Nature-based solutions for rural wastewater and agricultural waste management

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Abstract

For small communities in rural settings, nature-based, passive treatment systems such as constructed wetlands (CWs) provide alternatives to energy and chemical intensive centralised treatment works. However, there is insufficient information on CW performance across the seasons and wide range of environmentally relevant water quality parameters. Fieldwork was conducted at Northumbrian Water's sewage treatment plant (STP) at Birtley, England, which co-treats abandoned coal mine and STP effluents in CWs. This site represents a unique treatment challenge as it requires simultaneous removal of metals, nutrients, and pathogens. STP and coal mine effluent, CW influent and effluent, and receiving river water samples were comprehensively analysed for chemical and microbial quality in different seasons, followed by multivariate data analysis. Overall, chemical quality of the CW effluent was comparable to the river water. The CWs showed efficient removal of phosphate and iron and successfully converted treated sewage and mine water microbiomes into a freshwater microbiome. However, horizontal flow CWs require large land areas. It was therefore investigated if the performance of small-scale vertical flow CWs (biofilter) containing sand as biofilter medium could be improved by activated carbon (AC) amendment. It was found that 5% w/w AC-amendment in sand effectively removed putative human pathogens and micropollutants like diuron, diclofenac and enrofloxacin. Biochar is an AC-like material produced from agricultural waste biomass. Extending the lessons learned from nature-based wastewater management towards agricultural fields, this study tested the hypothesis that combined application of renewable energy generation by-products anaerobic digestate and coconut husk (CH) biochar can improve soil nutrient conditions, whilst minimizing groundwater pollution risks. Microcosms simulated digestate application to agricultural soil with and without CH biochar. Molecular microbiology techniques demonstrated that CH biochar retarded nitrate leaching via slower nitrification in digestate-amended soil. All these findings will contribute towards development of a more sustainable and circular rural economy.

Declaration

I hereby certify that this work is my own, except otherwise acknowledged, and that it has not been submitted previously for fulfilment of a degree at this or any other university.

Jidapa Plaimart

Contents

List of Tables

List of Figures

List of Acronyms

AC = activated carbon

AD = anaerobic digestion

AEC = anion exchange capacity

AMD = acid mine drainage

AOA = ammonia-oxidizing archaea

AOB = ammonia-oxidizing bacteria

ASP = activated sludge process

BOD = biological oxygen demand

CEC = cation exchange capacity

CH = coconut husk

COD = chemical oxygen demand

CSOs = combined sewer overflows

CWs = constructed wetlands

DO = dissolved oxygen

DOC = dissolved organic carbon

ECs = emergent contaminants

FIB = faecal indicator bacteria

FWSCWs = free-water surface flow constructed wetlands

HFBs = horizontal flow beds

HLR = hydraulic loading rate

HRT = hydraulic retention time

NOB = nitrite-oxidizing bacteria

PCA = principal component analysis

PNECs = predicted no effect concentrations

PRBs = permeable reactive barriers

RAPS = reducing and alkalinity producing systems

SDGs = sustainable development goals

SFCWs = subsurface flow constructed wetlands

SRT = solid retention time

STP = sewage treatment plant

TDS = total dissolved solids

TN = total nitrogen

TOC = total organic carbon

TP = total phosphorus

TSS = total suspended solid

VFBs = vertical flow beds

WFD = water framework directive

WSPs = waste stabilisation ponds

WWTPs = wastewater treatment plants

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Chapter 1

General introduction

Chapter 1. General introduction

In 2015, the United Nations established a universal policy agenda guiding all countries on shared actions over the next 15 years in the pursuit of sustainable global development (UN, 2015). The agenda includes 17 Sustainable Development Goals (SDGs) and 169 targets (UN, 2015) that encompass economic, social, and environmental dimensions of sustainable development (UN, 2015; Kroll *et al.*, 2019). Of all 17 SDGs, three aim towards addressing challenges that heavily affect rural communities, namely safe water and sanitation, sustainable agriculture, and access to sustainable and modern energy (UN, 2015). Wastewater and agricultural wastes are being generated on a large scale by rural societies where agriculture plays a major part in supporting society, however these wastes create environmental concerns unless properly managed (Zakaria, 2018; Gil *et al.*, 2019). Rural wastewater is often inadequately treated due to a lack of well-managed wastewater infrastructure in rural areas, thereby raising concerns over water pollution (Withers *et al.*, 2011; Singh *et al.*, 2019). For agricultural wastes, crop waste residues are often burnt or disposed into landfills/open dumping sites resulting in air/soil/water pollution and greenhouse gas emissions (Panyakaew and Fotios, 2008; Koul *et al.*, 2022). Livestock wastes (animal manures and slurries) raise similar concerns and issues as crop wastes (Koul *et al.*, 2022). Consequently, there is a need for innovative waste management practices that are economically suitable for rural areas and contribute towards sustainable rural development (Gil *et al.*, 2019). Employing suitable wastewater management practices in rural communities will contribute towards SDG 6 which intends to "ensure availability and sustainable management of water and sanitation for all" (UN, 2015). Wastewater treatment strategies can differ between urban and rural settings. Large-scale energy and chemical intensive centralised wastewater treatment systems are normally only established in urban areas, whereas lower cost treatment systems are preferred in rural areas (Nasr and Mikhaeil, 2015; Ullah *et al.*, 2020). Small sewage treatment plants or household treatment systems like septic tanks are typically implemented in rural settings across the world (Nasr and Mikhaeil, 2015; Bunce and Graham, 2019). However, inadequate treatment of a range of water pollutants and pathogens by some of these systems poses a significant threat to the environment and human health (Withers *et al.*, 2011; Schaider *et al.*, 2017; Yang *et al.*, 2017a). A further polishing step to improve the discharge quality may in such instances be required. Nature-based treatment systems are attractive for rural settings where affordability and low maintenance become the top priority for waste management. They provide a promising way forward for SDG target

6.2 to provide access to adequate sanitation and hygiene for all, and SDG target 6.3 to reduce water pollution and promote a safe reuse of water for agricultural irrigation and other purposes (UN, 2015). Nature-based treatment systems rely mostly on natural and freely available resources such as plants, sunlight, and microorganisms for water treatment in a relatively passive manner with low operational/maintenance requirements (Adrados *et al.*, 2014; Crites *et al.*, 2014). Well-known nature-based wastewater treatment systems include a range of water- and substrate-based systems e.g., ponds and surface flow constructed wetlands for water-based systems, and soil infiltration systems and subsurface flow constructed wetlands for substrate-based systems (Verbyla, 2017; Cross *et al.*, 2021). Horizontal free-water surface flow constructed wetlands have been successfully used for wastewater and mine water effluent polishing (Hoffmann *et al.*, 2011; Singh and Chakraborty, 2021) along with an additional role towards providing ecological benefits such as wildlife habitats (Zawadzka *et al.*, 2019). They effectively remove a range of water pollutants such as biological/chemical oxygen demands, heavy metals, emerging contaminants, and pathogens (Verlicchi and Zambello, 2014; Dufresne *et al.*, 2015; Wang *et al.*, 2017). Given these attributes, constructed wetlands (CWs) can eminently contribute towards SDG target 6.6, which aims to protect and restore aquatic ecosystems (UN, 2015). However, one drawback of horizontal flow CWs is that they are not suitable in a location where land availability is limited (Stefanakis, 2016). A smaller footprint of CWs would often be desirable. An optimized biofilter which mimics a small-scale vertical subsurface flow CW could be an alternative for nature-based treatment with a smaller footprint. It has been suggested that the performance of biofilters can be intensified using sorbent materials like activated carbon in the filter medium (Ulrich *et al.*, 2015; Wang *et al.*, 2020). Activated carbon can be generated from forestry and agricultural crop residues like wood or coconut shells that are available in the rural environment. However, the impact of sorbent amendments in biofilters on wastewater treatment performance needs more empirical evidence to support an improved performance across the wide range of environmentally relevant parameters (Ulrich *et al.*, 2015; Boehm *et al.*, 2020).

A more circular rural economy should broadly consider waste minimization and valorisation opportunities, in line with the SDG target 2.4 for resilient agricultural practices that strengthen sustainable natural resource utilisation and environmental protection, and SDG target 7.2 for increasing renewable energy generation (UN, 2015). Sustainable management of agricultural wastes can be considered as part of nature-based solutions that “protect,

sustainably manage and restore natural or modified ecosystems that address societal challenges effectively and adaptively, simultaneously providing human well-being and biodiversity benefits" (Cross *et al.*, 2021). For sustainable agricultural waste management, underutilised residues generated from crop and livestock farming can be transformed into value-added products (Koul *et al.*, 2022). Recalcitrant crop residues like woody plant matter can be utilised as biofuel to generate electricity via pyrolysis yielding biochar, an activated carbon-like sorbent material, as a useful by-product (Koul *et al.*, 2022). Manures and slurries from livestock and readily decomposable crop residues can be treated via anaerobic digestion giving useful by-products like anaerobic digestate and biogas (Koszel and Lorencowicz, 2015; Risberg *et al.*, 2017; Brown *et al.*, 2020). Anaerobic digestate is often utilised as biofertilizer, nevertheless, it sometimes contains over-concentrated nutrients leading to excess nutrient leaching from soils to groundwater after application on land (Akhiar *et al.*, 2017). Meanwhile, biochar can improve soil fertility and reduce nutrient leaching through its sorption property and its effect on soil microbiology (Atkinson *et al.*, 2010; Tan *et al.*, 2015). Therefore, it would be promising to apply biochar in digestate-amended soils which will also facilitate multi-use systems of agricultural wastes. This would subsequently return nutrients from waste to the fields, thus enhancing crop productivity and conserving natural resources whilst minimizing agricultural pollution of groundwater and surface water resources. Such practice can ensure nitrogen use efficiency which is an indicator for SDG target 2.4 (Gil *et al.*, 2019) whilst generating renewable energy to meet SDG target 7.2, and SDG target 6.3 to reduce water pollution.

This thesis therefore aims to demonstrate how nature-based solutions can help sustainably manage wastewater and agricultural wastes in rural settings.

Chapter 2

Literature review

Chapter 2. Literature review

Part of this literature review has been published as a book chapter. **Chapter 2: *Subsurface flow constructed wetlands as a post-treatment unit for emerging contaminants in municipal wastewater* in a book entitled *Contaminants of Emerging Concerns and Reigning Removal Technologies*** by CRC Press, Taylor & Francis group.
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2.1 Wastewater treatment

Wastewater treatment schemes consist of a range of treatment steps. They can be normally categorized into four steps involving preliminary, primary, secondary, and tertiary treatment (Crini and Lichtfouse, 2019). The requirement for each step relies on the source, characteristics, and intended use of wastewater. Each treatment step can treat different pollutants (Ullah *et al.*, 2020). Preliminary treatment is used to removed coarse suspended solids (Ullah *et al.*, 2020). Primary treatment is mainly for separation of solid organic matter via a sedimentation or flotation unit (Hreiz *et al.*, 2015; Rout *et al.*, 2020). Wastewater then flows to a secondary treatment governed by biological degradation which is used to remove the remaining solids and soluble organic matter escaping from a primary treatment (Rout *et al.*, 2020). In this process, microbial biomass (for example activated sludge) is responsible for the removal of pollutants under aerobic or anaerobic conditions (Hreiz *et al.*, 2015). Secondary effluent is then occasionally treated further in a tertiary treatment step. This may be known as a polishing unit which is mainly employed to remove nutrients, suspended solids, pathogens or, more recently, micropollutants (Rout *et al.*, 2020; Ullah *et al.*, 2020). These numerous treatment methods have been further categorized as physical, chemical and biological techniques or a combinational approach (Crini and Lichtfouse, 2019). Examples of the most widely applied technologies based on these four categories are listed in **Table 2.1**.

Table 2.1 Classification and examples of wastewater treatment technologies. Details were derived from Crini and Lichtfouse (2019) and El-Gendy et al. (2020).

Classification	**Example technologies**
Physical	Adsorption: activated carbon/zeolites
	Membrane separation: reverse osmosis, nanofiltration
Chemical	Chemical oxidation: ozonation, chlorination
	Photo-oxidation
	Coagulation: aluminium sulphate
	Ion exchange
Biological	Electrochemical reactors: microbial fuel cell
	Biodegradation: activated sludge, membrane bioreactor trickling filters
	Constructed wetlands
Biophysicochemical	Biofiltration

2.1.1 Activated sludge and tricking filter processes

The activated sludge process (ASP) is normally applied as a secondary treatment step in wastewater treatment plants (WWTPs) and is mainly governed by microbiological activities to degrade organic matter and nutrients in wastewater (Islam *et al.*, 2013; Meerburg *et al.*, 2015). Chemicals are not required for this process or maybe required in insignificant amounts (Islam *et al.*, 2013). The basic ASP consists of 2 main units, (1) an aerated-bioreactor operated continuously where suspended microbes consume the dissolved and colloidal organic matter (Hreiz *et al.*, 2015), and (2) a settling tank (also known as secondary clarifier) where gravitational separation of activated sludge and treated wastewater is employed. The effluent overflows from the settling tank into the receiving watercourses or perhaps undergoes tertiary treatment such as disinfection or media filtration in some WWTPs before being discharged (Hreiz *et al.*, 2015). There is also a sludge recycle line and a sludge waste line attached at the bottom of the settling tank. The former is to return the major proportion of the settled sludge to the bioreactor for maintaining a high microbial concentration in the bioreactor, while the latter is to dispose a small proportion of the sludge. Thus, the biomass concentration in the bioreactor is stabilized and an adequate solid retention time (SRT) is achieved (Hreiz *et al.*, 2015). Due to its efficiency and simplicity, ASP is by far the most well-known secondary wastewater treatment system (Hreiz *et al.*, 2015; Meerburg *et al.*, 2015; Guven *et al.*, 2019). In terms of ASP treatment performance ASP normally shows efficient removal (> 70%) of conventional water pollutants like chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen and ammonia, however the efficiencies

can be varied with SRT (Kim *et al.*, 2011; Hreiz *et al.*, 2015). Phosphorus (P) removal efficiency in a conventional ASP is fairly low, thus it is normally enhanced by an additional process like chemical coagulation and precipitation (Hreiz *et al.*, 2015; Ge *et al.*, 2018). ASP is not only capable of removing macro-organic matter but also organic micropollutants or emergent contaminants (ECs) although it is not specifically designed to do so (Alvarino *et al.*, 2018). ECs can be removed either through biomineralization by microbes or sorption onto particulate matter (sludges) (Alvarino *et al.*, 2018). The removal performance of ECs varies by different factors and ASP operating conditions, such as SRT, food to mass ratio, temperature and redox conditions or even the recalcitrant behaviour of ECs themselves (Collado *et al.*, 2014). For example, Alvarino *et al.* (2018) reported the removal efficiency of different pharmaceutical by an ASP. It was found that ibuprofen and naproxen were removed more than 80%, antibiotics like sulfamethoxazole were only removed by 20-50%, while a recalcitrant compound like diclofenac was removed less than 20%. Similarly, Collado *et al.* (2014) reported a high removal efficiency for ibuprofen and naproxen and moderate removal for sulfamethoxazole. They also reported that the removal performance varies by ambient temperature, with higher removal performance being observed in the summer than in colder periods. Furthermore, other biological processes can have an impact on the EC removal. There were reports on the enhanced elimination of ECs by nitrification (Fernandez-Fontaina *et al.*, 2012; Collado *et al.*, 2014; Alvarino *et al.*, 2016). ECs will be further discussed in **Section 2.3.7**. Apart from the ASP, tricking filter process is another biological wastewater treatment system typically being used as a secondary treatment step in WWTPs (Ullah *et al.*, 2020). The trickling filter is an aerobic attached growth reactor that consists of a tank filled with a highly permeable material such as rocks, gravel, slag, and plastic media to which microbes are attached (biofilm) to degrade water pollutants (Naz *et al.*, 2015; Bressani-Ribeiro *et al.*, 2018). Wastewater is applied on the top of the tank through a rotating arm sprinkler, allowing biofilm to develop on the support medium and air naturally moves upward or downward (Bressani-Ribeiro *et al.*, 2018). The system shows effective removal of biological/chemical oxygen demand, nitrogen, and faecal coliforms (EPA, 2000; Naz *et al.*, 2015; Maciejewski *et al.*, 2022). It has been widely implemented in developed and developing countries due to its operational simplicity and performance stability (Bressani-Ribeiro *et al.*, 2018). It has also been frequently used in small wastewater treatment plants in developed countries like the UK and Germany (Bressani-Ribeiro *et al.*, 2018; Bunce and Graham, 2019).

2.1.2 Household wastewater treatment systems

The on-site (household) systems are nowadays also gaining attention worldwide because they can extend treatment provision to rural households or urban areas without sewerage systems (Nasr and Mikhaeil, 2015). The centralised WWTPs normally consist of a biological treatment like the activated sludge process, while on-site municipal wastewater treatment systems generally use cesspits and septic tanks (Yates *et al.*, 2019). On-site domestic wastewater treatment systems such as cesspits and septic tanks are a kind of anaerobic reactor. The main aim of septic tank systems is to provide primary treatment for domestic sewage by intercepting and separating solid faecal matter from the liquid and the system needs to be de-sludged every four years to prevent sewage overflow to watercourses (Ting *et al.*, 2013; Singh *et al.*, 2019). Septic tank systems consist of two main components involving a septic tank and a drainfield, maybe also known as soil absorption system, in which the septic tank effluent is distributed via gravity or pressure from pipes or pits into well-draining unsaturated zone soils (Schaider *et al.*, 2017). The systems are normally suitable for a village population of up to 500 (Withers *et al.*, 2011). In developed countries e.g., the UK and the USA, such systems are still being used in some rural areas where the main sewerage network has not been extended to (Withers *et al.*, 2011; May *et al.*, 2015; Schaider *et al.*, 2017). However, unless maintained in a good working condition, discharges from such systems can pose a significant threat to the ecological quality of the local waterbodies, for example eutrophication from excess nutrients in the treated effluent (Withers *et al.*, 2011; May *et al.*, 2015). In the UK, there is a lack of evidence on the system performance as no authority is legally responsible for septic tank system monitoring (Withers *et al.*, 2011; Bunce and Graham, 2019). Many systems are not registered and often improperly maintained or monitored (Withers *et al.*, 2011). A study also reported that small treatment works tend to be less reliable compared to large-scale WWTPs and require more stringent management (Bunce and Graham, 2019). In developing countries, the septic tank system is a common treatment technique as a result of unproperly managed sewerage systems and lack of centralised treatment facilities in the rural area (Singh *et al.*, 2019). For instance, in a warm climate country like Thailand, septic tank systems are generally used to treat domestic wastewater (85%) even in the areas partially served by centralised WWTPs (Tsuzuki *et al.*, 2010; WHO, 2018). A septic tank can also be upgraded to a system called anaerobic baffled reactor (ABR). It consists of a series of vertical baffles that forces the wastewater to flow under and

over them as it passes from inlet to outlet. Such increased contact time with the active biomass (sludge) leads to enhanced treatment (Wang *et al.*, 2004; Yulistyorini *et al.*, 2019). Conventional septic tanks can remove settleable solids, oils, greases, and floating debris from the raw sewage at a range of 60-80% and the removed solids are stored in sludge and scum layers (Nasr and Mikhaeil, 2015). Nevertheless, faecal coliforms and some organic compounds are poorly removed (Nasr and Mikhaeil, 2015; Schaider *et al.*, 2017). The effluent from septic tank systems known as settled sewage is normally allowed to seep into soil or directly discharged into nearby watercourses, thereby becoming a source of groundwater and surface water contamination (Surinkul and Koottatep, 2009; Withers *et al.*, 2011; Yang *et al.*, 2017a). For example, in Thailand high levels of several pharmaceuticals, antimicrobial resistance genes or microorganisms, nutrients and pathogens were detected in the canal system around Bangkok, as a result of inadequately treated effluent from septic tanks and possibly also the direct discharge from households, suggesting potential ecological risks (Tewari *et al.*, 2013; Mrozik *et al.*, 2019; Thongsamer *et al.*, 2021). In the USA, endocrine disrupting compounds were detected in the septic tank effluent at trace concentrations ranging from a few ng/L to several μg/L (Yang *et al.*, 2017a). More common ECs like caffeine and ibuprofen were detected at levels ranging from 0.12 μg/L to 12.04 μg/L in the groundwater down-gradient from a septic tank systems in North Carolina (Del Rosario *et al.*, 2014). Schaider *et al.* (2017) reported that although some organic micropollutants may be removed within the septic tank via physical separation of solid particles and oil-associated organic micropollutants as well as anaerobic degradation, the greatest micropollutant removal seems to occur in drainfields via sorption, volatilization, and aerobic degradation. However, another study found that some pharmaceutical compounds increased or remained relative constant 30 metres downgradient of a drainfield (Phillips *et al.*, 2015). It is therefore desirable that a post-treatment unit is installed after septic tank systems to further reduce the remaining contaminants of concerns to acceptable levels before discharging treated effluent into natural waterbodies.

2.1.3 Nature-based wastewater treatment systems

Nature-based wastewater treatment systems rely mostly on natural processes such as gravity forces and natural components such as plants, sunlight and microorganisms for wastewater treatment in a relatively passive manner (Crites *et al.*, 2014). The systems sometimes also apply pumps and pipe works for wastewater conveyance and distribution, but require little to no external energy (Crites *et al.*, 2014). Consequently, they can reduce the need for energy

from fossil fuels as well as create wildlife habitats. Natural treatment systems such as ponds, lagoons and wetlands are some of the oldest and have been the most globally used technologies in past decades (Verbyla, 2017). There are several types of ponds that are used to treat wastewater at different stages in the treatment process. The most common pond types include anaerobic ponds, facultative ponds, and maturation ponds (Verbyla, 2017). These ponds can be combined into sequences known as waste stabilisation ponds (WSPs) (Mara, 2004). An anaerobic pond is designed as the first treatment step to reduce biological oxygen demand (BOD) and suspended solids, the effluent then enters a secondary facultative pond for further BOD reduction, and lastly enters a maturation pond for pathogen and nutrient removal (Mara, 2004; Verbyla, 2017). Nitrogen in a maturation pond can be removed via several mechanisms including ammonia volatilization, algal uptake, and nitrification-denitrification (Camargo Valero *et al.*, 2010). Apart from solely natural systems, there are also combined natural and engineered systems (Zawadzka *et al.*, 2019). Such systems involve constructed wetlands, riverbank filtration and managed aquifer recharge (Zawadzka *et al.*, 2019). The systems can be used for primary, secondary, and/or tertiary wastewater treatment depending on individual purposes (Verbyla, 2017). Constructed wetlands (CWs) are very effective as a tertiary treatment system after activated sludge plants (Hoffmann *et al.*, 2011). Systems such as sand filtration and constructed wetlands have gained recognition for wastewater treatment in small rural communities as compared to conventional WWTPs due to their efficiency, low construction and operating/maintenance costs (Adrados *et al.*, 2014). Nevertheless, in order to effectively treat wastewater, numerous factors have to be taken into account, including the system's capacity, plants used, microbial characteristics and the interactions of microbial-mediated and wastewater contaminants (Adrados *et al.*, 2014). CWs can perform a vital role in ecosystem service supply if they are implemented in suitable environmental settings, and if land cover management for ecological functioning is in place (Zawadzka *et al.*, 2019). Moreover, people's perception in terms of aesthetic value is highly positive for CWs as compared to their engineered equivalents (Zawadzka *et al.*, 2019). De Feo and Ferrara (2017) conducted a life cycle assessment on two on-site systems namely CWs and an activated sludge compact system and it was found that CWs were claimed to be a better option than an activated sludge compact system in terms of environmental impacts due to their lower electricity consumption. Several forms and modifications of CW technology have been invented (Nuamah *et al.*, 2020). Generally, CW configurations are based on wetland hydrology and flow pattern which include free-water surface flow, subsurface flow, and a hybrid system (Stefanakis, 2016; Verbyla, 2017) **(Figure 2.1)**.

Free-water surface flow CWs are mostly similar to wastewater treatment ponds with the only difference that they contain submerged/emergent vegetation and floating macrophytes (Verbyla, 2017). Subsurface flow constructed wetlands (SFCWs) can be classified into two types relative to flow pattern which are horizontal and vertical flow (Wang *et al.*, 2017). Hybrid CWs consist of those aforementioned types of CWs connected in sequences and they have now been implemented in many countries across Europe and Asia (Vymazal, 2013). The main characteristics of constructed wetlands and conventional wastewater treatment systems are compared in **Table 2.2**. More details on CW systems are provided in **Section 2.3**.

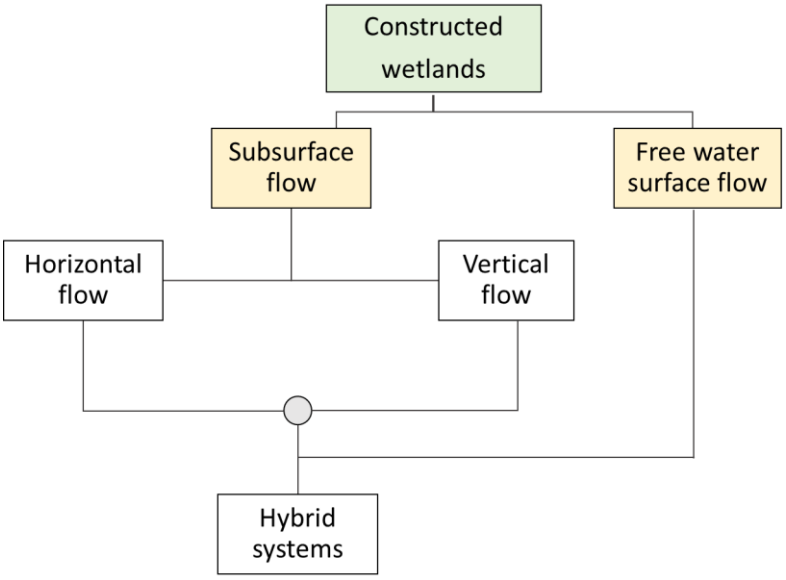

Figure 2.1 Classification of constructed wetlands. Adapted from Stefanakis (2016).

Table 2.2 Comparisons of the main features of constructed wetlands (CWs) and conventional wastewater treatment systems (Stefanakis, 2016).

	Constructed wetlands	**Conventional wastewater treatment systems**
Infrastructure	No mechanical components (or only pumps)	Many complex mechanical parts
Investment/Operational costs	Low particularly when there is available land	High
Demand of land area	High	Low
Raw materials	Nearly exclusive use of renewable resources (wind, solar, etc.)	Use of non-renewable materials for construction (steel, concrete) and operation (electricity, chemicals)
Energy consumption	Low	High
Staff during operation	No specialized personnel needed	Demand for specialized personnel
Performance	Similar to conventional systems with small fluctuations depending on temperature variations	Continuous effluent of high quality
By-products	No by-products	Large daily volumes of sludge production that require daily management

Nature-based wastewater treatment systems, particularly CWs, would therefore be a low-cost alternative solution for wastewater quality improvement whilst providing ecological, technical, and societal benefits.

Apart from common chemical pollutants, wastewater also contains a wide range of pathogens that present a major human health risk. More details about pathogens in water and the detection techniques are provided in the following section.

2.2 Human pathogens in the environment

A pathogen is known as an organism that causes disease to its host. Pathogens comprise viruses, bacteria, fungi, and parasites (Balloux and Dorp, 2017). Most viruses and bacteria are harmless and can be often useful, and only approximately one in a billion microbial species is considered a human pathogen. To date, around 1400 human pathogens have been identified (Balloux and Dorp, 2017). Pathogens can be classified into two main categories including facultative and obligate pathogens (Balloux and Dorp, 2017). Facultative pathogens are organisms that can reproduce themselves via various niches not only the host e.g., environmental bacteria and fungi that can occasionally cause infection. Whereas, obligate pathogens rely on a host to fulfil their life cycle e.g., viruses (Balloux and Dorp, 2017). Pathogens produces toxins to damage their host's tissues or cells during replication causing

the host's illness (Mara and Horan, 2003; Balloux and Dorp, 2017). Pathogenic bacteria can be transmitted through water, food, air and excreta (Mara and Horan, 2003). The faecal excretion by infected hosts is a key contributor to disease transmission as it is potentially introducing pathogens into the environment which normally contaminates natural waterbodies. Consequently, waterborne transmission is a significant pathway for spreading pathogens to a large portion of human population (Aw, 2018). Major water- and excreta-related bacterial human pathogens include *Legionella pneumophila, Campylobacter jejuni*, *Helicobacter pylori*, and *Vibrio cholerae* (Mara and Horan, 2003). Faecal pollution in water can also be indicated using faecal indicator bacteria (FIB) as a proxy for bacterial pathogens (Rochelle-Newall *et al.*, 2015). This group of bacteria are for example faecal coliforms, thermotolerant coliforms, *E. coli*, and faecal enterococci. FIB are found in the intestinal tracts of warm-blooded animals and regularly excreted in faecal matter (Rochelle-Newall *et al.*, 2015). Nowadays, several techniques are in-use to detect pathogenic bacteria including traditional plate count and modern techniques. The modern strategies consist of biosensor methods, DNA-amplification methods and metagenomics (Gorski *et al.*, 2019). Biosensors generally reveal interaction with biological components such as antibodies or nucleic acids, with the analyte then being detected by a transducer which generates electrical signal. DNA-amplification methods includes quantitative polymerase chain reaction (qPCR) techniques that detect the amplification of a targeted DNA sequence of a specific bacterium in real-time, while metagenomics technique is the comprehensive sequencing of all DNA in a sample (Gorski *et al.*, 2019).

As mentioned earlier in Section 2.1.3, constructed wetlands are one of the nature-based wastewater treatment systems, and more information on constructed wetlands is therefore provided in Section 2.3 below.

2.3 Constructed wetlands

2.3.1 Free-water surface flow constructed wetlands (FWSCWs)

Free-water surface flow constructed wetlands (FWSCWs) are shallow sealed basins or channels with soil at the base (substrate) to support the rooted vegetation (Stefanakis, 2016). They contain shallow-depth water above the substrate with low flow velocity (Vymazal and Kropfelova, 2008). However, their physical structure is diverse depending on their potential application. The size, depth, and lining system can be varied. They can be fully or partially planted, while the vegetation can also be emergent, submerged or floating (Dotro *et al.*,

2017). In terms of application, they are normally used to treat domestic wastewater, stormwater runoff, landfill leachate and mine drainage (Yeh *et al.*, 2009; Younger and Henderson, 2014; Austin and Yu, 2016; Sánchez, 2017). For wastewater, they are often used as a tertiary treatment step for secondary treated effluent from WWTPs or used after on-site treatment systems like septic tanks in rural communities (Austin and Yu, 2016; Dotro *et al.*, 2017). A pre-treatment step is normally required to reduce organic load into the CWs thus increasing CW longevity (Austin and Yu, 2016). FWSCWs with emergent macrophytes are the most commonly used system for sewage treatment (Vymazal and Kropfelova, 2008). The most commonly used plants species are *Phragmites australis* (common reed), *Typha* spp. (cattails), bulrush (*Scirpus* spp) and *Juncus* spp. (herbs) (Stefanakis, 2016). FWSCWs should be designed such that a consistently aerobic condition of the water column is maintained to prevent odour releases. More areas of open water also allow more sunlight penetration, which facilitates photo-degradation and solar disinfection (Dotro *et al.*, 2017).

The design

Geo-textile or clay material is used as a liner at the bottom of a CW basin to avoid water leakage (Stefanakis, 2016). The height of the water column above the soil layer is normally in a range of 15-60 cm and the size of CW is decided based on discharge targets or desired levels of pollutant removal (Vymazal and Kropfelova, 2008; Austin and Yu, 2016). For large CW systems, they are often divided into a sequence of cells in order to collect and redistribute the water uniformly, in which cells can be arranged in series or parallel (Dotro *et al.*, 2017). The water flows horizontally through the plant stems then comes into contact with the top layer of the sediment and the plant components, which allows for the pollutant removal via various mechanisms like adsorption, precipitation and biodegradation (Austin and Yu, 2016; Stefanakis, 2016) **(Figure 2.2)**. The main recommended design parameters are listed in **Table 2.3**.

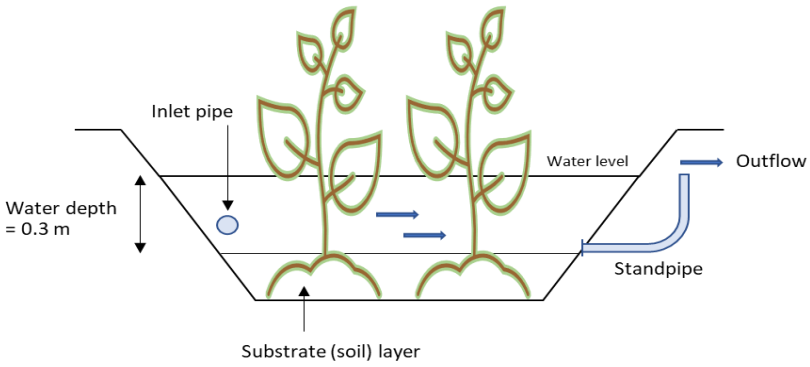

Figure 2.2 Free-water surface flow constructed wetlands design. Adapted from Stefanakis (2016).

Table 2.3 Main design parameter of free-water surface flow constructed wetlands (Verhoeven *et al.*, 2006; Austin and Yu, 2016).

Parameter	Value
Area requirement (m^2/person)	4.5-5
Maximum areal organic loading rate (g $BOD_5/m^2.d$)	6
Hydraulic loading rate (mm/d)	40-100
Soil depth (cm)	20-40
General water depth (cm)	15-60

2.3.2 Subsurface flow constructed wetlands (SFCWs)

The design

SFCWs mainly contain shallow basins with a seepage barrier and water inlet/outlet (Crites *et al.*, 2014). SFCWs are normally designed for horizontal or vertical flow. The former requires a larger area than the latter, but is easier to design and suitable for locations without energy supply (Vymazal, 1998; Mara, 2004). Nevertheless, if evapotranspiration is an issue, a vertical flow bed is preferable to a horizontal flow bed, because it has an unsaturated upper layer in the bed, which is more suitable for warm climate countries. Additionally, it also has been reported to show higher treatment efficiency (Vymazal, 1998; Hoffmann *et al.*, 2011). SFCWs traditionally consist of a sand or gravel bed, which acts as a filter medium where the water level is kept below its surface (Austin and Yu, 2016). The gravel in the bed does not perform a filtering function, but is mainly used to cover the influent distribution and drainage pipes, and avoid puddles on the surface (Hoffmann *et al.*, 2011). SFCWs require a pre-treatment prior to wetland treatment, which can for example be a pond or septic tanks (Crites *et al.*, 2014). The main aim for this is to decrease the concentrations of suspended solids and

organic matter that otherwise would accumulate in the inlet zone and clog SFCW systems (Hoffmann *et al.*, 2011; Crites *et al.*, 2014).

I. Horizontal flow beds (HFBs)

HFBs are the most common type of SFCWs. They are usually planted with common reeds *(Phragmites australis)* in Europe and the USA, while *Cyperus, Typha,* and *Heliconia* are commonly used in tropical climate countries (Vymazal and Kropfelova, 2008; Dotro *et al.*, 2017). In HFBs the wastewater flows horizontally through the filter bed medium under the surface of the bed until it approaches the outlet area, and the water level is kept 5-10 cm below the gravel layer surface (Hoffmann *et al.*, 2011) **(Figure 2.3)**. The water level at the outlet is controlled with an adjustable standpipe (Hoffmann *et al.*, 2011; Dotro *et al.*, 2017). The systems consist of an integration of a plastic liner and a geotextile membrane to isolate the whole bed from surrounding land (Dotro *et al.*, 2017). Generally, a maximum width of 25-30 m is in use to enable even flow distribution into a single wetland cell. The depth of HFBs is normally designed at 60 cm with an additional 15 cm freeboard for water accumulation, which is based on the assumed maximum root depth penetration (Dotro *et al.*, 2017). However, the implementation of a maximum cross-sectional area loading (the load applied at the inlet per width and depth) is instead considered as a design criterion nowadays, providing an opportunity to alter bed depth and length (Dotro *et al.*, 2017). The main recommended design parameters are listed in **Table 2.4**.

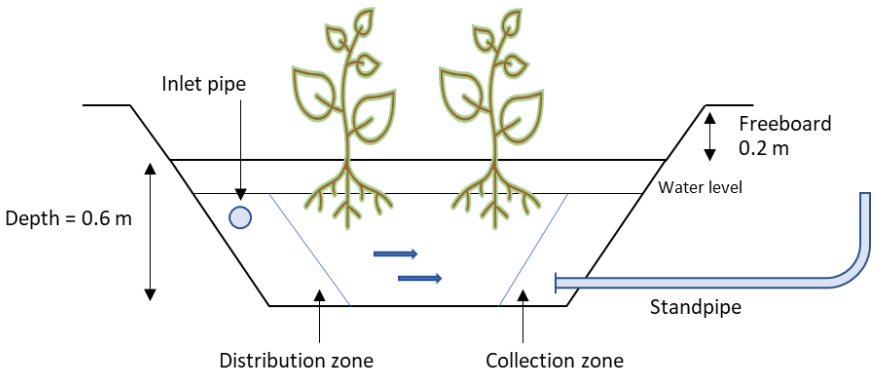

Figure 2.3 Horizontal subsurface flow constructed wetlands design. Adapted from Dotro et al. (2017).

Table 2.4 Main design components of horizontal subsurface flow constructed wetlands as a secondary or tertiary treatment step (Dotro et al., 2017).

	Treatment purpose	
	Secondary	**Tertiary**
Pre-treatment	Septic tank	Primary settling + biological treatment
Specific surface area requirement (m^2/population equivalent)	5-10	0.7
Maximum areal organic loading rate (g BOD_5/m^2.d)	4-8	2-13
Hydraulic loading rate (mm/d)	20-40	200
Gravel size (mm)	> 4	10-12
General filter bed depth (m)	0.5-0.7	0.6

II. Vertical flow beds (VFBs)

Vertical flow beds are usually implemented where there is a space constraint as they only require about half of the area of HFBs (Austin and Yu, 2016). VFBs look simply like a biofilter/sand filter and can be planted or left unplanted (Sylla, 2020). The most common design for VFBs consists of a basin where the wetland influent is intermittently applied onto the surface using pumps and then percolates vertically down through the filter medium (sand/gravel) to the drainage area at the bottom (Austin and Yu, 2016; Dotro *et al.*, 2017) **(Figure 2.4)**. The influent needs to be pre-treated normally in a septic tank to avoid wetland systems failure from filter bed clogging (Austin and Yu, 2016). The wetlands are typically receiving wastewater at 4 to 12 doses daily and go through long resting periods when the influent percolates down to the bottom and the surface dries out (Hoffmann *et al.*, 2011). The intermittent loading increases the oxygen transfer and aerobic degradation processes in the filter bed (Dotro *et al.*, 2017). VFBs have high capability of removing organic carbon because of the highly oxidizing conditions in their filter bed. They are also appropriate when stringent aerobic activities such as nitrification are required (Dotro *et al.*, 2017; Wang *et al.*, 2017). VFBs are usually planted with *Phragmites australis* (common reed), which is the same as the HFB type. The roles of vegetation with regard to pollutant removal in VFBs are mostly related to physical mechanisms such as protecting surface from erosion, maintaining hydraulic properties of the filter and providing surface area for biofilms attached to plant roots (Dotro *et al.*, 2017).

Basic design recommendations for VFBs treating domestic wastewater are as follows:

(1) The distribution pipes should provide even distribution of wastewater over the VFB. The drainage pipes are covered with gravel to allow good drainage. (2) The depth of the sand filter bed should be at least 50 cm with an additional 10 cm of gravel at the top to avoid free water accumulation on the surface, and 10 cm gravel at the bottom (Hoffmann *et al.*, 2011). However, the top layer also has disadvantages because it can reduce oxygen supply to the main layer leading to less organic matter degradation at the main layer hence higher risk of clogging (Dotro *et al.*, 2017). More specific design components depending on climate conditions are provided in **Table 2.5**.

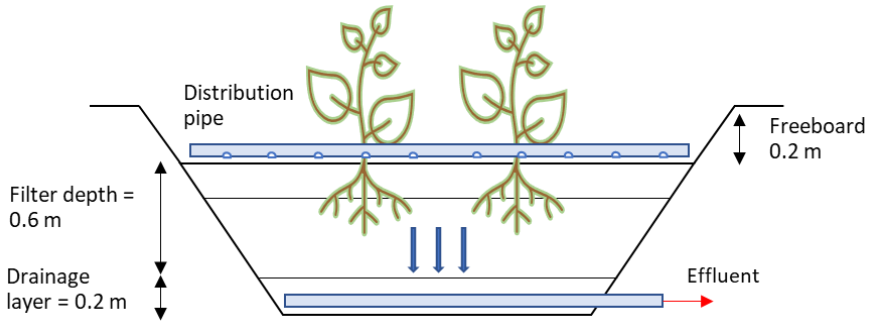

Figure 2.4 Vertical subsurface flow constructed wetlands design. Adapted from Dotro et al. (2017).

Table 2.5 Main design components of vertical subsurface flow constructed wetlands depending on cold or warm climate condition (Hoffmann et al., 2011; Dotro et al., 2017).

Design parameter	**Cold climate**	**Warm climate**
Specific surface area requirement (m^2/population equivalent)	3-4	1-2
Maximum organic loading rate (g COD/m^2.d)	20	60-70
Hydraulic loading rate (mm/d)	100-120	100-200

2.3.3 Hybrid CW systems

A sequence of free-water surface flow CWs and HFB/VFB SFCWs as a hybrid system is gaining interest, and has been utilised in Europe and Asia to achieve the highest water quality for high strength wastewater (Nuamah *et al.*, 2020). Integrating different types of constructed wetlands capitalizes on the capacity of each to efficiently remove specific compounds, which can often be undertaken at a much lower capital and operating cost than for conventional wastewater treatment systems (Austin and Yu, 2016). Hybrid systems are initially designed to enhance nitrogen removal as the different types of wetland environments allow various redox

conditions which are suitable for nitrification (oxidation of ammonia to nitrate) and denitrification (reduction of nitrate to N_2O and N_2) (Vymazal, 2013; Wang *et al.*, 2017). In general, HFBs can provide good anaerobic conditions for denitrification, however the capability for nitrification is very limited. In contrast, VFBs can remove ammonia due to their better aerobic condition, however denitrification hardly occurs in these systems. A combination of both types therefore strengthens the performance of the individual systems (Wang *et al.*, 2017). A VFB-HFB arrangement was reported to be the most widely used type for both industrial and municipal wastewater treatment among other combinations with high efficiency in ammonium removal (Vymazal, 2013; Nuamah *et al.*, 2020). Melián *et al.* (2010) conducted a pilot-scale VFB-HFB SFCWs in the Canary Islands, Spain and found that such system achieved very high treatment efficiency for BOD and ammonium. In addition, high (>99.5%) removal of faecal coliforms was also reported. The same combination has also been implemented in a warm tropical climate country like Thailand and it performed very well for BOD and nutrient removal, but, unfortunately the lack of staff taking responsibility for the system management caused problems later (Brix *et al.*, 2011).

2.3.4 Substrate materials in CWs

Substrates, filter media or support materials in CWs are one of the key components that define a successful operation of CWs (Austin and Yu, 2016; Yang *et al.*, 2018; Khalifa *et al.*, 2020). They play an important role as follows: (1) Serving as a substrate for the growth of bacteria that perform the biochemical treatment of the wastewater that pass through the systems (Austin and Yu, 2016); (2) providing physical support for the materials to directly interact with the contaminants via sorption mechanisms (Gupta *et al.*, 2016); and (3) supporting plant growth (Stefanakis, 2016). A suitable permeable filter media should be selected in accordance with the hydraulic and organic loading to the systems as most of the treatment issues occur when the permeability is inadequately chosen for the applied load (Austin and Yu, 2016). Additionally, Khalifa *et al.* (2020) suggested that the media should offer both aerobic and anaerobic pores for microbial processes as well as an internal carbon source.

Most studies have classified CW substrates based on their origin including natural substrates (soil, sand, and gravel), industrial substrates (slag and coal fly ash) and man-made substrates (activated carbon and ceramsite) (Cheng *et al.*, 2018; Yang *et al.*, 2018). Natural substrates such as sand and gravel have been mostly used among other types due to their abundance and

efficiency for pollutant retention with low purchasing cost (Yang *et al.*, 2018). Gravel did not perform well in nutrient removal but was found to have efficiency in heavy metal removal (Buddhawong *et al.*, 2005; Wang *et al.*, 2020). Coarse sand was recommended to be the most suitable filter material for SFCWs for wastewater treatment in developing countries (Hoffmann *et al.*, 2011). The sand should have a hydraulic conductivity of about 10^{-4} to 10^{-3} m/s with grain size of 0.6 mm (Hoffmann *et al.*, 2011; Austin and Yu, 2016). Sand-like material revealed higher removal efficiency for ammonium and total phosphorus relative to gravel in SFCWs (Abdelhakeem *et al.*, 2016). A wide range of alternative substrates has also been considered based on local availability together with achieving enhanced universal or specific pollutant treatment performance with cost effectiveness (Yang *et al.*, 2018). An emerging substrate, zeolite, has gained wide recognition as it contains high micropore and macropore volumes which increase its ability of nitrogen adsorption (Wang *et al.*, 2020). It has been recommended as an ideal substrate for nitrogen removal in CWs (Zou *et al.*, 2012). Another example is activated carbon or activated charcoal. It is an amorphous carbon obtained by physical and chemical processes that provides a high adsorption capacity for organic or inorganic matter in the wastewater due to its large specific surface area (Wang *et al.*, 2020). It has gained increasing recognition in the field of environmental pollution control (Wang *et al.*, 2020). Concurrently, in recent years a more sustainable sorbent material known as biochar sourced from agricultural wastes has also gained interest in water remediation in addition to activated carbon, as it is a cost-effective product with a considerable potential in various pollutant removal applications (Mohan *et al.*, 2014). Activated carbon and biochar amendments are also implemented in wetlands to reduce the mobility and availability of persistent chemicals for uptake into the aquatic or terrestrial food chains (Ghosh *et al.*, 2011). Such emerging materials could be integrated with natural media to optimize SFCW performance. Ulrich *et al.* (2015) showed that only a small amount (0.2-2% weight) of biochar or activated carbon added to sand significantly enhances trace organic micropollutant retention. On the other hand, de Castro *et al.* (2018) compared polishing units for activated sludge effluent with the filter media sand, or 9:1 v:v mixtures of sand with vermiculite, charcoal or granular activated carbon, and found similar EC removal efficiencies by all four filter media, ranging from > 90% for estrogens to 10-30% for ibuprofen, diclofenac, and paracetamol. Selection of filter media is one of the major concerns in CW wastewater treatment. Suitable media selection leads to a better treatment efficiency and a lower risk of system failure (Wang *et al.*, 2020). When selecting media, key points to consider include their source and cost, pollutant removal performance, hydraulic and engineering feasibility,

support for microbial and plant growth, substrate clogging, and recovery/disposal of exhausted substrate (Wang *et al.*, 2020). Substrate clogging is one of the main concerns when designing SFCWs. The main contributors to clogging are the small particle sizes, low porosity and poor hydraulic conductivities which can be prevented by choosing big particle media and trying to avoid using substrates that can react with the pollutants to form precipitates, thus occupying the pore space (Yang *et al.*, 2018).

2.3.5 Plants in CWs

Plants or macrophytes play an important role in CWs by maintaining the hydraulic conductivity of the substrate, reducing excessive nutrients, removing contaminants and also facilitating growth of bacterial communities and other microorganisms which form a biofilm attached to the surface of roots and substrate particles (Shelef *et al.*, 2013; Verlicchi and Zambello, 2014). Additionally, the plants play an indirect role in transferring oxygen from leaves to the rhizosphere via the roots, releasing chelating agents and antibiotics that increase precipitation of metals present in wastewater (Khalifa *et al.*, 2020). Other functions of plants include improving aesthetic appearance, and the elimination of pathogens, insects, and offensive odours (Shelef *et al.*, 2013). Nevertheless, the presence of plants can also provide some adverse effects such as increased mosquito reproduction, enhanced methane emissions and nitrous oxide emissions produced by anaerobic denitrification via aerenchyma tissue (Shelef *et al.*, 2013). The selection of CW plants is dependent on several factors such as climate conditions, types and characteristics of influent water, and types of SFCW (Austin and Yu, 2016; Nuamah *et al.*, 2020). In any cases, native plant species should be specified before selection (Austin and Yu, 2016). *Phragmites australis* is the most used plant for both subsurface flow and free-water surface constructed wetlands. It can be widely found in Europe and Asia and tolerates municipal wastewater that has been pre-treated in a septic tank (Austin and Yu, 2016; Dotro *et al.*, 2017). *Typha latifolia* or broadleaf cattail is a widely distributed North American native. It is capable of treating sewage and other effluents high in organic nitrogen (Austin and Yu, 2016). Meanwhile, in tropical hot climate countries, *Canna* and *Heliconia* plants are widely used (Brix *et al.*, 2011; Marín-Muñiz *et al.*, 2020; Zang *et al.*, 2021). The removal effect of plants on wastewater pollutants varies greatly by environmental conditions, number and type of plants, and the nature and chemical structure of such pollutants (Wang *et al.*, 2014). Several studies found higher removal efficiency of conventional water quality parameters such as BOD and nutrients in planted SFCWs than unplanted beds (Shelef *et al.*, 2013; Abdelhakeem *et al.*, 2016; Marín-Muñiz *et al.*, 2017;

Marín-Muñiz *et al.*, 2020). Contrarily, some studies also revealed that SFCWs without plants showed higher or equal removal of TOC, dissolved oxygen (DO), COD, BOD as well as some pharmaceuticals like carbamazepine and diclofenac compared to those with plants (Tanner, 2001; Baptista *et al.*, 2003; Zhang *et al.*, 2011).

2.3.6 Microbial communities in CWs

Interactions occur among water, plants and media through microbial processes (Adrados *et al.*, 2014). The most stable microbiota in CWs are found in the biofilm attached to plant's roots and/or to the surface of filter media (Adrados *et al.*, 2014). Sánchez (2017) explained that the wetland rhizosphere tends to perform a key role in the formation of oxic-anoxic interfaces where aerobic and anaerobic microbes can perform in proximity, increasing elemental cycling thus enhancing microbial activity. Microbial density and activity were enhanced in planted CWs (Sánchez, 2017). Sidrach-Cardona *et al.* (2015) found that plants in CWs such as common reeds influenced the microbial community of the rhizoplane, interstitial water and gravel-related microbes beyond the plant's roots. However, many studies also reported a lack of significant effect of plants on microbial community structure (Baptista *et al.*, 2003; Iasur-Kruh *et al.*, 2010; Sánchez, 2017). Sidrach-Cardona *et al.* (2015) suggested that such insignificant effects of plants on microbial communities could have resulted from inadequate redox conditions from root development. In any case, the underlying mechanisms of plants on microbial activities in different components of CW systems are still unclear.

Microbial communities can additionally be differentiated by other factors such as temperature, dissolved oxygen, hydraulic design, availability of organic matter and filter material type (Sánchez, 2017). Microbial activities involved in wastewater treatment are strongly influenced by climate and seasonal changes (Sperling and Chernicharo, 2005; Sánchez, 2017). The high temperature condition provides a conducive environment for higher treatment efficiencies particularly of such nature-based systems compared to those in cold temperature (Sperling and Chernicharo, 2005; Bateganya *et al.*, 2016). Wang *et al.* (2016) revealed that the treatment performance and microbial structure of SFCWs varied greatly with seasonal changes for both planted and unplanted beds. Planted beds showed more dominant phyla (e.g., *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*) in the summer than winter while unplanted beds revealed higher *Cyanobacteria* and photosynthetic bacteria in the summer than winter. Additionally, the abundance of microbes is generally decreased

during wintertime. Substrate is also another factor that shapes microbial population in CWs. It provides optimal conditions for microbial growth thus a variety of microbial species are present and contribute to a better treatment system performance (Meng *et al.*, 2014; Rajan *et al.*, 2019). Guan *et al.* (2015) conducted a study on microbial communities in different wetland substrate types (sand, zeolite, gravel), and found that structure and community of bacteria showed prominent spatial variations in zeolite and sand wetlands, but only slightly changed in gravel wetlands. Several phyla were present in CWs, including *Bacteroidetes*, *Proteobacteria*, *Nitrospirae*, *Cyanobacteria* etc. *Proteobacteria* dominated among other phyla and this group of microbes drives more effective organic pollutant reduction.

Moreover, enhanced nitrogen removal efficiency is one of the important targets for further development of CWs. Microbes involved in the N-cycle, particularly nitrification and denitrification, play a crucial role in nitrogen transformation in CWs (Adrados *et al.*, 2014). Rajan *et al.* (2019) reported that *Nitrosomonas* species of nitrifying bacteria were found in the vicinity of plant roots which therefore had better nitrogen removal than unplanted beds. Paranychianakis *et al.* (2016) reported on the seasonal shift in the composition of denitrifying microbes towards to a community with a lower genetic potential for N_2O emission in planted SFCWs.

2.3.7 CW performance on wastewater treatment

I. Removal of conventional water quality parameters

FWSCWs performed well on BOD and total suspended solid (TSS) removal, while their removal of nutrients particularly phosphorus (P) is typically limited depending on hydraulic loading rate (HLR) and sizes of CWs (Stefanakis, 2016; Wang *et al.*, 2017). Generally, in all climate conditions, FWSCWs showed 70-90% removal of BOD, COD and TSS, whilst removal of nitrogen (N) and P can be greatly varied from 20-80% (Verhoeven *et al.*, 2006; Wang *et al.*, 2017; Varma *et al.*, 2021). Removal efficiency of heavy metals (Fe, Cu, Zn, Pb) are on average around 50% (Parde *et al.*, 2021). Gunes *et al.* (2012) studied the one-year performance of FWSCWs in treating high strength domestic wastewater like septic tank effluent in Turkey and found that the CWs removed approximately 86%, 92%, 56% and 43% TSS, BOD, total nitrogen (TN), and total phosphorus (TP), respectively. Similarly, high removal of TSS, BOD with limited removal of TP was also found from FWSCWs in tropical climate (Jinadasa *et al.*, 2006).

For subsurface systems, SFCWs showed high treatment efficiencies for organic matter, nutrients, and pathogens (Hoffmann *et al.*, 2011; Wu *et al.*, 2015). In general, removal of BOD and TSS were found to be highly efficient and consistent across all types of SFCWs (Zhang *et al.*, 2015). In tropical environmental conditions, HFBs could efficiently reduce on average more than 80% of BOD and COD, 70% of TP, and 50% of TN (Varma *et al.*, 2021). For VFBs, the average BOD and COD removal was reported to be around 87% which is slightly higher than HFBs (Varma *et al.*, 2021). Similar removal (80%) in the two system types was found for TSS (Zhang *et al.*, 2015). Although, low denitrification occurred in VFBs as a result of prevailing aerobic conditions, TN removal was still observed at 68% which is higher than HFBs (Varma *et al.*, 2021). In terms of the hybrid systems, higher efficiency was found for removal of BOD, COD, TN, ammonia, nitrate, nitrite, etc., as compared to other systems. The average BOD, COD and TN reduction was approximately 90%, 80% and 70%, respectively (Vymazal, 2013; Zhang *et al.*, 2015; Varma *et al.*, 2021). The high removal rate of BOD and COD could be attributed to filtration or sedimentation of suspended solids as well as biodegradation. VFBs performed better than HFBs because the former is intermittently loaded resulting in a higher oxygen transfer to the filter medium as compared to HFBs (Zhang *et al.*, 2015). Besides, the removal efficiency is highly dependent on ambient temperature. Poorer treatment efficiency can be found at low temperature as a consequence of multiple variables such as the microbial metabolism rate, the plant's oxygen transfer capability, the sedimentation and adsorption velocity of the filter material etc. (Ji *et al.*, 2020). For pathogen removal, SFCWs and hybrid systems were reported to eliminate a considerably higher number of bacterial pathogens than surface flow wetlands (Shingare *et al.*, 2019). 3-log removal of faecal/total coliforms was typically found in SFCWs (Rajan *et al.*, 2019; Shingare *et al.*, 2019; Khalifa *et al.*, 2020). SFCWs also showed highest removal performance for helminths eggs. This is because parasitic eggs are typically bigger than bacterial pathogens hence a satisfying filtration by screening out and attachment of such eggs was found in CWs with filter media like SFCWs. (Shingare *et al.*, 2019).

II. Removal of ECs

Emerging contaminants (ECs), also known as contaminants of emerging concerns (CECs) refer to a broad range of chemicals such as pharmaceuticals, hormones, personal care products, pesticides etc. (Mailler *et al.*, 2016; McLain and Gachomo, 2019), which have a potential of endangering ecosystems and human health (Geissen et al., 2015).

ECs can be classified into more than 20 classes based on their origin, use, potential effects or environmental fate (Nassar and Younis, 2019). Most reported EC groups include (1) pharmaceuticals e.g. human and veterinary antibiotics, analgesics, anti-inflammatory drugs, and β-blockers, (2) personal care products e.g., fragrances and insect repellents, (3) hormones and steroids, (4) disinfectants, (5) flame retardants, (6) herbicides and pesticides, (7) industrial additives and agents, and (8) gasoline additives (Stefanakis and Becker, 2015; Wang and Wang, 2016; Tran *et al.*, 2018; Nassar and Younis, 2019). ECs are not commonly included in routine water monitoring programmes, and consequently their fate and behaviour are often not well-understood (Geissen *et al.*, 2015). They can be released to the environment through several routes including wastewater treatment plants (WWTPs), landfill leachate, agricultural activities, improper industrial disposal and direct discharge of household wastewater in some countries (Tewari *et al.*, 2013; Geissen *et al.*, 2015; Yang *et al.*, 2017b; Tran *et al.*, 2018). Their release to the environment has likely occurred for many years but not noticed until proper detection methods were developed (Geissen *et al.*, 2015; Gomes *et al.*, 2020).

Among the aforementioned routes, ECs released from WWTPs are of particular interest due to their continuous discharge to waterbodies. ECs can be detected in both influent and treated effluent of WWTPs at trace levels (ng/L- μg/L) (Tran *et al.*, 2018). Many of them, e.g. organic micropollutants, are known to be persistent in the environment and not easily degradable (Stefanakis and Becker, 2015). Moreover, although some ECs are not lasting in the environment and can be transformed or eliminated through natural processes, their continuous release from WWTPs maintains their existence in natural waterbodies thus adversely affects aquatic ecosystems (Stefanakis and Becker, 2015). Tran *et al.* (2018) compared the occurrence of ECs in municipal wastewater from different geographical locations and found that similar pharmaceuticals were present in both European and Asian municipal wastewater, only differing in the detected concentration levels. A wide range of antibiotics was detected in WWTP effluents at higher concentrations in Asian countries than those found in European countries, which is presumably because in several Asian countries such as Thailand, India, and China, antibiotics can be purchased without a doctor's prescription giving easier accessibility and subsequently higher consumption rates.

For a regulatory point of view, the environmental risk assessment evaluated based on an exposure concentration of each EC is required to avoid adverse effects of such EC on the aquatic environment (Minguez *et al.*, 2016). Predicted no-effect concentrations (PNECs) are often used as permissible limits for the EC concentrations in surface water in European

countries (Minguez *et al.*, 2016; Gredelj *et al.*, 2018). The PNECs are derived from eco-toxicity testing and are defined as "the concentration of a substance below which an unacceptable effect will most likely not occur" (Chapman and Elphick, 2015). Such concentrations are used in risk assessments and in environmental policy and regulation (Chapman and Elphick, 2015). The PNECs can then be used to obtain a hazard (risk) quotient for risk assessment purposes (Minguez *et al.*, 2016). The risk quotient of less than 1 means low ecological risk and no further assessment is necessary (Minguez *et al.*, 2016). In recent years, PNECs for antibiotics in the environment have also been studied to assess the risks of antibiotic resistance gene development based on minimal inhibitory concentration at which antibiotics inhibit the growth of bacteria (Bengtsson-Palme and Larsson, 2016).

Constructed wetlands have been widely investigated for EC removal from wastewater over the past decades (Li *et al.*, 2014; Dotro *et al.*, 2017; Ilyas and van Hullebusch, 2020). CWs implemented as a post-treatment system to remove pharmaceutical residuals from wastewater are receiving increased attention in many countries across the world (Li *et al.*, 2014). They can be potentially used as both secondary or tertiary treatment unit based on treatment requirements and suitability in each location. Reported removal efficiencies of the most commonly monitored pharmaceuticals in secondary/tertiary FWSCW and SFCW units are summarized in **Table 2.6**.

Table 2.6 Removal efficiencies (%) of pharmaceuticals in FWSCW and SFCW applied as an alternative secondary or tertiary wastewater treatment system. Data were derived from Verlicchi and Zambello (2014), Li et al. (2014), Ávila et al. (2015) and Ilyas and van Hullebusch (2020).

Pharmaceuticals	**Secondary treatment**			**Tertiary treatment**		
	FWS	**HFBs**	**VFBs**	**FWS**	**HFBs**	**VFBs**
Diclofenac	0-50 %	0-55 %	63-73 %	73-96 %	5-7.5 %	78-79 %
Ibuprofen	45-95 %	50-70 %	85-99 %	96%	28-96 %	66-72 %
Naproxen	25-75 %	50-90 %	84-92 %	52-92 %	14-36 %	39-45 %
Acetaminophen	99%	47-99 %	94-97 %	97-99%	45%	96-98 %
Salicylic acid	35-90 %	50-98 %	60-90 %	5-50 %	0-25 %	0-25 %
Carbamazepine	0-50 %	20-30 %	20-26 %	30-47 %	60-88 %	0-26 %
Caffeine	25-99 %	60-90 %	82-99 %	95%	0-25 %	0-25 %
Sulfamethoxazole	59-92 %	73-87 %	0-25 %	0-45 %	0-25 %	0-25 %

Even though CWs are not specifically designed for micropollutant treatment, the systems were still capable of removing micropollutants. As shown in **Table 2.6**, in general, CWs performed well for removing most of the pharmaceuticals reported. Better removal efficiencies were observed for most reported compounds in secondary treatment than tertiary treatment for all types of wetlands. However, no conclusion can be drawn as to which type

of CWs is the best option for EC removal as there were huge variations among the removal efficiencies of different compounds and system types. Noticeably, each compound tends to have its own specific behaviour. The removal efficiency can be affected by various conditions (temperature, plants, substrate) in each SFCW system. Removal of ECs happens in a combination of complex physical, chemical, and biological interactions by several wetland components. Such mechanisms include biodegradation, sorption, and plant interactions (Verlicchi and Zambello, 2014). Nevertheless, the primary removal mechanism is still not easily ascertained (Zhang *et al.*, 2011). For example, Zhang *et al.* (2011) found that planted SFCWs, through rhizosphere effects, revealed significantly increased removal efficiencies of ibuprofen and naproxen compared to unplanted beds. However, planted beds showed no significant difference for removal of recalcitrant compounds like diclofenac and carbamazepine, if compared to unplanted beds. The removal mechanism for these compounds likely came from sorption to organic surfaces instead of plant effects. For the reported pharmaceuticals **(Table 2.6)**, three mechanisms took place for removal, but the dominant mechanism was thought to be via biodegradation (Ilyas and van Hullebusch, 2020). CWs were also found to perform well in elimination of antibiotic resistance genes (ARGs) whereby biodegradation and adsorption onto filter medium were observed to be the major removal mechanisms, however this is still being researched (Pei *et al.*, 2019). Hybrid wetlands are also increasingly investigated for ECs treatment in recent years. A full-scale study of a hybrid CW system in southern Spain by Ávila *et al.* (2015) showed that such a system was capable of removing 89-99 % of pharmaceuticals such as ibuprofen and diclofenac. Ilyas and van Hullebusch (2020) who conducted a critical comparison of different types of CWs revealed that hybrid systems performed comparatively better for EC removal as compared to HFBs or VFBs alone. This is because of the synchronization of aerobic/anaerobic conditions and longer hydraulic retention time from multiple compartments of the hybrid systems. Moreover, in comparison with conventional treatment systems, Dotro *et al.* (2017) reported that CWs could remove a range of micropollutants better than an activated sludge system, which still can be attributed to the coexisting aerobic-anoxic-anaerobic microenvironments of biofilms and a long sludge retention time in the CW systems. Furthermore, researchers also investigated the impact of nitrification on ECs removal. Co-metabolic oxidation by the ammonia oxidation enzyme (ammonium monooxygenase (AMO)), taking part in the first step of nitrification processes, is thought to be initiating the biotransformation of several ECs (Fernandez-Fontaina *et al.*, 2012; Alvarino

et al., 2016). However, it should be noted that nitrification is not the only metabolic pathway that controls EC biodegradation (Rattier *et al.*, 2014).

2.3.8 Technology comparison

In terms of a technology comparison, among free-water surface flow CWs, SFCWs and ponds, there is no universal conclusion that one type of these systems is better than the others, hence the advantages and disadvantages of these three systems are still a subject of debate. Sperling and Chernicharo (2005) stated that SFCWs can be susceptible to clogging if improperly managed. Mara (2004) found that a horizontal SFCW required 22% larger area than a secondary facultative pond for the same BOD removal at 25 °C ambient temperature in a tropical climate country like Brazil. Contrarily, Mburu *et al.* (2013) carried out a pilot-scale study in Kenya, which also has a tropical climate, and it was found that a facultative pond required a three times larger area than a horizontal SFCW when water was treated to the same standard. Moreover, Kadlec (2009) revealed that a horizontal SFCW performed well in cold climates as it is less cold sensitive and easier to insulate for winter operation than a free-water surface treatment system. SFCWs also provide some other advantages over surface flow wetlands and waste stabilisation ponds such as lower risk of mosquito breeding and unpleasant odours (Mara, 2004; Austin and Yu, 2016). For financial perspective, the selected technology for a small rural community should be that which produces a compliant effluent at the least cost (Johnson *et al.*, 2007).

Apart from sewage treatment, CWs have also been used to treat mine water. More details of mine water pollution challenges and related treatment options are provided in Section 2.4.

2.4 Mine water treatment

With a long history of mining activity, there is a legacy of contamination of land and water resources in many countries (Brown *et al.*, 2002). Apart from active mines, abandoned mines have become one of the most important environmental pollution concerns due to their discharge of heavy metals and other pollutants into waterbodies (Mayes *et al.*, 2009; Potter *et al.*, 2009). This is because, when a mining site is closed, pumping is normally turned off and mine water from the rebound of groundwater and leachate of spoil heaps gets discharged in an uncontrolled manner through drainage streams into the environment (Potter *et al.*, 2009; Tran *et al.*, 2022). The main heavy metal pollutants associated with mine water include iron (Fe), zinc (Zn), lead (Pb), cadmium (Cd), manganese (Mn), copper (Cu), and arsenic (As) (Mayes *et al.*, 2009; Potter *et al.*, 2009). In most abandoned mine water discharges, Fe is the

main concern, whereas pH, Mn and other metals are of concern in more acidic mine waters (Younger and Henderson, 2014). The weathering of sulphide ores containing minerals such as sphalerite (ZnS), galena (PbS) and arsenopyrite (FeAsS) lead to the release of heavy metal ions. While acidity primarily came from the weathering of pyrite (FeS_2) in solutions containing dissolved oxygen. Ochre which is a yellow to red-brown solid will be generated as part of dissolved iron precipitation under oxic conditions (Younger *et al.*, 2002). Moreover, one major concern of mine water discharges is acid mine drainage (AMD). AMD is a polluted mine water generated from complex chemical, biological, physical processes under ambient conditions (Gao *et al.*, 2019; Ighalo *et al.*, 2022). It is extremely acidic (pH < 4) and contains high levels of sulphate ions, Fe and Zn, posing significant threats to the environment (Ighalo *et al.*, 2022). Two main treatment methods including active and passive treatments were established to tackle polluted mine water issue (Younger *et al.*, 2002; Jarvis *et al.*, 2006). The active treatment is basically a conventional wastewater treatment plant involving the use of artificial energy and chemicals, whereas the passive treatment mainly relies on natural components such as sunlight, plants and microbes to treat mine water (Younger *et al.*, 2002; Trumm, 2010; Dufresne *et al.*, 2015). Generally, active treatment is often implemented to treat mine water with high acid load from active mines, while passive treatment is more commonly used to treat low-acidity mine water from closed and abandoned mines (Trumm, 2010). Examples of active and passive treatment technologies derived from Younger *et al.* (2002) and Ighalo *et al.* (2022) are listed as follows

2.4.1 Active treatment technologies

- ODAS (Oxidation, Dosing with Alkali & Sedimentation): although its commonly known as ODAS for industrial wastewater treatment, a proper sequence for AMD treatment is DAOS in which the first step is dosing with alkali (DA), followed by oxidation (O) and sedimentation (S). pH is raised beforehand to increase reaction rate of the following oxidation step
- Sulfidisation and Biodesalination: mainly to reduce sulphate to form sulphide species
- Advanced oxidation processes (AOPs): mainly to oxidise organic compounds
- Sorption-based treatment method e.g., activated carbon/zeolite adsorption
- Membrane separation processes e.g., reverse osmosis and filtration
- Bioreactors
- Chemical precipitation e.g., barium sulphate precipitation

2.4.2 Passive treatment technologies

Inorganic media passive systems: a dissolution of limestone can be used to raise pH

- Anoxic Limestone Drains (ALDs)
- Oxic Limestone Drains (OLDs)

Wet-land type passive systems

- Settlement ponds/lagoons
- Aerobic wetland (reed beds): normally used for treating net-alkaline water
- Compost wetland: normally used for treating net-acidic water via sulphate reducing bacteria which consume acidity and generate bicarbonate alkalinity
- Reducing and Alkalinity Producing Systems (RAPS): they are compost wetlands with limestone aggregate layer underneath, and designed to generate alkalinity via calcite dissolution
- Other types such as Permeable Reactive Barriers (PRBs) which is an in-situ treatment of polluted groundwater

The options of passive treatment systems can be decided based on the water chemical properties such as DO, acidity and concentration of metals (Younger *et al.*, 2002; Trumm, 2010). For example, for an acidic and metal-rich mine water, the suitable treatment options are RAPS, compost wetlands and PRBs. The systems generate alkalinity to elevate pH, consequently facilitate precipitation of metal pollutants (Jarvis *et al.*, 2006). Moreover, a combinational approach can also be an option. Matthies *et al.* (2010) investigated the performance of a combined RAPS and aerobic wetland system treating net-acidic coal mine drainage and found that the system performed reasonably well for the removal of iron, aluminium and acidity, and constantly elevated pH and alkalinity.

2.5 Co-treatment of wastewater and mine water in CWs

CWs are typically designed to treat separate sources of pollution e.g., wastewater or mine water alone. The principal pollutant of concern varies between the two waters e.g., BOD and nutrients in wastewater versus heavy metals in mine water (Younger and Henderson, 2014). It would be beneficial if wastewater and mine water could be co-treated in the same CW unit since effluents of abandoned mine are often being discharged in or close to populated areas that historically developed around mines (Johnson and Younger, 2006). The co-treatment will provide great opportunities not only in terms of synergetic treatment, but also for a financial point of view (Johnson and Younger, 2006). Wang *et al.* (2021) reported that co-treating acid

mine drainage and domestic wastewater by CWs performed effectively in elevating effluent pH (from 2.5 to 8.1) and in removing heavy metals especially Fe, Zn, Cd and Cu as well as organic and nutrient pollutants. In the UK, there is only one full-scale FWSCWs for a co-treatment of mine water and secondary treated sewage effluent which has been operated for several years in the Lamesley area, North East England (CoalAuthority, 2018). It was found that the co-treated wastewater and mine water in CWs showed efficient removal of BOD and ammoniacal nitrogen and phosphate. However, such co-treatment by CWs exhibits a unique treatment challenge as it requires simultaneous removal a wide range of pollutants, namely nutrients, metals, organic micropollutants, and pathogens, which have only been partially investigated. Younger and Henderson (2014) studied the full-scale CWs performance in cotreating mine water and wastewater, but only reported on the performance of the CWs on the removal of BOD, ammoniacal nitrogen, phosphorus, iron, and suspended solids. Therefore, there is insufficient information of such full-scale CW performance on removal of nutrients like nitrate and total nitrogen, heavy metals like zinc, copper and lead, organic micropollutants like antibiotics, painkillers and pesticides, and pathogens like faecal bacteria causing gastroenteritis

Extending the research from the traditional wastewater treatment systems to interventions in agriculture fields will enhance the overall sustainability of rural waste management. A discussion of sustainable agricultural waste management is provided in Section 2.6.

2.6 Agricultural waste management

Nature-based wastewater treatment in CW is especially attractive in rural settings, where the required land area is more readily available, and wastewater loads are less than in urban settings. To further enhance the overall sustainability of waste management in rural settings, waste minimization and valorisation technologies should be considered broadly towards the aim of building a more circular rural economy. Due to the rapidly increasing world population, there has been an increasing demand of agricultural products for human well-being (Zakaria, 2018). Consequently, large volumes of agricultural wastes are being generated especially in developing countries where agriculture is an influential sector to their economy (Zakaria, 2018; Durga *et al.*, 2021; Koul *et al.*, 2022). Agricultural wastes can be classified into four main groups including crop waste, animal waste, food processing waste, and hazardous and toxic wastes (Zakaria, 2018). Residues generated particularly from crops and livestock, unless properly managed, create several detrimental effects to the environment

(Zakaria, 2018; He *et al.*, 2019a). Crop waste residues are often burnt or disposed into landfills/open dumping sites resulting in environmental impacts such as greenhouse gas (GHG) emissions, water, and soil contamination (Panyakaew and Fotios, 2008; Koul *et al.*, 2022). Similarly, wastes generated from livestock production such as animal manures and slurries also raise concerns over the same issues as crop wastes, but with concerns more geared towards groundwater pollution and GHG emissions especially methane (Scholten *et al.*, 2013; Obi *et al.*, 2016; Koul *et al.*, 2022). Several strategies via chemical, biological and physical process have been used to combat these issues. For crop wastes, many crops can be utilised as animal feed, fibre for textile industry, compost, fertilizer, biofuel and biochar (Koul *et al.*, 2022). For animal wastes, manures and slurries can be treated via anaerobic digestion giving useful by-products like anaerobic digestate (biofertilizer) and biogas (Koszel and Lorencowicz, 2015; Risberg *et al.*, 2017; Brown *et al.*, 2020). In this study, two of these management approaches, and their integration, are further discussed. Sustainable management of agricultural wastes can be considered as part of nature-based solutions that "protect, sustainably manage and restore natural or modified ecosystems, that address societal challenges effectively and adaptively, simultaneously providing human well-being and biodiversity benefits" (Cross *et al.*, 2021).

2.6.1 Biochar from pyrolysis

Biochar has been earlier mentioned in **Section 2.3.4** as an emerging substrate material being implemented in CWs and water biofilters (Mrozik *et al.*, 2021). Biochar, a carbon-rich material, is produced by heating biomass feedstock such as wood and agricultural waste through pyrolysis for renewable energy generation (Cole *et al.*, 2012; Tan *et al.*, 2015; Koul *et al.*, 2022). Pyrolysis occurs in the absence of oxygen and is classified as fast or slow pyrolysis (Cole *et al.*, 2012). The fast pyrolysis operates at heating temperatures of 400-600 °C for less than 2 seconds of heating time, whereas the slow pyrolysis undergoes temperatures of 300-800 °C with at least 1 hour of heating time (Cole *et al.*, 2012). Different feedstock sources and pyrolysis process conditions contribute to different structural and physical characteristics of biochar including structural complexity, surface area, porosity, particle size distribution, density and mechanical strength (Lehmann and Joseph, 2015). Biochar has gained interest in the multidisciplinary areas of global warming mitigation, soil amendment, crop production enhancement, water treatment and carbon sequestration (Glaser *et al.*, 2002; Laird, 2008; Tan *et al.*, 2015). Biochar can play a crucial role in enhancing nutrient retention in soil mostly due to its surface charge density (Kongthod *et al.*, 2015). The

retention or adsorption property of the biochar has been increasingly investigated not only for soil but also for water treatment (Kizito *et al.*, 2015; Tan *et al.*, 2015; Ulrich *et al.*, 2015; Trinh *et al.*, 2019; Koul *et al.*, 2022). Biochar's physical and chemical properties including porous structure, large surface area, enriched surface functional group and mineral components are key factors towards its adsorption capacity (Lehmann and Joseph, 2015; Tan *et al.*, 2015). Besides, in recent years, biochar has gained attention as a low-cost and effective adsorbent alternative to activated carbon as it requires relatively low energy/cost for the production (Tan *et al.*, 2015). It has been reported to show great potential for improving soil fertility and crop yield (Rutherford *et al.*, 2012; Yao *et al.*, 2012; Ahmad *et al.*, 2014). Apart from adsorption property, this can be partially attributed to its effects on soil microbiology that reduce fertilizer losses via leaching (Atkinson *et al.*, 2010; Tan *et al.*, 2015).

2.6.2 Biofertilizer from anaerobic digestion

Anaerobic digestion (AD) is a biological process in which organic matter is being decomposed by microorganisms in the absence of oxygen (Lukehurst *et al.*, 2010). Various types of organic material can be used as AD feedstock such as livestock manure, crop residues, food waste, and sludge from wastewater treatment (Risberg *et al.*, 2017; Aragón-Briceño *et al.*, 2020). AD is initially designed to process livestock manures alone, however the co-digestion of manures with crop residues has also gained interest due to improved performance of digesters (Brown *et al.*, 2020). AD generates two useful by-products namely biogas and anaerobic digestate (Koszel and Lorencowicz, 2015; Risberg *et al.*, 2017). Biogas is normally used for heat and electricity production while digestate, a nutrient-rich material, can be utilised as biofertilizer (Holm-Nielsen *et al.*, 2009). Digestate appears in a form of a liquid to thick slurry which can be either directly used as fertilizer or refined by solid–liquid separation to gain most benefit of the solid and liquid fraction of the digestate (Möller and Müller, 2012; Akhiar *et al.*, 2017). The nutrient contents in digestate varies with the origin of feedstocks. Digestate typically has higher ammonium to total nitrogen ratios, decreased organic matter content, decreased total and organic carbon content and higher pH value than original manure/slurries (Möller and Müller, 2012). Typical nutrient content in digestate is 1390-1450 mg/L for ammonium-nitrogen, 47–54 mg/L for nitrate-nitrogen, 3600- 4800 mg/L for total nitrogen and 15-20 mg/L for phosphate (Kizito *et al.*, 2015; AHDB, 2017). It has also been reported that digestate-fertilizer improves soil fertility, plant growth and plant immunity to biotic and abiotic agents (Koszel and Lorencowicz, 2015). On the other hand, with its nutrient-rich property, digestate can also pose threats to the environment. There have

been concerns over ammonia volatilization and excess nutrient leaching from soils to groundwater after digestate application (Lukehurst *et al.*, 2010; Akhiar *et al.*, 2017). This is attributed to rapid ammonification of organic nitrogen followed by nitrification of ammonia into the more soluble and leachable nitrogen compound, nitrate (Wang *et al.*, 2015). Svoboda *et al.* (2013) reported leachate nitrate levels above the drinking water threshold value after digestate application on an agricultural land.

2.6.3 Co-application of biochar and anaerobic digestate

As biochar is a well-known adsorbent material for soil amendment and water treatment, it could provide a solution to the concern over nutrient leaching from soils to groundwater after digestate application. Several studies have shown that the nitrification process in soil could be altered by biochar amendment due to its effect on soil geomicrobiology (DeLuca *et al.*, 2006; Song *et al.*, 2014; Bi *et al.*, 2017). There was also a report on reduced nitrification following biochar amendment in soil (Wang *et al.*, 2015). Therefore, the utilisation of biochar as a nitrification inhibitor could be a promising option for N-management in agriculture, which would be particularly relevant in co-application with a rich source of reduced nitrogen such as anaerobic digestate. Such co-application would facilitate multi-use systems of waste by integrating two residues (biochar and digestate) of bioenergy generation from different types of agricultural waste for re-use in sustainable agriculture.

2.7 Research gap

Although CWs have shown a great potential as a nature-based solution for wastewater and mine water treatment, there is still insufficient information of the full-scale CW performance on simultaneous removal of nutrients, heavy metals, organic micropollutants and pathogens in either separate or co-treatment of wastewater and mine water (more details in Section 2.5). Furthermore, full-scale horizontal flow CWs require large land area e.g., 5 ha CWs in the UK (Younger and Henderson, 2014) which might not be suitable in a location where there is a space constraint and land prices are high. Small-scale vertical SFCWs, a biofilter-like system containing sand as filter medium, could then provide an alternative treatment option. Sand filtration and biological activated carbon are reported to be the most commonly used biofilter technologies (Reungoat *et al.*, 2011). It is promising that biofilter performance can be intensified using activated carbon (AC). Several studies reported the potential of biofilters amended with AC for environmental pollutant removal, including EC removal, via adsorption and biodegradation mechanisms (Luo *et al.*, 2014; Ulrich *et al.*, 2015; Mailler *et al.*, 2016; de

Castro *et al.*, 2018). However, the impact of AC-amendment in biofilters on nutrient, pathogen and micropollutant mitigation in wastewater needs more empirical investigation to provide evidence for improved performance across a wide range of relevant parameters (Ulrich *et al.*, 2015; Boehm *et al.*, 2020). Extending the research from the traditional water treatment systems to agriculture fields, sustainable agricultural waste management has gained increasing attention in past decades. Bioenergy generation such as pyrolysis and biogas production from different types of agricultural waste is one of the well-known management options. Two valuable by-products including biochar and anaerobic digestate are generated from such systems. Digestate is normally utilised as biofertilizer, nevertheless, it sometimes contains over-concentrated nutrients leading to excess nutrient leaching from soils to groundwater after application on land (Akhiar *et al.*, 2017). This is attributed to rapid ammonification of organic nitrogen followed by nitrification of ammonia into the more soluble and leachable nitrogen compound, nitrate (Wang *et al.*, 2015). Svoboda *et al.* (2013) reported on leached nitrate level above the drinking water threshold value after digestate application on an agricultural land.Biochar has been reported to improve soil fertility and reduce nutrient leaching through its sorption property and its effect on soil microbiology (Atkinson *et al.*, 2010; Tan *et al.*, 2015). Therefore, it is promising to apply biochar in digestate-amended soil, and this would also facilitate multi-use systems of agricultural wastes. However, very little is known regarding the nutrient retention in soil, and soil microbial community response following combined application of digestate and biochar. All these nature-based solutions for wastewater and agricultural waste management will contribute towards achieving several sustainable development goals, including the development of a more sustainable and circular rural economy.

2.8 Aim and objectives

Aim

This thesis aims to demonstrate how nature-based solutions can help sustainably manage wastewater and agricultural wastes in rural settings.

Objectives

Objectives 1, 2 and 3 are associated with thesis Chapter 3, 4 and 5, respectively, and seek to fill the research gaps identified in Section 2.7.

1. To assess the performance of combined treatment of coal mine water and secondary treated wastewater in free-water surface flow constructed wetlands (CWs) in protecting the river

water environment from chemical and microbiological pollution.
Hypothesis: The CWs cotreating mine water and wastewater removes heavy metals, nutrients, and pathogens to meet the desired river water quality standards.

Contributorship statement: Jidapa Plaimart: Methodology, Investigation, Formal analysis, Data Curation, Writing - Original Draft, Visualization. **Kishor Acharya**: Investigation, Validation. **Adrian Blackburn**: Investigation, Validation **Wojciech Mrozik**: Supervision, Writing - Review & Editing. **Russell J. Davenport**: Supervision, Writing - Review & Editing. **David Werner**: Conceptualization, Methodology, Investigation, Software, Validation, Supervision, Writing - Review & Editing.

2. To investigate the effect of coconut shell activated carbon (AC) amendment in sand bed biofilters operated under tropical conditions on micropollutant and bacterial pathogen removal from secondary treated wastewater.
Hypothesis: The AC-amended biofilter improves residual micropollutant and pathogen removal relative to the biofilter without AC amendment.

Contributorship statement: Jidapa Plaimart: Methodology, Investigation, Formal analysis, Data Curation, Writing - Original Draft, Visualization. **Kishor Acharya**: Validation, Formal analysis. **Adrian Blackburn**: Validation, Formal analysis. **Wojciech Mrozik**: Formal analysis, Supervision, Writing - Review & Editing. **Russell J. Davenport**: Supervision, Writing - Review & Editing. **Soydoa Vinitnantharat**: Resources. **David Werner**: Conceptualization, Methodology, Software, Validation, Supervision, Writing - Review & Editing.

The effluent from the trickling filter and mine water were used as the CW influent (Chapter 3), while the effluent from the activated sludge treatment system was used as the biofilter influent (Chapter 4). In both cases, the nature-based solutions provide tertiary treatment of municipal wastewater. The initial plan for the CW work was to use the column study data for the design of a pilot-scale subsurface flow CW. However, due to the Covid-19 pandemic, the commissioning of the pilot-scale CW set-up was delayed, we instead decided to study the full-scale CW cotreating tricking filter effluent and mine water which has already been operated. Although heavy metal levels (Fe and Mn) between the two types of influents were different, the organic matter and nutrient concentrations were comparable.

3. To investigate the effect of combined application of digestate with coconut husk (CH) biochar on nutrient retention, nitrification, and nitrifying microbe abundance in agricultural soil.
Hypothesis: CH biochar improves nutrient retention in digestate-amended soil relative to the digestate-amended soil without biochar application.

This chapter includes and extends the work I completed for my MSc dissertation submitted for the MSc Environmental Engineering degree at Newcastle University in 2018. While the experiments and nutrient leaching/volatilization measurements were carried out during my MSc study, the molecular microbiology work was conducted during my PhD study.

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Chapter 3

Effective removal of nutrients, metals, and pathogens in constructed wetlands cotreating mine water and sewage treatment plant effluent

Chapter 3. Effective removal of nutrients, metals, and pathogens in constructed wetlands cotreating mine water and sewage treatment plant effluent

3.1 Abstract

In the UK, small wastewater treatment works in rural settings show variable performance which raises concerns about impacts on the water environment. Concurrently, discharge from abandoned mines has long raised concerns over excess heavy metals input into surface waters. Under the Environment Act 2021, more ambitious targets are being set for the UK water environment that will require significant investment into upgraded pollution control systems. Constructed wetlands (CWs) are nature-based, passive treatment systems that provide a water treatment solution suitable for the rural environment. In some settings, it would be advantageous, if domestic wastewater and mine water could be co-treated. However, there is insufficient information on such co-treatment performance of CWs across the wide range of water quality parameters affecting the status of receiving rivers. We therefore comprehensively analysed the performance of full-scale CWs located at Lamesley in Northeast England, which co-treat abandoned coal mine and secondary treated sewage treatment plant (STP) effluents. Overall, the CWs effectively removed phosphate and iron by 73% and 87%, respectively, to achieve the discharge standards through synergistic interaction between phosphate from sewage and iron from the abandoned mine. The CWs also effectively reduced ammoniacal nitrogen concentrations by 80%, but were ineffective in reducing dissolved manganese, lead, zinc, and copper. Conventional and molecular microbiology methods showed that CWs successfully converted sewage and mine water microbiomes into a freshwater microbiome and reduced faecal pollution and putative human pathogen indicators by 82-99%. Accordingly, the CW discharge had no detrimental impacts relative to the upstream nutrient and recreational status of the receiving River Team. But it further elevated the high manganese levels found upstream of the discharge towards the limit for good chemical status of rivers. Overall, CWs and the innovative monitoring methods demonstrated in this study can help sustainably protect the water environment in rural settings.

3.2 Introduction

Rural wastewater is normally treated by small sewage treatment plants (STPs) or household treatment systems like septic tanks (Nasr and Mikhaeil, 2015; Bunce and Graham, 2019). A

study of twelve small STPs in the UK found their performance is variable, poorly understood, and their ecological impacts may be underestimated (Bunce and Graham, 2019). The evaluation of STPs has focused on nutrient removal to meet Water Framework Directive (WFD) criteria for the ecological status of receiving waters (Withers *et al.*, 2011; Bunce and Graham, 2019). In this context, Bunce and Graham reported poorer mean removal of soluble chemical oxygen demand, total suspended solids, and ammonia-nitrogen in small STPs compared to large STPs (Bunce and Graham, 2019). The Water Environment (Water Framework Directive) (England and Wales) Regulations 2017 have no standards for the microbiology of receiving rivers (WFD, 2017), which are set instead in the Bathing Water Regulations 2013 (BWR, 2013). But most designated bathing waters in England and Wales are in coastal locations or inland lakes, and only one stretch of river in Yorkshire is currently a designated inland bathing water (Laville, 2021). This may be changing as citizen initiatives seek to declare more rivers for recreational use as bathing rivers (WaterUK, 2021). Bathing water designation will make the microbiological river status an important consideration since most rivers in England and Wales are impacted by sewage pollution (EnvironmentalAuditCommittee, 2022). In follow-on work from their 2019 study, Bunce *et al.* (2020) comparatively assessed the removal of genetic markers for faecal pollution in small and large UK STPs. They found significantly higher abundance of faecal marker genes from the genus *Bacteroides*, including marker genes HF183 and HumM2 for human-host-associated *Bacteroides* bacteria, in the effluent of small relative to large STPs. HF183 distinguishes human sewage pollution of surface water from other faecal pollution sources (Ahmed *et al.*, 2008). Scientists in the United States have proposed ambient water quality standards for recreation that use the HF183 marker gene concentration as a proxy for various pathogen concentrations (Boehm and Soller, 2020). For all these reasons, improved performance of small STPs may soon be required to meet heightened public expectations and more stringent river water quality standards.

Besides STPs, a major threat to river water quality in the UK is abandoned mines. One of the environmental targets being proposed under the Environment Act by the UK Government is to reduce the length of English rivers that are polluted from abandoned metal mines by 50% by 2037 (DEFRA, 2022). When a mining site is closed, pumping is normally turned off, and mine water from the rebound of groundwater gets discharged in an uncontrolled manner through drainage streams (Potter *et al.*, 2009; Tran *et al.*, 2022). The main heavy metal

pollutants associated with mine water include iron (Fe), zinc (Zn), lead (Pb), cadmium (Cd), manganese (Mn), copper (Cu), and arsenic (As) (Mayes *et al.*, 2009; Potter *et al.*, 2009).

Nature-based water treatment systems, also known as passive treatments, are an attractive option to tackle pollution in rural settings because they have low operational/maintenance requirements (Adrados *et al.*, 2014) and mainly rely on natural and freely available resources such as sunlight, plants, and microbes (Younger *et al.*, 2002; Jarvis *et al.*, 2012; Dufresne *et al.*, 2015). Well-known nature-based systems include ponds, lagoons, and constructed wetlands (CWs) (Verbyla, 2017). CWs successfully remove heavy metals like Fe, Zn, Cu from mine water making them a widely implemented mine water treatment system (Nyquist and Greger, 2009; Yeh *et al.*, 2009; Dufresne *et al.*, 2015; Singh and Chakraborty, 2021). Apart from mine water treatment, CWs have also been successfully used for sewage treatment plant effluent polishing, riverbank filtration and managed aquifer recharge (Zawadzka *et al.*, 2019). CWs can additionally perform an important role towards providing ecological benefits such as wildlife habitats (Zawadzka *et al.*, 2019). CWs can be designed in three different ways which include free-water surface flow, subsurface flow, and a hybrid system of the two types of flow (Stefanakis, 2016). The free-water surface CWs are the most widely implemented system. They look similar to ponds except that they contain emergent vegetation (Verbyla, 2017). *Phragmites australis* or common reed is the most frequently used plant for free-water surface CWs across Europe and Asia, which are then also known as reed beds (Austin and Yu, 2016). CWs effectively remove a range of water pollutants such as TSS, BOD, COD, heavy metals, emerging contaminants, and pathogens (Verlicchi and Zambello, 2014; Dufresne *et al.*, 2015; Wang *et al.*, 2017).

CWs are typically designed to treat one source of pollution e.g., wastewater or mine water, separately. The principal pollutant of concern then varies greatly according to the pollution source e.g., BOD and nutrients in wastewater versus heavy metals in mine water (Younger and Henderson, 2014). In some settings where they are found in proximity, it would be beneficial if wastewater and mine water could be co-treated in one CW unit, not only from a financial point of view, but also in terms of synergetic treatment (Johnson and Younger, 2006). Wang *et al.* (2021) reported that co-treating acid mine drainage and domestic wastewater by CWs performed effectively in elevating effluent pH (from 2.5 to 8.1) and in removing heavy metals especially Fe, Zn, Cd and Cu as well as organic and nutrient pollutants. In the UK, there is one full-scale free-water surface CW system for a co-treatment of mine water and secondary treated sewage treatment plant effluent which has been

operating since 2005 in the Lamesley area, Northeast England (Welsh, 2005; CoalAuthority, 2018). This Lamesley CW system was built to improve the receiving river (River Team) water quality (CoalAuthority, 2018). Younger and Henderson (2014) investigated the performance of the Lamesley CWs a decade ago in terms of the removal of BOD, ammoniacal nitrogen, suspended solids, phosphate, and iron (Fe). They found appropriate removal of BOD and ammoniacal nitrogen. As a synergistic effect of the cotreatment, phosphate from the sewage treatment plant effluent was effectively removed via precipitation with Fe from the mine water to form ferric phosphate solids in the CW sediments. We were interested in this CW system because it represents a unique treatment challenge that requires simultaneous removal of metals, nutrients, and putative pathogens. To date, there is a lack of comprehensive studies which report on the CW cotreatment performance across the wide range of chemical and microbial water quality parameters relevant for protection of the water environment. Nowadays, the impacts of discharges on receiving rivers and their ecological and recreational status needs much closer attention given the citizen initiatives to designate more rivers as bathing waters and new targets in the Environment Act 2021 for surface water quality in the UK. We therefore used state-of-the-art methods, including trace metal analysis and molecular microbiology to gain comprehensive understanding of CW cotreatment performance.

3.2.1 Aim

To assess the performance of combined treatment of coal mine water and secondary treated wastewater in free-water surface flow constructed wetlands in protecting the river water environment from chemical and microbiological pollution.

3.2.2 Objectives

1. To investigate whether the CW treatment improves the CW influent to meet all effluent discharge standards by determining chemical parameters including pH, DO, COD, TOC, TDS, conductivity, temperature, alkalinity, salinity, fluoride, ammonium (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N), total nitrogen (TN), phosphate (PO_4^{3-}-P), total phosphorus (TP) and heavy metals of Birtley sewage treatment plant (STP) influent and effluent, mine water, CW influent, CW effluent, river water upstream and river water downstream of the discharge point and comparing measurements with water quality standards.
Hypothesis: The CWs cotreating sewage treatment plant effluent and mine water improve influent quality to meet all effluent discharge standards.

2. To assess the CW treatment efficiency for the pollutants of concern including COD, ammonium (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N), total nitrogen (TN), phosphate (PO_4^{3-}-P), total phosphorus (TP), heavy metals (Fe, Mn, Pb, Zn, Cu, As), faecal coliforms, and micropollutants in 4 different months (March, May, July, August).
Hypotheses: The CWs reduce COD, nutrients, heavy metals, faecal coliforms, and micropollutants beyond the mere dilution effect of blending secondary treated wastewater with mine water, and consistently achieve the required discharge standards under changeable weather conditions.

3. To assess the status of the receiving river upstream and downstream of the CW effluent discharge according to the Water Environment (Water Framework Directive) and Bathing Water Regulations.
Hypothesis: The CW discharge has no detrimental impact on the chemical, ecological, and bathing water status of the receiving river.

4. To investigate whether the CWs can convert mine water and treated sewage microbiomes into a freshwater microbiome.
Hypothesis: The CWs improve influent microbiome characteristics to produce effluent microbiomes resembling those of the receiving river.

3.3 Materials and methods

3.3.1 Study site and sampling schedule

We conducted the study at Lamesley CWs located in Gateshead, Northeast England (Latitude 54°54’19.30”N, Longitude 1°35’57.8”W). The Lamesley CW cells cover a total area of 5.4 ha. They comprise of nine cells with impermeable bunds incorporating bentonite sealants. The site is split by a footpath. Four of the cells are arranged in two parallel series to the north of the footpath while the five-cell design is located to the south of the footpath and arranged in two parallel series of the four cells plus one cell at the end. They create two pairs of treatment streams which each converge on one of the two final outfalls to the River Team. We investigated the performance of the five-cell wetland. The outfall of this five-cell wetland is located upstream of the outfall of the four-cell wetland. The arrangement was designed to allow any one treatment stream to be taken out of service for maintenance, with the diverted flow being accommodated into the other still-active streams. The CWs were planted with *Typha latifolia*, *Phragmites australis*, and *Iris pseudacorus*. The target water depth was

around 15-50 cm. The CWs receive treated wastewater effluent from Northumbrian Water's Birtley sewage treatment plant (STP) where municipal wastewater of 30,872 population equivalent is treated by a combination of pre/post settling and trickling filter processes (Birtley STW, UKC22) (EnvironmentAgency, 2022). The STP effluent is blended with mine water from Kibblesworth mining site in an average ratio of about 1:4 (0.1:0.4 m^3/s wastewater:mine water) **(more details in Table A1, Appendix A)**. This shows that there was a dilution effect from the mine water on the STP effluent as the mine water contributed 80% of the total flow to the CWs. The hydraulic retention time (HRT) was then estimated from the CW area (5.4 ha) multiplied by the average dept of the wetland at 32.5 cm and divided by the flow rate of 0.5 m^3/s which gives an HRT of about 10 hours. The two waters are mixed in underground header tanks, then routed into the CWs via aeration cascades. Although the mining site is closed, pumping has been maintained from one of the deep mine shafts of Kibblesworth Colliery to prevent uncontrolled flooding of mine-workings in the densely populated urban area of Gateshead. The quantity of water pumped at Kibblesworth is very high and previously resulted in high loadings of iron into the River Team. The CWs were therefore constructed to treat the combined STP effluent and mine water to protect the River Team. More detailed information is provided by Welsh (2005) and Younger and Henderson (2014).

In this study, we collected samples from 7 locations around the CW area **(Figure 3.1)** comprising STP influent, STP effluent, mine water effluent, CW influent, CW effluent, river upstream and downstream of the CW discharge. The sampling points are illustrated in **Figure 3.1**. We conducted the sampling in March, May, July, and August 2021 covering the spring period (March & May) and summer period (July & August) for every sample except STP influent that was only obtained in May, July, and August. Weather (rainfall) conditions for the sampling events are summarised in **Table 3.1**. A preliminary scoping study was conducted in the winter of 2019 (before the outbreak of the Covid-19 pandemic and related lockdowns) for STP effluent, mine water, CW effluent, river upstream and river downstream sampling locations **(Table A14, Appendix A)**. However, these winter data were excluded from the main analysis due to the lack of CW influent data.

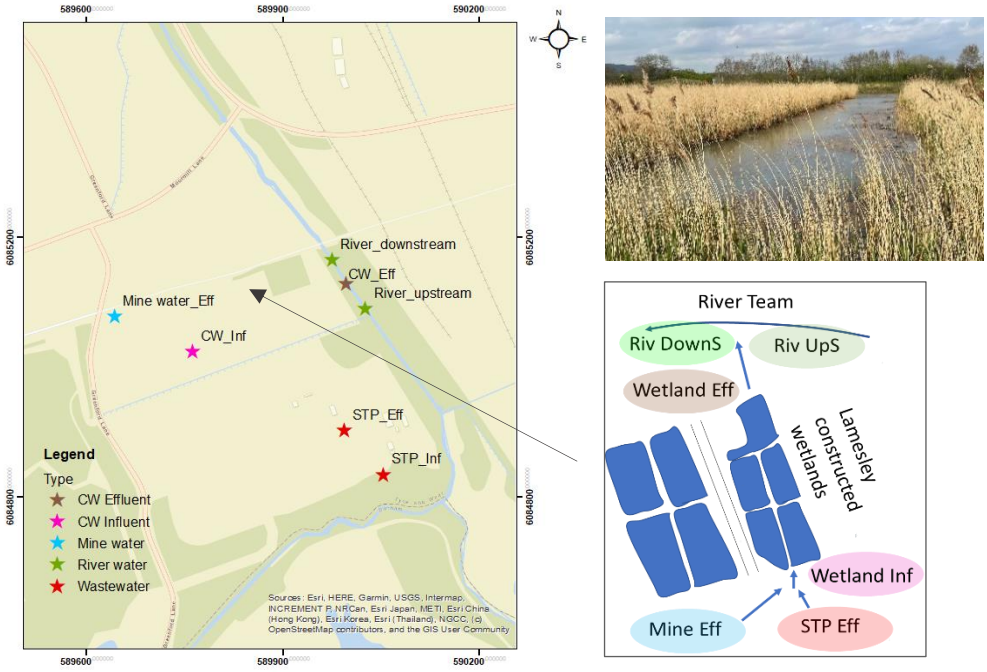

Figure 3.1 Sampling locations: STP influent, STP effluent, mine water effluent, CW influent, CW effluent, river upstream and river downstream.

Table 3.1 Rainfall data (mm) for one-week preceding and including the sampling date (indicated in bold), and in the 24h preceding the sampling on March 10th, May 12th, July 21st, and August 25th 2021. Data were obtained from the Urban Observatory data base for the Birtley area (Urbanobservatory, 2022). Also included is the STP effluent flow to the CWs (m^3/s) on the sampling day.

March		May		July		August	
Date	Rainfall (mm)	Date	Rainfall (mm)	Date	Rainfall (mm)	Date	Rainfall (mm)
4-Mar-21	2.8	6-May-21	0.4	15-Jul-21	0	19-Aug-21	0
5-Mar-21	1	7-May-21	3.6	16-Jul-21	0	20-Aug-21	1.2
6-Mar-21	0	8-May-21	4.4	17-Jul-21	0	21-Aug-21	16.8
7-Mar-21	0	9-May-21	1	18-Jul-21	0	22-Aug-21	1.6
8-Mar-21	0	10-May-21	2.4	19-Jul-21	0	23-Aug-21	0
9-Mar-21	0.2	11-May-21	5.2	20-Jul-21	0	24-Aug-21	0
10-Mar-21	3.6	**12-May-21**	1.2	**21-Jul-21**	0	**25-Aug-21**	0
Total rainfall in the seven days prior to and incl. the sampling day	7.6		18.2		0		19.6
Total rainfall in the 24 h before sampling	0.6		6.4		0		0
STP effluent flow (m^3/s)	0.11		0.16		0.08		0.07

3.3.2 Conventional water quality analysis

We analysed the water samples for temperature, pH, and electrical conductivity, total dissolved solids (TDS), salinity and dissolved oxygen (DO) in-situ using a pre-calibrated ExStik handheld probe (Extech Instruments, Nashua, NH, USA) and HQ40D Digital two channel multi meter (HACH, Manchester, UK). We measured alkalinity using a HACH digital titrator from HACH LANGE (HACH, Manchester, UK). Chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N), total nitrogen (TN), phosphate (PO_4^{3-}-P), total phosphorus (TP) and fluoride (F^-) were determined using HACH cuvette test kits LCI400, LCK339, LCK341, LCK238, LCK349, LCK349, and LCK 323, respectively. Cuvettes tests were performed following the manufacturer's instructions and evaluated in a HACH DR6000 Ultraviolet and Visible Spectrum Spectrophotometer. For quality assurance, we verified the cuvette tests with a blank solution (DI water) and known concentration standards prepared from the respective nutrient salts to assure that the result from cuvette tests agreed with the standard concentration by ±5%. We also filtered water samples through a cellulose acetate syringe filter (0.45 μm, 25 mm; VWR International, UK) to measure for anions using a Dionex High Pressure Ion Chromatography instrument (ThermoFisher, UK) and for dissolved organic carbon (DOC) using a carbon analyser (Vario TOC cube, Elementar Analysen Systeme GmbH, Germany). Additionally, filtered water samples were acidified with 1% v/v concentrated nitric acid and analysed for metals using a Varian Vista-MPX Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) or Agilent ICP-MS 7700 Series instrument, as appropriate for the metal concentration (Mayes *et al.*, 2021). Certified 1000 ppm standards (accuracy of ≤ ± 1.0%; VWR Chemicals, VWR International, UK) were diluted using 1% nitric acid solution for preparing calibration standards. Blanks and standards were run every 7 samples to check analytical accuracy and precision.

3.3.3 Micropollutant analysis

We performed micropollutant analysis according to EPA method 1694. Triplicate CW influent and effluent samples were analysed for each sampling event. We collected 100 mL samples and mixed them with 900 mL of distilled water making a 1000 mL (10 times dilution) sample ready for the next step. We then passed the 1000 mL through a 1 μm glass fiber filter (GF/B, Whatman, UK) and acidified the filtrate with diluted HCl to pH 2.5. After that we added surrogate standards atrazine-D5, enrofloxacin-D5, sulfamethoxazole-D4 and ibuprofen-D3 (from QMX Laboratories, Dunmow, UK) at concentrations of 1 ng/L, 5 ng/L, 5

ng/L and 5 ng/L, respectively, and left the samples for 30 minutes to equilibrate. Prior to solid-phase extraction (SPE), we added 150 mg $Na_4EDTA \cdot 2\ H_2O$ to each sample and equilibrated them for 30 minutes. The extraction was carried out on a Waters Oasis HLB cartridge (200 mg, 6cc, USA). The cartridge was conditioned with 6 mL methanol, 6 mL ultra-pure water, and 6 mL of acidified ultra-pure water (pH=2.5, HCl from Sigma-Aldrich St. Louis USA). Next, we loaded the water samples at a flow rate of 10 mL min^{-1}. After extraction, the cartridges were washed with 10 mL of 5% methanol and dried for 30 minutes under vacuum, then stored at -20 ^{0}C for further analysis. After the last sampling event, we eluted all the cartridges with 10 mL of methanol (LCMS grade, VWR, Lutterworth, UK). The eluates were concentrated to complete dryness at 35°C under a gentle stream of nitrogen in the Vertex evaporator (Labconco, Missouri, USA). Finally, the samples were reconstituted in a 1 mL of the mobile phase, ready for LC-MS analysis.

We shipped a portion of the samples to be screened for the occurrence of emerging contaminants using an LC-HRMS system (high resolution mass spectrometry) at the University of Bath, UK. Detailed methods are provided in **Section A1, Appendix A**. Once screened, we selected the most notable compounds plus caffeine for quantitative analysis with a UPLC-MS/MS system (Waters, Elstree, UK) at Newcastle University. Detailed chromatographic and MS conditions are presented in our previous work (Mrozik *et al.*, 2019). The only modification is the use of chromatographic column of Acquity C18-BEH (2.1 x 100 mm, 1.7 µm, Waters, Elstree, UK) instead of ACE C-18 PFP column (2.1x100 mm, 1.7 µm, HiChrom, Theale, UK). All data were acquired and processed using MassLynx 4.1 software (Waters, Elstree, UK). There were 8 selected compounds including acetaminophen, DEET, caffeine, carbamazepine, sulfapyridine, venlafaxine, sulpiride, and cetirizine. The Optimized MS/MS parameters for the analysis of micropollutants are presented in the **Appendix A, Table A3.**

3.3.4 Microbial analysis

We analysed faecal coliforms by membrane filtration according to Method 8074 from HACH LANGE (HACH, Manchester, UK). We also performed molecular microbiology methods to analyse the microbial communities using a combination of MinION nanopore sequencing and qPCR methods, as previously described (Acharya *et al.*, 2020a; Zan *et al.*, 2022). 100 mL of water samples were filtered through 0.22 µm membranes (Sartorius UK Limited, Surrey, UK) on the sampling day. We stored the membranes with the retained biomass immediately at -20

^{0}C to preserve DNA for analysis. We then extracted the retained DNA on the filter membrane the next day using a PowerWater® DNA Isolation Kit as per the manufacturer's instructions (QIAGEN, Crawley, UK). We measured DNA concentration using a Qubit dsDNA HS Assay Kit (Life Technologies, UK). The sequencing library for 16S rRNA gene sequencing was generated from 20 ng of DNA in triplicates for each sample using a 16S barcoding kit (SQK-16S024 from Oxford Nanopore Technologies (ONT), Oxford, UK) as per the manufacturer's instructions and loaded onto a MinION flow cell (R9.4.1, FLO-MIN106). We placed the flow cell into the MinION for the sequencing and controlled it using ONT's MinKNOW software. The raw reads (i.e. HDF5 raw signals) were base-called with GUPPY (Version; v.5.0.11) software (ONT, Oxford, UK) producing .fastq files. This step converted the electrical signals generated by a DNA strand passing through the nanopore into the corresponding base sequence. We then uploaded base-called data to the EPI2ME interface (v.5.0.4961252), a platform for cloud-based analysis of base-called MinION data. Data interpretation was performed with the FASTQ 16S workflow, using a quality score 7 for filtering. The FASTQ 16S workflow revealed the taxonomic classification of base-called reads along with their frequency. For quality assurance, a blank and a MOCK community (Zymo Research, Irvine, California) consisting of genomic DNA from eight bacterial species were included in the analysis as previously described by Acharya *et al.* (2019). We compared the theoretical composition (% relative abundance) of the MOCK community provided by the supplier (Zymo Research, Irvine, California) with the actual composition obtained from the MinION sequencing at genus level **(Table A6, Appendix A)**. It shows that 88% of the reads were correctly classified to a genus of the MOCK community with good reproduction of the relative genus abundances, which is greatly improved from the 60% reported by Acharya *et al.* (2019) for an older MinION flow cell type and base-calling software. Nonetheless, the FASTQ 16S workflow still miss-classified 12% of reads, likely because read errors occur during nanopore sequencing. Most of the misclassified reads were assigned to genera closely related to members of the MOCK community, such as *Shigella*, which has members sharing >99% 16S rRNA sequence similarity with *Escherichia* species (Acharya *et al.*, 2019). Consequently, the MinION data in this study were considered to provide an adequate representation of the overall bacterial community composition and its predominant membership. But no diversity metrics were calculated because these would be inflated by read errors and resulting misclassifications. An initial cluster analysis of the studied samples with the MOCK community and method blank samples is shown in **Figure A8, Appendix A**.

It shows that the MOCK community and blank control samples were clearly separated from the water samples.

To complement and validate the MinION nanopore sequencing data, we quantified genes for total bacteria (16S) and specific bacteria of interest comprising *E. coli* (rodA), human-host-associated *Bacteroides* (HF183, targeting *B. dorei* and closely related organisms) and *Vibrio cholerae* (ompW) by real time PCR assays (qPCR) on a portable qPCR machine (Quantabio, Beverly, MA, USA) using previously published primers and probes (Harms *et al.*, 2003; Chern *et al.*, 2011; Garrido-Maestu *et al.*, 2015; Ahmed *et al.*, 2019). More details are provided in **Table A4, Appendix A**. For the 16S rRNA gene qPCR assay, 2 µL of the DNA samples, 7.5 µL of SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad), 4 µL of nuclease free water (Invitrogen, Life Technologies, Paisley, UK), and 0.75 µL of each forward and reverse primer solutions (@ 10 micromol·L^{-1}) were combined for a 15 µL final volume with 500 (nmol·L^{-1}) of each primer. For specific genes of interest, 2 µL (5 ng·uL^{-1}) template DNA was used in a reaction mixture containing 5 µL PerfeCTa qPCR ToughMix (Quantabio,Beverly, MA, USA), 1.75 µL of nuclease free water (Invitrogen, Life Technologies, Paisley, UK), 0.25 µL of the probe solution (@ 10 micromol·L^{-1}), and 0.5 µL of each forward and reverse primer solutions (@ 10 micromol·L^{-1}) for 10 µL of final volume with 500 (nmol·L^{-1}) of each primer and 250 (nmol·L^{-1}) of the probe. Reaction conditions for quantification of each target gene were 98 °C for 3 minutes (1x), then 98 °C for 15 seconds, and the Primer Annealing Temperature (T_a) for 30 seconds **(Table A4, Appendix A)** (40 cycles). We produced standard curves using synthesized nucleotide sequences of the target genes (Invitrogen, Life Technologies, Paisley, UK) every time a qPCR analysis was performed, in parallel with the amplification of test samples. Melt-curve analysis from 72 °C to 95 °C was performed for the 16S assays to validate qPCR products. Serial dilution (10-fold) of the standards was performed to obtain standard solutions in the range of 10^8–10^1 target gene copies/µL. All samples were run in duplicate and molecular grade H_2O replaced template in control reactions. There were amplifications in the control reactions of the 16S assay likely due to spurious contaminants. But the quantification-cycle (Cq) values of the controls were higher than the highest Cq value of the standards (standard 1 for 10 gene copies/µL template) and substantially higher than the highest Cq values of the samples, meaning that 16S gene concentrations in controls were far below those of samples. There was no amplification in the control reactions for the probe-based assays.

Additionally, we also analysed for antibiotic resistant *E.coli* or also known as extended-spectrum beta-lactamase (ESBL)-producing *E.coli* using membrane filtration and a classical plate count technique in the month of July. The membrane filters were incubated on agar plates containing ESBL ChromoSelect Agar Supplement (Product 61471, Sigma Aldrich, UK) at 44 °C for 24 hours prior to plate counting. More details are provided in our previous publication (Hiruy *et al.*, 2022).

3.3.5 Data processing and statistical analysis

We processed sequencing data using Matlab© (Version R2019b, Mathworks, Portola Valley, CA, USA) for multivariate data analysis (cluster, principal component analysis (PCA) and ANOSIM). We downloaded the taxonomic classification and quality of barcoded reads from the EPI2ME dashboard as a CSV file which contained information on run and read IDs and read accuracy, barcodes, and NCBI taxa IDs for classified reads. Then, we processed the CSV file with Matlab© scripts published elsewhere (Thongsamer *et al.*, 2021). In brief, the script first generated root level OTU tables by matching NCBI taxa IDs to lineages and counting the number of reads per NCBI taxa ID, with and without rarefication. If required, these scripts also enabled combining root level OTU tables from different runs into a single table. Then, OTU tables with grouping of reads at genus level were created. We rarefied sequencing libraries at 45,000 reads per sample and performed multivariate statistical analysis for OTUs classified to genus level, and grouped at this level, using Matlab© for cluster and PCA of the square-root transformed relative abundance data with Euclidean distance as the similarity metric. Five samples, comprising STPEffluent3_March, Minewater3_March, RiverUpstream2_July, RiverDownstream2_August and the blank sample, had less than 45,000 reads and were therefore excluded from further analysis by this rarefaction step. Rarefaction still retained at least 2 replicates for each sample. We performed one-way analysis of similarities (ANOSIM) on Matlab© with the Fathom Toolbox developed by the Marine Resource Assessment Program at the University of South Florida's College of Marine Science (Jones, 2015). Additionally, one of the scripts allowed extracting species or genera of interest from root and genus level OTU tables, respectively. We used these scripts to extract bacterial genera containing putative human pathogens and faecal indicator bacteria. More details of this data processing are described in our previous publication (Acharya *et al.*, 2020a).

We used two-sided sign test function in Matlab© to investigate statistically significant difference of the overall chemical quality of the CW effluent and river upstream, and the difference between water quality in the spring and summer period. For conventional water quality parameters, we used two-tailed t-tests to evaluate the null hypothesis that there is no difference between the mean values of two sample groupings of interest. We also used one-tailed z-tests to investigate if the mean value of each parameter meets the desired standard. All calculations, t-tests and z-tests were performed using Excel. We used Primer7 software (primer-e, Auckland, New Zealand) to investigate the linkage between environmental parameters and microbial communities using the BEST (Bio-Env) procedure as described by Clarke *et al.* (2014).

An initial screening of the data found that there was suspected contamination of the mine water sampling tap by ingress of STP effluent in May and August, as explained in more detail in **Appendix A**. We therefore excluded these two outlier samples (mine water samples in May and August) from the final analysis.

3.4 Results and discussion

3.4.1 Characteristics of sewage influent and effluent, mine water, and constructed wetland influent and effluent

Table 3.2 Conventional water quality parameters of the STP influent and effluent, mine water and CW influent and effluent relative to the UK wastewater treatment work's compliance limits. Results were reported to two decimal places as Mean±S.D. STP effluent and CW influent and effluent was sampled in March, May, July and August, STP influent in May, July and August, and mine water in March and July.

	STP influent	STP effluent	Mine water	CW influent	CW effluent	Standard
NH_4^+-N (mg/L)	47.78±27.06	3.07±1.90	0.21±0.26	0.91±0.50	0.13±0.16	< 40 and 3.5[(ii), (iii)]
NO_2^--N (mg/L)	0.19±0.15	0.98±1.43	0.004±0.00	0.17±0.18	0.06±0.06	N/A
NO_3^--N (mg/L)	1.95±1.38	24.97±9.07	0.29±0.24	6.81±2.28	5.14±2.30	N/A
TN (mg/L)	80.73±19.11	35.75±9.26	1.40±0.40	9.42±4.1	6.43±2.73	< 15[(i)]
PO_4^{3-}-P (mg/L)	5.87±2.71	2.70 ±0.93	0.25 ±0.35	0.86±0.14	0.23±0.07	N/A
TP (mg/L)	6.89±2.96	2.86±0.96	0.23±0.32	1.00±0.20	0.26±0.08	< 2[(i)]
Fluoride (mg/L)	1.22±0.47	0.64±0.18	0.60±0.10	0.59±0.15	0.59±0.15	N/A
Alkalinity (mg/L $CaCO_3$)	273.00±47.84	51.00±19.24	545.00±7.07	408.25±71.51	436.50±84.08	N/A
Salinity (mg/L)	530.00±29.82	396.75±61.67	1525±35.36	1205.00±283.37	1320.00±154.92	N/A
pH	8.41±0.13	7.20±0.31	7.25±0.23	7.12±0.14	7.54±0.19	N/A
Conductivity (µS/cm)	1072.67±64.27	804.00±105.87	2985±49.5	2335.00±523.10	2595.00±238.12	N/A
TDS (mg/L)	749.00±44.19	578.75±84.55	2100.00±28.28	1656.25 ±374.37	1815.00±154.16	N/A
DO (% saturation)	22.10±11.21	52.75±15.81	7.14±3.20	6.47±1.29	62.17±9.91	N/A
COD (mg/L)	636.83 ±362.25	65.89±23.69	11.88±0.38	22.41±4.71	16.38±4.33	< 125[(i)]
DOC (mg/L)	22.77±2.98	15.61±2.01	3.44±2.51	7.27±3.12	6.72±2.00	N/A
Temperature °C	17.13±2.81	14.03±5.95	11.60±9.33	13.40±5.99	13.73±5.23	N/A
Faecal coliform (Log_{10}CFU/100 mL)	6.49±0.35	4.98±0.37	0 CFU/100 mL	3.87±0.46	3.28±0.27	N/A
*Heavy metals (µg/L)**						
Fe	40.00±10.00	45.00±17.32	2057.5±689.43	880.00±635.94	18.75±8.54	< 2000[(ii), (iv)]**
Mn	26.67±15.28	43.75±11.09	1137.50±38.89	756.25±95.95	516.25±296.18	N/A
Pb	0.43±0.15	0.00±0.00	0.05±0.07	0.07±0.08	7.50±5.00	N/A
Zn	35.00±8.66	57.50±9.57	5.00±7.07	27.50±9.57	27.50±28.72	N/A
Cu	18.80±12.65	10.00±11.55	0.10±0.15	1.66±1.29	2.50±5.00	N/A
As	0.88±0.19	0.75±0.29	0.21±0.29	0.32±0.23	0.17±0.11	N/A

*Dissolved metal concentration (µg/L) **For total iron (Fe) (µg/L) [(i)] The Environment Agency's compliance limits for treated wastewater discharge. [(ii)]The Water Resources Act (1991): Consent to Discharge from the Enviroment Agengy (consent number 235/1891): site-specific consent for Birtley sewage treatment work and Lamesley CWs. [(iii)] The standard for STP effluent from (i) and CW effluent from (ii) respectively. [(iv)] The standard for CW effluent.

Table 3.2 compares conventional water quality parameters at different treatment stages with the relevant standards. The untreated sewage (STP influent) had pH of 8 with high NH_4^+-N, TN, TP, unfiltered COD, and faecal coliform levels. After primary/secondary settling and trickling filter treatment, the treated sewage was of neutral pH, and the NH_4^+-N concentration was below the permissible limit for STP effluent (40 mg/L) (z-test, p-value < 0.01). But the TN and TP concentrations would ordinarily exceed the discharge limits of 15 mg/L and 2 mg/L, respectively (z-test, p-value > 0.05) if they did not receive further treatment from the CWs. The COD level of STP effluent was at 65.89±23.69 mg/L which met the desired standard (135 mg/L) during the sampling period (z-test, p-value < 0.01). Faecal coliforms were reduced by approximately 1.5 log units relative to the STP influent. As for the mine water, it was not acidic (pH of 7). The mine water also had substantial alkalinity levels, presumably because the mine water had been in contact with sandstone in overlaying geological strata, which helps establish a stable pH level. The pH and alkalinity levels are in line with previous studies reporting pH of 7.1 with high alkalinity level at 755 mg/L $CaCO_3$ for mine water discharge from Kibblesworth mine (Banks *et al.*, 1997; Younger and Henderson, 2014). In terms of heavy metals in the mine water, there were high levels of Fe and Mn but lower Zn, Cu, and As levels as compared to the STP influent and effluent. We measured metals as dissolved concentration to avoid damaging analytical instruments. The permissible limit for Fe was set for the total Fe concentration. But the dissolved Fe level in the mine water still exceeded the permissible limit (z-test, p-value > 0.05) for total Fe. Similarly, the Fe level in this mine water was also reported to be high (6000 μg/L, for total Fe) in a previous study (Younger and Henderson, 2014). Further treatment would thus ordinarily be required to reduce TN and TP levels in the STP effluent and the Fe level in the mine water.

3.4.2 Blending effects and CW influent characteristics

The STP effluent and mine water are blended in a ratio of about 1:4 (STP effluent: mine water) to produce the CW influent. The CW influent showed similar alkalinity, salinity, conductivity, and TDS characteristic to the mine water, and the pH remained at 7. High level of Fe and Mn were also maintained. This is because mine water had higher Fe, Mn, conductivity, TDS, salinity, and alkalinity than the STP effluent and contributed 4 of 5 parts of the blended water. Only slight changes of these parameters are therefore expected. Meanwhile, the STP effluent had much higher nutrient and faecal coliform, Cu and Zn levels than the mine water. After the blending, an 80% reduction of these metrics would be

expected simply because of the dilution from the blending with the mine water. All of this can be seen in **Table 3.2** by comparing STP effluent, mine water, and CW influent characteristics.

3.4.3 CW treatment effects and effluent compliance with discharge standards

After the CW treatment, nutrient levels were further reduced. The NH_4^+-N concentration was reduced from 0.91±0.50 mg/L in the influent to 0.13±0.16 mg/L in the effluent, which is well below the permissible limit specified for Lamesley CW discharge (3.5 mg/L) (z-test, p-value < 0.01). There was significantly lower TP concentration in the CW effluent than the CW influent (t-test, p-value < 0.01). Contrary to the STP effluent, CW effluent concentrations met the TN and TP limits set for treated wastewater discharge (z-test, p-value < 0.01). The COD level in the CW effluent was also lower than in the STP effluent (t-test, p-value < 0.05) and met the standard. For heavy metals, Fe concentration was at 18.75±8.54 μg/L which was lower than Fe level in the CW influent, and more than a factor of 100 below the limit for total Fe at 2000 μg/L (z-test, p-value < 0.01). There was no significant difference of Mn level in the CW effluent compared to the CW influent (t-test, p-value > 0.05). Mn level was high from the mine water contribution to the CW influent and not effectively removed in the CWs. The persistent level of Mn is likely because the hydroxide solubility product of Mn is higher than for other monitored metals, and very high pH (≈10) is required to immobilise Mn as hydroxide (Jarvis *et al.*, 2012). It is therefore more difficult for Mn to get precipitated in the CWs in comparison with Fe.

Table 3.3 Concentration of COD, nutrients, heavy metals, and faecal coliforms in the constructed wetland influent (CW Influent) and effluent (CW Effluent) in March, May, July, and August. Results were reported to two decimal places as Mean±S.D. for duplicate samples in each month. Numbers in parentheses represent percent removal of COD, nutrients, and heavy metals, and log removal of faecal coliforms in each month.

Sampling month	COD (mg/L)	Nutrient (mg/L)					Faecal coliform (Log_{10}CFU /100 mL)
		NH_4^+-N	NO_3^--N	TN	PO_4^{3-}-P	TP	
CW Influent March	18.55 ±0.49	0.53 ±0.00	9.89 ±0.05	14.40 ±0.14	0.71 ±0.00	0.79 ±0.04	3.77±0.01
CW Effluent March	14.45 ±0.21 (21.6%)	0.02 ±0.00 (95.3%)	7.48 ±0.00 (24.0%)	9.31 ±0.01 (35.3%)	0.29 ±0.00 (60.1%)	0.32 ±0.00 (60.0%)	2.89±0.27 (0.88)
CW Influent May	24.70 ±0.99	0.59 ±0.01	6.51 ±0.09	8.21 ±0.18	0.99 ±0.01	1.26 ±0.14	4.07±0.01
CW Effluent May	22.75 ±0.21 (7.2%)	0.37 ±0.00 (37.0%)	6.70 ±0.05 (-3.0%)	8.25 ±0.60 (0.9%)	0.26 ±0.00 (73.8%)	0.31 ±0.01 (74.9%)	3.70±0.01 (0.37)
CW Influent July	27.95 ±0.49	1.61 ±0.00	4.39 ±0.43	4.62 ±0.08	0.77 ±0.00	0.92 ±0.00	4.43±0.04
CW Effluent July	16.20 ±2.26 (42.1%)	0.04 ±0.00 (97.3%)	2.78 ±0.01 (36.3%)	3.97 ±0.33 (14.1%)	0.12 ±0.00 (83.9%)	0.14 ±0.00 (84.6%)	3.23±0.01 (1.20)
CW Influent August	18.45 ±0.21	0.92 ±0.05	6.47 ±0.04	10.45 ±0.21	0.95 ±0.01	1.04 ±0.01	3.26±0.12
CW Effluent August	12.90 ±0.99 (30.1%)	0.08 ±0.00 (91.9%)	3.59 ±0.01 (44.4%)	4.24 ±0.23 (59.4%)	0.25 ±0.00 (73.4%)	0.26 ±0.00 (74.6%)	3.23±0.06 (0.03)

Table 3.3 shows how the concentrations and removal efficiencies (%) of the key pollutants by the CWs varied between the four sampling events in March, May, July, and August. From the rainfall data in the 24 hours prior to sampling, May stood out as the sampling event with the wettest weather conditions and also had the highest STP effluent loading **(Table 3.1)**. From **Table 3.3**, COD removal was in a range of approximately 7%-42% across all sampling events, and the highest removal was found in July, when weather conditions were dry for the whole week prior to and including the sampling day **(Table 3.1)**. For nutrients, there was high removal (> 90%) of NH_4^+-N in all sampling events, except for May, when weather conditions were the wettest. NO_3^--N removal was in a range of small negative percentages to 44% and followed the trend for TN removal. This shows that TN removal occurs by

denitrification (a reduction of NO_3^- to nitrogen gas (N_2)) (Vymazal, 2007). A slightly negative removal of NO_3^--N occurred in May. This is likely due to the nitrification process in which organic nitrogen is first converted into NH_4^+ (ammonification), and nitrifying microbes then convert NH_4^+ into NO_2^- and finally NO_3^- (Vymazal, 2007). High abundance of nitrifying bacteria are reported in association with plant roots (Rajan *et al.*, 2019). Nitrification coupled with denitrification can play a crucial role for total nitrogen removal in CWs (Vymazal, 2007; Younger and Henderson, 2014; Wang *et al.*, 2021). It was also reported to be an important mechanism for nitrogen removal in other nature-based systems like waste stabilisation ponds (Camargo Valero *et al.*, 2010). There was high PO_4^{3-}-P and TP removal across all sampling months ranging between 60% and 84%. This is likely due to sorption of phosphate by ferric hydroxide and precipitation as ferric phosphate (Dobbie *et al.*, 2009; Younger and Henderson, 2014). Relatively high PO_4^{3-}-P and Fe concentrations **(Table 3.3 and 3.4)** in the CW influents are due to the mixing of wastewater with mine water, then providing a clear benefit of the co-treatment, i.e., phosphate coupled with Fe removal. Moreover, N and P removals could also be attributed to plant uptake (Ji *et al.*, 2020). The plant uptake process normally happens in spring and summer in temperate climates like the UK (Vymazal, 2007). We found higher NH_4^+-N and PO_4^{3-}-P removal than a previous study by Younger and Henderson (2014). They reported that the average annual mean removal of NH_4^+-N and PO_4^{3-}-P in the Lamesley CWs were 66% and 59%, respectively. This could be explained by the sampling period of this study being in the spring and summer. In terms of faecal coliforms, we observed the lowest removal in August, but this was because faecal coliform counts were in August already unusually low in the CW influent. Lower removal of faecal coliforms was observed in May (wet weather) as compared to July (dry weather). Additionally, we also analysed for the abundance of *E.coli* producing extended-spectrum β-lactamases (ESBL) which gives them resistance to commonly used antibiotics, including penicillins and cephalosporins (Rawat and Nair, 2010), in July **(Table A7, Appendix A),** and found substantial lower abundance of them in the CW effluent as compared to the CW influent, indicating that the CW was capable of removing antibiotic resistant *E.coli.* Hydraulic retention time (HRT) and loading rate are the two crucial factors for coliform removal (Tunçsiper *et al.*, 2012). Prolonged HRT and reduced cloud coverage improves coliform removal in free-water surface flow CWs via increased exposure of sunlight/UV radiation in the open water areas not covered by plants (Shingare *et al.*, 2019). HRT and cloud coverage are affected by a rainfall. Heavy rainfall diminishes HRT in CWs which consequently reduces the coliform removal efficiency (Shingare *et al.*, 2019). For anions like chloride and

sulphate, their levels were also lowest in the May as compared to other months, not only in the CW influent but also in other samples **(Table A13, Appendix A),** indicating the dilution effect of rainfall.

Table 3.4 Concentration (µg/L) of dissolved Fe, Mn, Pb, Zn, Cu, and As in the constructed wetland influent (CW Influent) and effluent (CW Effluent) in March, May, July, and August. Results were reported to two decimal places as Mean±S.D. for duplicate samples in each month. Numbers in parentheses represent percent removal of each metal in each month.

	Fe	Mn	Pb	Zn	Cu	As
CW Influent_March	60.50±0.71	755.00±7.07	0.06±0.00	40.50±0.71	1.84±0.11	0.32±0.00
CW Effluent_March	30.00±0.00 (50.4%)	280.00±0.00 (62.9%)	0.03±0.00 (40.6%)	10.00±0.00 (75.3%)	1.57±0.03 (14.2%)	0.18±0.01 (44.3%)
CW Influent_May	1175.00 ±7.07	640.50±0.71	0.19±0.02	30.50±0.71	3.13±0.13	0.47 ±0.02
CW Effluent_May	15.00±7.07 (98.7%)	470.00±0.00 (26.6%)	0.10±0.01 (49.9%)	20.00±0.00 (34.4%)	3.57±0.17 (-13.9%)	0.24 ±0.02 (49.1%)
CW Influent_July	745.00±7.07	755.00±7.07	n.d.	20.50±0.71	n.d.	n.d.
CW Effluent_July	20.00±0.00 (97.3%)	945.00±7.07 (-25.2%)	n.d. (-)	70.00±0.00 (-241.7%)	1.60 ±0.03 (-)	n.d. (-)
CW Influent_August	1540.50 ±0.71	875.00±7.07	0.04 ±0.00	20.50±0.71	5.27±0.19	0.50 ±0.00
CW Effluent_August	10.00±0.00 (99.4%)	370.00±0.00 (57.7%)	0.03±0.00 (22.3%)	10.00±0.00 (51.2%)	4.44±0.15 (15.7%)	0.25 ±0.00 (50.2%)

n.d. = not detected

Table 3.4 shows concentrations and removal efficiencies of the six key heavy metal pollutants. Fe and Mn in the CW influent were associated with mine water, while Zn, Cu, and As were from the STP effluent and Pb could be from either source **(Table 3.2)**. Fe concentration in the CW influent was fluctuating widely across sampling events ranging from 60.5 µg/L to 1540.5 µg/L. In comparison, Mn was less variable, being the lowest following rainfall in May. We speculate that, given the high alkalinity and neutral pH of the mine water, iron oxide precipitation may already occur to some extent as the mine water moves through overlaying geological strata containing sandstone before it is pumped to the CW for treatment. Mn, being still highly soluble at neutral pH, would be less likely to precipitate before the mine water reaches the CWs. There was high removal (>90%) of Fe in May, July, and August and good removal in March, when influent concentrations were the lowest. This agrees with the previous work on the CWs by Younger and Henderson (2014) showing an annual mean removal of Fe of 89%. The combined findings show continuous high performance of the wetlands between 9 and 15 years after their construction. Removal of Fe could be attributed to precipitation of Fe as ferric hydroxide and ferric phosphate (Younger

and Henderson, 2014). This CW system is of neutral pH and includes aeration cascades, and with this artificial aeration Fe removal through oxidation and precipitation should be enhanced (Wang *et al.*, 2021). Ferrous iron ($Fe^{2+)}$ is rapidly converted to ferric iron (Fe^{3+}) in oxidising conditions and ferric iron can form ferric hydroxide at pH of more than 3.5 (Jarvis *et al.*, 2012). Moreover, with the mixture of wastewater and mine water, suspended solids in the wastewater could also provide nuclei and counter-ions (phosphate) for the generation of iron flocs hence accelerating the precipitation of ferric hydroxide (ochre) and ferric phosphate (Johnson and Younger, 2006). Concurrently, ochre particles could also provide attachment sites for nitrifying bacteria and promote ammonia removal (Demin *et al.*, 2002), as mentioned earlier. There were high Mn concentrations in both CW influent and CW effluent in every sampling event. This is presumably because Mn removal is affected by Fe concentrations in water (Neculita and Rosa, 2019). High level of Fe could interfere with Mn removal (Neculita and Rosa, 2019). Also, Pb, Zn, Cu and As were not efficiently removed in the CWs, except for Zn removal in March. The removal of Cu and Zn could be ascribed to hydroxide precipitation and co-precipitation with Fe oxyhydroxide (Wang *et al.*, 2021). Cu can form its hydroxide precipitate at pH of 6.8, while Zn could be adsorbed by the Fe/Al hydroxide (Jarvis *et al.*, 2012; Olds *et al.*, 2013). Dissolved metals could also be reduced via plant uptake and accumulated in the reed's roots as well as through phytoremediation with microbial activities (Yeh *et al.*, 2009; Wang *et al.*, 2017; Sharma *et al.*, 2021). Additionally, chelating agents released by plants could bind with these metals thus increasing metal precipitation (Khalifa *et al.*, 2020). Concentration of Mn, Zn, and Cu were higher in the CW effluent than in the CW influent in July showing negative removals, perhaps due to mobilisation from the sediment. In summary, there was no clear seasonal trend (spring vs summer) or rainfall effect (May versus July) for heavy metal removal by the CWs (sign test, p-value > 0.05, for both groupings). Overall, metals were most likely removed via precipitation, but at the same time the vegetation in CWs could also be an important contributor to metal treatment.

The removal of P and heavy metals **(Table 3.3 and 3.4)** were relatively stable throughout the sampling events as they are mainly removed via geochemical processes e.g., iron hydroxide and iron phosphate precipitation. This demonstrated consistent effectiveness of the CWs in removing Fe and P from the influent. In contrast, the removal of COD and nutrients (N species) (Table 3.3) which happens via biological processes, and faecal coliforms were more variable. The performance of this biological removal and coliform removal appears to be

influenced by rainfall and implications for solar disinfection and HRT, in line with previous reports (García *et al.*, 2003; Tunçsiper *et al.*, 2012; Shingare *et al.*, 2019). There was an overall significant difference of COD, nutrients (N species), and faecal coliform removal between the dry and wet weather (i.e., May versus July) as well as the spring and summer (sign test, p-value < 0.05, for both groupings), but insignificant difference for the removal of P and heavy metals (sign test, p-value > 0.05, for both groupings).

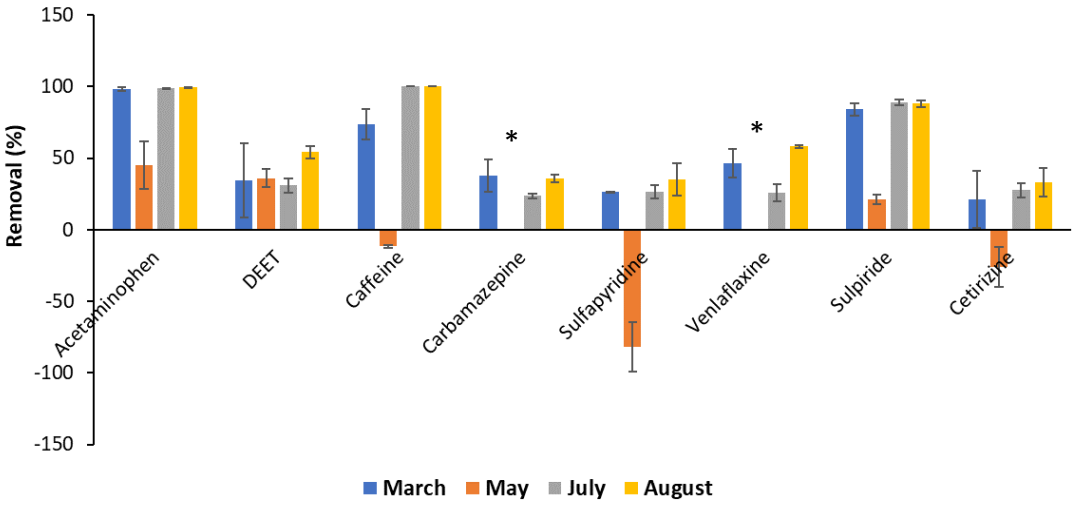

Figure 3.2 Removal efficiency (%) of acetaminophen, DEET, caffeine, Carbamazepine, sulfapyridine, venlaflaxine, sulpiride and cetirizine by the CWs in March, May, July and August. Error bars were calculated as standard deviation of triplicate samples. *Data were excluded as the removal was not significantly different from zero.

Figure 3.2 shows that the CWs were capable of removing the 8 reported micropollutants however the removal efficiencies varied among different months. There were high removals (70%-100%) of acetaminophen, caffeine and sulpiride, whilst low to moderate removals of DEET, carbamazepine, sulfapyridine, venlafaxine and cetirizine in March, July and August when the weather was dry and decreased removals of these compounds in May when the weather was at the wettest. This implies the reduced micropollutant removal in the CWs due to dilution effect from rainfall. The negative removals were likely caused by the diluted CW influent which would have reduced the concentrations of these compounds in the inlet while the levels at the outlet could still be high from the pre-rain higher influent concentrations (Sossalla *et al.*, 2020). Similarly, Ilyas and van Hullebusch (2020) reported on high removal of acetaminophen and caffeine in free-water surface flow CWs which is most likely to be from aerobic biodegradation. Presumably, the aeration cascades of the studied CWs could

enhance the removal of these compounds due to the improved aerobic conditions. In contrast, poor to moderate removals were reported for carbamazepine, venlafaxine, and DEET that is likely a consequence of poor biodegradation (Ilyas and van Hullebusch, 2020). DEET was poorly removed in CWs due to its low light sensitivity thus poor degradation (Li *et al.*, 2017).

3.4.4 Impacts of the CW discharge on water quality in the receiving river

Table 3.5 Conventional water quality parameters of the river upstream and downstream of constructed wetland discharge relative to the Water Framework Directive's standards. Results were reported to two decimal places as Mean±S.D or percentile (in italic) of four sampling events. Values in percentile were provided for comparing with the standard as required by the directive.

	River upstream	River downstream	Standard[1]
NH_4^+-N (mg/L) *90^{th} percentile*	0.65±0.24 *0.96*	0.33±0.12 *0.48*	Quality 0.3 = high, 0.6 = good, 1.1 = moderate, 2.5 = poor
NO_2^--N (mg/L)	0.17±0.09	0.10±0.03	N/A
NO_3^--N (mg/L)	6.69±1.96	5.52±1.09	N/A
TN (mg/L)	9.09±2.92	7.20±1.70	N/A
PO_4^{3-}-P (mg/L)	0.46±0.19	0.31±0.14	Quality[(i)] 0.04 = high, 0.08 = good 0.19 = moderate, 1.03 = poor
TP (mg/L)	0.50±0.19	0.33±0.15	N/A
Fluoride (mg/L)	0.39±0.15	0.53±0.20	N/A
Alkalinity (mg/L $CaCO_3$)	130.75±34.64	312.00±77.49	N/A
Salinity (mg/L)	414.00 ±56.26	987.25±168.81	N/A
pH *5^{th} and 95^{th} percentile*	8.02±0.30 *7.54-8.02*	7.64±0.21 *7.30-7.64*	6-9
Conductivity (µS/cm)	852.25±111.36	1960.50±305.28	N/A
TDS (mg/L)	595.25±88.94	1371.75±206.80	N/A
DO (% saturation) *10^{th} percentile*	83.15±6.08 *75.36*	72.59±5.20 *65.93*	Quality 70 = high, 60 = good 54 = moderate, 45 = poor
COD (mg/L)	25.53±9.45	20.31±3.24	N/A
DOC (mg/L)	9.24±2.25	7.22±1.09	N/A
Temperature °C *98^{th} percentile*	11.98±5.02 *22.30*	12.15±4.81 *22.00*	Quality 25 = high, 28 = good 30 = moderate, 32 = poor
Faecal coliform (Log_{10}CFU/100 mL) *90^{th} percentile*[+] *95^{th} percentile*[+]	3.38±0.48 *10147.10 (8554.00)*[#] *15265.47 (12868.79)*[#]	3.31±0.39 *6508.83 (5486.95)*[#] *9062.40 (7639.60)*[#]	Quality[(ii)] (CFU/100 mL) 500 = excellent[(iii)] 1000 = good[(iii)] 900 = sufficient[(iv)]
*Heavy metals (µg/L)**			
Fe	17.50±5.00	25.00±12.91	< 1000
Mn	140.00±92.01 *106.50±94.47***	435.00±250.13 *121.51±61.27***	< 123 bioavailable
Pb	5.00±5.77 *0.18±0.19***	5.00±4.08 *0.19±0.19***	< 1.2 bioavailable
Zn	35.00±12.91 *9.14±4.20***	33.75±4.79 *10.80±1.50***	< 12.3 bioavailable
Cu	4.10±1.04 *0.16±0.04***	2.53±1.16 *0.08±0.04***	< 1 bioavailable
As	0.34±0.23	0.26±0.20	< 50

[1]The Water Framework Directive (WFD) Standards (England and Wales) 2015 for river.
[(i)]Based on the standard for river upstream. [(ii)]The Bathing Water Regulations 2013 for *E.coli* in inland surface waters. [#]Not all, but most faecal coliforms (about 75-93%) are *E. coli* (Hamilton et al., 2005). Hachich *et al.* (2012) recommended 84.3% for the conversion.
[+]CFU/100 mL. Estimated numbers of *E.coli* (CFU/100 mL) are shown in parentheses after the faecal coliform numbers.
[(iii)]Based upon a 95-percentile evaluation, [(iv)]Based upon a 90-percentile evaluation.
The River Team is currently not a designated bathing river.
*Dissolved metal concentration (µg/L). **Bioavailable concentration (µg/L) was calculated using UKTAG tool. "bioavailable" means the fraction of the dissolved concentration of such metal likely to result in toxic effects as determined using the UKTAG Metal Bioavailability Assessment Tool.

Table 3.5 shows the water quality of the River Team upstream and downstream of the CW discharge in relation to surface water quality standards. The combined mine water and STP effluents contribute some 40% of flow in the River Team in dry weather conditions (Welsh, 2005), meaning that there is limited dilution of the discharge. From **Table 3.5**, there was no significant difference between NH_4^+-N concentration in the river upstream and river downstream of the discharge (t-test, p-value > 0.05). The NH_4^+-N concentration was indicative of good status for the upstream and of high status for the downstream of the discharge meaning that the discharge may even have improved the river water quality in terms of ammonia concentrations. The presence of ammonia in the upstream is presumably because of agricultural or Combined Sewer Overflows (CSOs) and storm drain related inputs to the catchment. PO_4^{3-}-P concentration in both upstream and downstream river samples indicated moderate status with respect to this nutrient. Overall, there was no significant difference of TN and TP concentration in the river upstream and downstream samples (t-test, p-value > 0.05 for both TN and TP) implying no significant impact of the CW discharge on the nutrient status in the receiving river. There were significantly higher alkalinity, salinity, conductivity, and TDS in the river downstream relative to the river upstream (t-test, p-value < 0.01, for all) showing the effect of CW discharge on these parameters in the receiving river. This is a consequence of the mine water characteristics and poor removal of the main soluble ions in water like calcium, magnesium, chloride, and sulphate in the CWs **(Table A13, Appendix A)**. pH of both, upstream and downstream samples, was in the desired range. DO as % saturation at the 10^{th} percentile was indicative of high status of the river in terms of its oxygenation. Water temperature in both upstream and downstream samples were also indicative of high status. The numbers of faecal coliform were high in both river upstream and downstream samples, but lower in the downstream. They were converted to estimated numbers of *E. coli* to compare with the Bathing Water regulations. The *E. coli* numbers

exceeded the limit for sufficient bathing water status (900 CFU/100 mL) by an order of magnitude. In terms of heavy metals, the Fe, Pb, Cu, and As concentration were below the permissible limits (z-test, p-value < 0.01, for all) for both river upstream and downstream samples, while the mean values of Zn and Mn were marginally below the limit without statistical significance (z-test, p-value > 0.05, for both). More detailed data of these parameters in the STP influent, STP effluent, mine water, CW influent, CW effluent, river upstream, and river downstream for all sampling events are provided in **Section A2, Appendix A**. In summary, from **Table 3.2** and **Table 3.5**, some water quality parameters of the STP effluent did not meet the permissible limits, but with further polishing by the CWs, the overall effluent quality met the desired discharge standards, but with a risk of detrimentally affecting the receiving river due to the bioavailable metal (Mn) levels from the mine water. Overall water quality of River Team receiving CW treated effluent was of moderate to high status, with PO_4^{3-}-P achieving only moderate status. High PO_4^{3-}-P levels were already noted in the river upstream of the discharge, and therefore attributed to upstream sources. If the STP effluent had been discharged directly into the river, it would have been a further substantial P source into the river as there was higher PO_4^{3-}-P concentration in the STP effluent **(Table 3.2)** than in the river upstream **(Table 3.5)**. Co-treatment of STP effluent and mine water thus demonstrated a clear benefit of excellent P removal that will benefit the River Team. The P concentration in the CW effluent was much lower than the STP effluent, lower than in the CW influent, and lower than the river upstream. For heavy metals, if the mine water were discharged directly, Fe standards would be grossly exceeded. However, there was an issue around limited removal of Mn in the CWs which still ended up in the river downstream, increasing its concentration relative to the upstream to only narrowly meet WFD standards. There was no significant difference in the overall water quality between the CW effluent and the river upstream as well as between the river upstream and the downstream (sign test, p-value > 0.05, for both).

In addition, winter sampling in a scoping study **(Table A14, Appendix A)** showed similar trends for nutrients, heavy metals, and faecal coliforms in STP effluent, mine water, CW effluent, river upstream and river downstream samples as compared to the spring and summer sampling **(Table A9-A12, Appendix A)**. However, further research is required to investigate the CW performance more comprehensively for winter conditions, when temperatures are near or below freezing.

3.4.5 Additional insights from molecular microbiology methods

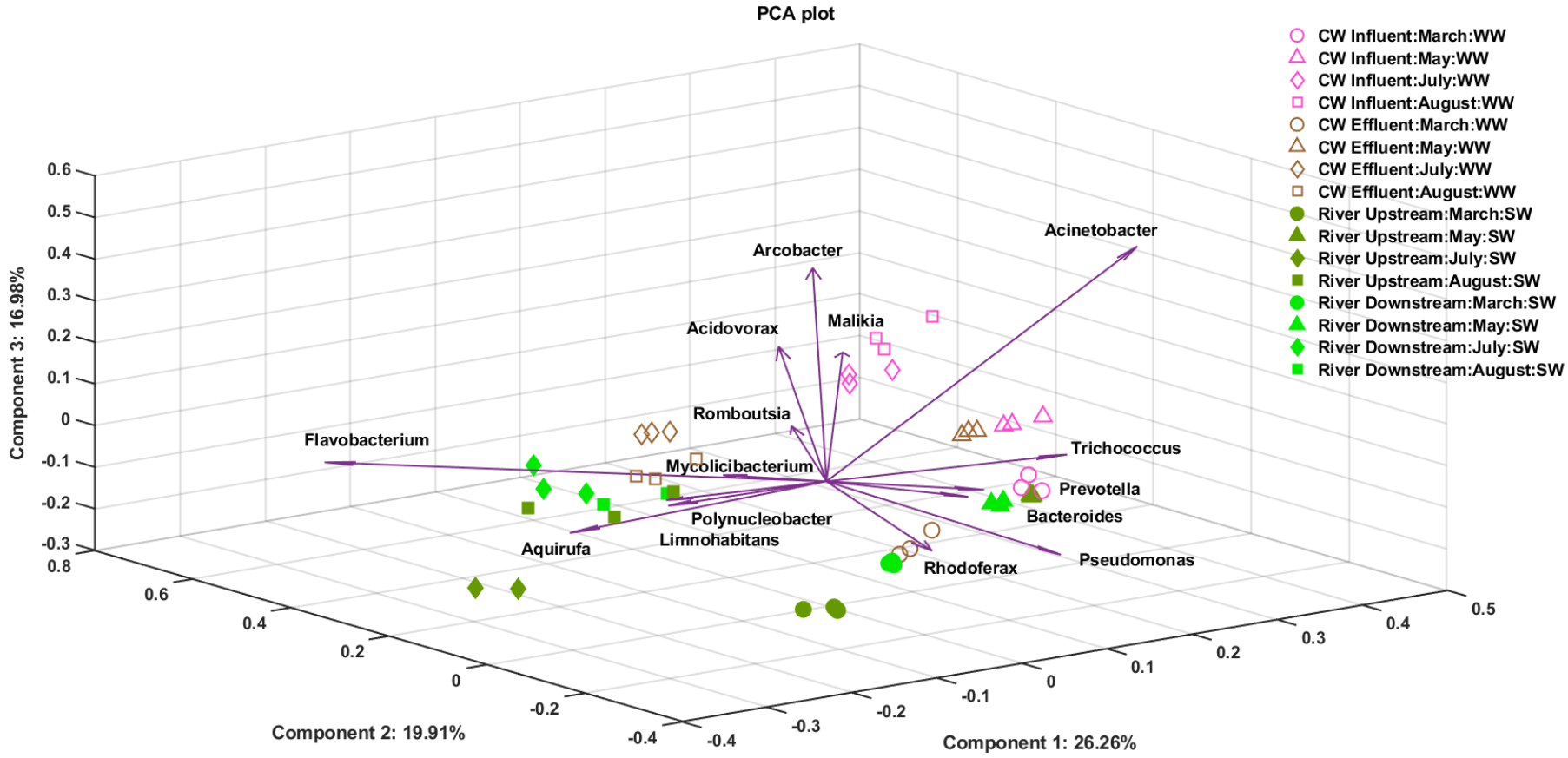

Figure 3.3 Principal component analysis (PCA) plot of the microbial community dissimilarity among CW influent, CW effluent, River upstream and River downstream of 4 sampling events (March, May, July, August). WW and SW indicate type of water samples which are wastewater (empty symbols) and surface water (filled symbols), respectively. The three principal components (PC) (Component 1, 2 and 3) were plotted showing the scores (circles, triangles, diamonds, and squares) and top 15 loadings (genera), (arrows) explaining the variance in the three-dimensional space. Percentage of variation accounted for by each principal component is shown with the axis label.

With the advent of molecular methods much more detailed insight can be obtained with regards to microbial water quality, although there are currently no related standards. This may change in the future as molecular methods are being used by scientists to monitor wastewater treatment and more specifically attribute faecal pollution to its sources (Ahmed *et al.*, 2019; Bunce *et al.*, 2020). Also, standard based on genomic markers are being proposed for ambient waters (Boehm and Soller, 2020). 16S rRNA sequencing can comprehensively characterize microbial communities in water samples, while qPCR can sensitively and selectively target genetic markers of interest such as those specifically found in *E. coli* (rodA) or human-host associated *Bacteroides* (HF183) (Acharya *et al.*, 2019; Hiruy *et al.*, 2022).

In a principal component analysis of our 16S rRNA gene sequencing data (PCA, **Figure 3.3**), the three principal components (PC) accounted for approximately 63% of the observed variance between samples. The 15 most notable microbial genera (i.e., variables) explaining the variance in the three-dimensional space were illustrated by the purple arrows. These included for the CW influent notable genera containing putative human pathogens that can be isolated from sewage samples such as *Arcobacter* (Fisher *et al.*, 2014) which can also be present in the human gut (Banting and Figueras Salvat, 2017). The genus *Acinetobacter* was equally notable in CW samples. It contains denitrifying bacteria that use Mn^{2+} as an electron donor (Su *et al.*, 2015). This supports the chemical evidence that denitrification occurred in the CWs **(Section 3.4.3)**. However, overall abundance of denitrifying bacteria should be confirmed further in future work by using a specific-gene qPCR targeting denitrifiers e.g. nitrite reductase genes (*nirS* and *nirK*) (Mrkonjic Fuka *et al.*, 2007; Camargo Valero *et al.*, 2010). The plot also highlighted many genera containing faecal indicator bacteria such as *Acidovorax*, *Prevotella*, *Romboutsia*, and *Bacteroides*, that were characteristic for the CW influent samples (Ricaboni *et al.*, 2016; Kho and Lal, 2018; Feng *et al.*, 2019). And genera containing freshwater bacteria such as *Aquirufa*, *Polynucleobacter*, *Flavobacterium*, *Mycolicibacterium* and *Rhodoferax* were most notable in the river samples (Hoetzinger *et al.*, 2019; Pitt *et al.*, 2019; Jin *et al.*, 2020; Dahl *et al.*, 2021; Hiruy *et al.*, 2022). Similar faecal pollution indicator genera amongst the freshwater bacterial communities of river systems were also found at sewage polluted locations in other countries across the world including Malaysia, Nepal, and Ethiopia (Ho *et al.*, 2021; Pantha *et al.*, 2021; Hiruy *et al.*, 2022).

PC1 explained 26.26% of variance and separated the wastewater from the surface water samples (empty versus filled symbols), with positive loadings of faecal bacteria like *Prevotella* and *Bacteroides*, and negative loadings of typical freshwater bacteria like *Aquirufa*

and *Polynucleobacter*. The CW effluent samples in March, July, and August were clearly shifted in a negative sign direction relative to the CW influent samples in these months, which demonstrates the CW treatment benefit in converting human gut like microbiomes into freshwater microbiomes. This outcome was in line with our previous study of this CW in April 2019, when we demonstrated the feasibility of onsite sequencing with the MinION at the Birtley sewage treatment works (Acharya *et al.*, 2020a). The same trend also applied in May (pink and brown triangles), although with a much smaller shift than in the other months, presumably due to the short HRT following heavy rainfall. There were 6.4 mm rainfall in the 24 hours prior to the sampling event in May **(Table 3.1)**. In May, the upstream and downstream river samples (green triangles) were also shifted significantly in a positive sign direction along PC1, having a much stronger faecal pollution signature as compared to the samples from March, July, and August. This is highly likely due to faecal pollution of the river in the upstream via discharge from CSOs and other rainfall related runoff in the upstream. PC2 explained 19.91% of variance and showed seasonal shift in both the CW influent and river microbiomes in a positive sign direction over time i.e., spring (March and May) versus summer (July and August). When comparing summer with spring, there was lesser influence of *Prevotella* and *Bacteroides*, and greater influence of *Romboutsia* for the wastewater bacteria, and lesser influence of *Rhodoferax* and greater influence of *Flavobacterium* and *Mycolicibacterium* for the river bacteria. Finally, PC3 explained 16.98% of variance and highlighted further seasonal change in the community of wastewater bacteria in the CW influent for the May, July, and August samples relative to March with greater prominence of *Arcobacter* and *Acidovorax* in the former samples. In summary, there were a treatment (PC1) and seasonal (PC2&3) effect in differentiating the microbial communities amongst the samples. Evidently and encouragingly, the CWs could turn sewage/faecal bacteria into communities more akin to a freshwater microbiome.

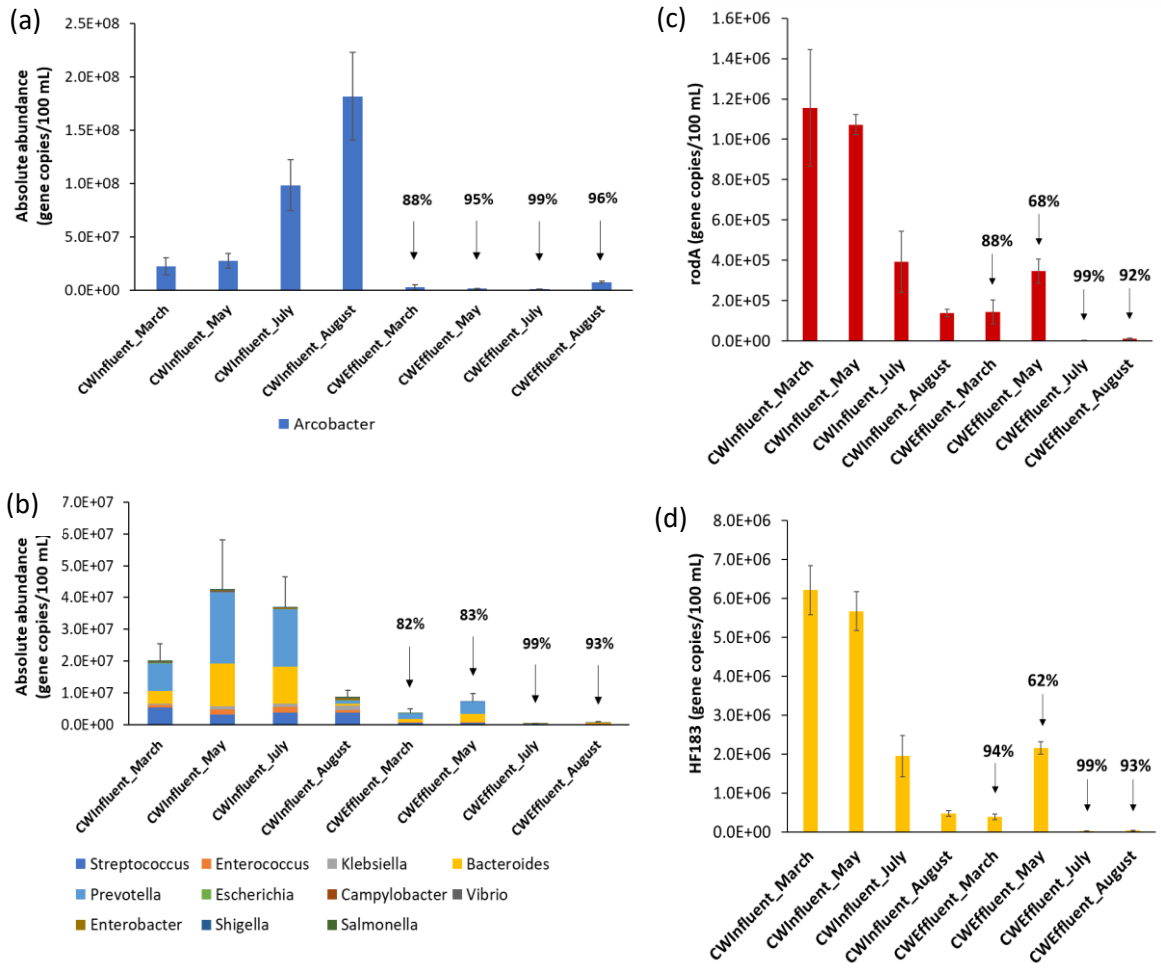

Figure 3.4 Absolute abundance (gene copies/100 mL) of selected genera containing a putative human pathogen (*Arcobacter*) (a), faecal indicator bacteria (b), and qPCR data for rodA (*E. coli*) (c), and HF183 (*Bacteroides*) (d). (a) and (b) were obtained from multiplying relative abundance from MinION 16S rRNA gene sequencing with qPCR quantification of 16S rRNA gene copy numbers in each sample of the CW influent and CW effluent of 4 sampling events (March, May, July, August). (c) and (d) were obtained from a specific gene by qPCR. Error bars were calculated as standard deviation of the total absolute abundance of the selected bacteria of triplicate samples in each month. Percentage above each bar indicates overall % removal by the CWs in each sampling event.

From **Figure 3.4**, there was overall higher absolute abundance of bacterial genera containing both, putative human pathogens (i.e., *Arcobacter*) and faecal indicator bacteria derived from 16S rRNA gene sequencing, as well as a specific gene by qPCRs for *E. coli* (rodA) and human host associated *Bacteroides* (HF183) in the CWs influent compared to the CW effluent in all sampling months. This indicates efficient removal of faecal indicator bacteria and associated pathogens in the CWs. The qPCR for *Vibrio cholerae* (ompW) was also carried out, but there was no detection of ompW genes in any of the samples, contrary to our findings in Ethiopia (Hiruy *et al.*, 2022). There was > 80% removal of the absolute abundance of both *Arcobacter* and faecal indicator bacteria genes, by the CWs in all

sampling months. For a putative human pathogen **(Figure 3.4a)**, the genus *Arcobacter* showed higher abundance in July and August relative to March and May. *Arcobacter* is normally found in sewage even in the treated effluent and comprises several pathogenic species (Fisher *et al.*, 2014; Banting and Figueras Salvat, 2017). Some species of *Arcobacter* remain viable in sewage discharge as they are aerotolerant (do not require oxygen for growth but can tolerate its presence) and can survive in a range of water temperatures (Fisher *et al.*, 2014; Banting and Figueras Salvat, 2017). For faecal indicator bacteria **(Figure 3.4b)**, the genus *Prevotella* predominated in the 16S rRNA gene amplicon libraries of CW influent samples in March, May, and July. *Prevotella* is also reported to be highly abundant in sewage (Fisher *et al.*, 2015). There were several mechanisms that could be responsible for removal of these bacteria in the CWs including sedimentation, filtration, adsorption, oxidation, solar disinfection, root exudation of biocides and predation by protozoa (Wu *et al.*, 2016). Plants and submerged parts in free-water surface flow CWs also play a role allowing some mechanisms to happen. They provide an additional surface area for biofilm development, the biofilm can then assist in pathogen filtration and adsorption (Wu *et al.*, 2016; Shingare *et al.*, 2019). Plants can also release antimicrobial exudates through roots which then reduce the abundance of pathogens (Tunçsiper *et al.*, 2012; Wu *et al.*, 2016). The exudates might also change chemical environment of the rhizosphere giving unsuitable condition for pathogen survival (Wu *et al.*, 2016). The plant used in this study i.e., *Phragmites australis* is reported to produce bactericidal substances that killed pathogenic/faecal indicator bacteria (Shingare *et al.*, 2019). Several studies reported higher removal efficiencies of pathogens by planted CWs than unplanted CWs (Hench *et al.*, 2003; Martin *et al.*, 2012; Avelar *et al.*, 2014; Alufasi *et al.*, 2017; Shingare *et al.*, 2019). Pathogen removal in CWs can be varied with seasons and weather conditions (Wu *et al.*, 2016; Shingare *et al.*, 2019). Pathogen removal is higher in the summer than colder periods which could be attributed to the increased temperature and UV radiation (Shingare *et al.*, 2019). Similarly, in this study the removals appeared to be higher in the summer (July and August) than in spring (March and May). The effect of sunlight/UV radiation enhances pathogen elimination by damaging DNA of bacteria (Jasper *et al.*, 2013; Shingare *et al.*, 2019). Mayo (2004) reported efficient bacterial reduction in free-water surface flow CWs associated with solar intensity. Moreover, CWs showed higher pathogen removal in the dry/warm weather than in the wet weather (Makvana and Sharma, 2013; Alufasi *et al.*, 2017; Shingare *et al.*, 2019). This agrees with the rodA (*E. coli*) and HF183 (for human host associated *Bacteroides*) qPCR results **(Figure 3.4c and d)** in this study, since the lowest removal was found in May when there was substantial rainfall the day before

sampling. Also, the highest removal was in July when there was no rainfall in the week before sampling **(Table 3.1)**. There was high removal of rodA in August which is different from the low removal of faecal coliform obtained from culturing method in August **(Table 3.3)**. But such low removal could be due to an artefact because faecal coliform counts were in August already unusually low in the CW influent. Bacterial abundance estimates typically differ between culturing and qPCR methods and are generally higher by qPCR. This is because the culturing methods demonstrate the viability of the cells, while in genomic methods the targeted genes could be from both viable and damaged cells or extracellular DNA (Garza and Dutilh, 2015; Figueroa-González and Pérez-Plasencia, 2017; Acharya *et al.*, 2020b). The elevated bacterial abundances obtained using qPCR thus may not always represent viable bacteria (Bunce *et al.*, 2020). On the other hand, not all viable bacteria can be isolated in culturing assays, so the two methodologies are complementary. The rodA and HF183 results were in line with a previous study by Bunce *et al.* (2020) who found similarly high abundance of these two genes (10^6-10^7 gene copies/100 mL) in small STP influent in the UK. In that study, small STPs showed mean removal of around 98% and 95% for rodA and HF183, respectively. In summary, the CWs could efficiently remove pathogens and faecal bacteria across all sampling events, but with reduced efficiency due to rainfall in May.

3.4.6 Relationships between chemical and microbial water quality

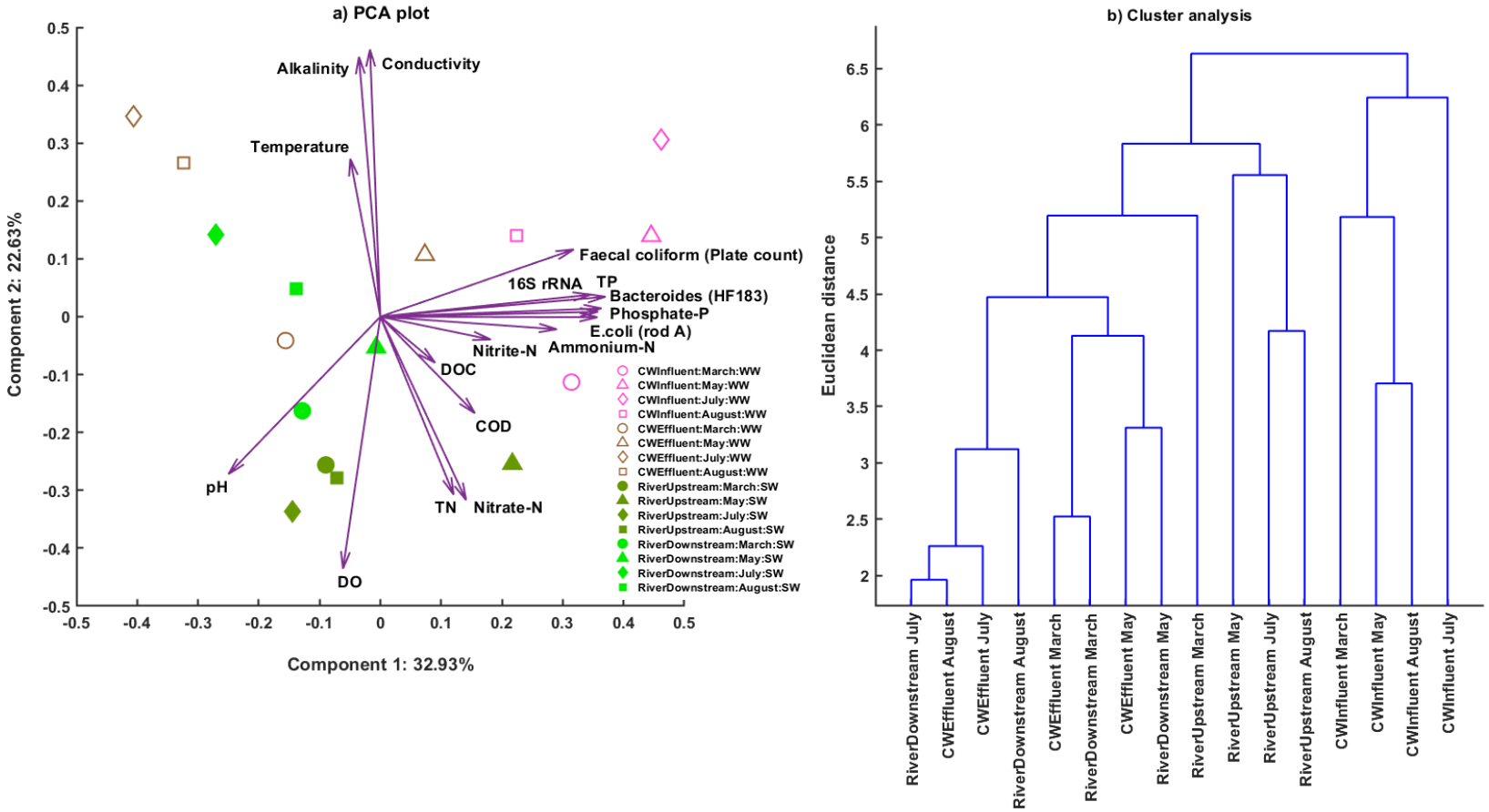

Figure 3.5 Principal component analysis (PCA) (a) and cluster analysis (b) combining chemical with microbial parameters to assess dissimilarity among CW influent, CW effluent, River upstream and River downstream samples from 4 sampling events (March, May, July, August). WW and SW indicate type of water samples which are wastewater (empty symbols) and surface water (filled symbols), respectively. For the PCA, the first two principal components (PC) (Component 1 and 2) were plotted showing the scores (circles, triangles, diamonds, and squares) and 17 loadings (arrows) of 13 physico-chemical parameters and 4 microbial parameters obtained from plate counting and qPCRs of specific genes explaining the variance in the two-dimensional space. Percentage of variation accounted for by each principal component is shown with the axis label.

Figure 3.5 shows a PCA and cluster analysis of physico-chemical parameters and microbial parameters derived from culturing (faecal coliforms) and qPCR of bacterial marker genes (16S for total bacteria, rodA for *E. coli*, and HF183 for human host associated *Bacteroides*). In the PCA plot **(Figure 3.5a)**, PC1 and 2 accounted for approximately 56% of the observed variance between samples. PC1 showed the impact of the CW treatment by clearly separating CW influent samples (pink empty symbols) from the CW effluent samples (light brown empty symbols). Along component 2, the river upstream samples (dark green filled symbols) were clearly separated from the CW effluent samples (light brown empty symbols) with the river downstream samples (light green filled symbols) from different sampling months scattering in between the two groups. This illustrates that the river downstream samples are a mixture of the CW effluent and river upstream samples, both in terms of their chemistry and microbiology. The four different types of samples were overall a significant microbial community shaping factor (one-way ANOSIM, p-value < 0.01 and R = 0.44), while sampling month overall had a lower effect on shaping the microbial community (p-value < 0.05 and R = 0.19).

The 17 variables explaining the variance in the PC1 and 2 space are illustrated by the purple arrows in **Figure 3.5a**. The CW influent samples were characterized by positive loadings of the nutrient parameters including TP, phosphate-P, ammonium-N, and nitrite-N, along with microbial parameters indicative of faecal matter, such as faecal coliforms obtained from both plate count and qPCR (rodA) methods, and human host associated *Bacteroides* obtained from the qPCR assays. This demonstrates a clear link between these nutrients, the overall size of the microbial community as measured by 16S rRNA gene copies, and the abundance of faecal bacteria (rodA), including from human hosts (HF183). In contrast, the CW effluent samples were characterized by positive loadings of the parameters alkalinity and conductivity due to the minerals dissolved in the mine water and higher temperature, but comparatively lower amounts of nutrients. This showed that the CWs could remove such nutrients and faecal bacteria from the influent. River upstream samples were characterized by a positive loading of the parameter dissolved oxygen, which was likely due to the faster and more turbulent flow characteristics of the river as compared to the CWs. ANOSIM confirmed statistically significant differences between the CW influent and effluent sample characteristics (p-value < 0.05 and R = 0.55). Cluster analysis **(Figure 3.5b)** shows that the greatest dissimilarities across chemical and microbial parameters were between the CW influent samples from all sampling months and the other samples. Compared with the CW

influent, the CW effluent was in all months more similar to the river water samples, which illustrates the benefit of the CW water treatment. Nonetheless, the river downstream samples clustered more closely with the CW effluent than the river upstream samples, which shows that the CW discharge still had an impact on the river water characteristics, mainly in terms of its physicochemical characteristics (conductivity, alkalinity, pH, temperature DO) and total and nitrate-nitrogen. Overall, there was however, no significant dissimilarity between the river upstream versus downstream sample groupings (one-way ANOSIM, p-value > 0.05 and R = 0.32).

Table 3.6 Combinations of up to 6 environmental variables from CW influent, CW effluent, river upstream and river downstream samples of the 4 sampling events, taken k variables at a time, yielding the best matches of 16S rRNA gene sequencing derived microbial community similarity matrices, and physico-chemical parameter similarity matrices for each k, as measured by weighted Spearman rank correlation ρ_s. The highest Spearman rank correlation was highlighted in bold.

k	Best variable combinations (ρ_s)		
1	pH (0.308)	Temperature (0.221)	PO_4^{3-}-P (0.213)
2	pH, DO (0.417)	pH, Temperature (0.387)	PO_4^{3-}-P, pH (0.370)
3	PO_4^{3-}-P, pH, DO (0.449)	TP, pH, DO (0.430)	PO_4^{3-}-P, pH, Temperature (0.419)
4	**PO_4^{3-}-P, pH, DO, Temperature (0.463)**	NO_3^--N, PO_4^{3-}-P, pH, DO (0.444)	TP, pH, DO, Temperature (0.439)
5	NO_3^--N, PO_4^{3-}-P, pH, DO, Temperature (0.452)	PO_4^{3-}-P, Alkalinity, pH, DO, Temperature (0.445)	TN, PO_4^{3-}-P, pH, DO, Temperature (0.441)
6	NO_3^--N, PO_4^{3-}-P, Alkalinity, pH, DO, Temperature (0.446)	TN, PO_4^{3-}-P, Alkalinity, pH, DO, Temperature (0.441)	PO_4^{3-}-P, TP, Alkalinity, pH, DO, Temperature (0.428)

Global test, p-value = 0.02

Table 3.6 shows combinations of environmental variables which were considered at steadily increasing levels of complexity, i.e., *k* variables at a time (*k* = 1, 2, 3, 4, 5, 6), to explain the dependency of the microbial community composition on environmental parameters. In this analysis the microbial communities were characterized by the relative abundance OTU table at genus level obtained from the sequencing data. The single environmental variable (k=1) which best linked to the microbial community similarities was pH (ρ_s = 0.308) and the next best was temperature, then PO_4^{3-}-P. The best 2-variable combination involved pH and DO and showed higher correlation than any single variable. The best 4-variable combination involved PO_4^{3-}-P, pH, DO, and temperature, and showed the highest Spearman rank

correlation (ρ_s = 0.463) between environmental and microbial community characteristics in the analysis. The correlations decreased for combinations of more than 4 variables. Microbial communities in the CW influent, CW effluent, river upstream and river downstream samples were therefore found to be highly associated with PO_4^{3-}-P, pH, DO, and temperature. PO_4^{3-}-P is a critical nutrient that often limits ecosystem productivity and has for example been associated with algae blooms in lakes through excess P input (eutrophication) (Schindler *et al.*, 2016; Withers *et al.*, 2020). As discussed earlier in **Section 3.4.3**, there was high PO_4^{3-}-P removal by the CWs **(Table 3.3)** which is likely due to precipitation as ferric phosphate. Certain microbes can contribute towards the formation of such phosphate minerals (Gadd, 2010). For example, biodegradation of organic phosphate to form orthophosphate enables reaction with the Fe from the mine water (Gadd, 2010). Concurrently, temperature, pH and DO are important factors for microbial activity in addition to the phosphorus cycle (Rosso *et al.*, 1995; Robinson, 2019). pH has a great influence on microbial metabolism and microbial diversity (Zhalnina *et al.*, 2015). DO determines electron acceptor availability and redox conditions for microbial metabolisms (Robinson, 2019). Microbial diversity can be strongly affected by varations in DO and temperature (Beman and Carolan, 2013). Microbial processes hence depend on and also alter these four parameters between the CW influent, CW effluent, river upstream, and river downstream sampling points.

3.5 Conclusions

Through this work, we arrived at the following conclusions:

- The STP effluent would not meet the desired standard for TN and TP and the mine water would not meet the desired standard for Fe, while the CW effluent quality met all the corresponding permissible limits. We therefore confirmed the hypothesis that the CWs cotreating sewage treatment plant effluent and mine water improve influent quality to meet all effluent discharge standards.
- The CW effluents showed lower COD, nutrient, heavy metal, faecal coliform, and micropollutant levels than the CW influents and consistently achieved the required discharge standards under changeable weather conditions. The hypotheses that the CWs reduce COD, nutrients, heavy metals, faecal coliforms, and micropollutants beyond the mere dilution effect of blending secondary treated wastewater with mine water, and consistently achieve the required discharge standards under changeable weather conditions are both accepted.

- The CWs could remove the 8 reported micropollutants. And there was higher micropollutant removal when there was little/no rainfall in the 24 hours before sampling (March, July, August) compared to May (wet weather).
- The CW discharge did not detrimentally affect the nutrient status of the receiving river but could detrimentally affect the river due to the bioavailable concentration of heavy metal like Mn which was near the desired limit. There were high faecal coliform numbers in the river downstream that exceeded the desired standard for bathing water status but were still lower than in the upstream. We therefore could only partly accept the hypothesis that the CW discharge has no detrimental impact on the chemical, ecological and bathing water status of the receiving river.
- Molecular microbiology methods revealed greater similarity of the CW effluent and river upstream microbial communities, as compared to CW influent and river upstream microbial communities. We therefore confirmed the hypothesis that CWs improved influent microbiome characteristics to produce effluent microbiomes more like those of the receiving river.
- The CW treatment resulted in further removal of putative human pathogen and faecal indicator bacteria and could consequently reduce impacts of the discharge on the recreational value of the river.
- By using innovative monitoring approaches like next generation sequencing and qPCR, much more detailed insight for microbial water quality was obtained for assessing wastewater treatment performance and impacts of discharges into the aquatic environment, as compared to culturing methods. Additionally, the use of a specific-gene qPCR will assist in wastewater-based infectious disease surveillance for pathogens of concern and in implementing microbial water quality standards based on genetic markers in the future.
- Given that land area is more readily available in rural settings, CWs would be a suitable nature-based treatment option addressing water pollution issues such as insufficient wastewater treatment in small STPs and pollution from abandoned mines, with opportunity for synergistic co-treatment of such waste streams in a single CW system.

Data availability: 16S sequencing data generated in this project has been submitted to the NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA837409. Additional data created during this research are openly available (https://doi.org/10.25405/data.ncl.20102783). Please contact Newcastle Research Data Service at rdm@ncl.ac.uk for access instructions.

Chapter 4

Activated carbon amendment in sand biofilters enhances micropollutant and pathogen removal from wastewater treatment plant effluent

Chapter 4. Activated carbon amendment in sand biofilters enhances micropollutant and pathogen removal from wastewater treatment plant effluent

4.1 Abstract

Biofiltration can improve effluent quality of wastewater treatment systems as a tertiary treatment stage. We investigated if activated carbon (AC) addition to sand in biofilters could enhance removal of residual micropollutants and pathogens after conventional, two-stage wastewater treatment. Duplicated column experiments were conducted to polish activated sludge treated effluent from the Tung Kru wastewater treatment plant in Bangkok, Thailand. We compared the removal of residual pollutants after activated sludge treatment in sand-alone biofilters (Control) versus 5% w/w AC amended sand biofilters. Conventional water quality parameters and micropollutants including acetaminophen, oxytetracycline, tetracycline, enrofloxacin, atrazine, sulfamethoxazole, diuron, and diclofenac which were previously detected in a canal in Thailand were analysed in the column influents and effluents following standard methods and EPA method 1694 with a UPLC-MS/MS system. The column influents, effluents, and filter media were also analysed for putative human pathogens and faecal indicator bacteria using a combination of MinION nanopore sequencing and quantitative PCR. Biofiltration improved the activated sludge effluent quality to meet Thailand's surface water quality standards. The differences between the AC amended and Control columns were not significant for overall nutrient and heavy metal removal. Concentrations of the monitored micropollutants in both column effluents also met the desired predicted no effect concentrations (PNECs), except for enrofloxacin. The AC columns improved the removal of ammonia-nitrogen, diuron, diclofenac, enrofloxacin putative human pathogens, and faecal indicator bacteria relative to the Control columns. AC amendment is thus a promising technology to reduce biofilter footprints and enhance treatment of pollutants of emerging concern.

4.2 Introduction

The presence of micropollutants or emerging contaminants like pharmaceuticals and personal care products in domestic wastewater has raised worldwide concerns since they are inadequately removed in conventional wastewater treatment plants (WWTPs) (Paredes *et al.*, 2016). Moreover, they are not commonly included in routine water monitoring programmes, and consequently their fate and behaviour are not well-understood (Geissen *et al.*, 2015;

Gomes *et al.*, 2020). Micropollutants released from WWTPs are of particular interest due to their continuous discharge into waterbodies. Many micropollutants are persistent in the environment and not easily degradable (Stefanakis and Becker, 2015). Some micropollutants can be transformed or eliminated through natural processes, but may still exist in natural waterbodies due to their continuous release from WWTPs, and adversely affect aquatic ecosystems (Stefanakis and Becker, 2015). Apart from micropollutants, untreated or partially treated wastewater is also a source of pathogens which may be transmitted via the environment, and this is a particular concern in developing countries without well-managed wastewater treatment facilities (Møller *et al.*, 2012; Kataki *et al.*, 2021). Even in a properly engineered WWTP, some pathogens can survive the treatment and end up in the effluent (Newton and McClary, 2019; Kataki *et al.*, 2021). Where residual human pathogens are being released to the aquatic environment they pose a risk to human and ecosystem health, unless additional treatment steps are implemented (Newton and McClary, 2019).

Several technologies including centralised and decentralised systems have been investigated to tackle inadequate treatment of micropollutants and pathogens (Hube and Wu, 2021). Ozonation, sand filtration, sorption, membrane filtration, and constructed wetlands have been investigated as tertiary treatment units to remove residual micropollutants and pathogens which survived the conventional two-stage treatment such as primary sedimentation followed by activated sludge. Among tertiary treatment options, constructed wetlands were found to be the most frequently reported nature-based treatment strategy (Hube and Wu, 2021). However, one drawback of horizontal flow constructed wetlands is that they are not suitable in a location where land availability is limited (Stefanakis, 2016). A smaller footprint would therefore be desirable. Vertical subsurface flow constructed wetlands are biofilter-like treatment systems containing sand as filter medium. They provide an alternative option to conventional constructed wetlands, especially for rural wastewater treatment. Vertical flow beds provide higher treatment efficiency for organic matter and ammonia, and require less space as compared to horizontal flow beds (Hoffmann *et al.*, 2011; Austin and Yu, 2016). Their influent is applied onto the surface, then percolates vertically down through the filter medium (Austin and Yu, 2016). Such biofiltration systems have been robustly and successfully used for wastewater treatment over decades as they are easy to construct and have low energy requirements (Reungoat *et al.*, 2011). They can contribute towards the United Nations Sustainable Development Goal 6 (SDG6) to ensure availability and sustainable management of water and sanitation for all (Hube and Wu, 2021). Besides sand,

biological activated carbon is the most commonly used biofilter technology (Reungoat *et al.*, 2011). Activated carbon (AC) is produced from a carbonaceous source material such as coconut shell, soft wood, or coal. AC has been widely used in WWTPs particularly as tertiary treatment step to adsorb organic compounds (Luo *et al.*, 2014; Castro *et al.*, 2019). Pollutant removal by biological AC occurs not only via biodegradation, but also via adsorption mechanism as AC has high sorptive affinities for solutes from its large surface area, pore size, and surface chemistry (Luo *et al.*, 2014; Ulrich *et al.*, 2015). It is thus expected that biofilter performance can be intensified using AC, ultimately enabling smaller treatment footprints. Several studies reported the potential of biofilters amended with AC for micropollutant removal via adsorption mechanism (Luo *et al.*, 2014; Ulrich *et al.*, 2015; Mailler *et al.*, 2016; de Castro *et al.*, 2018). However, the impact of AC amendment in sand bed biofilters on micropollutant and pathogen removal during tertiary wastewater treatment needs more empirical investigation to substantiate an improved performance. This is particularly pertinent in tropical countries with limited wastewater infrastructures like Thailand, where there is the lack of empirical studies on the occurrence and treatment of micropollutants (Tewari *et al.*, 2013). For example, rapid growing population and limited wastewater treatment in Bangkok, Thailand, presents a growing risk to the environment and coastal food production systems (Mrozik *et al.*, 2019; Thongsamer *et al.*, 2021). This study was therefore conducted in Bangkok to address a need for innovative, resilient, and sustainable wastewater treatment options in tropical countries.

4.2.1 Aim

To investigate the effect of 5% w/w coconut shell activated carbon (AC) amendment in sand bed biofilters operated under tropical conditions on micropollutant and bacterial pathogen removal from a secondary treated wastewater.

4.2.2 Objectives

1. To compare the treatment efficiency for chemical parameters including pH, DO, BOD, COD, ammonia (NH_3-N), nitrate (NO_3^--N), nitrite (NO_2^--N), phosphate (PO_4^{3-}-P), TSS and heavy metals, for sand bed biofilters without (Control) and with AC amendment. Hypothesis: AC amendment improves overall nutrient and heavy metal removal relative to the Control.

2. To compare the treatment efficiency for 8 micropollutants (acetaminophen, oxytetracycline, tetracycline, enrofloxacin, atrazine, sulfamethoxazole, diuron, and

diclofenac) for sand bed biofilters without (Control) and with AC amendment.
Hypothesis: AC amendment improves overall micropollutant removal relative to the Control.

3. To investigate the treatment efficiency for putative human pathogens and faecal indicator bacteria for sand bed biofilters without (Control) and with AC amendment.
Hypothesis: AC amendment improves putative human pathogen and faecal indicator removal relative to the Control.

4.3 Materials and methods

4.3.1 Activated carbon production and characterization

Commercial activated carbon produced from coconut shells was used in this study. However, there is a lack of detailed information on the AC properties as it was purchased from a small local company in Bangkok. We therefore assumed similar properties of this AC to the coconut shell AC used in our previous study. The AC has pH of 9.24 and the point of zero charge (PZC) of 10.4. More details of coconut shell AC properties are provided in our previous publication (Han *et al.*, 2015).

4.3.2 Biofiltration study

We conducted this biofiltration study at KMUTT University, in Bangkok, Thailand. Activated sludge effluent was collected from a secondary clarifier at the Tung Kru wastewater treatment plant in Bangkok, Thailand. It was kept at 4 ^{o}C and used for the whole filtration experiment. The experimental set-up consisted of two AC-amended columns (AC) and two columns without AC amendment (Control). The filter media of the AC columns comprised a 30 cm layer of coarse sand mixed with AC (20:1 or 5% w/w), sandwiched in between two 5 cm layers of gravel (grain size – 2 mm) **(Figure 4.1)**. We chose the proportion of Sand:AC as a midrange of proportions reported in previous studies (0-10% w/w) (Ulrich *et al.*, 2015; de Castro *et al.*, 2018; Mrozik *et al.*, 2021). The Control columns had the same configurations as the AC columns except that there was no addition of AC to the sand. We operated the columns for 15 days and loaded them once daily with 785 mL of activated sludge treated effluent based on a hydraulic loading rate (HLR) of 10 cm/d. The rate was chosen based on a recommended HLR for vertical subsurface flow constructed wetlands (Hoffmann *et al.*, 2011). The hydraulic retention time for the column was about 2 hours for each feeding cycle. We collected column effluent samples on day 2, 4, 8, 10, 12, and 15, whilst collecting column influent samples on days 2, 4 and 10.

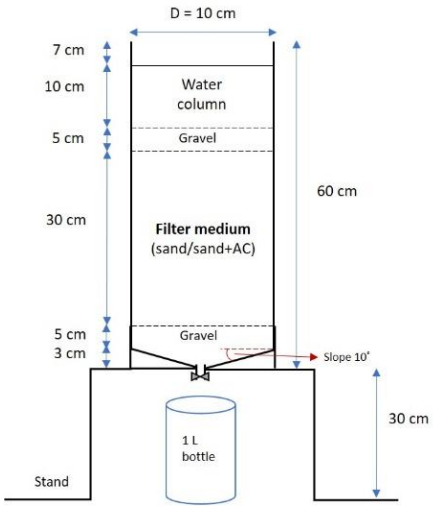

Figure 4.1 Schematic diagram of the biofilter containing sand or sand plus activated carbon.

4.3.3 Conventional water quality analysis

We analysed the column influent and effluent samples for pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), ammonia (NH_3-N), nitrate (NO_3^--N), nitrite (NO_2^--N), phosphate (PO_4^{3-}-P), total suspended solids (TSS) at KMUTT following Standard Methods for the Examination of Water and Wastewater (APHA, 2015). We analysed faecal coliforms by membrane filtration according to Method 8074 from HACH LANGE (HACH, Manchester, UK). We also filtered water samples through a cellulose acetate syringe filter (0.45 μm, 25 mm; VWR International, UK), acidified with 1% v/v concentrated nitric acid and stored at 4° C for metal analysis at Newcastle University using a Varian Vista-MPX Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) as previously described (Gozzard *et al.*, 2011). Certified 1000 ppm standards (accuracy of ≤ ± 1.0%; VWR Chemicals, VWR International, UK) were diluted using 1% nitric acid solution for preparing calibration standards. Blanks and standards were run every 13 samples to check analytical accuracy and precision.

4.3.4 Molecular microbiology

We analysed the bacterial community of column influent, effluent, and filter medium samples using a combination of MinION nanopore sequencing and qPCR methods, as previously described (Thongsamer *et al.*, 2021). 100 mL of water samples were filtered through 0.22 μm membranes (Sartorius UK Limited,Surrey, UK). The total DNA was immediately stored at -20 ^{0}C to preserve DNA for analysis. We extracted the total DNA retained on the filter

membranes at the end of the experiment at KMUTT using a PowerWater® DNA Isolation Kit as per the manufacturer's instructions (QIAGEN, Crawley, UK). For the filter media, 500 mg of sand/sand+AC sample were extracted at the end of experiment at KMUTT using a PowerSoil® DNA Isolation Kit as per the manufacturer's instructions (QIAGEN, Crawley, UK). We measured the DNA concentration using a Qubit dsDNA HS Assay Kit (Life Technologies, UK). The sequencing library for 16S rRNA gene sequencing was generated from 20 ng of DNA using a 16S barcoding kit (SQK-16S024 from Oxford Nanopore Technologies (ONT), Oxford, UK) as per the manufacturer's instructions and loaded onto a MinION flow cell (R9.4.1, FLO-MIN106). We placed the flow cell into the MinION for the sequencing and controlled it using ONT's MinKNOW software. The raw reads (i.e. HDF5 raw signals) were base-called with GUPPY (Version; v4.4.2) software (ONT, Oxford, UK) producing .fastq files. This step converted the electrical signals generated by a DNA strand passing through the nanopore into the corresponding base sequence. We uploaded base-called data to the EPI2ME interface (v.3.4.2), a platform for cloud-based analysis of base-called MinION data. Data interpretation was performed with the FASTQ 16S workflow, using a quality score 7 for filtering. The FASTQ 16S workflow revealed the taxonomic classification of base-called reads along with their frequency.

We quantified genes for total bacteria 16S and specific bacteria of interest including total coliforms (Eco1457F/ Eco1652R), total *E.coli* (rodA), human *E.coli* (Hu100), and *Vibrio cholerae* (EpsM) by real time PCR assays (qPCR) on a BioRad CFX C1000 system (BioRad, Hercules, CA USA) using the primers shown in **Table B2, Appendix B**. Regarding quantification of the target genes, 2 µL of the DNA samples, 7.5 µL of SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad), 4 µL of nuclease free water (Invitrogen, Life Technologies, Paisley, UK), and 0.75 µL of each forward and reverse primer solutions (@ 10 micromol·L^{-1}) were combined for a 15 µL final volume with 500 (nmol·L^{-1}) of each primer. Reaction conditions for quantification of each target gene were 98 °C for 3 minutes (1x), then 98 °C for 15 seconds, and the Primer Annealing Temperature (T_a) for 30 seconds **(Table B2, Appendix B)** (40 cycles). We produced standard curves using synthesized nucleotide sequences of the target genes (Invitrogen, Life Technologies, Paisley, UK) every time a qPCR analysis was performed, in parallel with the amplification of test samples. Serial dilution (10-fold) of the standards was performed to obtain standard solutions in the range of 10^8–10^1 target gene copies/µL. All samples were run in duplicate and molecular grade H_2O replaced template in control reactions. There was no amplification (the

quantification-cycle (Cq) values) in any control reactions in both 16S and specific-gene assays.

4.3.5 Micropollutant analysis

We analysed the selected micropollutants according to EPA method 1694. The influent and effluent of duplicate Control/AC columns were analysed for each sampling event. We collected 30 mL of samples and mixed them with 270 mL of distilled water making a 300 mL (10 times dilution) sample ready for the extraction step. We then passed 300 mL through a 1 µm glass fiber filter (GF/B, Whatman, UK) and acidified the filtrate with diluted HCl to pH 2.5. Then, we added surrogate standards atrazine-D5, enrofloxacin-D5, sulfamethoxazole-D4 and ibuprofen-D3 (from QMX Laboratories, Dunmow, UK) at concentrations of 10 ng/L, 50 ng/L, 50 ng/L and 50 ng/L, respectively and left samples for 30 minutes to equilibrate. Prior to solid-phase extraction (SPE), we added 150 mg $Na_4EDTA \cdot 2\ H_2O$ to the sample and equilibrated them for 30 minutes. The extraction was carried out on a Waters Oasis HLB cartridge (200 mg, 6cc, USA) at KMUTT. We first conditioned the cartridge with 6 mL methanol, 6 mL ultra-pure water, and 6 mL of acidified ultra-pure water (pH=2.5, HCl from Sigma-Aldrich St. Louis USA). Next, we loaded the water samples at a flow rate of 10 mL min^{-1}. After extraction, the cartridges were washed with 10 mL of 5% methanol and dried for 30 minutes under vacuum, then transported frozen to Newcastle University. Samples were eluted from the cartridges with 10 mL of methanol (LCMS grade, VWR, Lutterworth, UK). After that, we concentrated the samples to complete dryness at 35ºC under a gentle stream of nitrogen in the Vertex evaporator (Labconco, Missouri, USA). Finally, they were reconstituted in a 1 mL of the mobile phase, ready for LC-MS analysis. We analysed micropollutants in the water samples at Newcastle University with a UPLC-MS/MS system (Waters, Elstree, UK). All data were acquired and processed using MassLynx 4.1 software (Waters, Elstree, UK). LC-MS experimental condition, method parameters and validation data are described in our previous work (Mrozik *et al.*, 2019). We analysed samples for 8 micropollutants including (1) analgesic: acetaminophen ; (2) herbicides: atrazine, diuron; (3) antibiotics: oxytetracycline, tetracycline, enrofloxacin, sulfamethoxazole, and (4) anti-inflammatory: diclofenac based on our previous work in Thailand, which detected these micropollutants in canal water and sediments (Mrozik *et al.*, 2019).

4.3.6 Data processing and statistical analysis

We processed sequencing data using Matlab© (Version R2019b, Mathworks, Portola Valley, CA, USA) for multivariate data analysis (cluster and principal component analysis (PCA)). We downloaded the taxonomic classification and quality of barcoded reads from the EPI2ME dashboard as a CSV file which contained information on run and read IDs and read accuracy, barcodes, and NCBI taxa IDs for classified reads. Then, we processed the CSV file with Matlab© scripts published elsewhere (Thongsamer *et al.*, 2021). In brief, the script first generated root level OTU tables by matching NCBI taxa IDs to lineages and counting the number of reads per NCBI taxa ID, with and without rarefication. If required, these scripts also enable combining root level OTU tables from different runs into a single table. Then, OTU tables with grouping of reads at genus level were created. We rarefied sequencing libraries at 45,000 reads per sample and performed multivariate statistical analysis for OTUs classified to genus level, and grouped at this level, using Matlab© for cluster and PCA with Euclidean distance as the similarity metric. We performed analysis of similarities (ANOSIM) on Matlab© with the Fathom Toolbox developed by the Marine Resource Assessment Program at the University of South Florida's College of Marine Science (Jones, 2015). Additionally, one of the scripts allows extracting species or genera of interest from root and genus level OTU tables, respectively. We used these scripts to extract bacterial genera containing putative human pathogens and faecal indicator bacteria. More details of this data processing are described in our previous publication (Acharya *et al.*, 2020a).

We used the two-sided sign test function in Matlab© to investigate statistically significant difference of the overall removal of nutrients (N&P), heavy metals, micropollutants, and pathogens between the AC and Control column effluents. For chemistry data (conventional water quality parameters), we used two-tailed t-tests to evaluate the null hypothesis that there is no difference between the mean values of two sample groupings of interest. For example, pH in the Control column effluent versus pH in the AC column effluent. We also used one-tailed z-tests to investigate if the mean value of each parameter meets the desired standard. All calculations, t-tests and z-tests were performed using Excel.

We used Primer7 software (primer-e, Auckland, New Zealand) to investigate the linkage between environmental parameters and microbial communities in the water samples using the BEST (Bio-Env) procedure as described by Clarke *et al.* (2014).

4.4 Results and discussion

4.4.1 Characteristics of biofilter influent and effluent

Table 4.1 Conventional water quality parameters of the influent and effluent of the Control and AC column as compared to Thailand's surface water quality standards. Results were reported as Mean±S.D. for duplicates of 3 samples for the influent and 6 samples for the effluent collected throughout the entire experimental period.

Parameter	Influent	Control effluent	AC effluent	Standard [1]
pH	7.47±0.12	7.83±0.06	8.19±0.06	5-9
DO (mg/L)	7.52±0.58	5.03±0.20	4.64±0.30	> 4
BOD (mg/L)	4.88±0.92	2.01±1.29	1.19±0.62	< 2
COD (mg/L)	72.00±10.60	40.20±11.65	30.00±12.86	N/A
NH_3-N (mg/L)	0.60±0.16	0.29±0.13	0.16±0.07	< 0.5
NO_3^--N (mg/L)	0.60±0.02	0.73±0.12	0.84±0.28	< 5
NO_2^--N (mg/L)	0.13±0.02	0.00±0.00	0.04±0.04	N/A
PO_3^{4-}-P (mg/L)	6.92±0.22	0.17±0.07	1.42±0.19	N/A
TSS (mg/L)	7.83±3.66	4.17±1.97	15.83±9.61	N/A
Faecal coliform (CFU/100 mL)	21667±7028	350±327	183±170	< 4000
Heavy metals (mg/L)				
Fe	0.009±0.000	0.002±0.001	0.004±0.000	N/A
Cu	0.003±0.000	0.004±0.002	0.003±0.000	< 0.10
Zn	0.047±0.003	0.007±0.002	0.005±0.002	< 1.00
Cd	0.001±0.000	0.000±0.000	0.000±0.000	< 0.005
Mn	0.016±0.005	0.003±0.003	0.002±0.001	< 1.00
Ni	0.014±0.002	0.003±0.001	0.004±0.001	< 0.10
Pb	0.015±0.002	0.018±0.006	0.012±0.004	< 0.05
As	0.019±0.003	0.016±0.010	0.014±0.010	< 0.01

[1] Thailand's surface water quality Class 3, medium clean fresh surface water resources used for consumption but passing through an ordinary treatment process before using, and for agricultural purposes (WEPA, 2017).

As is evident in **Table 4.1**, pH was in a range of approximately 7-8 in the influent and effluent of both Control and AC columns in compliance with the standards for surface water quality. The pH of AC effluents was significantly higher than for the Control columns (t-test, p-value < 0.01). DO concentrations were overall decreased after filtration, likely because of biodegradation processes consuming oxygen, but did not drop below the permissible limit (z-test, p-value < 0.01 for both columns). BOD and NH_3-N concentrations in the column influent were above the permissible limit, but after filtration BOD and NH_3-N levels in the effluents were reduced to below the limit in only the AC columns for BOD (z-test, p-value < 0.01), and in both column types for NH_3-N (z-test, p-values < 0.01, for both columns). Lower NH_3-N following biofiltration could be attributed to the nitrification process in which nitrifying microbes converted NH_3 into NO_2^- then NO_3^- (Vymazal, 2013). The microbiology

results confirmed that the nitrifying bacteria abundance increased in the effluents compared to the influent for both column-media types **(Figure B3, Appendix B)**. The AC columns showed higher removal of NH_3-N relative to the Control columns. There was lower NO_2^--N level after biofiltration in both columns. However, no significant difference of NO_3^--N was found between influent and effluents for both column types, while the effluent concentrations still complied with the standard. COD and PO_3^{4-}-P concentrations were substantially lower in effluents of both column types than in the influent. Notably, there was a significant lower PO_3^{4-}-P concentration in the Control column effluents than AC effluents (t-test, p-value < 0.01) suggesting better PO_3^{4-}-P removal efficiency by sand-only biofiltration than sand with AC amendment. This could be attributed to the precipitation of PO_3^{4-}-P with Ca^{2+} that is present in the sand (Del Bubba *et al.*, 2003). The Control column effluent contained higher calcium concentration than the AC columns **(Table B1, Appendix B)**, which therefore could give higher precipitation capacity for PO_3^{4-}-P than the AC columns. TSS level in the effluent of the AC columns was increased presumably because of the leaching of fine AC particle fragments (Hale *et al.*, 2012). For faecal coliforms, their number in the influent largely exceeded the permissible limit of faecal coliforms set at 4000 CFU/100 mL. Encouragingly, after biofiltration by either the Control or AC columns, faecal coliforms were reduced to significantly below the permissible limit, without a significant difference between the Control and AC columns (t-test, p-value > 0.05). In terms of heavy metals, there were significant lower concentrations of Fe, Zn, Mn and Ni in both Control and AC column effluents relative to the influent (t-test, p-value < 0.05). However, no significant difference between the Control and AC columns was found (sign test, p-value > 0.05). All metals except As were already under the permissible limits in the influent and still were so in the effluents of all columns, while the As concentration marginally exceeded the limit (z-test, p-value < 0.05, for all). More detailed data of these parameters for all sampling events are provided in **Section B1, Appendix B.** In summary, some water quality parameters of the column influent, which is the effluent of activated sludge treatment, did not meet the permissible limits, but with further polishing steps by biofiltration with sand only or sand plus AC media, the overall effluent quality met the desired standards. However, the hypothesis that AC amendment improves overall nutrient and heavy metal removal relative to the Control is rejected since there was no significant difference of overall nutrient and heavy metal removal between AC and Control columns (sign test, p-value > 0.05).

4.4.2 Micropollutants in the influent and effluent of biofilters

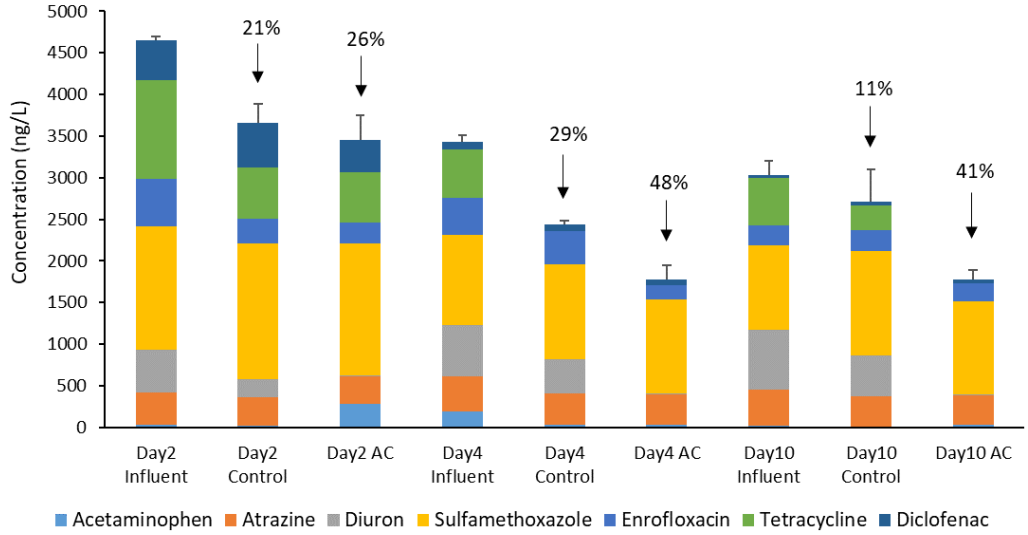

Figure 4.2 Concentration (ng/L) of the selected micropollutants in the column influent, Control and AC column effluents on days 2, 4 and 10. Error bars were calculated as standard deviation of the total concentrations of the seven reported compounds for duplicates. Oxytetracycline was not detected in any of the samples. Percentage above each bar indicates overall % removal by the columns.

Table 4.2 Concentration (ng/L) of the selected micropollutants in the column influent, Control and AC column effluents on days 2, 4 and 10. Results were reported as Mean±S.D. for duplicates. Results were compared against predicted no effect concentrations (PNECs). PNEC levels were derived from Minguez *et al.* (2016), Luo *et al.* (2014) and Bengtsson-Palme and Larsson (2016).

Compound (ng/L)	Acetaminophen	Atrazine	Diuron	Sulfamethoxazole	Enrofloxacin	Tetracycline	Diclofenac
Day2 Influent	27.50±4.95	386.83 ±14.85	513.50 ±35.59	1487.50±92.16	575.17 ±461.27	1179.00 ±601.98	482.33 ±65.05
Day2 Control	23.00±1.89	340.67 ±74.95	215.50 ±53.03	1632.00±313.01	291.00 ±34.88	617.83 ±21.45	541.5 ±130.34
Day2 AC	286.50±391.97	329.33 ±17.91	10.50 ±0.71	1585.17±287.79	244.67 ±44.78	606.17 ±4.48	394.83 ±497.1
Day4 Influent	188.33±141.42	420.50 ±102.53	618.67 ±65.53	1089.83±65.76	441.00 ±0.94	586.00 ±11.31	90.33 ±5.19
Day4 Control	36.00±24.98	375.83 ±18.15	406.33 ±4.24	1146.17±5.89	394.33 ±68.83	30.00 ±0.00	80.33 ±2.36
Day4 AC	29.67±19.33	365.00 ±58.45	9.33 ±1.89	1135.67±26.87	173.17 ±138.83	30.00 ±0.00	62.5 ±1.65
Day10 Influent	15.50±6.84	441.17 ±31.35	710.17 ±97.35	1024.17±98.76	232.67 ±9.43	577.67 ±0.94	26.33 ±20.27
Day10 Control	7.83±1.65	368.33 ±0.00	486.33 ±69.30	1256.50±0.24	255.33 ±33.00	302.67 ±385.61	47.67 ±16.97
Day10 AC	26.33±17.91	357.67 ±76.84	9.67 ±1.41	1123.67 ±52.33	211.33 ±63.64	30.00 ±0.00	47.5 ±12.49
PNEC	*814*	*2000*	*1800*	*16000**	*64**	*1000**	*10000*

*PNEC level for antimicrobial resistance

Overall, from **Table 4.2**, the majority of the selected micropollutants were detected at below 1000 ng/L (1 μg/L) except for sulfamethoxazole, and tetracycline in day 2 influent. Sulfamethoxazole showed the highest concentration, in a range of 1000-1600 ng/L, relative to the other compounds. Sulfamethoxazole is an antibiotic that has been previously detected in wastewater and canals in Bangkok (Tewari *et al.*, 2013). Additionally, a study on antimicrobial resistance in aquaculture water and surrounding canals in central Thailand revealed high levels of sulfa-resistant bacteria in a wastewater polluted canal draining central Bangkok versus less wastewater polluted locations (Thongsamer *et al.*, 2021). This implies that the sulfa-antibiotic class might be widely used in Bangkok, leading to high antibiotic concentrations and resistant bacteria in wastewater, both of which end up in the environment. Acetaminophen showed the lowest concentration ranging between 7-200 ng/L. The total concentrations of the selected micropollutants were lower in the effluent than the influent of both Control and AC columns in every sampling event showing an overall micropollutant removal capability of the biofilters ranging from 11-29% by the Control columns and 26-48% by the AC columns **(Figure 4.2)**. Noticeably, the AC column effluents had lower total concentrations of the selected micropollutants than the Control column effluents in every sampling event. The performance of AC-amended columns was improved on day 4 and 10 as compared to day 2 with an approximately 20% increase in removal. The overall concentrations of all non-antibiotic compounds in the influent and effluent of both columns were below the PNEC levels for surface water (z-test, p-values < 0.01) **(Table 4.2)** and unlikely to cause adverse effects in the aquatic environment. For antibiotics, the concentrations were compared against the estimated PNEC levels for antimicrobial resistance. All concentrations of sulfamethoxazole and tetracycline in the influent and effluent of both columns, except for tetracycline in day 2 influent were below such PNEC levels, but enrofloxacin concentrations exceeded the desired level of 64 ng/L in both influent and effluents for all sampling events **(Table 4.2)**. Enrofloxacin is widely used in pig farms, aquaculture, and domestic animals in Thailand, which could then result in high concentrations in wastewater and a development of enrofloxacin resistance genes (Udomkusonsri *et al.*, 2007; Lukkana *et al.*, 2016; Jansomboon *et al.*, 2018; Huber *et al.*, 2021).

Table 4.3 Removal efficiency (%) of acetaminophen, atrazine, diuron, sulfamethoxazole, enrofloxacin, tetracycline, and diclofenac by the Control and AC column on day 2, 4, and 10. Results were reported as Mean±S.D. for duplicates of each column on each day.

% Removal	Acetaminophen	Atrazine	Diuron	Sulfamethoxazole	Enrofloxacin	Tetracycline	Diclofenac
Day2 Control	16.36 ±4.85	11.93 ±13.70	58.03 ±7.30	-9.71 ±14.88	49.41 ±4.29	47.60 ±1.2	-12.26 ±19.11
Day2 AC	66.06 ±0.00	14.86 ±3.27	97.96 ±0.10	-6.57 ±13.68	57.46 ±5.51	48.59 ±0.27	18.14 ±72.87
Day4 Control	80.88 ±9.38	10.62 ±3.05	34.32 ±0.48	-5.17 ±0.38	10.58 ±11.03	94.88 ±0.00	11.07 ±1.85
Day4 AC	84.25 ±7.26	13.20 ±9.83	98.49 ±0.22	-4.21 ±1.74	60.73 ±22.26	94.88 ±0.00	30.81 ±1.29
Day10 Control	49.46 ±7.53	16.51 ±0.00	31.52 ±6.90	-22.7 ±0.02	-9.74 ±10.02	47.61 ±47.2	-81.01 ±45.57
Day10 AC	-69.90 ±81.72	18.93 ±12.32	98.64 ±0.14	-9.72 ±3.61	9.17 ±19.34	94.8 ±0.00	-80.38 ±33.54

Table 4.3 shows that there was overall significantly higher removal of the monitored micropollutants across day 2, 4, and 10 by the AC columns as compared to the Control columns (sign test, p-value < 0.01). Sulfamethoxazole was not removed in either of the column types for all sampling events. This was presumably because it is recalcitrant (not easily biodegraded), and not readily treatable by adsorption (Shen *et al.*, 2020). Tetracycline was generally easily removed also in the Control columns suggesting that it is readily biodegradable, since the sand medium lacks significant adsorption capacity. For the other compounds, the combined effect of sorption and biodegradation can explain greater removal by the AC columns. Poor removal of atrazine was observed in both columns throughout the experimental period, while the removal of acetaminophen was fluctuating. The AC columns showed better removal than the Control column for diuron and diclofenac. Diuron and diclofenac are known as recalcitrant compounds (Paredes *et al.*, 2016; Beltrán-Flores *et al.*, 2020), whereas the main removal mechanism of micropollutants in sand-only biofilters tends to be via biodegradation. Therefore these two compounds were hardly removed by the Control columns (Reungoat *et al.*, 2011). In addition, several studies have found that the main removal mechanism of recalcitrant compounds in biofilters is by adsorption (Serrano *et al.*, 2010; Rattier *et al.*, 2014; Paredes *et al.*, 2016). Consequently, higher removal of diuron and diclofenac by the AC amended biofilters can be explained by the sorption capacity of the AC. The relationship between the solution pH and the point of zero charge (PZC) of an adsorbent is an important factor affecting the solute removal by adsorption. PZC is the pH where the net total surface charge of a material is zero (Appel *et al.*, 2003). When the pH of the solution is below a sorbent material's PZC, such material will be positively charged and adsorb anions

from the solution (Appel *et al.*, 2003; Pereira *et al.*, 2019). A PZC of 10.4 for coconut shell AC was previously reported (Han *et al.*, 2015) which is substantially higher than the pH 8.19±0.06 of AC column effluent **(Table 4.1)**. This suggests that the AC should have a net positive surface charge, while diuron and diclofenac are negatively charged molecules at this effluent pH (Rossi *et al.*, 2013). The AC columns were therefore capable of retaining these two compounds, nevertheless it can be noticed that after 10 days of filtration there was a negative removal of diclofenac by the AC columns suggesting breakthrough of diclofenac after the adsorption capacity of the AC had been exhausted. Contrarily, de Castro *et al.* (2018) conducted a similar biofiltration test with sand columns and AC-amended sand columns, and reported that there was 15 % removal of diclofenac by both column types. Enrofloxacin was also better removed by the AC column which is likely due to adsorption (Fu *et al.*, 2017). Similarly, previous studies found high adsorption of enrofloxacin onto coconut shell activated carbon and biochar (DasSharma *et al.*, 2020; Mrozik *et al.*, 2021). Noticeably, there was no removal of sulfamethoxazole for all sampling events in both Control and AC columns. Each antibiotic will have a different chemical structure which may or may not bind to AC. The seemingly negative removal of this compound, albeit small, could be due to the formation of unmeasured metabolites that might be converted back to the parent compound during filtration (Tewari *et al.*, 2013; Achermann *et al.*, 2018). The behavior of most micropollutants can be in accordance with their fate in WWTPs, where biodegradation and sorption to biosolids contribute to the removal (Reungoat *et al.*, 2011). Tewari *et al.* (2013) investigated pharmaceutical residues in WWTPs and receiving waters in Bangkok, Thailand, and reported negative removal of sulfamethoxazole in all studied WWTPs. Contrarily, some studies reported high removal of sulfamethoxazole by AC amended biofilters via biotransformation and adsorption (Reungoat *et al.*, 2011; Paredes *et al.*, 2016). Overall, the results agree with the hypothesis that AC amendment improves micropollutant removal relative to the Control.

4.4.3 Cluster and PCA analysis of the overall microbial community

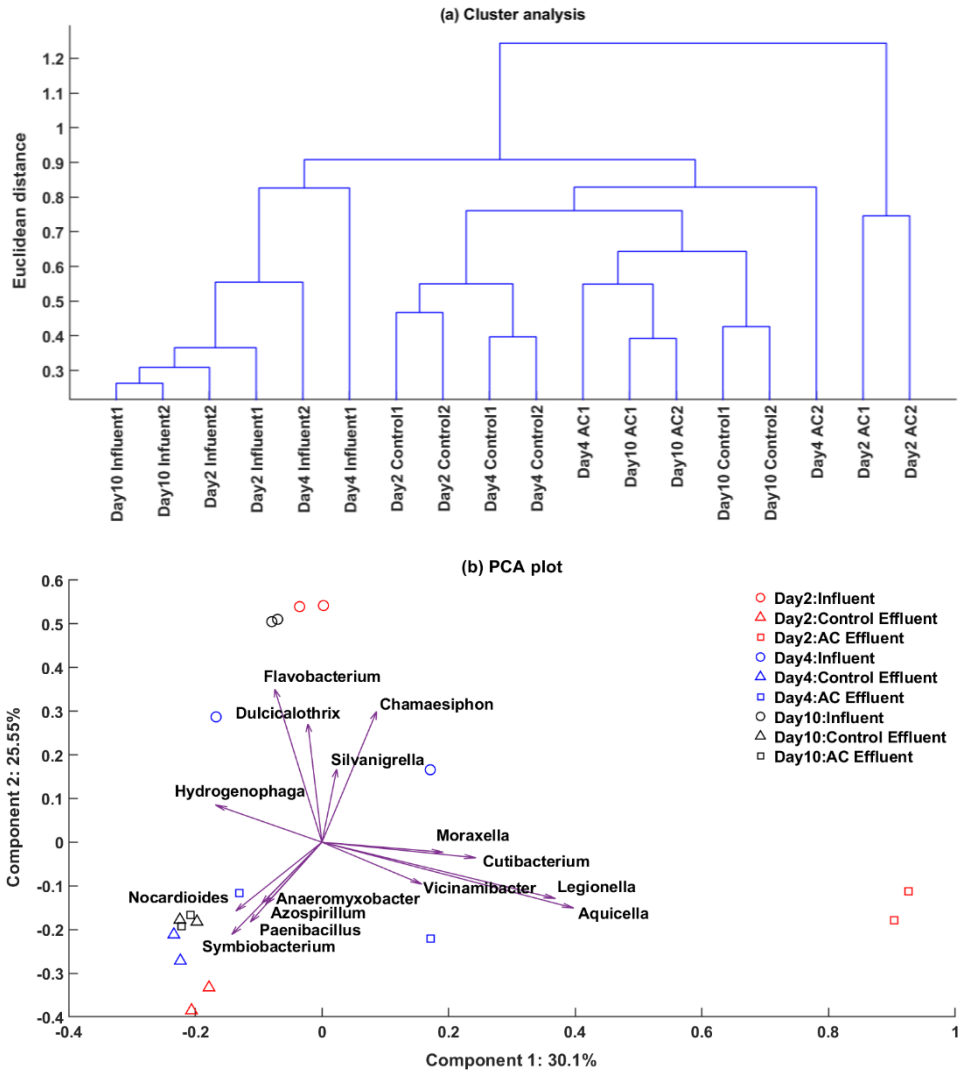

Figure 4.3 Cluster analysis (a) and Principal component analysis (PCA) (b) plots of the microbial community dissimilarity between influent and effluent of sand columns (Control) and AC-amended sand columns (AC) on day 2,4, and 10. For PCA, the first two principal components (Component 1 and 2) were plotted showing the scores (circles, triangles, and squares) and top 15 loadings (genera, arrows) explaining the variance in the two-dimensional space. Percentage of variation accounted for by each principal component is shown with the axis label.

Cluster analysis **(Figure 4.3a)** shows the greatest dissimilarities for microbial communities were between day 2 AC effluent and the other samples. Influent samples in every sampling event were clustered together and separated from the effluents. Sample replicates clustered closely, except for day 4 AC. In the principal component analysis (PCA, **Figure 4.3b**), components 1 and 2 accounted for almost 56 % of the observed variance between samples. Along component 1, day 2 AC effluent samples were clearly separated from the other samples, while component 2 clearly separated all influent samples from the effluent samples.

Biofiltration was overall a significant microbial community shaping factor (one-way ANOSIM, p-value < 0.01 and R = 0.57 for influents versus effluents) while sampling time overall had a weaker effect on shaping the microbial community (p-value < 0.05 and R = 0.15 for sampling events). There was no clear separation of microbial communities by AC amendment. The Control and AC effluent communities differed starkly on day 2 AC, but converged over time and became very similar by day 10 (black triangles and black squares). The 15 most notable microbial genera (i.e., variables) explaining the variance in the PC1 and 2 space are illustrated by the purple arrows in **Figure 4.3b**. These genera included *Legionella* which is a genus containing pathogenic bacteria causing respiratory disease (Ariyadasa *et al.*, 2021), but also many harmless bacteria. *Legionella* was predominant in the day 2 AC effluent community. The genus *Flavobacterium* was notable in the influents. These bacteria can be found in a variety of environments including water and soil. While most of them are harmless, some species can be pathogenic to humans and fish (Loch and Faisal, 2015). However, after 10 days of biofiltration, effluent microbial communities from both column types were characterized by harmless bacteria that can be mostly found in sand/soil including *Paenibacillus, Symbiobacterium* and *Nocardioides* (Ueda et al., 2001; Grady et al., 2016; Kwak et al., 2017) as shown in **Figure 4.3b**.

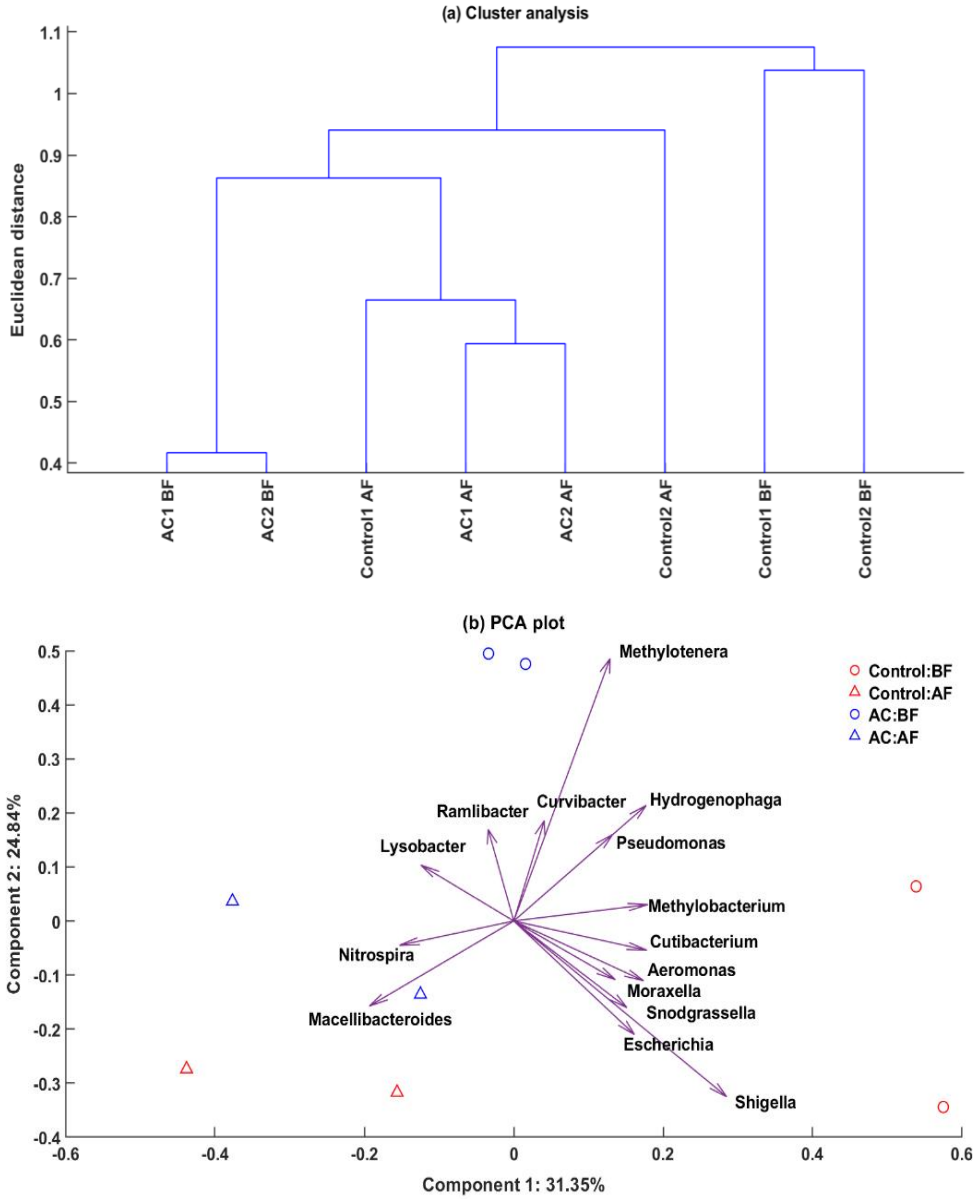

Figure 4.4 Cluster analysis (a) and Principal component analysis (PCA) (b) plots of the microbial community dissimilarity between filter media of sand columns (Control) and AC-amended sand columns (AC) before filtration (BF) and after filtration (AF). For PCA, the first two principal components (Component 1 and 2) were plotted showing the scores (circles and triangles) and top 15 loadings (genera, arrows) explaining the variance in the two-dimensional space. Percentage of variation accounted for by each principal component is shown with the axis label.

Cluster analysis **(Figure 4.4a)** shows the greatest dissimilarities for microbial communities were between the filter medium of the Control columns before filtration (Control BF) and the other samples. In the principal component analysis (PCA, **Figure 4.4b**), components 1 and 2 accounted for approximately 56% of the observed variance between samples. Along component 1, the filter medium samples from the Control columns before filtration were clearly separated from other samples, while component 2 separated filter medium samples

from the AC columns from the others. This suggests an initial difference in microbial communities in the Control and AC column media before the filtration, with greater resemblance by the end of the experiment. One-way ANOSIM confirmed that media before filtration versus after filtration was a significant factor in shaping the medium microbial communities (one-way ANOSIM, p-value < 0.05 and R = 0.32). The 15 most notable microbial genera (i.e., variables) explaining the variance in the PC1 and 2 space are illustrated by the purple arrows in **Figure 4.4b**. These genera included *Shigella, Escherichia,* and *Aeromonas* which are genera containing pathogenic bacteria (Maurya *et al.*, 2020). They were predominant in the community of the Control columns before filtration. *Methylotenera* predominated in the community of the AC columns before filtration. *Methylotenera* is a genus of methylotrophic bacteria that gain energy from compounds without carbon-carbon bonds e.g., methane (Kalyuzhnaya *et al.*, 2010). A previous study reported the substantial presence of a methylotrophic community in activated carbon (Tae Gwan and Kyung-Eun, 2013). Notably, after filtration, *Nitrospira* which is a genus containing nitrifying bacteria (Pjevac *et al.*, 2017) was present in the filter media. This provides evidence of nitrification during biofiltration as was discussed earlier in **Section 4.4.1**.

4.4.4 Removal of putative human pathogens and faecal indicator bacteria by Control and AC columns

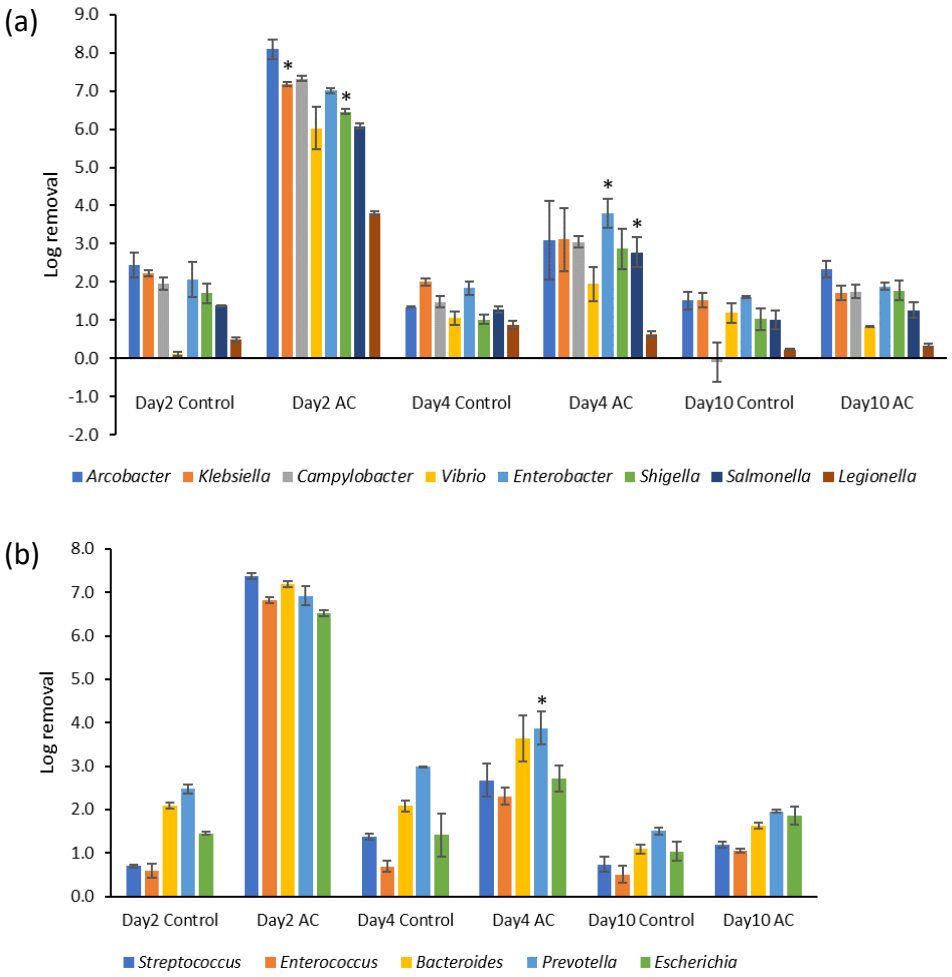

Figure 4.5 Log removal of bacterial genera containing putative human pathogens (a) and genera containing faecal indicator bacteria (b) by Control columns and AC columns on day 2, 4, and 10. Error bars were calculated as standard deviation of duplicate Control and AC columns. (*) The removal was calculated based in a detection limit of 1 gene copy per 100 mL.

From **Figure 4.5**, overall, there was higher removal of bacterial genera containing putative human pathogens and faecal indicator bacteria by the AC-amended columns than the Control columns across days 2, 4, and 10 (sign test, p-value < 0.01). There was a trend of decreasing removal of these bacteria by the AC column over time which could be attributed to an exhaustion of the adsorption capacity of the AC and the growth of biofilms. Attachment of organic matter to the AC may contribute towards the biofilm formation. Hence the removal efficiency of pathogens by AC sorption tends to decrease over time whilst biosorption becomes more dominant (Hube and Wu, 2021). From **Figure 4.5a**, after 10 days *Enterobacter* were still removed by both Control and AC columns, while there was a little

removal of *Legionella*. This agrees with a previous study showing that *Enterobacteriaceae* were still removed by sand column and AC-amended sand columns treating pond water after 56 days, but not *Legionella* (Vignola et al., 2018). High abundance of *Legionella* was previously found in biofilter media (Vignola *et al.*, 2018). The abundance of the selected putative human pathogens and faecal indicator bacteria in the filter media will be further discussed in the following section. The hypothesis that AC amendment improves putative human pathogen and faecal indicator removal relative to the Control was proven in this study.

4.4.5 Abundance of putative human pathogens and faecal indicator bacteria in the filter media

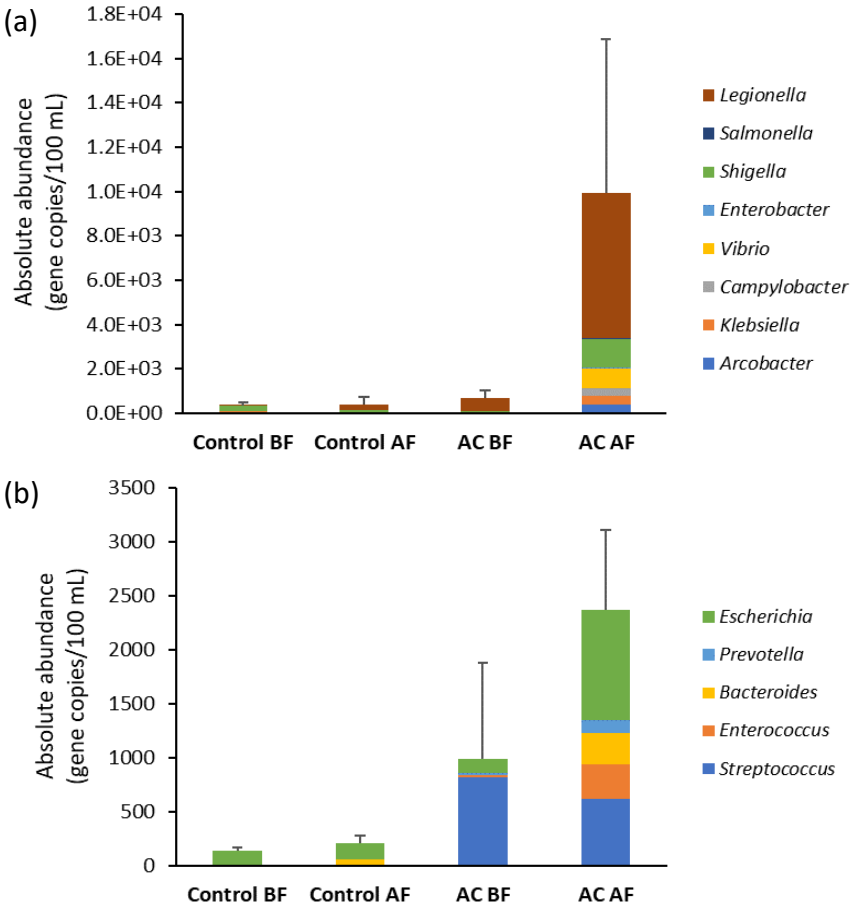

Figure 4.6 Absolute abundance (gene copies/100 mL) of selected bacterial genera containing putative human pathogens (a) and faecal indicator bacteria (b) obtained from multiplying relative abundance from MinION 16S rRNA gene sequencing with qPCR quantification of 16S rRNA gene copy numbers in each sample in the media of Control (C) and AC columns before filtration (BF) and after filtration (AF) (end of the experiment). Error bars were calculated as standard deviation of the total absolute abundance of the selected bacteria of duplicate Control and AC columns.

As is evident in **Figure 4.6**, there was overall higher absolute abundance of bacterial genera containing both, putative human pathogens and faecal indicator bacteria in the AC-amended sand column after filtration (AC AF) compared to the same column before filtration (AC BF) as well as to the sand column after filtration (Control AF). For the sand column, both figures clearly show that there was no obvious difference between the overall absolute abundances in the sand column after filtration and before filtration. These observations suggested that the microbes could be retained better in the AC-amended sand than in sand alone. **Figure 4.6a** shows that *Shigella* predominated in the original sand (Control BF) but then was taken over by *Legionella* after the filtration experiment (Control AF). Whereas in the AC medium, *Legionella* predominated both before and after filtration, but with substantial growth over the duration of the experiment. For faecal indicator bacteria **(Figure 4.6b)**, *Escherichia* predominated in Control columns before and after filtration. *Streptococcus* predominated in the AC columns before filtration and was taken over by *Escherichia* after filtration. A range of factors influence microbial migration in biofilters such as size, cell surface hydrophobicity, morphological characteristics and surface charge of microbes (Zhang *et al.*, 2022). Large-size and rod-shaped bacteria migrate slower than small-size and spherical or spiral bacteria, and elongated cells are more likely to attach to media particles than spherical types (Ma *et al.*, 2020; Zhang *et al.*, 2022). Consequently, rod-shaped bacteria like *Legionella* and *Escherichia* could more readily attach to the AC. In the view of surface hydrophobicity and surface charge, hydrophobic bacteria typically have weaker migration abilities than hydrophilic bacteria, while the surface charge of bacteria also determines their adsorption in the filter media (Liu *et al.*, 2020; Zhang *et al.*, 2022). *Legionella* has hydrophobic cell surface (Ariyadasa *et al.*, 2021), which decreases their transportability in the filter media. *Escherichia* are negatively-charged (Zhang *et al.*, 2022), which could attract them to the positively-charged AC used in this study **(Section 4.4.2)**.

4.4.6 Linkage of environmental parameters and microbial communities

Table 4.4 Combinations of up to 6 environmental variables from Column influent, Control column effluent and AC column effluent on days 2, 4 and 10, taken k variables at a time, yielding the best matches of 16S rRNA gene sequencing derived microbial community similarity matrices, and physico-chemical parameter similarity matrices for each k, as measured by weighted Spearman rank correlation ρ_s. The highest Spearman rank correlation was highlighted in bold.

k	Best variable combinations (ρ_s)		
1	NO_2^--N (0.464)	PO_4^{3-}-P (0.302)	pH (0.282)
2	**pH, NO_2^--N (0.571)**	COD, NO_2^--N (0.543)	DO, NO_2^--N (0.541)
3	DO, COD, NO_2^--N (0.553)	BOD, COD, NO_2^--N (0.548)	pH, NH_3-N, NO_2^--N (0.547)
4	pH, DO, NH_3-N, NO_2^--N (0.547)	DO, NH_3-N, NO_2^--N, PO_4^{3-}-P (0.532)	pH, DO, NO_2^--N, PO_4^{3-}-P (0.525)
5	pH, DO, NH_3-N, NO_2^--N, PO_4^{3-}-P (0.540)	pH, DO, COD, NO_2^--N, PO_4^{3-}-P (0.527)	pH, DO, BOD, COD, NO_2^--N (0.503)
6	pH, DO, COD, NH_3-N, NO_2^--N, PO_4^{3-}-P (0.493)	DO, BOD, COD, NH_3-N, NO_2^--N, PO_4^{3-}-P (0.484)	pH, DO, BOD, COD, NO_2^--N, PO_4^{3-}-P (0.482)

Global test, p-value = 0.17

Table 4.4 shows the outcome of BEST analysis in which combinations of environmental variables were considered at steadily increasing levels of complexity, i.e. *k* variables at a time (*k* = 1, 2, 3, 4, 5, 6), to explain the dependency of the microbial community composition on environmental parameters. In this analysis the microbial communities were characterized by the relative abundance OTU table at genus level obtained from the sequencing data. The single environmental variable (k=1) which best linked to the microbial communitity similarities was NO_2^--N (ρ_s = 0.464) and the next best was PO_4^{3-}-P, then pH. The best 2-variable combination involved pH and NO_2^--N. It showed higher correlation than any single variable and the highest Spearman rank correlation (ρ_s = 0.571) in the analysis. The correlations decreased for combinations of more than 2 variables. Microbial community characteristics in the column influent and effluents were found to be highly associated with the environmental variables pH and NO_2^--N, supporting the earlier discussions in **Section 4.4.1** that microbial processes depend on and alter pH and NO_2^--N concentrations between the column influent and effluents.

4.5 Conclusions

Through this work, we arrived at the following conclusions:

- AC-amended sand columns did not significantly improve overall nutrient and heavy metal removal relative to the sand-alone columns (Control). However, both types of biofilters polished the effluent of activated sludge treatment to meet Thailand's surface water quality standards.

- Faecal coliforms were effectively removed below 4000 CFU/100 mL standard in both Control and AC columns.

- Concentrations of acetaminophen, atrazine, diuron, sulfamethoxazole, tetracycline, and diclofenac in both AC and Control column effluents met the desired PNEC levels. Overall, there was a significantly higher removal of the monitored micropollutants by the AC columns as compared to the Control columns.

- AC-amended sand columns improved the removal of ammonia-nitrogen, diuron, diclofenac, enrofloxacin as compared to the Control columns.

- AC-amended sand columns showed higher removal of bacterial genera containing putative human pathogens and faecal indicator bacteria than the Control columns.

- The biofilters which mimic unplanted vertical SFCWs could remove chemical pollutants such as ammonia-nitrogen, nitrite, phosphate and a range of micropollutants, and microbiological pollutants such as faecal coliforms, putative human pathogens and faecal indicator bacteria with improved removal by the AC-amended biofilters for ammonia-nitrogen, microbiological pollutants and organic micropollutants. This shows similar outcomes as the CW treatment (Chapter 3), despite different types of wastewater influent.

- An AC-amended sand biofilter could be an alternative nature-based treatment technology to a horizontal flow CW in a location where land availability is limited. However, the biofilter treatment efficiency can still vary with different wastewater influent characteristics and the implementation should be considered with regards to final effluent standards required in an individual location.

Data availability: 16S sequencing data generated in this project has been submitted to the NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA843575. Additional data created during this research are openly available (https://doi.org/10.25405/data.ncl.20102765). Please contact Newcastle Research Data Service at rdm@ncl.ac.uk for access instructions.

Chapter 5

Coconut husk biochar amendment enhances nutrient retention by suppressing nitrification in agricultural soil following anaerobic digestate application

Chapter 5. Coconut husk biochar amendment enhances nutrient retention by suppressing nitrification in agricultural soil following anaerobic digestate application

5.1 Abstract

Anaerobic digestate and biochar are by-products of the biogasification and pyrolysis of agricultural wastes. This study tested the hypothesis that combined application of anaerobic pig/cattle manure digestate and coconut husk (CH) biochar can improve soil nutrient conditions, whilst minimizing atmospheric and groundwater pollution risks. Microcosms simulated digestate application to agricultural soil with and without CH biochar. Ammonia volatilization and nutrient leaching were quantified after simulated heavy rainfalls. Archaeal and bacterial community and abundance changes in soils were quantified via next generation sequencing and qPCR of 16S rRNA genes. Nitrifying bacteria were additionally quantified by qPCR of functional genes. We found that CH biochar retarded nitrate leaching via slower nitrification in digestate-amended soil. CH biochar reduced both nitrifying archaea and bacteria abundance in soil by 74-83 percent in the top 4 cm soil layer and 66-73 percent in the deeper soil layer one month after the digestate application. Methanotroph abundances were similarly reduced in the CH biochar amended soils. These findings demonstrate combined benefits of anaerobic digestate and CH biochar application which are relevant for the development of a more circular rural economy with waste minimization, renewable energy production, nutrient recycling and reduced water pollution from agricultural land.

5.2 Introduction

Due to the rapidly increasing world population, there has been an increasing demand of agricultural products for human well-being (Zakaria, 2018). Consequently, large volumes of agricultural wastes are being generated especially in developing countries where agriculture is an influential sector to their economy (Zakaria, 2018; Durga *et al.*, 2021; Koul *et al.*, 2022). Global demand for livestock products is expected to double by 2050 in Asia, Africa and Latin-America (Scholten *et al.*, 2013). There will therefore be a significantly higher number of livestock farms with waste generation as animal manure and slurry. These readily biodegradable agricultural wastes can be transformed into biogas through anaerobic digestion (Holm-Nielsen *et al.*, 2009). Anaerobic digestion also creates a nutrient-rich liquid by-product known as anaerobic digestate (Holm-Nielsen *et al.*, 2009; Brown *et al.*, 2020). The digestate can be used as a bio-fertilizer and save farmers the cost of artificial fertilizers

(Lukehurst *et al.*, 2010). According to previous research, digestate provides higher potential benefits for nitrogen (N) availability and crop yields compared to untreated animal manures (Möller and Müller, 2012). However, the application of digestate as fertilizer needs to comply with the codes of good agricultural practice and regulation in each country as a digestate can contain harmful contaminants such as pathogens and heavy metals which could pose environmental concerns, and animal and human health issues (Lukehurst *et al.*, 2010). Besides, there have been concerns over ammonia volatilization and nutrient leaching from soils to groundwater after digestate application (Lukehurst *et al.*, 2010). This is attributed to rapid ammonification of organic nitrogen followed by nitrification of ammonia into the more soluble and leachable nitrogen compound, nitrate (Wang *et al.*, 2015). About 50-70% of nitrogen in fertilizer may be lost to nitrification related processes (Singh and Verma, 2007). Nitrification involves the oxidation of nitrogen compounds in a two-step process in which ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB), e.g., *Nitrosomonas* and *Nitrosospira*, and ammonia-oxidizing archaea (AOA). Subsequently, nitrite is converted to nitrate by nitrite-oxidizing bacteria (NOB), e.g., *Nitrobacter* and *Nitrospira* (Singh and Verma, 2007; Wang *et al.*, 2015; Han *et al.*, 2018). Some *Nitrospira* species are also capable of oxidizing ammonia to nitrate on their own in both, water and soil systems (Pjevac *et al.*, 2017).

Water pollution control and nutrient recovery via adsorption is feasible using a wide variety of biosorbents derived from waste biomass (Takaya *et al.*, 2016). Biochar, a carbon-rich material, is one of these biosorbents. It is produced by heating biomass feedstock such as wood and agricultural waste through pyrolysis or biogasification for renewable energy generation (Cole *et al.*, 2012). Different feedstock sources and pyrolysis process conditions contribute to different structural and physical characteristics of biochar including structural complexity, surface area, porosity, particle size distribution, density and mechanical strength (Lehmann and Joseph, 2015). Biochar can play an important role in enhancing nutrient retention in soil mostly due to its surface charge density (Kongthod *et al.*, 2015). Biochar mostly has negatively charged surfaces which increases the adsorption capacity of cation species (Lou *et al.*, 2016). Biochar has gained interest in the multidisciplinary areas of global warming mitigation, soil amendment, crop production enhancement and carbon sequestration (Glaser *et al.*, 2002; Laird, 2008; Tan *et al.*, 2015). It has great potential for improving soil fertility (Ahmad *et al.*, 2014). This can be partially attributed to effects on soil microbiology that reduce fertilizer losses via leaching (Atkinson *et al.*, 2010; Tan *et al.*, 2015).

In past decades, nitrogen-related problems and their remediation have preoccupied many researchers. Several strategies such as using slow-release fertilizers and the addition of synthetic nitrification inhibitors to fertilizer have been investigated to reduce the risk of nitrate leaching and improve N-use efficiency in agricultural systems (Singh and Verma, 2007; Lu *et al.*, 2019). However, nitrification inhibitors are considered too expensive for large-scale applications and nitrification inhibitors synthesized from chemical compounds may also cause phytotoxicity problems (Zerulla *et al.*, 2001). Several studies have shown that the nitrification process in soil could be altered by biochar amendment due to its effect on soil geomicrobiology (DeLuca *et al.*, 2006; Song *et al.*, 2014; Bi *et al.*, 2017). Wang *et al.* (2015) found that nitrification was retarded by peanut shell biochar amendment in an acidic orchard soil. The utilisation of biochar as a nitrification inhibitor could be a promising option for N-management in agriculture, which would be particularly relevant in co-application with a rich source of reduced nitrogen such as anaerobic digestate. Such a co-application would facilitate multi-use systems of waste by integrating two residues (biochar and digestate) of bioenergy generation from different types of agricultural waste for re-use in sustainable agriculture. There have been reports on the effect of biochar or digestate application alone on soil microbiology (Anderson *et al.*, 2011; Xu *et al.*, 2016a; Gielnik *et al.*, 2019) and on the impact of combined biochar and digestate application in soil on aspects such as greenhouse gas reduction, carbon sequestration, plant growth and microbial respiration (Marchetti *et al.*, 2012; Martin *et al.*, 2015; Mukherjee *et al.*, 2015; Udall *et al.*, 2017; Cardelli *et al.*, 2018). However, very little is known regarding the soil microbial community response, especially nitrification, following combined application of digestate and biochar.

5.2.1 Aim

To investigate the effect of combined application of digestate with coconut husk (CH) biochar on nutrient retention, nitrification and nitrifying bacteria and archaea abundance in agricultural soil.

5.2.2 Objectives

1. To determine sorption coefficients (K_d) for ammonium (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N), total nitrogen (TN), organic nitrogen (N_{org}) and phosphate (PO_4^{3-}-P) in the biochar and soil-amended synthetic digestate solution and to estimate K_d of 10% (w/w) biochar amended soil.

Hypothesis: CH biochar amended soil enhances all nutrient sorption.

2. To investigate the impact of CH biochar in digestate-amended soil on ammonia volatilization and nutrient leaching.
Hypothesis: CH biochar reduces ammonia volatilization and nutrient leaching in digestate-amended soil.

3. To investigate the impact of CH biochar in digestate-amended soil on the abundance of nitrifying and methanotrophic microbes.
Hypothesis: CH biochar reduces the abundance of nitrifying and methanotrophic microbes in digestate-amended soil and thereby the rate of nitrification.

5.3 Materials and methods

5.3.1 Biochar production

This project was initiated as part of a UK-Thailand collaborative investigation into the valorisation opportunities for coconut husk (CH) biochar produced by an inexpensive oil drum kiln method that is accessible to low-income farmers. The details of biochar production at Kasetsart University/KMUTT University, in Thailand are discussed in our previous study (Khawkomol *et al.*, 2021) and also summarised in **Section C1, Appendix C** in this study. The biochar had pH of 9.8 with elemental composition including 68.4% C, 3.53% H, 27.8% O, 0.06% N and 0.15% S. The biochar BET surface area was 11 m^2/g and total porosity was 0.92. A well-homogenized, composite biochar sample was used for the experiments. The biochar was ground using a mortar and pestle and then sieved, and the <212 μm particle size fraction was used for the experiments.

5.3.2 Sampling of soil and digestate

Due to foreign soil and biohazardous waste import restrictions, anaerobic dairy/pig slurry digestate and an agricultural clay loam soil were obtained from Cockle Park farm in Morpeth, Northeast England (Latitude 55°12’56.7”N, Longitude 1°41’02.6”W). However, biogas technology is nowadays also well developed in the Thai swine farm industry (Wongsapai *et al.*, 2008), and clay loam is a common soil type in Thailand (Tsubo *et al.*, 2007). Clay loam soil with a pH of 6.4 was collected from arable land at approximately 0-10 cm depth. The digestate was collected from a 650 m^3 mesophilic anaerobic digester (41 ^oC), which was digesting mainly slurries of pig and cattle manure, which was sometimes augmented with energy crop and food residues. The digester was a continuous stirred-tank reactor (CSTR), in which feedstock materials were fed in every hour at a rate of approximately 0.4 m^3 per hour.

5.3.3 Characterization of digestate

We measured digestate pH using a pre-calibrated Jenway pH Meter 3310 and nutrient characteristics including total nitrogen (TN), ammonium (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N) and phosphate (PO_4^{3-}-P) from 1:100 distilled water diluted digestate using cuvette tests LCK338, LCK302, LCK340, LCK341, and LCK350, from HACH LANGE (Laser House, Manchester, UK), respectively. Tests were performed following the manufacturer's instructions and measured in a HACH DR6000 Ultraviolet and Visible Spectrum Spectrophotometer. Organic nitrogen (N_{org}) was calculated according to $N_{org} = TN - (NH_4^+ - N + NO_3^- - N + NO_2^- - N)$. For quality assurance, we verified the cuvette tests with a blank solution (DI water) and known concentration standards prepared from the respective nutrient salts to assure that the result from cuvette tests agreed with the standard concentration by ±5%. In addition, we prepared a synthetic digestate solution for the sorption experiments **(Section 5.3.4)** to facilitate mass balance and sorption coefficient calculations in a well-defined system. The synthetic digestate was prepared from NH_4Cl, $NaNO_3$, $NaNO_2$, urea and Na_2HPO_4 salts as explained in **Table C1, Appendix C** based on typical digestate nutrient characteristics (Kizito *et al.*, 2015; AHDB, 2017; Wrap, 2018). Although, oxidized forms of nitrogen (NO_3^- and NO_2^-) would not be favoured in anaerobic digesters, they were also added to the synthetic digestate solution and measured for the N_{org} calculation. Its nutrient compositions and pH were measured directly from the solution following the same procedures as for real digestate. The characteristics of the digestate and synthetic digestate solution used in this study were comparable with literature reports **(Table 5.1)**.

Table 5.1 Comparison of nutrient characteristics and pH values of real digestate used in this study, digestate reported in literatures and a synthetic digestate solution used for sorption batch experiments in this study. Results were reported as Mean±S.D for duplicates of the real digestate and the synthetic digestate solution used in this study.

Parameter	Unit	Real digestate used in this study	Real digestate reported values (Kizito *et al.*, 2015; AHDB, 2017)	Synthetic digestate solution
NH_4^--N	(mg/L)	1630 ± 298	1390-1450	1410 ± 73.7
NO_3^--N	(mg/L)	135 ± 20.5	47-54	54.7 ± 4.51
NO_2^--N	(mg/L)	13.4 ± 4.15	34-56	52.6 ± 1.42
TN	(mg/L)	3450 ± 500	3600-4800	3810 ± 161
N_{org}	(mg/L)	1680 ± 773	2129-3240	2300 ± 219
PO_4^{3-}-P	(mg/L)	281 ± 153	15-20	12.3 ± 3.53
pH	-	8.05 ± 0.250	8-8.3	7.78 ± 0.110

5.3.4 CH biochar and soil sorption experiments

We conducted sorption experiments using the synthetic digestate solution, which was initially prepared with $NaNO_3$, $NaNO_2$ and Na_2HPO_4. We then autoclaved the solution in a Rodwell autoclave at 121 °C for 15 minutes to minimize the potential of biodegradation in the sorption batch tests. NH_4Cl and urea, which were found to be unstable in the autoclaving process, were added to the autoclaved solution. We adjusted the solution pH to mimic typical digestate pH around 8 using NaOH 1 N at 0.91 mL. We measured the pH using a Jenway pH Meter 3310. We measured the concentration of each nutrient parameter in the synthetic digestate solution again after preparation **(Table C7, Appendix C)** by HACH LCK cuvette tests, as detailed in **Section 5.3.3**. We used the synthetic solution for sorption experiments promptly after the addition of urea and NH_4Cl. We sterilized the soil sample in an oven at 104 °C for 20 hours. We mixed 1.5 g of CH biochar or soil with 30 mL of the synthetic digestate solution. We measured the pH of each mixture before placing them on a Stuart Orbital Shaker SSLI running at 101 RPM for 16 hours. At the end of 16 hours, we measured the pH of each mixture again. We then filtered each sample using a sterile 0.20 μm cellulose acetate syringe filter (0.20 μm, 25 mm; VWR International, UK). We performed the batch experiment in duplicate and with two sets of controls. Control A contained only 30 mL the synthetic digestate solution and Control B contained 1.5 g of CH biochar or soil with 30 mL of distilled water to measure the nutrient release from the biochar or soil. The same procedures as in **Section 5.3.3** were followed for nutrient measurement from the filtrates, but no dilution was necessary for Control B. The concentration of each nutrient parameter from the biochar/soil sorption experiment was used to calculate a linear sorption coefficient (K_d)

(Eq.1). The derivation of the K_d equation, which considered in a mass balance both, the amount of nutrient associated with the biochar/soil and added to the batches as synthetic digestate, is shown in **Section C1, Appendix C**.

$$K_d = \frac{(C_i \times V_w + C_{eq,cont} \times V_w - C_{eq} \times V_w)}{m \times (C_{eq} - C_{eq,cont})} \quad \text{(Eq.1)}$$

Where: K_d = Sorption coefficient (L/kg), C_i = Initial concentration of nutrient in solution (mg/L), V_w = Volume of solution (L), $C_{eq,cont}$ = Equilibrium concentration of the nutrient in control batches with the sorbent and distilled water (mg/L), m = Mass of adsorbent (kg) and C_{eq} = Equilibrium (final) concentration of the nutrient in batches with the sorbent and synthetic digestate solution (mg/L).

5.3.5 Ammonia volatilization and leaching experiments

We conducted the experiments with soil microcosms placed over glass beakers for the leachate collection. Two soil microcosms with CH biochar (CH systems), and two soil microcosms without the biochar amendment (Control systems), were set up within closed polyethylene containers, which additionally contained an acidified distilled water trap for capturing gaseous ammonia during volatilization experiments **(Figure 5.1)**. The CH system contained 300 g of soil homogeneously mixed with 30 g of CH biochar and 30 mL of digestate was then applied on the soil surface based on a recommended application rate of 30 m^3/ha (Clarke, 2018) in clay flowerpots. The Control system contained 300 g of soil with 30 mL of digestate applied on top in clay flowerpots. These pots had a hole for water drainage by gravity at the bottom and were placed on a glass beaker to collect leachate. We conducted two types of experiments as follows:

(a) Ammonia volatilization experiment: to trap ammonia (NH_3) lost by volatilization from the soil as ammonium, a liquid trap containing 100 mL of distilled water acidified with 0.54 µL of 0.1 N HCl to a pH value of 3 was added to the enclosed systems. We opened the container's lid briefly to take the liquid sample for ammonium measurements and closed the lid immediately. The ammonium in the trap was measured as Mean±S.D from the duplicate CH and Control systems using cuvette test LCK302 every 2 hours for a 6-hour period, except for the first and last experiments which were monitored longer **(Table C9, Appendix C)**.

(b) Leaching experiment: rainfall was simulated by adding 70 mL of distilled water to soil every 1 hour for a 4-hour period, based on universal high rainfall intensity at approximately 7 mm/hour and the rate in Thailand at about 28-35 mm/day (TMD, 2016; Prakosa *et al.*, 2018).

Leachate freely draining from the flowerpots into beakers was then collected to be analysed for volume, pH and nutrient concentrations (TN, NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P and N_{org}) as Mean±S.D from the duplicate CH and Control systems using the same procedure for pH and nutrient measurement as detailed in **Section 5.3.3**. We carried out the experiments over a period of one month. Volatilization following digestate application to the CH and the Control systems was measured on days 1, 2, 11 and 28, and leaching following simulated heavy rainfall events was measured on days 7, 9, 16 and 30 to investigate CH biochar effects on nutrient losses from the digestate fertilized soil. The total amounts of each nutrient parameter initially in the CH and Control systems were calculated from the sum of the mass of each nutrient species (TN, NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P and N_{org}) in soil, CH biochar and digestate at the beginning of the experiments in order to account for the ammonia volatilization and nutrient leaching data as a percentage of the total nutrient mass initially present in the system. Results of the total mass of each nutrient in each system are provided in **Table C2, Appendix C**.

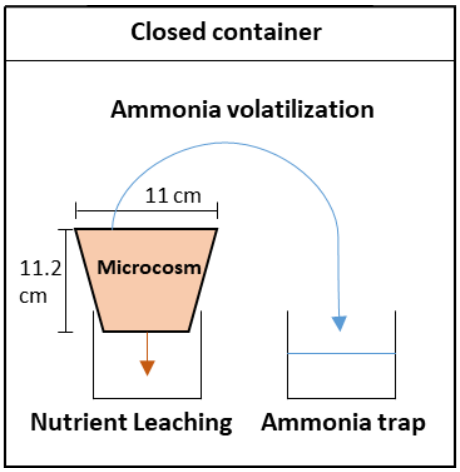

Figure 5.1 Schematic diagram of the sampling system for ammonia volatilization and nutrient leaching.

5.3.6 Molecular microbiology analysis

After 30 days, we extracted DNA from the top and bottom half of the soil microcosms and analysed it using 16S rRNA gene sequencing for microbial community characterization and qPCR of marker genes (amoA) to quantify microorganisms involved in the N cycle. We extracted the total DNA from the biomass contained in the top and bottom soils using the FASTDNA Spin Kit for soil according to the manufacturer's instructions (MPBiomedicals, Santa Ana, CA, USA). We then measured DNA concentration using a Qubit® dsDNA HS Assay Kit (Invitrogen, Life Technologies, Paisley, UK) and stored the DNA samples at -20

^{o}C until further use. Total extracted DNA was submitted for sequencing (paired end sequencing; 2 × 250 bp) in duplicate with an Illumina Miseq platform at the Department of Applied Biology, Cellular and Molecular Sciences, Northumbria University, UK using the primer set targeting the V4 region of the prokaryotic16S rRNA gene, as described elsewhere (Kozich *et al.*, 2013; Acharya *et al.*, 2019). The amplicon sequencing data from Illumina were processed using an open-source software package; Quantitative Insight Into Microbial Ecology, QIIME 2 (http://www.qiime.org). Denoising and de-replication of pair end sequencing, including chimera removal and trimming of reads based on positional quality scores, were performed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (Callahan *et al.*, 2016). The quality filtered sequences were clustered into ASVs (amplicon sequencing variants) by using the VSEARCH clustering method, which were then converted into OTUs (operation taxonomic units), with a threshold of 97% identity (Rognes *et al.*, 2016). Finally, the taxonomy for each OTU was assigned by matching against the GreenGenes database (v13_8), based on a naïve Bayesian classifier with default parameters.

We quantified genes for total bacteria (16S) and ammonia oxidizing bacteria (AOB) (amoA gene) by real time PCR assays (qPCR) on a BioRad CFX C1000 system (BioRad, Hercules, CA USA) using the primers shown in **Table C3, Appendix C**. Regarding quantification of the target genes, DNA samples were firstly diluted to a working solution of 10 ng/uL to prevent inhibitor effects. 2 µL of the DNA samples, 7.5 µL of SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad), 4 µL of nuclease free water (Invitrogen, Life Technologies, Paisley, UK), and 0.75 µL of each forward and reverse primer solutions (@ 10 micromol·L^{-1}) were then combined for a 15 µL final volume with 500 (nmol·L^{-1}) of each primer. Reaction condition for quantification of 16S genes was 98 °C for 3 minutes (1x), then 98 °C for 15 seconds, and the Primer Annealing Temperature of 60 °C for 30 seconds (40 cycles). While reaction condition for quantification of amoA genes was 98 °C for 3 minutes (1x), then 98 °C for 5 seconds, and the Primer Annealing Temperature of 56 °C for 10 seconds (40 cycles). We produced standard curves using synthesized nucleotide sequences of the target genes (Invitrogen, Life Technologies, Paisley, UK) every time a qPCR analysis was performed, in parallel with the amplification of test samples. Serial dilution (10-fold) of the standards was performed to obtain standard solutions in the range of 10^{8}–10^{1} target gene copies/µL. All samples were run in duplicate and molecular grade H_2O replaced template in control reactions. There were amplifications in the control reactions in the 16S assay likely due to spurious contaminants. But the quantification-cycle (Cq) values of

these controls were higher than the highest Cq value of the standards (standard 1 for 10 gene copies/μL) and were also all substantially higher than the highest Cq values of the samples. There was no amplification in the control reactions for the amoA assays.

We rarefied sequencing libraries at 45,000 reads per sample and performed multivariate statistical analysis for OTUs classified to genus level, and grouped at this level, using Matlab© (Version R2019b, Mathworks, Portola Valley, CA, USA) for cluster and principal component analysis with Euclidean distance as the similarity metric. We performed one-way analysis of similarities (ANOSIM) on Matlab© with the Fathom Toolbox developed by the Marine Resource Assessment Program at the University of South Florida's College of Marine Science (Jones, 2015) to predict the p-value and R-value of ranked dissimilarities between versus within groups of samples. For sequencing data processing, in order to calculate absolute abundance of the studied microorganisms, the accurate number of 16S rRNA genes per genome of each microorganism was required. The data were obtained from the ribosomal RNA operon copy number database (http: //rrndb.umms.med.umich.edu/) with the following details
AOB, AOA, NOB: 1 copy of 16S/genome**,** Methanogens: 3 copies of 16S/genome
Methanotrophs: 2 copies 16S/genome.

We performed two-tailed t-tests in excel to evaluate the null hypothesis that there is no difference between the mean values of two sample groupings of interest.

5.4 Results and discussion

5.4.1 CH biochar and soil sorption experiments

Table 5.2 Comparison of K_d (L/kg) of NH_4^+-N, NO_3^--N, NO_2^--N, TN, Urea-N_{org} and PO_4^{3-}-P in the biochar/soil-amended synthetic solution batch experiments and estimated K_d (L/kg) of the 10% (w/w) biochar amended soil. Results (Mean±S.D.) were reported to two decimal places.

	NH_4^+-N	NO_3^--N	NO_2^--N	TN	Urea-N_{org}	PO_4^{3-}-P
$K_{d,biochar}$ (L/kg)	3.43±0.99	1.90±1.07	n/a	3.08±1.66	2.99±2.85	n/a
$K_{d,soil}$ (L/kg)	0.80±0.43	0.57±1.38	0.56±0.22	0.78±0.50	0.78±0.71	68.11±20.40
$K_{d,amended\ soil}$ (L/kg)	1.06±0.40	0.70±1.25	0.51±0.19	1.01±0.48	1.00±0.71	61.30±18.36

*n/a = not available

K_d measurements were performed to characterize the sorption of NH_4^+-N, NO_3^--N, NO_2^--N, TN, Urea-N_{org} and PO_4^{3-}-P by CH biochar and soil, and to estimate the CH amendment effect on nutrient sorption **(Table 5.2)**. For NH_4^+-N, NO_3^--N, TN and Urea-N_{org} the measurements supported the initial research hypothesis that soil amendment with biochar (K_d, amended soil) could enhance nutrient retention. However, the anticipated impact, calculated using **Eq.4 in Appendix C** for K_d, in amended soil was small **(Table 5.2)**. The biochar K_d values for NH_4^+-N, NO_3^--N, TN and Urea-N_{org} showed similar and low K_d values, measuring 3.43±0.99 L/kg, 1.90±1.07 L/kg, 3.08±1.66 L/kg and 2.99±2.85 L/kg, respectively. Adsorption of NO_2^--N, and PO_4^{3-}-P were too low for the derivation of a K_d value **(Table C7, Appendix C)**.

Biochar has heterogeneous surface properties with both, hydrophobic and hydrophilic characteristics, containing polar and non-polar surface sites which therefore can attract both polar and non-polar compounds (Hale *et al.*, 2013; Ebrahimzadeh Omran *et al.*, 2020). The adsorption of nutrients is normally controlled by the biochar surface chemistry (Lehmann and Joseph, 2015). NH_4^+-N adsorbed on CH biochar could be by electrostatic adsorption to negatively charged oxygen-containing surface functional groups, associated with cation exchange capacity (CEC) (Liu and Zhang, 2009; Tan *et al.*, 2015). The CH biochar had H and O contents of 3.53% and 27.8%, respectively **(Section 5.3.1)**, implying the existence of hydroxyl (O-H) and other oxygen-containing functional groups such as C-O to form complexes on the biochar surface. These functional groups provided opportunity for cation, e.g., NH_4^+-N adsorption (Lui and Zhang, 2009).

CH biochar could also moderately adsorb NO_3^--N. Although it was earlier explained that sorption to biochar is mainly governed by its CEC, anion exchange sites may coexist on the heterogeneous biochar surfaces. The condensed aromatic structures on the biochar are capable of generating positive surface charge, which presents some anion exchange capacity (AEC) (Lehmann and Joseph, 2015). Urea-N_{org} was also adsorbed by the biochar. Biochar contains both polar and non-polar surface sites which allows N_{org} attraction to both sites. Beesley *et al.* (2010) reported high adsorption of organic compounds to black carbon sorbents. However, urea is a small and polar organic molecule, which may explain its weak sorption to CH biochar.

Soil adsorbed all nutrients, however less than the biochar, except for PO_4^{3-}-P. A high K_d value was observed for PO_4^{3-}-P from soil. This is likely due to the soil physicochemical characteristics such as clay content, pH, and surface functional groups, e.g. Fe or Al oxides/hydroxides (Sparks, 2003). The adsorption of phosphate normally happens as inner-sphere complexes through a ligand exchange mechanism. The exchange is facilitated by elevating acidity and abundance of positive charges (Sparks, 2003). Additionally, Foth and Ellis (1996) reported that adsorption capacity of anions of monoprotic conjugate acids (a compound that can donate one proton) reaches a maximum when solution pH is close to the anion's pK_a. This agrees with the scenario for this experiment that the synthetic solution contained $H_2PO_4^-$ with a pK_a of 7.2 and the solution pH was 7.66 **(Table C8, Appendix C)**.

5.4.2 Ammonia volatilization and nutrient leaching

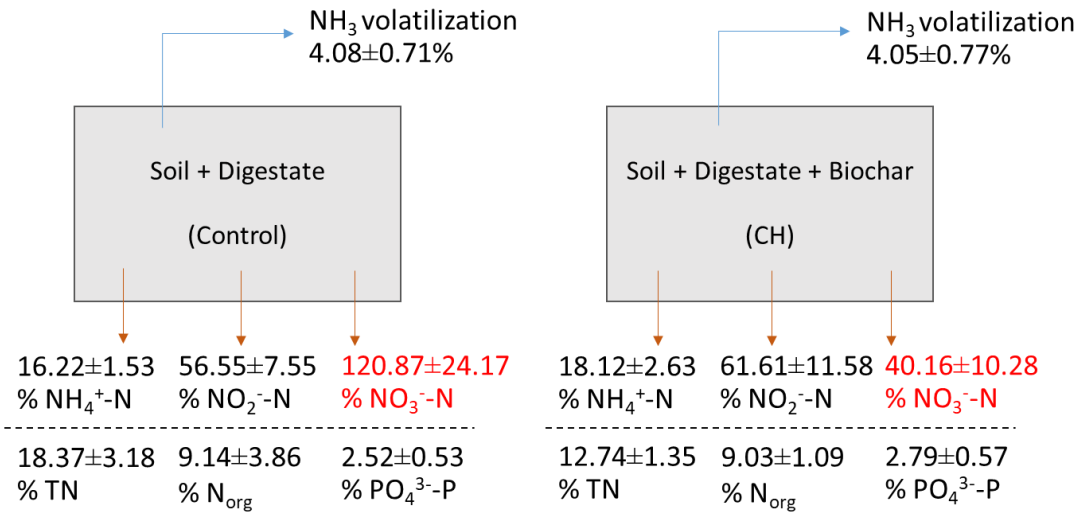

Figure 5.2 Total percentage of NH_4^+-N, NO_2^--N, NO_3^--N, TN, N_{org}, PO_4^{3-}-P initially in the systems which was lost by leaching (orange arrows) and ammonia (NH_3) volatilization (blue arrows) from digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH) after four repeated volatilization and leaching experiments. Results (Mean±S.D.) were reported to two decimal places. %NO_3^--N was coloured in red to emphasize the significant difference of the values between the two systems (t-test, p-value=0.0007).

Figure 5.2 shows the loss of each nutrient from the digestate-amended soil, and the digestate-amended soil with CH biochar, expressed as percentage of the amount of each nutrient initially present in each system. There was no significant difference between the ammonia volatilization from the CH and Control system (t-test, p-value=0.96), which was contrary to our hypothesis. This could be because the biochar did not significantly alter NH_4^+-N sorption **(Table 5.2)**, therefore had little impact on volatilization. Also, ammonia mainly volatilized from the digestate on the soil surface which had not had opportunity to interact with the soil or biochar amended soil, hence no difference could be noticed in the volatilization rates. Similarly, Sha *et al.* (2019) reported that on average, biochar addition to soil had no impact on ammonia volatilization. However, this varied with different soil, biochar and experimental conditions. Biochar applied to acidic soils following ammonium-based fertilizer could increase volatilization as a result of elevated soil pH and urea hydrolysis (Sha *et al.*, 2019). In contrast, using wood-based or acidified biochar at appropriate rates could mitigate ammonia volatilization following application of poultry litter or urea N fertilizer (Doydora *et al.*, 2011; Feng *et al.*, 2017). This could be attributed to several mechanisms including ammonia adsorption and microbial activities like microbially induced ammonia immobilization and nitrification (Mandal *et al.*, 2016).

For leaching, among the six nutrient parameters in each system, the lowest percent nutrient loss was for PO_4^{3-}-P in both systems, due to high PO_4^{3-}-P sorption by the soil **(Table 5.2)**. The highest percent nutrient loss via leaching was for NO_3^--N in the Control system and NO_2^--N in the CH system. TN showed the most significant loss in terms of absolute mass **(Figure C2, Appendix C)**. When looking at the effect of biochar, there was only one significant difference between the CH and Control system for the parameter NO_3^--N. Leached nitrate-N was significantly higher from the Control system at 120.87±24.17% of the amount initially present as compared to 40.16±10.28% in the CH system (t-test, p-value=0.0007). Our initial hypothesis of reduced nutrient leaching from the biochar amended soil was thus confirmed for this parameter only. It is noteworthy that NO_3^--N leaching in the Control system is more than 100% of the mass initially present in the system. This implies nitrate production which could be attributed to the nitrification of ammonia to nitrate. In most soils the nitrite produced by ammonia oxidizers does not accumulate but is quickly oxidized to nitrate by the nitrite-oxidizing bacteria, suggesting that complete nitrification can occur within a short period of time (Paul, 2007). Notably, NO_3^--N in the CH system leached less than in the Control system which implies that CH biochar could retard nitrification in digestate-amended soil. N_{org} adsorption by the biochar could have reduced the rate of microbial N mineralization and hence the rate of NO_3^--N leaching from the CH system (Laird *et al.*, 2010). Yao *et al.* (2012) studied nutrient leaching in sandy soil using peanut hull and Brazilian pepperwood biochar and found that both biochars, pyrolysed at 600 °C, could reduce ammonium and nitrate leaching, while peanut hull biochar showed no phosphate sorption ability. These effects of peanut hull biochar on leaching were consistent with the present study results using CH biochar. Another study using poultry litter-amended soil with pinewood biochar also found that such amendments reduced ammonium and nitrate leaching from sandy loam soil (Bohara *et al.*, 2019). In contrast, increased N leaching was also reported with biochar amendment associated with its application rate (Li *et al.*, 2018). Enhanced net N mineralization was observed in soil amended with N fertilizer and manure biochar which could cause higher nitrate leaching (Yoo and Kang, 2012). To better understand the microbiology of the Control and CH systems, 16S rRNA gene amplicon sequencing was performed for DNA extracted from the top and bottom soil layers at the end of the experiments.

5.4.3 Cluster and PCA analysis of the overall microbial community in the soils

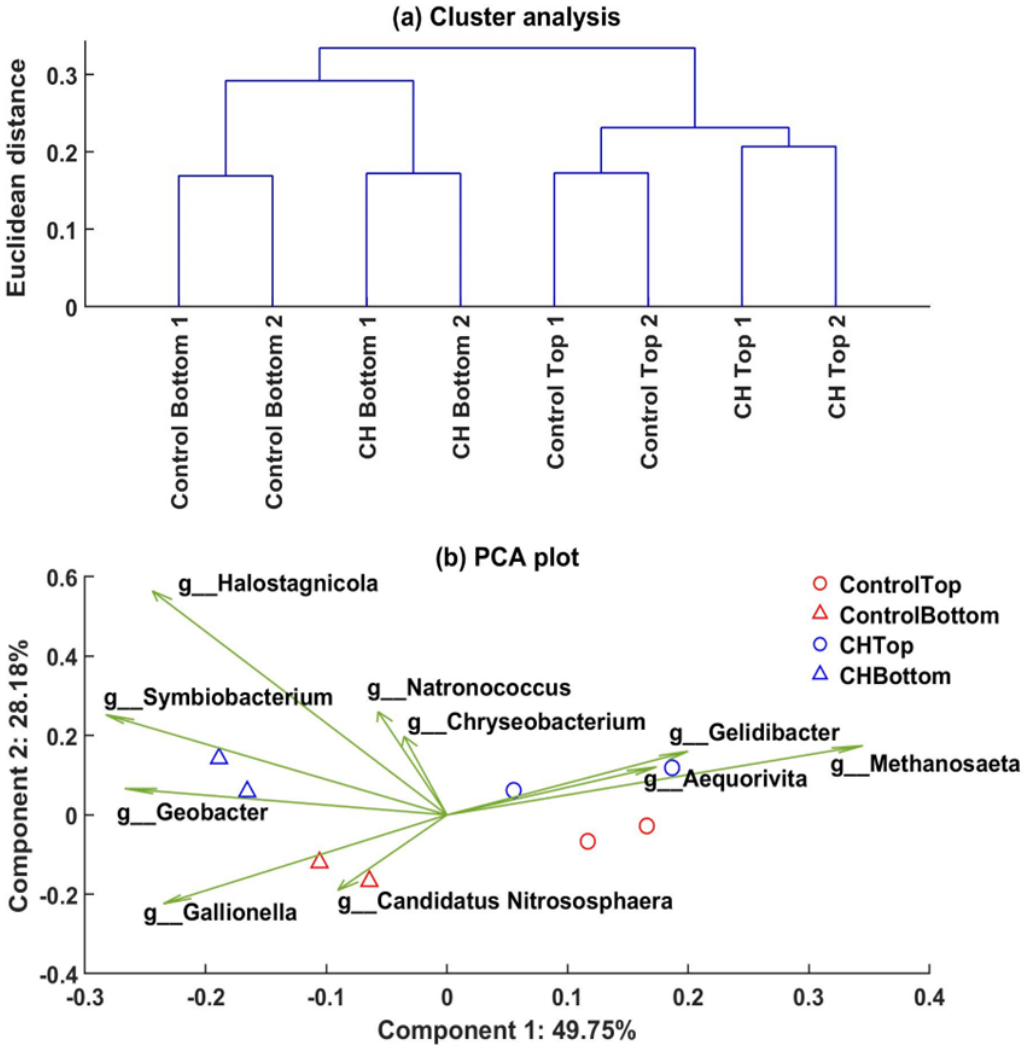

Figure 5.3 Cluster analysis (a) and Principal component analysis (PCA) (b) plots of the microbial community dissimilarity between the top and bottom soil samples of digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH). For PCA, the first two principal components (PC) (Component 1 and 2) were plotted showing the scores (circles and triangles) and top 10 loadings (genera, arrows) in the two-dimensional space. Percentage of variation accounted for by each principal component is shown with the axis label.

Cluster analysis **(Figure 5.3a)** shows the greatest dissimilarities for microbial communities were between samples from top and bottom soils, and then to a lesser extent in response to the biochar amendment. Sample replicates clustered most closely. One-way ANOSIM confirmed that top versus bottom soil was a significant factor in shaping the soil microbial communities (one-way ANOSIM, p-value < 0.05 and R= 0.80). In the principal component analysis **(PCA, Figure 5.3b)**, components 1 and 2 accounted for almost 78% of the observed variance between samples. Samples from top and bottom soils were separated along

component 1, while component 2 separated the Control from the CH system samples. Evidently, the digestate application to the surface of the soils was the most significant microbial community shaping factor, while biochar amendment became influential in shaping the microbial community response to the digestate application within each soil layer.
The ten most notable microbial genera (i.e., variables) in the PC1 and 2 space are illustrated by the green arrows in **Figure 5.3b**. These genera included *Candidatus Nitrososphera* and *Methanosaeta*, nitrifying archaea and methanogenic archaea, respectively. *Candidatus Nitrososphaera* was predominant in the Control bottom soil microbial community, while *Methanosaeta* was predominant in topsoil. The top 10 loadings for each PC 1 and 2 separately are presented in **Figure C3, Appendix C**. Clearly, the PCA highlighted nitrifying and methanogenic microbes as variables contributing to the sample dissimilarity showing that nitrifying and methanogenic microbes played an important role in the soil microbial community response to the digestate application. To confirm whether nitrification had likely occurred, and to study the biochar impacts on this process in more detail, the abundance of nitrifying microorganisms (AOB, AOA and NOB) was evaluated with a combination of sequencing and qPCR methods.

5.4.4 Abundance of nitrifying microorganisms

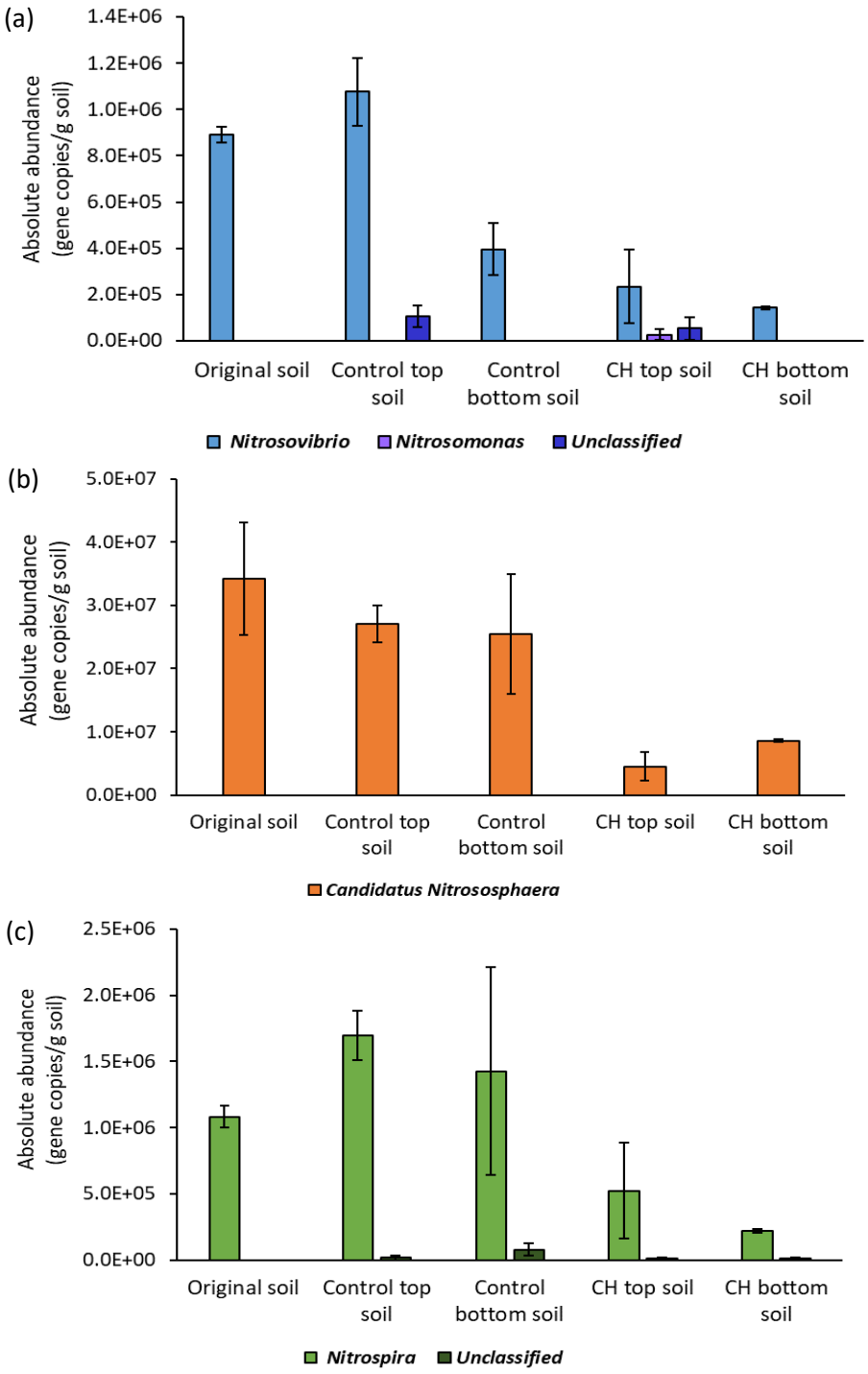

Figure 5.4 Absolute abundance (gene copies/g. of soil) of AOB (a), AOA (b) and NOB (c) in original soil, digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH). Data obtained from Illumina MiSeq 16S rRNA gene sequencing were combined with qPCR quantification of 16S rRNA gene copy numbers in each soil sample. Error bars were calculated as standard deviation in duplicate CH and Control systems.

There were fewer nitrifying microorganisms overall in both the top and bottom soil of the CH system compared to the Control system **(Figure 5.4)**, and one genus of each nitrifier was driving the abundance differences between Control and CH systems. **Figure 5.4a** shows that there was a lower mean AOB abundance in the CH system compared to the Control system. This demonstrates that the application of CH biochar with digestate led to the suppression of nitrifier populations, however, the difference was marginally not statistically significant (t-test, p-value=0.09). The ammonia-oxidizing genus of *Nitrosovibrio* was predominantly detected. *Nitrosomonas* was only detected in CH topsoil, and an unclassified species of the *Nitrosomonadaceae* family was only detected in Control/CH topsoil, indicating that some nitrifiers might have been introduced with the biochar and/or digestate. The abundance of AOA **(Figure 5.4b)** was generally more than one order of magnitude larger than the AOB in all soil samples. Overall, there was a significantly lower AOA abundance in the CH system compared to the Control system (t-test, p-value=0.01). CH topsoil had significantly lower AOA abundance than Control topsoil (t-test, p- value=0.03). Only the genus of *Candidatus Nitrososphaera* was found in both systems. Leininger *et al.* (2006) suggested that AOA are more numerous than AOB in soil, as was found in this study. The absolute abundance of NOB **(Figure 5.4c)** was similar to that of AOB. Overall, the NOB abundance was significantly reduced in the system with CH biochar compared to the Control system and the original soil (t-test, p-value=0.03 and 0.02, respectively). The *Nitrospira* genus was predominantly presented in both systems. There are alternative explanations for smaller nitrifier abundances in the CH system: (1) NH_4^+-N content is reduced through N_{org} immobilization by biochar, and the adsorption of NH_4^+-N as well as N_{org} by the biochar, which slows down N_{org} ammonification and ammonium availability for nitrification. Consequently, there is less NH_4^+ availability for oxidation by ammonia-consuming microbes and weakened nitrification in the soil (Wang *et al.*, 2015). (2) Leachable bio-oil compounds were formed during the biochar production, and released from the biochar into soil which may inhibit microbial activity and impede nitrification (Lee *et al.*, 2003; Wang *et al.*, 2013; Wang *et al.*, 2015). Clough *et al.* (2010) reported that nitrification rates decreased by adding wood biochar in pasture soils, which was attributed to a nitrification-inhibiting compound (α-pinene), a condensate product on the fresh biochar. (3) Biochar amendment to soil can affect moisture contents, hydraulic properties, and aeration in the soil (Novak *et al.*, 2012; Barnes *et al.*, 2014), which can all indirectly influence the fate of nutrients, soil microbiology and ultimately plant growth. The addition of biochar can increase or decrease soil water-holding capacity depending on biochar type and application rate as well as soil type (Devereux *et al.*,

2012; Barnes *et al.*, 2014), which will affect oxygen availability for nitrification. Complex soil-biochar-microbiota interactions may explain the variable literature reports of how biochar affects nitrification. Dempster *et al.* (2012) found that the rate of nitrification significantly decreased with *Eucalyptus marginata* biochar with either fertilizer N or compost amendment to soil, because of the limited substrate (NH_4^+-N) level in the presence of biochar in soil. However, Bi *et al.* (2017) found that soil nitrification was enhanced through the increased abundance of AOB in the combined application of rice straw biochar and nitrogen fertilizer like urea and $(NH_4)_2SO_4$. Prommer *et al.* (2014) also reported that the AOB community increased with wood biochar amendment to arable soils thus accelerated nitrification. Xu *et al.* (2014) indicated that the AOB abundance had not been affected by the rice straw biochar pyrolysed at 500 °C amended to an acidic soil.

Functional gene-specific qPCR (amoA) was also carried out **(Figure 5.5)** to confirm the abundance results of AOB derived from 16S rRNA gene sequencing.

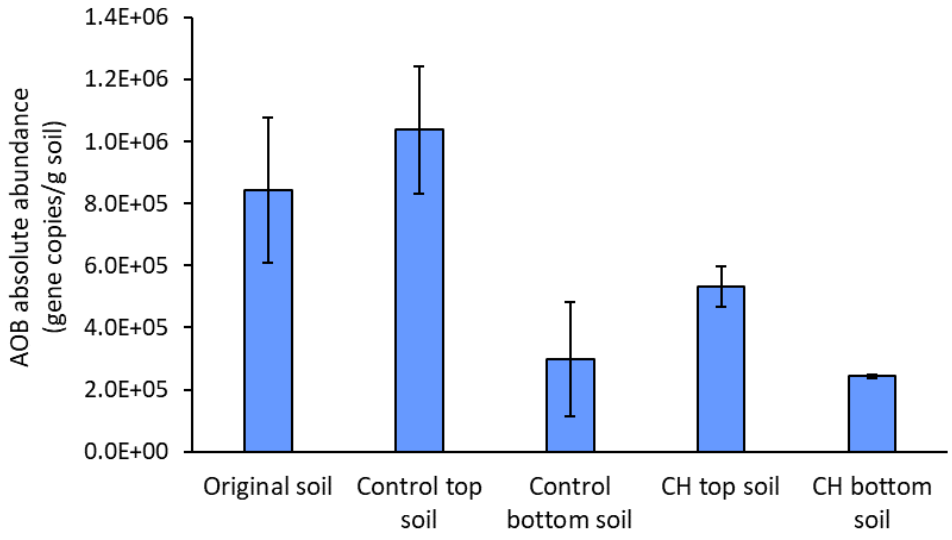

Figure 5.5 AOB absolute abundance (gene copies/ g. of soil) obtained using amoA qPCR in original soil, digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH). Error bars were calculated as standard deviation in duplicate CH and Control systems.

There was significantly lower amoA gene abundance in CH topsoil relative to Control topsoil (t-test, p-value=0.002). The abundances of AOB in every samples obtained by amoA-based methods yielded abundance estimates very similar to those obtained from 16S rRNA gene sequencing using Illumina MiSeq **(Figure 5.4a)**. Song *et al.* (2014) conducted a study using qPCR of the amoA genes targeting AOA and AOB, and reported contrary results to those in this study. In their study, the abundance of both AOA and AOB increased in soil amended with cotton stalk biochar after four-week incubation and the AOB were more abundant than

the AOA. Clearly, outcomes differ between studies, which may be attributed to variable biochar properties such as the characteristics of condensates formed from each biomass material under different pyrolysis conditions. A summary of the literature findings for different biochar types is provided **Table C11, Appendix C**. The summary shows that it is important to evaluate each biochar type separately before agricultural application.

5.4.5 Abundance of methanogens and methanotrophs

Methanogens are anaerobic prokaryotes belonging to the domain Archaea, which are responsible for methane production (methanogenesis) (Lew and Glińska-Lewczuk, 2018). Methanotrophs are microorganisms that oxidize methane as their sole carbon and energy source (methanotrophy) (Lew and Glińska-Lewczuk, 2018). Methanogenesis and methanotrophy take place simultaneously in the soil and such processes are associated with nitrification via ammonia oxidizers (Serrano-Silva *et al.*, 2014). The enzyme MMO used for methanotrophy is capable of binding to NH_4^+ and react with it, and methanogens can use NH_4^+ as their N source (Serrano-Silva *et al.*, 2014). Consequently, lower NH_4^+ substrate availability may reduce both methanotrophy and nitrification. Methanogens (genus *Methanosaeta*) were highlighted in the PCA of the overall microbial community **(Figure 5.3b)**. The abundance of methanogens and methanotrophs was therefore also analysed in more detail **(Figure 5.6)**.

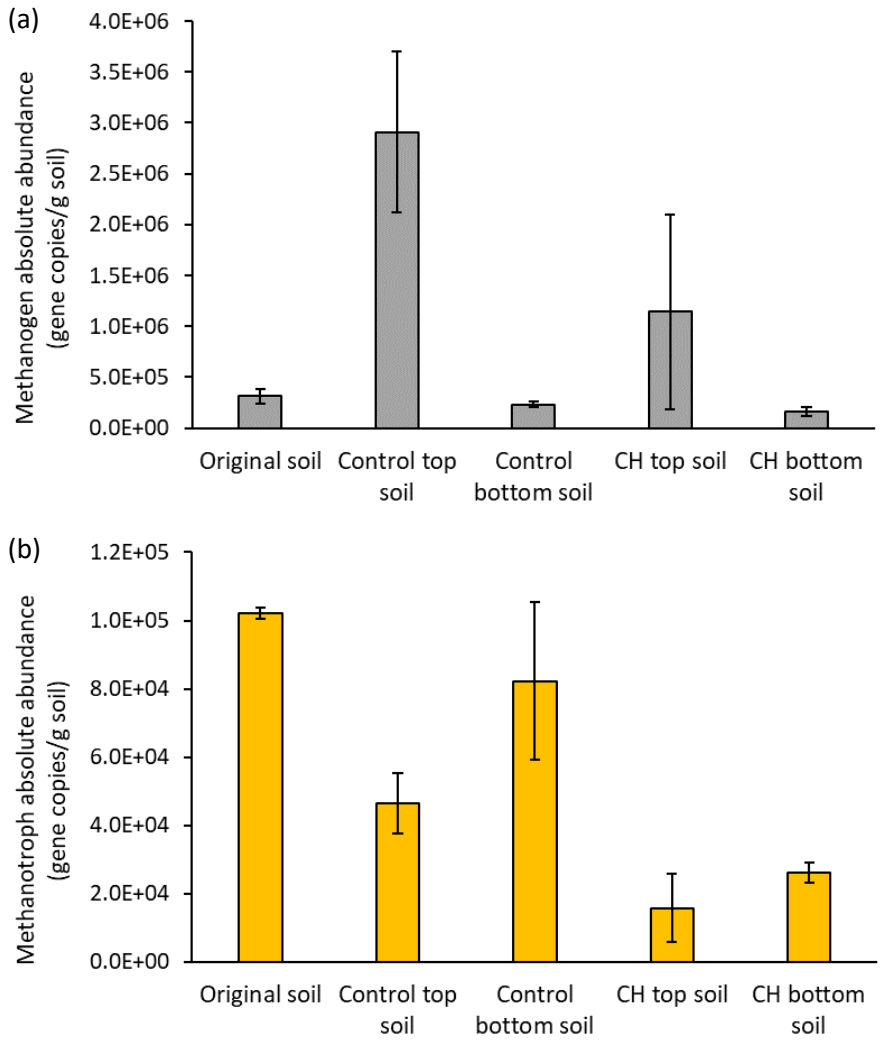

Figure 5.6 Absolute abundance (gene copies/ g. of soil) of methanogens (a) and methanotrophs (b) in original soil, digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH). Data obtained from Illumina MiSeq 16S rRNA gene sequencing were combined with qPCR quantification of 16S rRNA gene copy numbers in each soil sample. Error bars were calculated as standard deviation in duplicate CH and Control systems.

Figure 5.6a shows a higher mean methanogen abundance in the topsoil than in the original soil and in the bottom soil of the Control system. However, the differences were marginally not statistically significant (t-test, p-value=0.19 and 0.08, respectively). The application of digestate that contains methanogens to the soil surface can explain higher methanogen abundance in the topsoil layer. Additionally, most methanogens are able to function well in mesophilic environments (Garcia *et al.*, 2000) and the digestate used in this study was obtained from a mesophilic digester. Similarly, it was reported that the top 7-cm soil layers were the primary methane production and diffusion sites, whilst the deeper soil layers acted as the sink (Xu *et al.*, 2016b). However, even though the mean methanogen abundance was

higher in the topsoil of the Control as compared to the CH system, there was no statistically significant difference (t-test, p-value=0.39), indicating no impact of the biochar on methanogen abundances. Yuan *et al.* (2018) reported diverse biochar effects on methanogenesis. Wood chip biochar had little effect on methanogenesis in soil, whilst rice straw and manure biochar additions to soil enhanced methanogenesis remarkably due to the functional groups, mainly quinones, on the biochar surface. For methanotrophs **(Figure 5.6b)**, overall, there was lower methanotroph abundance in the CH system compared to the Control system (t-test, p-value=0.05). Thus, CH biochar reduced methanotroph abundance, which could be attributed to the following reasons: (1) the organic compounds released from biochar that inhibited nitrifiers also inhibited methanotrophs (Spokas, 2013). (2) Binding of organic compounds containing C/N from the digestate to the biochar led to less substrate availability for both methane and ammonium production, thus less substrate for methanotrophs and ammonium oxidizing bacteria, therefore less methanotroph and AOB abundances were detected in the CH systems. However, He *et al.* (2017) suggested that soils can also be more favorable for aerobic methanotrophs due to increased soil aeration by biochar addition. The addition of organic materials such as crop residues can diversely affect methanotrophic activity, depending on the C:N ratio of the materials (Serrano-Silva *et al.*, 2014). Notably, methanotrophs could be found in both top and bottom soil of the Control system, but with slightly higher abundance in the bottom soil. Taipale *et al.* (2009) revealed that methanotrophs can adapt to microaerophilic conditions as well as anaerobic conditions. Moreover, Hu and Lu (2015) indicated that nitrate addition promoted the abundance and activity of methanotrophs in soil. In this present study, enhanced nitrate that was produced by the nitrifiers percolated down through the bottom soil, as was evident from the leaching results **(Figure 5.2)**. This may have promoted higher methanotroph abundance at the bottom. The abundance of methanotrophs in both Control and CH systems would likely be smaller than that in the original soil because of the applied digestate, since ammonium, methane oxidizers, as well as aerobic metabolizers of the digestate would compete for oxygen as an electron acceptor. Consequently, the addition of a rich substrate (digestate) would likely impede methanotroph abundance and thus methane oxidation (Serrano-Silva *et al.*, 2014). Furthermore, CH biochar had higher pH than the soil **(Section 5.3.1 and 5.3.2)**, thereby raising the soil pH. As methanotrophs are more sensitive to elevated soil pH than methanogens (Jeffery *et al.*, 2016), CH biochar could have had a larger impact on the methanotrophs than the methanogens. A long-term study by Wang *et al.* (2019) reported that wheat straw biochar increased the abundances of both methanogens and methanotrophs in the

first year of study, mainly due to enhanced in-soil dissolved organic carbon, NH_4^+-N, and porosity. However, after three years, the abundances of methanogens decreased.

5.5 Conclusions

This is the first study applying chemical measurements and molecular microbiology tools in combination to report the effects of the combined application of biochar and anaerobic digestate on ammonia volatilization, nutrient leaching, and nitrification. Through this work, we arrived at the following conclusions:

- CH biochar amended soil slightly enhanced NH_4^+-N, NO_3^--N, TN and Urea-N_{org} sorption. We therefore rejected the hypothesis that CH biochar amended soil enhances all nutrient sorption.
- CH biochar addition had no effect on ammonia volatilization, but reduced nitrate leaching by slowing down nitrification in digestate-amended soil. We therefore could only partly accept the hypothesis that CH biochar reduces ammonia volatilization and nutrient leaching in digestate-amended soil.
- There were lower nitrifying and methanotrophic microbe abundances in the biochar-amended soil following digestate application. We therefore accepted the hypothesis that CH biochar reduces the abundance of nitrifying and methanotrophic microbes in digestate-amended soil.
- CH biochar could thus ultimately retard the rate of nitrification, retain nutrients longer for plant growth in the topsoil, reduce nitrate leaching during heavy rainfall events, and minimize groundwater pollution risks.
- The combined application of digestate with CH biochar is a promising biotechnology for sustainable agriculture, promoting the circular re-use of agricultural waste residues, in addition to renewable energy generation.

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Chapter 6

Conclusions, broader implications, and recommendations for future research

Chapter 6. Conclusions, broader implications, and recommendations for future research

6.1 Conclusions

This thesis provides potential nature-based solutions towards sustainable rural wastewater and agricultural waste management. With raising demands for water, food and energy by a growing global population and the rapid economic development of Asia, the water-food-energy nexus has become central to sustainable rural development (Biggs *et al.*, 2015; Zhang and Vesselinov, 2017). Conventional technologies to treat water are energy intensive, food production became the world's largest consumer of freshwater resources, and energy production not only consumes water for the mining of fossil fuels and cooling in thermal power stations, but increasingly also threatens agricultural production via man-made climate change (Zhang and Vesselinov, 2017; UN, 2018). It is therefore essential for innovation to provide more holistic solutions that break such detrimental interdependencies and invert them into win-win opportunities for more sustainable development.

For chapter 3, the co-treatment of mine water and wastewater in CWs removed heavy metals, nutrients, and pathogens to meet the desired river water quality standards under changeable weather conditions. The CWs showed efficient removal of phosphate and iron through synergistic interaction between phosphate from sewage and iron from mine water. They also effectively removed micropollutants like acetaminophen, caffeine and sulpiride during dry weather conditions. They effectively converted the distinct sewage treatment plant effluent/mine water microbiomes into freshwater microbiomes more similar to those of the receiving river. The CW treatment could consequently reduce impacts of the discharge on the ecological and recreational value of the river. This study filled in the research gap on simultaneous removals of nutrients, heavy metals, organic micropollutants and pathogens in CW cotreating wastewater and mine water.

For chapter 4, the AC-amended biofilter did not significantly improve overall nutrient and heavy metal removal, but improved the removal of putative human pathogens and micropollutants like diuron, diclofenac and enrofloxacin as compared to the sand-alone biofilter. Both types of biofilters polished the effluent of activated sludge treatment to meet Thailand's surface water quality standards. These findings therefore contributed to filling the research gap on the impact of AC-amendment in biofilters on improving the performance across a wide range of water contaminants.

For chapter 5, coconut husk biochar addition in digestate-amended soil had no effect on ammonia volatilization, but reduced the abundance of nitrifying and methanotrophic bacteria, and nitrate leaching via slower nitrification. The biochar amendment could thus help retain nutrients longer for plant growth in the agricultural topsoil and reduce nitrate leaching that results in groundwater pollution during heavy rainfall events. This study therefore contributed to reducing the knowledge gap regarding the nutrient retention in soil, and soil microbial community response following combined application of digestate and biochar

6.2 Broader implications of this research

As a first example for such win-win opportunities, this study showed co-treatment synergies for municipal wastewater and mine water in nature-based horizontal flow constructed wetlands (CWs) that will offer low energy and inexpensive treatment wherever the two waste streams are found in proximity, in addition to biodiversity benefits. The CWs showed efficient removal of phosphate and iron through synergistic interaction between phosphate from sewage and iron from mine water, and high removal of ammonia-nitrogen. They effectively converted the combined sewage treatment plant effluent/mine water microbiomes into freshwater microbiomes whilst removing putative human pathogens and faecal indicator bacteria. Wastewater polishing in CWs will thus better protect the ecosystem and recreational value of rivers receiving discharge, as compared to the direct discharge of mine water and secondary treated wastewater. However, since the horizontal flow CWs require large land area, the vertical flow biofilter with activated carbon amendment was then investigated as an alternative option. By realizing synergies between adsorption and biodegradation processes, activated carbon-amended sand biofilter as a polishing step for secondary treated municipal wastewater achieved the desired surface water quality standards for all the conventional water quality parameters and showed effective removal of a wide range of micropollutants, putative human pathogens, and faecal indicator bacteria. The activated carbon biofilter improved the removal of ammonia-nitrogen, diuron, diclofenac and enrofloxacin as compared to the sand-only biofilter. This means that activated carbon amendment can either improve the performance of sand-only biofilters or achieve similar treatment effectiveness at a shorter hydraulic retention time, which would enable a smaller biofilter footprint. It therefore provides a promising alternative nature-based treatment technology to a horizontal flow CW in a location where land availability is limited. Both CW and biofilter technologies can eminently contribute towards the UN SDG 6 to ensure availability and sustainable management of water and sanitation for all, which is particularly pertinent in rural areas

where affordability and low maintenance become the main criteria for wastewater management. The investigated systems provide promising technologies for achieving the targets in SDG 6 including target 6.2 to provide access to adequate sanitation and hygiene for all, target 6.3 to reduce water pollution and promote a safe reuse of water for agricultural irrigation and other purposes, and target 6.6 to protect and restore aquatic ecosystems. Extending the research from end of pipe wastewater management towards addressing diffuse pollution from agriculture fields and to further enhance the overall sustainability of waste management in rural settings, the combined application of two useful by-products namely biochar and anaerobic digestate generated from crop and livestock wastes via renewable technologies was investigated as another win-win opportunity. Biochar produced from coconut husk residues via pyrolysis was co-applied in agricultural soil with anaerobic digestate produced from livestock slurries via anaerobic digestion. Coconut husk biochar retarded nitrate leaching via slower nitrification in digestate-amended soil. The biochar could thus retain nutrients longer for plant growth in the agricultural topsoil, reduce nitrate leaching during heavy rainfall events, and minimize groundwater pollution risks. This co-application of biochar and digestate can thus play an important role towards achieving sustainable agriculture whilst minimizing related nutrient pollution problems. It promotes the circular re-use of agricultural waste residues in addition to renewable energy generation. This could contribute towards SDG 2 to end hunger, achieve food security and improved nutrition and promote sustainable agriculture, particularly by addressing the nitrogen use efficiency challenge which is an indicator of SDG target 2.4 for resilient agricultural practices. This illustrates a strategy that could concurrently ensure access to affordable, reliable, sustainable, and modern energy for all (SDG 7), whilst also contributing towards water pollution control (SDG target 6.3). It realizes win-win-win opportunities between interrelated SDGs, i.e., SDG 2, 6, and 7. The nature-based wastewater and agricultural waste management strategies investigated in this study thus proved to be promising sustainable rural development solutions. Not only can they protect the environment but also secure human-welling in rural societies.

As another example for a win-win opportunity, this research promoted international cooperation for rural wastewater and clean energy infrastructure, and agricultural development through Thailand-UK collaboration. International collaboration contributes towards SDG target 2.a (agricultural productive capacity), target 6.a (water management), and target 7.a (clean energy technology) that all recognize how we live on One Planet, and

therefore aim to enhance international cooperation for sustainable global development. When I return to my home country (Thailand), I will be able to transfer knowledge and a range of skills gained throughout my PhD study to several stakeholders in Thailand which globally one of the main exporters of agricultural produce. For example, I can engage with Thai farmers on the multi-use of agricultural wastes for enhancing crop production whilst minimizing environmental pollution and saving them the cost for artificial fertilizers. I can also promote the use of low-cost nature-based wastewater treatment technologies in Thailand's remote areas where there is a lack of wastewater infrastructure to ensure availability and sustainable management of water and sanitation for all citizens in the country. I can also pass on my skills on innovative chemical and molecular microbiological methods to the Thai academia to facilitate faster and more efficient analytical methods as compared to the traditional methods commonly in use in the Thai education.

The use of complementary investigative methods is another win-win opportunity that was realized throughout this study. By using innovative molecular microbiology approaches for water and soil monitoring throughout this work, much more detailed insight for microbial functions such as nitrification and microbial water quality was obtained for understanding and assessing the performance of nature-based treatment systems on ecological and public health targets as compared to traditional culturing methods. Furthermore, multi-variate statistical methods like PCA and BEST analysis were used to integrate insights gained from microbiological with physicochemical data sets. The use of gene sequencing and a specific-gene qPCR for example to quantify ammonia-oxidizing bacteria provided deeper understanding of nitrification processes and resulting nutrient leaching risks than could be obtained when using only chemical techniques for nitrogen measurements. The innovative molecular microbiology methods enabled much more comprehensive analysis of wastewater treatment performance across a wide range of potential bacterial hazards than could be obtained from conventional plate counts. However, the culturing method remains important as an assessment of faecal bacteria reduction as it clearly reveals the viability of bacteria while the genomic methods detect genetic material from both viable and inviable cells. Comprehensive microbial hazard assessment is crucial for a more circular rural economy to robustly assess whether the treated wastewater and wastes can be safely reused for agricultural irrigation, as fertilizer and for other purposes in rural communities. The molecular microbiology methods can also assist in wastewater-based infectious disease surveillance for pathogens of concern and in implementing new microbial water quality

standards based on genetic markers in the future. This is of particularly important nowadays towards overcoming the challenges of new infectious diseases, as was highlighted by the global pandemic.

Successful sustainable global development requires inclusive partnerships from the local to global level. Continuing international cooperation and capacity building to develop and disseminate innovative methods for agriculture, water, and energy management in the least developed countries (SDG target 2.a, 6.a, 7.a) will help establish their internal capacity for comprehensive and sustainable development for all. Knowledge/skill sharing and team working through international collaboration are keys to achieve the United Nations SDGs and their main purpose of prosperity for all people living on our planet.

6.3 Recommendations for future research

Continuing from the conclusions drawn from my research, I recommend the following work should be conducted in the future to close the remaining knowledge gaps and enhance progress towards sustainable development goals for water management and sustainable agriculture.

- The current CW research was only conducted in spring and summer, therefore there is still a need to investigate the performance of the CWs for in all four seasons, especially in winter when ambient temperature is low.
- The effect of plants on the CW treatment performance should also be investigated.
- Further studies should investigate the removal performance of new emerging contaminants like corona viruses and microplastics by the CWs.
- Continuous monitoring approaches to better monitor in real time the performance of CW in removing micropollutant and pathogen of concerns to robustly prevent serious environmental and human health impacts.
- Research on secondary benefits of CWs such as the use of biomass from CWs like harvested plants as an energy source for renewable energy generation and biochar production for water treatment.
- Investigating the wastewater treatment performance of different types of CWs including hybrid systems.
- Investigating the performance of CWs situated in agricultural areas on combined domestic and agricultural wastewater treatment, for example on farms.
- Scrutinizing the impact of planted and unplanted CWs on greenhouse gas emissions.

- Comprehensive review of utilising CWs for wastewater treatment in terms of economic, social, and environmental dimensions.
- Further studies should investigate different types of substrates that could be used in the biofilters, for example biochar or zeolite.
- Further studies should investigate the effects of different types of wastewater applied to the biofilters.
- The biofiltration study was conducted in a lab and under tropical conditions in Thailand, therefore there is a need for a study of biofilter performance in real life situation and in different climates.
- Longer-term studies (several years) of the performance of AC-amended biofilters and the combined application of biochar and digestate in soil on both chemical and microbiological aspects of ecosystem health.
- Investigating the effect of biochar produced from a wider variety of locally available agricultural waste residues on nutrient retention in soil following digestate application.
- Further studies should investigate the impact of combined application of biochar and digestate in soil on the leaching of other pollutants such as heavy metals.
- Long-term effect on crop productivity and nutrient retention efficiency of the combined application of biochar and digestate in a crop field being irrigated with treated wastewater effluent from either CWs or biofilters.
- Research towards implementing microbial water quality standards that are also based on genetic markers to support safe reuse of reclaimed wastewater in agriculture.
- Waste management strategy should be considered in a way that suits community needs. A local community survey should be conducted along with laboratory-based experiments to ensure suitability and tangible benefits of the technology to local communities.

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Appendices

Appendix A

A1: Methods

Flow data

Table A1. Monthly average flow rate (m^3/s) for mine water and STP effluent flowing to the CWs, and for the River Team in March, May, July, and August. Flow data were obtained from The Coal Authority, Northumbrian Water Limited and Environment Agency for mine water, STP effluent and River Team, respectively.

2021	Mine water (m^3/s)	STP effluent (m^3/s)	River Team (m^3/s)
March	0.495	0.096	0.527
May	0.427	0.115	0.698
July	0.401	0.090	0.579
August	0.326	0.080	0.510

LC-HRMS analysis

The LC-HRMS analyses were performed using an Agilent QTOF 6545 with Jetstream ESI spray source coupled to an Agilent 1260 Infinity II Quat pump HPLC with 1260 autosampler, column oven compartment and variable wavelength detector (VWD). The MS was operated in positive ionization mode with the gas temperature at 250 °C, the drying gas at 12 L/min and the nebulizer gas at 45 psi (3.10 bar). The sheath gas temperature and flow were set to 350°C and 12 L/min, respectively. The MS was calibrated using reference calibrant introduced from the independent ESI reference sprayer. The VCap, Fragmentor and Skimmer was set to 3500, 100 and 45 V respectively. The MS was operated in all-ions mode with 3 collision energy scan segments at 0, 20 and 40 eV. Chromatographic separation of a 10 µL sample injection was performed on a InfinityLab Poroshell 120 EC-C18 (3.0 x 50 mm, 2.7 µm) column using H20 (Merck, LC-MS grade) with 0.1 % formic acid (FA, Fluka) v/v and acetonitrile (MeOH, VWR, HiPerSolv) with 0.1 % FA v/v as mobile phase A and B, respectively. The column was operated at flow rate of 0.3 mL/min at 40°C starting with 2 % mobile phase B, as in **Table A2**.

Table A2. Column operating condition.

Time (min)	Mobile phase B (%)
0.0	2
3.0	2
5.0	100
8.0	100
8.10	2
12.0	2

The VWD was set to detect at 220 and 280 nm wavelengths at a frequency of 2.5 Hz. Data processing was automated in Qual 10 MassHunter software (Agilent, CA, USA) with molecular feature extraction set to the largest 20 compounds for $[M+H]^+$ and $[M+Na]^+$ ions. The results were searched against a Pesticide database (containing 1750 compound entries) with 5 ppm mass error tolerance, in which qualified compounds required 2 or more fragment ions to match.

LCMS method parameters

Table A3. Optimized MS/MS parameters for the analysis of micropollutants.

Analytes	Parent ion (m/z)	Daughter ion (m/z)	Fragmentor (V)	Collision Energy (eV)
Acetaminophen	151.9	110 64.8	8 8	16 24
DEET	192	91.06 90.99	20 20	19 13
Caffeine	195	69.01	20 20	18 25
Carbamazepine	237	194.01 179.16	20 20	29 18
Sulfapyridine	250	108.06 91.74	20 20	16 30
Venlafaxine	278	58.04 121.09	20 20	15
Sulpiride	342	112.05 213.9	20 20	16 13
Cetirizine	390	202.09 166.97	20 20	16 24
Caffeine C13	198	139.97	8	20
Sulfamethoxazole d4	257.9	95.87 159.74	40 40	26 16

Study site

Figure A1. Lamesley constructed wetlands.

Figure A2. CW inlet (a) and CW discharge into the river Team (b).

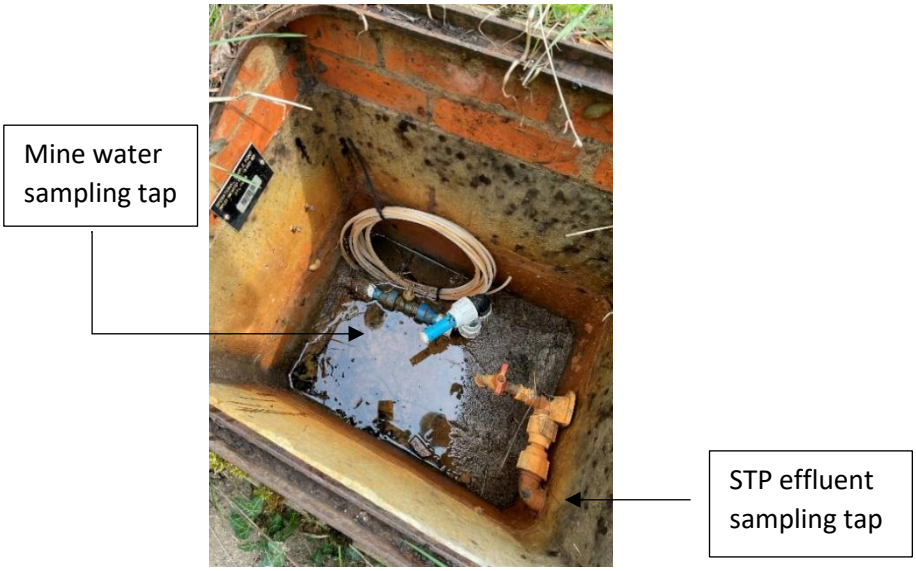

Figure A3. Sampling point for mine water.

Outlier analysis for mine water samples

For mine water, the average results were obtained from only two (March and July) out of the four sampling events. There appeared to be an issue with the sampling of the mine water in May and August, when samples showed unexpected characteristics. We performed cluster analysis and principal component analysis (PCA) for both chemistry and microbiology data of the mine water samples **(Section A2)** to investigate this further. From the chemistry data, the mine water sample in August had very low Mn content **(Table A12)** and was clustered most closely with the STP effluent samples **(Figure A5)** implying contamination by the STP effluent. For the microbiology data **(Figure A6)** only the mine water samples from March and July were clearly separated from the surface water samples (main cluster). The mine water samples from May and August were clustered with the STP effluent and CW influent samples. Additionally, the PCA plot **(Figure A7)** showed that the genus *Thiothrix*, a genus of sulfur-oxidizing bacteria normally found in mine water and assisting in the oxidation of sulfide minerals (Bhandari and Choudhary, 2021), was predominant in the mine water samples only in March and July, while human gut bacteria i.e., *Bacteroides* and *Acidovorax* were prevalent in May and August. These results imply that the mine water samples in May and August were likely contaminated with STP effluent, which has much higher microbial content than the normal mine water, resulting in microbial communities resembling those of STP effluent. Moreover, we also performed source tracking analysis to confirm whether STP effluent was the source of mine water contamination **(Section A1)**. And we found that the main contributor shaping microbial communities in the mine water samples in May and

August was the STP effluents from the respective months **(Figure A4)**. Mine water samples were obtained from a sampling tap on the mine water inlet pipe into a tank which combines the mine water and STP effluent for treatment at the inlet of the CWs **(Figure A3)**. We suspect that there were occasional backups of STP effluent from the tank into the mine water inlet pipe, perhaps because STP effluent had lesser density from its lower salinity and would float on top of the tank. All the evidence indicated that mine water samples in May and August did not have true mine water characteristics, and we therefore removed them from the analysis. If the average mine water chemistry data were derived from the four sampling events, all the standard deviations as well as the means of nutrient concentrations would be considerably higher than the average data from the two 'valid' sampling events **(Table A8)**.

Source tracking analysis

We used source tracking analysis as described elsewhere (Knights *et al.*, 2011) to evaluate the relative contribution of microbial communities from STP effluent samples ('source') to the mine water samples ('sink'). Sources consist of the STP effluent samples (n = 2) from 4 sampling months (March, May, July, August), While the sinks consist of the mine water samples (n = 2) from 4 sampling months (March, May, July, August). Source tracking uses Gibb's sampling (Markov chain Monte Carlo-algorithm) to evaluate the relative contribution of sources, while all the undefined operational taxonomic units in the sinks will be assigned as from unknown sources. The analysis was performed with a sequencing depth of 45,000 with 100 iterations, ten restarts and the auto-tuning functionality.

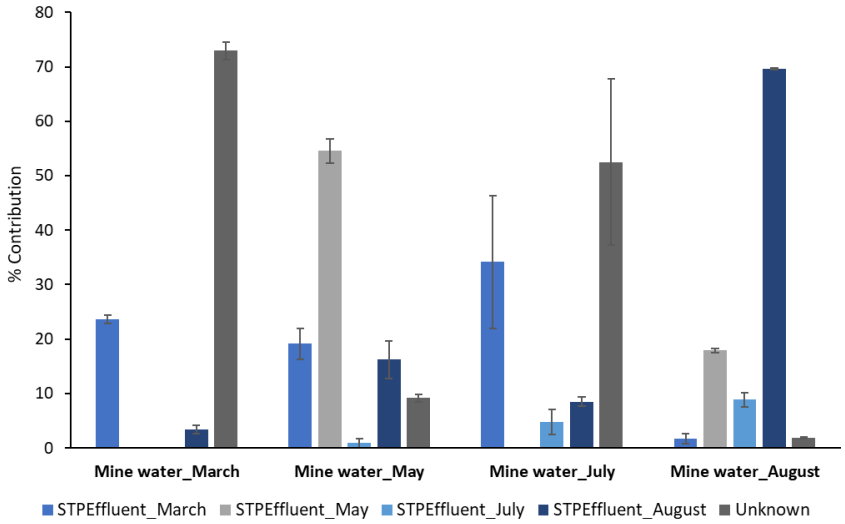

Figure A4. Source contribution (%) for mine water samples from 4 sampling events.

Table A4. Real-time qPCR primers for different genetic markers.

Target organisms	Primer	Sequence (5'>>>3')	Annealing Temperature (T_a)	Reference
Total Bacteria (16S rRNA); qPCR	1055 F	ATGGCTGTCGTCAGCT	60 ºC	Harms *et al.* (2003)
	1392 R	ACGGGCGGTGTGTAC		
Total *E.coli* (Faecal coliforms)	rodA-F	GCAAACCACCTTTGGTCG	60 ºC	Chern *et al.* (2011)
	rodA-R	CTGTGGGTGTGGATTGACAT		
	probe	AACCCCTACAACCGGCAGAATACC		
Human host associated *Bacteroides*	HF183-F	ATCATGAGTTCACATGTCCG	60 ºC	Ahmed *et al.* (2019)
	HF183-R	CTTCCTCTCAGAACCCCTATCC		
	probe	CTAATGGAACGCATCCC		
Vibrio cholerae	ompW-F	TCA ATG ATA GCT GGT TCC TCA AC	60 ºC	Garrido-Maestu *et al.* (2015)
	ompW-R	CGA TGA TAA ATA CCC AAG GAT TGA		
	probe	TGG TAT GCC AAT ATT GAA ACA ACG		
16S rRNA amplicon sequencing; MinION	27 F	AGAGTTTGATCMTGGCTCAG	55 ºC	Oxford Nanopore Technologies, UK
	1492 R	CGGTTACCTTGTTACGACTT		

Table A5. Metrics of the calibration curves on the qPCR instrument for different target bacteria.

Target organism	Slope (Cq/log (Genes/µL))	R^2	Efficiency (%)
Total Bacteria (16S rRNA); qPCR	-3.51±0.17	0.968±0.032	93.0±6.1
E.coli (rodA)	-3.34±0.02	0.991±0.007	99.2±0.8
Human host associated *Bacteroides* (HF183)	-3.50±0.13	0.996±0.002	93.5±4.8
Vibrio cholerae (ompW)	-3.70±0.25	0.993±0.007	87.1±8.1

A2: Results

PCA and cluster analysis for chemistry data

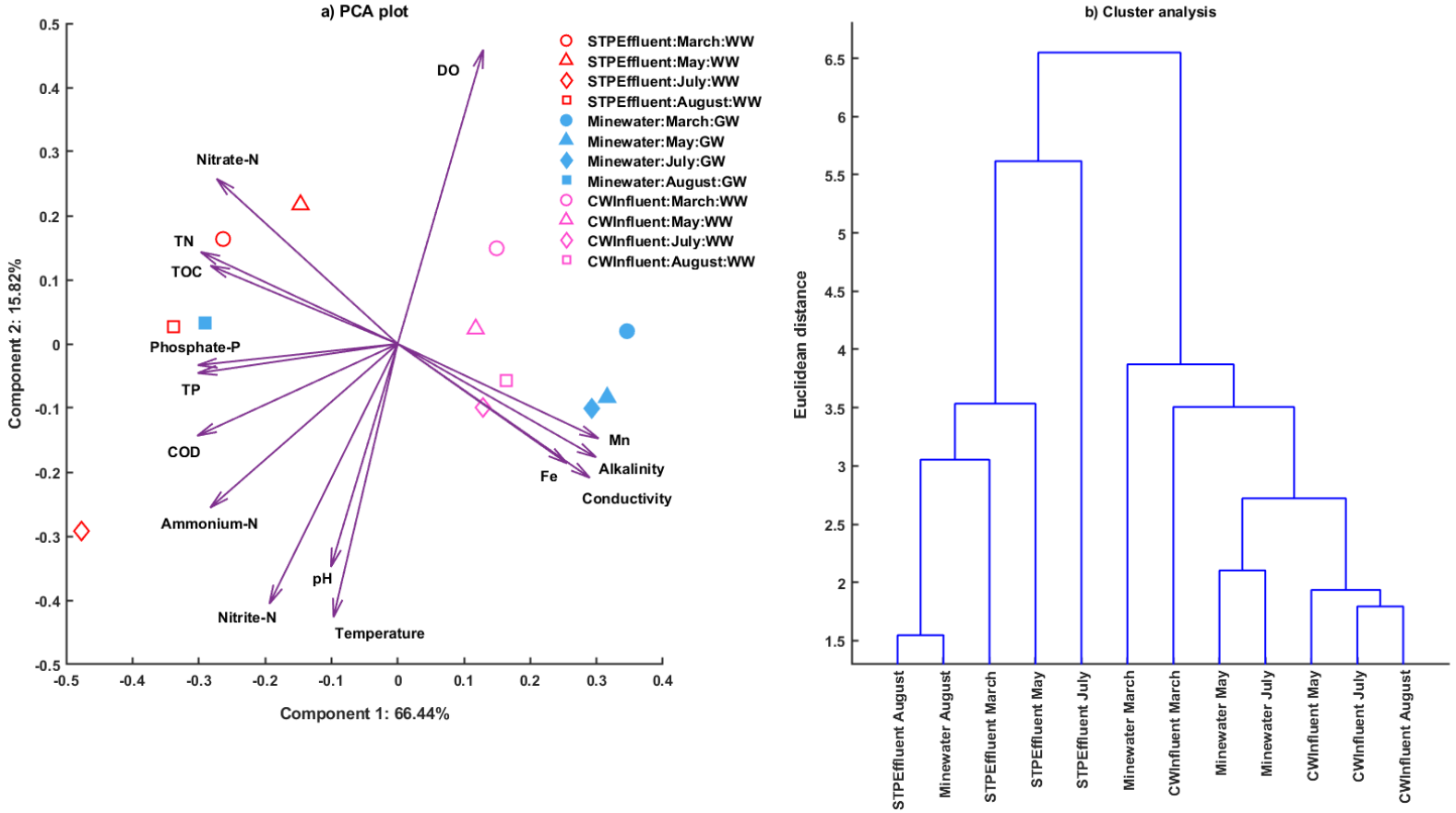

Figure A5. Principal component analysis (PCA) (a) and cluster analysis (b) for chemical parameters assessing dissimilarity among STP effluent, mine water and CW influent samples from 4 sampling events (March, May, July, August). WW and GW indicate type of water samples which are wastewater (empty symbols) and groundwater (filled symbols), respectively. For the PCA, the first two principal components (PC) (Component 1 and 2) were plotted showing the scores (circles, triangles, diamonds, and squares) and 15 loadings (arrows) of physico-chemical parameters explaining the variance in the two-dimensional space. Percentage of variation accounted for by each principal component is shown with the axis label.

Cluster and PCA analysis for microbiology data

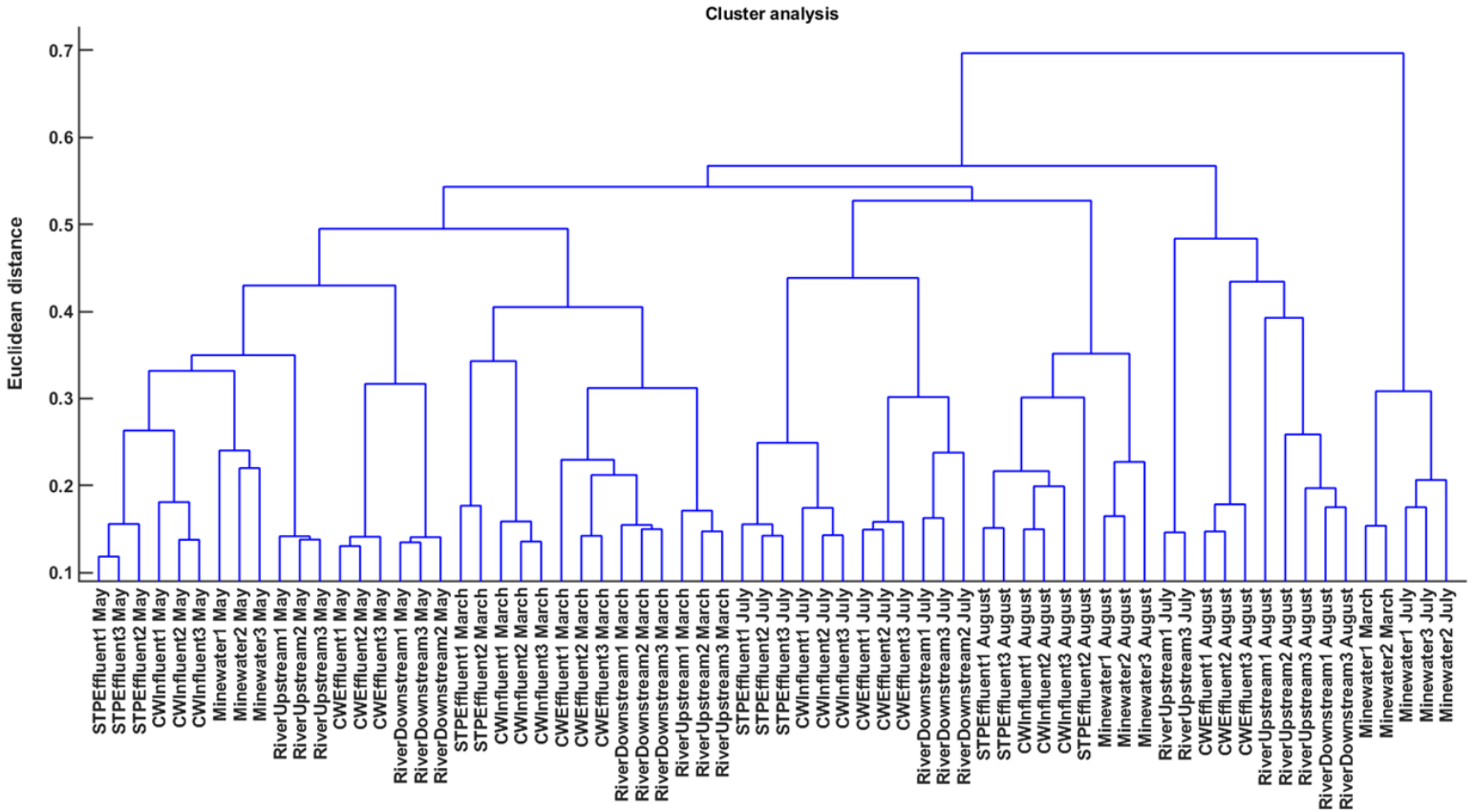

Figure A6. Cluster analysis plot of the microbial community dissimilarity among STP effluent, Mine water, CW influent, CW effluent, River upstream and River downstream of 4 sampling events (March, May, July, August).

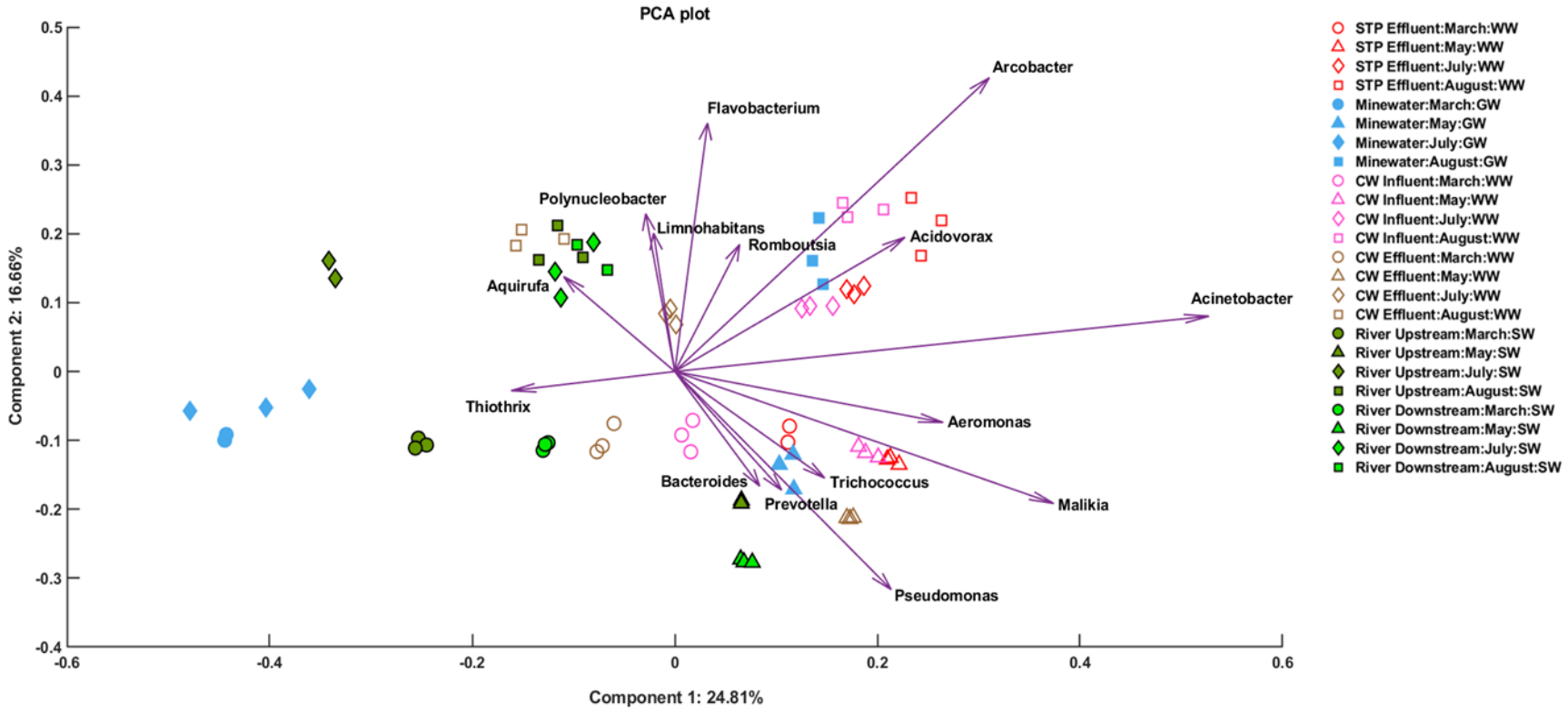

Figure A7. Principal component analysis (PCA) plot of the microbial community dissimilarity among STP effluent, Mine water, CW influent, CW effluent, River upstream and River downstream of 4 sampling events (March, May, July, August). WW, GW, and SW indicate type of water samples which are wastewater, ground water, and surface water, respectively. The first two principal components (PC) (Component 1 and 2) were plotted showing the scores (circles, triangles, diamonds, and squares) and top 15 loadings (genera), (arrows) explaining the variance in the two-dimensional space. Percentage of variation accounted for by each principal component is shown with the axis label.

Table A6. Relative abundance (%) of 8 bacterial genera in the theoretical composition in the MOCK community (ZymoResearch, 2019) and in the actual composition obtained from the MinION sequencing in this study**.**

Genus	Theoretical composition (% relative abundance)	MinION result (% relative abundance)
Bacillus	17.4	21.6±0.2
Enterococcus	9.9	7.1±0.3
Escherichia	10.1	10.4±1.3
Lactobacillus	18.4	10.4±0.7
Listeria	14.1	9.2±0.4
Pseudomonas	4.2	3.4±0.5
Salmonella	10.4	12.8±1.5
Staphylococcus	15.5	12.6±0.9
Others (genus >1%)		12.4±0.3 (*Shigella* 3.2±0.2, *Citrobacter* 1.2±0.2)

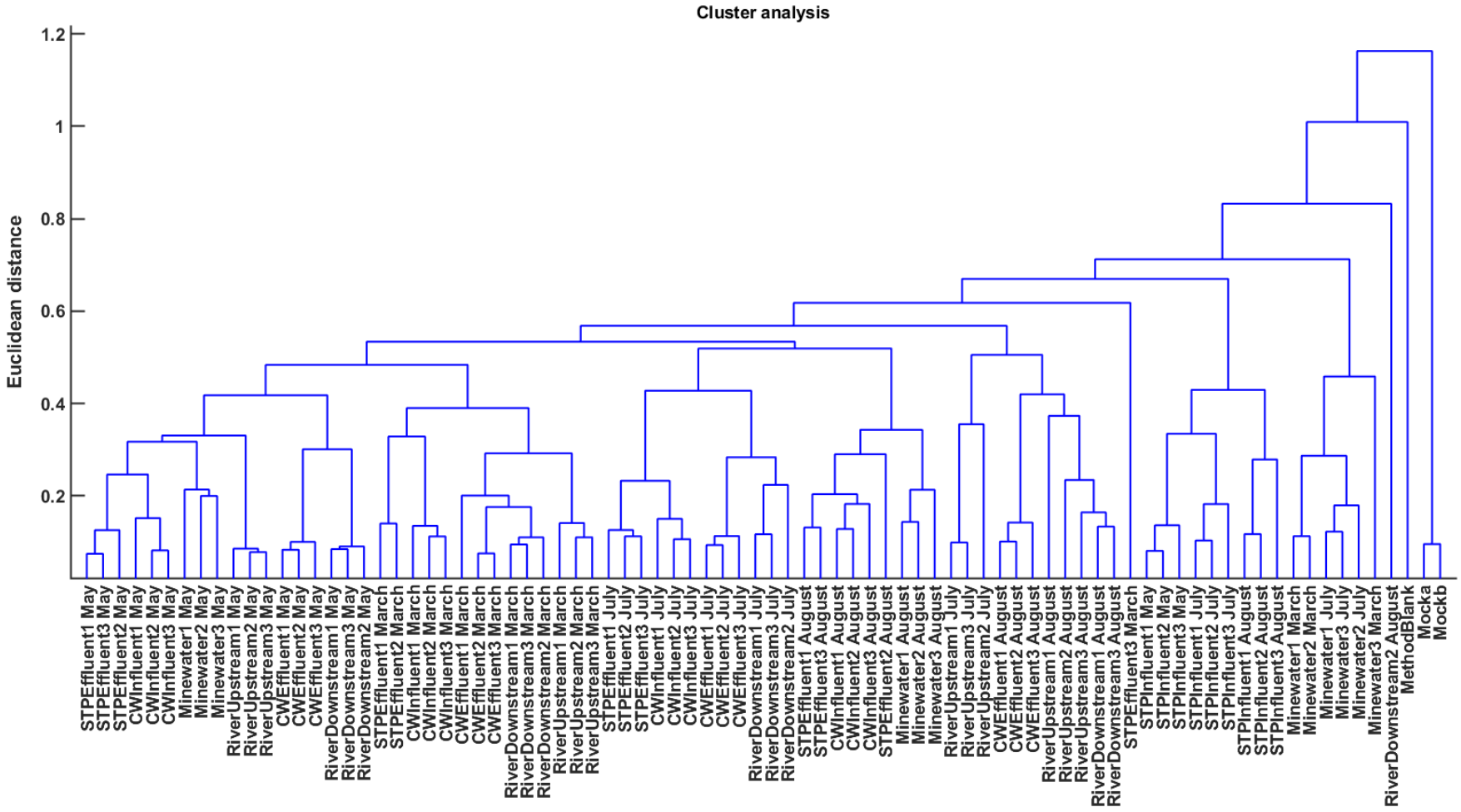

Figure A8. Cluster analysis plot of the microbial community dissimilarity among STP influent in May, July, August, and STP effluent, Mine water, CW influent, CW effluent, River upstream and River downstream of 4 sampling events (March, May, July, August) in triplicates, together with method blank (only reagent), and mock community samples. Sequencing data without rarefaction were used for this analysis. Rarefied data excluded the MethodBlank sample because of an insufficient number of sequencing reads.

Antibiotic resistant *E.coli* estimation

We analysed for antibiotic resistant *E.coli* or also known as extended-spectrum beta-lactamase (ESBL)-producing *E.coli* using a classical plate count technique and membrane filtration in the month of July. The membrane filters were incubated on agar plates containing ESBL ChromoSelect Agar Supplement (Product 61471, Sigma Aldrich, UK) at 44 °C for 24 hours. We then performed the plate counting.

Table A7. Extended-spectrum beta-lactamase (ESBL)-producing *E.coli* abundance (CFU/100 mL) in STP influent and effluent, mine water, CW influent and effluent, river upstream and downstream. Results were reported as Mean±S.D. of duplicate samples.

	ESBL-producing *E.coli* (CFU/100 mL)
	July
STP Influent	TNTC
STP Effluent	6200±600
Mine water	0±0
CW Influent	790±10
CW Effluent	10±0
River Upstream	0±0
River Downstream	15±5

TNTC indicates too numerous to count.

Chemistry data

Table A8. Conventional water quality parameters of mine water. Results were reported to two decimal places as Mean±S.D. of 4 sampling events for the mine water. TNTC indicates too numerous to count.

	Mine water
NH_4^+-N (mg/L)	0.81±1.17
NO_2^--N (mg/L)	0.07±0.12
NO_3^--N (mg/L)	7.46±14.36
TN (mg/L)	10.89±19.41
PO_4^{3-}-P (mg/L)	0.87±1.15
TP (mg/L)	0.81±1.06
Fluoride (mg/L)	0.56±0.11
Alkalinity (mg/L $CaCO_3$)	404.50±249.44
Salinity (mg/L)	1288.50±617.62
pH	7.15±0.21
Conductivity (μS/cm)	2544.25±1248.99
TDS (mg/L)	1801.25±847.21
DO (% saturation)	53.68±14.23
COD (mg/L)	24.77±22.92
DOC (mg/L)	6.45±5.77
Temperature °C	13.75±6.25
Faecal coliform (Log_{10}CFU/100 mL)	TNTC
Heavy metals (μg/L)	
Fe	1823.75±1323.39
Mn	811.25±518.24
Pb	0.10±0.07
Zn	28.75±44.42
Cu	5.49±10.48
As	0.48±0.45

Table A9. Conventional water quality parameters of STP effluent, mine water, CW influent and effluent, river upstream and downstream in March.

	STPEffluent_March	Minewater_March	CWInfluent_March	CWEffluent_March	RiverUpstream_March	RiverDownstream_March
NH_4^+-N (mg/L)	2.91 ±0.07	0.03 ±0	0.53 ±0	0.02 ±0	1.03 ±0.09	0.43 ±0.04
NO_2^--N (mg/L)	0.37 ±0	0 ±0	0.12 ±0	0.05 ±0	0.08 ±0	0.06 ±0
NO_3^--N (mg/L)	34.45 ±1.06	0.12 ±0.01	9.89 ±0.05	7.48 ±0	6.02 ±0.01	6.9 ±0.06
TN (mg/L)	45.9 ±0.14	1.12 ±0.05	14.4 ±0.14	9.31 ±0.01	9.04 ±0.54	9.61 ±0.01
PO_4^{3-}-P (mg/L)	1.92 ±0.01	0.5 ±0	0.71 ±0	0.29 ±0	0.69 ±0	0.28 ±0
TP (mg/L)	2.04 ±0.04	0.46 ±0.01	0.79 ±0.04	0.32 ±0	0.71 ±0	0.3 ±0
Fluoride (mg/L)	0.6 ±0.04	0.53 ±0.02	0.52 ±0	0.5 ±0.04	0.32 ±0.01	0.43 ±0.01
Alkalinity (mg/L $CaCO_3$)	58 ±0	550 ±0	398 ±0	399 ±0	166 ±0	286 ±0
Salinity (mg/L)	471 ±0	1550 ±0	780 ±0	1260 ±0	442 ±0	979 ±0
pH	7.25 ±0	7.41 ±0	7.01 ±0	7.58 ±0	7.72 ±0	7.61 ±0
Conductivity (μS/cm)	929 ±0	2950 ±0	1560 ±0	2480 ±0	906 ±0	1947 ±0
TDS (mg/L)	680 ±0	2080 ±0	1095 ±0	1720 ±0	636 ±0	1367 ±0
DO (mg/L)	6.48 ±0	9.4 ±0	7.87 ±0	8.96 ±0	9.46 ±0	8.83 ±0
COD (mg/L)	55.45 ±7.42	11.61 ±3.52	18.55 ±0.49	14.45 ±0.21	11.75 ±0.21	25.15 ±6.29
DOC (mg/L)	17.51 ±0.12	1.66 ±0.8	3.88 ±1.13	3.95 ±0.17	7 ±0.47	5.89 ±0.15
Temperature °C	5.2 ±0	5 ±0	4.8 ±0	5.4 ±0	4.2 ±0	4.4 ±0
Faecal coliform (CFU/100 mL)	160000 ±0	0 ±0	5950 ±70.71	850 ±494.97	1050 ±70.71	600 ±141.42
Heavy metals (μg/L)						
Fe	70 ±0	2545 ±7.07	60 ±0	30 ±0	20 ±0	40 ±0
Mn	60 ±0	1110 ±0	755 ±7.07	280 ±0	140 ±0	300 ±0
Pb	0.3 ±0.01	0.11 ±0.01	0.06 ±0	0.03 ±0	0.07 ±0	0.14 ±0
Zn	50 ±0	0 ±0	40 ±0	10 ±0	50 ±0	30 ±0
Cu	4.59 ±0.02	0.21 ±0.04	1.84 ±0.11	1.57 ±0.03	4.44 ±0.04	1.8 ±0.05
As	0.95 ±0.02	0.41 ±0.04	0.32 ±0	0.18 ±0.01	0.39 ±0.03	0.24 ±0.02

Table A10. Conventional water quality parameters of the STP effluent, mine water, CW influent and effluent, river upstream and downstream in May.

	STPInfluent_ May	STPEffluent_ May	Minewater_ May	CWInfluent_ May	CWEffluent_ May	RiverUpstream_ May	RiverDownstream_ May
NH_4^+-N (mg/L)	16.63 ±0.04	0.99 ±0.01	0.26 ±0.03	0.59 ±0.01	0.37 ±0	0.49 ±0	0.44 ±0
NO_2^--N (mg/L)	0.37 ±0	0.17 ±0	0.03 ±0	0.06 ±0	0.16 ±0	0.11 ±0	0.14 ±0
NO_3^--N (mg/L)	3.54 ±0.04	18.95 ±0.14	0.27 ±0.02	6.51 ±0.09	6.7 ±0.05	5.51 ±0.01	5.89 ±0.02
TN (mg/L)	62 ±0.71	24 ±0.14	0.76 ±0.17	8.21 ±0.18	8.25 ±0.6	6.75 ±0.08	7.59 ±0.05
PO_4^{3-}-P (mg/L)	3.47 ±0	2.03 ±0.01	0.42 ±0	1.26 ±0.14	0.31 ±0.01	0.57 ±0	0.55 ±0.02
TP (mg/L)	3.47 ±0	2.03 ±0.01	0.42 ±0	1.26 ±0.14	0.31 ±0.01	0.57 ±0	0.55 ±0.02
Fluoride (mg/L)	0.97 ±0.02	0.45 ±0.03	0.43 ±0.03	0.45 ±0.03	0.47 ±0	0.24 ±0.03	0.38 ±0.01
Alkalinity (mg/L $CaCO_3$)	218 ±0	37 ±0	496 ±0	311 ±0	336 ±0	83 ±0	215 ±0
Salinity (mg/L)	505 ±0	320 ±0	1730 ±0	1350 ±0	1140 ±0	340 ±0	770 ±0
pH	8.55 ±0	6.8 ±0	6.9 ±0	7.01 ±0	7.3 ±0	7.87 ±0	7.4 ±0
Conductivity (µS/cm)	1017 ±0	670 ±0	3500 ±0	2670 ±0	2330 ±0	695 ±0	1575 ±0
TDS (mg/L)	710 ±0	473 ±0	2450 ±0	1860 ±0	1650 ±0	470 ±0	1100 ±0
DO (mg/L)	3.74 ±0	8.11 ±0	4.21 ±0	6.13 ±0	5.26 ±0	9.64 ±0	8.16 ±0
COD (mg/L)	274 ±7.07	47.5 ±4.95	16.3 ±0.71	24.7 ±0.99	22.75 ±0.21	26.4 ±0.42	17.05 ±1.63
DOC (mg/L)	19.33 ±0.44	16.42 ±0.21	4.09 ±0.45	11.35 ±2.13	8.71 ±0.95	12.16 ±0.27	7.83 ±0.26
Temperature °C	13.9 ±0	12 ±0	13.5 ±0	13.8 ±0	13.2 ±0	11.2 ±0	12 ±0
Faecal coliform (CFU/100 mL)	1050000 ±212132.03	25000 ±0	0 ±0	11800 ±282.84	5050 ±70.71	16300 ±1838.48	7600 ±1979.9
Heavy metals (µg/L)							
Fe	30 ±0	40 ±0	3100 ±0	1175 ±7.07	15 ±7.07	20 ±0	30 ±0
Mn	10 ±0	40 ±0	920 ±0	640 ±0	470 ±0	70 ±0	390 ±0
Pb	0.34 ±0.02	0.28 ±0.03	0.11 ±0.01	0.19 ±0.02	0.1 ±0.01	0.15 ±0.01	0.12 ±0
Zn	30 ±0	60 ±0	10 ±0	30 ±0	20 ±0	30 ±0	30 ±0
Cu	8.32 ±0.69	5.05 ±0.23	0.56 ±0.05	3.13 ±0.13	3.57 ±0.17	3.49 ±0.08	2.14 ±0.06
As	0.81 ±0.04	0.88 ±0.02	0.42 ±0.01	0.47 ±0.02	0.24 ±0.02	0.49 ±0.01	0.35 ±0.01

Table A11. Conventional water quality parameters of the STP effluent, mine water, CW influent and effluent, river upstream and downstream in July.

	STPInfluent_ July	STPEffluent_ July	Minewater_ July	CWInfluent_ July	CWEffluent_ July	RiverUpstream_ July	RiverDownstream_ July
NH_4^+-N (mg/L)	61.2 ±0.28	5.61 ±0.01	0.39 ±0.01	1.61 ±0	0.04 ±0	0.57 ±0	0.18 ±0
NO_2^--N (mg/L)	0.11 ±0	3.13 ±0.01	0 ±0	0.44 ±0	0.03 ±0	0.27 ±0	0.1 ±0
NO_3^--N (mg/L)	1.31 ±0.01	15.7 ±0.28	0.46 ±0.13	4.39 ±0.43	2.78 ±0.01	9.61 ±0.11	4.79 ±0.13
TN (mg/L)	100.2 ±1.13	35.2 ±1.84	1.69 ±0.33	4.62 ±0.08	3.97 ±0.33	13.3 ±0.28	6.4 ±0.3
PO_4^{3-}-P (mg/L)	7.56 ±0.23	3.34 ±0.03	0 ±0	0.77 ±0	0.12 ±0	0.24 ±0	0.15 ±0
TP (mg/L)	8.57 ±0.17	3.61 ±0.01	0 ±0	0.92 ±0	0.14 ±0	0.27 ±0	0.18 ±0
Fluoride (mg/L)	1.77 ±0.06	0.88 ±0.02	0.67 ±0.01	0.8 ±0.03	0.81 ±0.01	0.59 ±0.06	0.83 ±0.04
Alkalinity (mg/L $CaCO_3$)	296 ±0	75 ±0	540 ±0	462 ±0	515 ±0	137 ±0	387 ±0
Salinity (mg/L)	563 ±0	397 ±0	1500 ±0	1340 ±0	1380 ±0	470 ±0	1180 ±0
pH	8.29 ±0	7.55 ±0	7.09 ±0	7.14 ±0	7.53 ±0	8.1 ±0	7.64 ±0
Conductivity (µS/cm)	1143 ±0	810 ±0	3020 ±0	2480 ±0	2700 ±0	950 ±0	2320 ±0
TDS (mg/L)	797 ±0	580 ±0	2120 ±0	1840 ±0	1920 ±0	675 ±0	1600 ±0
DO (mg/L)	0.82 ±0	2.79 ±0	4.87 ±0	4.85 ±0	5.07 ±0	8.16 ±0	6.45 ±0
COD (mg/L)	998.5 ±27.58	101 ±0	12.15 ±0.21	27.95 ±0.49	16.2 ±2.26	23.6 ±3.54	18.5 ±0.57
DOC (mg/L)	24.62 ±0.6	15.71 ±0.1	5.22 ±0.5	6.26 ±0.24	7.35 ±0.77	9.76 ±0.22	6.79 ±1.26
Temperature °C	19 ±0	20 ±0	18.2 ±0	17.3 ±0	18.8 ±0	17.5 ±0	17 ±0
Faecal coliform (CFU/100 mL)	5000000 ±0	281250 ±44194.17	0 ±0	26750 ±2474.87	1700 ±47.14	1333.33 ±0	1866.67 ±47.14
Heavy metals (µg/L)							
Fe	40 ±0	30 ±0	1570 ±0	745 ±7.07	20 ±0	20 ±0	20 ±0
Mn	30 ±0	40 ±0	1165 ±7.07	755 ±7.07	945 ±7.07	80 ±0	800 ±0
Pb	0.6 ±0.43	-0.19 ±0.21	-0.3 ±0.17	-0.47 ±0.14	-0.4 ±0.2	-0.47 ±0.08	-0.55 ±0.1
Zn	30 ±0	50 ±0	10 ±0	20 ±0	70 ±0	40 ±0	40 ±0
Cu	15.23 ±0.64	20.45 ±0.17	-2.52 ±0.19	-2.27 ±0.21	5.27 ±0.19	5.32 ±0.42	1.62 ±0.07
As	1.1 ±0.69	0.33 ±0.29	-0.34 ±0.12	-0.33 ±0.32	-0.5 ±0.02	-0.24 ±0.09	-0.49 ±0.02

Table A12. Conventional water quality parameters of the STP effluent, mine water, CW influent and effluent, river upstream and downstream in August.

	STPInfluent_ August	STPEffluent_ August	Minewater_ August	CWInfluent_ August	CWEffluent_ August	RiverUpstream_ August	RiverDownstream_ August
NH_4^+-N (mg/L)	65.5 ±1.77	2.76 ±0.03	2.55 ±0.04	0.92 ±0.05	0.08 ±0	0.55 ±0.15	0.27 ±0
NO_2^--N (mg/L)	0.1 ±0	0.25 ±0	0.25 ±0	0.07 ±0	0.02 ±0	0.21 ±0	0.1 ±0
NO_3^--N (mg/L)	1 ±0.03	31 ±0	29 ±0	6.47 ±0.04	3.59 ±0.01	5.59 ±0.23	4.51 ±0.01
TN (mg/L)	80 ±2.55	38.05 ±2.33	40 ±0.14	10.45 ±0.21	4.24 ±0.23	7.47 ±0.04	5.46 ±0.12
PO_4^{3-}-P (mg/L)	7.3 ±0.08	3.66 ±0.03	2.57 ±0.02	0.95 ±0.01	0.25 ±0	0.41 ±0.01	0.3 ±0
TP (mg/L)	8.62 ±0.08	3.77 ±0.07	2.38 ±0.03	1.04 ±0.01	0.26 ±0	0.44 ±0	0.32 ±0.01
Fluoride (mg/L)	0.92 ±0.13	0.62 ±0.02	0.61 ±0.01	0.6 ±0.03	0.59 ±0.01	0.4 ±0.03	0.49 ±0.02
Alkalinity (mg/L $CaCO_3$)	305 ±0	34 ±0	32 ±0	462 ±0	496 ±0	137 ±0	360 ±0
Salinity (mg/L)	522 ±0	399 ±0	374 ±0	1350 ±0	1500 ±0	404 ±0	1020 ±0
pH	8.38 ±0	7.18 ±0	7.2 ±0	7.3 ±0	7.75 ±0	8.4 ±0	7.9 ±0
Conductivity (µS/cm)	1058 ±0	807 ±0	707 ±0	2630 ±0	2870 ±0	858 ±0	2000 ±0
TDS (mg/L)	740 ±0	582 ±0	555 ±0	1830 ±0	1970 ±0	600 ±0	1420 ±0
DO (mg/L)	1.98 ±0	4.98 ±0	4.57 ±0	7.02 ±0	6.95 ±0	8.69 ±0	7.95 ±0
COD (mg/L)	638 ±2.83	59 ±1.7	59 ±0.42	18.45 ±0.21	12.9 ±0.99	37.7 ±2.4	20.2 ±0.71
DOC (mg/L)	24.36 ±0.54	12.8 ±0.1	14.82 ±0.34	7.57 ±1.08	6.89 ±3.69	8.05 ±1.96	8.35 ±1.97
Temperature °C	18.5 ±0	18.9 ±0	18.3 ±0	17.7 ±0	17.5 ±0	15 ±0	15.2 ±0
Faecal coliform (CFU/100 mL)	6000000 ±0	62500 ±17677.67	TNTC	1875 ±530.33	1700 ±235.7	1683.33 ±117.85	2233.33 ±329.98
Heavy metals (µg/L)							
Fe	50 ±0	40 ±0	80 ±0	1540 ±0	10 ±0	10 ±0	10 ±0
Mn	40 ±0	35 ±7.07	50 ±0	875 ±7.07	370 ±0	270 ±0	250 ±0
Pb	0.34 ±0.04	0.14 ±0.03	0.17 ±0	0.04 ±0	0.03 ±0	0.49 ±0.01	0.5 ±0
Zn	45 ±0	70 ±0	95 ±0	20 ±0	10 ±0	20 ±0	35 ±0
Cu	32.85 ±1.65	15.46 ±0.03	21.2 ±0.29	1.69 ±0.03	4.44 ±0.15	2.81 ±0.05	4.52 ±0
As	0.74 ±0.03	0.86 ±0.03	1.08 ±0.02	0.5 ±0	0.25 ±0	0.46 ±0	0.47 ±0.01

TNTC: too numerous to count, Note: mine water data is not reliable for this month.

Table A13. Concentration (mg/L) of calcium, magnesium, chloride, and sulphate in the STP influent in May, July, August, and STP effluent, Mine water, CW influent, CW effluent, River upstream and River downstream of 4 sampling events (March, May, July, August).

	Calcium	Magnesium	Chloride	Sulphate
STP Effluent March	63.65	10.42	82.48	84.42
Mine water March	189.27	69.28	280.35	577.94
CW Influent March	151.29	51.41	229.97	410.71
CW Effluent March	161.83	56.28	228.56	443.43
River Upstream March	77.75	19.78	460.67	115.74
River Downstream March	105.98	34.32	790.34	265.62
STP Influent May	26.76	4.40	126.20	3.62
STP Effluent May	31.07	4.72	54.03	3.42
Mine water May	189.28	71.14	16.71	63.86
CW Influent May	117.29	40.05	48.45	34.16
CW Effluent May	101.41	34.86	88.97	31.49
River Upstream May	40.01	8.95	56.81	6.33
River Downstream May	100.97	31.68	8.48	26.37
STP Influent July	16.83	3.22	2712.42	53.00
STP Effluent July	26.61	4.33	2165.35	50.92
Mine water July	206.59	74.07	1451.41	240.83
CW Influent July	139.27	49.16	547.82	555.05
CW Effluent July	185.21	65.40	789.13	362.94
River Upstream July	73.84	19.22	325.94	411.68
River Downstream July	170.04	58.86	308.66	239.62
STP Influent August	16.89	3.18	2081.45	39.68
STP Effluent August	29.54	4.75	1592.43	43.93
Mine water August	46.28	7.30	1101.16	50.55
CW Influent August	168.21	55.75	188.43	200.42
CW Effluent August	178.87	60.42	433.68	481.03
River Upstream August	140.67	45.71	438.93	467.82
River Downstream August	132.45	42.92	378.23	365.78

Note: mine water data in August is not reliable.

Table A14. Conventional water quality parameters of the STP effluent, mine water, CW effluent, river upstream and river downstream in January 2020 (winter).

	STP effluent	Mine water	CW effluent	River upstream	River downstream
NH_4^+-N (mg/L)	6.12	0.17	0.02	1.51	0.71
NO_2^--N (mg/L)	1.28	0.05	0.05	0.21	0.12
NO_3^--N (mg/L)	122.05	1.00	24.45	28.83	27.24
TN (mg/L)	N/A	N/A	N/A	N/A	N/A
PO_4^{3-}-P (mg/L)	N/A	N/A	N/A	N/A	N/A
TP (mg/L)	N/A	N/A	N/A	N/A	N/A
Fluoride (mg/L)	0.94	0.53	0.55	0.38	0.39
Alkalinity (mg/L $CaCO_3$)	82.00	576.00	472.00	140.00	300.00
Salinity (mg/L)	512.00	1640.00	1370.00	418.00	890.00
pH	7.04	1.55	7.18	7.47	7.38
Conductivity (µS/cm)	1043.00	3070.00	2690.00	902.00	1810.00
TDS (mg/L)	720.00	2190.00	1810.00	614.00	1260.00
COD (mg/L)	N/A	N/A	N/A	N/A	N/A
DOC (mg/L)	N/A	N/A	N/A	N/A	N/A
Temperature ^oC	N/A	N/A	N/A	N/A	N/A
Faecal colifiorm (Log_{10} CFU/100 mL)	5.30	0 CFU/100 mL	3.11	3.15	2.85
DO % saturation	N/A	N/A	N/A	N/A	N/A
Heavy metals (µg/L)					
Fe	14.86	2458.41	0.00	0.00	0.00
Mn	58.12	909.49	423.02	206.03	284.30
Pb	0.00	0.00	0.00	0.00	0.00
Zn	18.26	57.01	0.00	63.90	147.83
Cu	8.98	0.00	53.30	52.31	0.00
As	0.00	0.00	0.00	0.00	0.00

N/A = not available

Appendix B

B1: Conventional water quality parameters

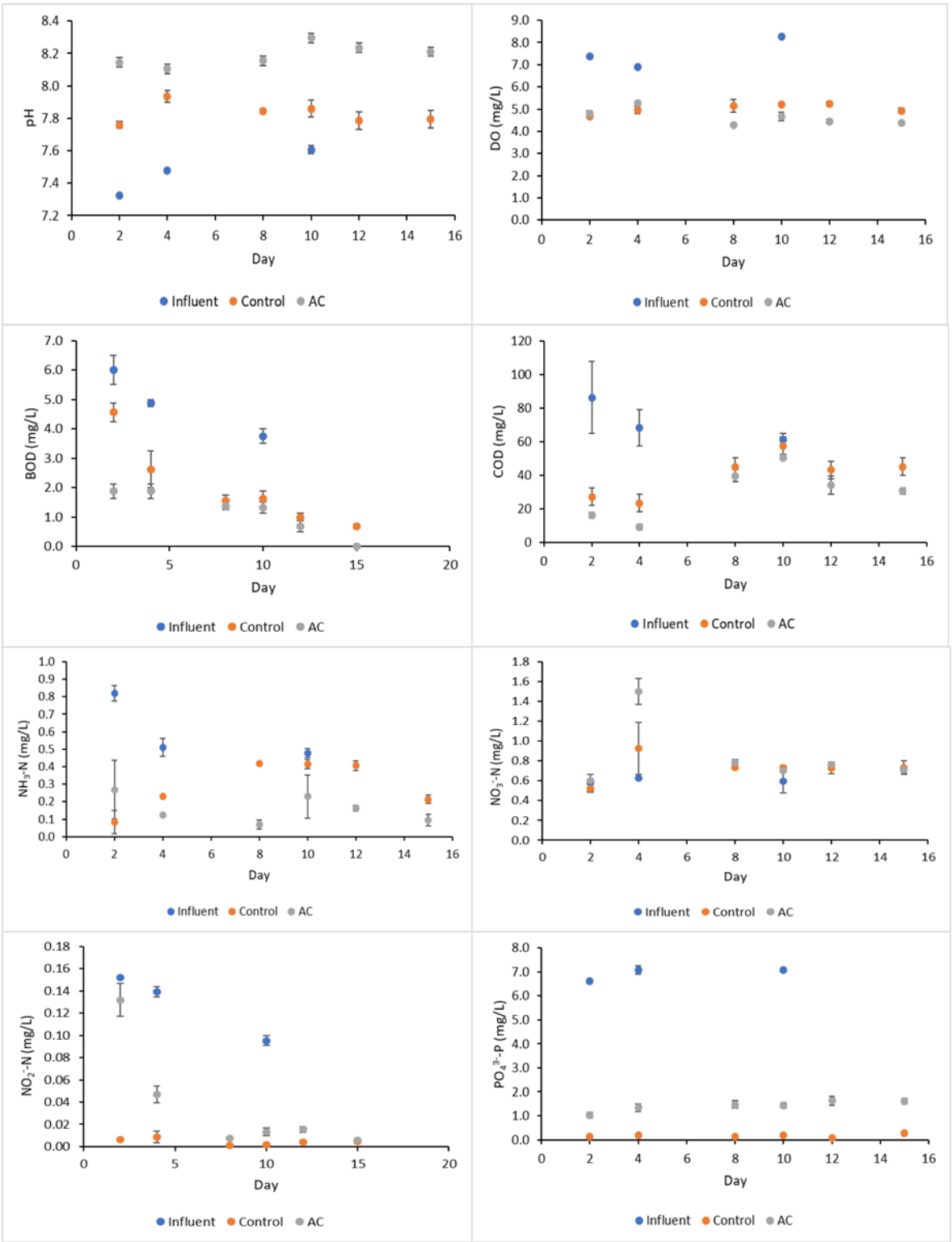

Figure B1. Conventional water quality parameters of the influent on day 2, 4 and 10, and effluent of the Control and AC columns on day 2, 4, 8, 10, 12 and 15. Error bars were calculated as the standard deviation in duplicate systems.

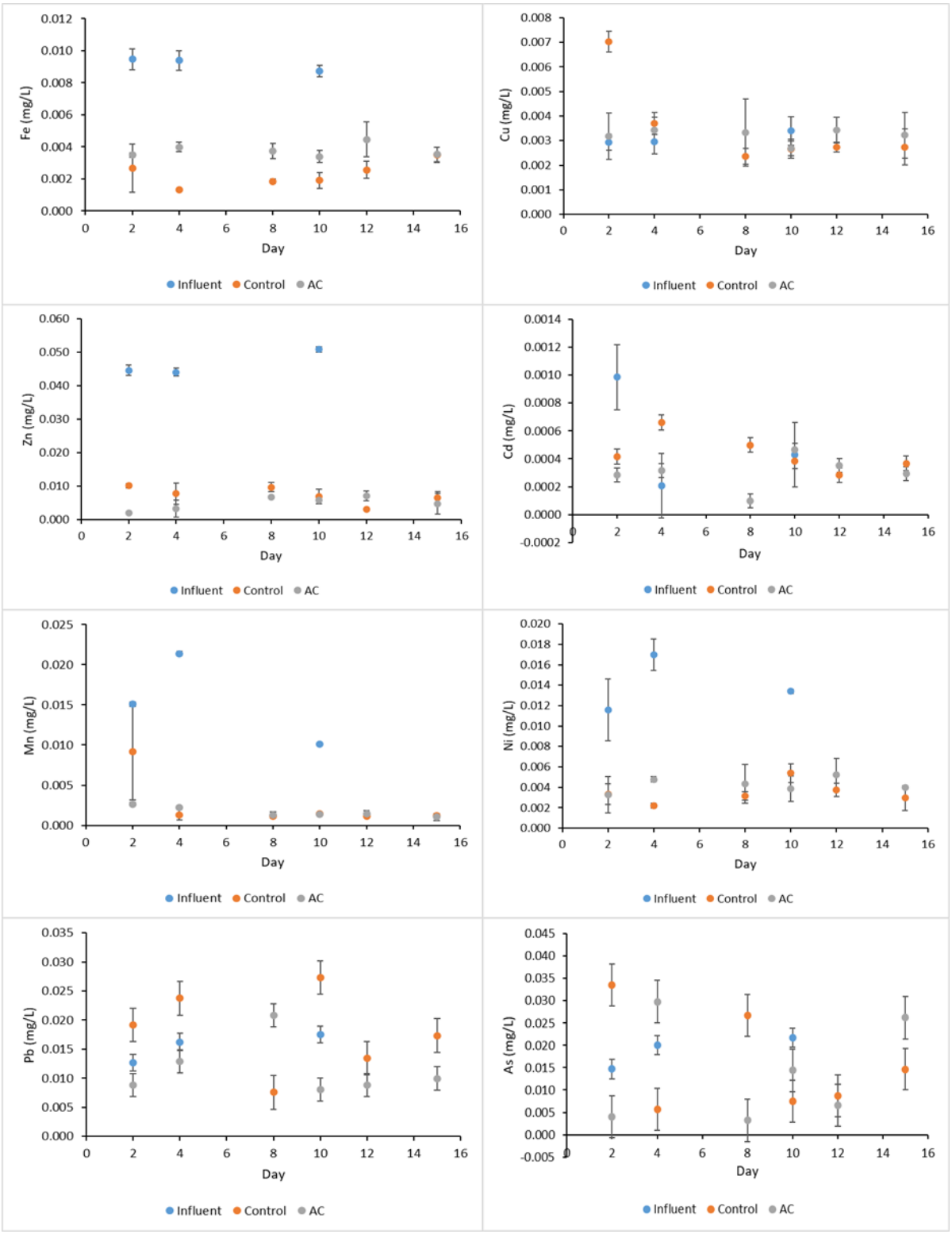

Figure B2. Heavy metal concentration (mg/L) (Fe, Cu, Zn, Cd, Mn, Ni, Pb, As) in the influent on day 2, 4 and 10, and effluent of the Control and AC columns on day 2, 4, 8, 10, 12 and 15. Error bars were calculated as the standard deviation in duplicate systems.

Table B1. Metal concentration (mg/L) (Ca, Mg, Na, K, Al, Si, Cr, S, Sr, Ba) in the influent on day 2, 4 and 10, and effluent of the Control and AC columns on day 2, 4, 8, 10, 12 and 15. Results were reported as Mean±S.D. for duplicates of 3 samples for the influent and 6 samples for the effluent collected throughout the entire experimental period.

Metal (mg/L)	Ca	Mg	Na	K	Al	Si	Cr	S	Sr	Ba
Day2_Influent	43.89 ±0.09	14.38 ±0.01	69.05 ±0.1	13.02 ±0.15	0.01 ±0	5.25 ±0	0 ±0	16.41 ±0.07	0.14 ±0	0.03 ±0
Day2_Control	392.55 ±17.78	33.3 ±0.87	60.26 ±1.61	12.43 ±0.26	0.02 ±0	2.93 ±0.01	0 ±0	331.66 ±11.75	0.43 ±0.01	0.02 ±0
Day2_AC	43.5 ±1	11.69 ±0.37	56.71 ±0.49	66.53 ±4.98	0.02 ±0	4.74 ±0.03	0.01 ±0	16.44 ±0.05	0.15 ±0.01	0.16 ±0.01
Day4_Influent	44.12 ±0.25	14.43 ±0.11	69.38 ±0.22	14.75 ±0.1	0.01 ±0	5.26 ±0.05	0 ±0	16.48 ±0.22	0.14 ±0	0.03 ±0
Day4_Control	153.67 ±3.7	10.48 ±0.91	70.9 ±0.21	6.66 ±0.02	0.02 ±0	3.15 ±0.03	0 ±0	99.04 ±0.23	0.18 ±0	0.02 ±0
Day4_AC	39.58 ±1.43	10.47 ±0.4	67.59 ±0.06	60.44 ±5.1	0.02 ±0	4.75 ±0.01	0 ±0	17.1 ±0.05	0.13 ±0.01	0.14 ±0.01
Day8_Control	73.37 ±0.97	7.36 ±0.7	70.26 ±0.09	12.49 ±4.36	0.02 ±0	3.46 ±0.02	0 ±0	27.56 ±2.29	0.11 ±0.01	0.03 ±0
Day8_AC	43.77 ±1.18	11.42 ±0.15	71.27 ±0.57	40.79 ±1.59	0.02 ±0	4.73 ±0.01	0 ±0	17.16 ±0.12	0.12 ±0	0.12 ±0
Day10_Influent	43.92 ±0.14	14.35 ±0.09	69.28 ±0.32	13.88 ±0.04	0.01 ±0	5.21 ±0.03	0.01 ±0	16.39 ±0.03	0.14 ±0	0.03 ±0
Day10_Control	67.06 ±0.41	8.03 ±0.43	71.51 ±0.35	11.46 ±0.08	0.01 ±0	3.7 ±0.03	0 ±0	24.8 ±2.11	0.11 ±0.01	0.03 ±0
Day10_AC	44.14 ±0.37	11.69 ±0.17	72.91 ±0.02	38.12 ±1.69	0.02 ±0.01	4.83 ±0.08	0 ±0	17.23 ±0.1	0.12 ±0	0.11 ±0
Day12_Control	61.27 ±0.3	8.9 ±0.18	71.9 ±0.39	13.09 ±1.63	0.02 ±0	3.89 ±0.05	0 ±0	21.82 ±1.62	0.1 ±0.01	0.03 ±0
Day12_AC	44.49 ±0.13	12.1 ±0.16	73.39 ±0	35.74 ±1.8	0.02 ±0.01	4.98 ±0.05	0 ±0	17.4 ±0.2	0.12 ±0	0.11 ±0
Day15_Control	55.29 ±0.41	10.59 ±0.21	70.08 ±0	11.78 ±0.32	0.02 ±0	3.97 ±0.03	0 ±0	19.19 ±0.79	0.1 ±0	0.03 ±0
Day15_AC	44.43 ±0.11	12.68 ±0.14	71.54 ±0.01	29.99 ±1.29	0.03 ±0	4.94 ±0.06	0 ±0	16.77 ±0	0.12 ±0	0.1 ±0

B2: Microbiology data

Table B2. Real-time qPCR primers for different genetic markers.

Target organism	Primer	Sequence (5'>>>3')	Annealing Temperature (T_a)	Amplicon size (bp)	Reference
Total Bacteria (16S rRNA); qPCR	1055 F	ATGGCTGTCGTCAGCT	60 ºC	337	Harms *et al.* (2003)
	1392 R	ACGGGCGGTGTGTAC			
Total Coliform	Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	60 ºC	190	Bartosch *et al.* (2004)
	Eco1652R	CTCTACGAGACTCAAGCTTGC			
Total *E.coli* (Faecal coliforms)	rodA -F	GCAAACCACCTTTGGTCG	60 ºC	194	Chandrashekhar *et al.* (2015)
	rodA -R	CTGTGGGTGTGGATTGACAT			
Human *E.coli*	Hu 100 –F	ACGGTTATCAGCTCACGTCG	60 ºC	98	(Robson and Davenport, in preparation)
	Hu 100 –R	TCGCCCCTCGAAAAGCATTA			
Vibrio cholerae	EpsM -F	GAATTATTGGCTCCTGTGCA	57 ºC	248	Kong *et al.* (2002)
	EpsM -R	ATCGCTTGGCGCATCACTGCCC			
16S rRNA amplicon sequencing; MinION	27F	AGAGTTTGATCMTGGCTCAG	55 ºC	1500	Oxford Nanopore Technologies, UK
	1492R	CGGTTACCTTGTTACGACTT			

Table B3. Metrics of the calibration curves on the qPCR instrument for different target bacteria.

Target organism	Slope (Cq/log (Genes/µL))	R^2	Efficiency (%)
Total Bacteria (16S rRNA); qPCR	-3.37±0.04	0.958±0.012	98.1±1.6
Total Coliform	-3.92±0.09	0.999±0.000	80.0±2.5
Total *E.coli* (Faecal coliforms)	-4.00±0.03	0.975±0.003	77.7±0.6
Human *E.coli*	-3.56±0.02	0.963±0.002	91.1±0.5
Vibrio cholerae	-4.33±0.09	0.995±0.003	70.3±1.8

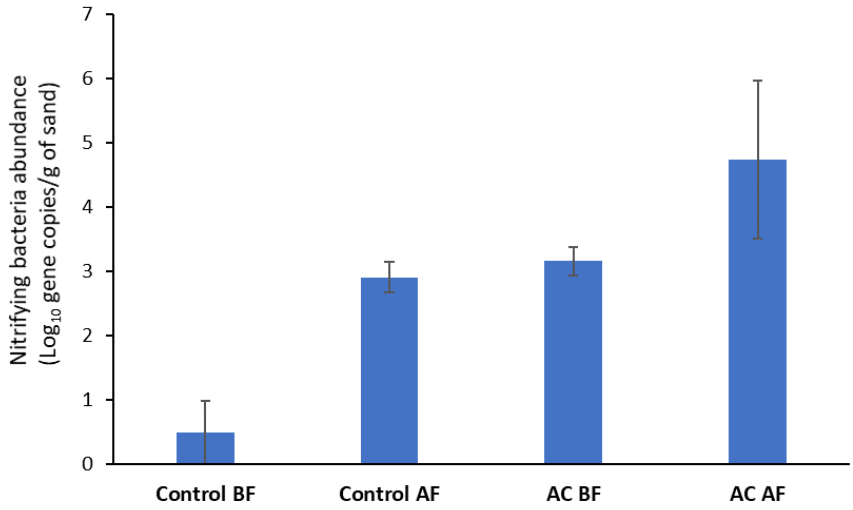

Figure B3. Nitrifying bacteria abundance (Log_{10} gene copies/g of sand) in the filter media of Control and AC columns before filtration (BF) and after filtration (AF).

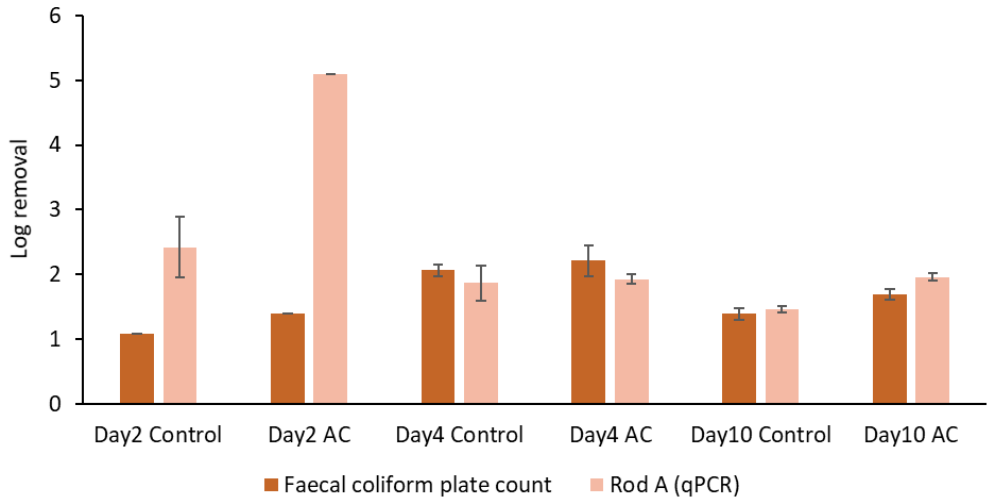

Figure B4. Comparison of log removal of faecal coliforms between plate count method and qPCR method using RodA as a marker gene.

Results from the qPCR method **(Figure B4)** showed similar results as the plate count method, except for day2 AC.

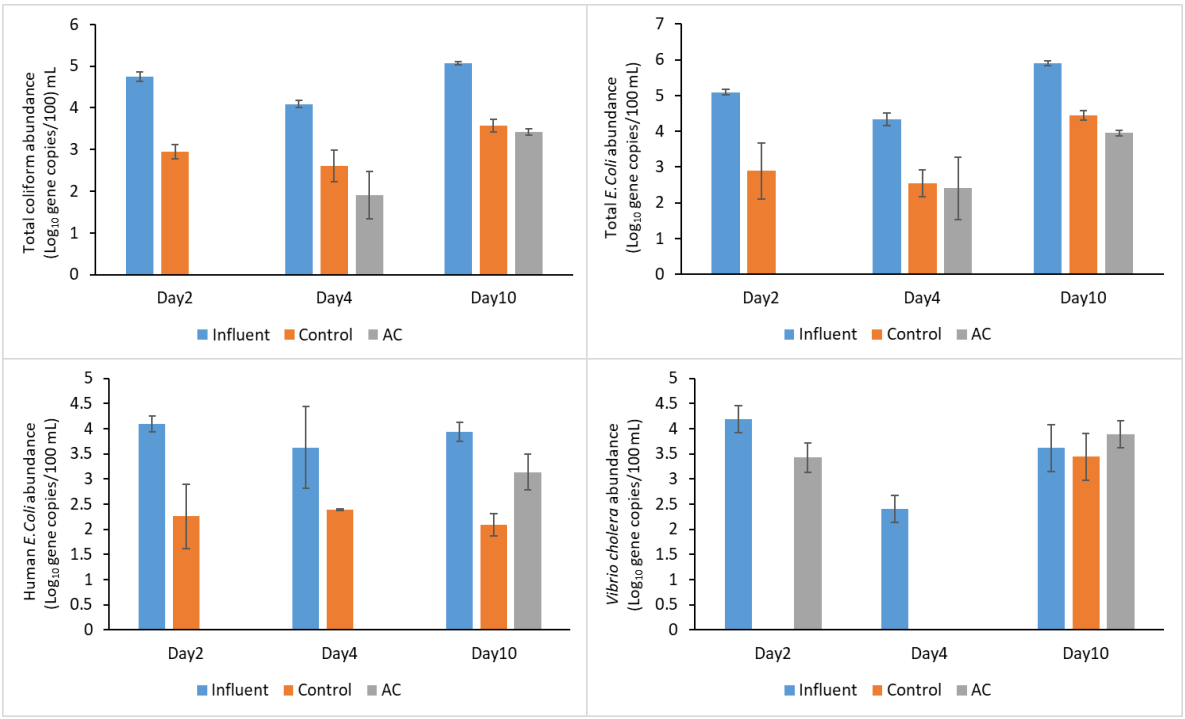

Figure B5. qPCR results (Log_{10} gene copies/100 mL) of total coliform, total *E. coli*, human *E. coli*, and *Vibrio cholerae* in the influent and effluent of the Control and AC columns on day 2, 4 and 10.

The qPCR results of all 4 bacteria **(Figure B5)** confirmed that both Control and AC biofilters performed well in removing these bacteria on day 2 and 4, then the number of gene copies of all 4 bacteria were increased on day 10 except for human *E.coli* in the control effluent. Of all 4 bacteria, AC column performed better than the Control in treating total coliform, total *E. coli and* Human *E. coli* on day 2.

Appendix C

C1: Materials and methods

Biochar production

Biochar was produced by heating coconut husk in a 200 L oil drum kiln under oxygen limitation at the Centre for Energy and Environmental Engineering, Kasetsart University, Kamphaeng Saen Campus, Thailand (Figure S1a). Prior to carbonization, coconut husk was air dried and weighed. To produce biochar in the oil drum kiln, a run was started by introducing 5 kg of dried coconut husks into the kiln. Then, the oven front was covered by clay, except for a square channel at the bottom front to feed in auxiliary fuels and allow some air and heat to circulate into the furnace. The size of the channel opening at the front was 20 x 20 cm^2 **(Figure C1a)**. The process of carbonization started with the burning of auxiliary fuel, such as wood chips or firewood, in the front channel of the kiln. The heat from the burning fuel then flowed into the kiln. The heat first evaporated residual moisture in the coconut husk, which would take two to three hours. When the carbonization occurred, it could be noticed by the smoke released from the chimney. If there was a lot of white smoke released, it indicated that the coconut husks were heated and starting to partially combust. Air intake was then reduced by covering the front channel to about one-fourth. The carbonization proceeded continuously via circulated heat inside the kiln. Once the smoke from the chimney was lessened and became clear smoke, or if there was only hot air released from the chimney, this showed that coconut husks were becoming biochar. Clay was then used to completely cover the front channel of the kiln and other leaks in order to prevent outside air to pass through the kiln. The kiln was left for about 1 night to complete the carbonization. The biochar was harvested once the kiln was cooled. The run was repeated three times. A biochar yield of 33.7% was obtained from each run, and thus a total of 5 kg biochar was obtained from the three runs. For one run, the temperature during biomass conversion to biochar of the first run was measured every 10 minutes using a thermocouple inside the kiln. The maximum chamber temperature of 378 °C was measured at 70 minutes **(Figure C1b)**.

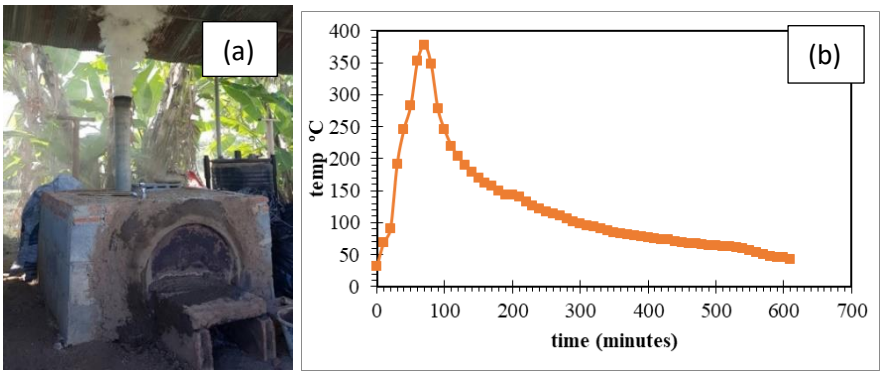

Figure C1. Oil drum kiln (a) and the chamber temperature profile (b).

Synthetic digestate solution

Table C1. Target concentration for each nutrient parameter and its chemical concentration in the synthetic digestate solution.

Parameter	Target concentration (mg/L)	Chemical	Molecular weight (g/mol)	Chemical concentration (mg/L)
NH_4^+-N (mg N/L)	1400	NH_4Cl	53.49	5349.10
NO_3^--N (mg N/L)	50	$NaNO_3$	85.00	303.55
NO_2^--N (mg N/L)	50	$NaNO_2$	69.00	246.41
TN (mg N/L)	3800	Urea	60.06	4933.5*
PO_4^{3-}-P (mg P/L)	15	Na_2HPO_4	141.96	68.69

* Urea concentration for TN was calculated from the target concentration of TN-(NH_4^+-N+NO_3^--N+NO_2^--N): 3800-(1400+50+50) = 2300 mg/L.

Sorption coefficient modelling

The concentration of each nutrient parameter from the biochar/soil sorption experiment was used to calculate a linear sorption coefficient (K_d) (Eq.1). The derivation of the K_d equation, which considered in a mass balance both, the amount of nutrient associated with the biochar/soil and added to the batches as synthetic digestate, is also shown below.

$$K_d = \frac{(C_i \times V_w + C_{eq,cont} \times V_w - C_{eq} \times V_w)}{m \times (C_{eq} - C_{eq,cont})} \quad \text{(Eq.1)}$$

Where: K_d = Sorption coefficient (L/kg), C_i = Initial concentration of nutrient in solution (mg/L), V_w = Volume of solution (L), $C_{eq,cont}$ = Equilibrium concentration of the nutrient in control batches with the sorbent and distilled water (mg/L), m = Mass of adsorbent (kg) and C_{eq} = Equilibrium (final) concentration of the nutrient in batches with the sorbent and synthetic digestate solution (mg/L).

K_d equation derivation

Control batches with distilled water and biochar or soil:

$$V_w \times C_{eq,cont} + m \times K_d \times C_{eq,cont} = M_{nutrient,solid} \quad \text{(Eq.2)}$$

Where: $M_{nutrient,solid}$ (mg) is the amount of nutrient initially associated with the biochar or soil.

Batches with synthetic digestate solution and biochar or soil:

$$C_{eq}\,(V_w + m \times K_d) = V_w \times C_i + M_{nutrient,solid} \quad \text{(Eq.3)}$$

Substitute (Eq.2) in (Eq.3).

$$C_{eq}\,(V_w + m \times K_d) = V_w \times C_i + V_w \times C_{eq,cont} + m \times K_d \times C_{eq,cont}$$

$$C_{eq} \times V_w + C_{eq} \times m \times K_d = V_w \times C_i + V_w \times C_{eq,cont} + m \times K_d \times C_{eq,cont}$$

$$C_{eq} \times m \times K_d - m \times K_d \times C_{eq,cont} = V_w \times C_i + V_w \times C_{eq,cont} - C_{eq} \times V_w$$

$$m \times K_d(C_{eq} - C_{eq,cont}) = V_w \times C_i + V_w \times C_{eq,cont} - C_{eq} \times V_w$$

$$K_d = \frac{(C_i \times V_w + C_{eq,cont} \times V_w - C_{eq} \times V_w)}{m \times (C_{eq} - C_{eq,cont})}$$

Mass balance

Table C2. Total mass (mg) of NH_4^+-N, NO_2^--N, NO_3^--N, TN, N_{org} and PO_4^{3-}-P in the Control and CH system. Results (Mean±S.D.) were reported to two decimal places.

	NH_4^+-N (mg)	NO_2^--N (mg)	NO_3^--N (mg)	TN (mg)	N_{org} (mg)	PO_4^{3-}-P (mg)
Control system	58.03±8.93	1.79±0.20	41.52±12.46	555.48±20.72	453.72±30.54	137.29±32.70
CH system	59.48±8.93	2.14±0.20	46.87±12.54	567.70±20.76	458.44±30.63	152.58±32.89

Table C2 shows that the amounts of nutrients in the CH system were initially up to 19.6% higher than those for the Control system because of the nutrient content of the biochar.

Table C3. Real-time qPCR primers for different genetic markers.

Target organisms	Primer	Sequence (5'>>>3')	Annealing Temperature (T_a)	Amplicon size (bp)	Reference
Total Bacteria (16S rRNA); qPCR	1055 F	ATGGCTGTCGTCAGCT	60 ºC	337	Harms *et al.* (2003)
	1392 R	ACGGGCGGTGTGTAC			
AOB	amoA-1F*	GGGGHTTYTACTGGTGGT	56 ºC	490	Stephen *et al.* (1999)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
16S rRNA gene sequencing; Illumina	515 F	GTGCCAGCMGCCGCGGTAA	55 ºC	291	Kozich *et al.* (2013)
	806 R	TAATCTWTGGGVHCATCAGG			

Table C4. Metrics of the calibration curves on the qPCR instrument for different target bacteria.

Target organism	Slope (Cq/log (Genes/µL))	R^2	Efficiency (%)
Total Bacteria (16S rRNA)	-3.58±0.00	0.987±0.000	90.0±0.0
AOB	-4.13±0.00	0.999±0.000	74.6±0.0

C2: Additional results

To estimate K_d of the biochar amended soil (L/kg)

$$K_{d,amended\ soil} = (1 - f_{bc}) \times K_{d,soil} + f_{bc} \times K_{d,biochar} \quad \text{(Eq.4)}$$

f_{bc} is the weight fraction of the biochar in the soil, K_d, soil (L/kg) is the soil K_d of each nutrient and $K_{d,biochar}$ (L/kg) is the biochar K_d of each nutrient

f_{bc} in this case is 0.09 calculated from the ratio of biochar to total weight in soil (30 g: 330 g)

$$K_{d,amended\ soil} = (1 - 0.09) \times K_{d,soil} + 0.09 \times K_{d,biochar} \quad \text{(Eq.5)}$$

Nutrient leaching

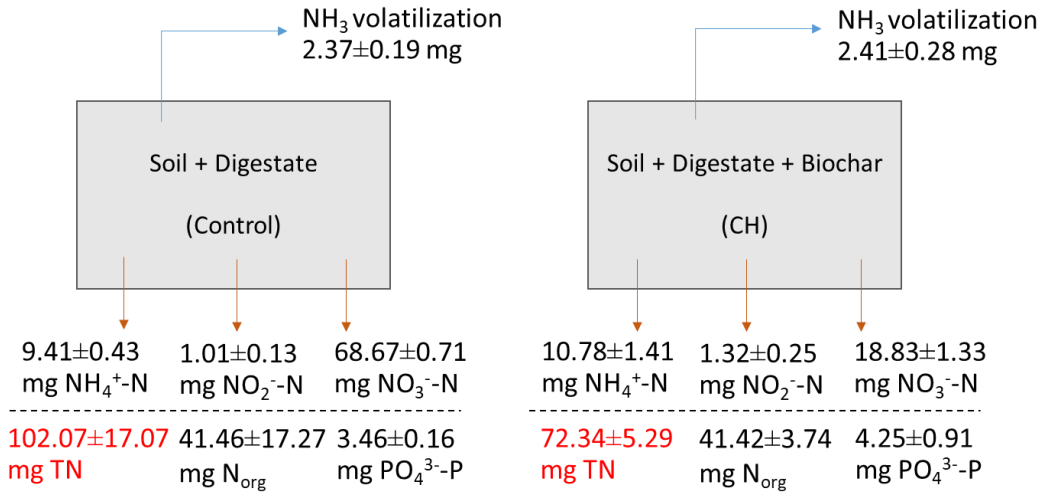

Figure C2. Total mass (mg) of NH_4^+-N, NO_2^--N, NO_3^--N, TN, N_{org}, PO_4^{3-}-P which was lost by leaching (orange arrows) and ammonia (NH_3) volatilization (blue arrows) from digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH) after four repeated volatilization and leaching experiments. Results (Mean±S.D.) were reported to two decimal places. mg TN was coloured in red to emphasize the highest loss in terms of absolute mass among all nutrients in both systems.

PCA analysis

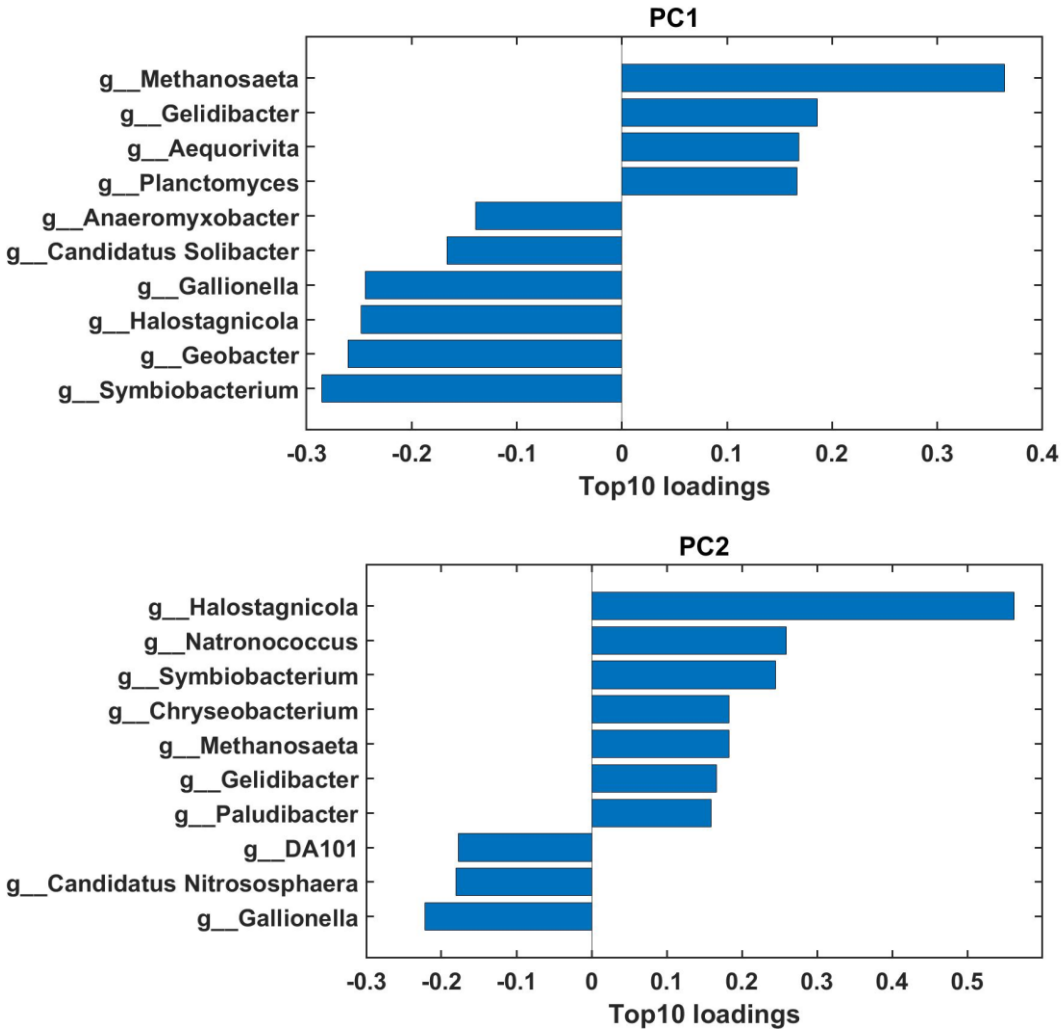

Figure C3. Variables (microbial genera) with the top 10 loadings for component 1 (PC1) and component 2 (PC2), respectively, of the principal component analysis (PCA).

DNA Sequencing and qPCR

Table C5. Relative abundances of different nitrifying microbes, methanogens and methanotrophs estimated from 16S rRNA genes sequenced with Illumina MiSeq sequencing in the original soil, top and bottom soil samples of digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH), and their replicates. n.d. indicates not detected.

	AOB			AOA	NOB		Methanogens	Methanotrophs
Sample	*Nitrosovibrio*	*Nitrosomonas*	Unclassified	*Candidatus Nitrososphaera*	*Nitrospira*	Unclassified		
Original soil 1	1.39E-03	n.d.	n.d.	6.50E-02	1.76E-03	n.d.	1.74E-03	3.03E-04
Original soil 2	1.81E-03	n.d.	n.d.	5.34E-02	2.11E-03	n.d.	1.53E-03	4.39E-04
Control topsoil 1	1.77E-03	n.d.	2.89E-04	4.61E-02	2.87E-03	n.d.	2.11E-02	2.10E-04
Control topsoil 2	2.75E-03	n.d.	1.36E-04	6.75E-02	4.24E-03	7.78E-05	1.43E-02	1.69E-04
Control bottom soil 1	1.05E-03	n.d.	n.d.	7.20E-02	4.57E-03	2.53E-04	1.60E-03	4.36E-04
Control bottom soil 2	1.07E-03	n.d.	n.d.	6.08E-02	2.43E-03	1.21E-04	2.33E-03	4.50E-04
CH topsoil 1	1.86E-03	2.35E-04	4.79E-04	3.20E-02	4.19E-03	8.24E-05	2.98E-02	2.43E-04
CH topsoil 2	1.68E-03	4.70E-05	1.10E-04	5.01E-02	3.55E-03	2.35E-04	1.21E-02	2.51E-04
CH bottom soil 1	9.21E-04	n.d.	n.d.	5.72E-02	1.59E-03	1.21E-04	2.45E-03	3.96E-04
CH bottom soil 2	9.01E-04	n.d.	n.d.	5.27E-02	1.22E-03	n.d.	3.78E-03	2.78E-04

Table C6. 16S rRNA gene copy numbers obtained from qPCR quantification in the original soil, top and bottom soil samples of digestate-amended soil (Control) and digestate-amended soil with CH biochar (CH), and their replicates.

Sample	gene copies/g soil
Original soil 1	6.63E+08
Original soil 2	4.73E+08
Control topsoil 1	5.25E+08
Control topsoil 2	4.44E+08
Control bottom soil 1	4.84E+08
Control bottom soil 2	2.63E+08
CH topsoil 1	2.12E+08
CH topsoil 2	4.50E+07
CH bottom soil 1	1.47E+08
CH bottom soil 2	1.67E+08

Raw data

Table C7. pH and nutrient concentrations (C_{eq}) of NH_4^+-N, NO_3^--N, NO_2^--N, TN, PO_4^{3-}-P and N_{org} (mg/L) in batch experiments containing biochar-amended synthetic solutions and two sets of control. Results were reported to two decimal places.

Sample	Biochar (g)	pH pre contact time	pH post contact time	NH_4^+-N (mg N/L)	NO_3^--N (mg N/L)	NO_2^--N (mg N/L)	TN (mg N/L)	PO_4^{3-}-P (mg P/L)	N_{org}* (mg N/L)
CH 1	1.5	8.51	8.21	1120.00	55.00	52.20	3690.00	39.40	2462.80
CH 2	1.5	8.54	8.18	1180.00	53.75	53.00	3210.00	42.00	1923.25
Control A1	X	7.88	7.81	1300.00	50.23	50.50	3890.00	8.60	2489.27
Control A2	X	7.88	7.76	1390.00	51.00	52.10	4030.00	9.00	2536.90
Control B1	1.5	9.78	9.75	2.13	10.30	0.60	16.50	32.20	3.47
Control B2	1.5	9.76	9.71	2.01	6.00	0.60	18.80	28.30	10.19

* N_{org} data were obtained from calculation

CH = synthetic solution + 1.5 g biochar

Control A = synthetic solution only

Control B = distilled water + 1.5 g biochar

Table C8. pH and nutrient concentrations (C_{eq}) of NH_4^+-N, NO_3^--N, NO_2^--N, TN, PO_4^{3-}-P and N_{org} (mg/L) in batch experiments containing soil-amended synthetic solutions and two sets of control. Results were reported to two decimal places.

Sample	Soil (g)	pH pre contact time	pH post contact time	NH_4^+-N (mg N/L)	NO_3^--N (mg N/L)	NO_2^--N (mg N/L)	TN (mg N/L)	PO_4^{3-}-P (mg P/L)	N_{org}* (mg N/L)
Soil 1	1.5	7.45	6.89	1420.00	65.00	52.80	3670.00	7.80	2132.20
Soil 2	1.5	7.37	6.82	1410.00	61.30	52.30	3520.00	9.10	1996.40
Control A 1	X	7.67	7.66	1440.00	61.50	54.30	3620.00	14.90	2064.20
Control A 2	X	7.67	7.66	1500.00	55.90	53.30	3700.00	16.60	2090.80
Control B 1	1.5	6.40	6.23	1.43	8.05	0.20	74.00	5.35	64.32
Control B 2	1.5	6.43	6.25	1.52	4.10	0.25	71.00	4.40	65.13

* N_{org} data were obtained from calculation

Soil = synthetic solution + 1.5 g soil

Control A = synthetic solution only

Control B = distilled water + 1.5 g soil

Table C9. NH_4^+-N (mg) in the acidic water trap as a function of time of the system containing digestate-amended soil with CH biochar (CH) and digestate-amended soil only (Control) on day 1, 2, 11 and 28. Results were reported to three decimal places.

Day	Hour	NH_4^+-N (mg)			
		CH 1	CH 2	Control 1	Control 2
1	0	0.000	0.000	0.000	0.000
	2	0.111	0.270	0.129	0.132
	4	0.318	0.586	0.308	0.297
	17	1.770	2.330	1.860	2.170
2	0	0.000	0.000	0.000	0.000
	2	0.073	0.081	0.066	0.106
	4	0.186	0.194	0.168	0.218
	6	0.287	0.279	0.290	0.338
11	0	0.000	0.000	0.000	0.000
	2	0.010	0.007	0.000	0.000
	4	0.047	0.045	0.017	0.027
	6	0.076	0.075	0.030	0.045
28	0	0.000	0.000	0.000	0.000
	2	0.002	0.000	0.000	0.000
	5	0.000	0.000	0.000	0.000
	8	0.000	0.000	0.000	0.000

Table C10. pH and mass of each nutrient (NH_4^+-N, NO_3^--N, NO_2^--N, TN, PO_4^{3-}-P and N_{org}) (mg) lost by leaching from the system containing digestate-amended soil with CH biochar (CH) and digestate-amended soil only (Control) on day 7, 9, 16 and 30. Results were reported to two decimal places.

Day	System	pH	NH_4^+-N (mg)	NO_3^--N (mg)	NO_2^--N (mg)	TN (mg)	PO_4^{3-}-P (mg)	N_{org}* (mg)
7	Control 1	6.11	2.82	31.20	0.10	77.04	0.66	42.93
	Control 2	5.75	3.16	31.27	0.11	42.93	0.62	8.40
	CH 1	7.45	3.83	12.57	0.08	40.92	0.38	24.43
	CH 2	7.52	3.19	14.25	0.06	36.23	0.29	18.72
9	Control 1	6.83	2.68	8.40	0.63	20.58	1.05	8.87
	Control 2	6.55	3.38	8.29	0.42	21.86	1.23	9.76
	CH 1	7.93	4.82	2.40	1.10	19.68	2.66	11.35
	CH 2	8.11	3.02	1.17	0.62	11.48	1.27	6.67
16	Control 1	7.50	2.90	7.30	0.29	16.63	1.15	6.14
	Control 2	7.11	3.11	7.51	0.21	17.33	1.40	6.50
	CH 1	7.98	3.65	2.82	0.28	17.01	2.06	10.26
	CH 2	8.15	1.89	1.18	0.28	12.51	1.03	9.17
30	Control 1	7.06	0.26	3.91	0.08	4.25	0.44	0.00
	Control 2	8.03	0.51	2.51	0.19	3.30	0.38	0.09
	CH 1	8.08	1.24	1.31	0.17	3.65	0.81	0.93
	CH 2	8.79	0.20	1.36	0.05	2.06	0.27	0.46

*N_{org} data were obtained from calculation

Literature comparisons

Table C11. Comparisons of biochar effects on ammonia volatilization, nutrient leaching and nitrification between this and literature studies.

	This study outcome	Studies with the same outcomes	Studies with different outcomes	Type of biochar used in each study
Ammonia volatilization	Coconut husk biochar had no significant effects on ammonia volatilization in soil	Sha *et al.* (2019) Sun *et al.* (2017)	Reduced ammonia volatilization: Doydora *et al.* (2011) Mandal *et al.* (2016) Taghizadeh-Toosi *et al.* (2012) Increased ammonia volatilization: Sun *et al.* (2014) Chen *et al.* (2013) Schomberg *et al.* (2012)	Sha *et al.* (2019): Ligno-cellulosic waste (Macadamia nutshell, walnut shells, peanut shells and maize cobs) Sun *et al.* (2017): Wheat straw Doydora *et al.* (2011): Wood Mandal *et al.* (2016): Poultry litter and Macadamia nutshell Taghizadeh-Toosi *et al.* (2012): Wood chips Sun *et al.* (2014): Wheat straw Chen *et al.* (2013): Green waste Schomberg *et al.* (2012): Peanut hull
Nutrient leaching	Coconut husk biochar reduced nitrate leaching in soil	Yao *et al.* (2012) Bohara *et al.* (2019) Laird *et al.* (2010) Zheng *et al.* (2013)	Increased nitrate leaching: Li *et al.* (2018) Yoo and Kang (2012) Eykelbosh *et al.* (2015)	Yao *et al.* (2012): Peanut hull and Brazilian pepperwood Bohara *et al.* (2019): Pinewood Laird *et al.* (2010): Hardwood Zheng *et al.* (2013): Giant reed Li *et al.* (2018): Apple branches Yoo and Kang (2012): Manure Eykelbosh *et al.* (2015): Filtercake
Nitrification	Coconut husk biochar retarded nitrification in soil	Wang *et al.* (2015) Clough *et al.* (2010) Dempster *et al.* (2012) Song *et al.* (2019)	Increased nitrification: Bi *et al.* (2017) Prommer *et al.* (2014) He *et al.* (2019b) Zhao *et al.* (2020)	Wang *et al.* (2015): Peanut shell Clough *et al.* (2010): Wood Dempster *et al.* (2012): *Eucalyptus marginata* Song *et al.* (2019): Bamboo leaf Bi *et al.* (2017): Rice straw Prommer *et al.* (2014): Wood He *et al.* (2019b): Rice straw Zhao *et al.* (2020): Wheat straw