE2 correlate with *Nannocystis* (Coello-Garcia, 2018) but were not found in this study. *Leucobacter* correlated negatively with E1, E2, E3 and EE2 but not E1-3S and require further investigation to understand.

CHAPTER SEVEN

THESIS CONCLUSION

7. THESIS CONCLUSIONS

- PLFA analysis revealed shifts in microbial diversity while the 16S rRNA analysis showed changes in bacterial diversity, hence useful information could be obtained from PLFA.
- The sensitivity of PLFA at detecting subtle changes in diversity was observable but difficult to quantify its significance. Therefore, the routine use of PLFA analysis for microbial diversity analysis by WWTP operators in mixed liquors is viable and could be adapted to establish a baseline for good microbial health and investigate deviations to prevent treatment failure.
- Generally, the microbial diversity in ASPs comprised of 60% Gram-negative bacteria, 30% fungi, 9% Gram-positive bacteria and 1% actinomycetes.
- The dominant taxa were *Proteobacteria*, *Betaproteobacteria* and 98% of the dominant species are uncultured bacteria. The abundant phyla were *Actinobacteria* (2.2 – 21%), *Bacteroidetes* (11.8 – 36.9%), *Chloroflexi* (0.6 – 12.1%), *Firmicutes* (2.1 – 25.8%), *Nitrospirae* (0.1 – 6.2%), *Planctomycetes* (0.5 – 9.9%), *Proteobacteria* (29.5 – 59.0%), *Spirochaetae* (0.1 – 4.9%) and *Verrucomicrobia* (0.4 – 2.2%).
- At low SRT *Thiothrix* dominated and at high SRT uncultured *Comamonadaceae* was dominant in pilot-scale ASP reactor.
- In full-scale WWTP the dominant genera were *Dechloromonas*, *Albidiferax*, uncultured *Saprospiraceae* and uncultured *Comamonadaceae* and *Flavobacterium*.
- The bacterial / microbial diversity and abundance are influenced by temperature, SRT, HRT and the inclusion of an MBBR pre-treatment reactor. Cold temperatures (<13 °C) increased the abundance of *Firmicutes* and *Bacteroidetes* and subsequently reduces the abundance of *Proteobacteria*. MBBR pre-treatment increased the *Planctomycetes*.
- Temperature increases corresponded with improved hazardous chemical removal. The improved removal at longer SRT and HRTs was not seen in all the full-scale WWTPs because the full-scale process parameters are not as controlled. DO across the WWTPs did not vary significantly and hence did not impact on bacterial diversity or HC removal.
- *Candidatus Accumulibacter* correlates positively with removal of trixylenyl phosphate.
- *Hyphomicrobium*, *Planctomyces*, *Mesorhizobium* and *Rhizomicrobium* positively correlated with the removals of E1, E2 and E3.
- *Thiothrix* correlates negatively with the removal of NP₂EC and NP₃₋₁₁EO.

8. REFERENCES

1. Abdel-Shafy, H.I. & Mansour, M.S.M., 2016. "A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation", *Egyptian Journal of Petroleum*, vol. 25, no. 1, pp. 107-123
2. Achermann, S., Bianco, V., Mansfeldt, C.B., Vogler, B., Kolvenbach, B.A., Corvini, P.F.X. & Fenner, K., 2018. "Biotransformation of sulphonamide antibiotics in activated sludge: the formation of pterin-conjugates leads to sustained risk", *Environ. Sci. & Technol.*, vol. 52, No. 11, pp. 6265-6274.
3. Achermann, S., Falås, P., Joss, A., Mansfeldt, C.B., Men, Y., Vogler, B. & Fenner, K., 2018. "Trends in Micropollutant Biotransformation along a Solids Retention Time Gradient", *Environ. Sci. Technol.*, vol. 52, pp. 11601-11611
4. Ahmed, Z., Cho, J., Lim, B., Song, K. & Ahn, K. 2007, "Effects of sludge retention time on membrane fouling and microbial community structure in a membrane bioreactor", *Journal of Membrane Science*, vol. 287, no. 2, pp. 211-218.
5. Akrotos, C.S. & Tsihrintzis, V.A., 2007. "Effect of temperature, HRT, vegetation and porous media on removal efficiency of pilot-scale horizontal subsurface flow constructed wetlands
6. Author links open overlay panel
7. Akarsubasi, A.T., Eyice, O., Miskin, I., Head, I.M. and Curtis, T.P. 2009, "Effect of sludge age on the bacterial diversity of bench scale sequencing batch reactors", *Environmental Science and Technology*, vol. 43, no. 8, pp. 2950-2956.
8. Aleksandra, Z., Sławomir, C., Anna, R. & Korneliusz, M. 2011, "Molecular analysis of microorganisms responsible for the first phase of nitrification in an anoxic environment", *Archives of Environmental Protection*, vol. 37, no. 1, pp. 89-98.
9. Almasi, A., Mousavi, S.A., Bahman, Z., Zolfaghari, M.R. & Zinatizadeh, A.A. 2016, "Effect of hydraulic retention time and aeration time on the performance and microbial diversity in an upflow aerobic/anoxic sequential bioreactor", *Desalination and Water Treatment*, vol. 57, no. 50, pp. 23589-23596.
10. Amin, M.M., Bina, B., Ebrahim, K., Yavari, Z. & Mohammadi, F. 2017, "Biodegradation of natural and synthetic estrogens in moving bed bioreactor", *Chinese Journal of Chemical Engineering*.
11. Amorim, C.L., Moreira, I.S., Maia, A.S., Tiritan, M.E. & Castro, P.M.L., 2014. "Biodegradation of ofloxacin, norfloxacin, and ciprofloxacin as single and mixed substrates by *Labrys portucalensis* F11", vol. 98, no. 7, pp. 3181-3190
12. APHA 2012, "Standard Methods for the Examination of Water and Wastewater". American Public Health Association, 22nd edition, Washington. D.C.
13. Aris A.Z., Shamsuddin A.S. & Praveena S.M., 2014, "Occurrence of 17 α -ethinylestradiol (EE2) in the environment and effect on exposed biota: a review", *Environ Int.*
14. Armstrong, D.L., Lozano, N., Rice, C.P., Ramirez, M. & Torrents, A. 2018, "Degradation of triclosan and triclocarban and formation of transformation products in activated sludge using benchtop bioreactors", *Environmental Research*, vol. 161, pp. 17-25.
15. Bai, N., Abuduaini, R., Wang, S., Zhang, M., Zhu, X. & Yuhua Zhao, Y., 2017, "Nonylphenol biodegradation characterizations and bacterial composition analysis of an effective consortium NP-M2", *Environmental Pollution*, vol. 220, pp. 95-104.
16. Barber, L.B., Loyo-Rosales, J.E., Rice, C.P., Minarik, T.A. & Oskouie, A.K. 2015, "Endocrine disrupting alkylphenolic chemicals and other contaminants in wastewater treatment plant effluents, urban streams, and fish in the Great Lakes and Upper Mississippi River Regions", *Science of the Total Environment*, vol. 517, pp. 195-206.
17. Bell, G., 2000. "The distribution of abundance in neutral communities", *The American Naturalist* vol. 155, pp. 606-617.

18. Bellucci, M., Ofițeru, I.D., Graham, D.W., Head, I.M. & Curtis, T.P. 2011, "Low-dissolved-oxygen nitrifying systems exploit ammonia-oxidizing bacteria with unusually high yields", *Applied and Environmental Microbiology*, vol. 77, no. 21, pp. 7787-7796.
19. Beneduce, L., Spano, G., Lamacchia, F., Bellucci, M., Consiglio, F. & Head, I.M. 2014, "Correlation of seasonal nitrification failure and ammonia-oxidizing community dynamics in a wastewater treatment plant treating water from a saline thermal spa", *Annals of Microbiology*, vol. 64, no. 4, pp. 1671-1682.
20. Berggren, D. R. V., Marshall, I. P. G., Azizian, M. F., Spormann, A. M., Semprini, L., 2013. "Effects of sulfate reduction on the bacterial community and kinetic parameters of a dechlorinating culture under chemostat growth conditions", *Environ. Sci. Technol.*, vol. 47, NO. 4, pp. 1879 – 1886.
21. Bhatia, A., Singh, N.K., Bhando, T., Pathania, R. & Kazmi, A.A. 2017, "Effect of intermittent aeration on microbial diversity in an intermittently aerated IFAS reactor treating municipal wastewater: A field study", *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, vol. 52, no. 5, pp. 440-448.
22. Bixio, D., Thoeue C., De Koning, J., Joksimovic, D., Savic, D., Wintgensd, T. & Melin, T., 2006, "Wastewater reuse in Europe", *Desalination*, vol. 187, no. 1–3, 5, pp. 89-101
23. Blanchfield, P.J., Kidd, K.A., Docker, M.F., Palace, V.P., Park, B.J. and Postma, L.D. 2015, "Recovery of a wild fish population from whole-lake additions of a synthetic estrogen", *Environmental Science and Technology*, vol. 49, no. 5, pp. 3136-3144.
24. Boer, J. L., Mulrooney, S.B. & Hausinger, R.P., 2014. "Nickel-dependent metalloenzymes", *Arch. Biochem. Biophys.*, vol. 544, pp. 142 – 152
25. Bowen, E.J., Dolfig, J., Davenport, R.J., Read, F.L. & Curtis, T.P. 2014, "Low-temperature limitation of bioreactor sludge in anaerobic treatment of domestic wastewater", *Water Science and Technology*, vol. 69, no. 5, pp. 1004-1013.
26. Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, I., Knight, R., Mills, D. A., Caporaso, J. G. 2013, "Quality Filtering Vastly improves Diversity Estimates from Illumina Amplicon Sequencing", *Nat Methods*, vol. 10, no. 1, pp. 57-59.
27. Bozzuto, C. & Blanckenhorn, W.U., 2017. "Taxonomic resolution and treatment effects – alone and combined – can mask significant biodiversity reductions", *International Journal of Biodiversity Science, Ecosystem Services & Management*, vol. 13, No. 1, pp. 86-99.
28. Bragg, L. M., Stone, G., Butler, M.K., Hugenholtz, P. & Tyson, G.W., 2013. "Shining a Light on Dark Sequencing: Characterising Errors in Ion Torrent PGM Data", vol. 9, no. 4
29. Cadwell, D.J., Mastrocco, F., Anderson, P.D., Lange, R., Sumpter, J.P., Aldwell, D.A.J.C., Astrocco, F.R.M., Nderson, P.A.U.L.D.A., Einhard, R.L.A., 2012. "Predicted No-Effect Concentration for Steroid Estrogens: Estrone, 17b-Estradiol, Estriol and 17a- Ethinylestradiol", *Environ. Toxicol. Chem.*, vol. 31, No. 6, pp. 1396-1406
30. Carvalheira, M., Oehmen, A., Carvalho, G. & Reis, M.A.M. 2014, "The effect of substrate competition on the metabolism of polyphosphate accumulating organisms (PAOs)", *Water Research*, vol. 64, pp. 149-159.
31. Chase, J.M., & Leibold, M.A., 2003. "Ecological Niches Linking Classical and Contemporary Approaches", Chicago: University of Chicago Press.
32. Chen, J., Huang, X. & Lee, D. 2008, "Bisphenol A removal by a membrane bioreactor", *Process Biochemistry*, vol. 43, no. 4, pp. 451-456.
33. Chen, Y., Ye, L., Zhao, F., Xiao, L., Cheng, S. & Zhang, X.-. 2017, "Bacterial community shift during the start-up of a full-scale oxidation ditch treating sewage", *Journal of Microbiology and Biotechnology*, vol. 27, no. 1, pp. 141-148.
34. Cheng, C., Hung, C., Lee, K., Liao, P., Liang, C., Yang, L., Lin, P. & Lin, C. 2008, "Microbial community structure of a starch-feeding fermentative hydrogen production reactor operated

- under different incubation conditions", *International Journal of Hydrogen Energy*, vol. 33, no. 19, pp. 5242-5249.
35. Chu, C., Chen, Q., Wang, C., Wang, H., Sun, Z., He, Q., He, J. & Gu, J. 2016, "*Roseomonas chloroacetimidivorans* sp. nov., a chloroacetamide herbicide-degrading bacterium isolated from activated sludge", *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, vol. 109, no. 5, pp. 611-618.
 36. Clara, M., Kreuzinger, N., Strenn, B., Gans, O. and Kroiss, H., 2005. "The solids retention time - A suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants", *Water Research*, vol. 39, no. 1, pp. 97-106.
 37. Coello-Garcia T., Curtis, T.P., Mroziuk, W. & Davenport, R.J., 2019. "Enhanced estrogen removal in activated sludge processes through the optimization of the hydraulic flow pattern," *Water Research*, vol. 164
 38. Coello-Garcia, T., 2018. Influence of microbial community structure and operational parameters on the degradation of estrogens in activated sludge processes", *PhD Thesis, Newcastle University*
 39. Comber, S., Gardner, M., Somner, P., Leverett, D. & Ellor, B., 2018. "Active Pharmaceutical Ingredients Entering the Aquatic Environment From Wastewater Treatment Works: A Cause for Concern?", *Science of the Total Environ.*, vol. 613-614, pp. 518-547.
 40. Comber, S., Gardner, M., Somner, P. & Ellor, B., 2019. "The Removal of Pharmaceuticals During Wastewater Treatment: Can it be Predicted Accurately?", *Sci. Of the Total Environment*, vol. 676, pp. 222-230.
 41. Corvini, P.F.X., Hollender, J., Ji, R., Schumacher, S., Prell, J., Hommes, G., Priefer, U., Vinken, R., and Schäffer, A., 2006. "The degradation of α -quaternary nonylphenol isomers by *Sphingomonas* sp. strain TTNP3 involves a type II ipso-substitution mechanism", *Applied Microbiology and Biotechnology*, vol. 70, pp. 114-122.
 42. Curds C.R., Cockburn A. and Vandyke J.M. 1968. "An experimental study of the role of the ciliated protozoa in the activated sludge process", *Water Pollution Control*, vol. 67, pp. 312-329
 43. Curtis, T.P., Head, I.M., Lunn, M., Woodcock, S., Schloss, P.D., Sloan, W.T., 2006. "What Is the Extent of Prokaryotic Diversity?", *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 361, pp. 2023-2037
 44. Cydzik-Kwiatkowska, A., Rusanowska, P., Zielińska, M., Bernat, K. & Wojnowska-Baryła, I. 2014, "Structure of nitrogen-converting communities induced by hydraulic retention time and COD/N ratio in constantly aerated granular sludge reactors treating digester supernatant", *Bioresource Technology*, vol. 154, pp. 162-170.
 45. Domaradzka, D., Guzik, U., Hupert-Kocurek, K. & Wojcieszynska, D., 2015. "Cometabolic Degradation of Naproxen by *Planococcus* sp. Strain S5", *Water, Air & Soil Pollution*,
 46. Damien, H.G., Batstone, J. & Keller, J., 2015. "Biological phosphorus removal from abattoir wastewater at very short sludge ages mediated by novel PAO clade Comamonadaceae", *Water Research*, vol. pp. 173-182.
 47. De Gussemé, B., Vanhaecke, L., Verstraete, W. & Boon, N. 2011, "Degradation of acetaminophen by *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* in a membrane bioreactor", *Water Research*, vol. 45, no. 4, pp. 1829-1837.
 48. Dharmadasa, K.H.S.M., Chairuangsi, S. & Muthukudaarachchi, K.H., 2018. "A Simplified Mathematical Model to Determine the Degradation Coefficient and Order of Decay for Activated Sludge Process", *International Forestry and Environment Symposium*, vol. 23
 49. Duan, L., Tian, Y., Liu, X., Song, Y., Yang, L., & Zhang, J., 2015. "Comparison between moving bed-membrane bioreactor and conventional membrane bioreactor systems. Part II: bacterial community, *Environmental Earth Sciences*, vol. 73, pp. 4891 - 4902
 50. Edwards, J., Santos-Medellin, C. & Sundaresan, V., 2018. "Extraction and 16S rRNA Sequence Analysis of Microbiome associated with Rice Roots", *Bio-protocol*, vol. 8, no. 12
 51. Fenner, K., Johnson, D.R., & Helbling, D.E., 2013, "Association of micropollutant biotransformation potential of WWTP microbial communities with process and community

- characteristics", in: *8th IWA Specialised Conference on Assessment and control of micropollutants and hazardous substances in water, 16 – 20 June 2013, Zurich*, pp. 18 – 19.
52. Feranchuk, S., Belkova, N., Potapova, U., Kuzmin, D. & Belikov, S., 2018. "Evaluating the use of diversity indices to distinguish between microbial communities with different traits", *Research in Microbiology*, vol. 169, no. 4-5, pp. 254-261
 53. Fitzgerald, C.M., Camejo, P., Oshlag, J.Z. & Noguera, D.R. 2015, "Ammonia-oxidizing microbial communities in reactors with efficient nitrification at low-dissolved oxygen", *Water Research*, vol. 70, pp. 38-51.
 54. Fornasier, F., Ascher, J., Ceccherini, M.T., Tomat, E. & Pietramellara, G., 2014. "A simplified rapid, low-cost and versatile DNA-based assessment of soil microbial biomass", *Ecological Indicators*, vol. 45, pp. 75-82.
 55. Forney, L.J., Liu, W-T, Guckert, JB, Kumagai, Y, Namkung, E, Nishihara, T & Larson, RJ 2001, 'Structure of microbial communities in activated sludge: Potential implications for assessing the biodegradability of chemicals', *Ecotoxicology and Environmental Safety*, vol. 49, no. 1, pp. 40-53.
 56. Forster, S., Snape, J. R., Lappin-Scott, H. M., Porter, J., 2002. "Simultaneous Fluorescent Gram Staining and Activity Assessment of Activated Sludge Bacteria" *Microbial Ecology* vol. 68 no. 10 pp. 4772-4779
 57. Fredriksson, J., 2013. "Investigations of the microbial diversity and dynamics in activated sludge using molecular methods", PhD Thesis, Chalmers University of Technology
 58. Frostegård, Å., Bååth, E. & Tunlid, A. 1993, "Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis", *Soil Biology and Biochemistry*, vol. 25, no. 6, pp. 723-730.
 59. Frostegård, A. and Baath, E., 1996, "The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil", *Biology and Fertility of Soils*, Vol. 22 (1–2), pp. 59–65
 60. Frostegård, A., Tunlid, A. & Bååth, E., 2011. "Use and misuse of PLFA measurements in soils", *Soil Biology and Biochemistry*, vol. 43, no. 8, pp. 1621-1625
 61. Fujii, K., Kikuchi, S., Satomi, M., Ushio-Sata, N., & Morita, N., 2002. "Degradation of 17 β estradiol by a Gram-negative bacterium isolated from activated sludge in a sewage treatment plant in Tokyo", *Japan. Appl. Environ. Microbiol.* 68, 2057–2060.
 62. Gabriel, F. L. P., Giger, W., Guenther, K., & Kohler, H-P. E., 2005. Differential Degradation of Nonylphenol Isomer by *Sphingomonas xenophaga* Bayram", *Appl. And Environmental Microbiology*, vol. 71, no 3, pp. 1123-1129.
 63. Galapate, R. P., Agustiani, E., Baes, A. U., Ito, K., Okada, M., 1999. "Effect of HRT and MLSS on THM precursor removal in the activated sludge process", *Water Research*, vol. 33, pp. 131-136.
 64. Gannoun, H., Khelifi, E., Omri, I., Jabari, L., Fardeau, M.-., Bouallagui, H., Godon, J.-. & Hamdi, M. 2013, "Microbial monitoring by molecular tools of an upflow anaerobic filter treating abattoir wastewaters", *Bioresource Technology*, vol. 142, pp. 269-277.
 65. Gao, R., Cao, Y., Yuan, X., Zhu, W., Wang, X. & Cui, Z. 2012, "Microbial diversity in a full-scale anaerobic reactor treating high concentration organic cassava wastewater", *Afr J Biotechnol*, vol. 11, no. 24, pp. 6494-6500.
 66. Gardner, M., Comber, S., Scrimshaw, M. D., Cartmell, E., Lester, J. and Ellor, B. 2012, "The significance of hazardous chemicals in wastewater treatment works effluents", *Science of the Total Environment*, vol. 437, pp. 363-372.
 67. Gallardo-Altamirano, M.J., Maza-Márquez, P., Montemurro, N., Rodelas, B., Osorio, F. & Pozo, C. 2019, "Linking microbial diversity and population dynamics to the removal efficiency of pharmaceutically active compounds (PhACs) in an anaerobic/anoxic/aerobic (A2O) system", *Chemosphere*, vol. 233, pp. 828-842.
 68. Gardner, M., Jones, V., Comber, S., Scrimshaw, M.D., Coello-Garcia T., Cartmell, E., Lester, J. & Ellor, B. 2013, "Performance of UK wastewater treatment works with respect to trace contaminants", *Science of the Total Environment*, vol. vol. 456-457, pp. 359–369.

69. Gaulke, L.S., Strand, S.E., Kalhorn, T.F., Stensel, H.D., 2008. 17 α -ethinylestradiol transformation via abiotic nitration in the presence of ammonia-oxidizing bacteria. *Environ. Sci. Technol.* Vol. 42, pp. 7622–7627.
70. Gómez-López, M.D., Bayo, J., García-Cascales, M.S. & Angosto, J.M. 2009, "Decision support in disinfection technologies for treated wastewater reuse", *Journal of Cleaner Production*, vol. 17, no. 16, pp. 1504-1511.
71. Gori, R., Cammilli, L., Petrovic, M., Gonzalez, S., Barcelò, D., Lubello, C. & Malpei, F. 2010, "Fate of surfactants in membrane bioreactors and conventional activated sludge plants", *Environmental Science and Technology*, vol. 44, no. 21, pp. 8223-8229.
72. Gros, M., Petrović, M., Ginebreda, A. & Barceló, D. 2010, "Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes", *Environment International*, vol. 36, no. 1, pp. 15-26.
73. Gruber-Dorninger, C., Pester, M., Kitzinger, K., Savio, D.F., Loy, A., Rattei, T., Wagner, M. & Daims, H. 2015, "Functionally relevant diversity of closely related *Nitrospira* in activated sludge", *ISME Journal*, vol. 9, no. 3, pp. 643-655.
74. Güven, H., Ersahin, M.E., Dereli, R.K., Ozgun, H., Suncar, D. & Ozturk, I., 2017. "Effect of Hydraulic Retention Time on the Performance of High-Rate Activated Sludge System: a Pilot-Scale Study", *Water Air and Soil Pollution*, vol. 228, no. 417, pp. 1-10.
75. Hamady, M., Knight, R., 2009. "Microbial Community Profiling for Human Microbiome Projects: Tools, Techniques and Challenges", *Genome Res*, Vol. 303 pp. 1141-1152
76. Han, H., Zhang, Y., Cui, C., Zheng, S., 2010. "Effect of COD level and HRT on microbial community in a yeast-predominant activated sludge system", *Bioresource Technology*, vol. 101, no. 10, pp. 3463-3465.
77. Haegeman, B., Hamelin, J., Moriarty, J., Neal, P., Dushoff, J., & Weitz, J.S., 2013. "Robust estimation of microbial diversity in theory and in practice", *ISME Journal*, vol. 7. pp. 1091-1102
78. Hao X., Heijnen J.J. & Van Loosdrecht M.C., 2002. "Model-based evaluation of temperature and inflow variations on a partial nitrification-anammox biofilm process. *Water Research*, vol 36, pp. 4839–4849.
79. Harris, J.A., Baptista, J.D.C., Curtis, T.P., Nelson, A.K., Pawlett, M., Ritz, K., and Tyrrel, S.F. 2012, "Engineering difference: Matrix design determines community composition in wastewater treatment systems", *Ecological Engineering*, vol. 40, pp. 183-188.
80. Hay, A.G., Dees, P.M. & Saylor, G.S., 2001. "Growth of a bacterial consortium on triclosan", *FEMS Microbiology Ecology*, vol. 36, no. 2-3, pp. 105–112
81. Helbling, D.E., Johnson, D.R., Honti, M. and Fenner, K., 2012, "Micropollutant biotransformation kinetics associate with WWTP process parameters and microbial community characteristics", *Environmental Science and Technology*, vol. 46, no. 19, pp. 10579-10588.
82. Hendrickx, T. L. G., Kampman, C., Zeeman, G., Temmink, H., Hu, Z., Kartal, B. & Buisman, C. J. N., 2014. "High specific activity for anammox bacteria enriched from activated sludge at 10°C", *Bioresource Technology*, vol. 163, pp. 214-221.
83. Henriët, O., Meunier, C., Henry P. & Mahillon, J., 2017, "Filamentous bulking caused by *Thiothrix* species is efficiently controlled in full-scale wastewater treatment plants by implementing a sludge densification strategy, *Scientific Reports*, vol. 7.
84. Ho, D., Jensen, P. & Batstone, D. 2014, "Effects of temperature and hydraulic retention time on acetotrophic pathways and performance in high-rate sludge digestion", *Environmental Science and Technology*, vol. 48, no. 11, pp. 6468-6476.
85. Hien Ho 1 and Tsunemi Watanabe, T., 2017. "Distribution and Removal of Nonylphenol Ethoxylates and Nonylphenol from Textile Wastewater—A Comparison of a Cotton and a Synthetic Fiber Factory in Vietnam", *Water*, vol. no. pp.
86. Hope, B. K., Pillsbury, L., Boling, B., 2012. "A state-wide survey in Oregon (USA) of trace metals and organic chemicals in municipal effluent", *Science of The Total Environment*, vol. 417–418, pp. 263-272.

87. Hu, M., Wang, X., Wen, X. & Xia, Y. 2012, "Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis", *Bioresource Technology*, vol. 117, pp. 72-79.
88. Hubbell, S.P., 2001. "The unified neutral theory of biodiversity and biogeography" Princeton Univ Dept of Arts & Sciences.
89. Isazadeh, S., Jauffur, S. & Frigon, D. 2016, "Bacterial community assembly in activated sludge: mapping beta diversity across environmental variables", *MicrobiologyOpen*, vol. 5, no. 6, pp. 1050-1060.
90. Ito, A., Mensah, L., Cartmell, E. & Nester, J.N. 2016, "Removal of steroid estrogens from municipal wastewater in a pilot scale expanded granular sludge blanket reactor and anaerobic membrane bioreactor", *Environmental Technology*, vol. 37, no. 3, pp. 415-421
91. Jarling, R., Kuehner, S., Janke, E. B., Gruner, A., Drozdowska, M., Golding, B. T., Rabus, R. & Wilkes, H., 2015. "Versatile transformations of hydrocarbons in anaerobic bacteria: substrate ranges and regio- and stereo-chemistry of activation reactions", *Front. Microbiol.* Vol. 6.
92. Janda, J.M. & Abbott, S.L., 2007, "16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls", *American Society for Microbiology*, vol. 45, No. 9, pp. 2761 -2764
93. Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M. & Barcelo, D. 2011, "Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment", *Water Research*, vol. 45, no. 3, pp. 1165-1176.
94. Jiang, Y., Qu, Y., Xu, P. & Tanga, H., 2015. "Genome Sequence of a Versatile Aromatic Hydrocarbon-Degrading Bacterium, *Arthrobacter* sp. W1", *Genome Announcements*, vol. 3
95. Jo, S.J., Kwon, H., Jeong, S.-., Lee, C.-. & Kim, T.G. 2016, "Comparison of microbial communities of activated sludge and membrane biofilm in 10 full-scale membrane bioreactors", *Water Research*, vol. 101, pp. 214-225.
96. Johnson, J.S., Spakowicz, D.J., Hong, B-Y., Petersen, L.M., Demkowicz, P., Chen, L., Leopold, S.R., Hanson, B.M., Agresta, H.O., Gerstein, M., Sodergren, E. & Weinstock, G.M., 2019. "Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis", *Nature Communications*, vol. 10, pp. 1-11.
97. Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., Mc Ardell, C.S., Ternes, T.A., Thomsen, A. and Siegrist, H. 2006, "Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme", *Water Research*, vol. 40, no. 8, pp. 1686-1696.
98. Ju, F. & Zhang, T., 2015. "Bacterial assembly and temporal dynamics in activated sludge of a full-scale municipal wastewater treatment plant", *The ISME Journal*, vol. 9, pp. 683–695
99. Kagle, J., Porter, A.W., Murdoch, R.W., Rivera-Cancel, G. & Hay, A.G., 2009. "Biodegradation of Pharmaceutical and Personal Care Products", *Advances in Appl Microbiol.*, vol. 67, pp. 65-108.
100. Kargi, F., & Eker, S., 2006. "Effect of sludge age on performance of an activated sludge unit treating 2,4 dichlorophenol-containing synthetic wastewater", *Enzyme and Microbial Technology*, vol. 38, no. 1–2, pp. 60-64.
101. Kessel, M., Speth, D., Albertsen, M., Nielsen, P., Op den Camp, H., Kartal, B., Jetten, M. & Lücker, S., 2015. "Complete nitrification by a single microorganism", *Nature*, vol. 528
102. Khelifi, E., Bouallagui, H., Fardeau, M., Touhami, Y., Godon, J., Cayol, J., Ollivier, B. & Hamdi, M. 2009, "Fermentative and sulphate-reducing bacteria associated with treatment of an industrial dye effluent in an up-flow anaerobic fixed bed bioreactor", *Biochemical engineering journal*, vol. 45, no. 2, pp. 136-144.
103. Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M. & Flick, R.W. 2007, "Collapse of a fish population after exposure to a synthetic estrogen", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 21, pp. 8897-8901.
104. Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S. & Aga, D.S. 2005, "Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process", *Environmental Science and Technology*, vol. 39, no. 15, pp. 5816-5823.

105. Knapp, C. W. and Graham, D. W., 2007. "Nitrite-oxidizing bacteria guild ecology associated with nitrification failure in a continuous-flow reactor", *FEMS Microbiology Ecology*, vol. 62, no. 2, pp. 195-201.
106. Koh, Y.K.K., Chiu, T.Y., Boobis, A.R., Scrimshaw, M.D., Bagnall, J.P., Soares, A., Pollard, S., Cartmell, E. & Lester, J.N. 2009, "Influence of operating parameters on the biodegradation of steroid estrogens and nonylphenolic compounds during biological wastewater treatment processes", *Environmental Science and Technology*, vol. 43, no. 17, pp. 6646-6654.
107. Koivuranta, E., Suopajarvi, T., Hattuniemi, J., Stoor, T. & Illikainen, M. 2017, "The effect of seasonal variations on floc morphology in the activated sludge process", *Environmental Technology* (United Kingdom), vol. 38, no. 24, pp. 3209-3215.
108. Kostich, M.S., Batt, A.L. & Lazorchak, J.M., 2014. "Concentrations of prioritized pharmaceuticals in effluents from 50 large wastewater treatment plants in the US and implications for risk estimation", *Environmental Pollution*, vol. 184, pp. 354-359
109. Kreuzinger, N.; Clara, M.; Strenn, B.; Kroiss, H. 2004. "Relevance of the sludge retention time (SRT) as design criteria for wastewater treatment plants for the removal of endocrine disruptors and pharmaceuticals from wastewater", *Water Sci. Technol.*, vol. 50, pp. 149-156.
110. Kruglova, A., Gonzalez-Martinez, A., Kråkström, M., Mikola, A. & Vahala, R. 2017, "Bacterial diversity and population shifts driven by spotlight wastewater micropollutants in low-temperature highly nitrifying activated sludge", *Science of the Total Environment*, vol. 605-606, pp. 291-299.
111. Kumar, V., Nakada, N., Yamashita, N., Johnson, A.C. & Tanaka, H. 2015, "Influence of Hydraulic Retention Time, Sludge Retention Time, and Ozonation on the Removal of Free and Conjugated Estrogens in Japanese Activated Sludge Treatment Plants", *Clean - Soil, Air, Water*, vol. 43, no. 9, pp. 1289-1294.
112. Kurisu, F., Ogura, M., Saitoh, S., Yamazoe, A., Yagi, O., 2010. Degradation of natural estrogen and identification of the metabolites produced by soil isolates of *Rhodococcus sp.* and *Sphingomonas sp.* *J. Biosci. Bioeng.*, vol. 109, pp. 576–582.
113. Langenhoff, A., Inderfurth, N., Veuskens, T., Schraa, G., Blokland, M., Kujawa-Roeleveld, K. & Riknaarts, H., 2013. "Microbial Removal of the Pharmaceutical Compounds Ibuprofen and Diclofenac from Wastewater", *BioMed Research International*, vol. 10.
114. Langford, K.H., Scrimshaw, M.D., Birkett, J.W. & Lester, J.N. 2005, "Degradation of nonylphenolic surfactants in activated sludge batch tests", *Water Research*, vol. 39, no. 5, pp. 870-876.
115. Larcher, S. & Yargeau, V., 2013, "Biodegradation of 17 α -ethinylestradiol by heterotrophic bacteria", *Environmental Pollution*, vol. 173, pp. 17-22.
116. Larsen, P., Nielsen, J.L., Svendsen, T.C., Nielsen, P.H., 2008, "Adhesion characteristics of nitrifying bacteria in activated sludge", *Water Research*, vol. 42, no. 10-11, pp. 2814-2826.
117. Lee, D.G., Zhao, F., Rezenom, Y.H., Russell, D.H. & Chu, K. 2012, "Biodegradation of triclosan by a wastewater microorganism", *Water Research*, vol. 46, no. 13, pp. 4226-4234.
118. Lee, S.-., Kang, H.-. & Park, H.-. 2015, "Influence of influent wastewater communities on temporal variation of activated sludge communities", *Water Research*, vol. 73, pp. 132-144.
119. Li, A.-., Yang, S.-., Li, X.-. & Gu, J.-. 2008, "Microbial population dynamics during aerobic sludge granulation at different organic loading rates", *Water Research*, vol. 42, no. 13, pp. 3552-3560.
120. Li, C., Lu, J., Liu, J., Zhang, G., Tong, Y. & Ma, N., 2016. "Exploring the correlations between antibiotics and antibiotic resistance genes in the wastewater treatment plants of hospitals in Xinjiang, China", *Environmental Science and Pollution Research*, vol. 23, no. 15, pp. 15111–15121
121. Li, H.-., Zhang, Y., Gao, F., Yu, T. & Yang, M. 2006, "Effects of hydraulic retention time (HRT) on nitrification performance and microbial community of conventional activated sludge (CAS)", *Huanjing Kexue/Environmental Science*, vol. 27, no. 9, pp. 1862-1865.

122. Li, H., Zhang, Y., Yang, M. & Kamagata, Y. 2013, "Effects of hydraulic retention time on nitrification activities and population dynamics of a conventional activated sludge system", *Frontiers of Environmental Science and Engineering in China*, vol. 7, no. 1, pp. 43-48.
123. Li, S., Liu, J., Sun, M., Ling, W. & Zhu, X. 2017, "Isolation, characterization, and degradation performance of the 17 β -estradiol-degrading bacterium *Novosphingobium* sp. E2S", *Int. J. of Environmental Research and Public Health*, vol. 14, no. 2.
124. Li, X.-., Ma, K.-., Meng, L.-., Zhang, J. & Wang, K. 2014, "Performance and microbial community profiles in an anaerobic reactor treating with simulated PTA wastewater: From mesophilic to thermophilic temperature", *Water Research*, vol. 61, pp. 57-66.
125. Li, B. & Wu, G., 2014. "Effects of Sludge Retention Times on Nutrient Removal and Nitrous Oxide Emission in Biological Nutrient Removal Processes", *Int J Environ Res Public Health*, vol. 11, no. 4, pp. 3553–3569.
126. Li, Y.M., Zeng, Q.L. & Yang, S.J., 2011. "Removal and fate of estrogens in an anaerobic-anoxic-oxic activated sludge system" *Water Science and Technology*, vol. 63, No. 1. pp. 51-56.
127. Liang, H., Ye, D. and Luo L. 2017, "Unravelling diversity and metabolic potential of microbial consortia at each stage of leather sewage treatment", *J. of Royal Society of Chemistry*, vol. 7, pp. 41727-41737
128. Lores M., Gomez-Brandon M., Dominiguez J., 2010 "Tracking down microbial communities via fatty acid analysis: analytical strategy for solid organic samples" *Current research, Technology and Education, Topic in Applied Microbiology and Microbial Biotechnology*, Mendez-Villas (Ed.)
129. Lozada, M., Itria, R.F., Figuerola, E.L.M., Babay, P.A., Gettar, R.T., De Tullio, L.A. & Erijman, L. 2004, "Bacterial community shifts in nonylphenol polyethoxylates-enriched activated sludge", *Water Research*, vol. 38, no. 8, pp. 2077-2086.
130. Lui, X., Yang, Y. and Freeman, D., 2007, "Laboratory Evaluation of relationships between solid retention time, microbial diversity and settling characteristics of activated sludge", *Proceedings of the Water Environment Federation*, WEFTEC Session 31-40, pp.2517.
131. Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D. & Hai, F.I., 2014. "A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment", *Science of the Total Environment*, vol. 473-474 pp. 619-641
132. Lust, M.J., Ziels, R.M., Strand, S.E., Gough, H.L. & Stensel, H.D., 2015. "Biodegradation Kinetics of 17 α -Ethinylestradiol in Activated Sludge Treatment Processes", *Environmental Engineering Science*, vol. 32, no. 7
133. Ma, C., Qin, D., Sun, Q., Zhang, F., Liu, H. & Yu, C.-. 2016, "Removal of environmental estrogens by bacterial cell immobilization technique", *Chemosphere*, vol. 144, pp. 607-614.
134. Ariel Marchlewicz, A., Dorota Domaradzka, D., Urszula Guzik, U. & Danuta Wojcieszńska, D., 2016. "Bacillus thuringiensis B1(2015b) is a Gram-Positive Bacteria Able to Degrade Naproxen and Ibuprofen", *Water, Air & Soil Pollution*, Open Acces
135. Magurran, A. E., 1988. "Why Diversity? In *Ecological Diversity and Its Measurement*", Springer Netherlands: Dordrecht, pp 1–5.
136. Manickum, T. and John, W., 2014. "Occurrence, fate and environmental risk assessment of endocrine disrupting compounds at the wastewater treatment works in Pietermaritzburg (South Africa)", *Science of The Total Environment*, vol.468–469, pp. 584-597.
137. Marathe, N.P., Shetty, S.A., Shouche, Y.S. & Larsson, D.G.J. 2016, "Limited bacterial diversity within a treatment plant receiving antibiotic containing waste from bulk drug production", *PLoS ONE*, vol. 11, no. 11.
138. McAdam, E.J.; Bagnall, J.P.; Koh, Y.K.K.; Chiu, T.Y.; Pollard, S.; Scrimshaw, M.D.; Lester, J.N.; Cartmell, E., 2010. "Removal of steroid estrogens in carbonaceous and nitrifying activated sludge processes", *Chemosphere*, vol. 81, 1-6.

139. McLellan, S. L., Huse, S. M., Mueller-Spitz, S. R., Andreishcheva, E. N. & Sogin, M. L., 2010. "Diversity and population structure of sewage-derived microorganisms in wastewater treatment plant influent", *Environmental Microbiology*, vol. 12, no. 2, pp. 378-392.
140. Men, Y., Han, P., Helbling, D.E., Jehmlich, N., Herbold, C., Gulde, R., Onnis-Hayden, A., Gu, A.Z., Johnson, D.R., Wagner, M. & Fenner, K., 2016. "Biotransformation of Two Pharmaceuticals by the Ammonia-Oxidizing Archaeon *Nitrososphaera gargensis*", *Environ. Sci. Technol.*, vol. 50, No. 9, pp. 4682-4692.
141. Mohamed, E.F., Andriantsiferana, C., Wilhelm, A.M. & Delmas, H., 2011. "Competitive adsorption of phenolic compounds from aqueous solution using sludge-based activated carbon", *Environmental Technology*, vol. 11-12, pp. 1325-1336.
142. Muller, M., Patureau, D., Godon, J.J., Delgenes, J.P., Hernandez-Raquet, G., 2010. "Molecular and kinetic characterization of mixed cultures degrading natural and synthetic estrogens", *Appl. Microbiol. Biotechnol.*, vol. 85, pp. 691–701.
143. Muyzer, G., De Waal, E.C. & Uitterlinden, A.G. 1993, "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA", *Applied and Environmental Microbiology*, vol. 59, no. 3, pp. 695-700.
144. Nagendra, H. 2002. Opposite trends in response for the Shannon and Simpson indices of landscape diversity", *Applied Geography*, vol. 22, no. 2, pp. 175-186
145. Napierska, D., Sanseverino, I., Loos, R., Cortés, L. G., Niegowska, M., & Lettieri, T., 2018. "Modes of action of the current Priority Substances list under the Water Framework Directive and other substances of interest" *JRC Technical Reports*, European Commission EUR 29008 EN.
146. Navas-Molina, J.A., Peralta-Sanchez, J.M., McMurdie, P.J., Vazquez-Baeza, Y., Xu, Z., Ursell, L.K., Lauber, C., Zhou, H., Song, S.J., Huntley, J., Ackermann, G.L., Berg-Lyons, D., Holmes, S., Caporaso, J.G., & Knight, R., 2013, "Advancing Our Understanding of the Human Microbiome Using QIIME", *Methods Enzymol.* Vol. 531, pp. 371–444
147. Ni, B., Zhang, Y., Chen, D.W., Wang, B.J. & Liu, S.J., 2013. "Assimilation of aromatic compounds by *Comamonas testosteroni*: characterization and spreadability of protocatechuate 4,5-cleavage pathway in bacteria. *Appl Microbiol Biotechnol*, vol. 97 no. 13, pp. 6031–6041.
148. Nielsen, P. H., De Muro, M. and Nielsen, J. L., 2001. "Studies of the *in situ* physiology of *Thiothrix* spp. present in activated sludge", *Environmental Microbiology*, vol. 2, no. 4, pp. 389-398.
149. Noophan, P., Phanwilai, S., Kasahara, T., Munakata-Marr, J. and Figueroa, L. A., 2017. "Comparison of Nitrogen Removal and Full-Scale Wastewater Treatment Plant Characteristics in Thailand and Japan", *Environment Asia*, vol. 10, no. 1, pp. 92-98.
150. Niu, L., Li, Y., Wang, P., Zhang, W., Wang, C. & Wang, Q. 2015, "Understanding the linkage between elevation and the activated-sludge bacterial community along a 3,600-meter elevation gradient in China", *Applied and Environmental Microbiology*, vol. 81, no. 19, pp. 6567-6576.
151. Niu T., Zhou Z., Shen X., Qiao W., Jiang L-M, Wei Pan W. & Zhou J., 2016 "Effects of dissolved oxygen on performance and microbial community structure in a micro-aerobic hydrolysis sludge in situ reduction process", *Water Research*, Vol 90, pp. 369-377.
152. Oehmen, A., Carvalho, G., Freitas F. & Reis, M. A., 2010. "Assessing the abundance and activity of denitrifying polyphosphate accumulating organisms through molecular and chemical techniques", *Water Science and Technology*, vol. 61 no. 8.
153. Ofiteru, I.D., Lunn, M., Curtis, T.P., Wells, G.F., Criddle, C.S., Francis, C.A., 2010. "Combined niche and neutral effects in a microbial wastewater treatment community", *Proc. Natl. Acad. Sci. U.S.A.* vol. 107, pp. 15345–15350.
154. Ouyang, E., Lu, Y., Ouyang, J., Wang, L. and Wang, X., 2017, "Bacterial community analysis of anoxic/aeration (A/O) system in a combined process for gibberellin wastewater treatment", *PLoS One*, vol. 12, no. 10, pp.1-15.

155. Paiva M.C., Ávila M.P., Reis M.P., Costa P.S., Nardi R.M.D., and Nascimento A.M.A., Editor: Aziz R.K., Editor, 2015, The Microbiota and Abundance of the Class 1 Integron-Integrase Gene in Tropical Sewage Treatment Plant Influent and Activated Sludge
156. Pauwels, B., Wille, K., Noppe, H., De Brabander, H., Van De Wiele, T., Verstraete, W. & Boon, N. 2008, "17 α -ethinylestradiol cometabolism by bacteria degrading estrone, 17 β -estradiol and estriol", *Biodegradation*, vol. 19, no. 5, pp. 683-693.
157. Pérez-Pantoja, D., Donoso, R., Junca, H., González, B. and Pieper, D. H. (2010), "Phylogenomics of aerobic bacterial degradation of aromatics", *Handbook of Hydrocarbon and Lipid Microbiology*, vol. 1, pp. 1355-1397.
158. Petrie, B., McAdam, E.J., Lester, J.N. & Cartmell, E. 2014, "Assessing potential modifications to the activated sludge process to improve simultaneous removal of a diverse range of micropollutants", *Water Research*, vol. 62, pp. 180 – 192.
159. Petrie, B., McAdam, E.J., Lester, J.N. & Cartmell, E. 2014, "Obtaining process mass balances of pharmaceuticals and triclosan to determine their fate during wastewater treatment", *Science of the Total Environment*, vol. 497, pp. 553-560.
160. Petrie, B., McAdam, E.J., Scrimshaw, M.D., Lester, J.N. & Cartmell, E. 2013, "Fate of drugs during wastewater treatment", *TrAC - Trends in Analytical Chemistry*, vol. 49, pp. 145-159.
161. Pholchan, M.K., Baptista, J. de C., Davenport, R.J., William T. Sloan, W.T., & Curtis, T.P., 2013. "Microbial community assembly, theory and rare functions", vol. *Front. Microbiol.*, vol. 4, pp. 1-9
162. Ponce, B.L., Latorre, V.K., Gonzalez, M. & Seeger, M., 2011. "Antioxidant compound improved PCB-degradation by Burkholderia xenovorans LB400, *Enzyme and Microbial Technology*, vol. 49, pp. 509 - 516
163. Poursat, B.A.J., van Spanning, R.J.M., Braster, M., Helmus, R., de Voogt, P., & Parsons, V.R., 2019. "Biodegradation of metformin and its transformation product, guanylurea, by natural and exposed microbial communities", *Ecotoxicology and Environmental Safety*, vol. 183
164. Qarni, H.A., Collier, P., O’Keeffe, J. & Akunna, J., 2016. "Investigating the removal of some pharmaceutical compounds in hospital wastewater treatment plants operating in Saudi Arabia", *Environ Sci Pollut Res Int*, vol. 23, pp. 13003–13014.
165. Quezada, M., Buitrón, G., Moreno-Andrade, I., Moreno, G. and López-Marín, L. M., 2007. "The use of fatty acid methyl esters as biomarkers to determine aerobic, facultatively aerobic and anaerobic communities in wastewater treatment systems", *FEMS Microbiology Letters*, vol. 266, no. 1, pp. 75-82.
166. Quince, C., Curtis, T.P. & Sloan, W.T. 2008, "The rationale exploration of microbial diversity", *The ISME Journal*, vol. 2, no. 10, pp. 997-1006.
167. Racz, L. & Goel, R.K. 2010, "Fate and removal of estrogens in municipal wastewater", *Journal of Environmental Monitoring*, vol. 12, no. 1, pp. 58-70.
168. Radjenović, J., Petrović, M. & Barceló, D. 2009, "Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment", *Water Research*, vol. 43, no. 3, pp. 831-841.
169. Rehman, A., Ayub, N., Naz, I. & Perveen, I., 2019 "Effects of Hydraulic Retention Time (HRT) on the Performance of a Pilot-Scale Trickling Filter System Treating Low-Strength Domestic Wastewater", *Polish Journal of Environmental Studies*, vol. 29, no. 1, pp. 1-11.
170. Ren, H., Ji, S., Ahmad, N.u.d., Wang, D., Cui, C., 2007. Degradation characteristics and metabolic pathway of 17 α -ethinylestradiol by *Sphingobacterium* sp. JCR5. *Chemosphere*, vol. 66, pp. 340–346.
171. Reyes, M., Borrás, L., Seco, A. & Ferrer, J. 2014, "Identification and quantification of microbial populations in activated sludge and anaerobic digestion processes", *Environmental Technology*, vol. 36, no. 1, pp. 45-53.
172. Rizvi, H., Ahmad, N., Abbas, F., Bukhari, I.H., Yasar, A., Ali, S., Yasmeen, T. and Riaz, M. 2014, "Start-up of UASB reactors treating municipal wastewater and effect of temperature/sludge age and hydraulic retention time (HRT) on its performance", *Arabian Journal of Chemistry*

173. Roh, H. and Chu, K.-H., 2011, "Effects of solids retention time on the performance of bioreactors bioaugmented with a 17 β -estradiol-utilizing bacterium, *Sphingomonas* strain KC8", *Chemosphere*, no. 84 (2), pp. 227-233.
174. Rubio-Rincon, F.J., Welles, L., Lopez-Vzquez, C. M., Nierychlo, M., Abbas, B., Geleijnse, M., Nielsen, P. H., van Loosdrecht, M. C. M. and Brdjanovic, D., 2017. "Long-term effects of sulphide on the enhanced biological removal of phosphorus: the symbiotic role of *Thiothrix caldifontis*", *Water Research*, vol. 116, no. 1, pp.53-64.
175. Saad, S.A., Welles, L., Abbas, B., Lopez-Vazquez, C.M., van Loosdrecht, M.C.M., & Brdanovic, D., 2016. "Denitrification of nitrate and nitrite by 'Candidatus Accumulibacter phosphatis' clade IC" *Water Research*, vol. 105, pp. 97-109
176. Sabirova, J.S., Cloetens, L.F.F., Vanhaecke, L., Forrez, I., Verstraete, W., Boon, N., 2008. Manganese-oxidizing bacteria mediate the degradation of 17 α -ethinylestradiol. *Microbiol. Biotechnol.*, vol. 1, pp. 507–512.
177. Saikaly, P.E., Stroot, P.G., and Oerther, D.B., 2005, "Use of 16S rRNA gene terminal restriction fragment analysis to assess the impact of solids retention time on the bacterial diversity of activated sludge", *Applied Environmental Microbiology*, vol. 71, no. 10, pp. 5814–22.
178. Santos, A., Barton, P., Cartmell, E., Coulon, F., Crane, R.S., Hillis, P., Lester, J.N., Stephenson, T. and Judd, S.J. 2010, "Fate and behaviour of copper and zinc in secondary biological wastewater treatment processes: II Removal at varying sludge age", *Environmental Technology*, vol. 31, no. 7, pp. 725-743.
179. Schloss, P.D., Gevers, D. & Westcott, S.L., 2011. "Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16s RRNA-Based Studies", *PLoS One*, vol .6 no.12.
180. Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M., Environmental Organic Chemistry, John Wiley & Sons: 2005.
181. Shi, S., Qu, Y., Ma, Q., Zhang, X., Zhou, J. & Ma, F. 2015, "Performance and microbial community dynamics in bioaugmented aerated filter reactor treating with coking wastewater", *Bioresource Technology*, vol. 190, pp. 159-166.
182. Shi, Y., Huang, J., Zeng, G., Gu, Y., Chen, Y., Hu, Y., Tang, B., Zhou, J., Yang, Y. & Shi, L. 2017, "Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: An overview", *Chemosphere*, vol. 180, pp. 396-411.
183. Shin, S. G., Han, G., Lim, J., Lee, C., Hwang, S., 2010. "A comprehensive microbial insight into two-stage anaerobic digestion of food waste-recycling wastewater", *Water Research*, vol. 44, no.17, pp. 4838-4849.
184. <https://www.sigmaaldrich.com/catalog/product/sigma/e9750?lang=en®ion=GB>
185. Siripong, S. and Rittmann, B. E., 2007, "Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants", *Water Research*, vol. 41, no. 5, pp. 1110-1120.
186. Snaidr, J., Amann, R., Huber, I., Ludwig, W. & Schleifer, K.-. 1997, "Phylogenetic analysis and in situ identification of bacteria in activated sludge", *Applied and Environmental Microbiology*, vol. 63, no. 7, pp. 2884-2896.
187. Soares, A., Murto, M., Guieysse, B. and Mattiasson, B., 2006. "Biodegradation of nonylphenol in a continuous bioreactor at low temperatures and effects on the microbial population", *Applied Microbiology and Biotechnology*, vol. 69, no. 5, pp. 597-606.
188. Stadler, L.B., Su, L., Moline, C.J., Ernstoff, A.S., Diana S.Aga, D.S. & Love, N.G., 2015. "Effect of redox conditions on pharmaceutical loss during biological wastewater treatment using sequencing batch reactors", *Journal of Hazardous Materials*, vol. 282, pp. 106-115
189. Stavarakis, C., Colin, R., Faur, C., Hequet, V. & Le Cloirec, P. 2008, "Analysis and behaviour of endocrine disrupting compounds in wastewater treatment plant", *Journal Europeen d'Hydrologie*, vol. 39, no. 2, pp. 145-155.

190. Stingley, R.L., Brezna, B., Khan, A.A. & Cerniglia, C.E., 2004. "Novel organization of genes in a phthalate degradation operon of *Mycobacterium vanbaalenii* PYR-1", *Microbiology*, vol. 150, pp. 3749-3761
191. Suarez, S., Carballa, M., Omil, L., Lema, J.M. 2008, "How are pharmaceutical and personal care products (PPCPs) removed from urban wastewaters?", *Reviews in Environmental Science and Bio/Technology*, vol. 7, no. 2, pp.125-138.
192. Szabó, E., Liébana, R., Hermansson, M., Modin, O., Persson, F. & Wilén, B.-. 2017, "Microbial population dynamics and ecosystem functions of anoxic/aerobic granular sludge in sequencing batch reactors operated at different organic loading rates", *Frontiers in Microbiology*, vol. 8.
193. Tchobanoglous, G., Burton, F.L. Stensel, D.H. (2002) *Wastewater Engineering: Treatment and Reuse* by Inc. Metcalf & Eddy, McGraw-Hill Higher Education; ISBN: 978-0071241403.
194. Tanghe, T., Dhooze, W. & Verstraete, W. 1999, "Isolation of a bacterial strain able to degrade branched nonylphenol", *Applied and Environmental Microbiology*, vol. 65, pp. 746-751.
195. Ternes, T.A., Herrmann, N., Bonerz, M., Knacker, T., Siegrist, H. and Joss, A. 2004, "A rapid method to measure the solid-water distribution coefficient (K_d) for pharmaceuticals and musk fragrances in sewage sludge", *Water Research*, vol. 38, no. 19, pp. 4075-4084.
196. Thornton, L.M., Path, E.M., Venables, B.J., & Jeffries, M.K.S., 2016. "The endocrine effects of dietary brominated diphenyl ether-47 exposure, measured across multiple levels of biological organization, in breeding fathead minnows", *Environmental Toxicology*, vol.35, pp.2048-2057
197. Tian, M., Zhao, F., Shen, X., Chu, K., Wang, J., Chen, S., Guo, Y. & Lui, H., 2015. "The first metagenome of activated sludge from full-scale anaerobic/anoxic/oxic (A2O) nitrogen and phosphorus removal reactor using Illumina sequencing", *J. of Environ. Sciences*, pp. 181-190
198. Tisler, S. & Zwiener, C., 2018. "Formation and occurrence of transformation products of metformin in wastewater and surface water", *Science of The Total Environment*, pp. 1121-1129
199. Thompson, K., Zhang, J. and Zhang, C. 2011, "Use of fugacity model to analyse temperature-dependent removal of micro-contaminants in sewage treatment plants", *Chemosphere*, vol. 84, no. 8, pp. 1066-1071.
200. Torresi, E., Fowler, S.J., Polesel, F., Bester, K., Andersen, H.R., Smets, B.F., Plósz, B.G. & Christensson, M., 2016. "Biofilm Thickness Influences Biodiversity in Nitrifying MBBRs - Implications on Micropollutant Removal" *Environ. Sci. Technol.*, vol. 50, no. 17, pp. 9279–9288
201. Valverde Pérez, B., Wágner, D. S., Lóránt, B., Gülay, A., Smets, B. F., & Plósz, B. G. 2016. "Low-sludge age EBPR process for resource recovery – microbial and biochemical process characterization", *Microbial ecology and water engineering & biofilms specialist groups (MEWE2016)* pp. 398-399. Copenhagen, Denmark.
202. Van Der Gast, C.J., Jefferson, B., Reid, E., Robinson, T., Bailey, M.J., Judd, S.J. & Thompson, I.P. 2006, "Bacterial diversity is determined by volume in membrane bioreactors", *Environmental Microbiology*, vol. 8, no. 6, pp. 1048-1055.
203. Verlicchi, P., Al Aukidy, M. & Zambello, E. 2012, "Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment-A review", *Science of the Total Environment*, vol. 429, pp. 123-155.
204. Veuliet, F., Lacroix, S., Bausseron, A., Gonidec, E., Ochoa, J., Christensson, M. & Lemaire, R. 2014, "Integrated fixed-film activated sludge ANITA™Mox process - A new perspective for advanced nitrogen removal", *Water Science and Technology*, vol. 69, no. 5, pp. 915-922.
205. Veum, K.S., Lorenz, T. & Kremer, R.J., 2019. "Phospholipid fatty acid profiles under variable handling and storage conditions", *Agronomy Journal*, vol. 111, no. 3, pp. 1090-1096
206. Vieno, N. & Sillanpää, M. 2014, "Fate of diclofenac in municipal wastewater treatment plant - A review", *Environment International*, vol. 69, pp. 28-39.
207. von der Ohe, P. C., Dulio, V., Slobodnik, J., De Deckere, E., Kühne, R., Ebert, R-U., Ginebreda, A., De Cooman, W., Schüürmann, G., & Brack, W., 2011. "A new risk assessment approach for the prioritization of 500 classical and emerging organic microcontaminants as potential river

- basin specific pollutants under the European Water Framework Directive”, *Science of The Total Environment*, Vol. 409, no. 11, pp. 2064-2077.
208. Vorkamp, K. and Sanderson, H. 2016, “EUROPEAN ENVIRONMENTAL QUALITY, STANDARDS (EQS) VARIABILITY STUDY” Analysis of the variability between national EQS values across Europe for selected Water Framework Directive River Basin-Specific Pollutants, Scientific Report from DCE, No.198, Aarhus University, Dept. of Environmental Science.
 209. Vuono, D. C., Benecke, J., Henkel, J., Navidi, W. C., Cath, T. Y., Munakata-Marr, J., Spear, J. R. & Drewes, J. E., 2015. “Disturbance and temporal partitioning of activated sludge metacommunity”, *The ISME Journal*, vol. 9, pp. 425-435.
 210. Wagner, M., and Loy, A., 2002. “Bacterial community composition and function in sewage treatments systems”, *Environmental Biotechnology*, pp. 218-227.
 211. Wang, D., Li, T., Huang, K., He, X. & Zhang, X-X., 2019. “Roles and correlations of functional bacteria and genes in the start-up of simultaneous anammox and denitrification system for enhanced nitrogen removal”, *Science of The Total Environment*, vol. 655, pp. 1355-1363.
 212. Wang, X., Xia, Y., Wen, X., Yang, Y. & Zhou, J. 2014, "Microbial community functional structures in wastewater treatment plants as characterized by GeoChip", *PLoS ONE*, vol. 9, no. 3.
 213. Wang, Y., Zhang, Z., Qiu, L., Guo, Y., Wang, X., Xiong, X. & Chen, S. 2015, "Effect of temperature downshifts on biological nitrogen removal and community structure of a lab-scale aerobic denitrification process", *Biochemical engineering journal*, vol. 101, pp. 200-208.
 214. Weber, S., Leuschner, P., Kämpfer, P., Dott, W., Hollender, J., 2005. Degradation of estradiol and ethinylestradiol by activated sludge and by a defined mixed culture. *Appl. Microbiol. Biotechnol.*, vol. 67, pp. 106–112.
 215. Wei, J., Sun, B., Zhao, S. & Li, Z. 2017. “Effect of pH on operational performance and microbial community structure of activated sludge of SBR”, *Chinese Journal of Environmental Engineering*, vol. 11no. 3, pp. 1953-1958
 216. Welles, L., Lopez-Vazquez, C. M., Hooijmans, C. M., van Loosdrecht, M. C. M. & Brdjanovic, D. 2016. “Prevalence of ‘Candidatus Accumulibacter phosphatis’ type II under phosphate limiting conditions”, *AMB Express*, vol. 6, no. 44
 217. Werker A.G., Becker, J., & Huitema, C., 2003. “Assessment of activated sludge microbial community analysis in full-scale biological wastewater treatment plants using patterns of fatty acid isopropyl esters (FAPEs), *Water Research*, vol. 37, no. 9, pp. 2162-217
 218. Whittaker, R. H., 1972. “Evolution and Measurement of Species Diversity”, *Int. Assoc. Plant Taxon.*, vol. 21 no. 2-3, pp. 213–251.
 219. Wilén B-M, Markiewicz A., and Nilsson Å. 2010, “Variation in dissolved oxygen concentration and its effect on the activated sludge properties studied at a full-scale wastewater treatment plant”, *Water Environment Technology*, Chalmers University of Technology, Göteborg, Sweden
 220. Winkler, M.K.H., Boets, P., Hahne, B., Goethals, P. & Volcke, E.I.P., 2017. “Effect of the dilution rate on microbial competition: r-strategist can win over k-strategist at low substrate concentration”, *Plos one*, vol. 12
 221. Winkler, M-K. H., Kleerebezem, R., de Bruin L. M. M., Verheijen, P. J. T., Abbas, B., Habermacher, J., van Loosdrecht, M., 2013. “Microbial diversity differences within aerobic granular sludge and activated sludge flocs”, *Applied Microbiology*, vol. 97, no. 16, pp. 7447-7458.
 222. Wojcieszńska, D., Domaradzka, D., Hupert-Kocurek, K. & Guzik, U., 2014. “Bacterial degradation of naproxen – Undisclosed pollutant in the environment”, *J. Environ. Management*, vol. 145, pp. 157-161
 223. Wu, L., Ning, D., Zhang, B., Li, Y., Zhang, P., Shan, X., Zhang, Q., Brown, M., Li, Z., Nostrand, J.D.V., Ling, F., Xiao, N., Zhang, Y., Vierheilig, J., Wells, G.F., Yang, Y., Deng, Y., Tu, Q., Wang, A., Global Water Microbiome Consortium, Zhang, T., He, Z., Keller, J., Nielsen, P.H., Alvarez, P.J.J., Criddle, C.S., Wagner, M., Tiedje, J.M., He, Q., Curtis, T.P., Stahl, D.A., Alvarez-Cohen, L., Rittmann, B.E., Wen, X. & Zhou, J., 2019. “Global diversity and biogeography of bacterial communities in wastewater treatment plants”, *Nature Microbiology*, vol.4. no. 1, pp.1183-1195.

224. Xia, Z., Xiao-Chun, W., Zhong-Lin, C., Hao, X., Qing-Fang, Z., 2015. "Microbial community structure and pharmaceuticals and personal care products removal in membrane bioreactor seeded with aerobic granular sludge", *Appl. Microbiology & Biotechnology*, vol. 99, pp. 425-433.
225. Xie, K., Li, J., Jia, R., Li, H., Duan, L., Xu, X. and Xia, S. 2011, "Microbial diversity and composition in two full-scale wastewater treatment plants (WWTPs) in Shanghai", *Fresenius Environmental Bulletin*, vol. 20, no. 6 A, pp. 1525-1534.
226. Xing, W., Zuo, J.-., Dai, N., Cheng, J. & Li, J. 2009, "Reactor performance and microbial community of an EGSB reactor operated at 20 and 15°C", *Journal of applied microbiology*, vol. 107, no. 3, pp. 848-857.
227. Xu, S., Yao, J., Ainiwaer, M., Hong, Y., & Zhang, Y., 2018. "Analysis of Bacterial Community Structure of Activated Sludge from Wastewater Treatment Plants in Winter", *BioMed Research International*, vol. no. pp.
228. Yao, Q. & Peng, D-C., 2017, "Nitrite oxidizing bacteria (NOB) dominating in nitrifying community in full-scale biological nutrient removal wastewater treatment plants", *AMB Express*
229. Yi, T., Lee, E.-., Kang, S., Shin, J. & Cho, K.-. 2012, "Structure and dynamics of microbial community in full-scale activated sludge reactors", *Journal of Industrial Microbiology and Biotechnology*, vol. 39, no. 1, pp. 19-25.
230. Yoshimoto, T., Nagai, F., Fujimoto, J., Watanabe, K., Mizukoshi, H., Makino, T., Kimura, K., Saino, H., Sawada, H., Omura, H., 2004. Degradation of estrogens by *Rhodococcus zopfii* and *Rhodococcus equi* isolates from activated sludge in wastewater treatment plants. *Appl. Environ. Microbiol.* Vol. 70, pp. 5283–5289.
231. Yu, C.-., Deeb, R.A. & Chu, K.-. 2013, "Microbial degradation of steroidal estrogens", *Chemosphere*, vol. 91, no. 9, pp. 1225-1235.
232. Yu, C.-P., Roh, H., Chu, K.H., 2007, 17 β -Estradiol-degrading bacteria isolated from activated sludge. *Environ. Sci. Technol.* 41, 486–492.
233. Yuan, L., Zhi, W., Liu, Y., Smiley, E., Gallagher, D., Chen, X., Dietrich, A.M. & Zhang, H. 2016, "Degradation of cis- and trans-(4-methylcyclohexyl) methanol in activated sludge", *Journal of hazardous materials*, vol. 306, pp. 247-256.
234. Zelles, L., 1999. "Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: A Review" *Biology and Fertility of Soils*, vol. 29 no. 2, pp. 111–129
235. Zeng, Q., Li, Y. M., Gu G.W., Zhao, J.M., Zhang, C.J., Luan, J.F., 2009. Sorption and biodegradation of 17 β -estradiol by acclimated aerobic activated sludge and isolation of the bacterial strain. *Eng. Sci.* 26. 783-790.
236. Zeng, Q., Li, Y. & Yang, S. 2013, "Sludge retention time as a suitable operational parameter to remove both estrogen and nutrients in an anaerobic-anoxic-aerobic activated sludge system", *Environmental Engineering Science*, vol. 30, no. 4, pp. 161-169.
237. Zhang, B., Xu, X. and Zhu, L., 2018. "Activated sludge bacterial communities of typical wastewater treatment plants: distinct genera identification and metabolic potential differential analysis", *Open Access*, vol. 8, pp.184
238. Zhang, C., Li, Y., Wang, C., Niu, L. & Cai, W. 2016. "Occurrence of endocrine disrupting compounds in aqueous environment and their bacterial degradation: A review", *Critical Reviews in Environmental Science and Technology*, vol. 46, no. 1, pp. 1-59.
239. Zhang, L., Hu J., Zhu R., Zhou Q. & Chen J., (2013) "Degradation of Paracetamol by pure bacterial cultures and their microbial consortium" *Appl. Microbiol Biotechnol.*, Vol. 97, no. 8, pp. 3687-3698.
240. Zang, K., Kurisu, F., Kasuga, I., Furumai, H. & Yagi, O., 2008. "Analysis of the phylogenetic diversity of estrone-degrading bacteria in activated sewage sludge using microautoradiography–fluorescence in situ hybridization". *Syst. Appl. Microbiol.*, vol. 31, pp. 206–214
241. Zhang, T., Shao, M-F. & Ye, L., 2011. "454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants", *The ISME Journal*, vol. 6, pp. 1137-1147.

242. Zhang, Y., McPhedran, K.N. & Gamal El-Din, M. 2015, "Pseudomonads biodegradation of aromatic compounds in oil sands process-affected water", *Science of the Total Environment*, vol. 521-522, pp. 59-67.
243. Zhang, Y., Wang, X., Hu, M. & Li, P. 2014, "Effect of hydraulic retention time (HRT) on the biodegradation of trichloroethylene wastewater and anaerobic bacterial community in the UASB reactor", *Applied Microbiology and Biotechnology*, vol. 99, pp. 1977-1987.
244. Zhao, Y.-., Guo, X., She, Z., Gao, M. & Guo, L. 2017, "Effect of gradual-increasing aeration mode in an aerobic tank on nutrients' removal and functional microbial communities", *Environmental Technology* (United Kingdom), vol. 38, pp. 2621-2628.
245. Zhang, Y., Gu, X., Zhang, J. & Yang, M. 2014, "Degradation pathways of low-ethoxylated nonylphenols by isolated bacteria using an improved method", *Environmental Science and Pollution Research*, vol. 21, no. 16, pp. 9468-9476.
246. Zhou, K., Xu, M., Dai, J. & Cao, H., "The microfauna communities and operational monitoring of an activated sludge plant in China", *European Journal of Protistology*, vol. 42, no. 4, pp. 291-295.
247. Zhou, Y., Zha, J. & Wang, Z. (2012), "Occurrence and fate of steroid estrogens in the largest wastewater treatment plant in Beijing, China", *Environmental monitoring and assessment*, vol. 184, no. 11, pp. 6799-6813.
248. Zornoza, R., Guerrero, C., Mataix-Solera, J., Scow, K.M., Arcenegui, V. & Mataix-Beneyto, J., (2009). "Changes in soil microbial community structure following the abandonment of agricultural terraces in mountainous areas of Eastern Spain" *Applied Soil Ecology*, vol. 42, no. 3, pp. 315–323.

APPENDED TABLES

Table AT1. Pilot plant sanitary performance in SRT study at constant HRT of 8 h

Operational conditions		BOD			COD			NH3			SS			Loading rate		
		Settled	Final effluent	% removal	Settled	Final effluent	% removal	Settled	Final effluent	% removal	Settled	Final effluent	% removal	MLVSS	F:M	
Varying SRT at constant HRT of 8 hours	3 d	1 st	127	34	73.2	465	158	66.0	48.3	46.2	4.3	136	87	36.0	1063	0.36
		2 nd	152	33	78.3	471	193	59.0	49.8	47.3	5.0	146	89	39.0	967	0.47
		3 rd	146	42	71.2	399	167	58.1	53.5	50.3	6.0	138	79	42.8	997	0.44
		4 th	109	27	75.2	461	179	61.2	55.4	51.4	7.2	140	88	37.1	1018	0.32
		5 th	113	27	76.1	450	158	64.9	51.6	48.6	5.8	138	91	34.1	976	0.35
		6 th	142	39	72.5	476	166	65.1	53.9	50.1	7.1	156	94	39.7	956	0.45
		7 th	103	27	73.8	481	180	62.6	47.9	45	6.1	129	90	30.2	998	0.31
	10 d	1 st	120	18	85.0	662	99.3	85.0	55.4	0.25	99.5	123	35	71.5	1405	0.26
		2 nd	118	14	88.1	589	94.2	84.0	51.8	0.43	99.2	129	31	76.0	1594	0.22
		3 rd	107	14	86.9	467	88.7	81.0	46.7	0.15	99.7	131	26	80.2	1630	0.20
		4 th	124	13	89.5	452	94.9	79.0	49.8	0.2	99.6	117	22	81.2	1576	0.24
		5 th	131	14	89.3	496	89.3	82.0	49.1	0.31	99.4	133	27	79.7	1586	0.25
		6 th	123	15	87.8	441	106	76.0	52.4	0.34	99.4	127	36	71.7	1674	0.22
		7 th	119	14	88.2	466	65	86.1	50.7	0.16	99.7	129	34	73.6	1398	0.26
	27 d	1 st	122	5.1	95.8	397	73.9	81.4	51.5	0.24	99.5	141	18	87.2	1316	0.31
		2 nd	141	6.2	95.6	421	81.8	80.6	52.5	0.19	99.6	136	14	89.7	1248	0.34
		3 rd	137	6.8	95.0	441	85.7	80.6	49.7	0.34	99.3	144	20	86.1	1248	0.38
		4 th	129	3.9	97.0	376	63.7	83.1	50.8	0.3	99.4	138	11	92.0	1316	0.29
		5 th	103	4.1	96.0	481	72.2	85.0	53.4	0.14	99.7	140	21	85.0	1378	0.22
		6 th	117	3.5	97.0	496	84.9	82.9	48.8	0.19	99.6	138	19	86.2	1502	0.23
		7 th	111	4.1	96.3	480	62.4	87.0	49.7	0.21	99.6	156	23	85.3	1428	0.23

BOD = biochemical oxygen demand; COD = chemical oxygen demand

Table AT2. Pilot plant sanitary performance in HRT study at constant SRT of 27 d

Operational conditions		BOD			COD			NH3			SS			Loading rate		
		Settled	Final effluent	% removal	Settled	Final effluent	% removal	Settled	Final effluent	% removal	Settled	Final effluent	% removal	MLVSS	F:M	
Varying HRT at a constant SRT of 27 days	8 h	1 st	122	5.1	95.8	397	73.9	81.4	51.5	0.24	99.5	141	18	87.2	1316	0.28
		2 nd	141	6.2	95.6	421	81.8	80.6	52.5	0.19	99.6	136	14	89.7	1248	0.34
		3 rd	137	6.8	95.0	441	85.7	80.6	49.7	0.34	99.3	144	20	86.1	1248	0.33
		4 th	129	3.9	97.0	376	63.7	83.1	50.8	0.3	99.4	138	11	92.0	1316	0.29
		5 th	103	4.1	96.0	481	72.2	85.0	53.4	0.14	99.7	140	21	85.0	1378	0.22
		6th	117	3.5	97.0	496	84.9	82.9	48.8	0.19	99.6	138	19	86.2	1502	0.23
		7th	111	4.1	96.3	480	62.4	87.0	49.7	0.21	99.6	156	23	85.3	1428	0.23
	16 h	1 st	106	6	94.3	344	51.6	85.0	32.1	0.11	99.7	103	27	73.8	1204	0.13
		2 nd	96	7	92.7	376	52.6	86.0	32.9	0.19	99.4	85	21	75.3	1086	0.13
		3 rd	119	9	92.4	353	56.5	84.0	40.9	0.16	99.6	153	33	78.4	1084	0.16
		4 th	99	6	93.9	395	63.2	84.0	37	0.14	99.6	103	23	77.7	1006	0.15
		5 th	102	3	97.1	354	56.6	84.0	39.7	0.17	99.6	110	29	73.6	1108	0.14
		6th	107	8	92.5	371	55.6	85.0	41.2	0.16	99.6	100	28	72.0	1142	0.14
		7th	92	4	95.7	345	58.7	83.0	35.3	0.13	99.6	112	34	69.6	1184	0.12
	24 h	1 st	141.8	5	96.5	334	57.4	82.8	27.2	0.11	99.6	89	30	66.3	1298	0.08
		2 nd	103.3	6	94.2	397	75.4	81.0	35.9	0.18	99.5	82	29	64.6	1276	0.08
		3 rd	146.8	3	98.0	374	59.7	84.0	40.7	0.16	99.6	102	33	67.6	1128	0.09
		4 th	82	4	95.1	389	75.6	80.6	31.5	0.11	99.7	94	37	60.6	1202	0.07
		5 th	79	1	98.7	339	54.9	83.8	42.5	0.12	99.7	83	24	71.1	1136	0.07
		6th	86	3	96.5	384	72.9	81.0	39.1	0.12	99.7	91	29	68.1	1040	0.08
		7th	73	7	90.4	349	62.8	82.0	32.5	0.17	99.5	84	25	70.2	1218	0.06

BOD = biochemical oxygen demand; COD = chemical oxygen demand

Table AT3. Bacterial diversity and abundance $\geq 1.00\%$ at 3 d SRT

Domain	Phylum	Class	Order	Family	Genus	Species	%	
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Other	3.60%	
		Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	uncultured	Other	1.20%	
		Other	Other	Other	Other	Other	1.31%	
	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Other	Other	1.09%	
	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Other	Other	1.09%	
	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	Other	1.31%		
				Tabrizicola	uncultured soil bacterium	1.53%		
				Other	Other	2.62%		
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Other	Other	7.21%	
							Thiothrix eikelboomii	11.90%
							Gammaproteobacteria	Thiotrichales
						Other	3.06%	
	Verrucomicrobia	OPB35 soil group	Other	Other	Other	Other	1.31%	

Table AT4. Bacterial diversity and abundance $\geq 1.00\%$ at 10 d SRT and 8 h HRT

Domain	Phylum	Class	Order	Family	Genus	Species	%
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Iamiaceae	Iamia	Ambiguous_taxa	1.09%
	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Ambiguous_taxa	1.09%
	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	Other	6.66%
	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Planctomyces	uncultured gamma pr	
						oteobacterium	1.09%
						Other	1.20%
					Other	Other	1.42%
					Rhodobacter	Other	1.75%
	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Tabrizicola	uncultured soil bacter	
						ium	1.86%
					Other	Other	2.07%
		Betaproteobacteria	Burkholderiales	Comamonadaceae	Sphaerotilus	Other	1.09%
					Other	Other	4.15%
		Deltaproteobacteria	Myxococcales	Phaselicystidaceae	Phaselicystis	uncultured bacterium	1.09%
		Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Thiothrix	Thiothrix eikelboomii	1.75%
			Xanthomonadales	Xanthomonadaceae	Dokdonella	uncultured bacterium	1.20%
	Spirochaetae	Spirochaetes	Spirochaetales	Leptospiraceae	Turneriella	Ambiguous_taxa	1.09%

Table AT5. Bacterial diversity and abundance $\geq 1.00\%$ at 27 d SRT and 8 h HRT

Domain	Phylum	Class	Order	Family	Genus	Species	%
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobiales	Candidatus Microthrix	Candidatus Microthrix parvicella	1.75%
	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	Ambiguous_taxa	2.40%
		Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium sp. S4	2.95%
						Other	1.42%
		Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Ambiguous_taxa	1.31%
					uncultured	Other	1.09%
	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus delbrueckii subsp. bulgaricus	1.09%
		Clostridia	Clostridiales	Peptostreptococcaceae	Other	Other	1.31%
	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Planctomyces	uncultured gammaproteobacterium	1.20%
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Other	Other	Other	1.31%
			Rhodobacterales	Rhodobacteraceae	Rhodobacter	Other	1.31%
					Other	Other	3.93%
			Sphingomonadales	Sphingomonadaceae	Novosphingobium	Other	1.09%
					Sphingopyxis	uncultured alpha proteobacterium	1.31%
					Other	Other	1.42%
					Hydrogenophaga	Ambiguous_taxa	1.64%
		Betaproteobacteria	Burkholderiales	Comamonadaceae	Other	Other	5.90%
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Prostheco bacter	uncultured bacterium	1.20%

Table AT6. Bacterial diversity and abundance $\geq 1.00\%$ at 8 h HRT and 27 d SRT

Domain	Phylum	Class	Order	Family	Genus	Species	%
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobiales Incertae Sedis	<i>Candidatus Microthrix</i>	<i>Candidatus Microthrix parvicella</i>	1.75%
	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	<i>Leadbetterella</i>	<i>Ambiguous_taxa</i>	2.40%
		Flavobacteriia	Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i>	<i>Flavobacterium sp. S4</i>	2.95%
						<i>Other</i>	1.42%
		Sphingobacteriia	Sphingobacteriales	Saprospiraceae	<i>Haliscomenobacter</i>	<i>Ambiguous_taxa</i>	1.31%
					<i>uncultured</i>	<i>Other</i>	1.09%
	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	1.09%
		Clostridia	Clostridiales	Peptostreptococcaceae	<i>Other</i>	<i>Other</i>	1.31%
	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	<i>Planctomyces</i>	<i>uncultured gammaproteobacterium</i>	1.20%
						<i>Other</i>	1.31%
						<i>Other</i>	1.31%
						<i>Other</i>	3.93%
	Proteobacteria	Alphaproteobacteria			<i>Novosphingobium</i>	<i>Other</i>	1.09%
					<i>Sphingopyxis</i>	<i>uncultured alpha proteobacterium</i>	1.31%
					<i>Other</i>	<i>Other</i>	1.42%
					<i>Hydrogenophaga</i>	<i>Ambiguous_taxa</i>	1.64%
		Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>Other</i>	<i>Other</i>	5.90%
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	<i>Prostheco bacter</i>	<i>uncultured bacterium</i>	1.20%

Table AT7. Bacterial diversity and abundance $\geq 1.00\%$ at 16 h HRT and 27d SRT

Domain	Phylum	Class	Order	Family	Genus	Species	%
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	<i>Haliscomenobacter</i>	<i>Ambiguous_taxa</i>	1.20%
				env.OPS 17	<i>uncultured bacterium</i>	<i>uncultured bacterium</i>	2.40%
				SJA-28	<i>Ambiguous_taxa</i>	<i>Ambiguous_taxa</i>	1.31%
	Chlorobi	Chlorobia	Chlorobiales	Caldilineaceae	<i>uncultured</i>	<i>uncultured bacterium</i>	1.09%
						<i>Other</i>	1.20%
	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<i>Other</i>	<i>Other</i>	1.31%
	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	<i>Nitrospira</i>	<i>Other</i>	9.39%
	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	<i>Planctomyces</i>	<i>uncultured bacterium</i>	1.31%
						<i>uncultured gamma proteobacterium</i>	1.09%
					<i>Other</i>	<i>Other</i>	1.97%
	Proteobacteria	Alphaproteobacteria	Rhizobiales	MNG7	<i>Ambiguous_taxa</i>	<i>Ambiguous_taxa</i>	1.09%
			Rhodobacterales	Rhodobacteraceae	<i>Tabrizicola</i>	<i>uncultured soil bacterium</i>	1.20%
					<i>Other</i>	<i>Other</i>	1.53%
		Betaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Other</i>	<i>Other</i>	1.09%
			Burkholderiales	Comamonadaceae	<i>Other</i>	<i>Other</i>	4.04%
		Gammaproteobacteria	Thiotrichales	Thiotrichaceae	<i>Thiothrix</i>	<i>eikelboomii</i>	2.73%
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	<i>Prostheco bacter</i>	<i>uncultured bacterium</i>	1.09%

Table AT8. Bacterial diversity and abundance $\geq 1.00\%$ at 24 h HRT and 27d SRT

Domain	Phylum	Class	Order	Family	Genus	Species	%
Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	<i>Mycobacterium</i>	<i>Other</i>	1.31%
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i>	<i>Flavobacterium sp. S4</i>	1.42%
		Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	<i>Pedobacter</i>	<i>Other</i>	1.64%
						<i>uncultured Sphingobacteriaceae bacterium</i>	1.09%
						<i>env.OPS 17</i>	2.51%
	Chloroflexi	Caldilineae	Caldilineales	Caldilineaceae	<i>uncultured</i>	<i>Other</i>	1.42%
	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<i>Other</i>	<i>Other</i>	1.64%
	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	<i>Planctomyces</i>	<i>uncultured bacterium</i>	1.42%
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Methylosula</i>	<i>uncultured alpha proteobacterium</i>	1.42%
			Rhodobacterales	Rhodobacteraceae	<i>Other</i>	<i>Other</i>	1.64%
		Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>Rhodoferrax</i>	<i>uncultured beta proteobacterium</i>	1.09%
					<i>Other</i>	<i>Other</i>	5.57%
		Deltaproteobacteria	Myxococcales	Nannocystaceae	<i>Nannocystis</i>	<i>Other</i>	1.31%
		Gammaproteobacteria	Thiotrichales	Thiotrichaceae	<i>Thiothrix</i>	<i>eikelboomii</i>	1.86%
						<i>uncultured bacterium</i>	1.75%
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	<i>Prostheco bacter</i>	<i>uncultured bacterium</i>	1.75%

Table AT9. Full-scale WWTPs process parameters and sanitary performance of WWTPs A, B and C

WWTP	Inlet flow (m ³ /d)	SAS (mg/L)	SAS (m ³ /d)	MLSS (mg/L)	DO (mg/L)	T (°C)	HRT (h)	SRT (d)	COD (mg/L)			SS (mg/L)			NH ₃ (mg/L)			NO ₃ (mg/L)			pH		
									Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem	Crud	Sett	Final
A1	25920	5410	518	2783	1.64	18.8	9.9	10.1	350	38	89	151	6	96	54.5	0.0	>99	1.1	21.7	95	7.9	7.9	7.5
A2	21344	6520	561	3004	2.41	18.5	12.0	8.5	303	36	88	107	6	94	50.0	0.0	>99	0.8	19.8	96	8.0	7.8	7.3
A3	31968	7120	605	3379	1.48	12.7	8.0	8.2	194	19	90	80	3	97	39.0	0.0	>99	0.5	14.4	97	7.2	7.5	7.0
A4	23328	6240	518	3748	2.22	12.4	11.0	11.8	376	35	91	135	7	95	54.8	0.0	>99	0.6	22.5	97	7.3	8.2	7.1
A5	19483	5480	518	2920	0.93	17.5	13.2	10.2	363	18	95	163	12	93	51.6	0.1	>99	0.0	21.9	100	7.6	8.0	7.3
B1	24926	6630	1150	2934	1.17	15.2	18.5	7.2	128	20	84	84	8	91	41.1	0.0	>99	0.3	15.4	98	7.7	7.9	7.3
B2	31860	6750	440	2861	1.07	13.2	14.5	17.4	143	26	82	59	6	90	48.7	0.0	>99	0.5	24.4	98	6.7	7.2	6.7
B3	44020	6820	1046	3278	1.35	12.7	10.5	8.4	313	19	94	151	8	95	39.1	0.0	>99	0.6	19.6	97	7.2	7.3	7.2
B4	36290	5150	730	3489	1.15	14.0	12.7	17.0	167	18	89	120	5	96	32.8	0.0	>99	3.5	26.8	87	7.9	7.4	6.9
B5	44496	4510	1620	2606	1.67	20.3	10.4	6.5	189	15	92	63	9	86	31.7	0.2	>99	0.6	22.1	97	7.8	7.7	7.2
B6	31580	4720	860	2746	2.32	19.9	14.6	12.4	42	10	76	74	6	92	28.0	0.2	>99	1.5	21.7	93	7.7	7.6	7.2
C1	11750	6200	500	4051	2.40	19.9	10.6	9.8	968	6	99	870	8	99	26.8	0.5	98	1.1	23.6	95	6.9	7.1	7.6
C2	8726.4	6260	1056	4329	0.46	19.4	15.1	5.2	601	44	93	356	10	97	30.6	0.6	98	1.0	24.0	96	6.9	7.0	7.4
C3	19612	8500	489	4676	0.44	14.8	9.5	8.5	47	12	74	43	5	88	16.3	0.0	>99	0.1	21.6	>99	6.5	7.0	7.5
C4	24192	6660	328	3565	0.60	16.2	7.7	12.2	129	45	65	58	4	94	14.7	0.0	>99	0.3	22.3	99	7.2	7.2	7.8
C5	13392	7320	375	4526	0.81	19.0	13.9	12.5	174	43	75	95	5	95	17.2	0.0	>99	0.7	25.3	97	7.6	7.3	7.9

Table AT10. Full-scale WWTPs process parameters and sanitary performance of WWTPs D, E and F

WWTP	Inlet flow (m ³ /d)	RAS (mg/L)	SAS (m ³ /d)	MLSS (mg/L)	DO (mg/L)	T (°C)	HRT (h)	SRT (d)	COD (mg/L)			SS (mg/L)			NH ₃ (mg/L)			NO ₃ (mg/L)			pH		
									Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem	Crud	Sett	Final
D1	8726	4500	450	4500	0.74	18.2	23.5	18.7	447	28	94	162	5	97	53.6	0.0	>99	0.6	4.2	86	8.1	7.7	7.2
D2	10540	7200	400	3671	1.55	15.2	19.5	10.6	470	56	88	147	9	94	55.6	1.2	98	0.7	9.7	93	7.7	7.6	7.5
D3	13867	6800	400	2695	1.73	15.5	14.8	8.1	333	56	83	153	10	94	32.5	0.7	98	1.7	11.2	85	8.2	7.8	7.3
D4		6380	500	3455	1.07	16.9		9.3	306	27	91	155	8	95	32.7	0.0	>99	0.4	10.6	97	6.6	7.4	7.2
E1	10590	6800	186	2202	0.46	13.7	20.0	6.0	174	22	87	114	8	93	16.7	0.1	>99	1.7	21.0	92	7.3	7.2	7.1
E2	7991	6100	162	3621	1.23	12.1	26.6	12.9	536	48	91	121	3	98	47.6	0.2	>99	0.9	29.0	97	7.0	6.3	6.8
E3	16323	6820	125	3277	2.19	10.6	13.0	13.1	71	25	64	63	5	93	20.8	0.0	>99	0.4	15.8	97	7.3	7.3	7.1
E4	16613	4400	181	2006	1.57	15.6	12.8	8.8	234	30	87	74	2	98	24.9	0.1	>99	0.2	12.4	98	7.3	7.2	7.1
E5	8102	4300	348	2072	0.91	18.0	26.2	4.9	322	18	94	98	4	96	34.8	0.2	>99	1.3	21.3	94	7.1	7.2	7.2
F1	124995	18000	650	3090	1.05	17.4	9.8	13.0	315	37	88	217	5	98	41.4	0.0	.	1.2	25.1	95	7.8	7.0	6.9
F2	150124	18440	800	3075	1.05	17.6	10.2	10.2	177	25	86	121	7	94	36.0	0.0	>99	0.4	13.3	97	-	-	-
F3	114262	11840	940	3535	1.13	12.0	14.7	19.0	244	24	90	123	5	96	40.3	0.0	>99	0.6	21.5	97	8.1	7.3	6.9
F4	127350	14500	851	3148	2.29	17.6	9.5	12.2	230	22	90	112	5	95	36.8	0.0	>99	0.4	23.1	98	7.8	7.9	7.0
F5	147510	7320	1333	3201	1.85	17.6	8.2	15.4	241	22	91	112	5	95	53.0	0.0	>99	0.1	23.9	>99	7.5	7.7	6.9

Table AT11. Full-scale WWTPs process parameters and sanitary performance of WWTPs G, H and J

WWTP	Inlet flow (m ³ /d)	RAS (mg/L)	SAS (m ³ /d)	MLSS (mg/L)	DO (mg/L)	T (°C)	HRT (h)	SRT (d)	COD (mg/L)			SS (mg/L)			NH ₃ (mg/L)			NO ₃ (mg/L)			Crud	pH	
									Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem		Sett	Final
G1	24498 ⁺	6680	1319	3863	0.81	15.1	15.0	6.3	547	34	94	240	26	89	52.4	0.4	>99	1.1	23.3	95	6.9	6.8	7.1
G2	23385 ⁺	6840	634	4827	1.25	16.4	15.5	15.5	494	59	88	130	18	86	44.3	0.1	99.8	0.7	30.2	98	7.4	7.2	7.2
G3	24983 ⁺	7520	626	4825	0.60	17.0	14.7	13.4	706	76	89	434	33	92	83.0	0.0	>99	1.6	25.8	94	7.6	7.6	7.4
G4	23458 ⁺	8280	629	4981	4.27	19.0	15.7	13.5	568	53	91	207	20	90	51.1	0.0	>99	2.0	29.6	94	7.2	7.2	7.3
G5	36038 ⁺	10160	530	6334	4.66	19.4	10.2	17.0	322	33	90	150	9	93	39.6	0.0	>99	0.7	19.7	96	7.0	6.9	7.0
G6	40059 ⁺	7760	623	4482	7.53	13.3	9.2	13.2	375	43	89	121	9	93	37.3	0.0	>99	0.5	17.7	97	6.9	6.9	7.1
G7	44781 ⁺	10400	351	4583	4.25	13.3	8.2	17.5	423	27	94	232	8	96	31.5	0.0	>99	0.4	18.3	98	6.6	6.6	6.8
H1	21761	6210	274	2141	1.77	16.4	8.2	8.7	233	32	86	82	6	93	47.8	0.0	>99	0.4	28.6	99	8.3	7.6	7.3
H2	22557	5500	346	2745	6.53	10.7	7.9	10.2	57	20	64	62	5	93	19.6	0.0	>99	0.3	13.3	98	7.3	7.7	6.9
H3	22075	6040	223	2991	1.08	16.1	8.1	14.9	422	28	93	183	6	97	47.7	2.8	94	0.6	12.1	95	7.8	7.0	7.0
H4	22136	6040	386	2941	1.01	17.6	8.1	8.7	248	22	91	170	9	95	37.4	3.1	92	0.2	15.3	99	8.1	7.2	6.8
H5	13456	6830	314	3453	2.60	17.7	13.2	11.7	175	18	90	105	4	96	34.2	1.8	95	0.0	13.9	>99	8.7	7.7	6.9
J1	13750	4200	400	2421	1.23	18.2	14.0	11.2	114	35	70	71	4	95	32.0	0.0	>99	0.7	15.4	96	7.6	7.5	7.1
J2	10720	5200	400	2241	5.10	15.6	18.1	8.5	197	24	88	57	3	94	33.9	0.0	>99	0.9	22.7	96	8.2	7.4	7.8
J3	11796	4000	532	1870	0.91	10.9	16.3	6.9	259	35	86	69	4	94	40.5	0.0	>99	0.6	22.3	98	6.8	7.3	6.7
J4	11823	4200	532	2162	0.75	11.4	16.2	7.5	228	56	75	76	7	91	28.6	0.0	>99	0.6	23.8	98	7.0	7.3	6.8
J5	19208	5100	532	2217	1.99	14.3	10.0	6.3	104	28	73	61	4	93	23.6	0.0	>99	1.3	18.8	93	7.7	7.6	7.0

Table AT12. Full-scale WWTPs process parameters and sanitary performance of WWTPs K, L and M

WWTP	Inlet flow (m ³ /d)	RAS (mg/L)	SAS (m ³ /d)	MLSS (mg/L)	DO (mg/L)	T (°C)	HRT (h)	SRT (d)	COD (mg/L)			SS (mg/L)			NH ₃ (mg/L)			NO ₃ (mg/L)			pH		
									Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem	Sett	Final	% Rem	Crud	Sett	Final
K1	25866	9050	384	3153	1.54	17.1	17.6	16.5	268	34	87	173	6	96	35.9	0.0	>99	1.5	19.6	92	7.6	7.6	7.3
K2	25217	5880	432	3000	1.86	13.0	18.1	20.9	526	42	92	160	7	96	60.2	0.0	>99	0.8	17.5	95	6.8	6.8	6.6
K3	24958	7260	432	2549	1.54	11.1	18.2	14.6	321	24	92	394	7	98	35.8	0.0	>99	0.8	18.9	96	7.2	7.3	7.3
K4	24800	7600	600	3368	1.69	12.2	18.4	13.5	407	19	95	210	6	97	60.2	0.0	>99	0.7	18.8	96	7.2	7.2	7.1
K5	28701	8450	346	3601	1.62	13.2	15.9	22.3	294	26	91	177	5	97	36.0	0.0	>99	2.9	15.9	82	7.6	7.2	6.8
L1	14688	3832	518	2833	2.30	18.7	15.4	13.0	137	30	78	89	5	95	27.3	0.0	>99	0.1	12.7	99	8.4	7.8	7.1
L2	15279	5584	518	2789	1.57	16.5	14.8	8.9	346	25	93	110	5	96	54.7	0.0	>99	0.4	14.4	97	8.5	7.9	7.5
L3	29818	6192	605	3044	6.16	10.9	7.6	7.5	179	19	89	51	3	95	11.6	0.0	>99	0.1	8.5	98	7.6	7.3	7.1
L4	28141	5935	691	2680	1.56	15.2	8.1	6.0	134	24	82	201	4	97	30.3	0.0	99	0.3	12.5	97	8.2	7.9	7.1
L5	36767	3072	605	2327	1.40	17.6	6.2	11.1	259	18	93	138	6	96	44.8	0.1	99	0.1	14.1	99	7.6	7.5	7.2
M1	82229	5050	1555	2764	0.74	18.4	11.2	11.9	226	48	79	89	13	85	37.5	0.3	99	0.2	18.7	99	7.4	7.5	7.3
M2	86832	5140	1555	2436	1.34	17.3	10.6	11.0	530	43	92	322	7	98	49.6	0.0	99	1.0	21.3	95	8.1	7.7	7.5
M3	71833	6490	1642	2854	1.08	11.5	12.9	10.1	77	29	60	79	3	96	22.5	0.0	>99	0.3	11.7	97	7.2	7.3	6.9
M4	100570	4510	1814	2589	1.05	18.1	9.2	10.8	150	33	78	97	10	90	32.5	0.1	99	0.3	19.0	99	7.4	7.4	7.0
M5	127773	5750	1469	2754	1.20	18.2	7.2	11.1	123	27	78	90	8	91	34.5	0.1	99	0.0	15.1	>99	7.9	7.7	7.1

Table AT13. P-values from the ANOVA of SRT from the twelve WWTPs

[illegible]

H3	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	1	1	1	1	0	0																		
H4	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0																	
H5	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	0	1	1	1	0	1	0	1	1	1	1																
J1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	0	1	0	1	1	0	1	1																	
J2	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1														
J3	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	1													
J4	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	1														
J5	0	1	1	0	0	1	0	1	0	1	0	1	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1											
K1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0								
K2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
K3	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	1	1	1	1	1	0	0	1	0	1	1	0	0	0	0	1	0							
K4	1	0	0	1	1	0	0	0	1	0	1	0	0	0	1	1	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	1	0	0	0	0	1	0	1						
K5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0					
L1	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	1	1	1	0	0	0	1	1	0	0	0	1	1	0	0	1	1	0	1	1	1	0	1	0	1	1	0	1	1	1	0											
L2	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	0	0	1	1	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	0	0	0	0	0	0						
L3	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1	0	0	0	0	0	0	1		
L4	0	1	1	0	0	1	0	1	0	1	0	0	1	1	0	0	0	0	1	1	1	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1						
L5	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	0	0												
M1	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	0	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1										
M2	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	0	1	1										
M3	1	1	1	1	1	1	0	1	0	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1									
M4	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	1	1	0	1	0	1	1	1	1	0	1	0	0	0	0	1	0	1	1	1	1							
M5	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0	1	1	0	1	1	1	1	1

Table AT14. P-values from the ANOVA of HRT from the twelve WWTPs

	A	A	A	A	A	B	B	B	B	B	B	C	C	C	C	C	D	D	D	D	E	E	E	E	F	F	F	F	F	G	G	G	G	G	G	H	H	H	H	H	J	J	J	J	J	K	K	K	K	K	L	L	L	L	L	M	M	M	M	M																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											
	1	2	3	4	5	1	2	3	4	5	6	1	2	3	4	5	1	2	3	4	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	6	7	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
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J1	0	1	0	1	1	0	1	1	1	0	1	1	0	0	0	1	0	0	1	1	0	0	1	1	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
J2	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
J3	0	0	0	0	1	1	1	0	0	0	1	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
J4	0	0	0	0	1	1	1	0	0	0	1	1	0	0	0	1	0	1	1	1	0	0	0	1	1	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											
J5	1	1	1	1	1	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
K1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	0																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											
K2	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
K3	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
K4	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
K5	0	0	0	0	1	1	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	0	1	1	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
L1	0	1	0	0	1	1	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	0	1	1	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	1	1	1	1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
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Table AT15. P-values from the ANOVA of temperature from the twelve WWTPs

[illegible]

H5	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
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Table AT16. P-values from the ANOVA of dissolved oxygen concentration from the twelve WWTPs

[illegible]

[illegible]

Table AT17. Tukey HSD test; variable SRT (d) (Spreadsheet3 in Individual site ANOVA - 1.stw); Homogenous Groups, alpha = .05000

	SRT (d)	Groups																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
E5	4.9	***																							
C2	5.2	***																							
E1	6.0	***	***																						
L4	6.0	***	***																						
G1	6.3	***	***	***	***	***	***	***																	
J5	6.3	***	***	***																					
B5	6.5	***	***	***	***																				
J3	6.9	***	***	***	***	***																			
B1	7.2	***	***	***	***	***																			
J4	7.5	***	***	***	***	***	***																		
L3	7.5	***	***	***	***	***	***																		
D3	8.1	***	***	***	***	***	***	***																	
A3	8.2	***	***	***	***	***	***	***	***																
B3	8.4	***	***	***	***	***	***	***	***	***															
J2	8.5	***	***	***	***	***	***	***	***	***	***														
A2	8.5	***	***	***	***	***	***	***	***	***	***	***													
C3	8.5	***	***	***	***	***	***	***	***	***	***	***	***												
H4	8.7	***	***	***	***	***	***	***	***	***	***	***	***	***											
E4	8.8	***	***	***	***	***	***	***	***	***	***	***	***	***	***										
L2	8.9	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***									
D4	9.0	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***								
H1	9.7		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***							
C1	9.8		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***						
F2	9.9		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***					
M3	10.1		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***				
A1	10.1		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***			
A5	10.2		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***		
H2	10.2		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
D2	10.6			***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
M4	10.8				***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
M2	11.0				***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
L5	11.1					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
M5	11.1					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
J1	11.2					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***

Table AT18. Tukey HSD test; variable HRT (h) (Spreadsheet3 in Individual site ANOVA - 1.stw); Homogenous Groups, alpha = .05000

Sample	HRT (h)	Groups																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
L5	6.2	****																			
M5	7.2	****	****																		
L3	7.6	****	****	****																	
C4	7.7	****	****	****	****																
H2	7.9	****	****	****	****																
A3	8.0	****	****	****	****																
H4	8.1	****	****	****	****																
L4	8.1	****	****	****	****																
H3	8.1	****	****	****	****																
H1	8.2	****	****	****	****																
G7	8.2	****	****	****	****																
F5	8.2	****	****	****	****																
G6	9.2	****	****	****	****	****															
M4	9.2	****	****	****	****	****															
C3	9.5	****	****	****	****	****	****														
F4	9.5	****	****	****	****	****	****													****	
F1	9.7	****	****	****	****	****	****	****												****	
A1	9.9	****	****	****	****	****	****	****												****	
J5	10.0	****	****	****	****	****	****	****												****	
G5	10.2	****	****	****	****	****	****	****	****											****	
F2	10.2	****	****	****	****	****	****	****	****											****	
B5	10.4	****	****	****	****	****	****	****	****	****										****	
B3	10.5	****	****	****	****	****	****	****	****	****	****									****	
M2	10.6		****	****	****	****	****	****	****	****	****	****								****	
A4	11.0		****	****	****	****	****	****	****	****	****	****	****							****	
M1	11.2			****	****	****	****	****	****	****	****	****	****	****						****	
A2	12.0				****	****	****	****	****	****	****	****	****	****	****					****	
B4	12.7					****	****	****	****	****	****	****	****	****	****	****				****	
E4	12.8					****	****	****	****	****	****	****	****	****	****	****	****			****	
M3	12.9					****	****	****	****	****	****	****	****	****	****	****	****	****		****	
E3	13.0					****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	
A5	13.2					****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	
H5	13.2					****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****

Table AT19. Tukey HSD test; variable Temp °C (Spreadsheet3 in Individual site ANOVA - 1.stw); Homogenous Groups, alpha = .05000

	Temp oC	Groups																														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
E3	10.6	**																														
H2	10.7	**	**																													
J3	10.9	**	**																													
L3	10.9	**	**																													
K3	11.1		**	**																												
J4	11.4			**	**																											
M3	11.5				**																											
F3	12.0					**																										
E2	12.1					**	**																									
K4	12.2					**	**																									
A4	12.4						**	**																								
B3	12.7							**	**																							
A3	12.7							**	**																							
K2	13.0								**	**																						
B2	13.2									**																						
K5	13.2									**																						
G7	13.3									**																						
G6	13.3									**																						
E1	13.7										**																					
B4	14.0										**	**																				
J5	14.3											**																				
C3	14.8												**																			
G1	15.1												**	**	**																	
L4	15.2													**																		
D2	15.2													**	**																	
B1	15.2													**	**																	
D3	15.5													**	**																	
E4	15.6														**																	
J2	15.6														**																	
H3	16.1															**																
C4	16.2															**	**															
G2	16.4															**	**	**	**													
H1	16.5																**															
L2	16.5																**															

D4	16.9
G3	17.0
K1	17.1
M2	17.3
F1	17.4
A5	17.5
L5	17.6
F4	17.6
F5	17.6
H4	17.6
F2	17.6
H5	17.7
E5	18.0
M4	18.1
M5	18.2
D1	18.2
J1	18.2
M1	18.4
A2	18.5
L1	18.7
A1	18.8
G4	19.0
C5	19.0
C2	19.4
G5	19.4
B6	19.9
C1	19.9
B5	20.3

[illegible]

Table AT20. Tukey HSD test; variable Dissolved O₂ (Spreadsheet3 in Individual site ANOVA - 1.stw); Homogenous Groups, alpha = .05000

	DO (mg/L)	Groups						
		1	2	3	4	5	6	7
C3	0.44	****						
C2	0.46	****						
E1	0.46	****						
G3	0.60	****	****					
C4	0.60	****	****					
M1	0.74	****	****					
D1	0.74	****	****					
J4	0.75	****	****					
C5	0.81	****	****					
G1	0.81	****	****					
J3	0.91	****	****					
E5	0.91	****	****					
A5	0.93	****	****					
H4	1.01	****	****					
M4	1.05	****	****					
F1	1.05	****	****					
F2	1.05	****	****					
D4	1.07	****	****					
B2	1.07	****	****					
M3	1.08	****	****					
H3	1.08	****	****					
F3	1.13	****	****					
B4	1.15	****	****					
B1	1.17	****	****					
M5	1.20	****	****					
J1	1.23	****	****					
E2	1.23	****	****					
G2	1.25	****	****					
M2	1.34	****	****					
B3	1.35	****	****					
L5	1.40	****	****					
A3	1.48	****	****					
K3	1.54	****	****					
K1	1.54	****	****					

D2	1.55	****	****					
L4	1.56	****	****					
E4	1.57	****	****					
L2	1.57	****	****					
K5	1.62	****	****					
A1	1.64	****	****					
B5	1.67	****	****					
K4	1.69	****	****					
H1	1.69	****	****					
D3	1.73	****	****					
F5	1.85	****	****					
K2	1.86	****	****					
J5	1.99	****	****					
E3	2.19	****	****	****				
A4	2.22	****	****	****				
F4	2.29	****	****	****				
L1	2.30	****	****	****				
B6	2.32	****	****	****				
C1	2.40	****	****	****				
A2	2.41	****	****	****				
H5	2.60		****	****	****			
G7	4.25			****	****	****		
G4	4.27			****	****	****		
G5	4.66				****	****	****	
J2	5.10				****	****		
L3	6.16				****	****	****	
H2	6.53					****	****	
G6	7.53							****

Table AT21. Tukey HSD test; variable GRAM+; Homogenous Groups, alpha = .05000

Sample	GRAM+ (%)	Groups																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A3	7.0	****																
J2	7.2	****	****															
M3	7.3	****	****	****														
M4	7.3	****	****	****	****													
F5	7.4	****	****	****	****	****												
J3	7.5	****	****	****	****	****	****											
H1	7.6	****	****	****	****	****	****	****										
B3	7.6	****	****	****	****	****	****	****	****									
L3	7.7	****	****	****	****	****	****	****	****	****								
B5	7.7	****	****	****	****	****	****	****	****	****	****							
F4	7.8	****	****	****	****	****	****	****	****	****	****							
B6	7.8	****	****	****	****	****	****	****	****	****	****							
J1	7.9	****	****	****	****	****	****	****	****	****	****	****						
M5	8.0	****	****	****	****	****	****	****	****	****	****	****	****					
L2	8.1	****	****	****	****	****	****	****	****	****	****	****	****	****				
D1	8.1	****	****	****	****	****	****	****	****	****	****	****	****	****	****			
A5	8.2	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****		
M2	8.2	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	
G7	8.2	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
G6	8.3	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
A4	8.3	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
E1	8.4	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
K2	8.4	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
K1	8.4	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
F2	8.4	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
D4	8.4	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
G4	8.4	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
G2	8.4	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
K4	8.5	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
D3	8.5	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
H3	8.7	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
B4	8.7	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****
H2	8.7	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****	****

Table AT22. Tukey HSD test; variable GRAM- (Spreadsheet1 in Workbook2); Homogenous Groups, alpha = .05000

	GRAM- (%)	Group																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
C2	45.6	***																								
C3	49.2	***	***																							
M1	50.4		***	***																						
B4	50.8		***	***																						
C1	51.2		***	***																						
E2	52.5		***	***	***																					
D2	54.0			***	***	***																				
B3	55.6				***	***	***																			
J4	55.8				***	***	***	***																		
J1	56.1				***	***	***	***	***																	
B2	56.4				***	***	***	***	***	***																
K3	56.4				***	***	***	***	***	***	***															
M4	56.8					***	***	***	***	***	***	***														
J5	56.8					***	***	***	***	***	***	***	***													
M5	57.0					***	***	***	***	***	***	***	***	***												
C4	57.2					***	***	***	***	***	***	***	***	***												
E1	57.4					***	***	***	***	***	***	***	***	***	***											
F2	57.5					***	***	***	***	***	***	***	***	***	***	***										
E3	57.6					***	***	***	***	***	***	***	***	***	***	***	***									
A3	57.7					***	***	***	***	***	***	***	***	***	***	***	***	***								
B5	57.7					***	***	***	***	***	***	***	***	***	***	***	***	***	***							
J2	57.7					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***						
L3	57.8					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***					
M3	57.8					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***				
H2	57.8					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***			
L5	58.1					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
B6	58.1					***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
L4	58.4						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
K1	58.5						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
K5	58.5						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
J3	58.6						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
F1	58.9						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
A4	58.9						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
F5	59.1						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
F4	59.3						***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	

Table AT23. Tukey HSD test; variable ACTS (Spreadsheet1 in Workbook2); Homogenous Groups, alpha = .05000

	ACTS (%)	Groups									
		1	2	3	4	5	6	7	8	9	10
G4	0.60	****									
G2	0.63	****	****								
C4	0.64	****	****								
K2	0.67	****	****								
G7	0.67	****	****								
J3	0.68	****	****								
E4	0.72	****	****	****							
G3	0.72	****	****	****							
G6	0.73	****	****	****							
H5	0.76	****	****	****	****						
C5	0.78	****	****	****	****	****					
K4	0.81	****	****	****	****	****					
G5	0.84	****	****	****	****	****					
F5	0.84	****	****	****	****	****					
J4	0.85	****	****	****	****	****					
H2	0.86	****	****	****	****	****					
G1	0.88	****	****	****	****	****	****				
M4	0.89	****	****	****	****	****	****				
C2	0.89	****	****	****	****	****	****				
H3	0.90	****	****	****	****	****	****				
F3	0.90	****	****	****	****	****	****				
A4	0.90	****	****	****	****	****	****				
H4	0.90	****	****	****	****	****	****				
F4	0.91	****	****	****	****	****	****	****			
M3	0.93	****	****	****	****	****	****	****			
E1	0.95	****	****	****	****	****	****	****	****		
M2	0.96	****	****	****	****	****	****	****	****	****	
M5	0.98	****	****	****	****	****	****	****	****	****	
C1	1.00	****	****	****	****	****	****	****	****	****	
K5	1.00	****	****	****	****	****	****	****	****	****	
E5	1.00	****	****	****	****	****	****	****	****	****	
E3	1.02	****	****	****	****	****	****	****	****	****	
H1	1.06	****	****	****	****	****	****	****	****	****	****
B6	1.08	****	****	****	****	****	****	****	****	****	****
D4	1.08	****	****	****	****	****	****	****	****	****	****

B5	1.10	****	****	****	****	****	****	****	****	****	****	
C3	1.11	****	****	****	****	****	****	****	****	****	****	
K3	1.12	****	****	****	****	****	****	****	****	****	****	
L3	1.13	****	****	****	****	****	****	****	****	****	****	
A5	1.14	****	****	****	****	****	****	****	****	****	****	
L5	1.14	****	****	****	****	****	****	****	****	****	****	
L4	1.15	****	****	****	****	****	****	****	****	****	****	
L2	1.15	****	****	****	****	****	****	****	****	****	****	
E2	1.16	****	****	****	****	****	****	****	****	****	****	
L1	1.16	****	****	****	****	****	****	****	****	****	****	
B3	1.17	****	****	****	****	****	****	****	****	****	****	
B2	1.21	****	****	****	****	****	****	****	****	****	****	
A3	1.31	****	****	****	****	****	****	****	****	****	****	****
D3	1.32	****	****	****	****	****	****	****	****	****	****	****
F1	1.33	****	****	****	****	****	****	****	****	****	****	****
D1	1.34	****	****	****	****	****	****	****	****	****	****	****
F2	1.38	****	****	****	****	****	****	****	****	****	****	****
K1	1.43		****	****	****	****	****	****	****	****	****	****
D2	1.49			****	****	****	****	****	****	****	****	****
B4	1.50			****	****	****	****	****	****	****	****	****
A1	1.55				****	****	****	****	****	****	****	****
B1	1.60					****	****	****	****	****	****	****
J2	1.68						****	****	****	****	****	****
A2	1.72							****	****	****	****	****
J1	1.77								****	****	****	****
M1	1.85									****	****	****
J5	2.07										****	****

Table AT24. Tukey HSD test; variable FUNGI (Spreadsheet1 in Workbook2); Homogenous Groups, alpha = .05000

	FUNGI (%)	Groups																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
G5	20.0	***																							
G7	22.4	***	***																						
H3	22.9	***	***																						
H4	23.8		***																						
G6	23.9		***																						
H5	24.3		***	***																					
G4	24.7		***	***																					
G2	24.8		***	***																					
G3	25.3		***	***	***																				
G1	25.3		***	***	***																				
K2	27.1			***	***	***																			
C5	27.3			***	***	***	***																		
A2	27.9				***	***	***	***																	
L1	28.3				***	***	***	***	***																
E4	28.3				***	***	***	***	***																
E5	28.5					***	***	***	***	***															
A1	28.6					***	***	***	***	***	***														
M2	28.6					***	***	***	***	***	***	***													
L2	28.8					***	***	***	***	***	***	***	***												
B1	28.9					***	***	***	***	***	***	***	***	***											
D1	29.4					***	***	***	***	***	***	***	***	***	***										
K4	29.7					***	***	***	***	***	***	***	***	***	***	***									
F3	29.7					***	***	***	***	***	***	***	***	***	***	***	***								
D4	29.8					***	***	***	***	***	***	***	***	***	***	***	***	***							
J5	30.2						***	***	***	***	***	***	***	***	***	***	***	***	***						
H1	30.3						***	***	***	***	***	***	***	***	***	***	***	***	***	***					
A5	30.6							***	***	***	***	***	***	***	***	***	***	***	***	***	***				
D3	30.7							***	***	***	***	***	***	***	***	***	***	***	***	***	***	***			
F1	30.7							***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***		
K5	31.3								***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
E3	31.5									***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
L4	31.5										***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
K1	31.6											***	***	***	***	***	***	***	***	***	***	***	***	***	***
L5	31.7												***	***	***	***	***	***	***	***	***	***	***	***	***

Table AT25. Average influent and effluent concentration of chemicals from WWTPs A and B in full-scale study

	influent (µg/L)				effluent (µg/L)				influent (µg/L)			Effluent (µg/L)		
	A1	A2	A3	A4	A1	A2	A3	A4	B1	B2	B3	B1	B2	B3
E1	0.0685	0.0423	0.0294	0.01882	0.0041	0.0042	0.00277	0.00153	0.04408	0.05618	0.01909	0.00673	<0.001	<0.001
E2	0.0173	0.0185	0.00506	0.00882	0.00249	0.0005	<0.0003	<0.0003	0.01052	0.01811	0.00748	<0.0003	<0.0003	<0.0003
EE2	0.00296	0.0011	0.00107	0.00027	0.00238	0.001	0.00012	0.00008	0.00128	0.00039	0.00016	0.00046	<0.00003	<0.00003
DCF	0.663	0.803	0.582	0.335	0.418	0.569	0.427	0.222	0.9157	0.396	0.391	0.3944	0.299	0.192
IBPF	33.532	29.432	21.64	28.331	0.041	<0.01	0.01	<0.01	26.8458	21.233	20.555	<0.01	0.012	<0.01
ATOV	0.589	0.482	0.502	0.326	0.062	0.024	0.089	0.147	0.435	0.318	0.0536	0.0149	0.086	0.319
ATOV_o	1.29	1.944	1.137	1.391	0.052	0.017	0.148	0.4	0.948	0.866	0.189	0.0294	0.25	1.269
ATOV_p	0.995	0.093	0.91	0.602	0.062	0.059	0.165	0.166	0.739	0.647	0.126	<0.01	0.11	0.608
PRPL	0.463	0.415	0.3625	0.298	0.516	0.284	0.4298	0.16	0.246	0.189	0.0557	0.263	0.184	0.0234
ATNL	4.788	5.236	4.305	2.993	0.147	0.0603	0.318	4.899	1.381	1.278	1.114	0.0557	0.09	0.0833
ERMY	8.022	0.869	1.439	1.256	0.844	0.496	0.74	0.478	1.42	0.562	0.146	<0.1	0.253	<0.1
ERMY_n	0.212	0.108	<0.1	0.138	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
AZMY	0.133	4.6585	2.913	0.943	1.235	1.9234	2.344	0.244	0.463	0.727	0.0166	0.201	0.237	0.019
CLMY	2.499	1.6	4.513	1.971	0.838	0.618	1.248	0.919	2.266	3.969	0.0158	0.132	0.623	<0.01
CIPR	1.933	1.6318	5.879	0.955	0.292	0.2265	0.88	0.0473	4.562	2.052	0.687	0.305	0.262	0.0823
METF	220.87	261.898	232.215	154.552	1.281	0.7368	1.723	5.002	136.594	100.089	78.351	0.667	0.409	0.48
RNTD	4.624	2.5113	2.249	2.683	0.427	0.339	0.466	4.904	1.666	1.92	1.796	0.148	0.295	0.194
CBAZ	1.302	0.793	0.643	0.496	1.163	0.691	0.916	0.522	0.572	0.52	0.126	0.768	0.714	<0.1
CBAZ_e	0.221	0.109	0.114	0.136	0.148	0.125	<0.1	<0.1	<0.1	0.123	<0.1	<0.1	<0.1	<0.1
SERT	0.247	0.18	0.42	0.116	0.105	0.05	0.126	0.0434	0.346	0.148	0.045	0.0461	0.04	0.144
SERT_n	0.182	0.258	0.403	0.172	0.048	0.018	0.048	0.0325	0.237	0.122	0.0494	0.0209	0.027	0.129
FLXT	0.186	0.139	0.296	0.139	0.085	0.097	0.142	0.0609	0.175	0.235	0.0615	0.0635	0.049	0.115
TMXF	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
TXP	<0.01	<0.01	<0.01	0.792	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
BZT	1.536	15.038	30.938	1.505	4.45	5.5	4.862	4.38	4.967	1.538	1.562	3.427	6.547	2.843
TZT	0.992	1.013	7.709	0.748	0.665	0.667	1.453	0.704	1.166	0.886	0.842	0.922	1.155	0.793

Table AT26. Average influent and effluent concentration of chemicals from WWTPs C and D in full-scale study

	Influent (µg/L)			Effluent (µg/L)			Influent (µg/L)				Effluent (µg/L)			
	C3	C4	C5	C3	C4	C5	D1	D2	D3	D4	D1	D2	D3	D4
E1	0.0778	0.05942	0.06957	0.00014	0.00047	0.00025	0.0759	0.0691	0.07577	0.0656	0.00134	0.0010	0.00178	0.00164
E2	0.0257	0.02654	0.02391	0.00013	0.00011	0.00013	0.0328	0.0423	0.03085	0.0262	0.00011	0.00004	0.00014	0.00008
EE2	0.00198	0.00051	0.00096	0.00014	0.0005	0.00038	0.00090	0.00054	0.00091	0.00061	0.00006	0.00013	0.00006	0.00008
ATOV	0.379	1.340	1.450	< 0.010	0.059	0.011	1.930	1.580	2.140	1.450	< 0.010	0.068	0.154	0.040
ATOV_o	1.260	1.220	1.470	0.017	0.044	< 0.010	2.130	1.870	2.000	1.540	0.014	0.085	0.145	0.040
ATOV_p	0.547	1.720	2.250	< 0.010	0.057	< 0.010	2.940	2.470	2.880	2.210	< 0.010	0.051	0.132	0.031
ERMY_n	0.110	0.377	0.156	< 0.100	< 0.010	0.011	0.062	0.057	0.083	0.026	< 0.010	0.010	0.011	< 0.010
AZMY	0.815	0.281	0.522	0.389	0.223	0.252	1.090	1.200	0.632	0.870	0.430	0.541	0.458	0.346
CLMY	0.321	0.563	0.338	0.012	0.062	0.039	1.750	0.389	1.960	2.460	0.164	0.142	0.168	0.093
CIPR	1.210	1.040	0.823	0.396	0.139	0.099	3.520	2.040	5.670	6.380	0.052	0.103	0.097	0.065
METF	114.000	134.000	136.000	0.513	0.656	0.992	210.000	187.000	211.000	228.000	0.570	0.254	2.290	1.090
SERT	0.118	0.198	0.197	0.011	0.020	0.024	0.693	0.312	0.309	0.328	0.063	0.068	0.064	0.045
SERT_n	0.129	0.154	0.199	0.014	0.015	< 0.010	0.698	0.301	0.285	0.215	< 0.010	0.020	0.019	0.016
TXP	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010
BZT	3.740	1.700	2.450	17.100	2.470	1.860	2.190	1.730	1.880	1.620	0.858	1.020	0.868	0.550
TZT	0.610	2.010	0.631	6.390	0.616	0.347	1.310	1.020	0.938	0.783	0.618	0.796	0.759	0.571
IBPF	22.600	28.400	19.100	< 0.010	< 0.010	< 0.010	27.000	34.100	33.100	28.600	< 0.010	< 0.010	0.045	< 0.010
ERMY	0.985	5.460	1.180	< 0.100	0.089	0.094	0.870	0.339	0.886	0.401	0.060	0.080	0.081	0.057
PRPL	0.366	0.538	0.323	0.209	0.192	0.144	0.562	0.568	0.497	0.584	0.299	0.315	0.251	0.265
FLXT	0.196	0.147	0.155	0.017	0.027	0.029	0.179	0.104	0.161	0.140	0.044	0.050	0.054	0.040
DCF	0.480	0.335	1.250	0.308	0.339	0.337	1.270	0.690	0.948	0.879	0.449	0.520	0.470	0.440
ATNL	1.760	1.960	2.410	0.114	0.197	0.296	5.920	3.950	4.990	4.550	0.140	0.327	0.466	0.193
CBAZ	0.903	0.775	0.827	0.743	0.693	0.682	0.826	0.583	1.970	0.751	1.070	0.836	0.857	0.825
CBAZe	0.186	0.264	0.161	< 0.100	0.069	0.087	0.106	0.104	0.134	0.358	0.139	0.107	0.092	0.096
RNTD	3.810	5.170	2.990	0.213	0.166	0.194	5.720	4.350	4.210	4.520	0.453	0.795	0.742	0.378
TMXF	< 0.005	0.012	0.014	< 0.005	< 0.005	< 0.005	0.007	< 0.005	0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005

Table AT27. Average influent and effluent concentration of chemicals from WWTPs H, K and M in full-scale study

	Influent (µg/L)		Effluent (µg/L)		Influent (µg/L)				Effluent (µg/L)			
	H1	H2	H1	H2	K1	K2	K3	K4	K1	K2	K3	K4
E1	0.0659	0.00737	0.01277	0.00941	0.0106	0.05622	0.03854	0.01114	0.0039	0.00364	0.00141	0.00262
E2	0.02176	0.0059	0.00186	0.00071	0.0265	0.0347	0.0143	0.00331	0.0054	0.00175	0.00172	0.00215
EE2	0.00066	0.000185	0.00021	0.00004	0.0062	0.00032	0.0003	0.00028	0.0037	0.00004	0.00013	0.00004
DCF	1.241	0.167	0.354	0.296	0.818	0.912	0.656	0.27	0.41	0.471	0.493	0.382
IBPF	36.8	15.599	<0.01	<0.01	23.589	25.137	23.664	8.148	<0.01	0.01	0.027	0.0476
ATOV	0.797	0.223	0.202	0.153	0.881	1.323	0.464	0.331	0.037	0.142	0.144	0.214
ATOV_o	1.959	0.817	0.482	0.61	2.183	3.532	1.769	0.986	0.234	0.402	0.551	0.527
ATOV_p	1.459	0.413	0.279	0.242	1.193	2.263	0.817	0.539	0.057	0.191	0.237	0.32
PRPL	0.381	0.135	0.234	0.187	0.905	0.181	0.261	0.089	0.529	0.282	0.291	0.254
ATNL	3.538	1.113	0.229	0.266	3.819	2.871	2.867	0.862	0.233	0.419	0.493	0.383
ERMY	2.864	0.639	0.919	0.649	2.417	1.37	1.004	0.578	0.792	0.886	0.812	0.599
ERMY_n	0.199	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
AZMY	0.563	0.386	0.708	0.542	1.425	1.611	1.08	0.113	0.516	0.586	0.697	0.223
CLMY	1.905	1.906	0.778	0.886	3.758	2.767	3.516	1.764	0.668	2.593	2.508	1.457
CIPR	2.624	0.993	0.135	0.155	1.201	1.735	3.401	0.104	0.043	0.196	0.182	0.01
METF	331.612	109.409	4.539	0.878	206.703	188.759	230.218	76.057	1.784	5.532	5.75	1.938
RNTD	3.907	1.363	0.464	0.328	3.957	2.538	2.263	0.713	0.271	0.54	0.555	0.493
CBAZ	0.529	0.356	0.682	0.367	0.832	0.385	0.648	0.2	0.954	0.685	0.62	0.609
CBAZ_e	0.125	<0.1	<0.1	<0.1	0.134	<0.1	0.163	<0.1	0.184	0.105	<0.1	<0.1
SERT	0.243	0.0828	0.052	0.0439	0.655	0.221	0.395	0.0732	0.096	0.064	0.073	0.0579
SERT_n	0.248	0.0652	0.037	0.0272	0.606	0.215	0.343	0.0811	0.065	0.043	0.026	0.0421
FLXT	0.261	0.0603	0.11	0.0445	0.366	0.13	0.399	0.0508	0.07	0.129	0.143	0.0792
TMXF	<0.005	<0.005	<0.005	<0.005	<0.005	0.008	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
TXP	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
BZT	1.4715	0.579	1.08835	1.14	2.276	3.048	2.77755	2.34	0.973	2.079	1.52228	1.494
TZT	0.81204	5.306	1.29382	2.171	8.579	1.155	0.28403	0.797	0.522	1.197	0.89397	1.262

Table AT28. Average influent and effluent concentration of chemicals from WWTP M in full-scale study

	Influent (µg/L)			Effluent (µg/L)		
	M1	M2	M3	M1	M2	M3
E1	0.059	0.07948	0.01538	0.0078	0.0109	0.00245
E2	0.0254	0.00907	0.00276	0.005	0.00084	0.00032
EE2	0.0075	0.00026	0.00037	0.0037	0.00019	0.0002
DCF	0.319	1.592	0.406	0.65	0.362	0.38
IBPF	9.378	16.9	12.948	<0.01	<0.01	<0.01
ATOV	0.338	2.229	0.365	0.051	0.0726	0.138
ATOV_o	0.658	3.356	1.114	0.07	0.0898	0.504
ATOV_p	0.42	1.747	0.621	0.06	0.0939	0.209
PRPL	0.169	0.244	0.138	0.24	0.183	0.108
ATNL	1.333	1.973	1.462	0.171	0.179	0.267
ERMY	0.916	1.555	0.725	0.434	0.648	0.477
ERMY_n	<0.1	0.106	<0.1	<0.1	<0.1	<0.1
AZMY	0.124	0.0748	0.849	0.539	0.26132	0.417
CLMY	0.702	0.748	0.947	0.202	0.306	0.357
CIPR	0.701	1.0248	0.577	0.234	0.2964	0.0683
METF	127.907	162.332	183.968	1.17	0.841	1.327
RNTD	3.324	2.468	2.055	0.64	0.668	0.384
CBAZ	0.458	0.648	0.593	0.748	0.609	0.452
CBAZ_e	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
SERT	0.056	0.3	0.108	0.036	0.0822	0.02
SERT_n	0.081	0.01	0.0894	0.011	0.0328	0.0132
FLXT	0.022	0.0837	0.0793	0.016	0.062	0.0221
TMXF	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
TXP	0.203	0.2688	0.0282	<0.01	<0.01	0.0268
BZT	1.001	39.106	11.522	3.587	5.085	6.673

Table AT29. m/z values of the estrogens and nonylphenols in pilot-scale study

Nonylphenols	m/z precursor	Estrogens	m/z precursor
NP	219.0 ^a	Estrone	269.10a
NP ₁ EC	263.5 ^a	Estradiol	271.10a
NP ₂ EC	307.0 ^a	Estriol	287.10a
NP ₃ EC	351.0 ^a	Ethinylestradiol	295.15a
NP ₁ EO	282.0 ^b	Estrone-1,3-sulfate	349.05a
NP ₂ EO	326.0 ^b		
NP ₃ EO	370.0 ^b		
NP ₄ EO	414.0 ^b		
NP ₅ EO	458.0 ^b		
NP ₆ EO	502.0 ^b		
NP ₇ EO	546.0 ^b		
NP ₈ EO	590.0 ^b		
NP ₉ EO	634.0 ^b		
NP ₁₀ EO	678.0 ^b		
NP ₁₁ EO	722.0 ^b		
NP ₁₂ EO	766.0 ^b		

^a[M-H]; ^b[M+NH₄]⁺

Table AT30. Concentrations (unnormalized) of nonylphenols in settled sewage and final effluent and their percentage removal from the pilot-scale study

	Solids retention time (SRT)									Hydraulic retention time (HRT)								
	3 d			10 d			27 d			8 h			16 h			24 h		
	Settled sewage (ng/L)	Final Eff. (ng/L)	% Rem	Settled sewage (ng/L)	Final Eff. (ng/L)	% Rem	Settled Sewage (ng/L)	Final Eff. (ng/L)	% Rem	Settled sewage (ng/L)	Final Eff. (ng/L)	% Rem	Settled sewage (ng/L)	Final Eff. (ng/L)	% Rem	Settled sewage (ng/L)	Final Eff. (ng/L)	% Rem.
NP	1583	1738	-10	1062	1379	-30	1665	1764	-6	1665	1764	-6	247.9	73.2	70	628.9	234.0	63
NP₁EC	48.8	207.1	-324	1374	443.4	68	45.3	101.5	-124	45.3	101.5	-124	629.7	2959	-370	416.2	1906	-358
NP₂EC	68.0	1044	-1435	774.3	691.4	11	76.9	357.4	-365	76.9	357.4	-365	2334	3133	-34	1867	6108	-227
NP₃EC	48.7	49.7	-2	48.8	681.0	-1295	45.3	94.6	-109	45.3	94.6	-109	155.1	832.5	-437	246.3	2117	-759
NP₁EO	1812	710.7	61	65.7	1635	-2391	1067	372.8	65	1067	372.8	65	238.5	14.9	94	376.5	15.6	96
NP₂EO	703.6	1066	-52	48.7	121.7	-150	1010	651.7	35	1010	651.7	35	51.4	3.5	93	153.2	3.4	98
NP₃EO	10.4	18.0	-73	13.9	8.5	39	18.8	8.6	54	18.8	8.6	54	7.8	1.6	80	18.5	1.3	93
NP₄EO	329.2	183.7	44	354.1	95.2	73	613.9	99.1	84	613.9	99.1	84	333.1	18.9	94	384.1	35.4	91
NP₅EO	104.6	52.9	49	113.8	17.2	85	172.3	19.4	89	172.3	19.4	89	112.7	9.4	92	112.7	8.5	92
NP₆EO	421.0	58.2	86	398.7	43.1	89	596.8	24.8	96	596.8	24.8	96	433.4	21.7	95	420.1	31.9	92
NP₇EO	228.8	28.0	88	207.9	19.7	91	255.0	12.0	95	255.0	12.0	95	224.3	12.1	95	230.9	15.2	93
NP₈EO	160.4	24.3	85	152.2	8.5	94	177.8	6.0	97	177.8	6.0	97	167.8	16.8	90	161.7	11.7	93
NP₉EO	281.9	23.9	92	263.6	15.6	94	320.9	13.0	96	320.9	13.0	96	283.8	18.0	94	301.6	21.9	93
NP₁₀EO	383.7	34.2	91	372.1	21.8	94	381.8	20.3	95	381.8	20.3	95	403.1	29.0	93	433.6	32.5	93
NP₁₁EO	451.8	45.3	90	445.5	24.8	94	547.3	32.4	94	547.3	32.4	94	509.8	45.4	91	534.0	44.2	92
NP₁₂EO	378.4	43.6	88	372.0	23.4	94	481.5	40.2	92	481.5	40.2	92	477.3	57.2	88	488.7	47.5	90

% Rem = % removal; Inf = influent; Eff = effluent

APPENDED FIGURES

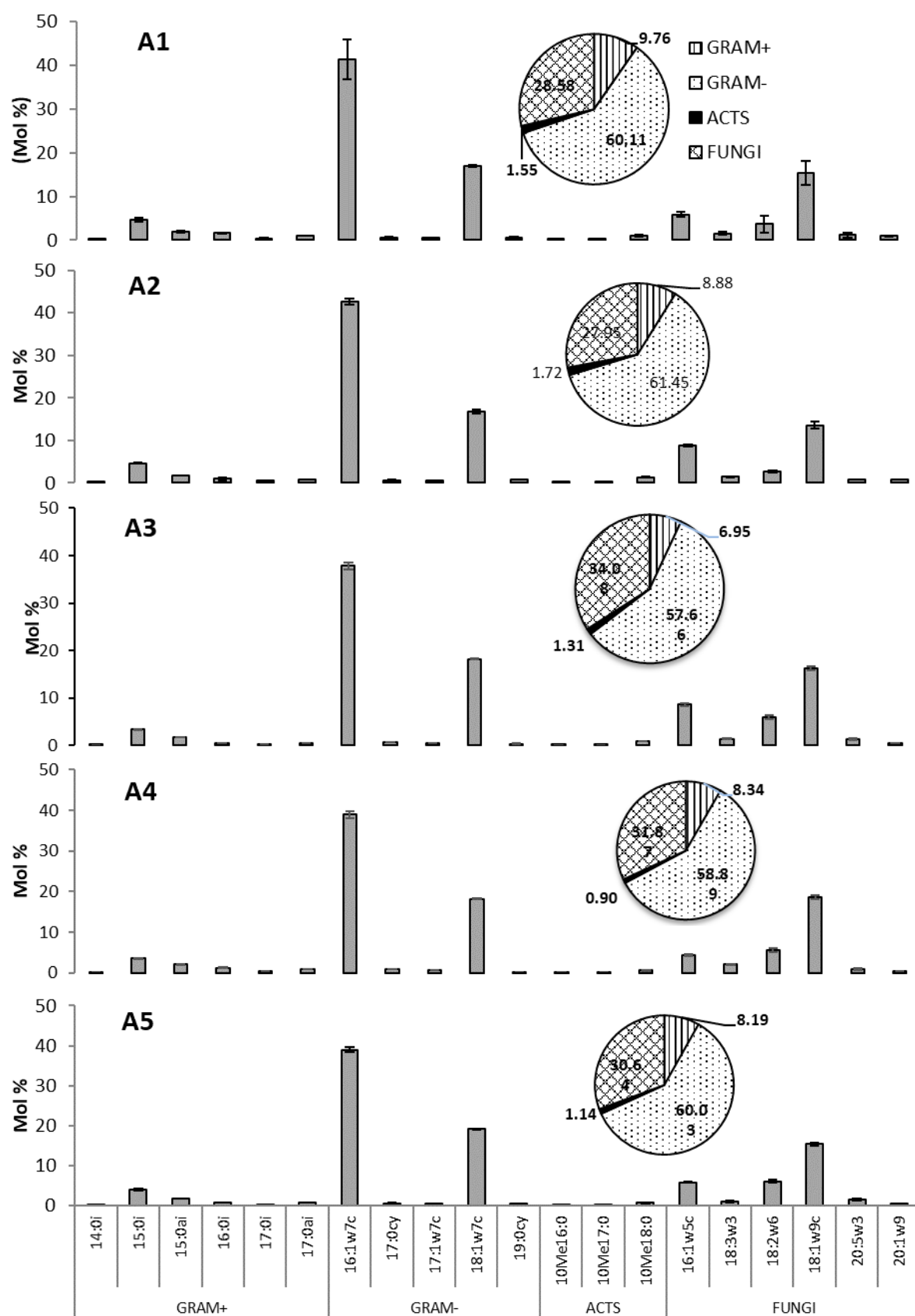


Figure AF1. Bar charts of the fatty acids profile from sampling event from site A, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

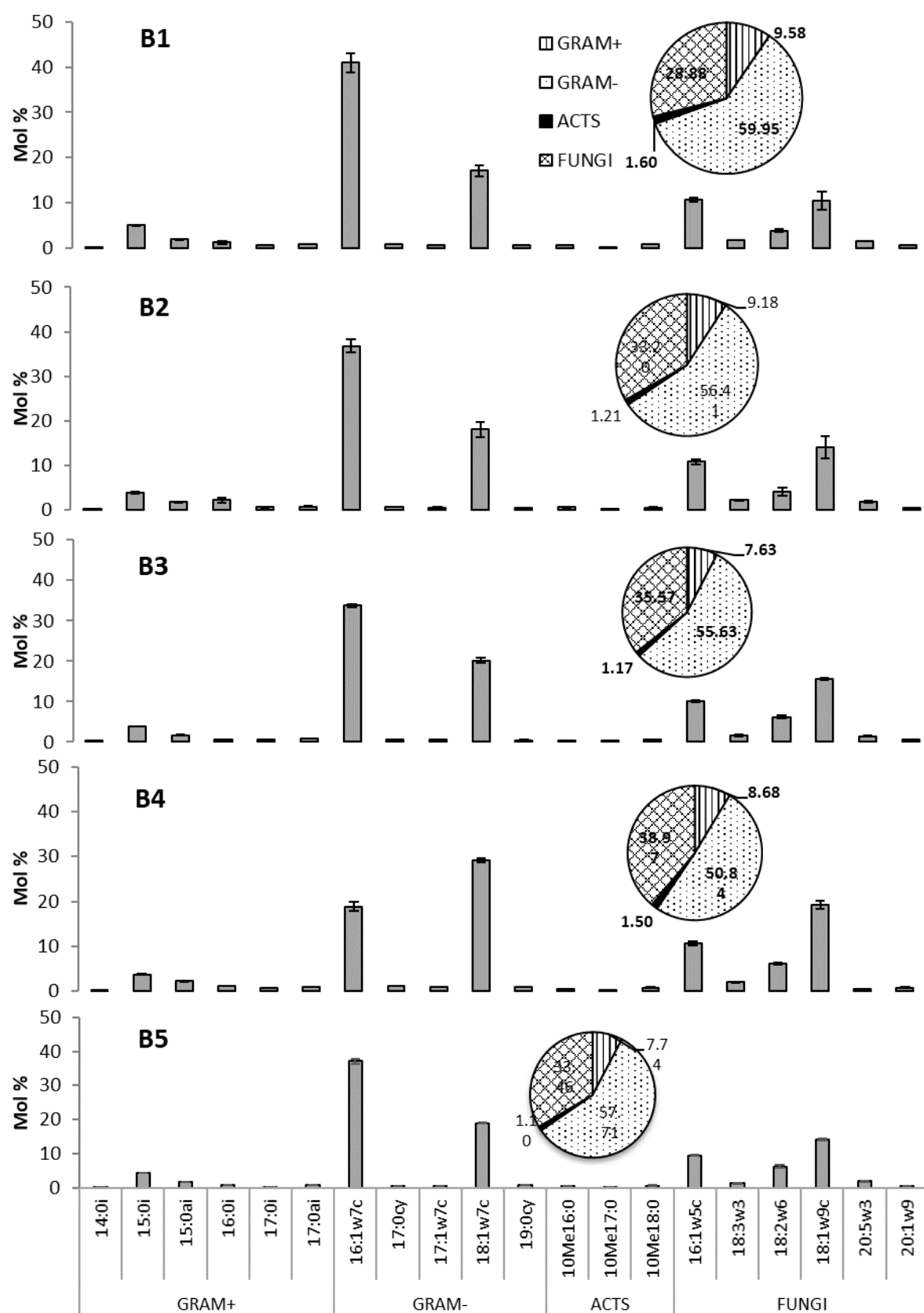


Figure AF2. Bar charts of the fatty acids profile from sampling event from site B, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

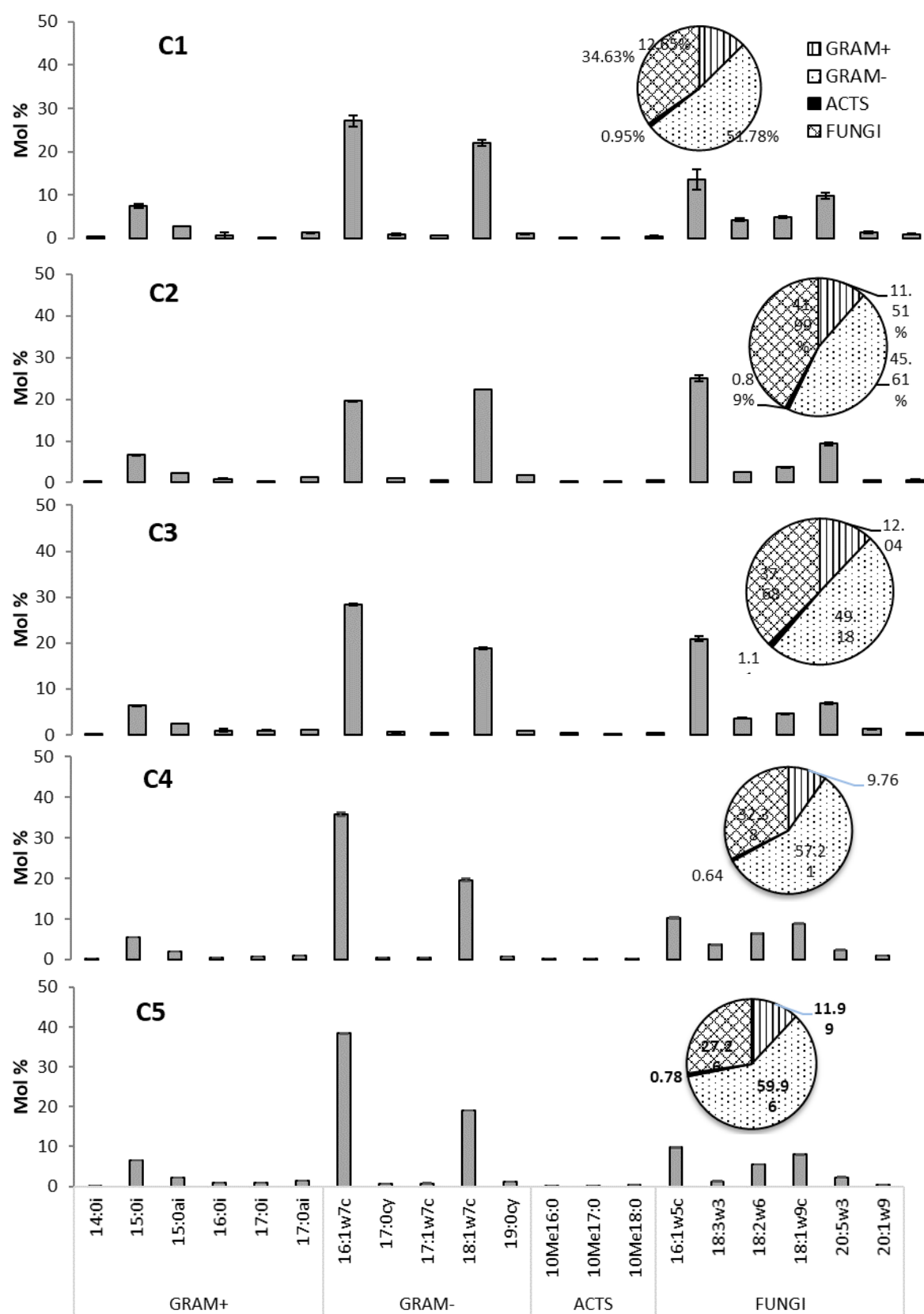


Figure AF3. Bar charts of the fatty acids profile from sampling event from site C, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

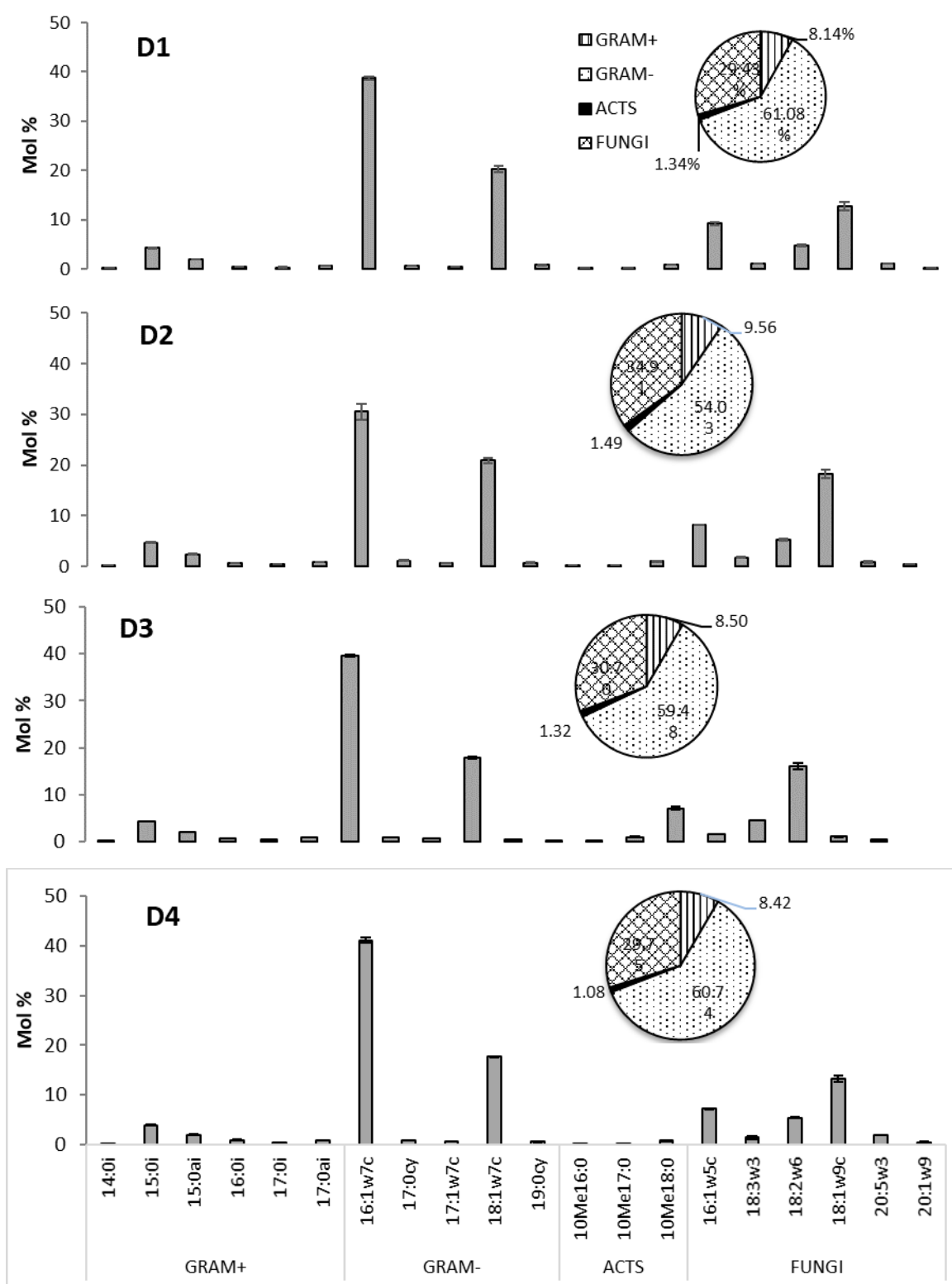


Figure AF4. Bar charts of the fatty acids profile from sampling event from site D, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

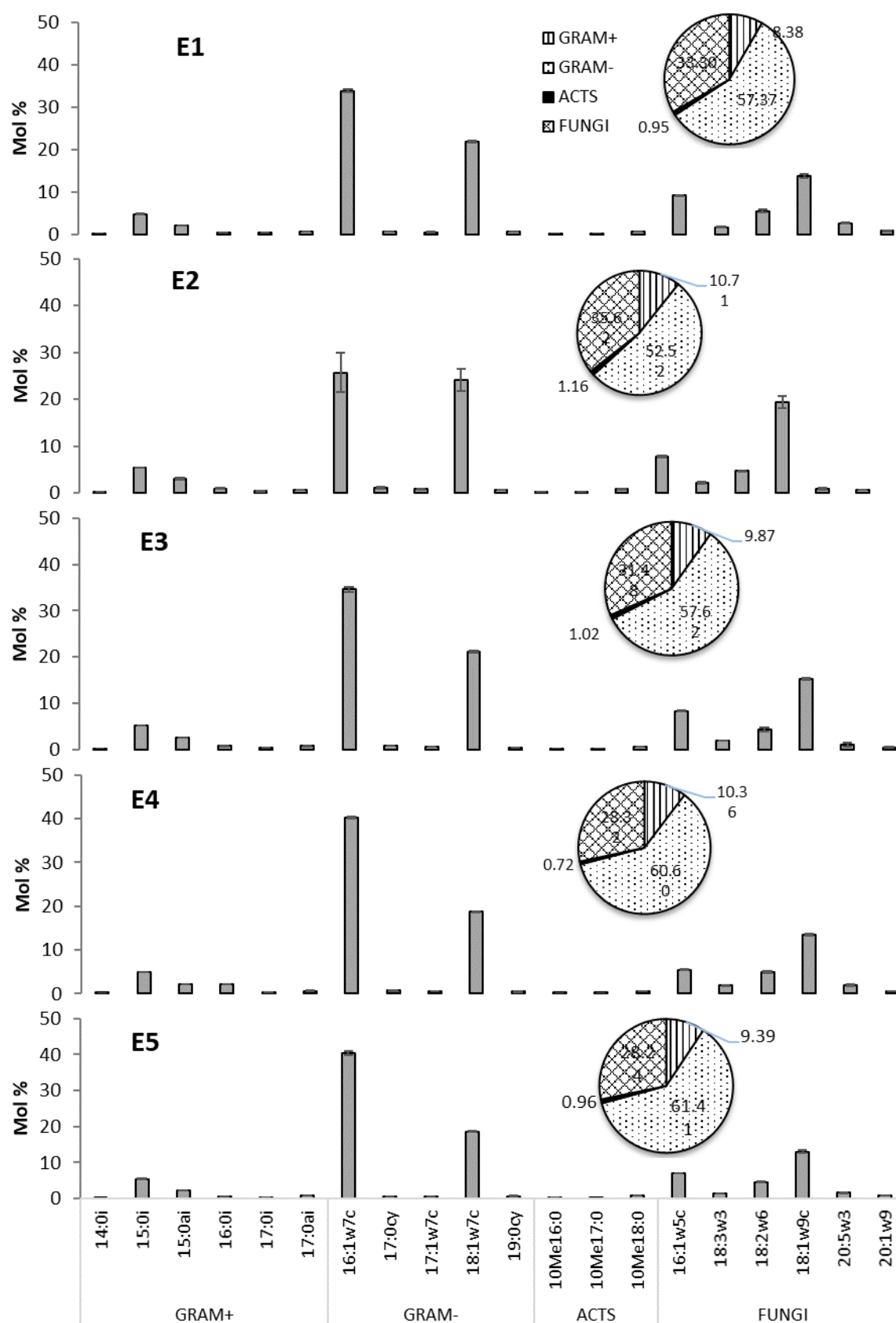


Figure AF5. Bar charts of the fatty acids profile from sampling event from site E, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

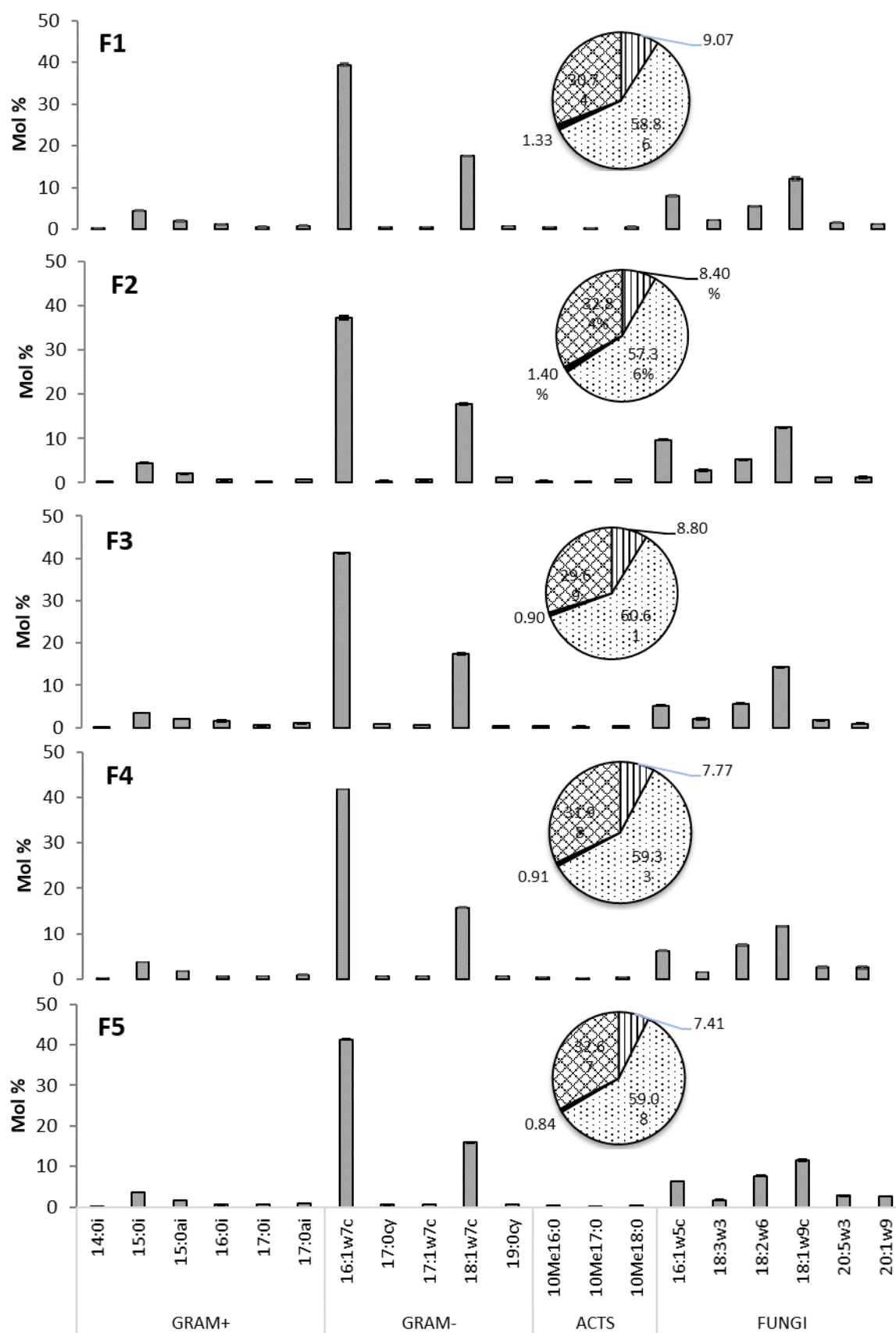


Figure AF6. Bar charts of the fatty acids profile from sampling event from site F, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

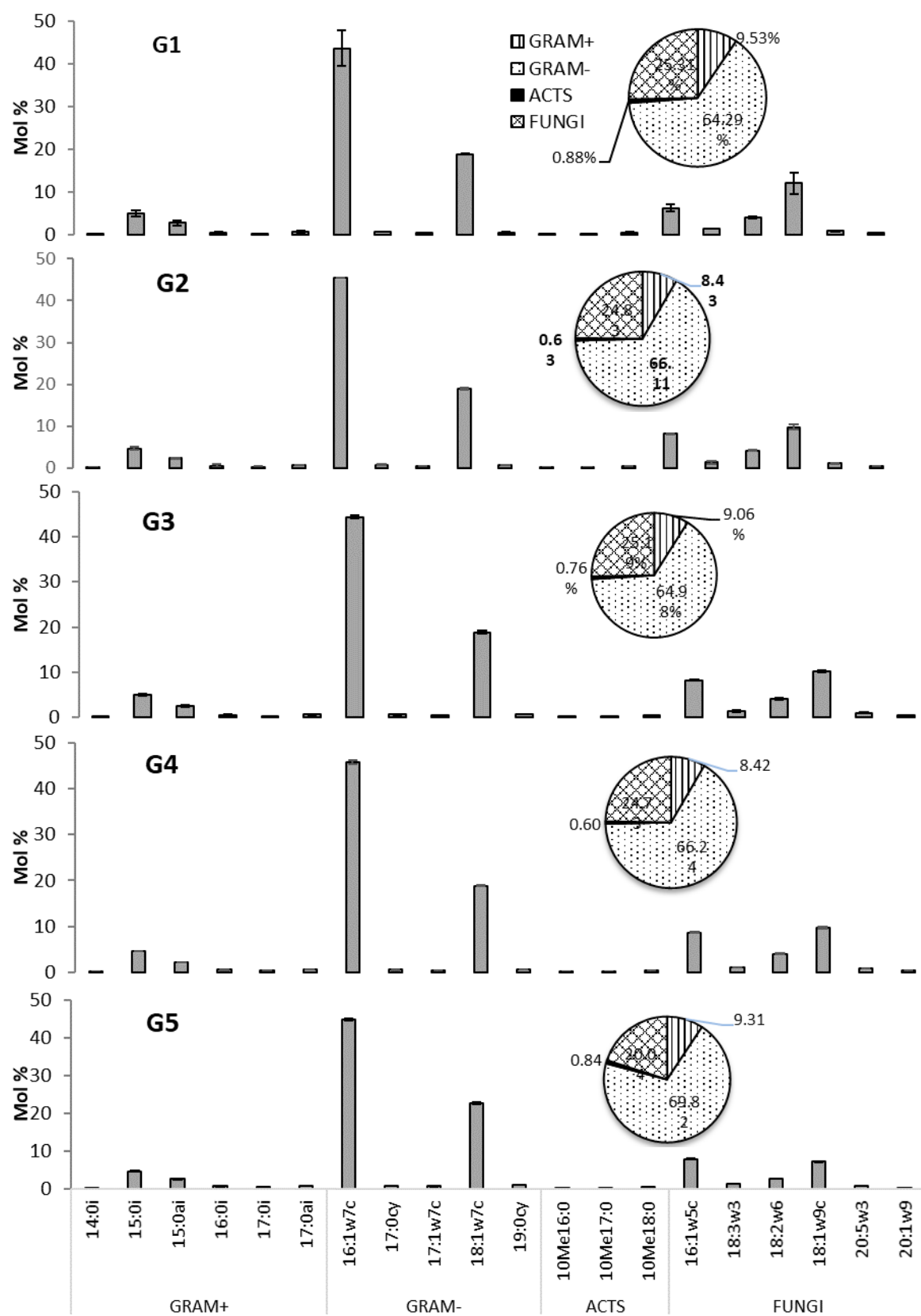


Figure AF7. Bar charts of the fatty acids profile from sampling event from site G, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

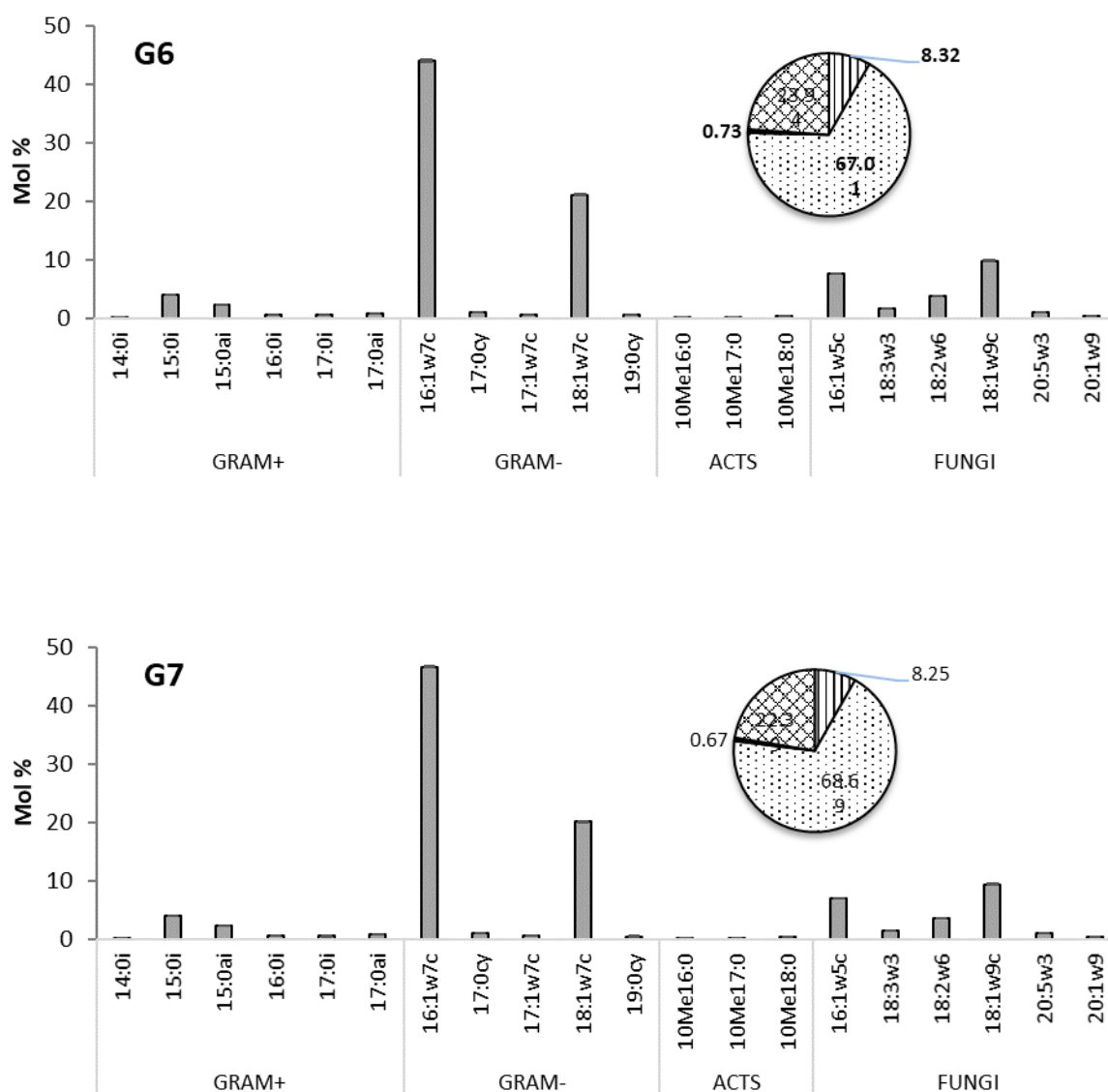


Figure AF8. Bar charts of the fatty acids profile from sampling event from site G (continued), and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

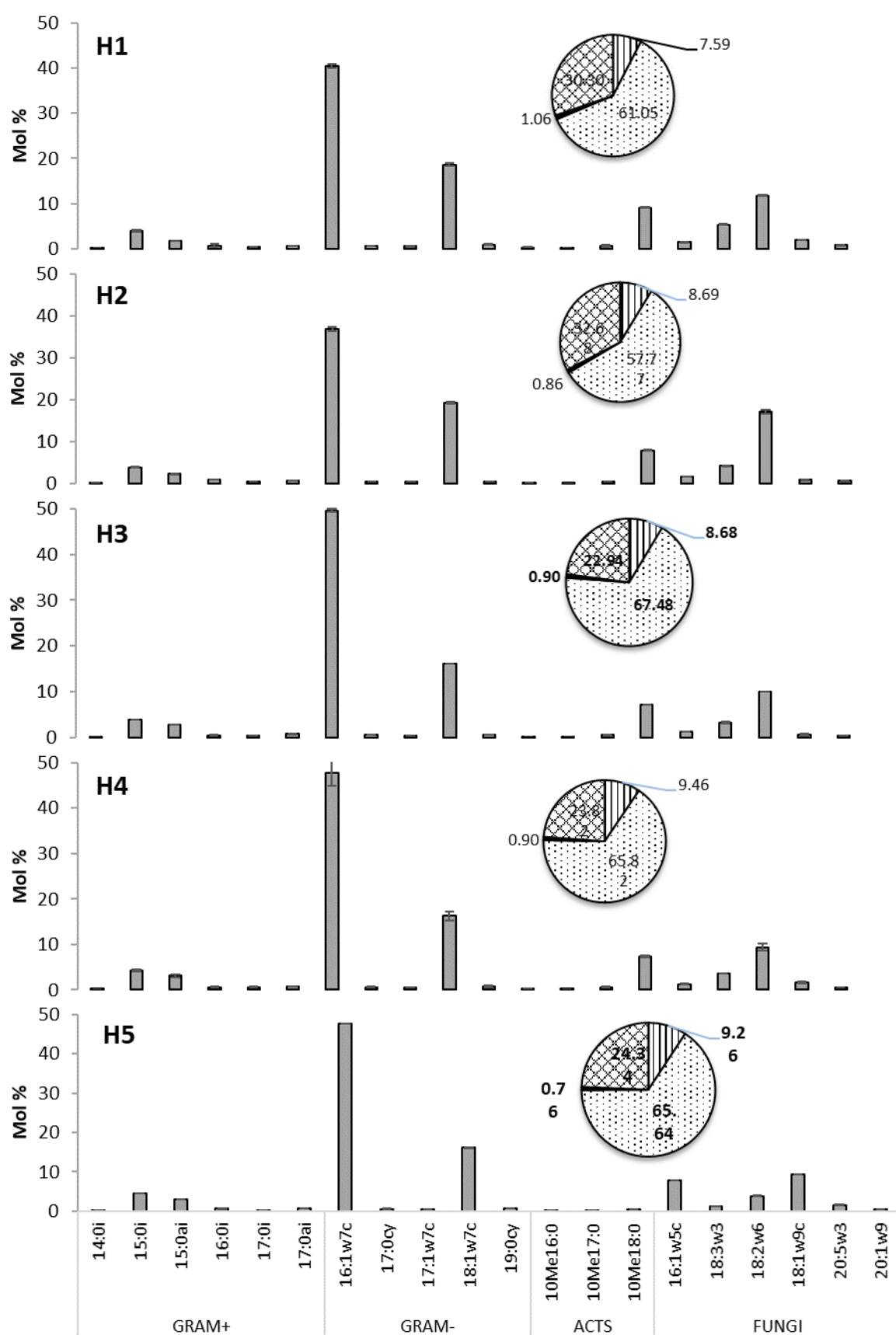


Figure AF9. Bar charts of the fatty acids profile from sampling event from site H, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

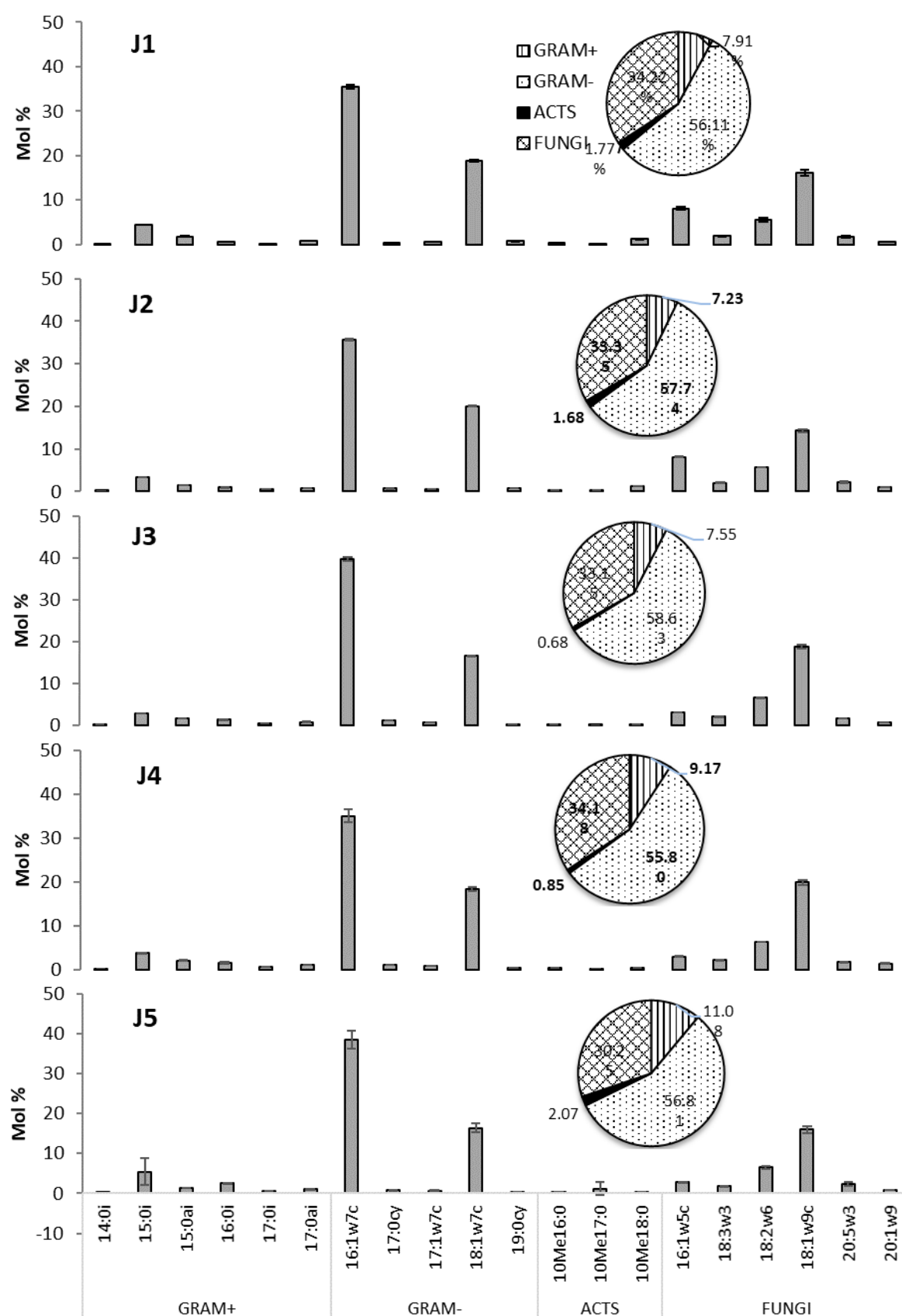


Figure AF10. Bar charts of the fatty acids profile from sampling event from site J, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

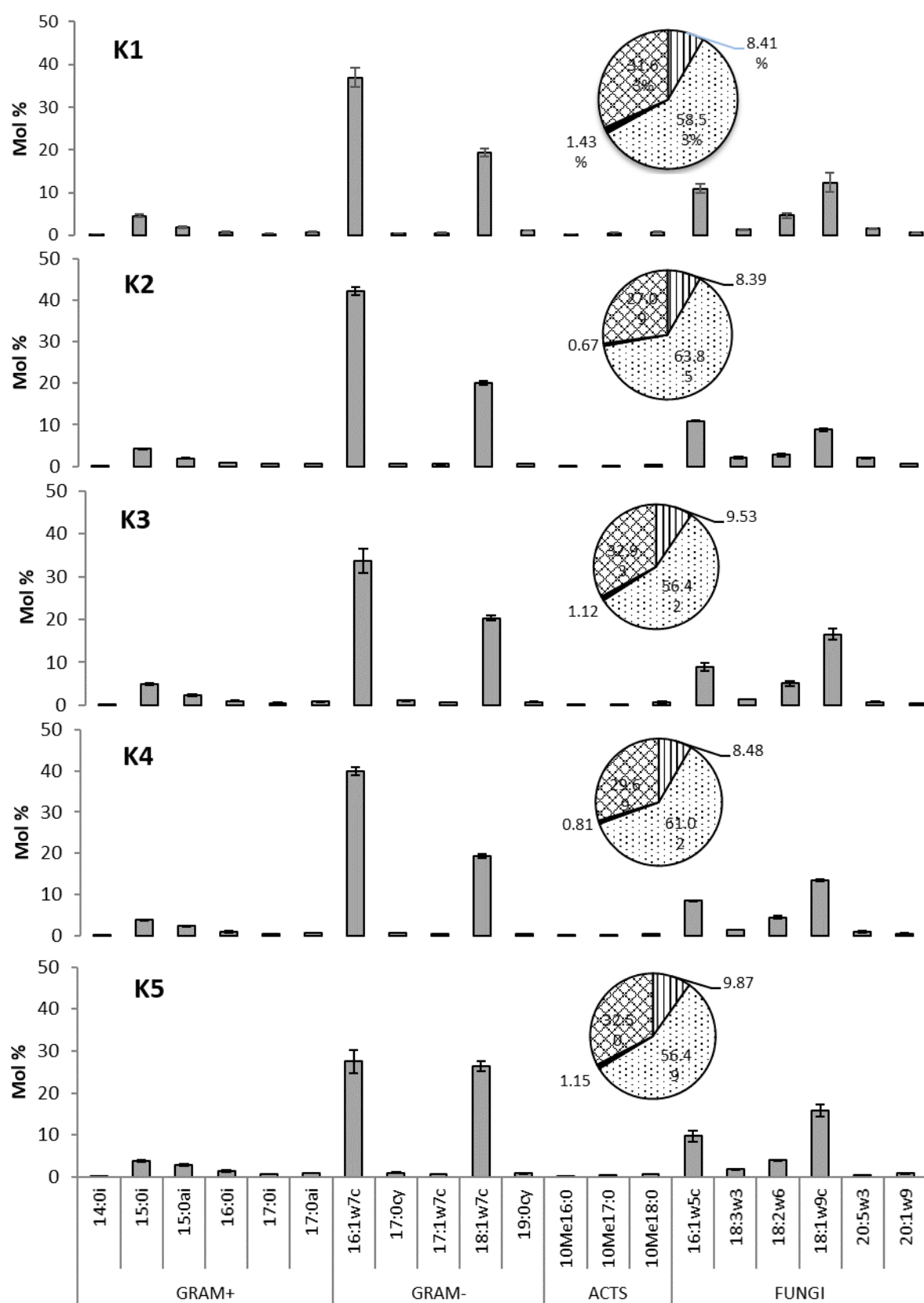


Figure AF11. Bar charts of the fatty acids profile from sampling event from site K, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

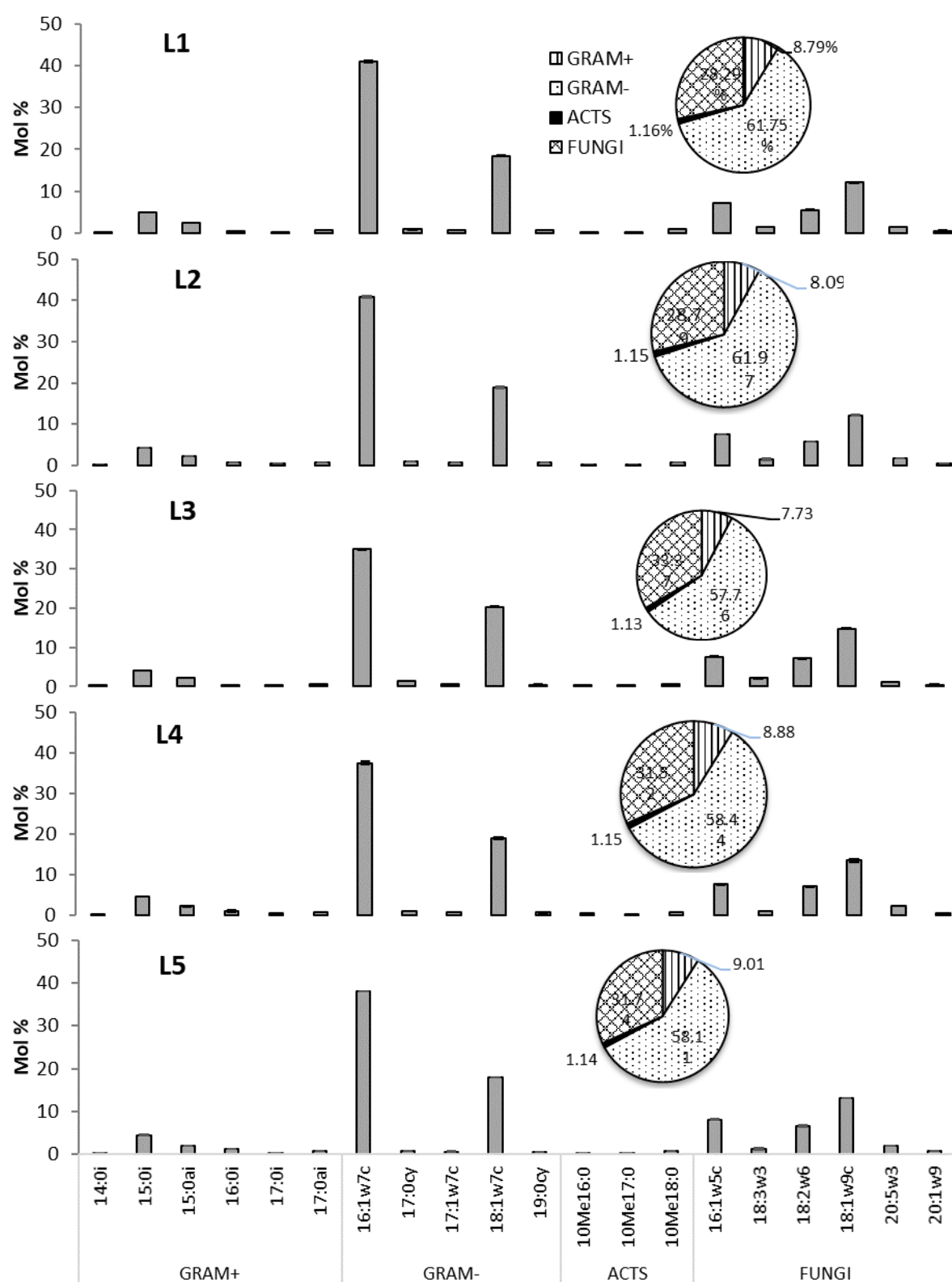


Figure AF12. Bar charts of the fatty acids profile from sampling event from sites A, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

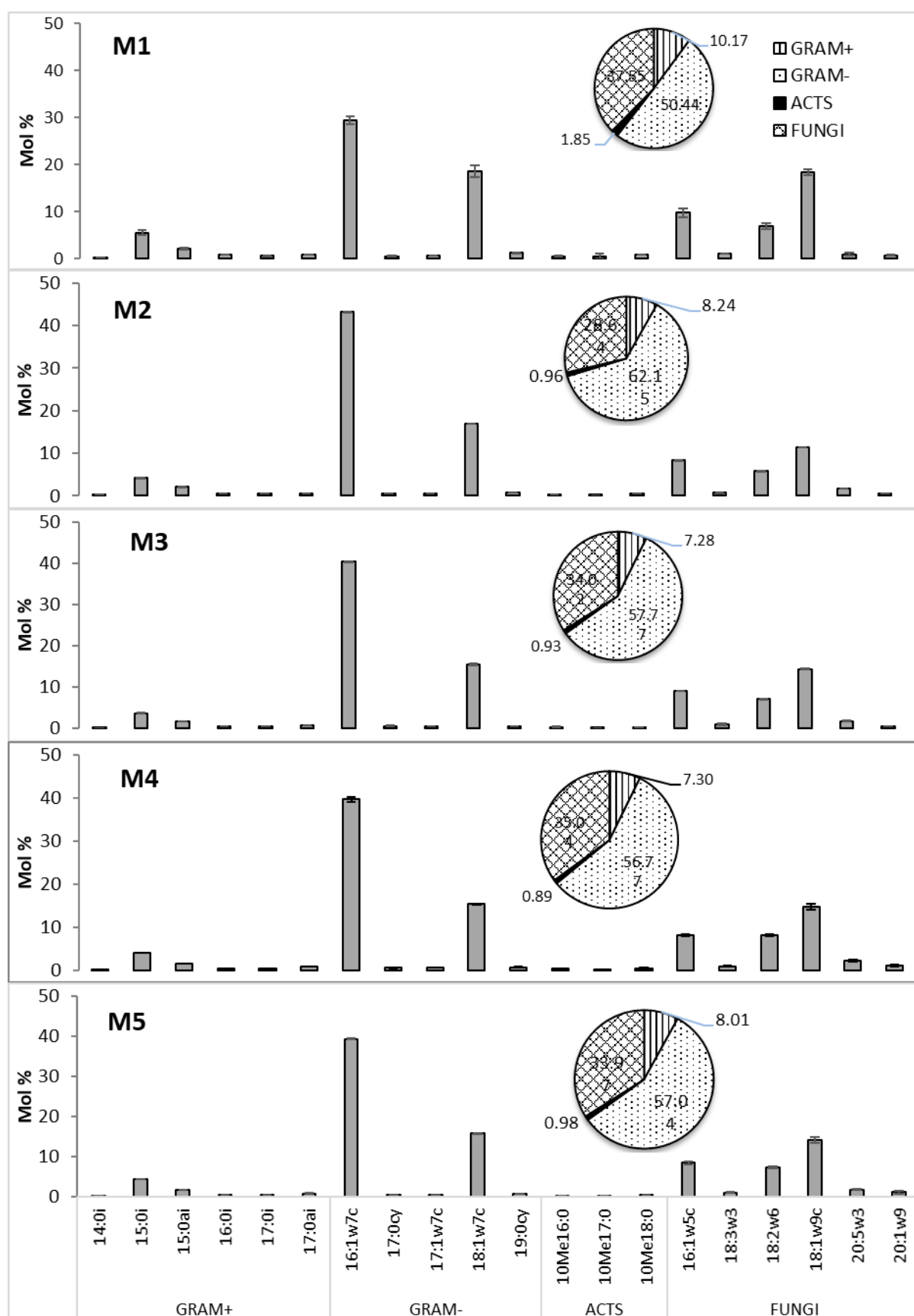


Figure AF13. Bar charts of the fatty acids profile from sampling event from sites A, and pie charts of the relative abundances of G+ bacteria, G- bacteria, actinomycetes and fungi.

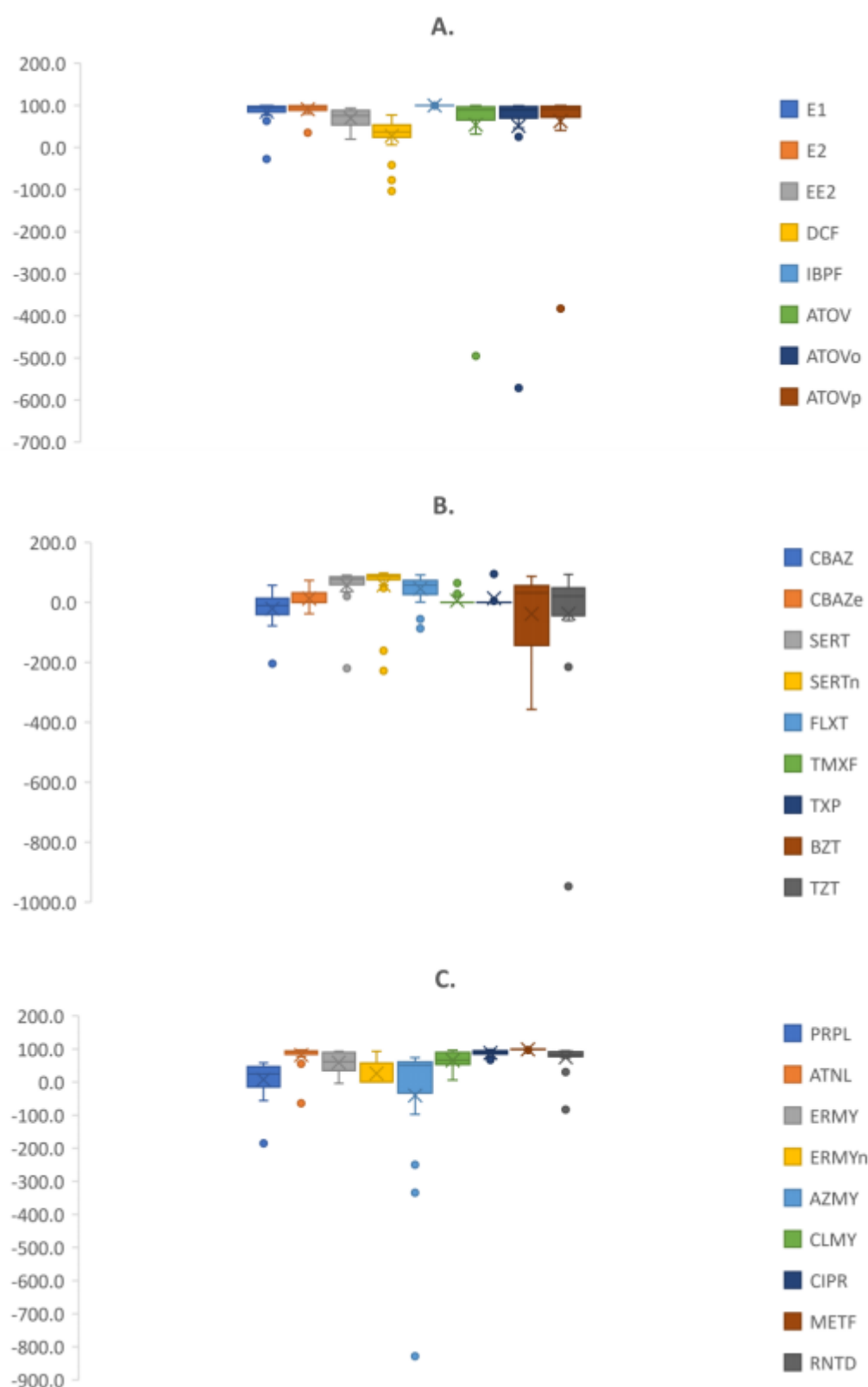


Figure AF14. Box and whiskers plot of average removal for each chemical across seven WWTP over 21 sample events to show outliers.

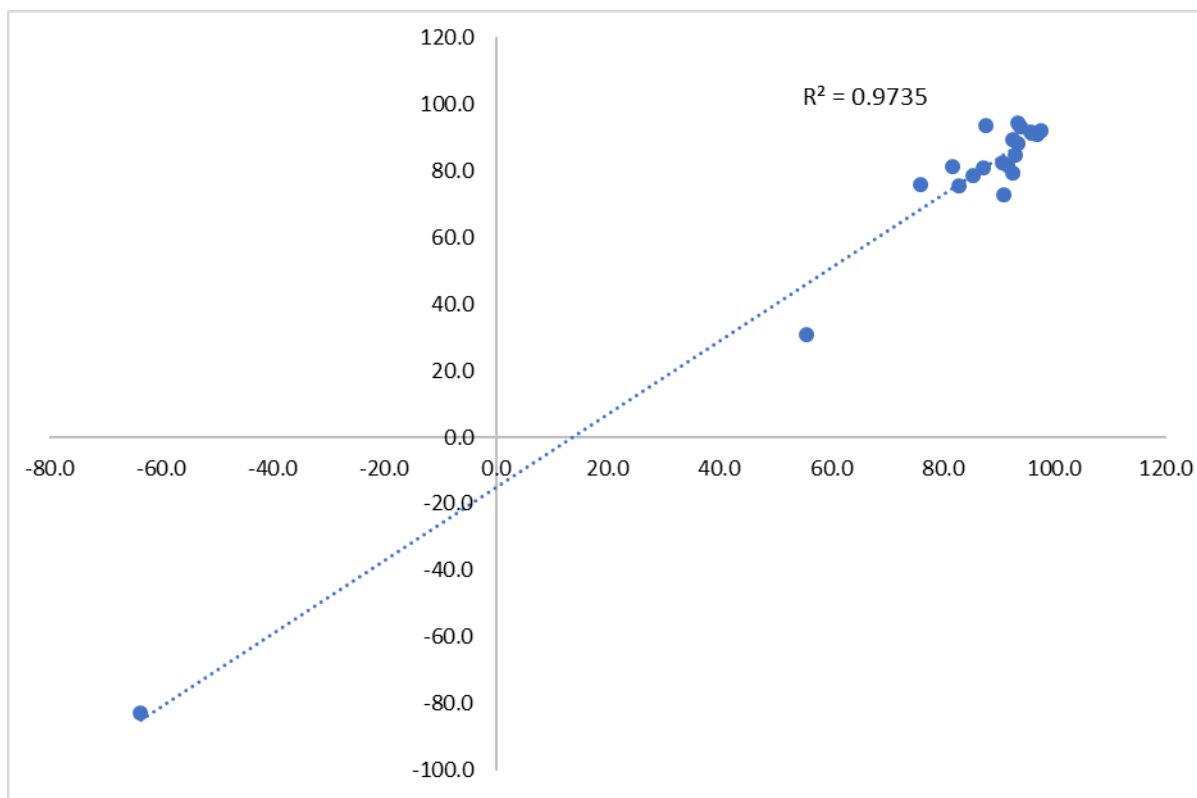


Figure AF15. The correlation of chemical removal between atenolol and ranitidine

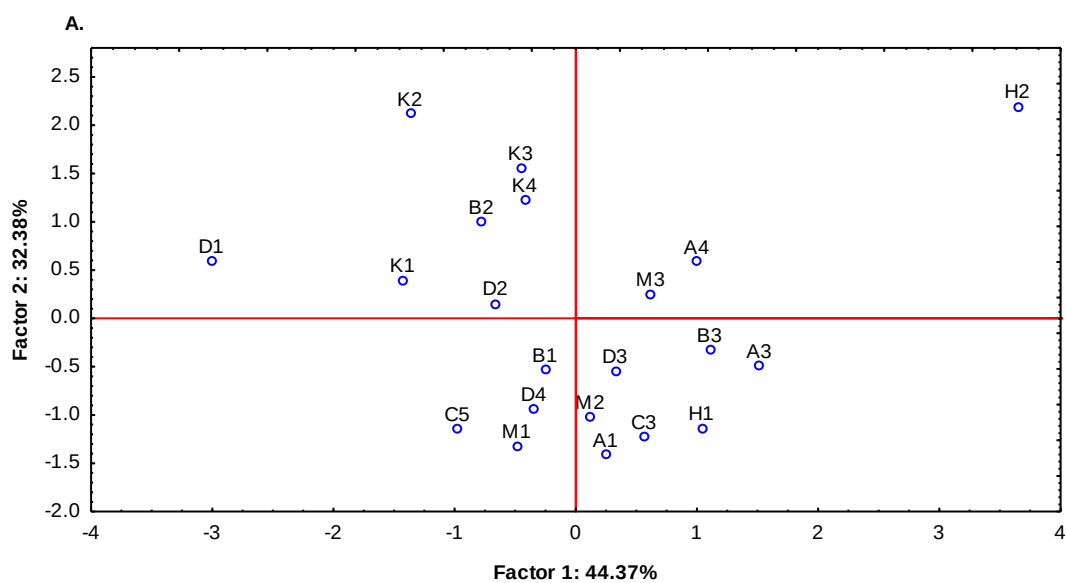


Figure AF16. Comparison of sampling events based on the operational process parameters

B.

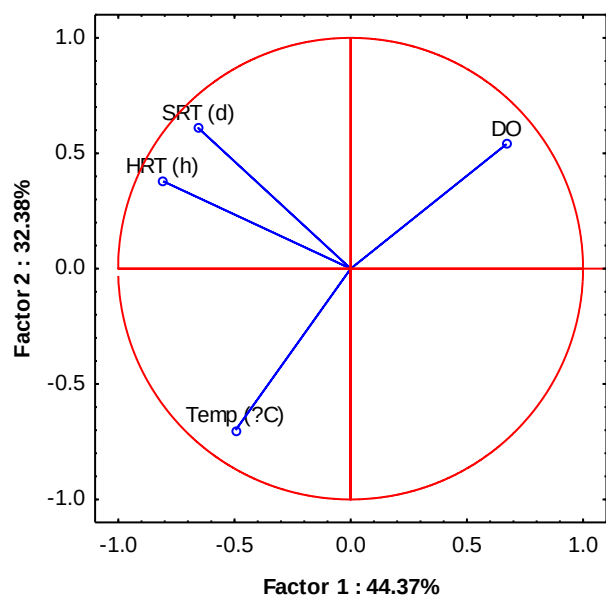


Figure AF17. Loading plot of PCA of process parameters revealing the factors underpinning the distribution of the sampling events.

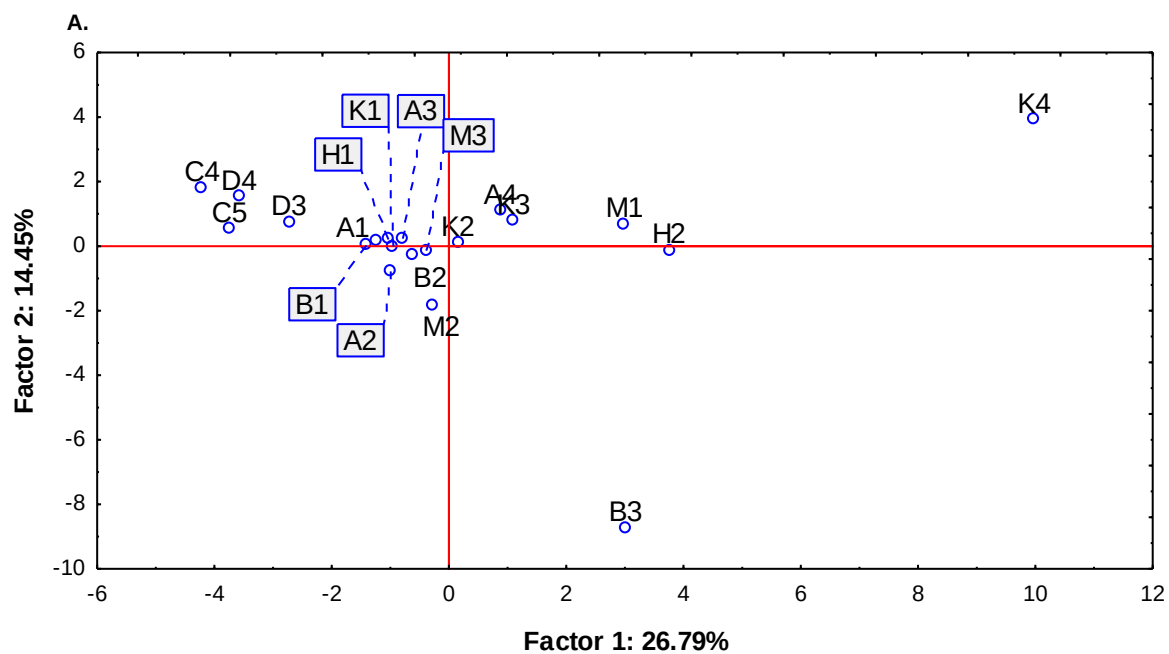


Figure AF18. Comparison of sampling events based on their removal of hazardous chemical removal and sanitary determinants

B.

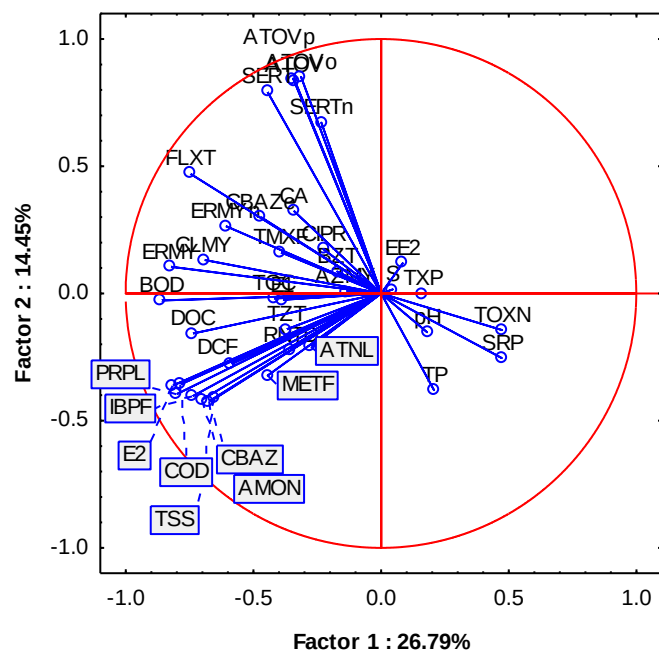


Figure AF19. Loading plot of PCA of hazardous chemical removal (above) to understand the contribution of each determinant in the distribution of sampling events.

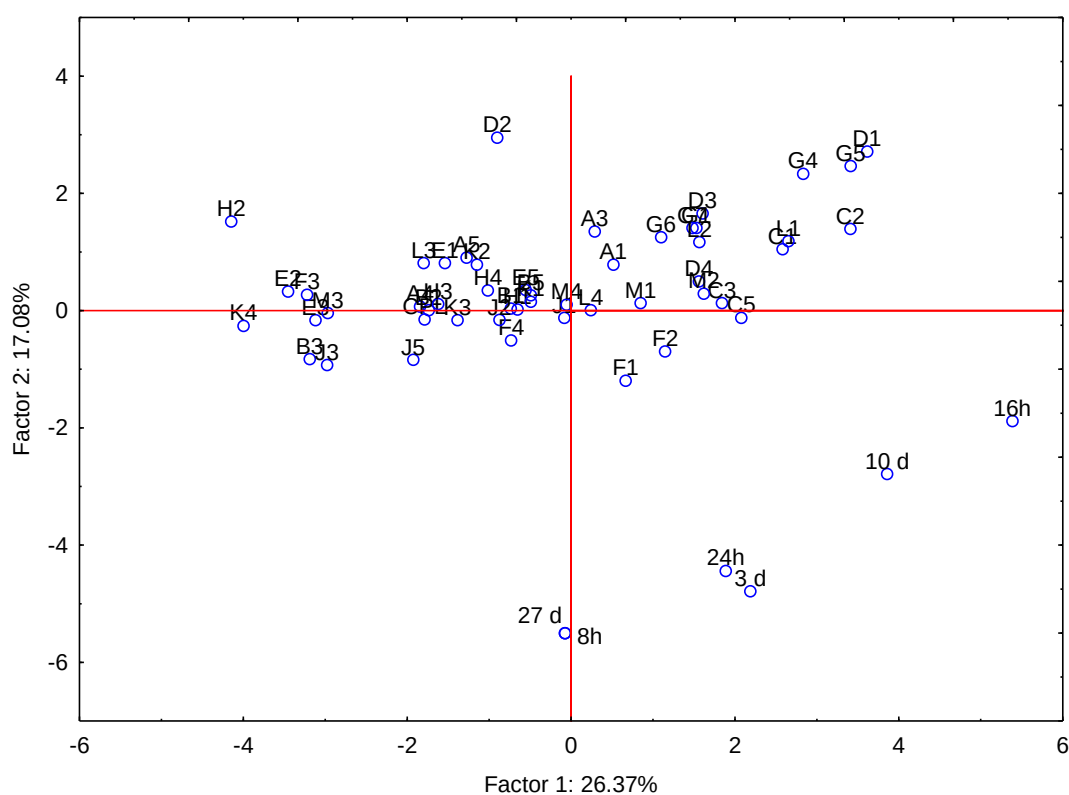


Figure AF20. PCA of pilot and full-scale bacteria phyla diversity. The pilot-scale samples are 3 d, 10 d, 27 d, 8 h, 16 h and 24 h. The rest are full-sale samples.

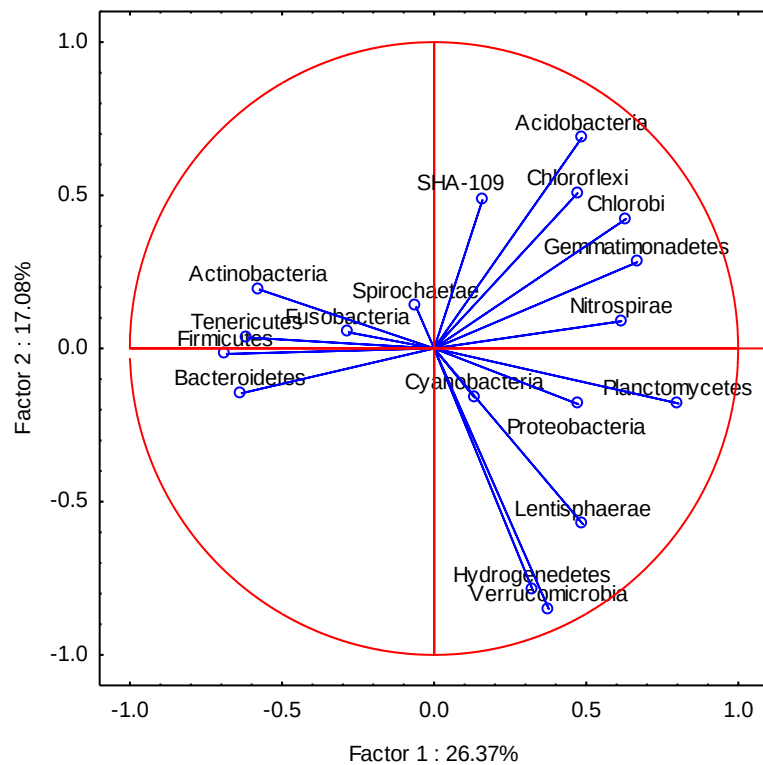


Figure AF21. Loading plot of PCA of showing how the bacterial diversity in the pilot plant compares with the diversity in the full-scale samples at phylum level.