Establishment of Anammox Wastewater Treatment Process in the Expanded Bed Biofilm Reactor (EBBR) with ABDite

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Establishment of Anammox Wastewater Treatment Process in the Expanded Bed Biofilm Reactor (EBBR) with ABDite

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Abstract

The anaerobic ammonia oxidation (Anammox) process plays a vital role in wastewater treatment by removing up to 90 % nitrogen at a lower cost: due to reductions in energy and oxygen consumptions by 60 %. The Anammox process has successfully been used by different bioreactors at laboratory, pilot, and industrial scale to treat wastewater that originated from agricultural, domestic, and industrial sources amongst others. The aim of this thesis is to determine whether the Anammox wastewater treatment process could be established using the lab scale expanded bed biofilm reactor (EBBR) successful at high-rate nitrification of wastewater. In a series of studies, the research will: i) establish the Anammox process in the EBBR; ii) optimize the EBBR-Anammox process pH and temperature conditions; iii) Examine the EBBR ABDite biofilm and compare the EBBR-Anammox performance with other available Anammox process technologies; and iv) identify key Anammox bacteria in the established Anammox process. Results indicated that the established lab scale EBBR-Anammox process achieved a maximum nitrogen removal rate (NRR) of 6.1 Kg N m^{-3} d^{-1} from a loading rate of the 9.8 Kg N m^{-3} d^{-1} with 80 – 100 % nitrogen removal efficiency (NRE). The optimization results suggested that pH 8.2, at 28 ºC as the optimum condition for the EBBR-Anammox process: with 2.23 ± 0.07 kg N m^{-3} EBBR d^{-1} NRR. SEM analysis detected biofilm with cauliflower structure, alongside filamentous, rod and cocci-like bacteria cell consistent with reported characteristic of Anammox bacteria; and yeasts-like communities on the EBBR ABDite bioparticles. Using molecular biology techniques, 3 Anammox bacteria genera (Candidatus *Brocadia,* Candidatus *Kuenenia* and Candidatus *Jettenia)* were identified in the established Anammox EBBR. Also identified were ammonia oxidizer *Nitrosomonas*, complete ammonia oxidizer *Nitrospira*, nitrite oxidizer *Nitrobacter*, ammonia oxidizing Archaea *Nitrosarchaeum limnium*; nitrate reducing bacteria *Nitratireductor,* anaerobic heterotrophic bacteria *Anaerolinea thermolimosa*, yeasts and others. The EBBR achieved comparable nitrogen removal rate and nitrogen removal efficiency, developed similar Anammox

granules, cauliflower biofilm and key bacterial communities reported by other Anammox technologies. These results suggest that the Anammox process was established in the EBBR and opens the possibility of further research in adapting the EBBR system to the complete Anammox process for wastewater treatment.

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List of Abbreviations

AGE: Agarose gel electrophoresis

Anammox: Anaerobic ammonia oxidation

AnAOB: Anaerobic ammonia oxidizing bacteria

AnBGE: Anammox bacteria granule that developed in the EBBR

AnMBR: Anaerobic membrane biofilm reactor

AOB: Ammonia oxidizing bacteria

ASR: Activated sludge reactor

BLAST: Basic local alignment search tool for DNA/protein

CANON: Completely autotrophic nitrogen removal over nitrite

DNA: Deoxyribose nucleic acid

DWW: Domestic wastewater

EBBR: Expanded bed biofilm reactor

EBC: Expanded bed column

EPS: Extracellular polymeric substance

FBR: Fluidized bed reactor

GLR: Gas lift reactor

HAT: Higher annealing temperature

HRT: Hydraulic retention time

ILAB: Internal-loop-airlift bioparticle reactor

LAP: Lower annealing temperature

MBR: Membrane biofilm reactor

MSBR: Membrane sequencing batch reactor

MWW: Municipal wastewater

NCBI: National Centre for Biotechnology Information

NGS: Next generation sequencing

NH_3-N: Ammonia nitrogen

NLR: Nitrogen loading rate

NO_2-N: Nitrite nitrogen

NO_3-N: Nitrate nitrogen

NOB: Nitrate oxidizing bacteria

NRB: Nitrate reducing bacteria

NRE: Nitrogen removal efficiencies

NRR: Nitrogen removal rate

OAT: Optimum annealing temperature

OTU: Operational taxonomic unit

PCM: Phase contrast microscope

PCR: Polymerase chain reaction

PDN-AMX: Partial denitrification - Anammox

PN: Partial nitrification

PN-AMX: Partial nitrification – Anammox

PSS: Primer stock solution

PWS: Primer working stock

RBC: Rotating biological contactor

SA: Single Anammox

SAnBG: Seed Anammox bacteria granule supplied by STW

SBR: Sequencing batch reactor

SEM: Scanning electron microscope

SHARON: Single high activity ammonium removal over nitrite

SPNA: Simultaneous partial-nitrification Anammox

STW: Severn Trent Waters

SWW: Synthetic wastewater

TN: Total nitrogen

PTC: - Temperature combination

UASB: Upflow anaerobic sludge blanket

WWT: Wastewater treatment

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CHAPTER ONE

Literature Review

1.0 Introduction

1.1 Wastewater, origins, and compositions

Water is very important to humans; the supply of safe drinking water is a major contributor to a healthy life. Between 50 and 100 L of water is required per person each day to meet drinking (1.6 – 7.5 L), personal hygiene (20 L), cooking and other basic needs; the water must be of good quality to avoid diseases and reduce health concerns (WHO, 2015a; Fawell and Nieuwenhuijsen 2003). In 2020, an average of 149 – 165 L of water was used per person, while a 2-person household consumed 523 L of water a day across the UK (Tiseo, 2021), generating wastewater.

Wastewater refers to any water, whose quality has been affected adversely by human activities (Tilley et al., 2014). Wastewaters originate from a variety of sources such as domestic, farming, commercial, industrial processes, storm water runoffs, infiltration from sewerage systems amongst other (Schroder et al., 2016; Margot et al., 2015; Henze and Comeau, 2008), as summarized in Table 1.1.

Table 1.1: Sources of wastewater (adapted from (Kumwimba et la., 2018, Henze and Comeau, 2008; Fawell and Nieuwenhuijsen 2003).

Main sources	Type of contaminants
Underground leakage from soil, oceans	Physical contaminants (e.g., suspended organic matter, sediment)
	Radiological contaminants (e. g., Radon, radium, uranium, lead
Agriculture	Fertilizer (ammonia/nitrogen, phosphorus), pesticides
Domestic	Nitrogen from human urea, ammonia from detergents, bleach, disinfectants etc, phosphorus in drinking water

industry	Chemical (e.g., dye, heavy metals, and pharmaceutical residues, etc.
Hospital effluents	Biological contaminants (pathogenic and non-pathogenic microorganisms)

There are variations in the composition of wastewater emanating from these sources (Table 1.1); and the quantity of wastewater is influenced by various factors and human activities (Henze and Comeau, 2008).

Municipal wastewater (MWW) contains a variety of physically suspended solids, dissolved organic and inorganic compounds, pathogenic and non- pathogenic microorganisms (Table 1.2). Some organic substances in sewage are carbohydrates, proteins, fats, lignin, soaps and detergents, natural and synthetic compounds (Rodriguez-Sanchez et al., 2014; Blancheton *et al.,* 2013; Henze and Comeau, 2008). Ellis (2004) stated that there is approximately 50 % proteins, 40 % carbohydrates, 10 percent fats and oils, with trace amount of other pollutants in municipal wastewater. However, other sources have found a range of 10 – 38 % (protein), 6 – 18 % (carbohydrate) and 7 – 45 % of lipids in MWW (Huang et al., 2010 Sophonsiri and Morgenroth, 2004). These chemical compounds if not removed before the wastewater is discharged into natural water bodies, would have negative effects on the environment.

Table 1.2: Physical and chemical content of untreated wastewaters (adapted from Rouwane et al., 2016; Henze and Comeau, 2008).

Constituent	Example	Negative Effect
Microorganism	Pathogenic bacteria, viruses, and warm eggs	Risk of infection when bathing and eating fish
Biodegradable organic materials	green waste, food waste	Oxygen depletion in rivers and lakes
Other organic materials	Detergents, pesticides, fats, oil and grease, solvent, colouring phenols, cyanide	Toxic effect, aesthetic inconveniences, bioaccumulation in the food chain
Metals	Mercury, lead, cadmium, chromium, copper, nickel	Toxic effects, bioaccumulation
Other inorganic materials	Acids and bases	Toxic effect, corrosion
Thermal effects	Hot water	Changes living conditions of aquatic organisms (flora and fauna)
Odour and taste	Hydrogen sulphide	Toxic effect, aesthetic inconveniences
Radioactive materials	-	Toxic effect, accumulation
Nutrients	Nitrate, phosphate, Ammonium	Eutrophication, oxygen depletion, toxic effect
	Nitrate and phosphate	Enhance the release of arsenic and antimony in oxic conditions; and nitrate help retain phosphate in sediment.

The origin and volumes of domestic wastewater (DWW) are more predictable than industrial sources. Approximately, daily per household, 50L of domestic wastewater come from toilet flushing; 13.5 L emanate from laundry. Baths and showers yield another 50 L; dishwashing and cleaning 13.5 L, gardening 7 L and 4.5 L contributed by cooking; these values have changed in the UK and other developed countries, owing to increased frequency of showering and use of power showers (Tiseo, 2021). The chemical composition of organic matter in DWW is estimated to be 20.64 %, Fibers, 12.38% proteins, 10. 65 %, and 30 % volatile and

soluble compounds (Man-hong et al., 2010); these figures do vary depending on the predominant activities that generated the organic matter in the wastewaters. Approximately, 21 % of DWW is faeces and urine (Racek, 2019; Raclavsky et al., 2013); human urine is the main source of wastewater nitrogen (Simha and Ganesapillai, 2017).

Around the world, about 80% of wastewater discharged into water bodies are without prior treatment. Such discharges have resulted in an increased deterioration in water quality, with detrimental effects on the ecosystem and human health (WWAP, 2018; Okereke et al., 2016). The discharge of ammonia rich wastewaters from human urea, nitrate fertilizer, metal finishing, processing crude oil and pharmaceutical products into lakes, rivers, and streams, upsets the nitrogen balance in these water bodies, causing several problems associated with the water pollution (Schroder *et al*., 2016; WHO, 2015a; Canfield et al., 2010).

Therefore, wastewater treatment process such as the anaerobic ammonia oxidation (Anammox) process is an eco-friendly and energy efficient process of treating wastewater rich in ammonia that could remove up to 90 % of total nitrogen in the form of NH_3-N and NO_2-N, with the production of about 10 % of NO_3-N is very important; the Anammox process can reduce the adverse effects caused the discharge of wastewater into aquatic environments (Hu et al., 2013; Van Hull et al., 2010; Mulder et al., 1995).

1.2 Problems associated with nitrogen species in wastewater

1.2.1 Effect of nitrogen species in wastewater on aquatic life

Three nitrogen species, which are ammonia nitrogen (NH_3-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N) and organic nitrogen bound in proteins and decomposing remains of animal and plant are the main forms of nitrogen found in wastewater (Szogi et al., 2015; Holland et al., 2005).

1.2.1.1 Ammonia nitrogen

Ammonia nitrogen (NH_3-N) contamination causes damage to aquatic life in receiving water bodies around the world (Rodriguez-Sanchez et al., 2014). The natural level of total ammonia in surface and ground water is about 0.2 g L^{-1}; however, anaerobic bacterial respiratory activity could sometimes increase the groundwater concentration up to 3.0 mg L^{-1} (Mouser et al., 2009). Concentration of ammonia above 0.2 mg L^{-1}, is an indicator of possible pollution from microbial activity, sewage, and animal waste (WHO, 2003). In fish, ammonia concentrations (e. g. 2 – 20 mg L^{-1}) prevents the transfer of oxygen from the gill to the blood, which causes a temporary to permanent damage to the gills. Fish exposed to such level of ammonia are usually sluggish and found on the surface of water gasping for air due to gill damage (Thangam et al., 2014; Benli et al., 2008). Ammonia, at a sub-lethal value of 1.05 ppm could cause up to 15 - 30 % decrease in red blood cell (RBC) and 25 - 51 % white blood cell (WBC) counts of *Cyprinus carpio* fish species (Thangam et al., 2014).

Some species of fish can tolerate high level of ammonia; for example, elasmobranchs and teleosts can convert ammonia to urea in their brain (Randell and Tsui, 2002). Ammonia concentration higher than 0.04 mg L^{-1} can reduce swimming performance or kill the Coho salmon (Levit, 2010). While 80 - 100 mg L^{-1} of ammonia has a chronic health damage on juvenile rainbow trout (Yang et al., 2019; Davidson et al., 2014). In Europe, the acceptable level of ammonia in European Commission designated waters, set by the Europe

Quality Services (EQSs) for protection of freshwater fish is 0.021 mg L^{-1} un - ionised ammonia (NH_3) and 0.78 mg L^{-1} for total ammonia – i.e., NH_3 and NH_4^+ (Killeen, 2007). The UK Environment Protection Agency (EPA) has set the threshold value for un-ionised ammonia in estuaries at 0.021 mg L^{-1} (EPA UK, 2007).

1.2.1.2 Nitrate nitrogen

Nitrate nitrogen (NO_3-N) toxicity to aquatic animals including invertebrates, fishes and amphibians is due to the conversion of haemoglobin to forms incapable of carrying oxygen. The level of toxicity is positively correlated with increase in nitrate concentration, length of exposure and size of organisms. While 10 mg L^{-1} of NO_3-N is toxic to freshwater animals, marine animals can tolerate higher level with toxicity occurring at about 20 mg L^{-1} (Camargo et al., 2005). Nitrate, at a concentration of 165 mg L^{-1} causes a chronic damage to *Oplegnathus punctatus* (Yang et al., 2019; Davidson et al., 2014).

1.2.1.2 Nitrite nitrogen

Nitrite nitrogen (NO_2-N), another wastewater nitrogen species is equally toxic to aquatic invertebrates and fish depending on the size, age, and fish species in addition to factors such as exposure length, temperature, and oxygen level amongst others (Kroupova et al., 2005). Svobodova et al., 2005, reported the death of catfish with brownish coloured gills in a water tank in which the oxygen level was around 12 mg L^{-1} and temperature of 24 °C. The exposure of two tropical fish species (*Astyanax altiparanae* and *Prochilodus lineatus)* to 30 mg L^{-1} for 96 - h resulted in a red blood cell counts decrease and the formation *of* methaemoglobin (Martinez and Souza, 2002). At a concentration of 50 mg L^{-1} nitrite causes a mass death of fish as it diffuses into red blood cells resulting in a permanent oxidation of iron present in haemoglobin. The increase in methaemoglobin reduces the ability of the red blood cells to carry oxygen, which gives the fish blood and gills the characteristic brownish colour leading to death (Alonso and Camargo, 2006; Kroupova et al., 2005).

1.2.2 Effects of wastewater pollution on humans

1.2.2.1 Pathogenic human diseases

The discharge of wastewater into aquatic environments constitutes a significant risk to public health because wastewaters contain pathogens that could cause gastrointestinal and other human diseases (Gerba and Smith, 2005). Infections from pathogenic microorganisms, occurs during bathing and fishing. The UK epidemiological studies have revealed that bathers have 3.2 times higher risk of suffering from enteric disease including nausea, stomach-ache, diarrhoea or vomiting than non-bathers (Defra, UK, 2014). It is estimated that each year, around 3.4 million people die from diseases (cholera, typhoid, infectious hepatitis, polio, cryptosporidiosis, ascariasis, and diarrhoeal diseases) linked to pathogens present in polluted water (WHO, 2015a). In USA, about 3.5 million who engage in recreational activities fall sick by swimming and boating in contaminated waters (Blaettle, 2018) each year.

1.2.2.2 Contamination of drinking water

Drinking water contamination by ammonia is common in water sourced from rivers (Hassan *et al.,* 2014). The presence of ammonia in drinking water does not cause an immediate human health problem; but when ammonia exposure is above 200 mg kg^{-1} of body weight, toxicity can occur (WHO, 2003). Ammonia and chlorine are used during the chloramination process of drinking water during; ammonia reacts with chlorine to form chloramines, which disinfects the water and kill pathogens. Inadequate chlorination could encourage nitrite formation in water distribution systems resulting in pathogens thriving in the drinking water (Table 1.1); leading to water borne infections (WHO, 2003; Regan et al., 2002).

1.2. 2.3 Aesthetic damage to aquatic environments

Ammonia nitrogen is a major contributor to eutrophication, leading to potential changes in water quality and reduction in biodiversity (European Environment Agency, 2015). The accumulation of nutrients e.g., nitrate in natural water bodies disrupts natural processes such as fish migration and destroys the aesthetic values of lakes and rivers, (WHO, 2015a; Rodriguez-Sanchez et al., 2014). Algal blooms reduce water quality; resulting in colour change and offensive odour that pollutes the air. The aesthetic damage inconveniences visitors, as the water is no longer suitable for swimming, with detrimental effect on tourism, which threatens jobs and local economy. Water pollution is the cause of up to a billion-dollar loss each year by the US tourism industry (EPA, 2019; Craig-Smith et al., 2006). Freshwater damage by eutrophication in England and Wales is estimated to cost between £75.0 − 114.3 m every year (Pretty et al., 2003).

The need to remove these nitrogen species (NH_3-N; NO_3-N and NO_2-N) from wastewater before discharge is extremely important to safeguard the aquatic environment (Van Kessel et al., 2015). Proper treatment of wastewater via the Anammox process amongst others is crucial in maintaining public health, preventing disease outbreaks and avoid environmental damage of recipient waters. The effective removal of ammonia from wastewater is a necessary water-quality management requirement to reduce the adverse effects in receiving waters bodies and meet wastewater discharge regulatory standards. (Du et al., 2015; Carey et al., 2009; Khopkar, 2004). In addition, treated effluent could have some beneficial applications, e.g., as fertilizer for growing agricultural crops, irrigation, and aquaculture for fish farming (Mara, 2013).

1.3. Stages of wastewater treatment

The level of treatment wastewater is subjected to is determined by the initial quality and the intended final use of the treated water. Wastewater treatment occurs in five stages (Table 1.3).

Table: 1.3 Stages for treatment of wastewater for different purposes.

Stages	Details	Reference
Screening	Removes large objects such as plastics, sanitary items, blocks of woods and broken bottles and grits washed down the sewer	Matamorose et al., 2009
Primary	Effluent (liquid water) is separated from solid organic matter such as human waste using separators.	Collin et al., 2020; Gagnon et al., 2008
Secondary	Wastewater pollutants are biodegraded to safe levels by aerobic microorganisms; and may involve biofiltration, activated sludge, oxidation ponds and trickling filters	Posadas et al., 2015; Daigger, and Boltz 2015; He and Xu, 2010.
Tertiary	Involves the removal of dissolved inorganic nutrients (e.g., phosphates) and nitrogenous compounds (ammonia, nitrite and nitrates) form the wastewater to improve the water quality and meet regulatory/discharge standards set by bodies e. g. Environment Agency for England and Wales; and the European Union (EU) Water Framework Directive. The conversion of nitrogenous compounds to nitrogen gas is carried out by the actions of different species of aerobic bacteria through nitrification and anaerobic denitrification processes e.g., Anammox.	Fatta-Kassinos, et al., 2011; Hamodal et al., 2004; Jaroszynski et al., 2012; Van der Star et al., 2007
Quaternary	Uses oxidation and fine filtration processes to remove micropollutants to the level of parts per million and parts per billion; and activated materials such as carbon powder coal or sand to adsorb contaminants	Tezel et al., 2007

1.4 Anammox and other wastewater treatment methods

Methods used in wastewater treatment (WWT) to remove nitrogen include nitrification – denitrification (Rodriguez-Sanchez et al. 2014; Langone et al. 2014); partial nitrification (PN) – the oxidation of ammonia to nitrite coupled to denitrification process (Ruiz et al. 2003) and the anaerobic ammonia oxidation (Anammox), which converts the nitrite from PN and ammonia to nitrogen gas (Kuenen, 2020).

1.4.1 Nitrification

Nitrification was initially described as a two-step oxidation of ammonia (NH_3) to nitrite (NO_2^-), and nitrite to nitrate (NO_3^-), carried out by ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), nitrite oxidizing bacteria (NOB) and Crenarchaea (Roy et al., 2017; Wang et al., 2017) (Eqns. 1.1- 1.4). However, in 2015, discovery of Nitrospira capable of complete ammonia oxidation (Commamox) caused a scientific shift in the established knowledge of nitrification and the nitrogen cycle (Koch et al., 2019; Daims et al., 2015; Van Kessel et al., 2015) (Eqn. 1.5).

$NH_3 + O_2 + 2H^+ \rightarrow 2e\text{-}\ NH_2OH + H_2O$ (*1.1)*

$NH_2OH + H_2O \rightarrow NO_2^- + 4e^- + 5H^+$ (*1.2)*

$½\ O_2 + 2\ H^+ + 2\ e^- \rightarrow H_2O$ (*1.3)*

$NO_2\text{-} + H_2O \rightarrow NO_3\ \text{-} + 2H^+ + 2e\text{-}$ *(1.4)*

$NH_3 + 2\ O_2 \rightarrow NO_3^- + H^+ + H_2O$ *(1.5)*

Partial nitrification (PN) that leads to the NO_2-N accumulation (Eqn.1.2) is carried out by ammonia-oxidizing bacteria (AOB); and includes species of *Nitrosomonas europea, Nitrosococcus oceanus* and *Nitrosospira* amongst others. The second stage, which completes the nitrification process, producing NO_3-N (Eqn. 1.4), is conducted by NOB species such as *Nitrobacter winogradsky* and *Nitrobacter hamburgensis* (Stempfhuber et al., 2016; Bitton, 2005). During PN, it is important to control NOB activity to prevent complete nitrification of ammonia to nitrate; this reduces the availability of nitrite for the Anammox process required to convert the remaining ammonia and nitrite to nitrogen gas (Rodriguez-Sanchez et al., 2014; Zhang et al., 2010). In addition, the presence of Comammox bacteria (e.g., *Nitrospira inopinata, Nitrospira nitrosa,* and *Nitrospira nitrificans* in an Anammox wastewater treatment bioreactor, could also increase the concentration of nitrate (NO_3-N) and reduce Anammox bacterial activity by competing for nitrite; the electron acceptor to the Anammox process (Kuenen, 2020; Koch et al.,2019; Daims et al., 2015).

1.4. 2 Denitrification

During denitrification, nitrate and nitrite are reduced to atmospheric nitrogen gas (Eqn. 1.6) Denitrifying heterotrophs which use organic carbon e. g., methanol as energy source; are anaerobic bacteria that live in oxygen depleted water-logged soils and sediments. and include *Caldilinea aerophile* and *Anaerolinea thermolimosa*. Denitrification is a very useful wastewater treatment process that reduces the concentration of nitrate in wastewater before it is discharged into receiving water bodies (Fajardo et al., 2014).

$$2\ NO_3^- + 10\ e^- + 12\ H^+ \rightarrow N_2 + 6\ H_2O \quad \textit{(1.6)}$$

In a single Anammox (SA) process, in which nitrite and ammonia are present in the influent, it is important to control the activities of AOB and NOB to prevent the accumulation of nitrite from partial and nitrate from complete ammonia oxidation;

both processes which could negatively affect Anammox bacterial activity (Bettazzi et al., 2010; Van der Star et al., 2007).

1.4.3. Anaerobic ammonia oxidation

Anammox wastewater treatment (Figures 1.1) is a biological process in which nitrite and ammonia are converted directly into dinitrogen gas (N_2) under anoxic conditions (Rodriguez-Sanchez et al., 2014). The Anammox process makes important contributions to the environment by producing up to 50% of the gaseous nitrogen (N_2) in the Earth's atmosphere (Van Teeseling et al., 2013); and accounts for nearly 50% of the nitrogen recycled in aquatic environments (Kartal et al., 2010).

$$NH_3 + NO_2^- + H^+ \rightarrow N_2 + 2H_2O \quad (1.7)$$

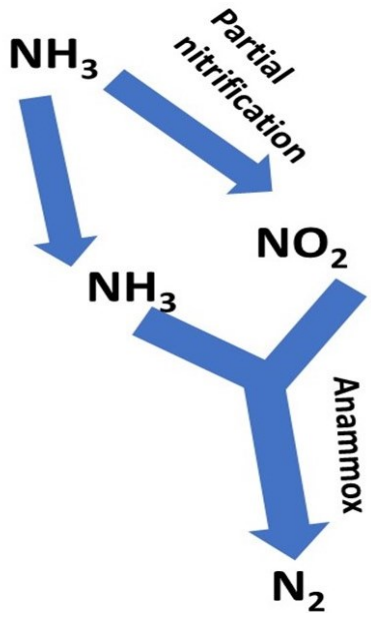

Figure 1.1The Anammox process (Adapted from Rodriguez-Sanchez et al., 2014).

1.4.3.1 The characteristics of Anammox bacteria

The anaerobic ammonia oxidation (Anammox) is a denitrification process that converts ammonia and nitrite to atmospheric nitrogen (N_2) (Eqn.1.7) Anammox bacteria are autotrophs that utilize inorganic carbon such as carbon dioxide and carbonates as energy source for metabolic activity. They belong to a group called Planctomycetes; with *Anammoxoglobus* Brocadia, Kuenenia, Jettenia and Scalindua as members (Jetten et al., 2001; Wang et al., 2015). Anammox bacteria are obligate anaerobes which produce N_2 from ammonia and nitrite, with hydroxylamine and hydrazine as intermediates (Park et al., 2010; Sonthiphand and Neufeld, 2013). Although Anammox bacteria share several features e. g., chromosomal genes with other bacteria and domains of archaea, and eukarya, Anammox organisms consists of a highly divergent community of bacteria with unique characteristics that are different from other identified bacteria (Van Niftrik and Jetten, 2012). Most known bacteria possess a single cell compartment (the cytoplasm) for chemical reactions. Gram-positive bacteria have a thick peptidoglycan layer with no outer lipid, which, differentiate them from gram

negative bacteria with a thin peptidoglycan layer and outer lipid membrane. Anammox bacteria, however, have three different compartments (paryphoplasm, riboplasm and anammoxosome), each surrounded by a bilayer membrane. The anammoxosome is the biggest organelle of the Anammox bacterial cell where energy conversion occurs (Kuenen, 2020; Van Teeseling et al., 2013; Reith and Mayer, 2011).

The anammoxosome is the site for Anammox metabolism, where intermediates such as hydroxylamine and hydrazine are produced; this organelle confines the powerful reducing agent, hydrazine until it is converted to nitrogen gas; thereby preventing it from damaging the components and contents of the cytoplasm (Boumann et al., 2009; Van Teeseling et al., 2013). Anammox bacteria are obligate anaerobic chemolithoautotrophic bacteria belonging to the order Planctomycetales. The characterised genera (Table 1.2), which have been described as Candidatus (Ca.) are Ca. *Anammoxoglobus* (Oshiki et al., 2015; Kartal et al., 2007b), Ca. *Brocadia*, (Kartal et al., 2008; Strous et al., 1999), Ca. *Kuenenia,* Ca. *Jettenia* and Ca. *Scalindua* (Jetten et al., 2001; Park et al., 2010; Sonthiphand and Neufeld, 2013; Wang et al., 2015).

The Anammox reaction is an environmentally sustainable wastewater treatment (WWT) method that does not require aeration because NH_3 - N and NO_2 - N are directly converted to N_2 gas, thereby bypassing the nitratation stage (Carvajal-Arroyo et al. 2013; Van Hulle et al., 2010).

The stoichiometry of the Anammox process reveals that for every mole of NH_4^+-N converted to 1.02 moles of N_2 gas, 1.32 moles of NO_2^--N is required (Suneethi et al., 2014; Kumar and Jih-Gaw, 2010 Van Haandel and Van der Lubble, 2007; Jetten et al., 2001). Hence, in the preparation of Anammox synthetic wastewater, this ratio is usually taken into consideration to maintain the balance between NH_3-N and NO_2-N concentrations in the influent.

$$1NH_4^+\text{-}N + 1.32\ NO_2^-\text{-}N + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}\ N_{0.15} + 2.03\ H_2O \quad (1.8)$$

1.4.3.2 Anammox bacterial communities

Bacteria can exist as distinct single entity or in multispecies microbial community called biofilm – microbial biomass attached to a solid surface (Figure 1.2) In many engineered environments like the wastewater treatment (WWT) bioreactors, living in a multispecies community is advantageous to most bacteria; but more importantly to the Anammox organisms (Niederdorfer et al., 2021).

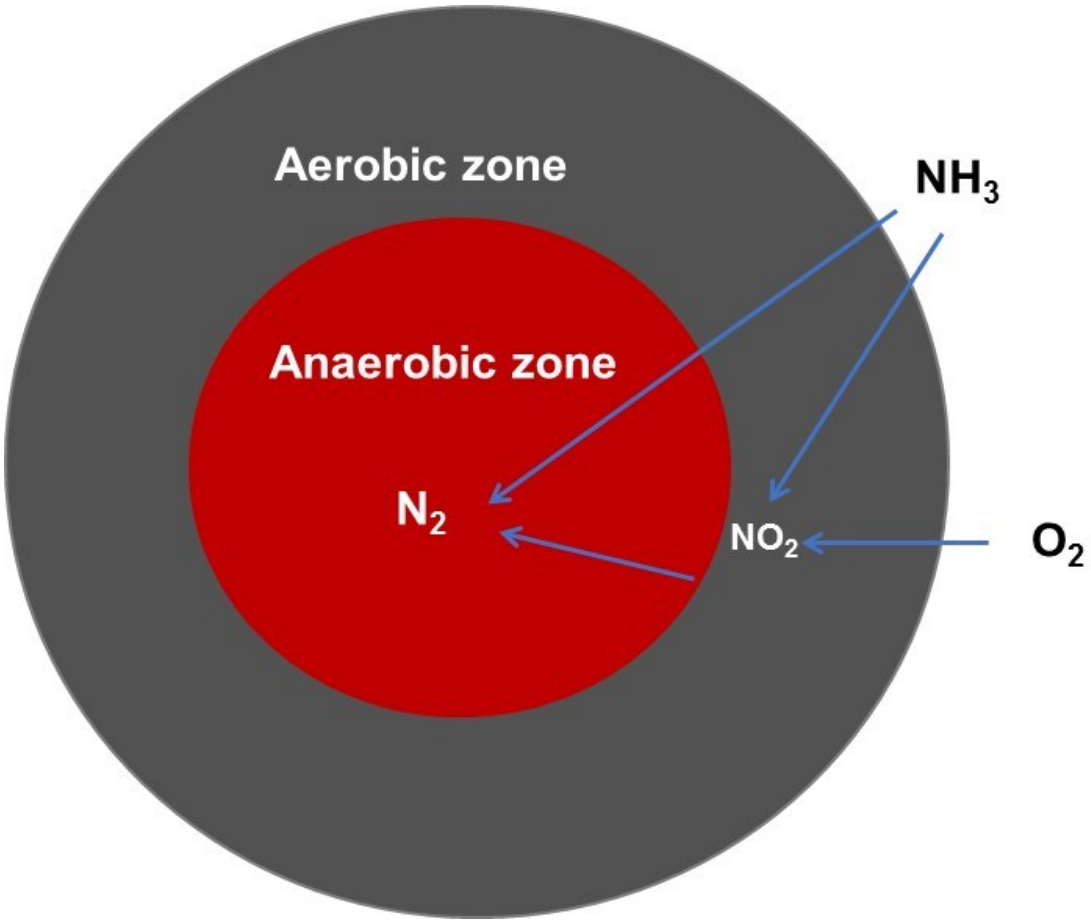

Figure 1.2: Schematic diagram of Anammox bacteria biofilm showing the co-existence of ammonia oxidizing bacteria (AOB) in the aerobic (grey) aerobic zone and Anammox bacteria (AnAOB) in the anaerobic (red) zone.

Anammox bacteria grow in a community of mixed microbial culture because they require the presence of other bacteria, particularly ammonia oxidizing bacteria (AOB) for partial nitrification (Table 1.4).

Table 1.4: Anammox bacterial community in engineered and natural ecosystems (Adapted from Wang et al., 2015 and Rodriguez-Sanchez et al., 2014).

Species	Ecological Role	Class
Nitrosomonas sp.	NH_4^+ oxidizer	β-Proteobacteria
Nitrosospira sp.	NH_4^+ oxidizer	β-Proteobacteria
Nitrososolobus sp.	NH_4^+ oxidizer	β-Proteobacteria
Nitrososovibrio sp.	NH_4^+ oxidizer	β-Proteobacteria
Nitrosococcus sp.	NH_4^+ oxidizer	γ-Proteobacteria
Nitrobacter sp.	NO_2^- oxidizer	α-Proteobacteria
Nitrococcus sp.	NO_2^- oxidizer	γ-Proteobacteria
Nitrospina sp.	NO_2^- oxidizer	Nitrospinae
Nitrospira sp.	NO_2^- oxidizer	Nitrospirae
Anammoxoglobus sp.	Anammox	Planctomycetia
Brocadia sp.	Anammox	Planctomycetia

Jettenia sp.	Anammox	Planctomycete
Kunenia sp.	Anammox	Planctomycete
Scalindua sp.	Anammox	Planctomycete

Anammox bacteria communities contains bacteria that are directly involved with the Anammox reaction such as the are ammonia oxidizing bacteria (AOB) that oxidize ammonia to nitrite; nitrite oxidizing bacteria (NOB), which oxidize nitrite to nitrate; complete ammonia oxidation (Commamox) bacteria, which can metabolize ammonia to nitrate and anaerobic ammonia oxidizing (Anammox) bacteria that finally convert ammonia and nitrite to nitrogen gas (Daims et al., 2015).

Other organisms which do not contribute directly to the Anammox process but might provide necessary support for the development of the complex and stable biofilm (to ensure retention of active Anammox bacteria that will remove the wastewater ammonia) on growth medium are heterotrophic bacteria (HB) and yeast (Daims et al., 2015; Laureni et al., 2015). Heterotrophs constitute about 23% of the total mixed Anammox bacterial culture that contribute to the efficiency of anaerobic biofilm reactors (Ni et al., 2012). The diverse microbiota within the biofilm plays specific roles, which result in the metabolization of the wastewater nitrogen pollutants (Fernandez et al., 2008). For example, heterotrophic bacteria found on Anammox biofilm played a very important part by initiating the creation of the first layer required for biofilm formation (Niederdorfer et al., 2021; Mozumder et al., 2014). Anammox biofilms might also include yeast cells that provide an anoxic microenvironment to support the growth of anaerobic bacteria cultured in environments where oxygen, which is naturally toxic to Anammox bacteria is available (Zekker et al., 2014).

The successful establishment of the Anammox process depends on the oxidation of ammonia to nitrite - the rate limiting and difficult step (Cho et al., 2011). The AOB and Anammox bacteria (AnAOB) are usually grown on the same biomass growth support material. A low oxygen concentration (e.g., 0.02 to 0.8 mg L^{-1}) must be maintained so that AOB can grow in the outer layer to allow oxygen utilization

and AnAOB in the anoxic core to form aggregates of biomass or biofilms (Hao et al., 2009). The growth of nitrite oxidizing bacteria (NOB), which compete with AnAOB for nitrite, must be controlled to promote AnAOB activities. Conditions to reduce NOB activity include maintaining low oxygen and the right nitrite concentrations in the bioreactor. Nitrite oxidizers have lower specific growth rates and oxygen affinity compared to AOB; they also have less affinity for nitrite relative to AnAOB; and can easily be washed out of the reactor at high temperatures e.g., 30 - 40 ^{o}C (Li and Sung, 2015).

1.4.3.3 Challenges of the Anammox process

Slow growth rate and low biomass yields; e.g., *Candidatus Brocadia fulgida* enriched from a wastewater sludge had a biomass yield of 0.046 g per g of nitrogen converted (Hendrickx et al., 2014); and the need to maintain oxygen concentration at a level that supports AOB (needed for partial nitrification of ammonia) and yeast (that help create anoxic environment), but will not inhibit the Anammox bacteria (AnAOB) are among the challenges of establishing the Anammox process to treat wastewater (Kuenen, 2020; Wang et al., 2015; Awata et al., 2015; Lage and Bondoso, 2012). The Anammox bacteria (AnAOB) grow extremely slowly; a growth rate of 0.003 h^{-1} and a biomass yield of 0.13g dry weight/g NH4+-N oxidized was reported by Suneethi et al., (2014). In reports by Van der Star et al., (2008), Anammox bacteria achieved growth rates of 0.0026 h^{-1} and 0.0035 h^{-1} with doubling times of 8.3 days respectively. Park et al., 2010 observed doubling time of 8.9 days; while a 14 - day average doubling time was recorded by Strous et al., (2006). Differences in growth rates and doubling times reflect the type of bioreactor, configuration, and process conditions. Another drawback of the Anammox wastewater treatment (WWT) method is that around 10 % NO_3-N produced during the operation requires additional treatment, which if released into water bodies without prior treatment leads to further water pollution (Du et al., 2015; Van Hull et al., 2010).

1.4.3.4 Anammox wastewater treatment process

The discovery of Anammox led to successful full-scale implementation of the process in the treatment of ammonia - rich wastewater that is cost effective with 60 % less energy requirements compared to existing technologies such as the nitrification-denitrification process (Abma et al., 2010; Joss et al., 2009; Wett, 2007).

Anammox wastewater treatment comprises of two processes, partial nitrification conducted by AOB and Anammox, which is a denitrification process carried out by AnAOB, in which nitrite and ammonia are converted directly to dinitrogen gas (N_2) under anaerobic conditions (Strous et al., 1999; Hu et al. 2013). The AnAOB depend on AOB to partially convert half of the supplied ammonia to nitrite; the remaining ammonia and nitrite are later reduced to N_2 by AnAOB, with nitrate as a by-product left in the bioreactor (Kartal et.al, 2007).

The advantages of the Anammox wastewater treatment process include the removal of up to 90 % wastewater nitrogen (Hu et al., 2013); there is about 60 % reductions in oxygen requirement (Wang et al., 2016; Guo et al., 2010; Van Hull et al., 2010); energy consumption by 60 % and greenhouse gas emissions (Kampschreur et al., 2008). Again, carbon source requirement for denitrification is reduced by 40 % (Guo et al., 2009; Egli et al., 2001). These benefits make the Anammox process both a cost effective and environment friendly wastewater treatment process (Kartal et al., 2010; Van Hull et al., 2010).

Comparatively, the Anammox process saves 60 % in aeration and partial nitrification (PN) 25 % (Rodriguez-Sanchez et al., 2014). While oxygen demand is reduced by about 58 % during partial nitrification - Anammox (PN-AMX), there is a 48 % reduction during partial denitrification - Anammox (PDN-AMX). Again, there is 84 % in sludge production reduction in using PN-AMX and 66 % for PDN-AMX (Zhang et al., 2019).

Additionally, although Anammox bacteria produce about 10 % nitrate by oxidation of nitrite because they possess the nitrite oxidoreductase (NXR) enzyme (Chicano

et al., 2021), nitrate is suggested to provide the electrons needed to fix carbon (CO_2) by Anammox bacteria (Hu et al., 019); Anammox bacteria are equally reported to conduct dissimilatory nitrate reduction to ammonia (DNRA) by using nitrate (NO_3^-) as an electron acceptor. For example, purified cells of Keunenia *Suttgartiensis* (Anammox bacteria) reduced $^{15}NO_3^-$ to $^{15}NH_4^+$ with $^{15}NO_2^-$ as the intermediate (Zhu et al., 2019; Bu et al., 2017; Castro-Barros et al., 2017). Previously Anammox studies have reported that Anammox reaction produce the greenhouse gas, nitrous oxide (N_2O), (Ma et al., 2013; Xu et al., 2014), but a recent study in Germany by Kartal research team has suggested that Anammox process does not emit N_2O (Hu et al., 2019). Instead, Anammox bacteria utilize the nitric oxide (NO) intermediate as electron acceptor coupled to ammonia, converting it atmospheric dinitrogen (N_2) gas. If the recent finding is confirmed following robust research; it might provide further evidence of the usefulness of the Anammox wastewater treatment process, as a greener technology capable of removing wastewater ammonia completely.

1.4.3.5 Factors that affect Anammox process in bioreactors

Bioreactors that successfully been used for the Anammox process include the popular sequencing batch reactor (SBR) amongst others (Table 1.5). To successfully establish the Anammox process for WWT, both chemical and environmental parameters within the reactor must be optimized to maximise the rate of bacterial activity and avoid inhibitory conditions The range of conditions that support the Anammox activity are pH (7 to 8); temperature (25 - 30 ºC); DO range (0.2 to 1.8 mg L^{-1}) and sparging with inert gas, e.g., 95% / 5% AR / CO_2 or N_2 / CO_2. (Table 1.5).

The factors that reduce Anammox bacterial activity and the process efficiency within the bioreactor include:

Temperature

In natural environments, Anammox bacteria grow at temperatures as low as -5 to 4 ºC and high as 60 - 80 ºC. For example, species of Candidatus *Brocadia* such as *sinica* and *fulgida*) were reported to grow at temperatures of 5 - 6 ºC (Cho et al.,

2020; Lotti et al., 2015). Anammox bacteria species living in marine environments are found at temperatures below 10°C (Hoekstra et al., 2018). Temperature has high impact on Anammox wastewater treatment in engineered systems (Rodriguez-Sanchez et al., 2016; Chamchoi and Nitisoravut, 2007). Temperature changes in such systems can trigger the accumulation of inhibitory concentrations of nitrite and ammonia, which affects the structure of the microbial community and ultimately reduce the nitrogen removal rate (Cho et al., 2020; Tomaszewski et al., 2017

Table 1.5: Operating conditions used in partial nitrification-Anammox bioreactors. **Key:** Anaerobic membrane biofilm reactor (**AnMBR**), Upflow anaerobic sludge bioreactor (**USAB**), Sequencing batch reactor **(SBR**). Nitrite nitrogen (NO_2-N), Ammonia nitrogen (NH_3-N), Dissolved oxygen (DO).

Reactor Type	Influent NO_2 - N: NH_3-N (mgL^{-1})	NH_3-N: NO2-N Ratio	DO (mgL^{1})	Ar / CO2/ N_2 (%)	pH	Temperature (°C)	NO_2 - N: NH_3- N removal (%)	Reference
SBR	150:150	1.0:1.0		95/5	7.5 - 8.0	30	98	Arrojo et al., 2008
SBR	50 - 70: 40 - 60	1.0: 1.25	< 0.02	95/5	7.7 - 8.4.	34	80	Chamchoi and Nitisoravut, 2007
SBR	630: 630	1.0:1.0		95/5	7.3	25-40	90	Hu et al., 2013
SBR	3 - 8: 1.9 - 10	1.0:1.6	0.8	-	7.8 - 8.0	30	95: 94	Langone et al., 2014
USAB	420: 350	1.2	0.4	N_2 (-)	7.3 - 7.5	31	88	Li and Sung 2015
AnMBR	100: 10,000	1.0: 1.0	0.2 – 0.5	-	6 - 8	32 - 35	96	Suneethi and Joseph, 2011
SBR	NA: 14		0.04	95/5	7.8	30	85	Sliekers et al, 2003

Temperature has a direct effect on the growth rate and activity of Anammox bacteria; as temperature increases, nitrogen removal efficiency (NRE) increases. A decrease in temperature below 15°C leads to a reduction in NRR (Hoekstra et al., 2019; Laureni et al., 2015), nitrite accumulation and loss of stability within the bioreactor (Isanta, et al., 2015). Anammox investigation carried out at 45 °C resulted in cell lysis and irreversible loss of activity (Isanta, et al., 2015).

The reported temperature ranges suggest that the Anammox process is mostly ideal for wastewater treatment (WWT) in tropical environments but could be adapted for the same use in colder climates like the UK. The wide variation in temperatures used in Anammox WWT partly depends on the reactor design (Tables 1.5); it is expected that the temperature could be different in the current study involving the EBBR-Anammox WWT system.

pH

Anammox bacteria are sensitive to variations in pH within the bioreactor; the best pH range is between 7- 8 (Suneethi et al., 2014). Changes in pH results in the accumulation of toxic compounds that inhibits Anammox bacterial activity, which reduces the efficiency of the process (Jung et al., 2007; Jetten et al., 2001). Low pH values hinder Anammox activity, particularly at lower temperature, leading to a reduction in nitrogen removal rate (Pooja et al., 2020; Tomaszewski et al., 2017). Lower pH, e. g., below 7.6, enhances the activity of NOB resulting in nitrate accumulation, and above 8.2, increase the growth of AOB to produce nitrite. Accumulation of nitrite in a bioreactor adversely affects the Anammox process (Rodriguez-Sanchez et al., 2014). pH between 7 - 8 promotes partial nitrification, which supports the Anammox bacterial activity (Cho et al., 2020; Jung et al., 2007).

In addition, the predominant Anammox bacteria species present in an Anammox wastewater treatment system is partly determined by the prevailing pH condition. For example, Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgartiensis* are usually enriched at pH range of 7.8 – 8.0 (Tomaszewsk, et al., 2017; Cho et al., 2011).

The optimum pH condition for the Anammox process reported for different bioreactors is 7.8 – 8.3 0 (Yang et al., 2011; Jetten et al., 2001). Achieving a high nitrogen removal efficiency in any Anammox system requires maintaining the pH within this favourable range (Table 1.5).

Dissolved oxygen

Aerobic ammonia oxidizers need oxygen for partial oxidation of ammonia, while Anammox bacteria grow in oxygen depleted environments. Maintaining the balance of dissolved oxygen (DO) concentration within the bioreactor to provide anoxic condition for AnAOB is another challenge to deal with during Anammox wastewater treatment (WWT) process (Table 1.5). Anammox activity is reversibly inhibited by dissolved oxygen at concentrations of 0.25 - 2% (Jetten et al., 2001). A DO concentration of 0.02 to 0.15 mg L^{-1} reversibly inhibited the growth AnAOB; but inhibition became irreversible at a level > 1.37 mg L^{-1} in a rotating disk contactor system (Egli et al., 2001). Using a granular sludge bed reactor (GSBR) and a SBR respectively, AnAOB activity was established at a DO concentration of 0.8 mg L^{-1} (Langone et al., 2014). Again, 2.3 to 3.8 mg L^{-1} of DO, resulted in a significant inhibition in a granular reactor (Carvajal-Arroyo et al., 2013). Therefore, maintaining the balance of oxygen level will be crucial in deploying the expanded bed biofilm reactor for the Anammox reaction.

Nitrite nitrogen concentration

The concentration of NO_2-N is critical to the stability of the Anammox process; nitrite can inactivate the Anammox bacteria, causing partial to complete inhibition of the process (Tables 1.6). Keeping nitrite concentration at the right level is important for effective Anammox wastewater treatment process. While very low nitrite concentrations (e.g., 5 mg L^{-1}) would slow down the growth rate of the organisms, nitrite could become toxic to the bacteria when concentration becomes high (e.g., from 50 mg L^{-1}) depending on the size and configuration of the reactor (Van der Star et al., 2007; Schalk, et al., 2000). For example, NO_2-N concentration at 100 mg L^{-1} caused a complete Anammox bacteria growth inhibition (Bettazzi et

al., 2010). A concentration of 384 mg L^{-1} caused a 50% inhibition in the presence of ammonia, and without ammonia, inhibition occurred at 53 mg L^{-1} nitrite, which was about 7- fold higher (Carvajal-Arroyo et al., 2014). Langone et al., (2014) found that 42 mg L^{-1} of nitrite did not result in an inhibition of Anammox process. These studies seem to suggest that to avoid inhibition, NO_2-N concentration in a bioreactor should be maintained below 50 mg L^{-1}, taking the size of the reactor into consideration and operation mode. Higher levels of NO_2-N also cause biomass flotation (Campos et al., 2017); and increased washout of suspended Anammox granules with average diameter of 0.4 cm (Suneethi et al., 2014). In continuous culture, while e.g., < 400 mg L^{-1} NO_2^-N in influent can support Anammox activity, about 750 mg L^{-1} could lead up to 90 % loss of activity. However, recovery of activity may be possible if influent NO_2^- concentration is reduced below 274 mg L^{-1} (Langone et al., 2014; Jin et al., 2012).

Free ammonia (FA)

Anammox bacteria can effectively treat wastewater containing high concentrations of ammonia and nitrite. For example, Anammox bacteria can remove ammonia from wastewater containing up to 1,500 mg L^{-1} ammonia and 500 mg L^{-1} of nitrite (Aktan, et al., 2012). However, in a high pH and temperature environments, free ammonia (natural form of ammonia - NH_3-N) accumulates causing inhibition of the Anammox process as previously discusses (Isanta, et al., 2015). Bacterial cells utilize ammonia rather than ammonium ion (NH_4^+) as the actual substrate; the concentration of available ammonia and the balance between free ammonia (FA) and NH_4^+ in wastewater depends on the pH and temperature. Again, FA instead of NH_4^+ inhibits Anammox bacterial activity because FA can easily penetrate the cell membrane, dissolve in lipid, and destabilize Anammox bacteria with up to 90 % reduction in activity (Aktan, et al., 2012). The rate of nitrogen removal increases with increase in temperature; but temperature rise, increases the concentration of FA leading to a decrease in nitrogen removal rate (Liu et al., 2019; Suneethi et al., 2014). For example, while 150 mg L^{-1} of FA did not inhibit Anammox activity, when the concentration rose to 190 mg L^{-1} nitrogen

removal decreased to 10 % (Aktan, et al., 2012; Jaroszynski et al., 2012); a 90% loss of Anammox activity.

Nitrogen loading rate

As with other factors that affect the Anammox process, Anammox bacteria need to adapt to a new low or high nitrogen concentrations conditions that will effectively support their metabolism and NRR. For example, Anammox bacteria adapted in a sequencing batch reactor (SBR) for more than 60 days removed about 70 % supplied nitrogen loaded at a concentration of 1 g NH_3 -N L^{-1} (Wang, et al., 2016). When fish canning wastewater was fed into an Anammox bioreactor at a loading rate of 0.105 and 0.203 g N L^{-1}, 80% of the nitrogen was removed in 30 days; 87 % of supplied nitrogen was successfully removed within 150 days at a NLR of 0.2 g N L^{-1} ; but when the nitrogen loading rate was increased to 1.7 kg N m^3 d^{-1} there was a significant decrease in Anammox activity and nitrogen removal rate (Val del Rio et al., 2018).

Hydrogen sulphide

Hydrogen sulphide is a common anaerobic wastewater constituent produced during sulphate reduction and organic matter degradation that inhibits Anammox bacterial activity (Cho et al., 2020). There is a positive correlation between increase in sulphide concentration and inhibition of Anammox bacterial activity. For example, while there was a 35.6% decrease in the activity of Anammox bacteria exposed to 192 mg L^{-1} sulphide concentration; complete inhibition occurred at 320 mg L^{-1} (Yang and Jin, 2012). Undissociated hydrogen sulphide (H_2S), which accounts for a third of total hydrogen sulphide present in wastewater could cause from 50 % to an irreversible inhibition of the Anammox process at a concentration of 0.03 mg - 1.02 mg L^{-1} (Carvajal-Arroyo, et al., 2013; Kimura et al., 2010).

Light

Ammonia oxidizers, (AOB, AOA, AnAOB) are sensitive to light; their activities are reduced in the presence of a continuous light with high intensity (Merbt et al., 2012). Light has a negative impact on partial nitrification conducted by AOB to

produce nitrite required for the Anammox reaction; light equally supports the growth of photosynthetic algae (Manser, 2016). Algal growth causes competition for inorganic carbon, increases oxygen level and inhibits the Anammox process (Cho et al., 2020; Suneethi et al., 2014). Light penetration could be prevented by covering the Anammox bioreactors with black plastic bag (Uyanik et al., 2011); and will be relevant in the EBBR-Anammox study.

Gas sparging

Anammox bacteria (AnAOB) require strict anaerobic condition for maximum nitrogen removal. However, when partial nitrification is coupled to Anammox, oxygen is required to support the growth of ammonia oxidizers to produce nitrite needed for the Anammox process. Sparging the reactor with inert gases such as argon (Ar), carbon dioxide (CO_2), nitrogen (N_2) or a mixture of the gasses deoxygenate the system and maintain conditions for AnAOB growth (Table 1. 5). The composition of inert gas used in Anammox bioreactors should contain at least 5% CO_2 to buffer the growth medium (Hue et al., 2013; Suneethi et al., 2014). However, simultaneous partial nitrification - Anammox (SPNA) process was established by alternating 2-hour sparging with air for the aerobic and argon for the anaerobic phase (Strous et al., 1997). Anammox reaction was operated by flushing the bioreactor with nitrogen (Li and Sung, 2015); suggesting that bubbling an Anammox reactor with a single inert gas could be effective at reducing oxygen concentration; and will be adopted in current EBBR-Anammox study.

1.5 Anammox biofilms, growth medium and bioreactors

1.5.1 Anammox biofilm

Biofilms are aggregate of biomass formed on solid surfaces by organism growing in a community; they are held together by extensive self-made extracellular polymeric substances (EPS) with water channels (Almstrand, 2012; Fernandez et al., 2008). The slimy biological matrix of EPS anchors the bacteria to the surfaces

of material in aqueous environments (Alpkvist et al., 2006). Examples of the hydrated EPS include polysaccharides, proteins, lipids, nucleic acids, uronic acids, humic substances, and others (Arundhati and Pau, 2008). The EPS is also a survival adaptation useful for accumulating nutrients (Decho and Gutierrez, 2017; Flemming and Wingender, 2010). The protective polymeric surface matrix is joined together by weak chemical bonds. If the colonies were not immediately detached from the surface, they become permanently anchored by protein adhesion molecules, which bind them to other cells (Fernandez et al., 2008).

Biofilm formation offers bacteria increased advantages, as they are in a better position to survive various adverse conditions such as being washed off in aqueous environments or eaten by grazing protozoa (Vyas et al., 2019; Singh et al., 2006). Anammox bacteria have a low biomass yield; for example, 0.046 g of biomass is produced per g of nitrogen metabolized (Hendrickx et al., 2014), slow growth rate, with a generation time > 10 days; and require very stringent conditions of temperature and pH (Cho et al., 2020; Niederdorfer et al., 2021; Van der Star et al., 2008). Hence biofilm formation is essential for their survival and metabolism.

In Anammox wastewater treatment (WWT) operations, the achievement of a shorter start-up time and high nitrogen removal is supported by the retention of the Anammox bacteria biomass within the bioreactor. Biofilms play a vital role in wastewater treatment (WWT) as an effective strategy for retaining Anammox bacteria biomass in a wastewater treatment system (Suarez et al., 2015; Almstrand, et al., 2013). Biofilm formation increases the concentration of active biomass within a bioreactor required for efficient nitrogen removal in biological wastewater treatment systems (Hibiya et al., 2004).

1.5.2 Anammox Installations and research around the world

Since the discovery of the Anammox process, over 100 full scale Anammox wastewater treatments (WWT) plants (Figure 1/3) have been built around the world (Cho et al., 2020; Zhang et al., 2018)

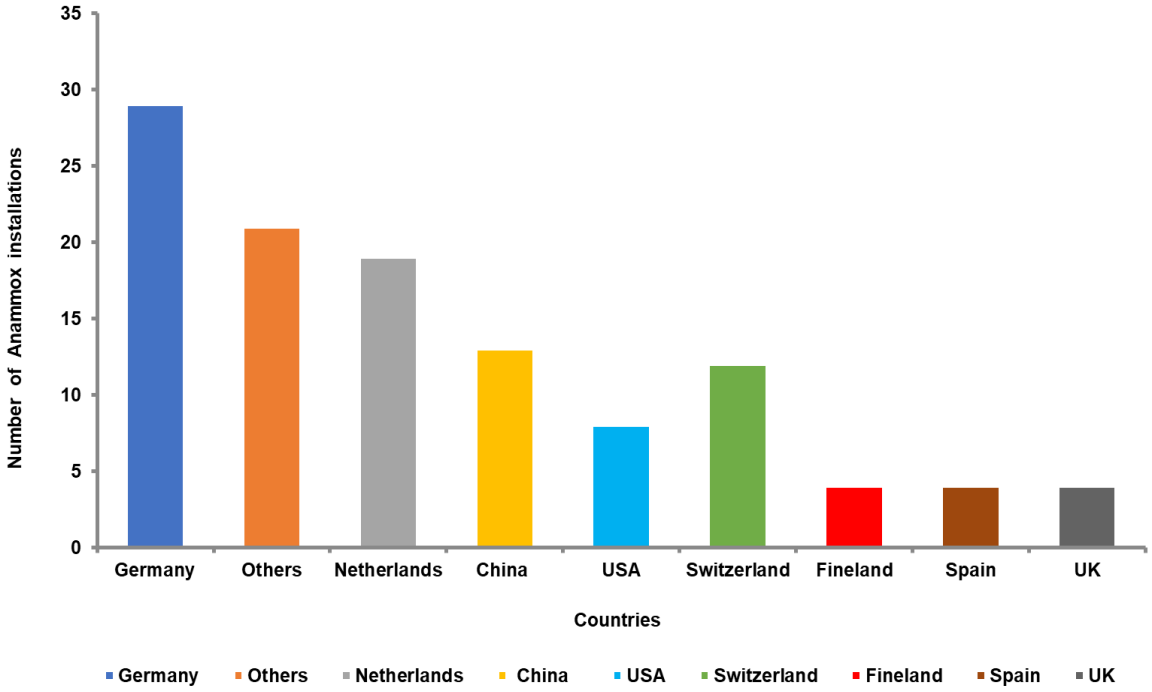

Figure 1.3 Anammox full scale installations around the world (adapted from Zhang et al., 2018).

While Germany has benefited greatly from Anammox wastewater treatment with about 25 % of total installations, countries including the USA (7 %) and UK (< 4 %) are yet to fully maximize the potentials of this wastewater treatment technology that reduces aeration energy by 63%s, sludge production between 70 – 80%, cost of external organic carbon (e.g., methanol) by 100% with almost 0 % CO_2 emission (Castro-Barros et al., 2017).

Evaluation of the top ten world Anammox research publications have revealed that although the Anammox process was discovered in the Netherlands; and the first industrial application developed there, most research publications are from China (27.71%), then Netherlands (23.8%) and followed by the USA 20.03% (Kumwimba et al., 2020; Zhang and Sitong, 2014). There is the need for increase in Anammox wastewater treatment research in the UK, which makes the current study relevant.

1.5.3 Anammox Growth Support Medium

Biofilms are useful in systems in which the capacity and process efficiency is limited by the biomass concentration and low biomass yield as in the case Anammox bacteria. Biofilm developed on support media can maintain a large concentration of biomass up to 30 g L^{-1} and provide large specific surface area (up to 3000 $m^2\ m^{-3}$) as reported by Nicollela et al., (2000). Biofilm growth support medium used for Anammox bacterial growth come from a variety of natural and synthetic sources; and include nylon, foam, coconut husk fibre, coke, wood chips, sand, glass, foam, volcanic rock amongst others (Zainab et al., 2020; Lu et al., 2018).

The type, size and shape of biomass support used in Anammox biofilm reactors is extremely important for the development of the stable biofilm to achieve a high nitrogen removal rate. This is because the surface texture and shape of the medium particle affects both bacterial attachment and the surface area of biomass formed.

Glassy coke growth support medium that was previously used for complete nitrification experiments (Akhidime and Dempsey, 2009).

The coke medium used in the expanded bed biofilm reactor (EBBR) technology is a rough, microporous biomass support medium that provides a greater surface area than a smooth medium support of the similar size. The 0.7 to 1.0 mm EBBR ABDite® biomass support particle provided a surface area of up to 2400 $m^2\ m^{-3}$ of (Dempsey, 2018; Akhidime and Dempsey, 2009).

Naturally, Anammox bacteria have an inherent adhesion ability that allow them to form continuous layers of immobilised biomass on the carrier materials. The growth medium provides the bacteria with support and protection from predation (Hoekstra et al., 2018; Suarez et al., 2015). The support media equally reduces the rate at which active bacteria biomass is washed out of the bioreactor (Zhang, 2013). Additionally, growth support medium that has microporous structure such as the ABDite® particles provides the Anammox bacteria with an anoxic environment in

the inner core, which shield them from direct contact with molecular oxygen toxic to them (Lu et al., 2018).

1.5. 4 Anammox bioreactor operation stages

Anammox biofilm reactors (Tables 1.7) have long been applied at Lab, pilot and full-scale to treat wastewaters from various sources (e.g., farming, industrial and municipal) rich in NH_3-N by using one or two bioreactors of different configurations and sizes (Wang et al., 2017; Rosche et al., 2009). The Anammox wastewater treatment process could be conducted as a single Anammox (SA) or simultaneous partial nitrification - Anammox (SPNA) operation in one or two stages (Table1.6).

Table 1.6 Mode and operation stages of Anammox bioreactors. **Key:** *CANON - completely autotrophic nitrogen removal over nitrite reactor *(CSTR - continuous stirred-tank reactor *SGR- granular sludge reactor *****SBR - sequencing batch reactor *RBC– rotating biological contactor *UASB-Up-flow anaerobic sludge bioreactor. * SHARON - single high activity ammonium removal over nitrite.

Reactor	Mode	Stage	Reference
SHARON (CSTR- SGR)	SPNA	Two	Abma et al., 2010; Van Dongen et al., 2001.
GLR	SA	One	Dapena-Mora et al., 2004a
SBR	SPNA	Both	Langone et al., 2014; Suneethi et al., 2014;
MBR	SPNA	One	Xie et al., 2017.
CANON	SPNA	One	Sliekers et al, 2003
UASB	SA	One	Li and sung, 2015

RBC	SA	One	Liu et al., 2008

The operation of simultaneous partial-nitrification - Anammox (SPNA) process is characterized by the removal of both NH_3-N and NO_2-N; and NO_3-N production (Suneethi et al., 2014). In the SPNA system, the wastewater contains only ammonia; AOB partially oxidize the NH_3 to NO_2. The remaining NH_3 and NO_2 are converted to N_2 gas by AnAOB; SPNA can be conducted in one or two bioreactors (Hu et al., 2013)

Advantages of SPNA in one bioreactor over conventional ammonia removal methods such as nitrification- denitrification include that additional source of carbon (e. g., methanol) is not required, nitrous oxide (N_2O), is not produced (Hu et al., 2019). The SPNA process reduces energy, aeration, and space requirement for separate reactors, with reduction in overall cost (Du et al., 2015; Lackner et al., 2014; Carvajal-Arroyo et al. 2013).

In SA operation, both ammonia and nitrite are supplied in the wastewater; (Li and Sung 2015; Langone et al., 2014). During SA operation, both partial-nitrification and Anammox reactions occur in one bioreactor (Suneethi et al., 2014; Abma et al., 2010).

In both SA and one-stage SPNA, the mixed Anammox microbial communities of AOB, NOB, AnAOB and others grow on the same growth support (Figure 1.2); with AnAOB in the anoxic core; and AOB and NOB on the outer core (Abma et al., 2010; Van der Star et al., 2007). The NOB grow when oxygen and nitrite levels are high; reduction in NOB activity is achieved by maintaining low DO and NO_2 levels; and temperature kept above 25 ^{o} C to enhance AnAOB growth and prevent NOB outcompeting Anammox bacteria for nitrite (Rodriguez-Sanchez et al., 2014; Munz, et al., 2011; Wouter et al., 2008).

Many bioreactors (Tables 1.5 and 1.6) such as SBR and MBR have successfully implemented the Anammox process in WWT; the lab scale EBBR has not been used, forming the basis for this research.

1.6 Expanded Bed Biofilm Reactor (EBBR)

The expanded bed biofilm reactor (EBBR) is a biological wastewater treatment (WWT) technology that uses ABDite® growth support medium to improve biomass retention, which in turn increases the efficiency of the treatment processes with a high substrate removal rate (Akhidime and Dempsey, 2009). The EBBR is compact and occupies less space compared to other reactors of the same size; it is highly resistant to toxic shock loads of wastewater nutrients; and pH and temperature variations (Akhidime and Dempsey, 2009; Dempsey et al., 2005). The expanded bed biofilm reactor (EBBR) has been successful at both laboratory and pilot-scale for high-rate tertiary nitrification process (1.7 ± 0.6 kg NH_3-N m^{-3} $_{EBBR}$ d^{-1}); and has met most criteria for sustainable WWT technology, such as simplicity, low-cost and energy-efficiency (Akhidime and Dempsey 2009; Dempsey et al., 2005). The EBBR will be adapted to conduct the Anammox wastewater treatment process in this study.

1.7 Tools for identification of Anammox bacteria biofilm and Anammox bacteria

1.7.1 Microscopy tool for identification of Anammox bacteria biofilm

Microscopy techniques such as the phase contrast microscope (PCM) and scanning electron microscopes (SEM) have been used to study the surface of Anammox bacterial biofilm formed on different growth support media (Sehar and Naz, 2016; Zeng et al., 2016). Both microscopes will be used for the visualization and analysis of the biofilm and bioparticles formed in the pores and surface of the EBBR-Anammox ABDite® growth support particles.

1.7.1.1 Phase contrast microscope

Light dependent microscopy e.g., the phase-contrast microscope (PCM) is applied in Anammox wastewater treatment to examine the surface structure of biofilm (Li et al., 2016; Botchkova et al., 2015). The PCM is equipped to convert small variations in phase shifts of light passing through a specimen to corresponding changes in brightness of the image (Maurer et al., 2008). The PCM enhances the image of a weak phase objects by using a quarter-wave phase plate to produce a high signal contrast by shifting the phase of the light scattered (Majorovitsa et al., 2007). The invisible phase shifts become visible when the brightness is displayed; allowing morphological imaging without the need for extensive sample preparation such as staining; unlike the scanning electron microscope (Toda et al., 2019).

1.7.1.2 Scanning electron microscope

The scanning electron microscope (SEM) uses high-energy focused beam of electrons to generate images by scanning the surface of a solid sample (Swapp, 2017; Drouin et al., 2007). In SEM analysis, data is collected over a selected area on the sample (area of 1 cm to 5 microns area is used to generate a 2 - dimensional image in a scanning mode), with magnification ranging between x 20 and x 30, 000. Spatial resolutions of 50 to 100 nm are easily achieved using SEM. The SEM has the capability to analyse selected point locations on the sample surface as interaction between the sample and electron generate signals that reveal the morphology and the orientations of the different materials the sample is made up of (Swapp, 2017; Denk and Horstmann, 2004).

1.7.2 Molecular tools for identification of Anammox bacteria

Molecular tools that have been applied in the study of Anammox bacteria include DNA extraction, polymerase chain reaction, Sanger and NGS nucleotide sequencing, BLAST analysis and construction of genetic trees amongst others.

The application of molecular techniques in the study of microbial populations in wastewater treatment saves time spent in culturing microorganisms using

traditional methods. Molecular biology-based studies have also led to the discovery of new microorganisms that remove wastewater nitrogen, such as the Commamox, *Nitrospira inopinata* (Koch et al., 2019; Daims et al., 2015 Ottman et al., 2012), *Nitrotoga arctica* and the Anammox bacteria (Boetius et al., 2015; Alawi et al., 2007) amongst others.

Molecular techniques have enhanced our understanding of the complex microbial diversity and interactions in environmental samples, allowing accurate and reliable identification of individuals or microbial communities of interest. Molecular tools are more beneficial in studying the DNA from the entire organisms present in samples taken from the environment or engineered systems, such as the Anammox bioreactors. The unculturable Anammox bacteria have only been studied by means of molecular techniques (Kuenen, 2020; Wang et al., 2015; Lage and Bondoso, 2012). Molecular analytical tools are very sensitive and rapid, producing results within hours (Mohini and Deshpande, 2010). Till now, Anammox bacteria have only been studied by means of molecular techniques (Kuenen, 2020; Wang *et* al., 2015*;* Lage and Bondoso, 2012); and will be employed in this research.

1.7.2.1 DNA extraction and quantification tools

Sample DNA could be extracted using commercially available extraction kits depending on the type of sample under investigation (Kennedy et al., 2014; Sambrook and Russell, 2001; Quinque et al., 2006).

The DNA extracted are quantified to determine the concentration and purity before downward applications. The quantification process is important because the presence of impurities in extracted DNA can result in inaccurate measurement when the DNA are used for other purposes (Shehzad et al., 2016; Desjardins and Conklin, 2010). Purity ratios and spectral profiles are important parameter that indicate the quality of the extracted DNA. Techniques such as the Nanodrop spectrophotometric technology is one of the reliable methods used to assess the purity and concentration of DNA. DNA has maximum absorbance at 260 nm while protein impurities absorb at 230 nm. Nanodrop records the concentration and

purity ratios for DNA samples at A260/A230 and A260/A280 respectively (Shehzad et al., 2016; Desjardins and Conklin, 2010).

A pure DNA sample usually has an approximate 260/280 purity ratio value of 1.8; and is dependent on the pH and ionic strength of the buffer used in DNA extraction. An acidic blank solution normally will under-represent the ratio by 0.2 - 0.3 while a basic solution will be over by 0.2 - 0.3 (Dilhari et al., 2017). DNA quantification provides information of DNA purity and concentration, which indicate the quality and usefulness of a DNA sample for downstream application such as during polymerase chain reaction (Dilhari et al., 2017).

1.7.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) is used widely in the study of slow-growing bacteria such as the Anammox bacteria (Yang., 2020). Polymerase chain reaction effectively amplifies DNA sequences extracted from samples; and has the capacity of making up to a billion copies of DNA from an initial small segment. PCR has a variety of applications because the reaction is fast, simple, sensitive, and specific; PCR uses a thermo-stable DNA polymerase as the key enzyme to mimic DNA replication in a test tube (Van Kessel et al., 2016; Wimbles et al., 2016; Dale et al., 2009).

During a PCR reaction, emphasis is placed on the annealing temperature at which the primers bind to the DNA template. The annealing temperature is very important, specific, and dependent on length and composition of each primer set (Harhangi et al., 2012; Li et al., 2009). An optimum annealing temperature (OAT) will produce the highest yield of the correct amplicon. At a lower annealing temperature (LAP), there is a mismatch as the primer set binds to the wrong DNA sequences. An increase in annealing temperature could sometimes improve the specificity at which the primer anneals with the template (Shao et al., 2011). However, a very high annealing temperature (HAT) will result in the breaking apart of the primer from the template (Evans et al., 2018).

The PCR products could be further analysed using agarose gel electrophoresis (AGE) to visualize the band and estimate the size of amplified DNA (Johnson et al.,

2019). During AGE analysis, interaction between DNA and the intercalating dye (e.g., Midori-green) produce visible band; the intensity of which depends on the initial concentration of DNA in a sample Lee et al., 2012).

1.7.2.3 DNA sequencing

The use of the16S DNA sequence to profile microbial communities present in environmental samples from wastewater treatment plants (WWT) and other industries has increased. The popularity of the DNA sequencing techniques has increased because they are fast; making it easy to study microbial communities such as the ones involved in the Anammox process, which cannot be studied using traditional culture technique (Wang et al., 2015). The sequencing of a DNA is useful for the identification and taxonomic classification of bacteria (Johnson et al., 2019 Kolpashchikov, 2019). Due to the diversity of the Anammox bacteria, specific primers are required to successfully screen for each genus in a sample (Pjevac et al., 2017).

Sequence techniques that have been used in the study of Anammox bacteria include Sanger (capillary electrophoresis sequencing) and next generation sequencing (NGS) amongst others (Kolpashchikov, 2019). Similar principles are applied in the use of Sanger and NGS technologies. For example, in both sequencing methods, the enzyme, DNA polymerase adds fluorescent nucleotides one by one onto a growing DNA template strand; the incorporated nucleotide is then identified by specific fluorescent tag. However, there are differences between the two techniques.

1.7.2.3.1 Sanger sequencing

The cost of Sanger sequencing is lower, providing a fast, cost-effective sequencing for low numbers (e.g., 1 - 20) of DNA targets (Johnson et al., 2019; Clarridge, 2004). Although Sanger sequencing can identify diversity of microbial communities in a system, the drawbacks include limited resolution, lower sensitivity and only one DNA fragment is sequenced at a time compared to next generation sequencing (Peker et al., 2019; Poretsky et al., 2014).

1.7.2.3.2 Next generation sequencing

Next generation sequencing (NGS) involves the direct study of the total genetic material isolated from samples and produce a comprehensive coverage of all the microbial species present within a bioreactor (Ciesielski et al., 2018); and will be used in the EBBR-Anammox system.

Compared to Sanger sequencing, NGS is more labour intensive and time consuming. However, it can sequence simultaneously millions of genes or gene regions present in DNA fragments in one run. NGS is more sensitive at detecting low-frequency variants with a faster turnaround time for high sample volumes; and in addition, has no limit of detection. With NGS, there is a greater chance of discovering novel or variants due to in-depth sequencing **(**Peker et al., 2019; Jamuar., 2014; Poretsky et al., 2014; Shendure and Ji, 2008).

Next generation sequencing occurs in stages including chastity testing, a preliminary filter test, which DNA reads must pass before they are sequenced. Other steps involve demultiplexing and primer clipping, Read FASTQ Statistics (FRS) and microbiome profiling.

The sequenced nucleotides are afterward aligned; the alignment is an important step in constructing phylogenetic trees to establish evolutionary relationships between the analysed sequences.

1.7.2.4 Phylogenetic tree

A phylogenetic tree is visual schematic diagrams used to communicate the biological evolutionary relatedness or diversity within groups of organisms (Dees et al., 2014). Phylogenetic trees show the positions of organisms retrieved from a sample and their clades. A clade or monophylogenic group is the natural group of organisms that come from a common ancestor and are shown on the same tree nodes. The tips of the phylogenetic tree indicate the taxa or microbial species present in the sample (Novick and Catley, 2013).

Taxa that are closely positioned on the phylogenetic tree, share a more recent common ancestor, and are closely related compared to the ones further apart

(Halverson et al., 2011). Software widely used to construct a phylogenetic include MEGA version 11 (Kumar et al., 2021) and MEGA X (Kumar et al., 2018).

1.8 Research Aim

The aim of this research is to determine whether the Anammox wastewater treatment process could be established using the EBBR technology; investigate, optimize, and evaluate the EBBR-Anammox performance with other Anammox process technologies available. Also, this work aims to identify key Anammox bacterial communities and explain their roles in the Anammox process.

Objectives

1. Investigation of the establishment of the Anammox wastewater treatment process in the Lab scale EBBR.
2. Examination of Anammox bacteria biofilm formation on the EBBR ABDite® biomass support medium using microscopy techniques.
3. Optimization of the EBBR-Anammox process conditions (pH and temperature).
4. Identification of the key bacterial communities of the EBBR-Anammox process with ABDite using molecular biology techniques.

CHAPTER TWO

Establishing Anammox Process with ABDite Biomass Medium in the Lab Scale Expanded Bed Biofilm Reactor

2.0 Research Question

Could the Anammox wastewater treatment process be established using the lab scale EBBR despite its challenges?

Sub questions and objectives

1. What evidence of the Anammox wastewater treatment process were found within EBBR?
2. How did the nitrogen removal rate (NRR) of the EBBR compare with other Anammox processes technologies?
3. How did the nitrogen removal efficiency (NRE) of the EBBR-Anammox process compare with other Anammox processes?
4. How did EBBR-Anammox process challenges face compare with those faced by other Anammox technologies?
5. How did the duration of the EBBR-Anammox process investigation compare with other Anammox technologies?

2.1 Introduction

2.1.1 Growth of Anammox biofilm and granules in bioreactors

Biofilms are microbial biomass attached to solid surface; the use of biofilm in Anammox wastewater treatment (WWT) helps to improve the nitrogen removal efficiency of the processes (Guest et al., 2007). The use of growth support material increases biomass retention by immobilizing the microbial population on the medium. Cell attachment ensures high biomass hold up within the reaction vessel and increases nitrogen removal from the wastewater being treated (Acharya et al., 2008; Nicolella et al., 2000).

Biofilms develop inside the pores and surfaces of the growth support material in a stationary state; this study will attempt to develop the Anammox biofilm using the EBBR ABDite® biomass support medium (Akhidime and Dempsey, 2009). Anammox bacteria could grow as attached biofilm or flocs, which develop into aggregates of suspended brick red granules in the liquid. Sometimes, both the attached biomass and granules develop within the same bioreactor (Li et al., 2021; Sehar et al., 2016; Ferreira et al., 2010). The physical presence of red granules, nitrogen gas production alongside simultaneous removal of ammonia and nitrite confirms the occurrence of the Anammox process within a system; as ammonia is oxidized to molecular nitrogen (N_2) gas and nitrite used as the electron acceptor is reduced (Meng et al., 2017).

2.1.2 The expanded bed biofilm reactor (EBBR) operation

Bioreactors used for lab, pilot, or industrial scale Anammox wastewater treatment could be fixed bed or fluidized bed reactors. A fixed bed reactor consists of a cylindrical tank that is packed with solid catalyst pellets loaded into the bed that do not move (Dixon and Partopour, 2020; Haffez et al., 2019.); this was not the focus of this study. The current research will be using fluidized bed reactor in which influent wastewater, pumped upwards through a packed bed of small growth

support medium at specific velocity, fluidizes the particles (Burghate and Ingole, 2013).

The expanded bed biofilm reactor (EBBR) is an example of a particulate fluidized bed reactor that uses small growth medium to support biofilm the development. The biomass growth particles are suspended in the wastewater flowing upward in a vertical column. The bed expansion is achieved when the upward velocity from the flowing wastewater exerts a drag force that is greater than the gravitational force, thereby suspending the biomass growth particles in the wastewater. The expansion of the particle within the EBBR column is below 100% to create space for biomass increase; and prevent the biomass growth medium from floating out of the bioreactor (Butler and Boltz, 2014; Dempsey, 2011).

The operation of the EBBR technology involves fluidizing the biomass support medium at a velocity of 1 cm s^{-1}. The suspension of the particles ensures the growth support particles are separated from each other. Biofilm form, as the bacteria adhere to the fluidized particles, colonize the inner pores first and overgrow to the surface, which further develops into individual bioparticles – growth medium completely covered with layer of biofilm (Dempsey et al., 2006). To achieve the right degree of fluidization and bed expansion, the upward velocity for fluidization must be maintained. The use of incorrect velocity results in sprouting and non-uniform expansion, causing the biomass support particles to rub against each other. This inter-particle abrasion could wear off attached biofilm; leading to reduction in bed heights and overall process efficiency (Khan et al., 2016; Dempsey et al., 2006).

The advantages of using the EBBR in wastewater treatment include process intensification resulting in a compact reactor that occupies 10 times less space compared to other technologies where the biomass is suspended in the wastewater (Akhidime and Dempsey 2009). Cell immobilization on the biomass medium separate them from the bulk liquid, eliminating the need for biomass recovery before the effluent is discharged (Akhidime and Dempsey, 2009). EBBR technology provides high concentration (e.g., 40 g L^{-1}) of very active biomass within

the bioreactor making the process more productive. With only active cells retained, unattached floating individual cells are washed out creating a selection process that ensures only immobilized cells remain in the system (Akhidime and Dempsey 2009). In addition, bed expansion provides excellent mixing of the up flowing polluted wastewater, which provides the immobilized cells with dissolved nutrients and oxygen, while mineralised nutrients are removed. Fluidization of the biomass growth support and bed expansion prevents channelling or blockage; therefore, the system does not require a backflushing operation, making the technology less complicated (Dempsey *et al.,* 2006; Nelson *et al.,* 2017).

Additionally, the use of small-sized biomass support medium to produce a large surface area of active biofilm for reaction, results in a low capital cost compact wastewater treatment plant. Examples of biomass growth support medium that have been used in Anammox wastewater treatment process include plastics, rocks, and charcoal amongst others (Niederdorfer et al., 2021; Lu et al., 2018).

In this study, the approximately 1mm (diameter) black ABDite® biomass medium will be used to develop the Anammox bacteria biofilm/bioparticles. ABDite® is a porous carbon made from bituminous coal that is abundant and readily available (Grammelis et al., 2016; Schumacher and Juniper, 2013). The ABDite medium is cheaper than other biofilm support media manufactured from other materials such as plastics (Balogun et al., 2003; Karayigit et al., 2018). Furthermore, unlike other biomass support materials including plastic that contributes to environmental pollution, ABDite® is made from natural material, hence, the EBBR is a more environment friendly technology (Dempsey, 2018).

The EBBR has been successful at laboratory and pilot-scales for high-rate tertiary nitrification process; and meets most criteria for sustainable WWT technology, e.g., simplicity, low-cost and energy-efficiency (Dempsey et al., 2005). Despite the successes achieved, the EBBR is yet to be deployed for the Anammox wastewater treatment; this, therefore, is the reason for the EBBR-Anammox wastewater treatment study.

2.1.3 Single and simultaneous partial nitrification Anammox processes bioreactors

The discovery of the Anammox bacteria has transformed wastewater treatment with increased nitrogen removal efficiency (Table 2.1); particularly in systems that use biomass support to immobilise cells; and meet the stringent growth conditions of the Anammox bacteria despite their slow growth rate, e.g., 0.0624 d^{-1} (Cho et al., 2020; Zainab et al., 2020; Kartal et al., 2013). The Anammox process is carried out either as a single Anammox (SA) or simultaneous partial-nitrification-Anammox (SPNA) reaction (Tables 1.7). In SA, the influent wastewater, which contains both NH_3-N and NO_2-N are fed into a single reactor at the same time (Van Dongen et al., 2001). However, in SPNA, the wastewater, which contains only NH_3-N is loaded into the bioreactor (Slieker at al., 2002). Details of SA and SPNA operations have been discussed in section 1.7.3 Anammox bioreactor operations (Table 1.6).

The single Anammox (SA) wastewater treatment investigations have been conducted in reactors of various configurations to achieve high nitrogen removal efficiency at different nitrogen loading and removal rates. The EBBR-Anammox study will conduct the SA process, in which the synthetic wastewater will contain ammonia and nitrite (Table 2.1).

Table 2.1: Nitrogen removal efficiency of Single Anammox reactors supplied with wastewater containing NH_3-N and NO_2-N. **Key:** *AnMBR–Anaerobic membrane biofilm reactor *FBR- fluidized bed reactor *GLR-gas lift reactor *HRT – Hydraulic retention time *MBR-membrane biofilm reactor *MSBR–membrane sequencing batch reactor *SBR - sequencing batch reactor *RBC– rotating biological contactor *UASB-Upflow anaerobic sludge blanket.

Type of reactor	Working Volume (L)	Duration (d)	Influent NO_2-N: NH_3-N (mg L^{-1})	N-Removal Rate (kg N L^{-1} d^{-1})	NO_2-N: NH_3-N removal efficiency (%)	References
SBR	12	-	20	0.00004	-	Laureni *et al.,* 2016
GLR	7	200	70:70	2.0	99:88	Dapena-Mora et al., (2004a)
UASB	5	280	420:350	-	88	Li and Sung (2015)
RBC	1.7	100	350:350	0.091	100:99	Liu et al., (2008)
FBR	23	150	< 840: <1100	1.5	81 - 99	Strous et al., (1997)
AnMBR	15	135	100:10,000	0.02-5	96	Suneethi and Joseph (2011)
MSBR	5	375	390:390	-	90:90	Trigo et al., (2006)
MBR	8	>250	552:552	-	-	Van der star et al., (2008)

2.1.4 Aim

The aim of the research in this chapter (chapter two) is to investigate if the Anammox wastewater treatment process could be established in a lab scale expanded bed biofilm reactor (EBBR).

The objectives to achieve the above aim were:

1. Design and inoculation of the lab scale EBBR with active Anammox bacteria; maintained with synthetic wastewater in continuous culture operation.

2. Examination of the lab scale EBBR for biofilm formation and gas production as evidence of Anammox process

3. Determination of EBBR-Anammox process nitrogen removal rate (NRR) and nitrogen removal efficiency (NRE)

4. Comparison of EBBR-Anammox investigation results with data from other Anammox process studies to determine if the Anammox process (ammonia and nitrite removal) was established within the EBBR.

2.2 Methods

2.2.1 Design and inoculation of the Expanded bed biofilm reactor

2.2.1.1 Expanded bed biofilm reactor design and set up

An expanded bed biofilm reactor (EBBR) constructed using borosilicate glass components (QVF Corning Process Systems, UK), comprised of two vertical columns for bed expansion and bulk wastewater recirculation (Figure 2.1). The expanded bed column (58 x 4 cm) and the recirculation column (45 x 2 cm) were connected using silicon tubing (Silex, UK) and the EBBR had a total reactor volume of about 700 cm^3 (0.7 L). The recirculation ensured the mixing of influent and effluent allowing the synthetic wastewater nitrogen to be reduced to nitrogen gas by Anammox bacteria (Castro-Barros et al., 2017; Winkler et al., 2012).

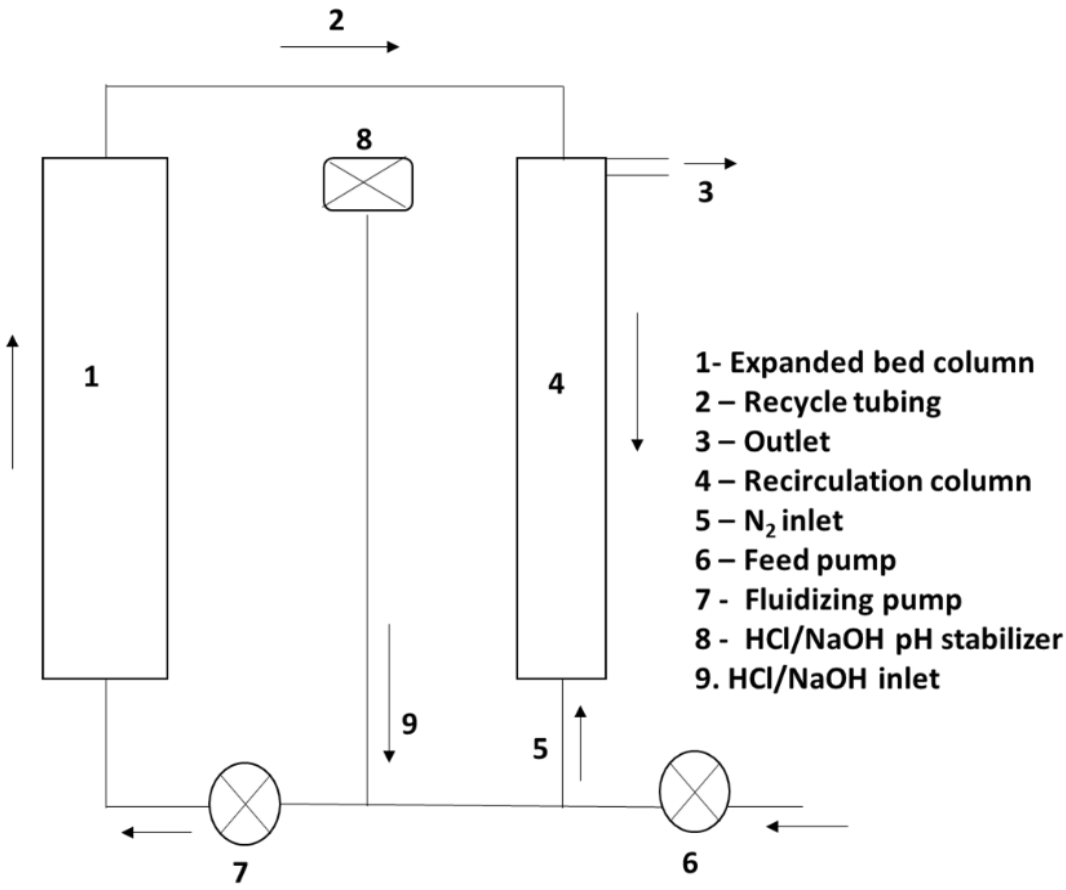

Figure 2.1: Schematic diagram of the lab scale expanded bed biofilm reactor (EBBR)

2.2.1.2 Synthetic wastewater composition

To simulate high-strength ammonia wastewater, the initial concentrations of NH_3-N (100 - 500 mg L^{-1}) and NO2-N, (130 - 660 mg L^{-1}) were adjusted to achieve the 1:1.32 (NH_3-N: NO2-N) ratio established by Delft University (Van Haandel and Van der Lubble, 2007; Jetten et al., 2001), the pioneer of the Anammox process.

The bioreactor was supplied with synthetic wastewater (SWW) adapted from Van de Graff et al., (1996) and Sliekers et al., (2002), (Table 2.2). The SWW medium was formulated by dissolving the right amount of each constituent (g L^{-1}) in deionised water and the solution sterilized by autoclaving for 15 min at 121 º C (15 psi). Upon cooling to room temperature, sterile trace element solution ((4 mL L^{-1}),

Table 2.2)) was added to the mineral medium aseptically. The synthetic medium was flushed with nitrogen gas for 30 min to reduce the oxygen dissolved in the SWW to support the growth of Anammox bacteria that grow in anaerobic condition (Suneethi et al., 2014).

Table 2.2: Composition of synthetic wastewater (SWW) and trace element (Graff et al., 1996 and Sliekers et al., (2002).

Synthetic wastewater		**Trace element solution**	
Chemical formula	Concentration (g L^{-1})	Chemical formula	Concentration (g L^{-1})
$(NH_4)_2SO_4$	2.82	EDTA	15
$NaNO_2$	2.46	$ZnSO_4.7H_2O$	0.43
$KHCO_3$	5	$CoCl_2.6H_2O$	0.24
KH_2PO_4	27.2	$MnCl_2.4H_2O$	0.99
$MgSO4.7H_2O$	3	$CuSO_4.5H_2O$	0.25
$CaCl_2.2H_2O$	180	$NaMO_4.2H_2O$	0.22
		$NiCl.6H_2O$	0.19
		$NaSO_4.10H_2O$	0.21

2.2.1.3 ABDite biomass support medium

The EBBR was filled to one third of bed height (12 cm) with of 0.7 – 1 mm porous ABDite® biomass support particles (ABD Ltd., Manchester, UK). A 5 cm (1.0 -1.71 mm) silica sand (Chelford (now Sibelco) UK) layer was added to reduce turbulence and stop the ABDite® biomass support medium from grinding (Figure 2.1).

2.2.1.4 Source, seeding and storage of the Anammox seed granules

2.2.1.4.1 Source of Anammox seed granules

The seed Anammox bacteria granules (SAnBG) were sourced from an active Anammox wastewater treatment plant operated by Severn Trent Water, UK (Figures 2.2).

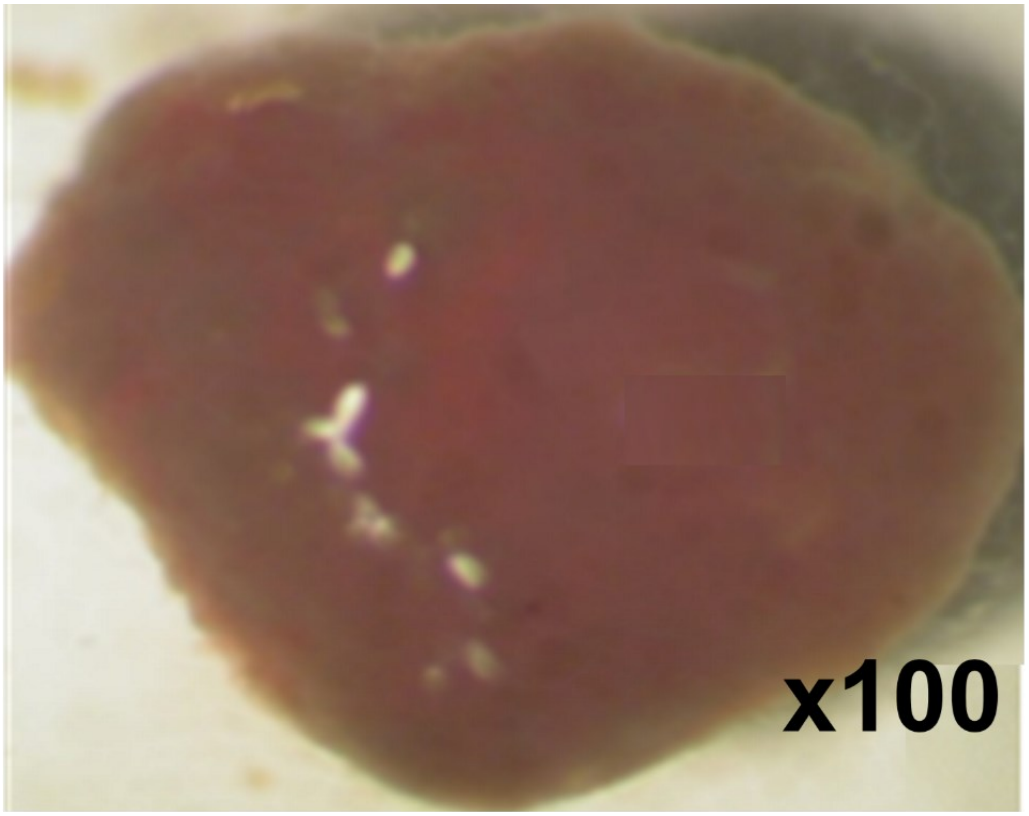

Figure 2.2 Phase contrast microscope (Standard 20, Germany) image of STW seed Anammox bacteria granule (SAnBG) inoculated into the EBBR expanded bed.

2.2.1.4.2 Seeding the bioreactor

The EBBR was seeded with 9 g of seed Anammox bacteria granules (SAnBG), washed 3 - times with fresh deionised water in sterile universal bottle to remove extra nutrient from the treatment plant.

2.2.1.4.3 Storage of replacement samples

The remaining granules were stored in the fridge at 4 ^{o} C for future use, such as for replacement after sampling. The viability of the remaining granules was maintained by washing and replacing the synthetic wastewater (SWW) once every 3 months (Ali and Okabi, 2015).

2.2.1.5. Maintaining the expanded bed biofilm reactor

The EBBR was maintained in continuous culture with synthetic wastewater (SWW) at 50 % bed expansion from the baseline height after the bioreactor was set up in static mode (Figure 2.1) using a peristaltic pump (Watson Marlow 120S, UK) by the upward flowing SWW at a velocity of 1 cm s^{-1} (Akhidime and Dempsey, 2009). At this level of bed expansion, the reactive column for the suspended ABDite® medium, is retained within the rig.

The EBBR operations were maintained at a pH 8.3, using a pH controller (Electrolab 260, UK) that delivered 0.1M HCl or 0.25 NaOH to the EBBR at the entry point of wastewater feed to ensure adequate dilution of the acid or alkali before it reached the immobilized biomass (Figure 2.1).

The EBBR was kept at a temperature of 30^{o} C by a water jacket connected to a thermo-circulator (Thermofisher, UK).

To reduce the level of dissolved oxygen (DO) in the EBBR that will inhibit the growth of Anammox bacteria, nitrogen (N_2) gas was injected continuously at the base of the recirculation column (Figure 2.1, point 5) at a rate of 100 m^3 min^{-1} to reduce oxygen concentration in the bioreactor that might have diffused across the silicone rubber tubing.

The expanded bed column (EBC) was covered with thick layers of black plastic bag (Banquet, UK) to prevent light penetration into the bioreactor. In the presence of light, phototrophic algae can produce oxygen, but algal growth is eliminated in

the dark (Babaei et al., 2013; Stepniewska et al., 2012; Schumacher and Sekoulov, 2002).

2.2.2 Monitoring of the Lab scale EBBR for evidence of Anammox process

2.2.2.1 Physical examination of EBBR for biofilm/bioparticle formation and gas production

After seeding, the EBBR was examined every 2 to 3 days to detect physical evidence (brick red granules or brown biofilm) of Anammox biofilm/bioparticle formation on the ABDite biomass medium and gas production in the EBBR expanded bed (Figure 2.1).

2.2.2.2 Measurement of physical parameters

2.2.2.2.1 Measurement of pH and temperature

The temperature and pH were recorded manually on a result table as indicated by the display panel on the individual controllers (Electrolab 260, UK).

2.2.2.2.2 Bed height measurements for biomass development

The expanded bed height (EBH) and static bed height (SBH) measurements were taken to monitor the increase in biomass development leading to formation of biofilm on the ABDite® medium. Biomass formation occurs as the bacteria utilize the wastewater nutrients, accumulate cell components, which cause cell size increase; cell replication results in biomass increase (Rosalind and Bartlomiej, 2019).

The expanded bed heights (EBH) were recorded with the fluidizing pump switched on. The collected EBH data were used for other calculations including the EBBR reaction volume (V), dilution rate (D) and Anammox nitrogen removal rates (NRR) Kg Nm^{-3} d^{-1}. The SBH readings were taken after the fluidizing pump was switched off, allowing the ABDite® particles to settle.

2.2.3 Determination of EBBR-Anammox process nitrogen removal rate (NRR) and nitrogen removal efficiency (NRE).

2.2.3.1 Collection of liquid samples from the expanded bed column

Samples (5 mL) of influent going int the bioreactor was collected after point 6, before the N_2 gas inlet at point 5). Effluent coming out of the EBBR was collect at point 3; the collection points were sealed to maintain anaerobic condition (Figure 2.1). Collected samples were filtered using a 0.4 µm syringe filter (Cronus, UK) into a 7 mm sterile Bijou bottle (Fisher Scientific, UK) and analysed immediately.

2.2.3.2 Chemical analysis to determine the concentration of nitrogen species

Anammox activity is commonly indicated by simultaneous consumption of ammonia and nitrite (Langone et al., 2014). The NRR was calculated as the difference between the influent nitrogen (ammonia + nitrite) load into and effluent nitrogen (ammonia + nitrite) from the Anammox bioreactor, which is based on the stoichiometry reaction of the Anammox (equation 2.1).

$$NRR = \frac{\text{Load } N_{in} - \text{Load } N_{out} \text{ (mg N L}^{-1\ d\ -1})}{V} \qquad 2.1)$$

Where:

- Load N_{in} (sum of inlet nitrogen fed into the reactor (ammonia + nitrite) (mg N d-1).

- Load N_{out} (sum of nitrogen leaving the reactor (ammonia and nitrite) (mg N d-1);
- V: volume of the Anammox bioreactor (L) (Jaroszynski et al., 2012).

In the EBBR, the volumetric Anammox rate was calculated and expressed as kg N m^{-3} EBBR d^{-1}. The active volumetric Anammox rate was based on the ammonia and nitrite removed in the total reaction volume (expanded bed), which was available for Anammox activity in the expanded bed column at sampling time. The ammonia and nitrite removed was an indication of the volumetric conversion

(Anammox) ability of the bioreactor. The EBBR-Anammox NRR was determined based on equations 2 to 5.

The total reaction volume was calculated based on the bioparticle bed height upon expansion by the fluidizing pump in the expanded column and is expressed as V(m^3).

EBBR reaction volume (V) = EBH x π x r^2 (m^3) *(2.2)*

The dilution rate D (d^{-1}) of the EBBR system was calculated as

dilution rate D = F/V (d^{-1}) *(2.3)*

Where:

F = influent flow rate (m^{-3} d^{-1}) *(2.4)*

Finally, the dilution rate (D) was multiplied by the concentration of nitrogen consumed by the Anammox biomass (kg N m^{-3}EBBR).

Therefore, the reactor volumetric Anammox rate =

Concentration of nitrogen [NH_3-N] and [NO_2-N] removed by the Anammox biomass (kg N m^{-3} EBBR) x D (d^{-1})

NRR (Kg N m^{-3} d^{-1}) = D x ([Total inlet N] – [Total Outlet N]) *(2.5)*

2.2.3.3 Determination of ammonia nitrogen [NH_3-N] concentration

The ammonia [NH_3-N] nitrite [NO_2-N] and nitrate [NO_3-N] concentrations were determined using the standard methods ISO 7150-1:1984; ISO 6777-1984 and (APHA 4500-NO_3-) respectively, which are based on manual spectrophotometric (Jenway model 6305, Bibby Scientific, UK) analysis. The concentration of the nitrogen associated with ammonia (NH_3-N) was determined and taken as the

ammonia concentration. The method involved measurement of absorbance at 655 nm of the blue compound formed when sample ammonia reacts with salicylate, hypochlorite ions and sodium nitroprusside.

2..2.3.4 Determination of nitrite [NO_2-N] concentration

The concentration of nitrite-nitrogen was determined using the molecular absorption spectrophotometric method (ISO 6777-1984). The method involves the reaction of nitrite present in the sample with 4-aminobenzene sulphonamide (colour regent) in the presence of orthophosphoric acid to form a diazonium salt. This salt forms a pink coloured dye in a reaction with N-(1-naphtyl)-1, 2-diamoethane dihydrochloride; the absorbance measurement was then made at 540 nm

2.2.3.5 Determination of nitrate-nitrogen [NO_3-N] concentration

The American Public Health Association standard method for examination of water and wastewater (APHA 4500-NO3-) was used to determine the nitrate-nitrogen concentration. The method is based on the measurement of the UV absorbance of nitrate at 220 nm after HCl (1M) was added to acidify the sample to reduce interference caused by carbonate and hydroxide. Measurements were also made at 275 nm to correct the reading obtained because organic matter absorb UV light at 220 nm (Cuidad et al., 2005). The absorbance of NO_3-N was derived from the calculation below:

NO_3-N = absorbance at 220 nm – (2 x absorbance at 275 nm)

(2.6)

2.3 Results

2.3.1 The lab scale EBBR designed; inoculated with Anammox bacteria granules; and maintained with synthetic wastewater in continuous culture operation.

The 0.7 L Lab scale expanded bed biofilm reactor (EBBR) designed for the Anammox process had two vertical columns for bed expansion and bulk wastewater recirculation respectively (Figure 2.3).

Figure 2.3: Image of EBBR designed, inoculated with Anammox bacteria granules, and covered with black plastic bag. **Key: 1**- Expanded bed column covered with black plastic bag; **2** – Recycle tubing; **3** – Outlet; **4** – Recirculation column; **5** – N2 inlet; **6** – Feed pump; **7** - Fluidizing pump; **8** - HCl/NaOH pH stabilizer; **9** – HCl/NaOH inlet; **10** – Temperature controller.

Legend: The image revealed the EBBR column covered with black plastic bag after synthetic wastewater, ABDite® biomass support medium and Anammox seed granules were added; and maintained in continuous culture.

2.3.2 Physical evidence of Anammox activity in the Lab scale EBBR

2.3.2.1 Biofilm development on the EBBR ABDite biomass medium to form bioparticles

After seeding, and physical examination for 6 months, brick red granules and brown biofilms, consistent with Anammox bacteria biofilm, rich in biomass were observed in the expanded bed in static mode, after the fluidizing pump was switched off. (Figures 2.4). These observations suggest that active Anammox bacteria biomass were developing in the EBBR.

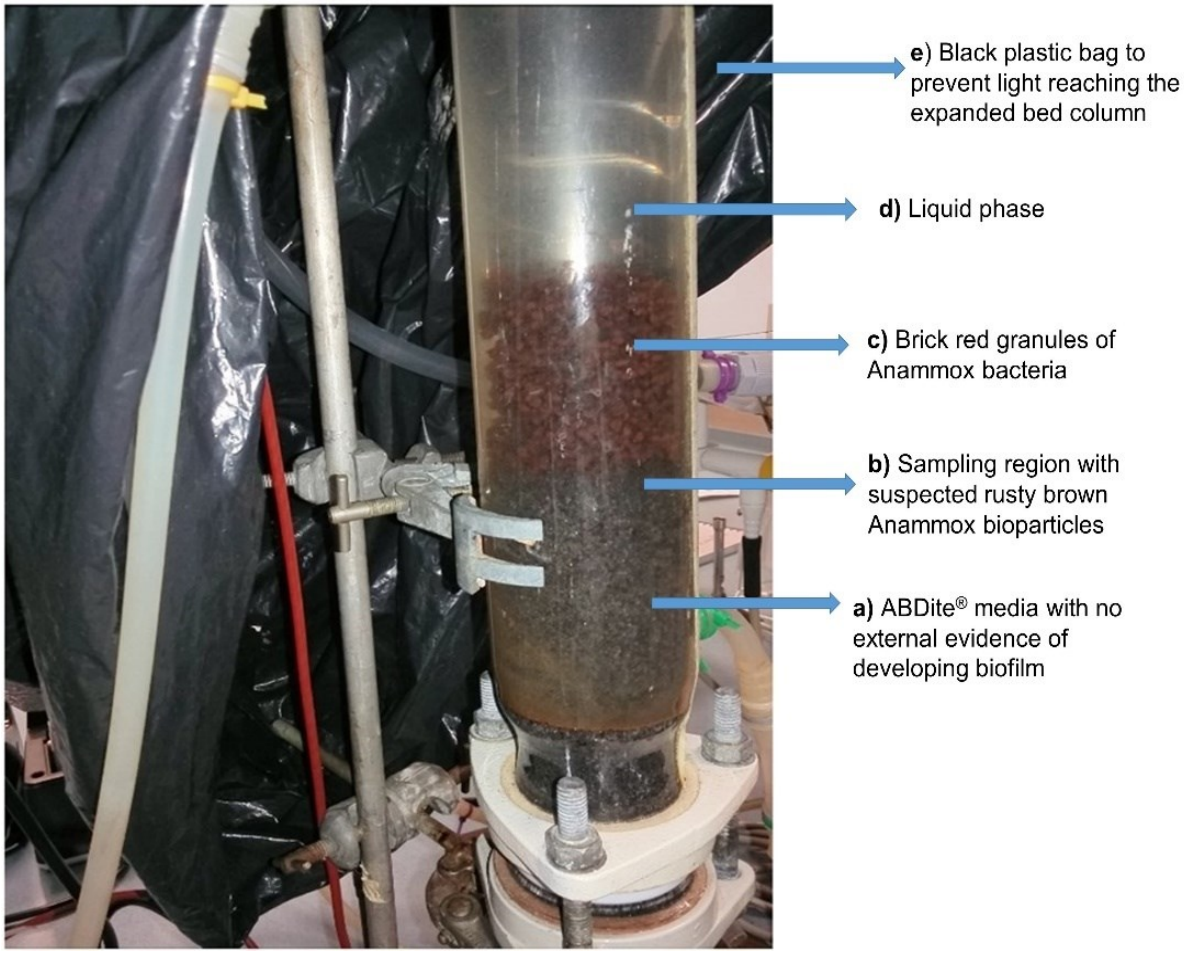

Figure 2.4: EBBR image with layers of brick red Anammox bacteria granules and developing bioparticles in the bed column with the fluidizing pump switched off. The Anammox bacteria granules alongside suspected Anammox biomass

developed on the ABDite® media, with the black plastic bag covering, 6 months, after seeding.

2.3.2.2 Suspected nitrogen gas production in the EBBR column

Furthermore, 8 months from the start-up of the EBBR-Anammox investigation, there was a continuous production of gas, suspected but unconfirmed to be molecular nitrogen (N_2) gas (Figures 2.5). Gas production indicated that N_2, the known main product of the Anammox wastewater treatment process was being produced in the EBBR; and therefore, Anammox process was taking place.

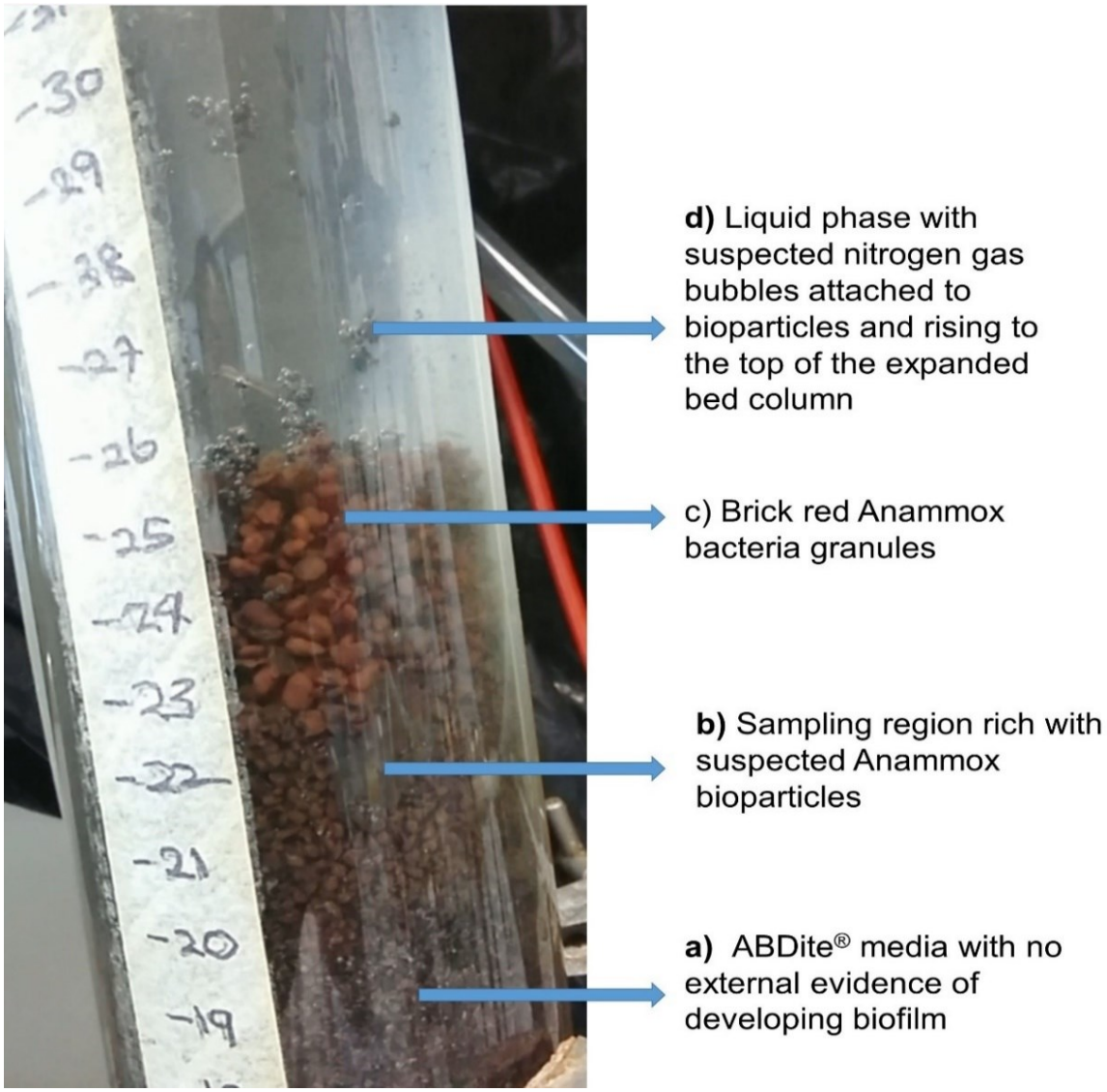

Figure 2.5: EBBR image with layers of Anammox red granules and suspected bioparticles (rusty brown) developing on the ABDite media and suspected nitrogen

gas bubbles in static mode. Anammox bacteria granules developed alongside suspected Anammox bioparticles with suspected nitrogen gas produced in the EBBR.

The gas bubbles were seen to steadily originate from layers that contained the suspected Anammox bioparticles (the sampling region); the gas bubbles, attached to the ABDite particles floated upward into the liquid phase (Figures 2.5). The bottom layer (a) of the EBBR had ABDite® medium that appeared to have no biofilm attached; a suggestion that the bed colonisation was incomplete (Figure 2.5).

2.3.2.3 Bed height increases

The expanded bed height (EBH) and static bed height (SBH) measurements were made to determine biomass development on the ABDite® medium. The results indicated an increase in both the EBH and SBH (Figure 2.6). Increase in EBH was about 63 % (180 to 294 mm) and SBH, 54 % (130 to 200 mm) from the start-up to 78 days of the EBBR-Anammox investigation (Figure 2.6).

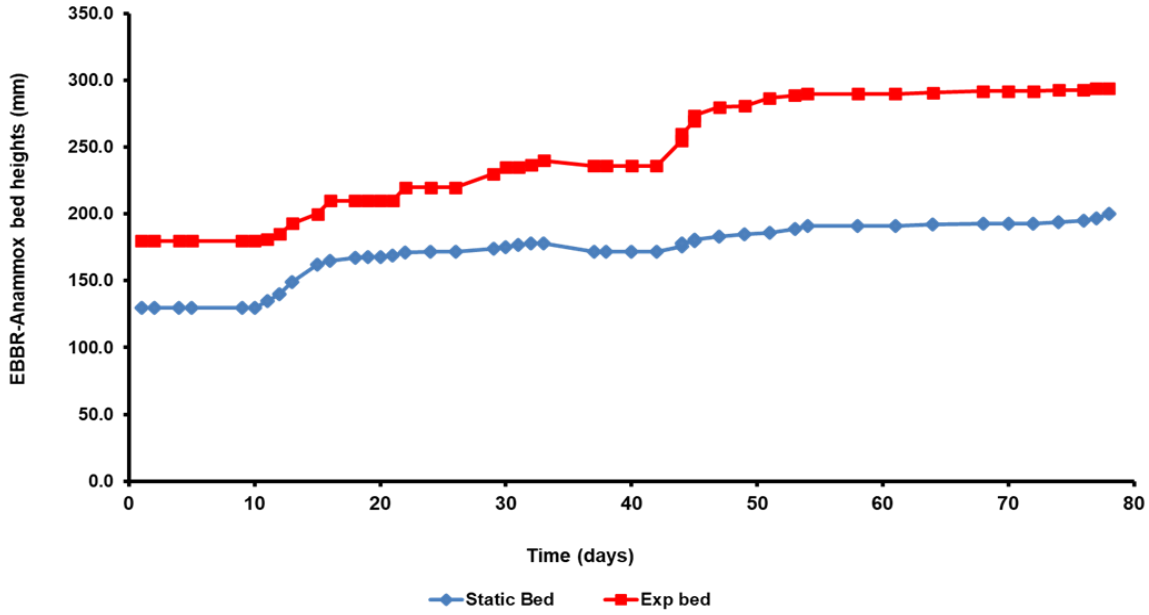

Figure 2.6: Increase in static bed height (SBH) and expanded bed height (EBH) over time (80 days). *Used as indicator of increase in Anammox bacteria biomass

and biofilm formation on the EBBR ABDite® media. The highest EBH achieved was 294 mm and 200 mm for the SBH from start-up to day 78. (Each data point represents a mean of 2 replicate samples (n=2)).

Between day 0 to about day 10, there was no apparent increase in average bed heights, indicating the lag phase. Day 12 to 33 saw a steady rise in bed heights as SHB rose from 135 to 178 mm and EBH from 185 to 240 mm; a positive sign suggesting that the Anammox organisms have started to grow in the EBBR column. The bed height increase was followed by a decrease to 172 mm (SBH) and 236 (EBH) from day 38 to 42 (Figure 2.6). The reduction was caused by a fault in the air flow meter which supplied nitrogen (N_2) gas through the recirculation column to reduce oxygen concentration in the bed (Figure 2.1, point 5). However, the bed quickly recovered, and bed heights gradually increased from 255 mm to a maximum height of 294 mm between day 45 and 78 and SBH rose from 195 to 200 mm. Approximately, the bed heights increased at an average of rate of 0.009 d^{-1} (EBH) and 0.014 d^{-1} (SBH) for the 78 - day period before the Christmas break (Figure 2.6).

2.3.3 Nitrogen removal activities in the EBBR-Anammox process

2.3.3.1 NH_3-N and NO_2-N removed from EBBR-Anammox synthetic wastewater

The chemical analysis of NH_3-N and NO_2-N removed, and NO_3-N produced in the EBBR-Anammox system were reported in two stages; from the set-up of the investigation to day 78 (Figures 2.7); and from day 139 to 317 (Figure 2.8). The results indicated that between 82 - 362 mg L^{-1} (NH_3-N) and 99 - 449 mg L^{-1} (NO_2-N) were removed from the SWW; and 14 - 26 mg L^{-1} NO_3-N produced from start-up to day 317 (Figures 2.7 & 2.8). The maximum nitrogen removal rate (NRR) was 6.1 Kg N m-3 d-1 from a loading rate (NLR) of 9.0 Kg N m-3 d-1 (Figure 2.10); and nitrogen removal efficiency (NRE) between 90 – 100 % (Figures 2.12 – 2.14). The simultaneous removal of NH_3-N and NO_2-N; and NO_3-N production indicated that the Anammox WWT reaction was occurring in the EBBR.

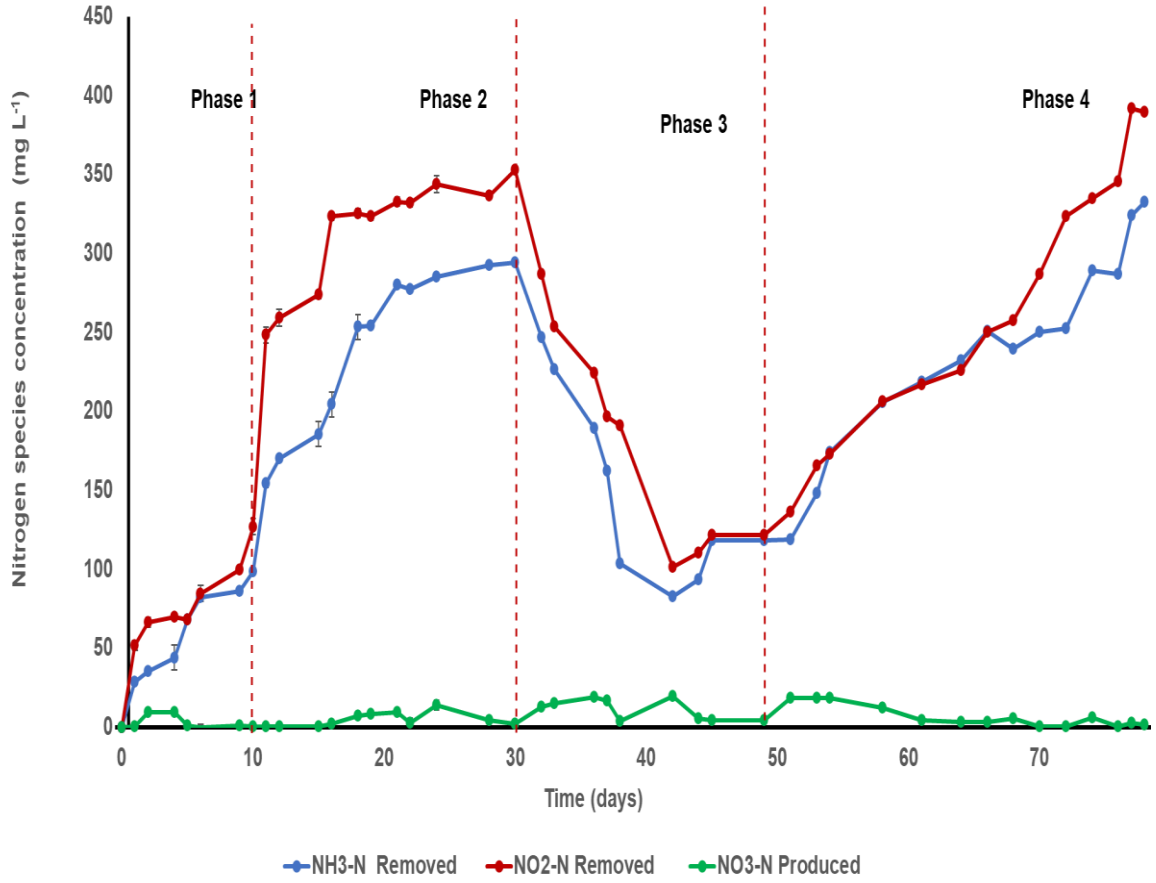

Figure 2.7: Ammonia nitrogen and nitrite nitrogen removed, and nitrate nitrogen produced in the first 78 days of EBBR-Anammox investigation after start-up. **Key**: **Phase 1**- start-up; **Phase 2-** stable operation; **Phase 3-** unstable operation - fault with air flow meter; **Phase 4 -** stable operation. The highest concentration of NH_3-N (332 mg L^{-1}) and NO_2-N (393 mg L^{-1}) were removed; and 20 mg L^{-1} (NO_3-N) produced. Nitrite 272 mg L^{-1}) accumulation and reduction in Anammox activity occurred in phase 3. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

In the first 78 days after the EBBR-Anammox investigation was set, the data indicated that there was a steady increase in the concentrations of NH_3-N (99 – 353 mg L^{-1}) and NO_2-N (127 – 294 mg L^{-1}) removed from day 0 to 30) in phases 1 and 2 respectively (Figure 2.7). Approximately, 86 mg L^{-1} (NH_3-N) and 99 mg L^{-1} (NO_2-N) were consumed by the Anammox organisms at a volumetric removal rate of 0.5 Kg N m^{-3} d^{-1}. The amount of NH_3-N and NO_2-N removed at this period was

less compared to the NO_2-N and 294 mg L^{-1} (NH_3-N) and 353 mg L^{-1} (NO_2-N) metabolized a rate of 1.4 Kg N m^{-3} d^{-1}, in phase 2; suggesting an increase in active biomass of Anammox bacteria that have adapted to the process conditions in the EBBR (Figures 2.6 and 2.7). A fault in the air flow meter in phase 3 (days 32 to 45) led to > 70 % decrease in the concentration of NH_3-N (82 mg L^{-1}) and NO_2-N (101 mg L^{-1}) removed from the EBBR influent (Figure 2.7); which was also marked by decrease in bed heights (Figure 2.6). Reduction in nitrogen removal suggests a partial inhibition of the Anammox process caused by increase in oxygen (O_2) level in phase 3 between day 40 – 45; and build-up of NH_3-N (222 – 267 mg L^{-1}) and NO_2-N (224 -272 mg L^{-1}). The full recovery of the bioreactor after the air flow meter was fixed resulted in an increased concentration of NH_3-N (332 mg L^{-1}) and NO_2-N (393 mg L^{-1}) removal, day 51 to 78 (phase 4). Conversely, < 20 mg L^{-1} NO_3-N was produced during these 78 days, indicating that Anammox bacterial activity occurred in the EBBR despite the mishap.

The nitrogen species concentration results from day 139 – 317 suggested that > 300 mg L^{-1} (NH_3-N) and about 400 mg L^{-1} (NO_2-N) were removed from the bioreactor in phases 1, 3 and 5, when the EBBR had a steady operation (Figure 2.8). There was a 16 % (NH_3-N) and 14 % (NO_2-N) rise in the amount of nitrogen removed between phase 1 and 5; and 24 mg L^{-1} (NO_3-N) produced, which suggests there was a progression in Anammox activity in the bioreactor. The result also revealed that instability caused by problem with the thermocirculator resulted in a 57 % (NH_3-N) and 55 % (NO_2-N) fall in the concentrations removed in phases 2; with 203 mg L^{-1} (NH_3-N) and 231 mg L^{-1} (NO_2-N) recovered in the effluent by day 198. Similar decrease in nitrogen removal was observed in phase 4, when the replacement of the pumps (Figure 2.2) caused NH_3-N and NO_2-N removal to decrease by 49 % and 47 % respectively (Figure 2.8). These results suggests that fluctuations in process conditions could cause up to 50 % reduction in the effectiveness of the EBBR-Anammox process to remove nitrogen; the threshold of concentrations of nitrogen species that partially inhibited the EBBR-Anammox activity was 200 – 260 mg L^{-1} (NH_3-N) and 220 – 270 mg L^{-1} (NO_2-N); in addition, the results demonstrates the resilience of the Anammox bacteria and the

EBBR system to recover quickly when normal process conditions were restored (Figures 2.7 & 2.8).

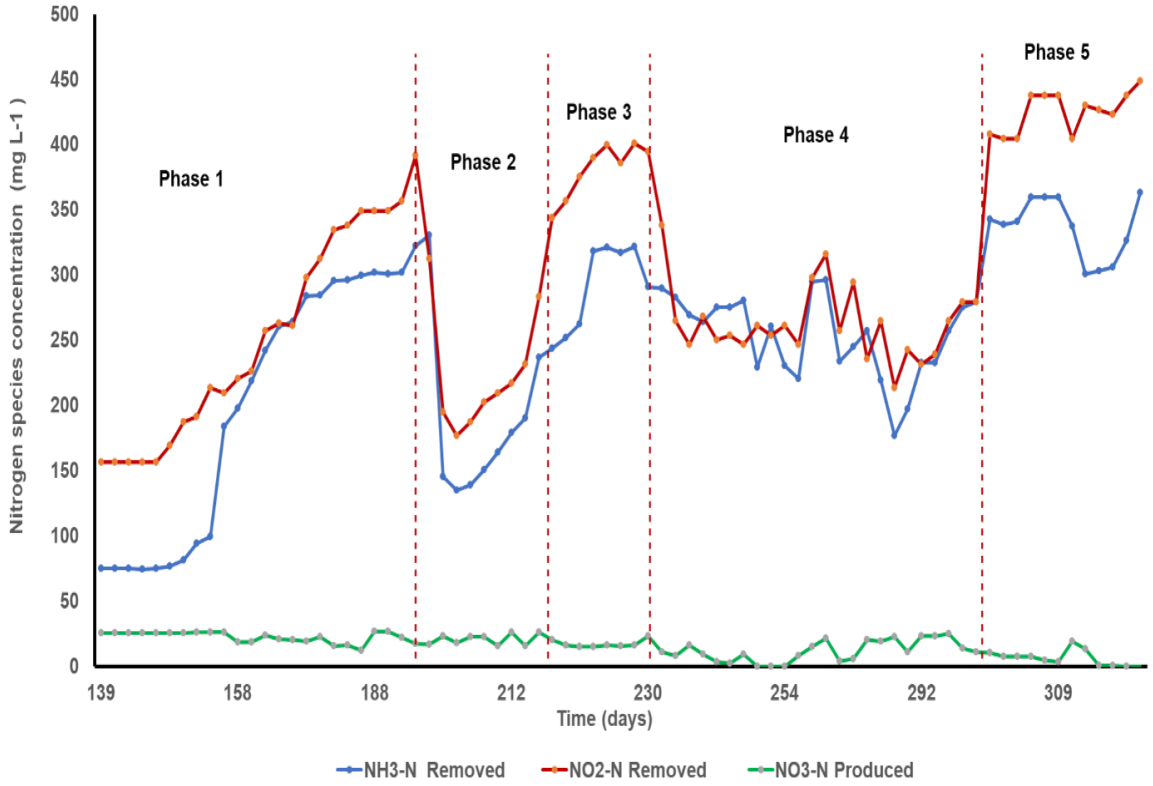

Figure 2.8: Ammonia nitrogen and nitrite nitrogen removed from effluent, and nitrate nitrogen produced between day 139 and 317 of EBBR-Anammox investigation. **Key**: **Phase 1**- stable operation; **Phase 2-** unstable operation - fault with temperature controller; **Phase 3-** stable operation - fault with air flow meter; **Phase 4-** unstable operation – pumps replacement; **Phase 5 -** stable operation. A maximum of 322 Mg L^{-1} of NH3-N and 392 mg L^{-1} of NO_2-N were removed; and 26 mg L^{-1} (NO_3-N) was produced day 139 - 317. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

2.3.3.2 Nitrogen removal rate of the EBBR-Anammox process

The performance of the EBBR-Anammox operation was evaluated by calculating the nitrogen loading rate (NLR) against nitrogen-removal rate (NRR) for 355 days. The EBBR-Anammox process achieved a nitrogen-removal rate (NRR) between 0.2 to 6.1 Kg N m^{-3} d^{-1} from a nitrogen-loading rate (NLR) of 0.4 to 9.8 Kg N m^{-3} d^{-1} (Figures 2.9 - 2.11). The wide range in the NLR and NRR measurements suggested that the system did not operate at steady state throughout the EBBR-Anammox wastewater treatment investigation. In the first 78 days (Figure 2.9), the maximum NLR was 1.8; and NRR of 1.4 Kg N m^{-3} d^{-1} was achieved by day 30 (Figure 2.9).

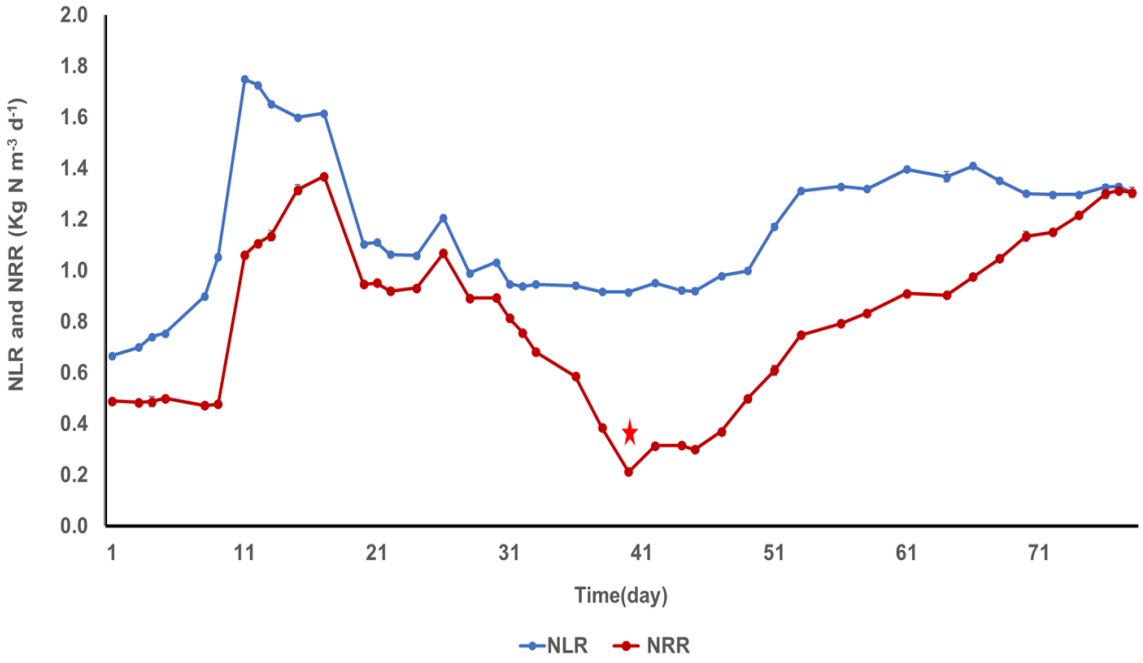

Figure 2.9: Nitrogen loading rate (NLR) and nitrogen removal rate (NRR) for the first 78 days after start-up. ★ **Key**: unstable operation - fault with air flow meter. Total nitrogen supplied at a rate of 1.7 Kg N m^{-3} d^{-1} was removed at the rate 1.4 Kg N m^{-3} d^{-1}; 90 - 100 % of supplied nitrogen was removed between day 70 and 78. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

The fluctuation in the NRR around day 40 due to faulty equipment already discussed reduced NRR to 0.2 1.3 Kg N m^{-3} d^{-1}. With the recovery of full operation, day 50 - 78 NRR increased steadily to 1.3 Kg N m^{-3} d^{-1} with 100 % (719 mg L^{-1}) of the total inlet nitrogen (NH_3-N and NO_2-N) supplied to the bioreactor removed (Figure 2.9)

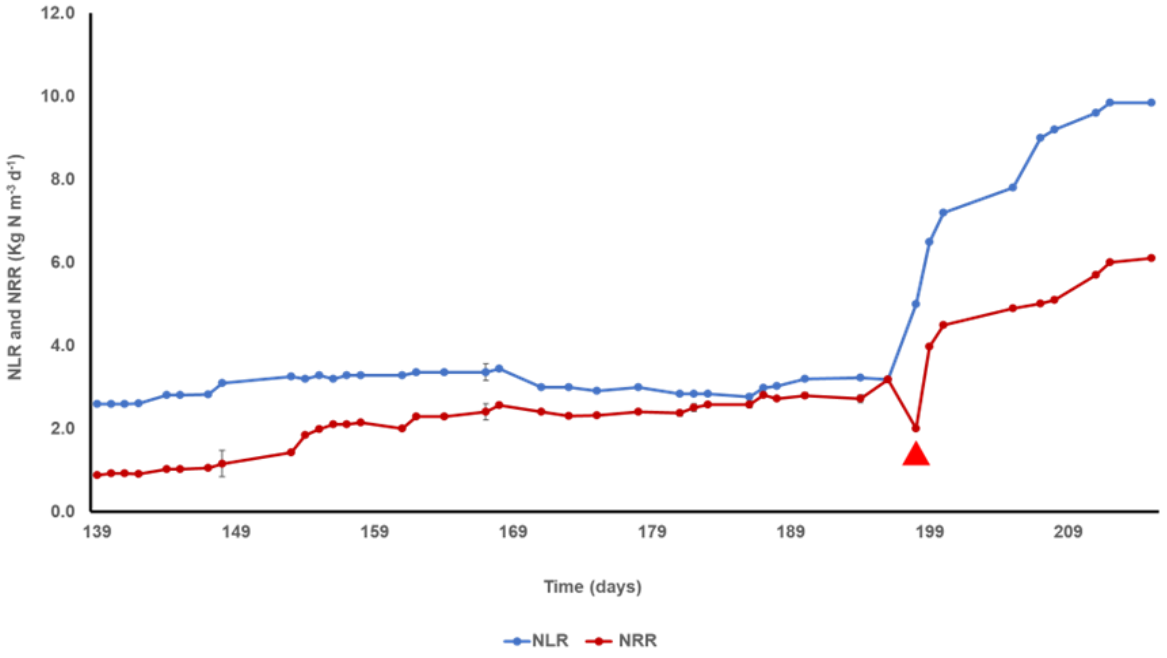

Figure 2.10: Nitrogen loading rate (NLR) and nitrogen removal rate (NRR) from day 139 of the EBBR-Anammox investigation. **Key** ▲Temperature decrease to 23 ° C (day 198), reduced NRR to 0.9 Kg N m^{-3} d^{-1}. The highest NLR and NRR were 9.8 Kg N m^{-3} d^{-1} and 6.1 Kg N m^{-3} respectively. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

The maximum NLR (9.8 Kg N m^{-3} d^{-1}) and NRR (6.1 Kg N m^{-3} $d^{-}1$) of the EBBR-Anammox process investigation was recorded between day 139 to 212 (Figure 2.10); approximately, 67 % of the inlet nitrogen was removed, indicating there was stability in the EBBR (Figure 2.10). The data also revealed that low temperature

(23 °) reduced NRR to 0.9 Kg N m^{-3} d^{-1} between day 138 to 144 (Figure 2.10). Installation of a new thermocirculator restored the temperature to 30 ° C: with a sustained increases in NLR (2.8 to 9.8 Kg N m^{-3} d^{-1}) and NRR (1.1 – 6.1 Kg N m^{-3} d^{-1}), for approximately 23 days (Figure 2.10). The steady increase in NRR further supports the development of the suspected brown Anammox bioparticles (Figure 2.4) and nitrogen gas production (Figure 2.5) and EBBR bed height increase results discussed previously (Figures 2.6). These results suggest that the EBBR-Anammox system was working well; and therefore, able to achieve a high NRR. From day 234 – 260 (Figure 2.11), NLR and NRR increased by 60 % and 72 % respectively; by day 262, there was a 90 % decrease in NRR due to faults with the pumps; the system still recovered fully and achieved a NRR of 93 % by day 355 at 1.6 Kg N m^{-3} d^{-1} (NLR) and 1.4 Kg N m^{-3} d^{-1} (NRR). This again, indicated that the EBBR-Anammox system had the capacity to recovery from challenges, with full operation restored (Figure 2.11).

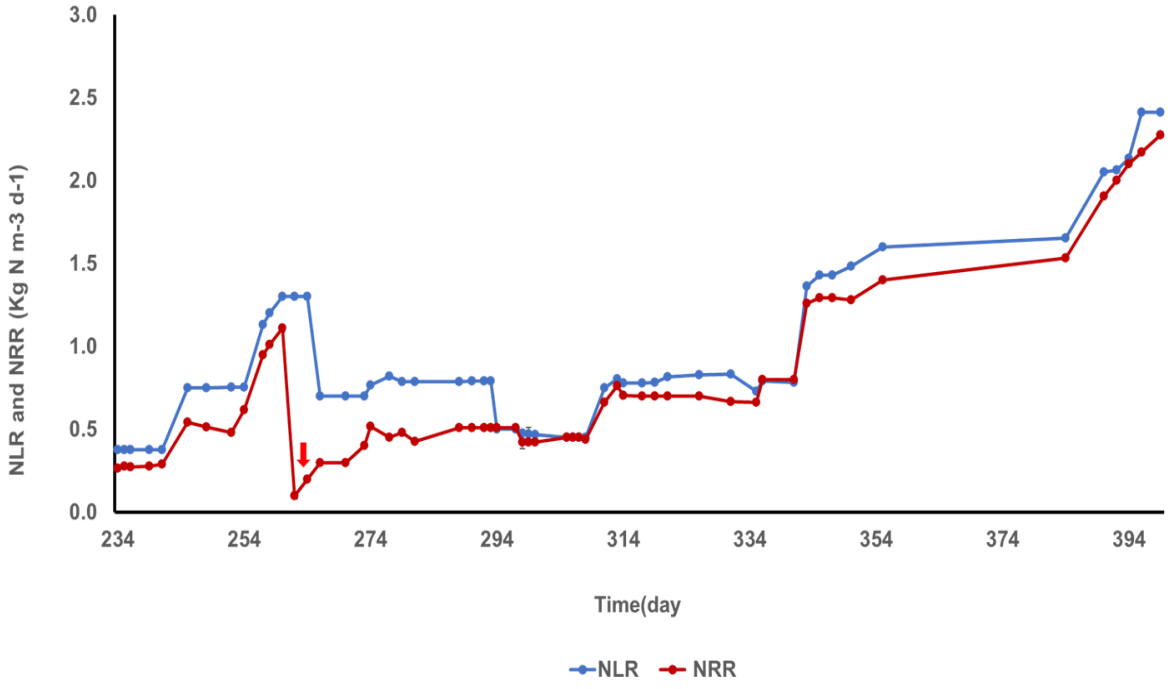

Figure 2.11: Nitrogen loading rate (NLR) and nitrogen removal rate (NRR) from day 234 - 355 of the EBBR-Anammox investigation. **Key** ⬇Faults in pumps (day 262), reduced NRR by 90 % The highest NLR and NRR were 1.6 Kg N m^{-3} d^{-1} and 1.4

Kg N m^{-3} respectively. NRR increased to 93 % after recovery (day 355). (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

2.3.3.3 Nitrogen removal efficiency (%) of the EBBR-Anammox process

The EBBR-Anammox process achieved 14 - 100 percentage (%) nitrogen-removal efficiency (NRE) over the > 560-day duration of the study (Figures 2.12 – 2.14). The % NRE showed variations depending on whether the reactor was operating steadily or not.

During steady operations, the maximum NRE was 90 – 100 %; for examples; days 26, 30 & 72-78 (Figure 2.11); 183 -196 & 229 - 307 (figure 2.12) and 341- 397 & 448 - 462 (Figure 2.14). These results suggest that EBBR-Anammox system can effectively remove the wastewater nitrogen and therefore, was working well during these periods. The NRE fell below 40 % during the start-up of operation days 1 - 10, (Figure 2.12); and when the reactor developed a fault e.g., (day 40) and days 198 and 262 (Figure 2.13) for reasons previously discussed.

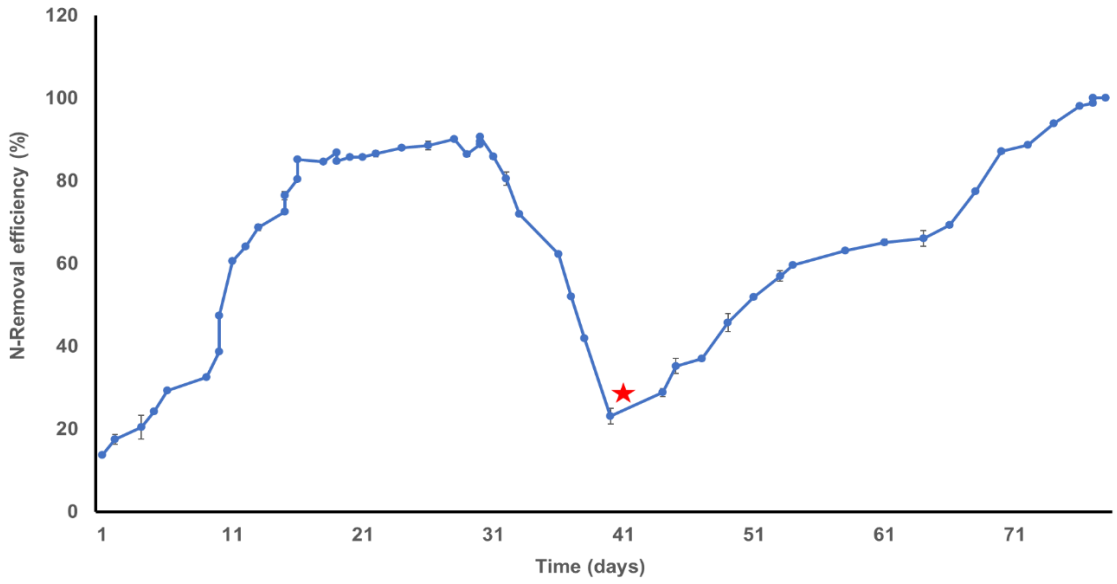

Figure 2.12: EBBR-Anammox % Nitrogen removal efficiency (NRE) day 0 to 78. **Key:**★Days 40 to 45 NRE decreased to 23 % (fault in air flow meter, day 40. The highest NRE the EBBR-Anammox process achieved between day1 – 78 was 80 – 100 %. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

These results demonstrate the negative impact changes in operating conditions such as increase in oxygen and nitrite accumulation (Figure 2.7 - 2.9) or low temperature (Figure 2.10) could have on the efficiency of the Anammox process such as in day 40 (Figure 2.12), and days 198 and 262 (Figure 2.13).

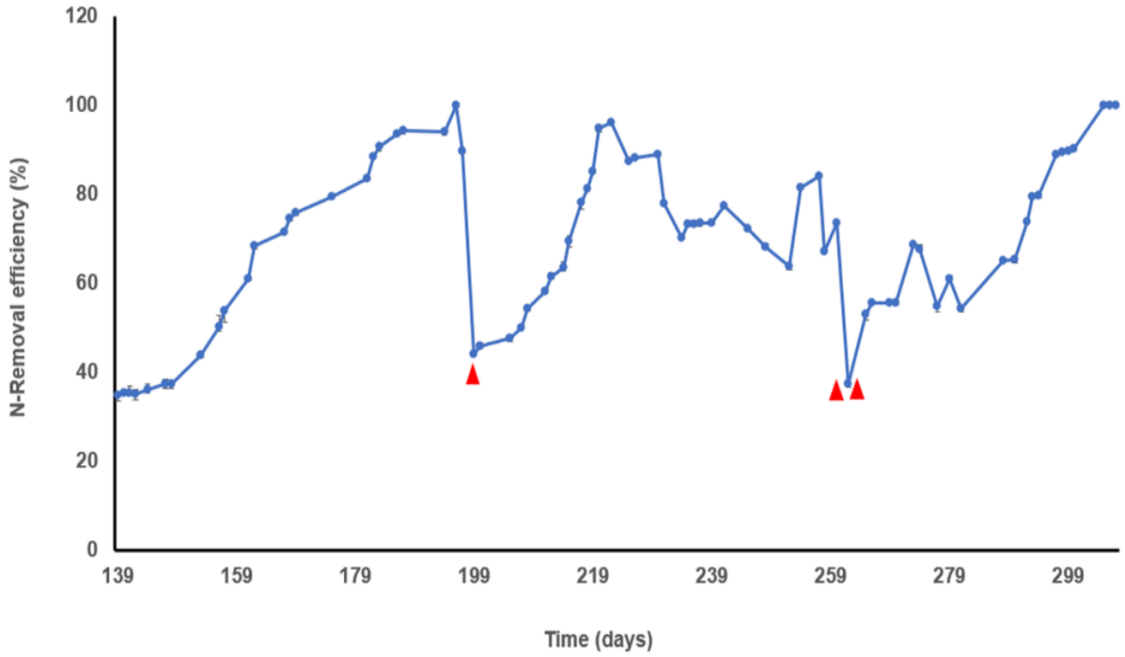

Figure 2.13: EBBR-Anammox % Nitrogen removal efficiency (NRE) day 139 to 307. **Key:** ▲Days 198 NRE decreased to 40 % decrease in NRE as temperature fell to 23 ° C before thermocirculator replacement. ▲▲ Problem with pumps caused NRE decrease to 38 % (day 262). The highest NRE the EBBR-Anammox process achieved was between day139 – 307 was > 90 - 100%. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

Approximately, for 140 days between 331- 470 (Figure 2.14), the EBBR achieved 60 - 100 % NRE; but the efficiency fell to about 50 % (days 475 - 561) as the bioreactor became unstable having been in continuous operation for almost 2 years (Figure 2.14); as part of both the expanded bed and recirculation glass column began to break. This indicates that while the design of the EBBR with glass, had advantages such as ability to physically monitor biofilm/bioparticle

formation on the ABDite growth medium and gas production; there is problem with durability. This has important implication for future design of the EBBR for longer Anammox wastewater treatment operation.

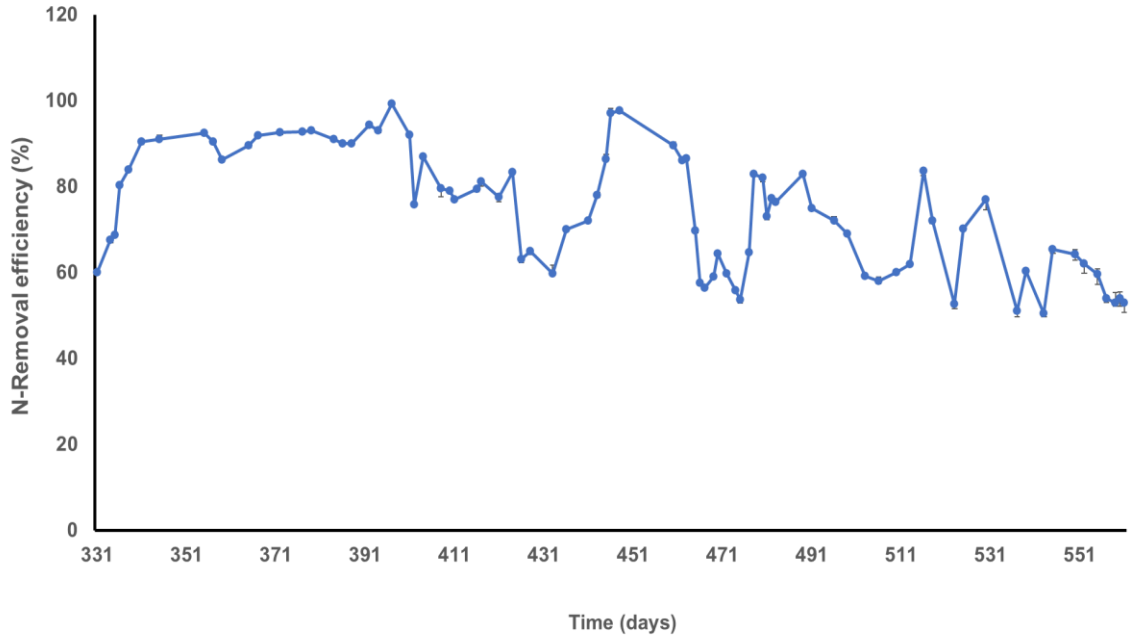

Figure 2.14: EBBR-Anammox % Nitrogen removal efficiency (NRE) day 331 to 561. Days 331- 470 the EBBR achieved 60 - 100 % NRE; and 50 % (days 475 – 561) as part of the glass bioreactor starts to break approximately 2 years in continuous operation. Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean.

The maximum NRE results achieved in this study indicates that EBBR-Anammox system had the capacity to achieve a high NRE between 90 to 100 % when the bioreactor is in a steady operation; and was therefore, working well (Figures 2.12 - 2.14).

2.4 Discussion

According to the pioneers of the Anammox process from Delft University (Van Haandel and Van der Lubble, 2007; Jetten et al., 2009), Anammox process is the partial oxidation of NH_3-N to NO_2-N by AOB. This is followed by the Anammox reaction conducted by the AnAOB, which convert the remaining NH_3-N to NO_2-N to nitrogen gas (N_2); producing NO_3-N, mediated by the NOB. In the EBBR-Anammox investigation, there was a simultaneous removal of NH_3-N and NO_2-N, production of NO_3-N (Figures 2.7 & 2.8) and suspected N_2 gas (Figure 2.5). These results were indication that the Anammox process was established within the EBBR. Using an Anammox enrichment system, Hu et al., (2013) found that nitrogen was the main product of the Anammox reaction. The evidence for the establishment of Anammox process by Zhang et al., (2019) and Laureni et al., (2016) in sequencing batch reactors (SBR) was the conversion of ammonia and nitrite directly to N_2 gas.

Anammox reaction produces nitrogen gas (N_2) through the oxidation reaction of NH_3-N using NO_2-N as the electron acceptor (Jetten, et al., 2009). The gas produced in the EBBR column suggest it was dinitrogen (N_2). While the identification of this gas was not the focus of the current study, its confirmation would have provided further evidence to validate the findings of this study. There is, therefore, the need to confirm the identity of this gas produced in future EBBR-Anammox investigation. If the gas is confirmed to be nitrogen, this will require a redesigning of the bioreactor to make provision for the gas collection, which currently escaped from the system with the effluent. The collection the nitrogen gas will constitute yet another area for further study. The advancement of the research work in these areas will be important because the collected nitrogen gas could be channelled to other uses such as the deoxygenation of feed bottle and the rig; or even sold to further reduce the overall cost of the Anammox process.

The EBBR-Anammox process achieved a maximum NRR of 6.1 Kg N m^{-3} d^{-1} supplied at the highest NLR of 9.8 Kg N m^{-3} d^{-1}(Figure 2.10). In comparison, the 0.2 - 6.1 Kg N m^{-3} d^{-1} NRR obtained using the 0.7 L the EBBR (Figures 2.10 & 2.11)

was within the range of nitrogen removal rates recorded by previous Anammox operations. For example, Dapena-Mora et al., (2004) reported that his 7.0 L gas lift reactor (GLR) had a NRR of 1.78 Kg N m^{-3} d^{-1} a 2 Kg N m^{-3} d^{-1} NLR. A 15 L AnMBR supplied with nitrogen at a rate of 10 Kg N m^{-3} d^{-1} recorded a maximum NRR of 5.0 Kg N m^{-3} d^{-1} (Suneethi et al., 201). Additionally, Zhang et al., (2019) achieved a NRR of 0.98 Kg N m^{-3} d^{-1} from 1.34 Kg N m^{-3} d^{-1} NLR in a SBR. The NRR results from these bioreactors suggest that the EBBR was functioning well to have achieved this comparative level of NRR recorded; and therefore, the Anammox process was established in the EBBR.

The EBBR-Anammox study, apart from the startup or periods of accidental faults, consistently achieved a maximum of 90 - 100 % nitrogen removal efficiency (NRE) when the bioreactor operation was uninterrupted (Figures 2.12 -2.14). The EBBR performed favorably well in line with other established Anammox bioreactors within the 2 years investigation. While a 24 L internal-loop-airlift bioparticle reactor (ILAB) recorded 91 % NRE (Abbas et al., 2015), for the 1.7 L rotating biological contactor (RBC), it was 99 % (Liu et al, 2008). In addition, Li and Song, (2015) reported using a 5 L Up-flow anaerobic sludge bioreactor (UASB) to achieve of 88% NRE; for a hybrid MBBR study by Laureni et al., (2016); it was 63 %; and 46 – 75 % from Anammox experiment in a SBR (Ma et al., 2016). Although the variations in NRE achieved by these reactors were directly linked to differences in process conditions and bioreactor configurations; the EEBR, which was a small compact reactor by volume still achieved a high NRE like the results reported for bigger sized Anammox bioreactors. The NRE data suggests that the EBBR-Anammox system was effective at removing the NH_3-N and NO_2-N pollutant from the synthetic wastewater.

The EBBR-Anammox investigation faced some challenges because this study marked the first time the EBBR technology was used to investigate the Anammox process; in addition to challenges common to Anammox bioreactors reported in literature. For example, Cho et al., (2020) and Kartal et al., (2013) reported the Anammox bacteria have stringent growth conditions, a very slow growth rate (e.g., 0.0624 d^{-1}) and slow to accumulate biomass. Hendrickx et al., (2014) reported that

the biomass yield of Anammox bacteria was about 0.046 g per g nitrogen metabolized. (The EBBR-Anammox bacteria spent <13 days in lag phase before increases in bed height due to biomass development on the ABDite growth medium was detected after the inoculation (Figure 2.6). The EBBR-Anammox doubling time aligned with the results from previous Anammox studies. For example, Kartal et al., (2013) found that Anammox bacteria reached exponential growth phase 10 – 22 days for biomass to increase. Using a membrane bioreactor (MBR), van der Star et al., (2008) reported that Anammox bacteria accumulate biomass between 15 – 30 days after the start-up of operation. Park et al., (2010) observed doubling time of 9 days; and 14 days was recorded by Strous et al., (2006). These reports indicate that the EBBR faced the same challenge; and that the process conditions supported Anammox bacterial growth resulting in biomass increase within similar periods reported by these other studies and therefore, was functional.

The EBBR-Anammox system encountered another challenge when a fault in nitrogen gas supply resulted in an increase in dissolved oxygen (DO) concentration. The EBBR-Anammox synthetic medium was continuously flushed with nitrogen gas to reduce the adverse effect of dissolved oxygen on Anammox bacteria. The problem with N_2 supply led to decrease in bed heights (Figure 2.4), > 86 % reductions in NRR (Figure 2.9) and NRE by 23 % (Figure 2.12). This negative impact on the EBBR- system is because DO is naturally toxic to Anammox bacteria (Zekker et al., 2014); and could cause from partial to complete inhibition of Anammox process. For example, Jetten et al., (2001) found that Anammox activity was reversibly inhibited by DO concentrations of 0.25 - 2%. An irreversible inhibition of AnAOB growth occurred at DO level > 1.37 mg L^{-1} in a rotating disk contactor system (Egli et al., 2001). Again, 2.3 to 3.8 mg L^{-1} of DO, inhibited Anammox bacteria activity in a granular reactor (Carvajal-Arroyo et al., 2013). These results suggest that although the DO concentration in the EBBR was not determined during this study, similar concentrations of DO could have partially inhibited the EBBR-Anammox process as reported by these established Anammox

technologies. Determination of the level of DO that partially inhibits the EBBR-Anammox process constitutes an area for future work.

Directly, linked to increase in DO level was the accumulation of NH_3-N and NO_2-N during days of instabilities previously discussed. Within those periods, partial inhibition of the EBBR-Anammox activity caused a build-up of approximately 200 – 260 mg L^{-1} (NH_3-N) and 220 – 270 mg L^{-1} (NO_2-N) in the bioreactor (Figures 2.7 & 2.8); with NRR reduced below 50 % (Figures 2.12 -2.14). NH_3-N and NO_2-N inhibition of the Anammox process have different threshold concentrations depending on the size, configuration, and process conditions within the bioreactor (Van der Star et al., 2007; Schalk, et al., 2000). For example, a study into nitrite inhibition of Anammox bacteria activity by Bettazzi et al., (2010) found that 100 mg L-1 NO_2-N caused a complete inhibition of the Anammox process. While a 90 % loss in Anammox activity occurred when the NO_2-N concentration reached 750 mg L-1 in a SBR (Langone et al., 2014). Carvajal-Arroyo et al., (2014) reported that 384 mg L^{-1} of NO_2-N caused a 50% loss of Anammox activity in an Anammox bioreactor. The result of NO_2-N inhibition of EBBR-Anammox process is a confirmation of the findings from the above studies discussed. In addition, EBBR-Anammox process evidence suggests that up to 80 % reduction in NRE occurred when ammonia and nitrite concentration reached 267 ± 0.0 and 272 ±1.1 mg L^{-1} respectively (Figure 2.7- 2.8). These results have implication for the EBBR-Anammox process; the need to maintain NH_3-N and NO_2-N concentrations below these levels for efficient Anammox operation by monitoring the oxygen level in the EBBR.

Additionally, a 7 ° C temperature decrease (from 30 – 23 ° C) affected the EBBR-Anammox NRR and NRE; for example, in day 198, NRR reduced to 0.9 Kg N m^{-3} d^{-1} (Figure 2.10) and NRE of 35 % (Figure 2.13). These values obtained at low temperature contrasted with the 2.3 - 6.1 Kg N m^{-3} d^{-1} (NRR) 80 – 100 % (NRE) recorded at 30 ° C during stable operation. These results, where therefore, a confirmation that the optimum temperature for the Anammox process was around 30 ° C depending on the bioreactor (Rodriguez-Sanchez et al., 2016; Li and Sung

2015; Langone et al., 2014). Fernandez et al., (2012) reported a similar reduction in Anammox activity at low temperature when a breakdown of the temperature controller in a lab-scale SBR inoculated with Anammox bacteria biofilm coincided with a decrease in process efficiency. Morales et al., (2015) recorded a 16% loss in Anammox bacterial as temperature fell to 15 °C; and a temperature below 20 ^{o} C reduced the ability of Anammox bacteria to remove nitrogen in a hybrid SBR-MBBR bioreactors (Laureni et al., 2016). These results suggest that the reduction in NRE observed in EBBR-Anammox process was caused by temperature decrease (23 ^{o} C) as was the case in the other bioreactors reported.

In comparison to the operational periods reported for continuous Anammox WWT in using lab-scale bioreactors, the EBBR-Anammox system operated for a longer period (560 days) than some other Anammox bioreactors (Figure 2.14). For example, a MBBR study ended after 240 days (Laureni et al., 2016); while a SBR Anammox investigation lasted 155 days before the Anammox bacteria were outcompeted by heterotrophic denitrifying bacteria (val del Rio et al., 2018). Chen, et al., (2016) reported a stable Anammox operation for 91 days in an ABR; and a lab partial nitrification-Anammox reactor had a 40-day operation (Ma et al., 2015). The EBBR system has shown potential for Anammox WWT; more studies are required to achieve improved performance for a longer period (Figure 2.8).

Fluctuations in DO, NO_2-N and temperature caused the NRE of the EBBR-Anammox process to fall below 50 % (Figure 2.11 - 2.12). Despite these challenges, which is common even in established Anammox systems, the EBBR achieved a maximum NRE of 80 - 100 % and NRR of 6.1 Kg N m^{-3} d^{-1} (Figure 2.10 & 2.11). A 100 % NRE were achieved by the EBBR-Anammox process several times during the investigation e.g., day 77 (Figure 2.7), day 196 (Figure 2.) and on day 305 (Figure 2.). during which the concentrations of NH_3-N concentration and NO_2-N removed from the EBBR influent were > 300 and 400 mg L^{-1} respectively. Xu et al., (2017) and Van Haandel and Van der Lubble, (2007) described a high strength ammonia wastewater as one that contains 50 – 500 mg L^{-1} of ammonia. The removal of up to 80 – 100 % with < 4. 0 % NO_3-N produced is a further

indication that the EBBR-Anammox system has the capacity to treat wastewater containing e.g., 700 mg L^{-1} total inlet nitrogen. This evidence suggest that the Anammox process was established in the EBBR, with an important implication for the Anammox wastewater treatment. The EBBR-Anammox process was effective at removing nitrogenous pollutants (NH_3-N and NO_2-N) from synthetic wastewater; and therefore, capable of meeting wastewater regulatory standards, aimed at reducing the adverse effects caused by a discharge of nitrogen rich wastewater into receiving natural waters bodies.

2.5 Conclusion

The data collected from this study do suggest that the single Anammox (SA) wastewater (containing NH_3-N and NO_2-N) treatment process was established in the lab scale EBBR. The development of brick red granules and biofilm consistent with Anammox bacteria rich biofilm on the ABDite® growth medium support this conclusion. There also was increase in the expanded bed heights as more biomass attached to the growth support particles with simultaneous HN_3-N and NO_2-N removal, limited NO_3-N and sustained gas production; the main signs of the Anammox wastewater treatment process. In addition, was the 6.1 Kg N m^{-3} d^{-1} NRR from a NLR of 9.8 Kg N m^{-3} d^{-1} under the following conditions: under hydraulic retention time HRT (0.1 d) and pH (8.3) at 30 º C with 14 to100 % NRE. With these results obtained it is reasonable to suggest that the EBBR technology could make a positive contribution to future wastewater treatment using the Anammox process. The EBBR-Anammox study encountered some bioreactor challenges, but the system quickly recovered full operation from each mishap.

CHAPTER THREE

Optimization of the EBBR-Anammox Process

3.0 Research question

What EBBR operating conditions (pH and temperature) condition will yield the optimum nitrogen removal rate from the EBBR-Anammox optimization investigation?

Sub questions

1. What are the effects pH and temperature on nitrogen removal rate (NRR) of the Anammox process?
2. What pH and temperature yielded the optimum NRR in the EBBR-Anammox process optimization investigation?
3. How did pH and temperature alone affect the EBBR-Anammox optimized NRR
4. How did EBBR-Anammox optimized NRR compare with other Anammox technologies

3.1 Introduction

3.1.1. Optimization of the lab scale EBBR-Anammox process conditions

The Anammox process outputs e.g., ammonia and nitrite concentrations removed from the influent and the nitrogen removal rate (NRR) are determined by the input variables such as pH, temperature (Cho et al., 2020; Mpofu et al., 2020; Qasim, 2017; Qizhen et al., 2016; Langone et al., 2014). Details of the factors that affect the Anammox process have been discussed previously in Chapter 1(Section 1.4.3.5).

Temperature has great impact on Anammox wastewater treatment in engineered systems (Table 3.1). The operation of the Anammox process at lower temperature (e.g., below 10°C) and very high temperatures (e.g., 45°C) usually results in decrease in Anammox bacterial activity, low biomass yield and reduced NRR (Liu et al., 2019; Isanta, et al., 2015; Suneethi et al., 2014).

Anammox wastewater treatment studies have reported that high nitrogen removal rates (NNR) are achieved at temperatures between 25 - 40 °C (Rodriguez-Sanchez et al., 2016; Kartal et al., 2007a); with 30 ^{o} C being the optimum (Lotti et al., 2015; Jetten et al., 2001). A SBR Anammox process obtained a 400 mg N L^{-1} d^{-1} NRR at pH 7.5 and 29 ^{o} C; but NRR decreased by 55 % (to 46 mg N L^{-1} d^{-1}) at 12.5 ^{o}C and the same pH (Laureni et al., 2015). Isanta et al., (2015) reported that an increase in temperature from 30 °C to 45 °C led to an irreversible loss of Anammox bacterial activity due to cell lysis; and when the temperature was decreased to 15 °C, nitrite accumulated in the system and inhibited the Anammox process (Isanta, et al., 2015).

There was > 62 % NRR reduction from 0.04 kg N m^{-3} d^{-1} to 0.015 kg N m^{-3} d^{-1} temperature decreased from 20 and 10 °C in a moving bed biofilm reactor (MBBR) investigation by Gilbert et al., (2014).

Table 3.1: Examples of Optimization temperature, pH and NRR of Anammox bioreactors. **Key:** ***SBR** - sequencing batch reactor ***AnMBR** - Anaerobic membrane biofilm reactor ***UBR**- Up-flow biofilm reactor ***UASB** - Up-flow anaerobic sludge blanket.

Reactor	**Volume (L)**	**pH**	**Temperature (° C)**	**Nitrogen removal rate (kg N L^{-1} d^{-1})**	**References**
SBR	-	7.5 - 8.0	30	0.0002	Arrojo et al., 2008
SBR	8.0	7.8	30	0.84	Daehee et al., 2019
SBR	10.0	7.5 - 8.5	35 - 46	0.25	Isanta, et al., 2015
SBR	12.0	7.5	29	0.0004	Laureni et al., 2015
SBR	1200	8.0 - 8.1	29 - 30	0.0006	Wang et al., 2016
AnMBR	15	6.0 - 8.0	32 - 35	0.02- 5	Suneethi and Joseph (2011)
UBR	1.25	7.86	37	0.22 – 0. 35	Cho et al., 2011
UASB	2.5	7.9	28	2.28	He et al., 2018
UASB	8.0	-	30	5.72	Ma et al., 2013

To achieve a high NRR (Table 3.1), the Anammox process is mostly operated at pH between 7.5 - 8 .5 (Suneethi et al., 2014; Arrojo et al., 2008). Rodriguez-Sanchez et al., (2014) found that a pH > 8.2 enhanced partial nitritation of

ammonia by ammonia oxidizing bacteria (AOB) resulting in an increase in nitrite-nitrogen (NO_2-N) concentration, which inhibited Anammox bacterial activity.

The optimization process is even more important when investigating a new system e.g., the EBBR-Anammox system for the first time. As a result, it is a necessity that the parameters are optimized to determine the highest rate of nitrogen removal and the overall efficiency of the Anammox process of any given bioreactor under those conditions (Ali and Okabi, 2015; Calderon et al., 2012)

3.1.2 The choice of optimization parameters

Temperature, and pH are reported to have the most profound impact on the activity of Anammox organisms (Cho et al., 2020; Pooja et al., 2020). Therefore, these two factors have been chosen as the focus for the EBBR-Anammox process optimization investigation. Even though the Anammox process could be conducted at pH below 6 and temperature above 35 °C (Isanta et al., 2015; Laureni et al., 2015; Lotti et al., 2014), despite variations in reactor configuration and design, most Anammox process optimizations have been carried out at pH 7.5 – 8.3 and temperatures between 25 - 30 °C (Table 3.1). These pH and temperature conditions are considered the ideal regimes to achieve maximum nitrogen removal rates (Daehee et al., 2019; Langone et al., 2014).

3.1.3 Aim

The aim of the research in this chapter (chapter three) is to optimize the EBBR-Anammox process to determine the combination of temperature and pH condition that will yield the highest nitrogen removal rate (NRR), which is dependent on the NH_3-N and NO_2-N removed as N_2 gas.

The objectives to achieve the aim were:

1) Conduct the Anammox-EBBR process investigations at different sets of temperature (28 to 30 ºC) and pH (7.7 – 8.3) combinations and determination of wastewater ammonia nitrogen (NH_3-N) and nitrite nitrogen (NO_2-N) concentrations removed; the nitrogen removal rates.

2) Statistical analysis of the optimization data to determine the optimal pH and temperature; and evaluation of the effect of pH at constant temperature and vice versa on nitrogen removal rate (NRR) of EBBR-Anammox process.

3) Comparison of effect of pH and temperature on EBBR-Anammox optimization NRR; and with the NRR from other lab scale Anammox bioreactors.

3.2 Methods

3.2.1. Investigation of the Anammox- EBBR process at different pH temperature sets to determine NRR

The EBBR-Anammox process optimization will be conducted using the established optimum pH (7.7 – 8.3) and temperature (28 to 30 ºC) ranges published in literature (Isanta et al., 2015; Lotti et al., 2015; Yang et al., 2011; Jetten et al., 2001); and followed by statistical analysis.

3.2.1.1 Set-up of the optimization experiments

The EBBR-Anammox optimization investigation was conducted after the lab scale bioreactor had been running in continuous operation mode at 30 ˚C and pH, 8.3 for at least 6 months. The optimization investigation involved 6 different pH and temperature combinations (PTC) - pH8.3, T30ºC; pH8.2, T30 ºC; pH8.1, T30 ºC; pH8.3, T28 ºC; pH8.2, T28 ºC and pH7.7, T30 ºC, replicated 7 times (described as runs) for each set.

The pH range investigated was between 8.3 and 7.7; and temperature, 30 ° C and 28 ° C. These conditions were chosen because they fell within the idea pH and temperature that gave the highest nitrogen removal rates (NRR) in published Anammox wastewater treatment investigations. The highest and lowest temperature investigated were 30 ° C and 28 ° C; and pH, 8.3 and 7.7 respectively.

3.2.1.2 Sampling the EBBR influent and effluent to determine NH_3-N and NO_2-N concentrations at steady state

The procedure involved first sampling the EBBR influent and effluent at 30 minutes intervals, to determine when the system was operating at a steady state.

Operation at steady state was determined when the ammonia nitrogen (NH_3-N) and nitrite nitrogen (NO_2-N) in outlet attained a constant concentration after 2 to 3 samplings. Following the establishment of steady state operation in the bed, the

input variables (temperature and pH) were changed to the next set of conditions. The sampling of the inlet and outlet wastewater were repeated out at 30 minutes intervals until the reactor achieved a steady state again.

When run 1, which involved investigating the Anammox process using the 6 different sets of pH - temperature combinations (pH8.3, T30ºC; pH8.2, T30 ºC; pH8.1, T30 ºC; pH8.3, T28 ºC; pH8.2, T28 ºC and pH7.7, T30 ºC) were completed; then run 2 experiments started. This regime was successfully repeated 7 times (n= 7) for each of the pH - temperature set listed above.

The difference in inlet and outlet ammonia nitrogen and nitrite nitrogen concentrations (nitrogen reduced to molecular nitrogen gas and lost from system) was used to determine the nitrogen removal rate (NRR) of the Anammox process in the expanded bed using the equation 2.5.

The concentration of total inlet nitrogen [NH_3-N] and [NO_2-N] removed by the Anammox biomass (kg N m^{-3} $_{EBBR}$) x D (d^{-1})

NRR (Kg N m^{-3} d^{-1}) = D x ([Total inlet N] – [Total Outlet N])

3.2.2. Statistical Analysis of NRR output

All the statistical analysis in this work were performed using IBM® SPSS® Statistics version 25.0. The data analyses carried out were normality test and a parametric test (one-way ANOVA) to determine the effects of different temperatures and pH combinations on the nitrogen removal rate. A Post hoc test was performed to determine which set of pH and temperature process conditions produced the nitrogen removal rate that was statically different from the other experimental conditions.

3.3. Results

3.3.1 EBBR – Anammox Optimization NRR conducted at different pH - temperature combinations

3.3.1.1 EBBR-Anammox optimized nitrogen removal rate at steady state

The optimization experiment to determine the process conditions that will give the highest rate of nitrogen removal involved a total of 42 experiments (Table 3.2); consisting of 7 runs (n=7), completed using 6 pH and 2 temperature combinations (PTC).

Table 3.2: Optimization nitrogen removal rates (Kg N m^{-3} $d^{-1)}$ at different pH and temperature combinations. A total of 42 investigation using six pH-temperature combinations and seven runs were carried for the NRR optimization process. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

Run	pH8.3, T30	pH8.2, T30	pH8.1, T30	pH8.3, T28	pH8.2, T28	pH7.7, T30
1	0.97 ± 0.04	1.39 ± 0.04	0.8 ± 0.23	0.92 ± 0.02	2.71 ± 0.01	0.34 ± 0.09
2	0.92 ± 0.01	1.34 ± 0.01	1.12 ± 0.00	0.89 ± 0.02	2.72 ± 0.07	0.47 ± 0.00
3	0.9 ± 0.00	1.36 ± 0.43	1.12 ± 0.02	0.92 ± 0.35	2.82 ± 0.00	0.47 ± 0.05
4	0.9 ± 0.12	0.75 ± 0.62	1.15 ± 0.42	1.41 ± 0.25	2.82 ± 1.19	0.404 ± 0.07
5	1.07 ± 0.02	1.63 ± 0.23	1.74 ± 0.38	1.05 ± 0.25	1.14 ± 0.35	0.51 ± 0.01
6	1.04 ± 0.04	1.3 ± 0.05	1.2 ± 0.21	1.41 ± 0.80	1.64 ± 0.10	0.52 ± 0.01
7	0.99 ± 0.01	1.37 ± 0.01	0.91 ± 0.08	2.69 ± 0.02	2.89 ± 0.02	0.51 ± 0.12

The NRR obtained at the different pH - temperature combinations indicated that the bioreactor achieved a steady state operation between 1.5 - 2.0 hours, which

was approximately 4 - 5 samplings after each change in the operating conditions (Figures 3.2 – 2.5, chosen as representative of all the runs) as the system needed time to adjust before a new steady state was reached for the process condition under investigation; and hence, none of the subsequent NRR data obtained were the same (Table 3.2).

The optimization data revealed differences in NRR when the pH - temperature process conditions were changed whether from a higher PTC set (i.e., pH 8.3 and 30 ºC) to a lower set throughout the investigations. In run 1, a change from pH 8.3, T 30ºC to pH 8.2, T 30 ºC, and then, to pH8.1, T30 ºC saw NRR increased from 0.97 ± 0.01 to 1. 39± 0.04 (43%) and then decreased by 42 % to 0.80 ± 0.23 Kg N m^{-3} d^{-1} respectively. A similar result pattern was observed in the final run (7), where NNR decreased by 7 % from 2.89 ± 0.02 Kg N m^{-3} d^{-1} (pH8.3, T28 ºC) to 2.69 ± 0.02 Kg N m^{-3} d^{-1} (pH8.2, T28 ºC); with a further 82 % decrease to 0.51 ± 0.12 Kg N m^{-3} d^{-1} at pH7.7, T30 ºC. These variations in nitrogen removal rates suggested that each PTC affected the EBBR-Anammox system differently; therefore, nitrogen is removed at different rates.

The observed duration at which steady state was attained appeared consistent for most of the optimization runs whether the change was from a low to a high temperature and pH or vice versa; The results also indicated that pH 8.2, 28 T ºC had the highest NRR (1.41 - 2.82 Kg N m^{-3} d^{-1}), while pH 7.7, T 30 ºC, achieved the lowest NRR (0. 34 – 0.52 Kg N m^{-3} d^{-1}) in all the runs (Figures 3.2 - 3.5). In run 1, the highest NRR obtained with pH 8.2, 28 T ºC were approximately 78 % more (Figure 3.2); 80 % in run 2 (Figure 3.3); for run 4 (Figure 3.4), it was 68 % and 79 % higher in run 7 (Figure 3.5) compared to NRR at pH 7.7, T 30 ºC. The steady state optimized NRR results suggests that the optimum pH for the EBBR-Anammox is above 7.7 and temperature below 30 ºC.

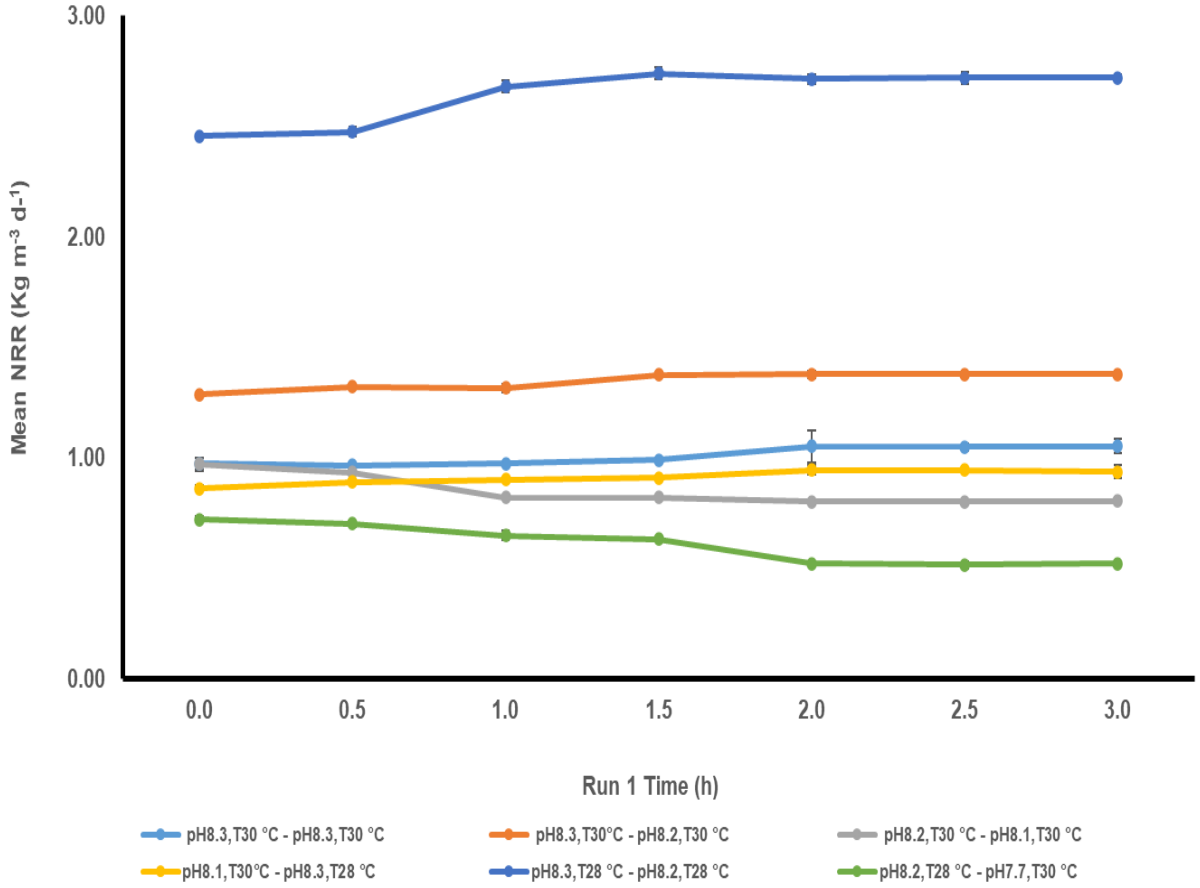

Figure 3.2 Run 1 steady state change nitrogen removal rate (NRR) at different pH-temperature combination **key**: **-** on the legend indicates a change from one set of pH/temperature to the next. The EBBR achieved steady state operation between 1.5 and 2.0 hours during the optimization investigation after a change in pH / temperature set. NRR obtained at PTC 8.2, T28 was approximately 78 % higher than NRR at PTC 7.7, T30. Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean.

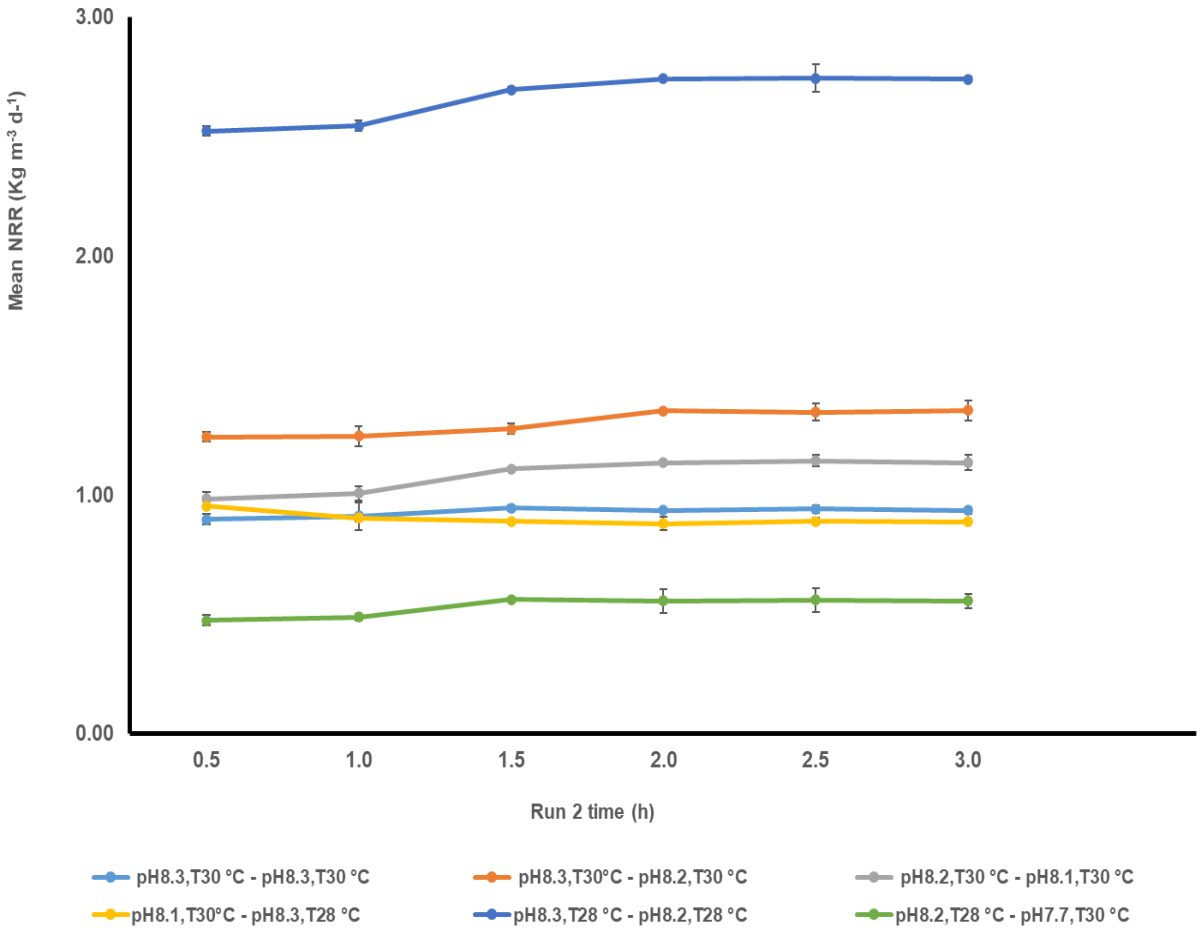

Figure 3.3 Run 2 steady state change nitrogen removal rate (NRR) at different pH-temperature combination **key**: **-** on the legend indicates a change from one set of pH/temperature to the next. The EBBR achieved steady state operation between 1.5 and 2.0 hours during the optimization investigation after a change in pH / temperature set. NRR obtained at PTC 8.2, T28 was approximately 80 % higher than NRR at PTC 7.7, T30. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

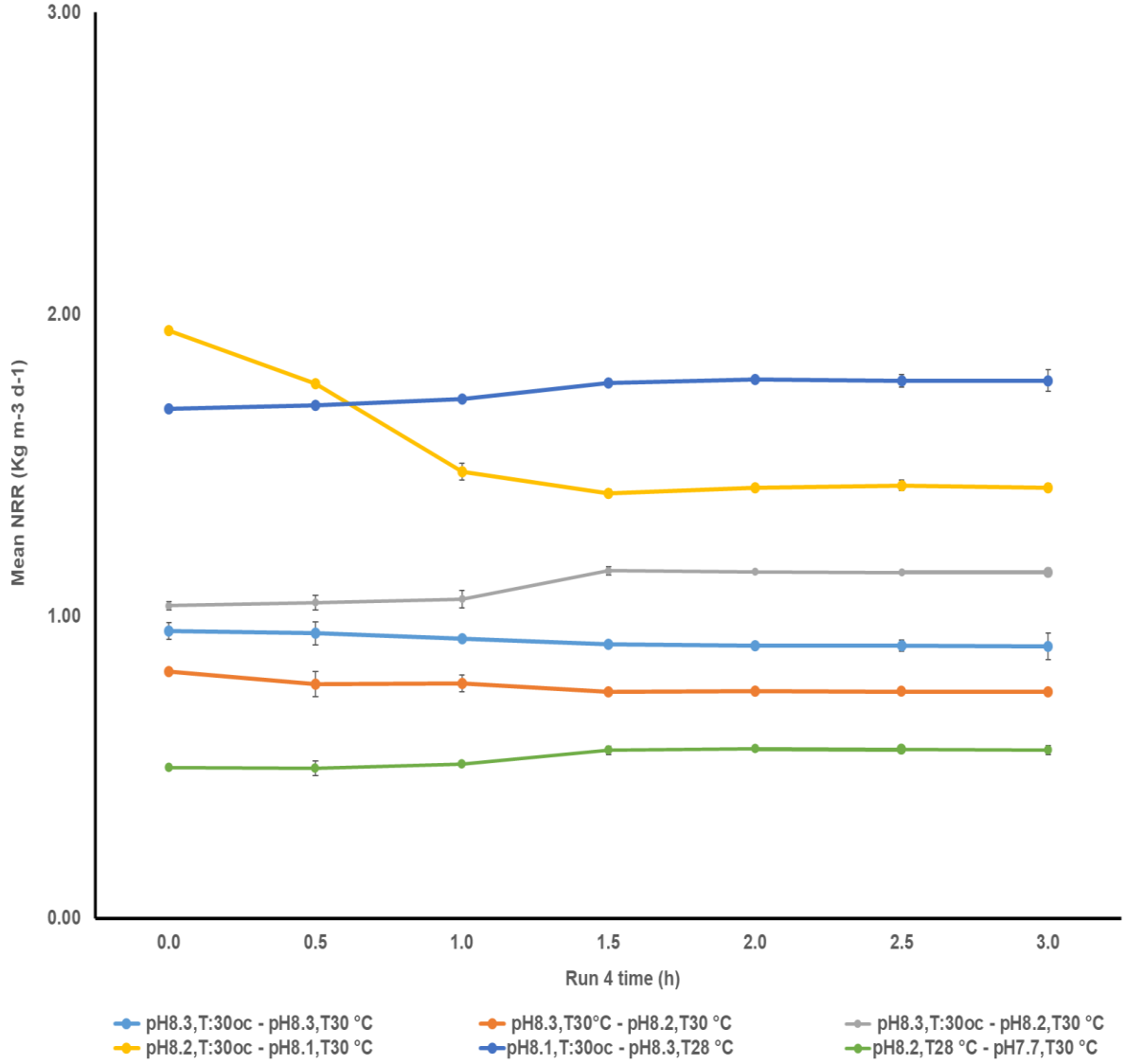

Figure 3.4: Run 4 steady state nitrogen removal rate (NRR) at different pH- temperature conditions during optimization process. **key**: **-** on the legend indicates a change from one set of pH/temperature to the next. The EBBR achieved steady state operation between 1.5 and 2.0 hours during the optimization investigation after a change in pH / temperature set, NRR obtained at PTC 8.2, T28 was approximately 68 % higher than NRR at PTC 7.7. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

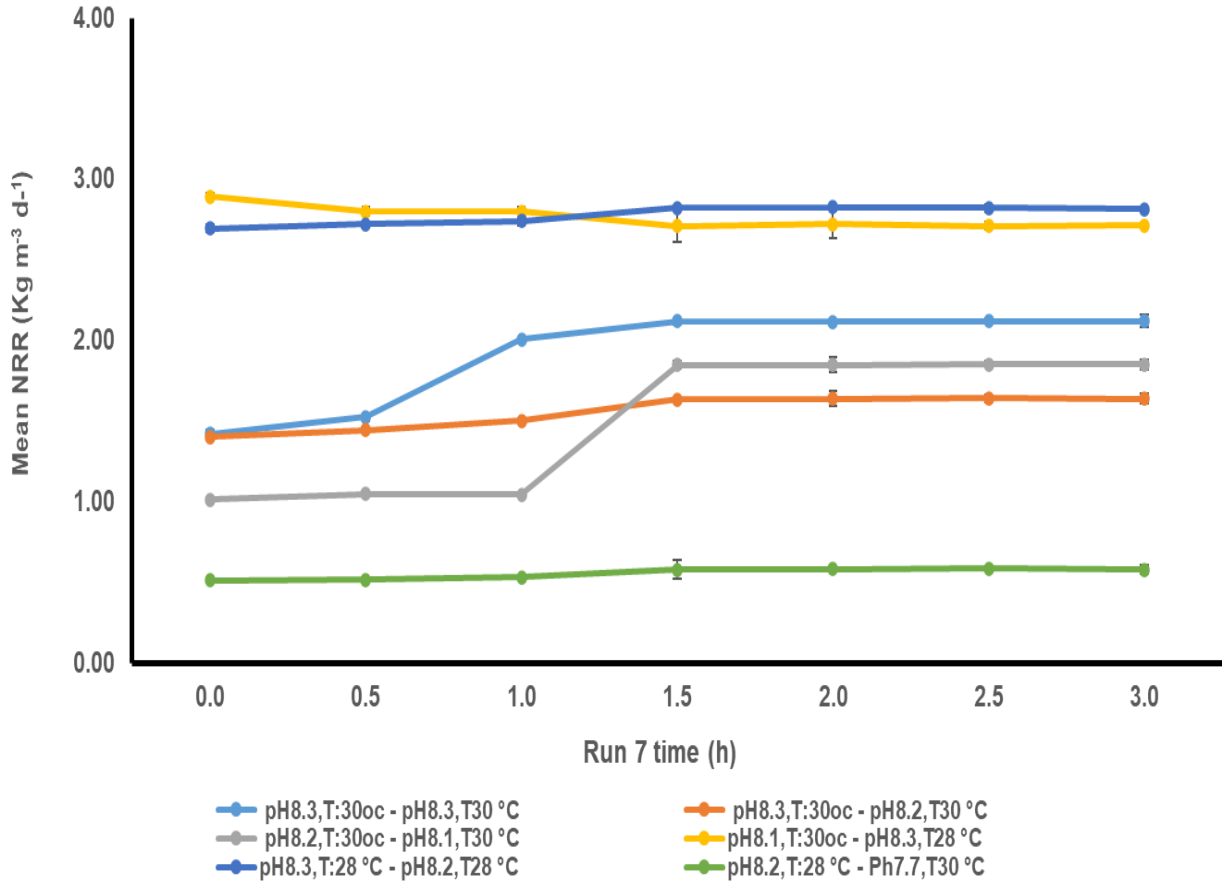

Figure 3.5: Run 7 steady state nitrogen removal rate (NRR) at different pH- temperature conditions during optimization process. **key**: **-** on the legend indicates a change from one set of pH/temperature to the next. The EBBR achieved steady state operation between 1.5 and 2.0 hours during the optimization investigation after a change in pH / temperature set, NRR obtained at PTC 8.2, T28 was approximately 79 % higher than NRR at PTC 7.7. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

3.3.1.2 Optimized mean nitrogen removal rates at different pH-temperature conditions

A marked differences was found in the mean rate at which nitrogen was removed from EBBR column obtained by conducting the Anammox process at different conditions during the optimization investigation (Figure 3.6).

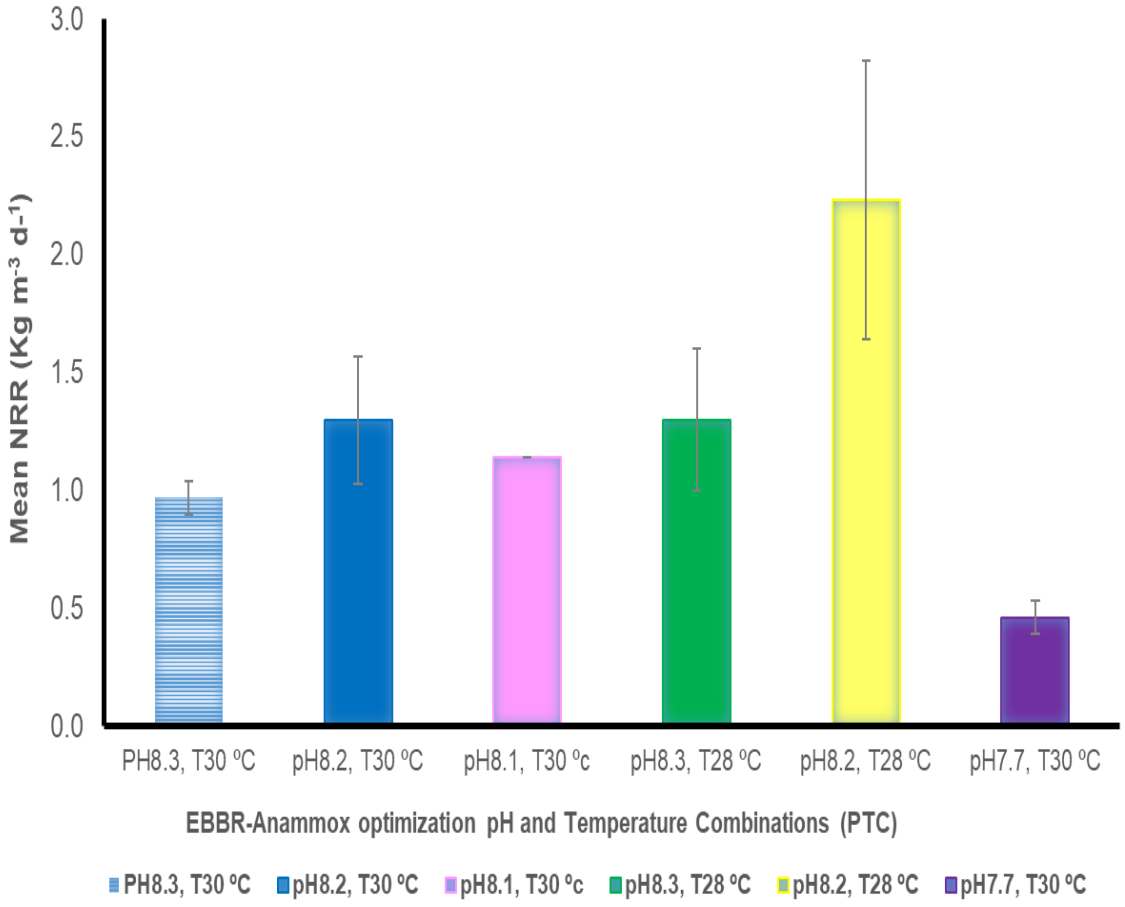

Figure 3.6: Optimization results of nitrogen removal rate at different temperature (°c) and pH combinations. **Key:** The pH8.2. T28 ºC test condition produced the highest mean NRR (2.23 ± 0.07 Kg N m^{-3} d^{-1}); and pH7.7, T30 ºC the lowest (0.46 ± 0.06 Kg N m^{-3} d^{-1}). (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

The lowest average NNR of 0.46 ± 0.06 kg N m^{-3} EBBR d^{-1} achieved was when the bioreactor was operated at pH 7.7, T 30 ºC; while the highest rate, 2.23 ± 0.07 kg N m^{-3} EBBR d^{-1} was recorded at pH 8.2, 28 ºC (Figure 3.6); this was around 5 – fold increase in NRR. The mean NRR at pH 8.2, T 28 ºC was over 79 % higher than the amount of nitrogen removed at PTC, pH 7.7, T 30 ºC. Additionally, it was found that the NRR was the same (1.3 Kg N m^{-3} EBBR d^{-1}), when the optimization experiment was conducted at pH8.3 T28 ºC. and pH8.2 T30 ºC respectively; this NRR was 41 % lower compared to the result (2.23 ± 0.07 kg N m^{-3} EBBR d^{-1}) obtained at pH 8.2, 28 ºC (Figure 3.6). The

results indicate that pH 8.2, T 28 ºC process condition, might be the optimum for the EBBR-Anammox system as the highest NRR consistently, has been achieved using this pH / temperature set.

3.3.1.3 Effect of pH on EBBR-Anammox NRR at constant temperature (30 º C)

A remarkable difference was found in the mean NRR obtained from the EBBR-Anammox process optimization experiment results when pH was changed, but temperature kept constant at 30 ºC (Figure 3.7); to evaluate the effect of change pH alone on the process . The NRR obtained using the four different pH values with a range 0.6 between (pH 8.3 – 7.7) them at 30 ºC, revealed 68 %, (pH 8.1- green); 84 % (pH 8.2 – purple) and 51 % (pH 8.3 – blue) increases in nitrogen removal compared to the 0.46 kg N m^{-3} EBBR d^{-1} NRR achieved at pH 7.7 in pink (Figure 3.7)

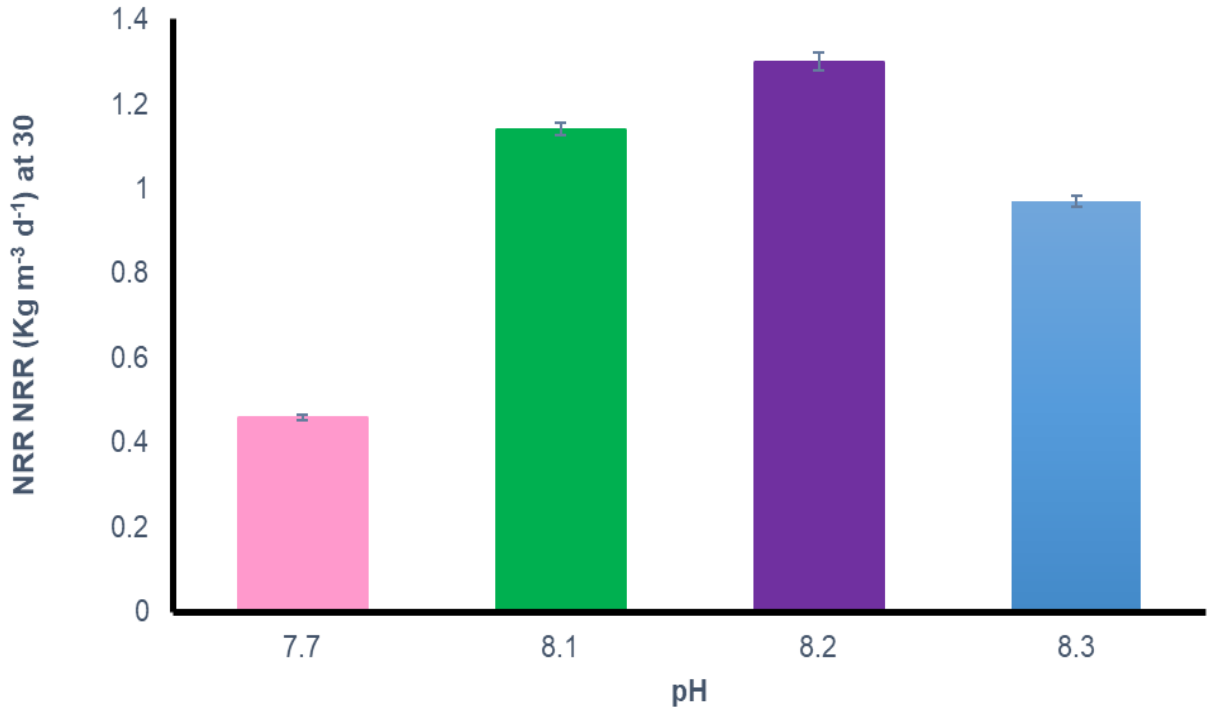

Figure 3.7: Effect of pH on Nitrogen removal rate at 30 °C. **Key:** Pink (pH 7.7); **Green**

(pH 8.1); **Purple** (pH 8.2); **Blue** (pH 8.3.) At 30 ° C NRR was 183 % higher at pH 8.2 than NRR at pH 7.7(Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

The comparison the mean NRR (Figure 3.6; Table 3.2) found that when the temperature was kept constant at 30 º C, the maximum NRR achieved were pH 8.2 (1.30 kg N m^{-3} EBBR d^{-1}), pH 8.1 (1.14 kg N m^{-3} EBBR d^{-1}); pH 8.3 (0.97 kg N m^{-3} EBBR d^{-1}) and (0.46 kg N m^{-3} EBBR d^{-1}) at pH 7.7 (Figure 3.7). The NRR results suggested that while a pH decrease or increase by a factor of 1.0 affected NRR; a by a 5-fold decrease in pH from 8.2 to 7.7 reduced the NRR by 65 %, indicating that the optimal pH of the EBBR-Anammox was above pH 8.0.

3.3.1.4 Effect of temperature on EBBR-Anammox NRR at constant pH

The effect of temperature on the EBBR-Anammox NRR at constant pH of 8.2, saw that a 2 º C decrease in temperature from 30 to 28 º C led to a 42 % increase in nitrogen removal rate, which was > 20 % per degree change (Table 3.3); and an increase from 28 to 30 led to a 25 % fall in NRR. The results indicated that 28 º C, is the EBBR-Anammox optimum temperature to achieve maximum NRR (Table 3.3).

Table 3.3: Effect of different temperatures on Nitrogen removal rate. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

Temperature (° C)	**28**	**30**	**% Difference**
(a) Nitrogen removal rate (Kg N m^{-3} d^{-1}); pH 8.2	2.23 ± 0.07	1.30 ± 0.27	41.7

(b) Nitrogen removal rate (Kg N m^{-3} d^{-1}); pH 8.3.	1.30 ± 0.59	0.97 ± 0.07	25

Furthermore, considering the effect of temperature separately on the EBBR-Anammox process at constant pH, the optimized NRR results indicated that Anammox activity was more efficient at 28 °C rather than at 30 °C. When the pH was maintained at 8.2, the highest NRR from the optimization experiments of 2.23 ± 0.70 Kg N m^{-3} d^{-1} was obtained at 28 °C, which is about 42 % higher compared to the 1.30 ± 0.27 Kg N m^{-3} d^{-1}, the highest NRR at 30 °C at the same pH condition. Again, with the pH kept at 8.3, a 2 °C reduction in temperature from 30 to 28 º C resulted in a 15 % rise in NRR from 0.97 Kg N m^{-3} d^{-1} to 1.14 ± 0.30 Kg N m^{-3} d^{-1} (Figure 3.4; Table 3.3).

3.3.2 Statistical analysis of EBBR-Anammox optimization data

The outcome of the statistical test (One way ANOVA) indicated that there was a significant difference in the mean nitrogen removal rates obtained at various test conditions ($p <= 0.0001$), which suggested that the changes made to the process conditions affected the activity of the Anammox bacteria differently (Figures 3.6 & 3.7; Table 3.3).

The Post hoc analysis compared the effect of pH and temperature conditions within the EBBR on NRR; and to confirm if the identified difference in the nitrogen removal rates were statistically different. The statistical analysis confirmed there was a significant difference in the optimized mean NRR achieved at the pH and combination sets investigated (Table 3.2).

The NRR at pH 8.2, T 28 was statistically significant ($p <= 0.0001$) in contrast to the NRRs at other experimental conditions. The lowest mean NRR (0.46 Kg N m^{-3} d^{-1}) produced at pH 7.7, T 30 was equally statistically different ($P <= 0.005$) from nitrogen removal rates obtained from the rest of others test conditions investigated; and was 79

% lower than the 2.23 ± 0.07 kg N m^{-3} EBBR d^{-1} NRR obtained at pH 8.2, T 28 (Figure 3.6). This finding has important implication for the EBBR-Anammox process; that is, for optimum NRR using the EBBR, the Anammox process should be conducted at pH 8.2, T 28; in addition, pH 7.7, T 30 has the worst negative effect on the EBBR, the Anammox NRR (Figure 3.6).

3.4 Discussion

Unfavourable pH and temperature conditions are the main factors that affect the nitrogen removal rate (NRR) of the Anammox process (Cho et al., 2020; Tomaszewski et al., 2017; Jung et al., 2007; Jetten et al., 2001).

The NRR results recorded from the EBBR-Anammox optimization experiments suggested that the chosen temperature and pH regimes were useful for the investigation; because 0.46 - 2.23 Kg N m^{-3} d^{-1} of inlet nitrogen was removed by conducting the Anammox process at pH (7.7 – 8.3) and temperature (28 – 30 ºC) combinations (Figures 3.2 - 3 .7; Table 3.2 & 3.3).

The EBBR-Anammox optimum NRR of 2.23 Kg N m^{-3} d^{-1} was achieved at pH 8.2, T 28 ^{o} C, which is within the optimum conditions (pH 7.8 - 8.3; and temperature (28 - 30 ^{o} C) reported in literature that have conducted the Anammox process using different technologies (Table 3.1). For example, Daehee et al., (2019) using a SBR obtained 0.84 Kg N m^{-3} d^{-1} NRR at pH 7.8 and 30 ^{o} C. He et al., (2018); optimized his UASB-Anammox process at pH 7.9 and 29 ^{o} C and achieved 2.28 Kg N m^{-3} d^{-1} NRR.

Anammox studies by Cho et al., (2020) and Langone et al., (2014) reported that changes in pH have a high impact on Anammox wastewater treatment process. Evaluation of the effect of 4 different pH values at 30 ^{o} C, on EBBR-Anammox NRR found that changes in pH had a profound influence on the rate nitrogen was removed (Figure 3.7). In the EBBR-Anammox investigation with ABDite, a pH change from 8.1-8.2 increased NRR by 14.0 %; and from pH 8.2 - 8.3, NRR fell by 25 %. Rodriguez-Sanchez et al., (2014), reported that the accumulation of nitrite at low pH in a bioreactor

adversely affects the Anammox process. Cao et al., (2013) in an investigation into the effects of pH on wastewater Anammox denitrification found that 250 mg L^{-1} NO_2-N accumulated at low pH (7.5) and 500 mg L^{-1} at a pH value of 8.5, causing reduction in NRR by inhibiting the Anammox bacterial activity. These reports suggest that the lowest NRR (0.46 Kg N m^{-3} d^{-1}) achieved in the EBBR-Anammox optimization investigation could have been caused by nitrite accumulation; in addition, the reports above highlight the importance of maintaining pH above 7.7 for maximum NRR using the EBBR-Anammox system.

Temperature changes also affect the NRR of the Anammox process as reported by Hoekstra et al., (2018); and can trigger the accumulation of inhibitory concentrations of nitrite and ammonia within an Anammox systems (Tomaszewski et al., 2017). The EBBR-Anammox process achieved a 42 % increase in NRR, at constant pH of 8.2 with a 2 °C decrease in temperature (30 - 28 º C); and when the temperature was increased by the same margin from (30 - 28 º C), NRR decreased by 5 % (Table 3.3). Laureni et al., (2015), using a SBR reported a 55 % decrease in NRR when temperature was change from 29 ºC to 12.5 º C. Lotti et al., (2014) investigated the effect of temperature on the Anammox in a MBB, with about 82 % decrease in NRR from 0.023 at 30 º C to 0.004 Kg N m^{-3} d^{-1} at 10 º C. The range of temperature change (2 °C), which affected NRR in the EBBR was shorter compared to the other Anammox studies; the variation could be due to differences in the sizes amongst other factors specific to each bioreactor. Data from the EBBR and other Anammox technologies agree that temperature changes have profound influence on the Anammox WWT process.

The activity of Anammox bacteria has a wide range of optimum conditions of pH and temperature when different lab scale bioreactors are used (Table 3.1). Variations in NRR caused by changing temperature and pH regimes is evident from the EBBR-Anammox optimization NRR results (0.46 - 2.23 Kg N m^{-3} d^{-1}).

In comparison with other lab scale Anammox technologies the 0.7 L EBBR-Anammox process was optimized at pH 8.2, 28 °C; and achieved 2.23 Kg N m^{-3} d^{-1} NRR. Daehee et al., (2019) using an 8 L SBR at pH (7.8, 30 °C) obtained a NRR of 0.84 kg N m^{-3} d^{-1}.

The NRR difference between the approximately 11- fold larger sized SBR and EBBR was about 265 %. The NRR of the EBBR was > 11, 000 % more compared to the 0.0002 Kg N m^{-3} d^{-1} from a SBR investigation by Arrojo et al., (2008). The EBBR NRR also varied with the NRR from two Upflow anaerobic sludge blankets (UASB). He et al., (2018) achieved 2.28 Kg N m^{-3} d^{-1} NRR with pH7.9 at 28 °C; and for Ma et al., (2015), the NRR was 5.72 kg N m^{-3} d^{-1} at 30 °C. The 2.5 L and 8 L UASB had 102 % and 349 % higher NRR compared to that of the EBBR respectively. With a 1.25 L Upflow biofilm reactor (UBR) operated at pH 7.86 and 37 °C, Cho et al (2011) reported achieving a 0.35 Kg N m^{-3} d^{-1} NRR, with 637 % difference from the EBBR - 2.23 Kg N m^{-3} d^{-1} result recorded at pH 8.2, T 28.

The NRR from the Anammox process studies using different bioreactors discussed above and that of EBBR (2.23 ± 0.70 Kg N m^{-3} d^{-1}) suggest that NRR of the Anammox process is affected by pH and temperature process conditions alongside other factors such as reactor type and size (Table 3.1); and Anammox bacteria. The optimized nitrogen removal rate obtained in this study has contributed evidence that temperature and pH affect nitrogen removal rate of the Anammox process.

3.5 Conclusion

The optimization investigation results confirmed that changes in temperature (28 – 30 °C) and pH (pH - 7.7 - 8.3) regimes affected the nitrogen removal rate (0.46 to 2.23 Kg N m^{-3} d^{-1}) of the EBBR-Anammox process (Tables 3.2 & 3.3; Figures 3.6 & 3.7). The results also demonstrated that the maximum nitrogen removal rate (2.23 Kg N m^{-3} d^{-1}) was recorded at 28 °C and pH 8.2; and is therefore, the optimum operating condition for the lab based EBBR-Anammox process; this result constituted a contribution made to knowledge from this research.

The implication of these results is that in future Anammox wastewater treatment, the EBBR technologies could make a useful contribution. In addition, the optimum process conditions established in the current study, i.e., a temperature of 28 °C and pH 8.2 alongside the characteristics of the wastewater and the species of Anammox bacteria (e.g., Candidatus *Brocadia*) used might be given due consideration. Further investigation using a wider range of pH and temperature conditions is needed to increase the robustness of the optimization data.

CHAPTER FOUR

Examination of the EBBR ABDite® biomass support medium for Anammox biofilm formation

4.0 Research question

Could Anammox biofilm be established on the lab scale EBBR ABDite® biomass support medium?

Sub questions

1. What is the physical evidence (brick red granules and cauliflowerlike biofilm) for the development of Anammox bacteria biofilm /bioparticles in EBBR expanded bed column?
2. What type of bacteria-like cells developed on the EBBR-Anammox biomass medium?
3. How did the EBBR-Anammox biofilm development data compare with other Anammox technologies.
4. What is the implication of the development of Anammox biofilm on the EBBR ABDite medium on Anammox wastewater treatment process?

4.1 Introduction

4.1.1 Formation of Anammox bacterial biofilms on support medium

The anaerobic ammonia oxidation (Anammox) reaction is mediated by one or more members of the five genera (*Candidatus* Brocadia, Candidatus *Kuenenia, Candidatus Anammoxoglobus, Candidatus Jettenia* and *Candidatus Scalindua)* of bacteria that belong to a single phylum, Planctomycetes (Wang et al., 2015; Hendrickx et al., 2014; Sonthiphand and Neufeld, 2013; Kartal et al., 2011).

The growth of active Anammox bacteria within a wastewater treatment (WWT) system is characterized by the formation of brick red granules and biofilm contributed by the various microbial groups; these bacteria play specific roles in the process; and has irregular cauliflower structure (Ni and Zhang, 2013; Campos et al., 2006). The unique red colour of the Anammox bacteria is due to the presence of the heme c group, which is a part of the cytochrome c protein required as an electron carrier for nitrite reduction (Lage et al., 2019; Lu et al., 2018; Ni and Zhang, 2013). While the presence of a bright red Anammox granules (1 - 3 mm in size) confirms healthy biofilm, pale red granules indicate a decrease in the heme c protein. On the hand, black granules caused by the accumulation of metal precipitate e.g., zinc (11) toxic to Anammox bacteria over a long period of time, with a concomitant decrease in activity, followed by cell death (Xu et al., 2021; Ni et al., 2018; 2018; Driessen et al., 2015; Ali et al., 2013; Ni and Zhang, 2013).

In a bioreactor, Anammox bacteria can grow in many forms; these include flocs, which are small cloudy collection of cell suspended in water; granules, these are suspended compact aggregates of bacteria biomass; biofilms – microbial mass attached to a solid surface and bioparticles, which consists of solid support materials that are entirely covered by a layer of biofilm (Manonmani and Joseph, 2018; Wang and Gao, 2018; Lotti et al., 2014; Weber et al., 2008).

To increase nitrogen removal efficiency (NRE), Anammox bacteria have been developed on several biomass support materials including sponge, volcanic rock, and charcoal (Niederdorfer et al., 2021; Lu et al., 2018). The current study will attempt to grow Anammox bacteria biomass on the EBBR ABDite® particles that has extremely small pores (microporous).

In Anammox bioreactors, the initial stage of the growth of these bacteria is marked by the appearance of flocculates (flocs) that later develop into granules used by industrial Anammox reactors or as attached biofilm on a growth support medium (Almstrand, et al., 2014; Botchkova et al., 2014). Like other bacterial species, Anammox bacteria have a strong ability for biofilm formation as a prerequisite for the growth of stable communities and a necessity for metabolic activities (Niederdorfer et al., 2021; Botchkova, et al., 2015). Biofilm formation on growth support materials e.g., volcanic rock and zeolite (microporous solids) particles is reported to occur in three main stages (Niederdorfer et al., 2021; Lu et al., 2018; Fernandez et al., 2008).

The first stage is characterized by bacterial attachment and colonization of the medium surface. This initial stage, which is controlled by weak electrostatic, van der Waals and hydrophilic interaction forces occurs within 0 to 36 h after the seed granules are added to the bioreactor.

The second stage is marked by the formation of microcolonies at the consolidation or succession phase after about 14 days (Niederdorfer et al., 2021; Fernandez et al., 2008). Finally, the biofilm reaches maturation and detachment stage. At maturation, the thick and extensive extracellular polymeric substances (EPS) for cell adhesion establishes the microcolonies (Crouzet et al., 2014; Fernandez et al., 2008). The length of time required to develop mature biofilm differs depending on the bioreactor and growth support medium used. Within 56 days, Anammox biofilm developed on glass slide biomass support reached the final stage in an upflow anaerobic sludge bed (UASB) reactor that treated brewery wastewater (Fernandez et al., 2008).

A mature Anammox bacteria biofilm has a complex structure that have been described as either mushroom, cauliflower, or tulip, which is supported by dense EPS. The biofilms

also contain channels for the liquid to move, allowing a wide range of metabolic abilities of the Anammox bacteria, which includes nitrogen removal from wastewater (Miller et al., 2012).

4.1.2 Microscopy techniques for Anammox biofilm examination

The surface structure of the Anammox biofilms developed on growth support medium can be investigated using different microscopy techniques including phase contrast microscope (PCM), scanning electron microscopes (SEM) and scanning confocal microscopes (SCM) amongst others.

4.1.2.1 Phase contrast microscopy

Phase-contrast microscopy is an important biological tool for the visualisation of living cell components and examination of biofilm formed on solid surfaces amongst other uses (Dingding et al., 2021). The advantage of using the PCM technique is that it is an easy, non-destructive approach to study Anammox biofilms without the need to stain the specimen. In contrast, the disadvantage of PCM is that it has lower spatial resolution (e.g., 50 nm); resulting in a low-quality image of the object examined (Vartiainen et al., 2014; Obara et al., 2013; Maurer et al., 2008). Improvement in the quality of images obtained with PCM requires the use of other microscopic techniques that offer higher resolution e.g., the scanning electron microscope (SEM).

4.1.3.2 Scanning electron microscopy

The scanning electron microscopy (SEM) is useful for observing the diversity and morphologies of biofilms formed by different microbial communities within a biological system (Table 4.1). SEM can produce good quality surface images of biofilm with details of the morphology of the microbes present in an Anammox wastewater treatment

bioreactor (Sehar and Naz, 2016). SEM has been used to examine the structure of biofilms and bacteria cell types developed in Anammox bioreactors (Table 4.1).

Table 4.1 Description of SEM analysed Anammox bacteria biofilm developed in Anammox bioreactors. Key: membrane reactor (MBR); sequencing batch reactor (SBR); moving bed biofilm reactor (MBBR; activated sludge reactor (ASR), micro fuel cell (MFC); anaerobic baffled biofilm reactor (ABBR); Up-flow anaerobic Sludge Bed (UASB).

An advantage of the SEM application is the ability to produce a 3 – dimensional (3-D) images of objects with high resolution (Swapp, 2017; Inkson, 2016; Denk and Horstmann, 2004). On the contrary, SEM sample preparation is more demanding in terms of specialist knowledge, skill and is time consuming. Other challenges include that SEM samples must be solid, dry and images can only be seen in black and white; in addition, strong insulator samples must be coated with gold or carbon to reduce the

Anammox Bioreactor	**Shape of Anammox biofilm**	**Anammox bacteria - like Cells**	**Reference**
MBR	Cauliflower	Cocci	Wang et al., (2018)
SBR	Cauliflower	Rod, filamentous and cocci -shaped,	Xu et al., (2018); Ni et al., (2018)
MBBR	Cauliflower	Cluster of cocci	Zekker et al., (2012)
MFC	-	Clusters of cocci	Domenico et al., (2015)
ABBR	Cauliflower	Rod, cocci, filamentous	Wang et al., (2019)
Anammox bioreactors	Broccoli	-	Ma et al., (2020)
UASB	Mushroom and tulip	-	Fernandez et al., (2008).

formation of artefacts, which can distort the image. The major drawback of using SEM for sample analysis, is that the desiccation process can damage the sample structure or alter the characteristics of the biofilm, making the interpretation of results more difficult (Mathias, 2020).

The usefulness of SEM technique has led to its successful applications to detect the morphology of the diverse microorganisms on biofilms from Anammox wastewater treatment systems (Sonthiphand and Neufeld, 2013; Wang et al., 2015). SEM images from previous Anammox studies have revealed the morphology of the mixed bacterial cells developed on biomass support martials from different bioreactor; the Anammox bacteria cell types that have been described include rods and cocci amongst others (Table 4.1). While rod bacteria developed in a SBR (Xu et al., 2018), cluster of cocci bacteria were enriched in MFC (Domenico et al., 2015), a MBR reported by Wang et al., (2017) and in a MBBR (Zekker et al., 2012) respectively.

The size of cocci bacteria found on Anammox biofilm ranges between 1.0 - 4.5 mm; although most are 2.0 - 5 mm (Yang et al., 2021; Lu et al., 2018; Feng et al., 2015; Xiong et al., 2013; Ni et al., 2010). The morphology of Anammox biofilm and bioparticles are however determined by the biomass support medium used for their development (Figures 4.1 and 4.3). Mature Anammox biofilm come in different shapes, which include mushroom architecture contributed by individual microbial communities that make the structures; this shape is developed under biologically controlled conditions (Huang et al., 2019; Suarez et al., 2019; Wang et al., 2017; Lopez-Palau et al., 2011). Other shapes of Anammox biofilm that have reported are broccoli (Ma et al., 2020) and cauliflower (Wang et al., 2018; Liu et al., 2017).

4.1.4 Aim

The aim of the research in this chapter (chapter four) is to investigate the Anammox bacteria biofilm and bioparticles development on the EBBR ABDite® growth support medium using microscopic methods and analyse the surface morphology of the biofilms and compare with biofilms from other Anammox bioreactors published in literature.

The objectives to achieve this aim were:

1. Sampling and visual inspection of EBBR ABDite® medium for biofilm/bioparticle formation.

2. Microscopic ((Phase contrast microscope (PCM) and scanning electron microscope (SEM) examination of ABDite® medium for formation of biofilm and bioparticles.

3. Morphological analysis of the EBBR biofilm and bioparticles; and comparison with biofilms from other Anammox bioreactors.

4.2 Methods

4.2.1 Physical examination of sampled of EBBR ABDite® growth support medium for biofilm formation

4.2.1.1 Physical examination of the EBBR for evidence of biofilm and bioparticles formation.

The lab scale EBBR expanded bed column was examined to identify the content of each layer within the bioreactor before sampling. Samples of ABDite® biomass support medium with the adhered biofilms (bioparticles) were harvested from the top of the expanded bed column (Figure 2.1) via suction by inserting a silicon rubber tube into the sampling depth (17 – 20 cm) of the expanded bed biofilm bioreactor (EBBR) containing suspected bioparticle rich in Anammox bacteria in static mode. The suction point was sealed to maintain anaerobic conditions within the EBBR-Anammox system.

The harvested ABDite® bioparticles, which were different from the larger bright red seed Anammox bacteria granules and the granules that developed within the EBBR (Figure 2.2), were examined by naked eyes for evidence of biofilm formation (Figure 4.1); and compared with biofilm reports from previous Anammox studies in literature (Driessen et al., 2015; Ni and Zhang, 2013; Cho et al., 2010).

4.2.2 Microscopic examination of ABDite® medium for formation of biofilm and bioparticles.

4.2.2.1 Phase contrast microscope (PCM) examination of ABDite® bioparticles

The freshly harvested samples were transferred to cavity slides and the excess water removed using a pipette. The bioparticles were observed under a phase contrast light microscope (Standard 20, Germany) at x100 and x400 magnifications (Figure 4.2). The images of the bioparticles were obtained using Nikon camera (Coolpix 4500 4.0 Mp, UK) attached to the microscope.

4.2.2.2 Scanning electron microscope (SEM) examination of ABDite® bioparticles

4.2.2.2.1 Glutaraldehyde fixation of Anammox biomass on ABDite® bioparticles for SEM analysis

Scanning electron microscope (SEM) analysis was conducted to determine the morphology (shape) of the microbes that colonized the ABDite® biomass support medium; uncolonized ABDite® particles used as control were also examined. The colonized samples were prepared for SEM by fixing the biomass on the ABDite® support medium with glutaraldehyde (2.5% v/v) in 0.2 M sodium cacodylate buffer (pH 7.1). The samples were rinsed twice with 5 mL of deionised water (DIW) to remove excess glutaraldehyde and then dehydrated with graded ethanol solutions (10, 30, 50, 70, 90, and 100% ethanol) at 10 min intervals (Fernandez et al., 2008; Figueiro et al., 2004). The dehydrated samples were air-dried to evaporate the ethanol and then stored in a 70 % ethanol sterilized desiccator containing oven dry silica gel to absorb moisture. The rim

of the desiccator was sealed with Vaseline to make it airtight, and the specimen kept away from light to preserve their integrity.

4.2.2.2.2 Sputter coating of ABDite® bioparticles for SEM examination

Uncolonized ABDite particles and glutaraldehyde fixed biomass on the ABDite® bioparticles were mounted onto aluminium pin stubs using adhesive carbon tabs and coated with a thin layer of Au/Pd metal (Polaron Uk; Model: SEM Coating System; Target: Au) under the following conditions - coating time: 30s; voltage: 800V; current: 5mA).

4.2.2.2.3 SEM examination of gold coated ABDite® bioparticles

Then SEM analysis was conducted by loading the stubs holding the gold coated bioparticles into the machine and visualized with the SEM (model: Supra 40VP; and the SmartSEM, Carl Zeiss Ltd, UK) for imaging. The SEM examination covered both the uncolonized ABDite®; the surfaces and pores of colonised ABDite® bioparticles. The secondary electron detector was used to obtain SEM images taken using an acceleration voltage of 2 kV and a working distance of approximately 7 mm; and images captured at several magnifications.

4.3 Results

After seeding with the STW Anammox bacteria granules, the physical observation of the lab scale EBBR through the transparent expanded bed column was made 2- 3 days of continuous culture operation to identify evidence of biofilm formation on the ABDite® biomass medium to form bioparticles. Images of uncolonized ABDite® biomass medium were compared with colonized bioparticles physically and by using phase contrast microscope (PCM) and scanning electron microscope (SEM) examinations.

4.3.1 Physical examination of biofilm formation on EBBR-Anammox ABDite® medium.

After for 180 days of the EBBR-Anammox investigation, it was observed different layers liquid (23 cm), Anammox bacteria granules (7 cm), suspected bioparticles (5 cm) and uncolonized ABDite® growth support medium (12 cm) developed in the 58 cm bed height (Figure 4.1).

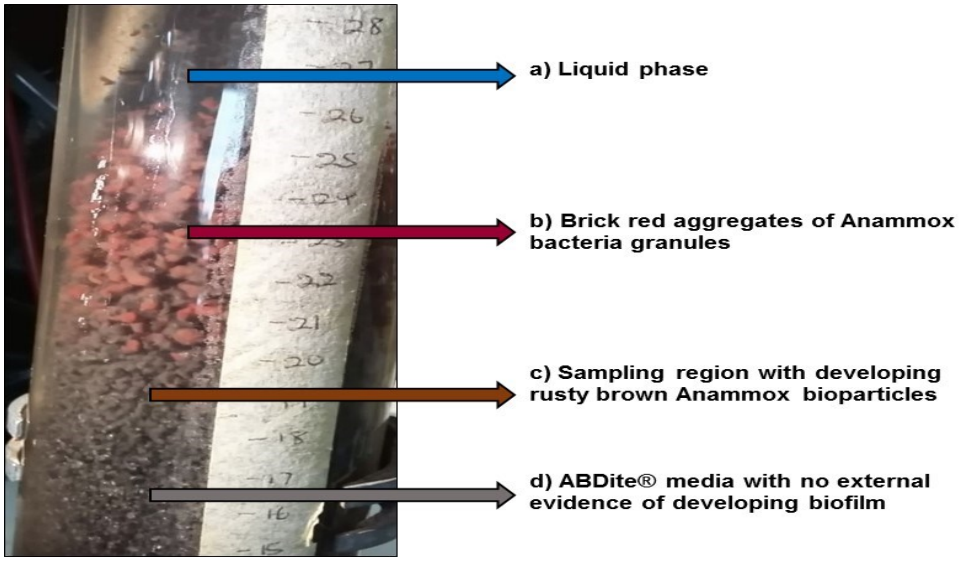

Figure 4.1: Section of active EBBR showing development of brick red and rusty brown aggregates of Anammox bacterial in different layers (Nikon, Coolpix 4500). The proportion of the different layers of the bed were about (a) 40 % liquid phase; (b) 11 % Brick red Anammox bacteria granules; (c) 6 % Rusty brown developing bioparticles and (d) 21 % black ABDite® media and 5 cm (1.0 -1.71 mm) silica sand (Chelford, UK) to prevent grinding.

Approximately, the layers within the 58 cm (height) EBBR column were (a), 40 % liquid phase in which Anammox bacteria flocs floated; (b), 11 % of brick red Anammox bacteria granules; (c), 6 % rusty brown layer suspected to be developing Anammox bioparticles and (d), 21 % black ABDite® media, with no observable evidence of biofilm development and sand layer. The near spherical granules, which consisted of compact microbial biomass with almost smooth surface were suspended under the liquid phase. Underneath the granular layer where suspected bioparticles that developed on individual ABDite® biomass support medium (Figure 4. 1). The Anammox granules and bioparticles formed in the EBBR column had different sizes (measurement were not made). The bioparticles in section c were smaller, but much denser than the granules in layer b. (Figures 4.1). However, the colour of the granules and bioparticles were consistent with the brick red colour of active Anammox bacteria biofilm. While the EBBR-Anammox bacteria granules had similar colour with the 2 mm seed granules inoculated into the bed at the start of the investigation, they were smaller in size (Figure 4.1b). The biofilms that formed on the 0.7 to 1.0 mm ABDite medium were mostly

spherical in shape (Figure 4. 1). At the bottom of the bioreactor were some ABDite® medium that appeared to have no biofilm attached, which indicated that the rig was yet to be fully colonized at that stage of the EBBR - Anammox investigation.

4.3.2 Microscopy analysis of biofilm development in the EBBR

4.3.2.1 Phase contrast microscope images of biofilm formed on EBBR - Anammox ABDite® medium.

The phase contrast microscope (PCM) images revealed that a layer of external biofilm developed on the surface of ABDite® biomass support particles seen under the x100 and x400 magnifications (Figures 4.2c and 4.2d). The external biofilm that developed on the ABDite® medium had smooth surface compared to the black uncolonized ABDite® medium with rough surface and sharp edges (Figure 4.2a). The distinct, orange-coloured biofilm (though not the true colour of biofilm) surrounding the ABDite® particles was different from both the uncolonized part of the growth medium and the image of the initial brick red 2 mm Anammox granules with which the bioreactor was seeded (Figure 4.2b).

While the seed granules were almost circular in shape, the EBBR biofilm was nearly spherical; and wrapped around the black ABDite® biomass medium embedded within the centre. The biofilm formed on the ABDite® medium appeared to be thin suggesting that the biofilm was at the early stage of its formation, which later developed into mature biofilms and individual bioparticles (Figures 4.7 - 4.12). Detection of biofilm development on the ABDite medium surface by PCM suggests that the ABDite particles provided the right surface for the Anammox bacteria from the seed granules to; and the EBBR-Anammox system was functional.

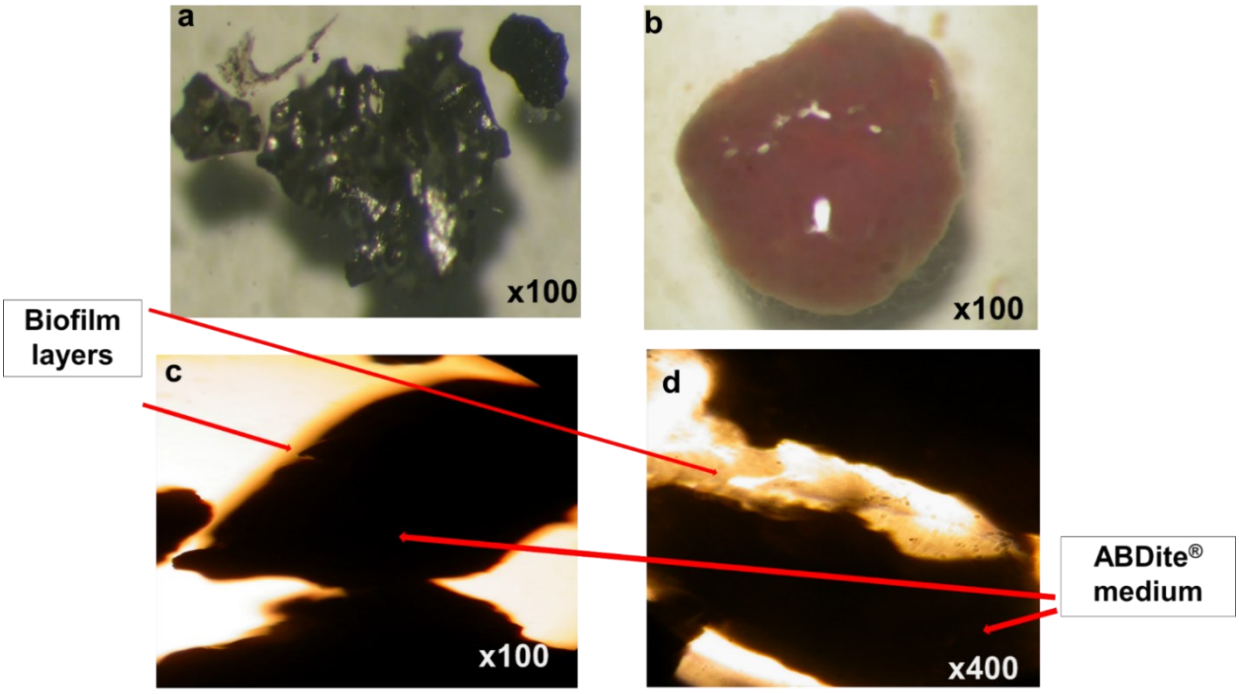

Figure 4.2 Phase contrast microscope (Zeiss Standard 20, Germany) images (Nikon, Coolpix 4500, 4.0 Mp, Uk) of bioparticle formed on the EBBR-Anammox ABDite® particles (a) Uncolonized 1.0 mm ABDite® medium before inoculation into the reactor; (b) Anammox granule (2.0 mm) before seeding; (c) Colonized ABDite® bioparticles taken from EBBR showing external biofilm (x100) and (d) Colonized ABDite® bioparticles taken from EBBR showing external biofilm (x400).

4.3.2.2 SEM analysis of biofilm development on EBBR-Anammox ABDite® medium

4.3.2.2.1 Unlocalized and colonized ABDite® biomass growth medium before SEM examination

The Images of uncolonized ABDite® particles were compared with colonized sputter gold coated ABDite® particles taken from the lab scale EBBR-Anammox bioreactor between 90 to 560 days before SEM observation (Figures 4.3a and 4.3b).

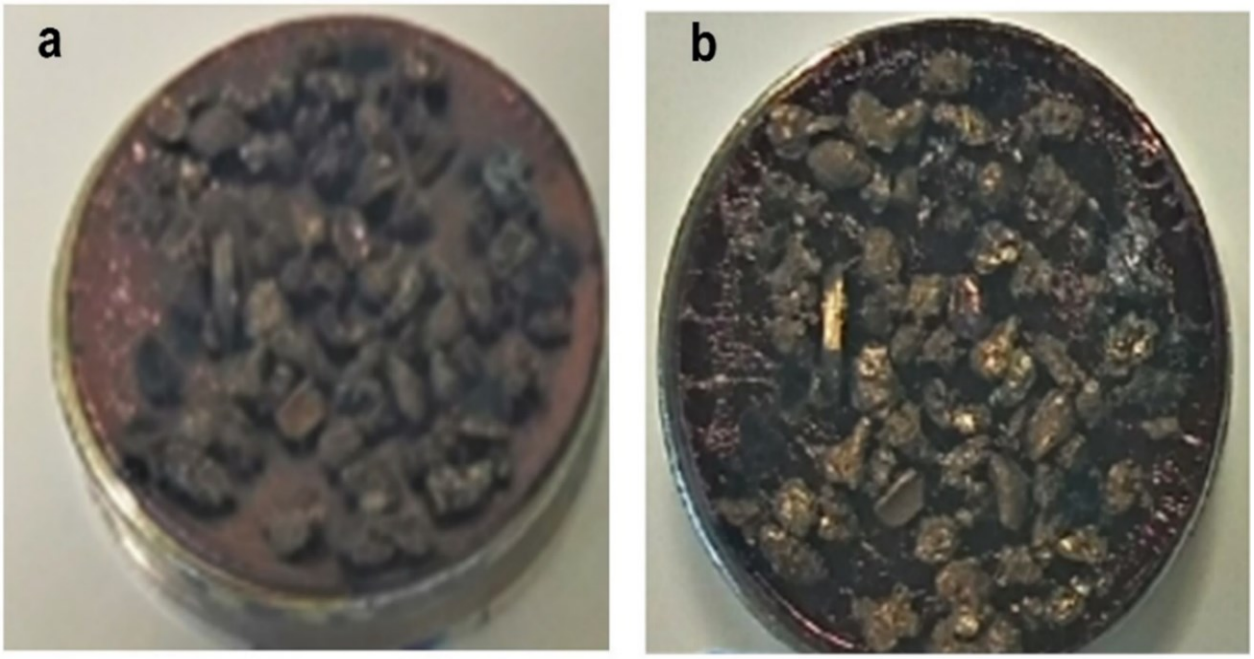

Figure 4.3: Images of the uncolonized and colonized EBBR-Anammox ABDite growth support medium prepared for SEM analysis (Nikon, Coolpix 4500). a) Uncolonized ABDite particle before SEM observation b) Colonised ABDite particle removed from the EBB SPUTTER coated with gold for SEM observation.

4.3.2.2.2 SEM micrographs of unlocalized ABDite® surface

The SEM micrographs revealed that the uncolonized ABDite® particles had a characteristic rough surface with sharp edges; and have no biological matter (4.4a & 4.4b).

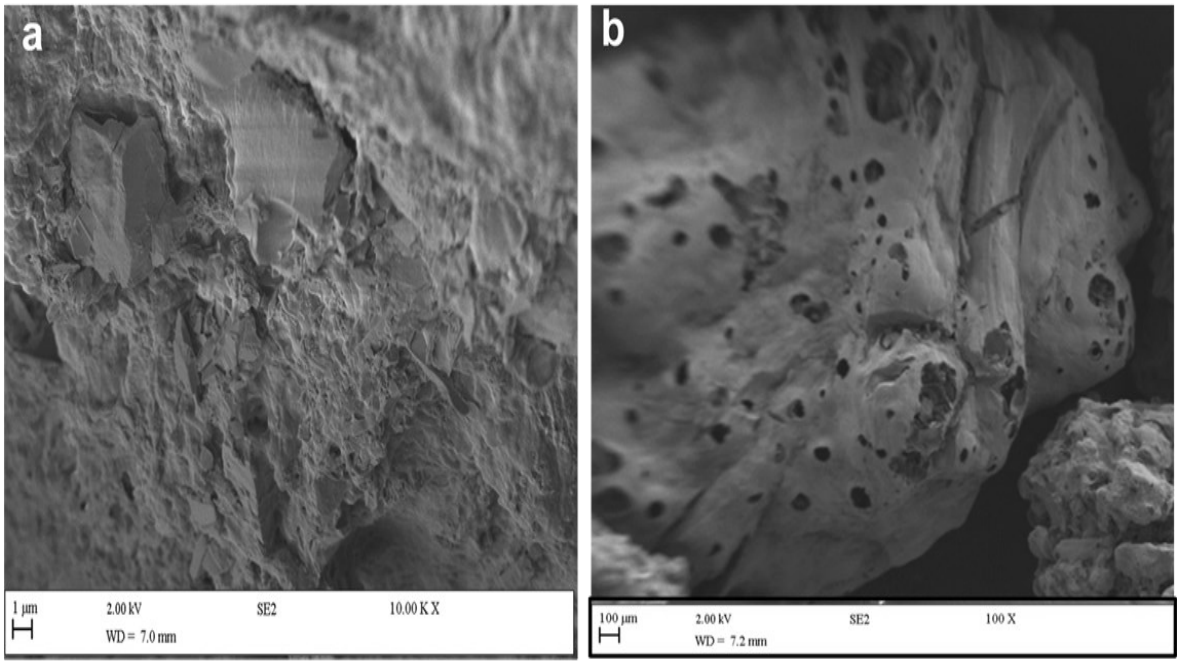

Figure 4.4: SEM micrograph of the uncolonized EBBR-Anammox ABDite growth medium surface. a) Uncolonized ABDite surface at 10,000 KX; a) Damaged uncolonized ABDite surface at 100 X.

The SEM image reveal that the surface of one of the uncolonized ABDite might have been damaged possibly by mechanical handling such as the use of forceps during sample preparation process in preparation for SEM observation (4.4b). This highlights one of the challenges of using SEM to analyse biofilm, as the distortion of the image could make interpretation difficult or lead to false conclusion.

4.3.2.2.3 SEM micrographs of fully colocalized ABDite® medium surface

The SEM images revealed that the surfaces of the ABDite medium were colonised by biofilm suspected to belong to Anammox bacteria (4.5a & 4.5b). The colonized ABDite growth medium surfaces were completed covered with biomass layers of biofilm. The biofilm was fully developed, forming individual ABDite® bioparticles (4.5a & 4.5b).

The shape of the EBBR bioparticles were influenced by the shape ABDite® particles, the biofilm developed on. The surface of bioparticles was suspected to be

colonized by the same type of bacteria – like cells; cocci-like cells appeared to be predominant (Figures 4.5ba &4.5b).

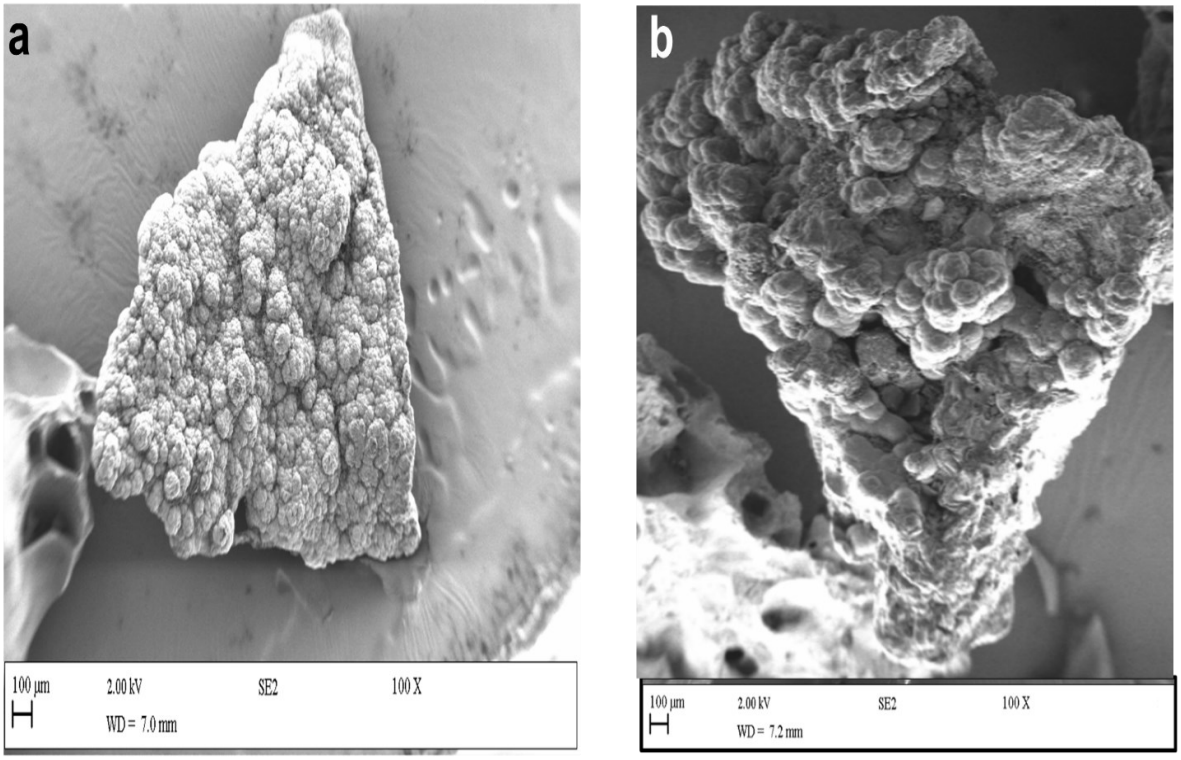

Figure 4.5: SEM micrographs of the fully colonized EBBR-Anammox ABDite growth medium surface to form individual bioparticle at 100 X. a) Colonized ABDite surface evenly covered with apparently the same type of b) Colonized ABDite surface covered with cocci-like bacteria cells.

The SEM images provided evidence suggesting that the Anammox bacteria biofilm and individual bioparticles developed on the EBBR-Anammox ABDite®, and therefore, the growth medium had enough surface area, and supported the growth of the Anammox bacteria.

4.3.2.2.4 SEM micrographs biofilm colonization of the ABDite® biomass pore

From the SEM images, evidence was found that biofilm developed on both the pores and surface of the EBBR-Anammox ABDite® biomass support medium (Figures 4. 5 and 4.6). The colonized pores are filled with biomass with different amounts of biological matter.

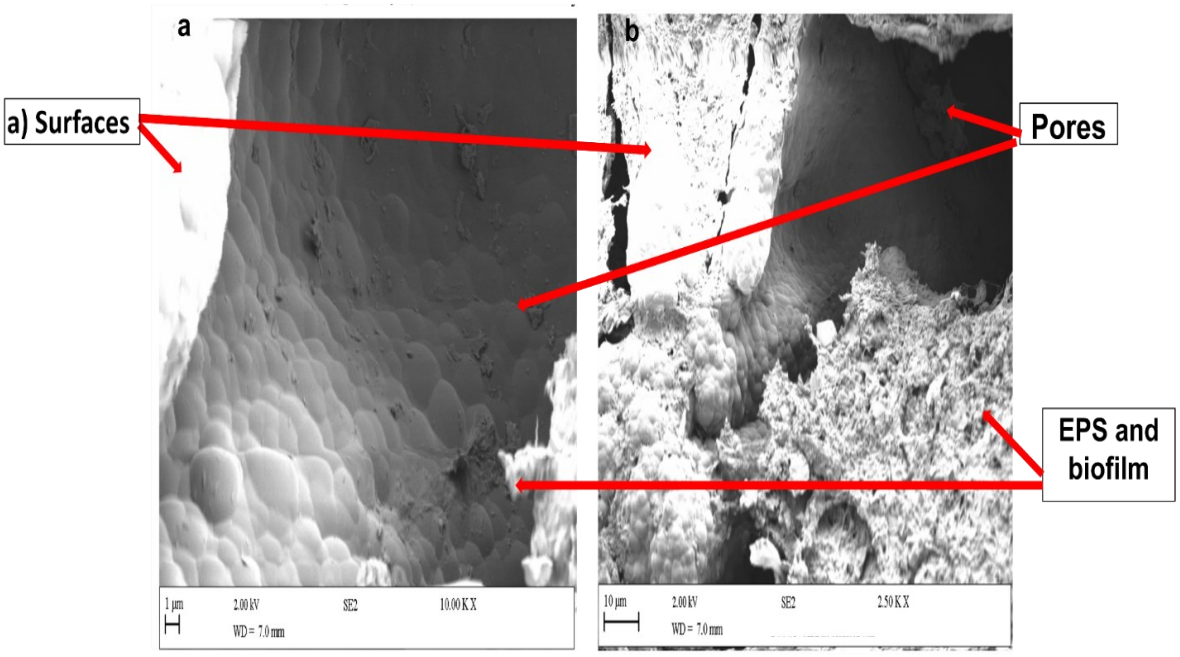

Figure 4:6 SEM images of developing formation in the pores and surfaces and of ABDite® biomass support medium. a) Colonized (1µm) ABDite® media pore at 10KX with small amount of extra polymeric substance (EPS). b) Colonized (10 µm) pore covered with extensive EPS at 2.5KX.

The observed colonization pattern indicated that the growth of the biofilm started from inside the pore and extended to the surface. The images suggested an early stage of biofilm attachment in the pores and outgrowth of biological material to the surface because the visualized biofilm was still thin and not well developed (Figure 4.6).

4.3.2.2.5 SEM micrographs of fully developed biofilm with cauliflower structure on the ABDite® biomass

The SEM images indicated that the EBBR-Anammox biofilms developed further with increased biomass layer attached on the ABDite medium (Figures 4.7 – 4.9). Compared to SEM images in (Figures 4.6) with thin layer of biofilm; in which part of the pore and surface of the ABDite® medium were not used; the advanced biofilms have both the pores and the entire surfaces completely covered (Figure 4.6 – 4) to form the continuous irregular cauliflower shape, which is characteristic of mature Anammox bacteria biofilm described by other established Anammox technologies (Table 4.1).

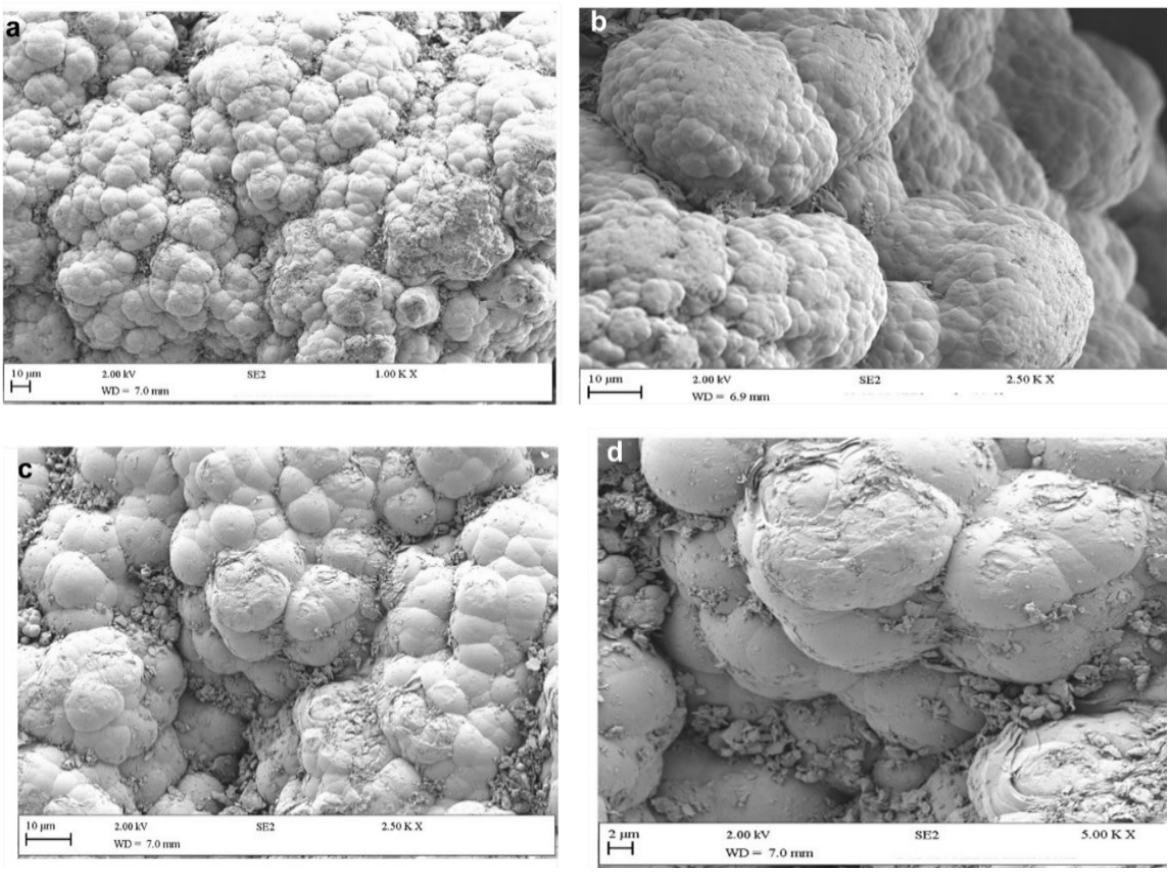

Figure 4.7 SEM micrographs of advanced Anammox bacteria biofilm on the ABDite medium with cauliflower structure. a) Biofilm (10 µm) at 1.00 KX; b) Biofilm (10 µm) at 2.50 KX; c) Biofilm (10 µm) at 2.50 KX; d) Biofilm (2 µm) at 5.00 KX.

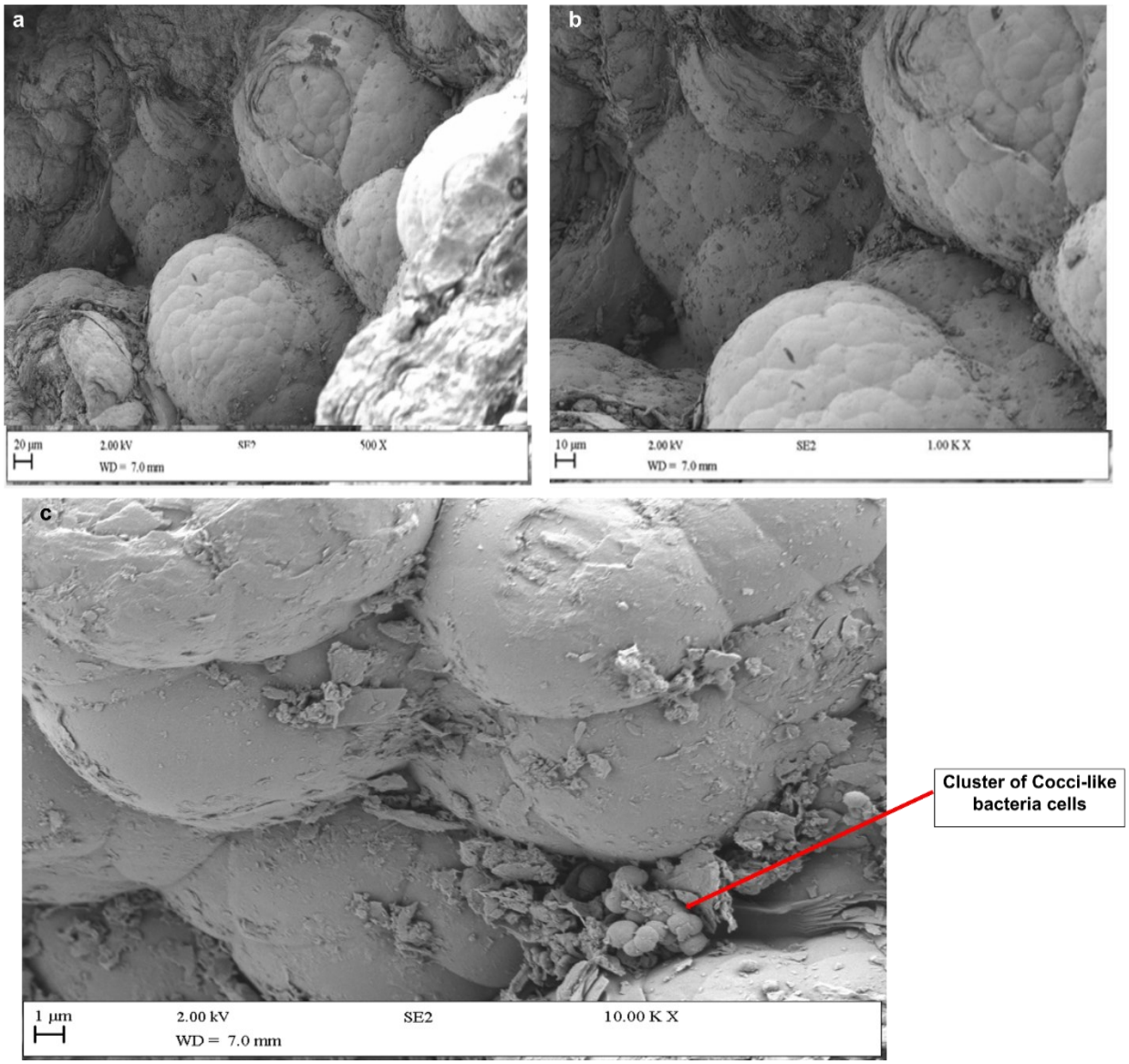

Figure 4.8: SEM micrographs of biofilm formed on the EBBR-Anammox ABDite medium with the cauliflower structure. a) Biofilm (20 µm) at 500 K; b) Biofilm (10 µm) at 1.00 KX; c) Biofilm (1µm) at 10.00 KX with cocci-like bacteria.

4.3.2.2.6 SEM micrographs of bacteria-like cells on the ABDite® bioparticle/ biofilm

In addition to the development of the cauliflower shaped biofilms were found cocci-like bacteria cells attached to the surface of the ABDIte medium (Figure 4.8); and others were embedded inside biofilm (Figure 4.9).

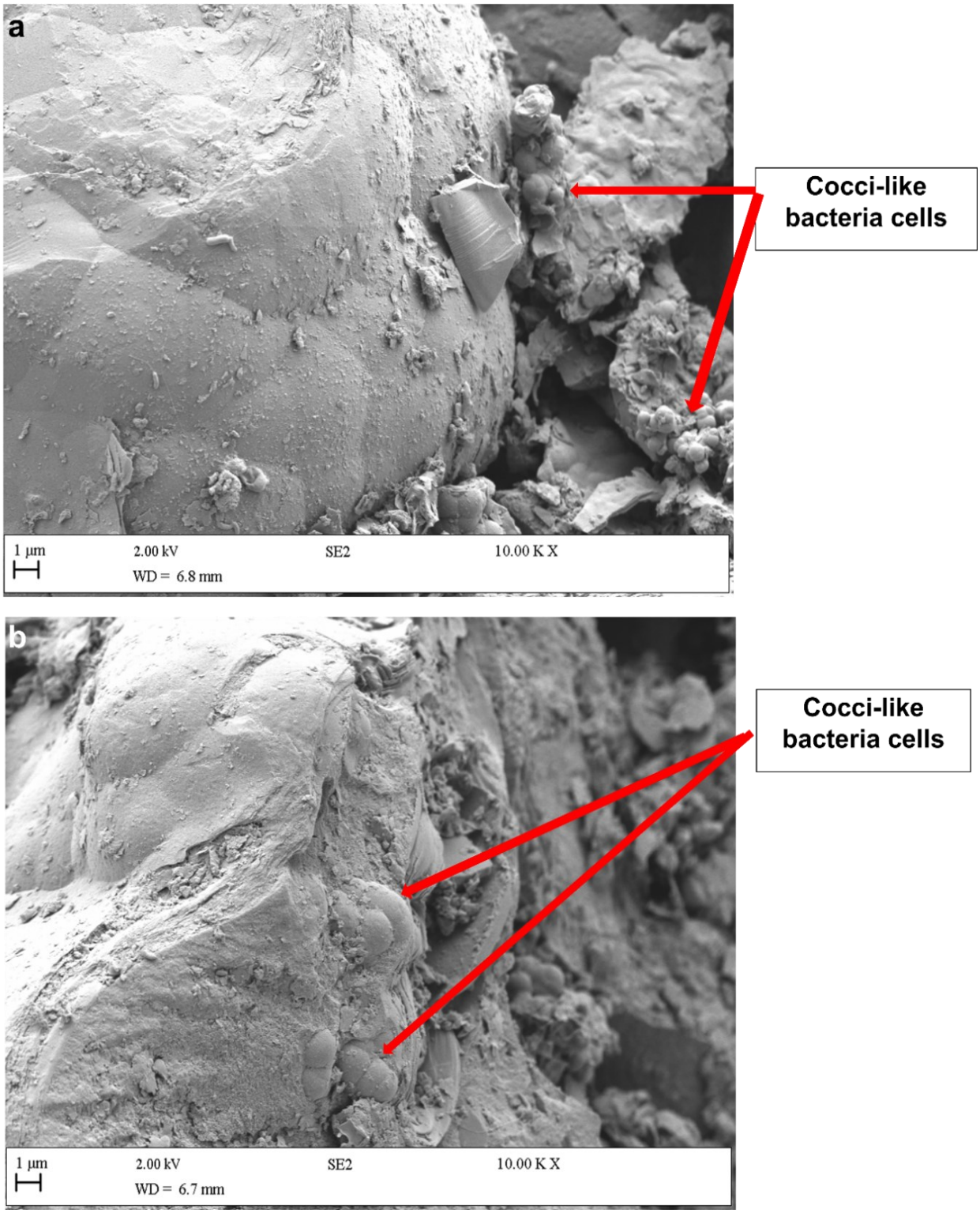

Figure 4.9: SEM micrographs of biofilm formed on the EBBR-Anammox ABDite medium with the cauliflower structure and cocci-like bacteria cells. a) Biofilm (1 µm) with cocci-like bacteria on the surface; b) Biofilm (1 µm) with embedded cocci-like bacteria cell.

Furthermore, in addition to the presence of the advanced biofilms with the cauliflower structure, the SEM micrographs also revealed morphological diversity in the suspected Anammox bacteria cell that colonised the EBBR ABDite® medium (Figures 4.10 – 4.14). The distinct bacteria–like cells found to have developed on the EBBR ABDite were filamentous-like, rod-like and cocci-like bacteria cells.

The suspected filamentous bacteria was about 1 - 2 µm in size (Figure 4.10). Equally present was a mixture of rods rod-like bacterial cells; some around 1 µm and other < 1 µm in size (Figures 4.10, 4.11 & 4.14). The surface of the biofilm layer developed on the ABDite surface was littered with rod-like cell, which were in more abundant over the filamentous cells. In addition, the layer appeared to be pilling off., possibly due to damage caused by the treatment process in preparation of the sample for the SEM observation. The biomass layer was very distinct from the uncolonized ABDite with sharp edges on which a is found an approximately 1 µm rod-like bacterial cell labelled c (Figure 4.10).

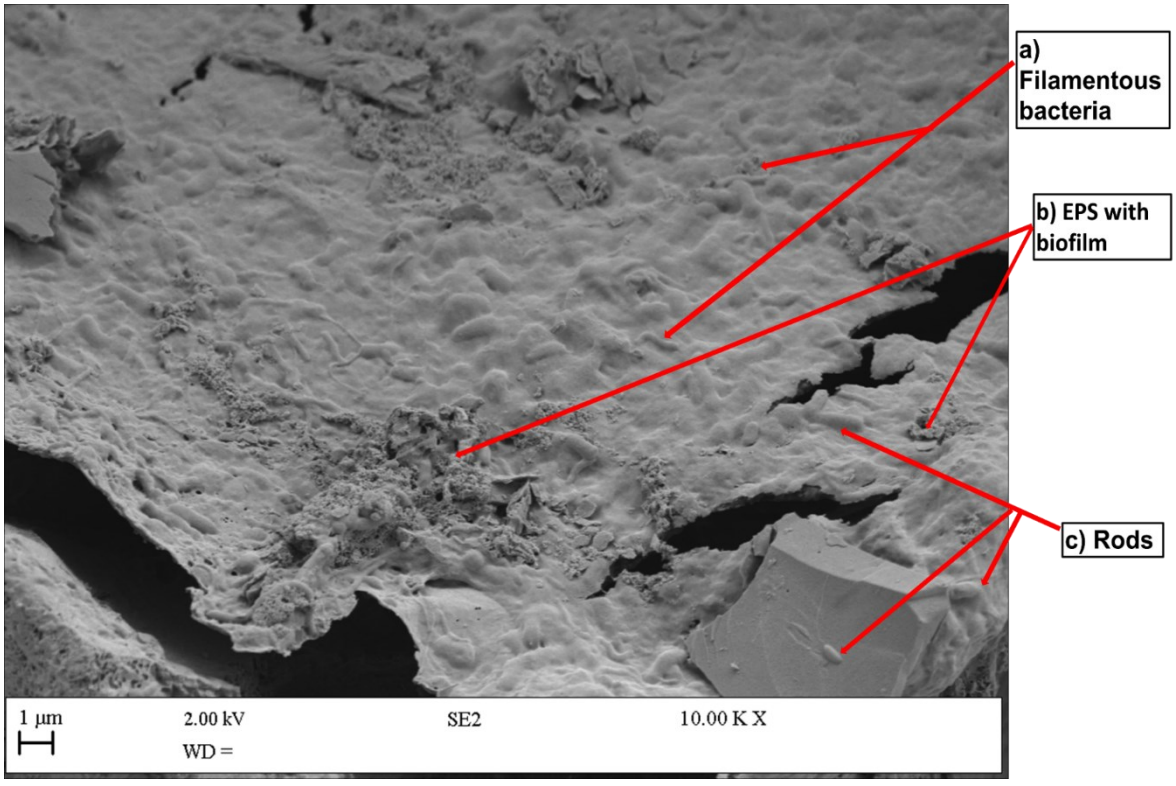

Figure 4.10: SEM micrographs of mixed culture of bacteria - like cells that developed on the surface of the 0.7- 1.0 mm EBBR-Anammox ABDite® medium. a) Filamentous bacteria like cells; **b)** EPS with biomass c) Rod - like bacteria cell.

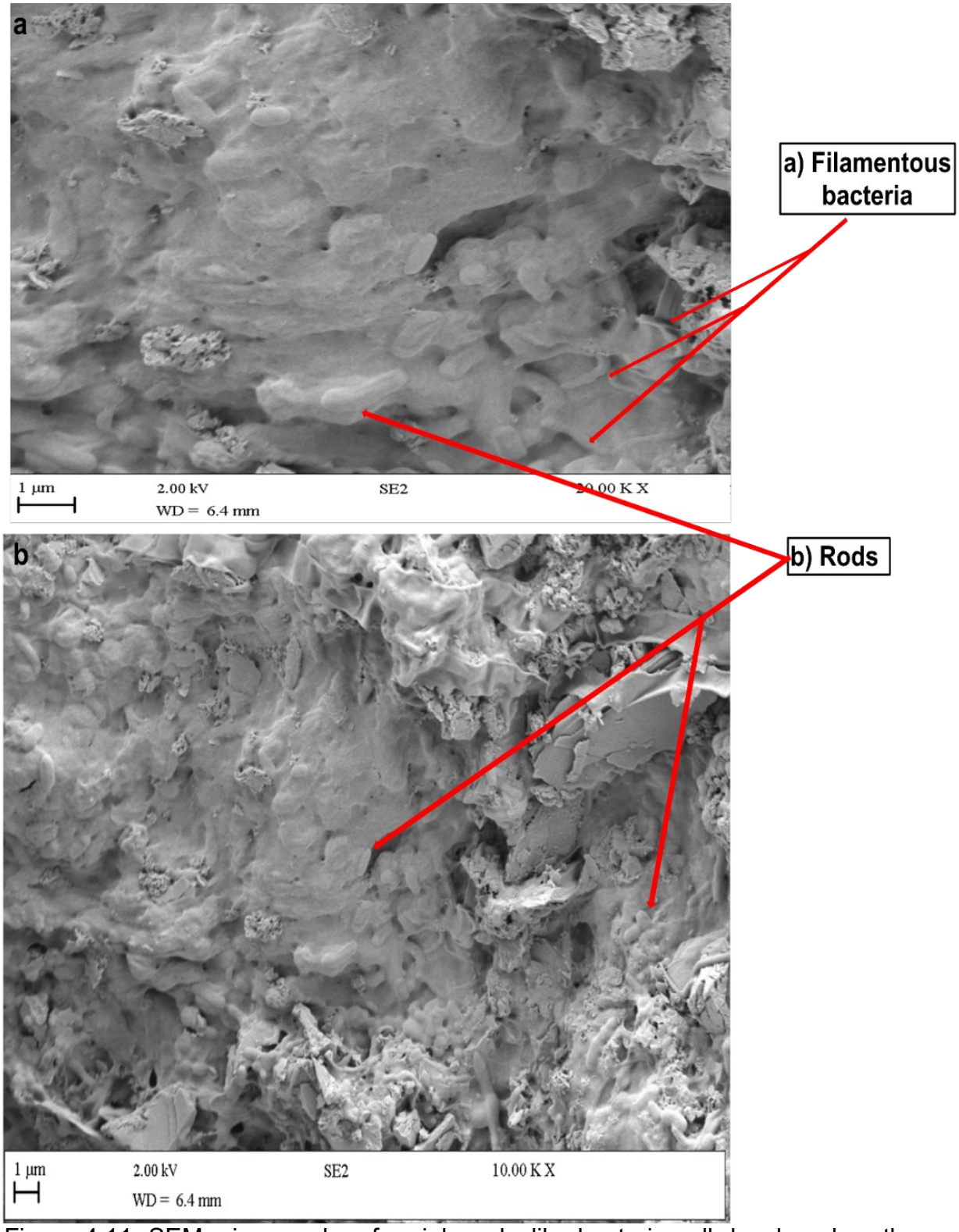

Figure 4.11: SEM micrographs of mainly rod - like bacteria cell developed on the surface of the 0.7- 1.0 mm EBBR-Anammox ABDite® medium. a) Filamentous-like cells at 40 000 KX **b)** Rod-like cells at 10 00 KX.

Two types of cocci-like bacteria cells were developed on the surface of the ABDite® medium (Figures 4. 12 & 4.13). While one of cocci-like cells grew as individua clusters containing around 6 cells or more (Figures 4. 12); the other was encased within as an aggregate with up to 100 cells (Figure 4.13)

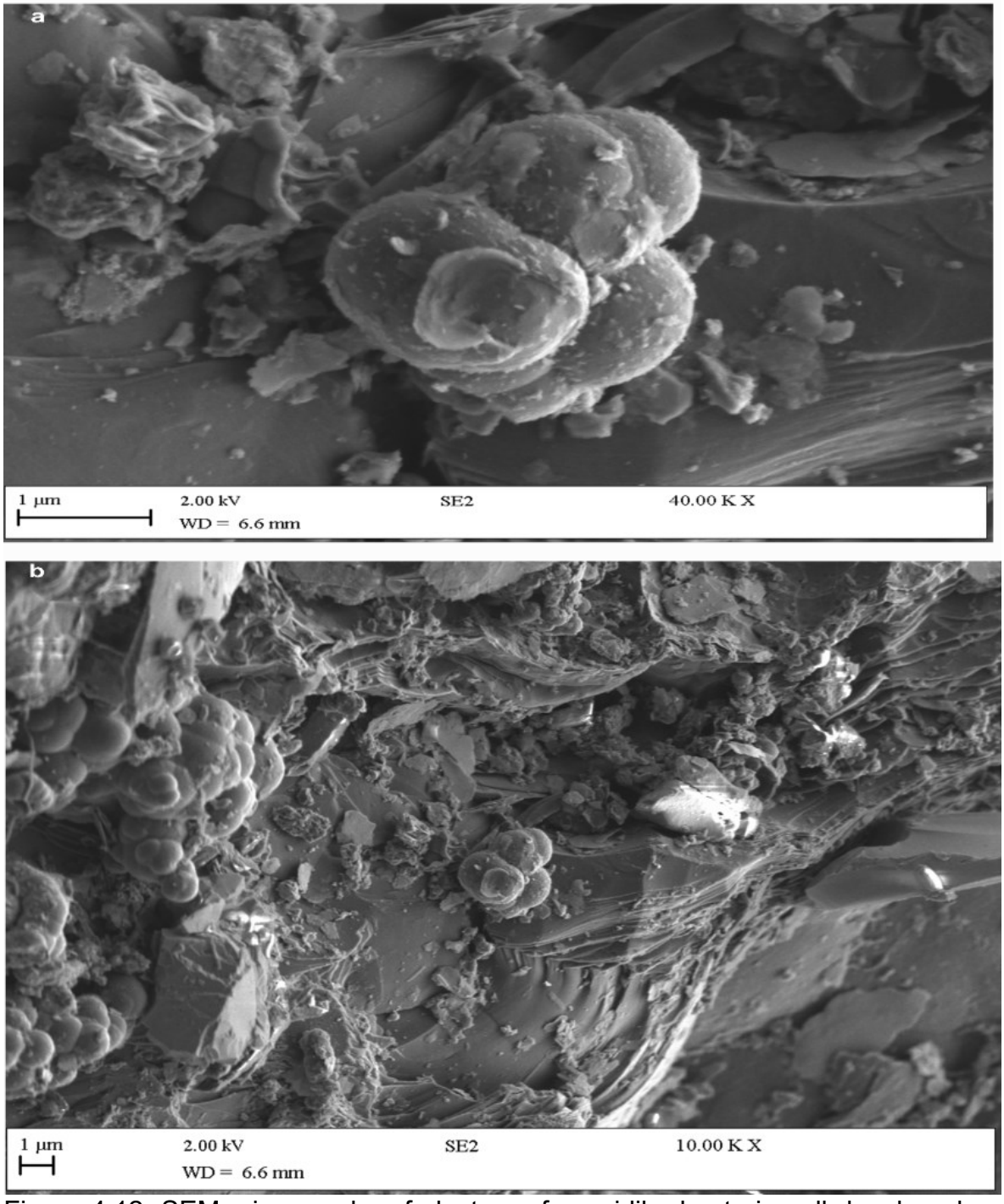

Figure 4.12: SEM micrographs of clusters of cocci-like bacteria cell developed on the surface of the 0.7- 1.0 mm EBBR-Anammox ABDite® medium. a) Cocci-like cells at 40 000 KX **b)** Cocci-like cells at 10 00 KX.

The SEM images indicated that each of the two suspected cocci bacteria; dominated the surfaces colonized.

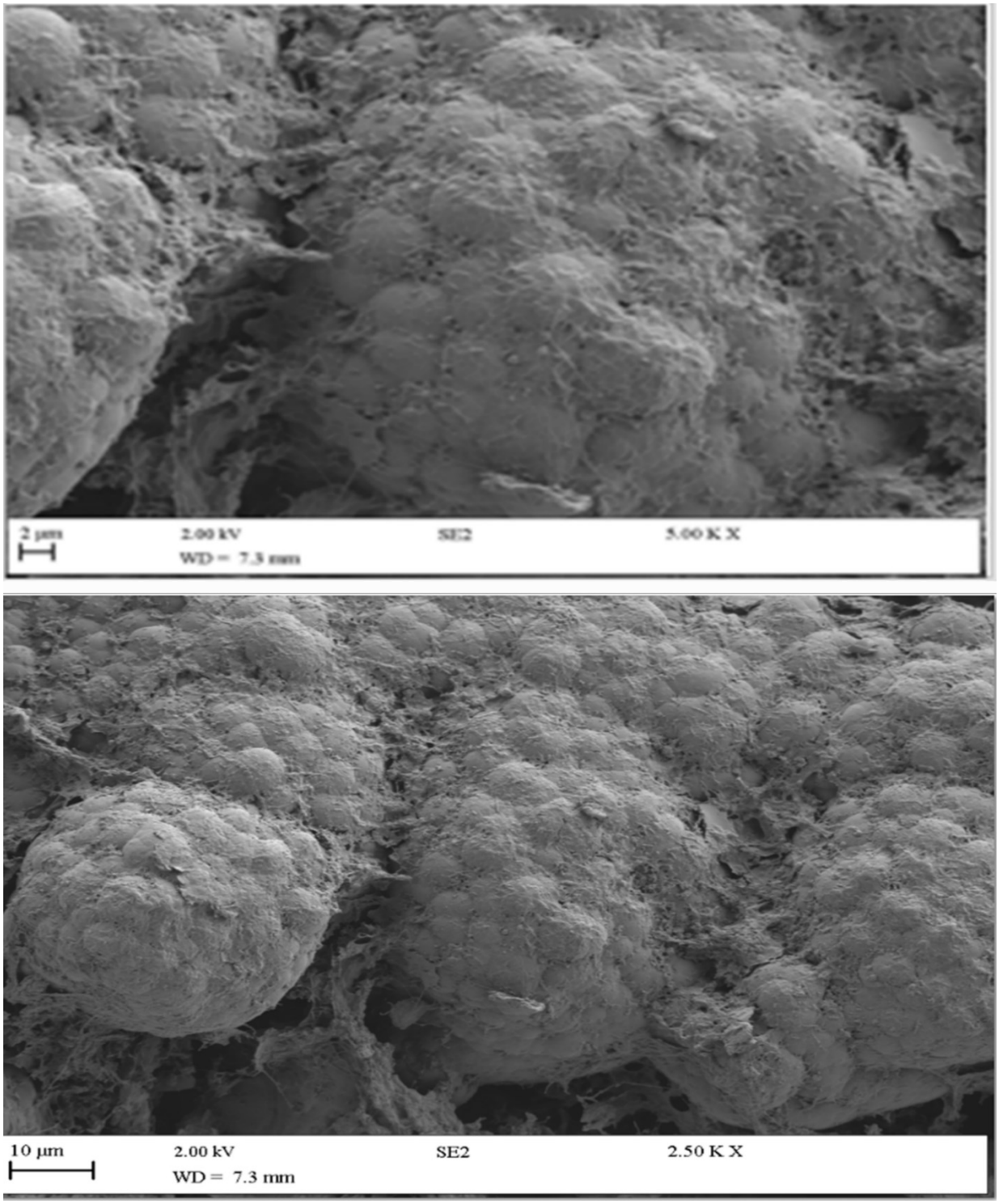

Figure 4.13: SEM micrographs of cocci-like bacteria cell aggregate developed on the surface of the 0.7- 1.0 mm EBBR-Anammox ABDite® medium. a) Cocci-like cells at 5 00 KX; b) Cocci-like cells at 2.50 K

Additionally, suspected yeasts-like cells grew alongside the cocci, filamentous and rod-like bacteria cells (Figures 4.14 & 4.15). The SEM images suggest there were two types of yeasts cells, which were about 5 - 10 µm in diameter and, therefore, much bigger than the all the approximately 1- 2 µm sized bacteria-like cells described above (Figures 4.10 -14). Each yeast-like cell type was dominant over the ABDite medium surface colonised.

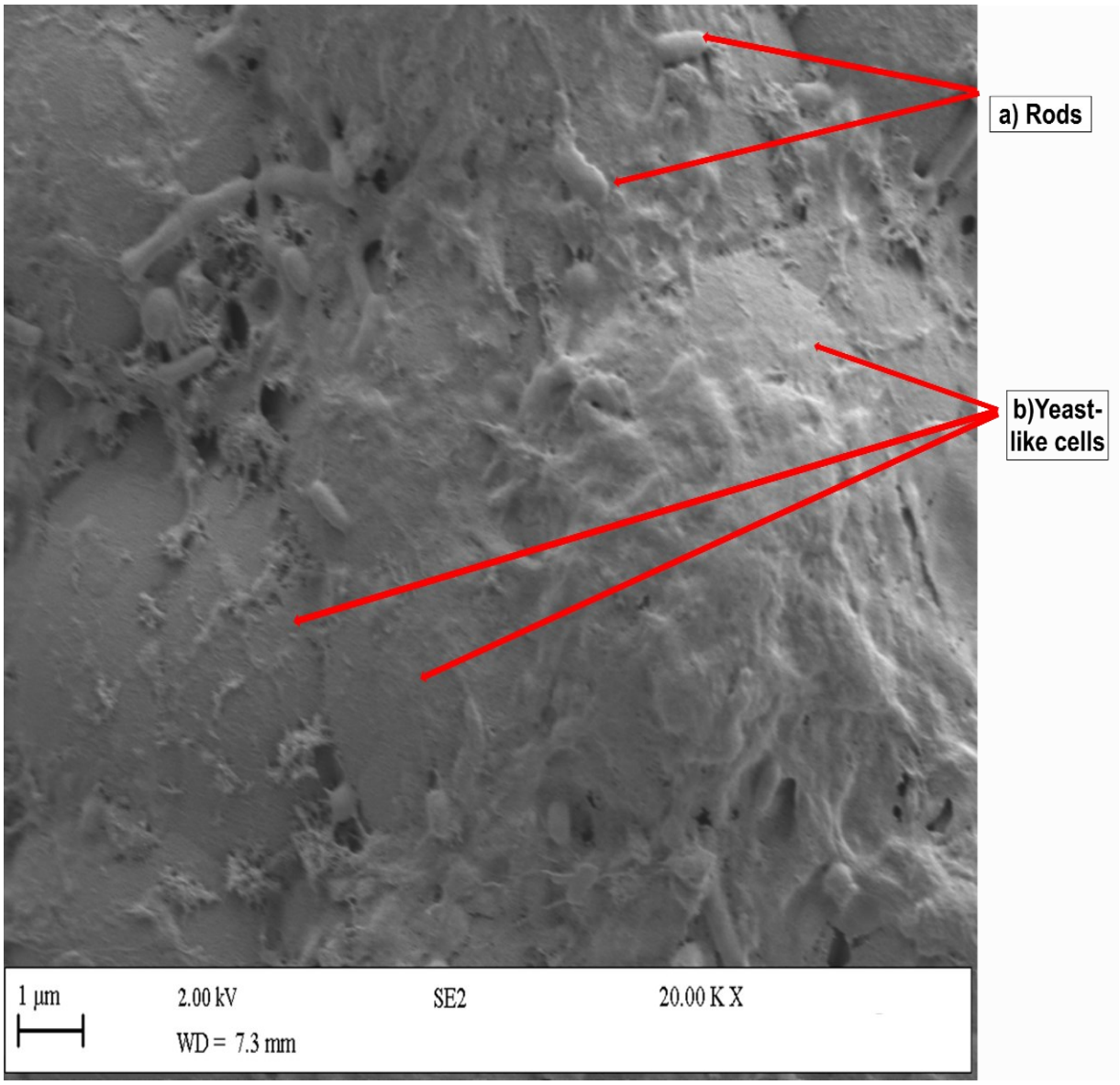

Figure 4.14: SEM micrographs of mixed culture of bacteria and yeast - like cells that developed on the surface of the 0.7- 1.0 mm EBBR-Anammox ABDite® medium a) Rod-like bacteria (1 µm); b) Yeasts cells (10 µm).

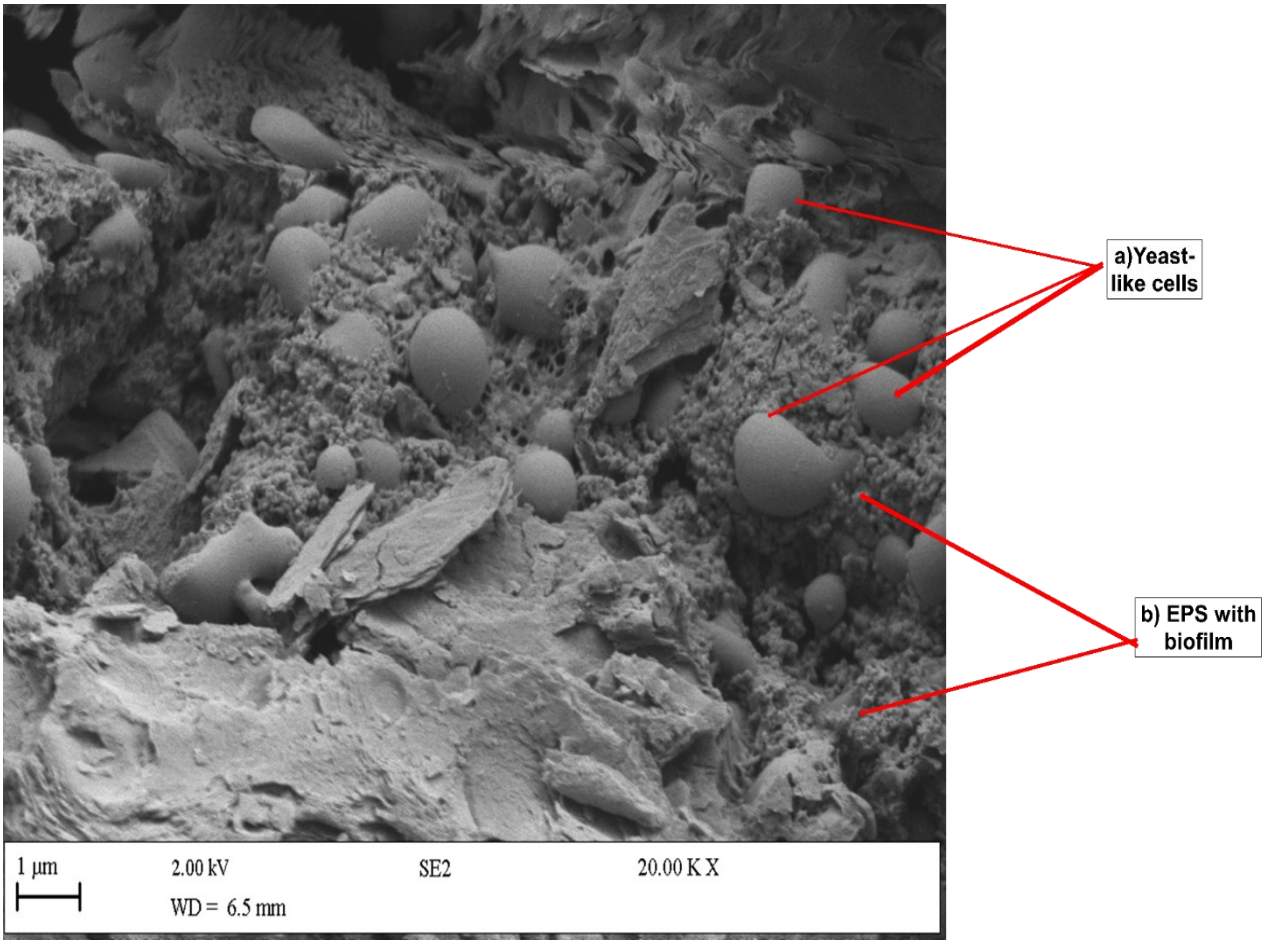

Figure 4.15: SEM micrographs of mixed yeast - like cells (about 5 µm) that developed on the surface of the 0.7- 1.0 mm EBBR-Anammox ABDite® medium. a) Yeasts cells; b) EPS with biofilm.

The development of these bacteria-like cells, in addition to the suspected yeast cells on the EBBR-Anammox ABDite medium suggest that the biomass growth support was effective for the attachment and retention of the suspected Anammox bacteria active biomass in the expanded bed column; hence the EBBR-Anammox system was working well.

The physical observation of red biofilms on the surface of ABDite medium, which was confirmed by detection of biofilm layer by PCM examination; alongside SEM finding of advanced cauliflower biofilm, individual bioparticles, filamentous, rod and cocci-like bacteria cells fulfilled objectives 1 and 2 of chapter 4 research aim.

4.4 Discussion

The current study marked the first time the lab scale expanded bed biofilm reactor (EBBR) and the ABDite biomass medium have been used for the Anammox synthetic wastewater treatment investigation.

The use of growth support medium such the EBBR ABDite® is important in Anammox wastewater treatment process is important because it supports and protects the Anammox bacteria from predation from grazing protozoa as reported by Hoekstra et al., (2018) and Suarez et al., (2015). Zhang, (2013) found that using biomass medium to develop biofilms reduced the rate active bacteria biomass was washed out of the bioreactor. Other benefits Anammox bacteria have from the use of growth support medium are stability and shielding from the toxic effect of molecular oxygen (Niederdorfer et al., 2021; Mozumder et al., 2014). In addition, biomass support materials provide suitable surface area for microbial attachment to form stable biofilms (Yang et al.,2021).

To observe the content of the bioreactor, the transparent property of the EBBR offered additional advantage; this is because the different layers, mainly, the liquid phase, Anammox bacteria granules and bioparticles developed within the expanded bed column were easily examined from the outside before sampling (Figure 4. 1). The physical examination EBBR-Anammox reactor showed that microbial flocs from the seed granules moved around the liquid phase; 40 % of the expanded bed column (Figure 4. 1, layer a). The appearance of flocs in the expanded bed column was an indication of the early stage of Anammox bacterial growth and subsequent aggregation to form granules or immobilized on the ABDite® medium to form biofilms described by (Laureni et al., (2015) and Almstrand, et al., (2014).

Anammox bacteria granules and biofilm are known to grow with a brick red to dark brown colour on the biomass support materials (Lu et al., 2018; Zenga et al., 2016; Ni and Zhang, 2013).The presence the of red bacteria granules and suspected bioparticles with rusty brown colour within the expanded bed column11 % and 6 %

respectively (layer b and layer c) (Figure 4.1) were taken as evidence suggesting the development of active Anammox bacterial granules, biofilm and or bioparticle on the 0.7 - 1 mm porous ABDite® medium.

The Anammox bacteria granules and biofilms that developed 6 months into the EBBR-Anammox investigation corroborated findings from previous studies, which have described the brick red or brown appearance of Anammox bacteria that colonised different support media. For example, the growth of a 1.0 mm reddish-brown biofilm on foam and charcoal biomass support media were the reported evidence for Anammox bacteria biofilm formation reported by Liu et al., (2018) using an up-flow column reactor. Brick red spherical biofilm of around 5 mm grew on microscopic slides carriers submerged in Anammox bioreactor (Botchkova et al., 2015). Using an Anammox non-woven membrane reactor Ni et al., (2010) recorded red Anammox bacteria biofilm of 2.0 - 3.5 mm in thickness.

Although the sizes of the EBBR Anammox granules (estimated to be 3 – 4 mm) and bioparticles were not determined as it was not the focus for this study, the development of the brick red – brown biofilm characteristic of Anammox bacteria in the EBBR-Anammox system as reported in the above studies, suggested that the Anammox bacteria attached to the ABDite medium could have had the same benefits provided by the other biomass particles reported above. For example, the microporous nature of the biomass support material might have shielded the Anammox bacteria from the toxic effect of molecular oxygen needed by ammonia oxidizing bacteria (AOB) for partial nitrification of ammonia to nitrite (Lu et al., 2018); and therefore, indicated that the EBBR system was functional.

Dingding et al., (2021) reported that that Phase-contrast microscope (PCM) is an important tool for visualisation of Anammox biofilm formed on growth support materials. The PCM images confirmed that suspected Anammox biofilms developed on the surface of the ABDite® particles in the EBBR expanded bed column (Figure 4.2). Botchkova et al., (2015) studied the morphology of Anammox biofilm grown on slides that were submerged in an Anammox bioreactor. Using a SBR, Kallistova (2020) found that Anammox bacteria biofilm was formed and

retained on the biomass growth carrier. These results suggests that the biofilm developed on the EBBR ABDite medium and detected by the PCM could be that of Anammox bacteria; and hence, the ABDite medium has the capacity to retain the biomass.

Furthermore, SEM micrographs revealed black and white layers of biofilm, which might belong Anammox bacteria developed on the EBBR ABDite® medium pores and surfaces (Figures 4.5- 4.9). In the EBBR system, biofilm formation on the ABDite® starts with bacterial adhesion within the pores. Biomass development continues outward until the support medium is totally enveloped by biological matter to from individual bioparticles (Figures 4.5) as reported by Akhidime and Dempsey, (2009), that used the ABDite medium to develop nitrification bacteria biomass. Using a SBR for the Anammox process, Kallistova (2020) studied the stages of Anammox bacteria biofilm formation on biomass growth medium. Mature Anammox bacteria biofilm are described to have structure such as cauliflower, tulip, mushroom, or broccoli depending on the system (Table 4.2).

In the EBBR, the suspected Anammox bacteria biofilm developed had the cauliflower structure (Figures 4.5-4.9) that has been described by other Anammox investigations. For example, a MBR Anammox experiment by Wang et al., (2018) reported the development of biofilm with the cauliflower structure. Xu et al., (2018) and Ni et al., (2018). In separate SBR investigations Xu et al., (2018) and Ni et al., (2018) reported Anammox biofilms with similar description. Additionally, in an ABBR that used honeycomb-like and a FBR with non-woven cloth as biomass medium; and Wang et al., (2019) and Liu et al., (2017), all developed Anammox bacteria biofilm that matched the same of cauliflower description respectively. These results suggested that the cauliflower biofilm developed on the EBBR ABDite, was that of Anammox bacteria; and in addition, that the ABDite medium supported the growth of these bacteria as was the case in the studies described above.

In literature, bacterial cell types reported to be consistent with Anammox bacteria are rods of different sizes, and cocci or clusters of cocci-like bacteria, which most

times coexist with filamentous bacteria (Domenico et al., 201; Malamis et al., 2013). Winkler et al., (2012) stated that Anammox bacteria have rod and cocci shapes; and Ali et al., (2013) reported that Anammox bacteria are mainly rod-shaped, cocci, and filamentous bacteria. In the EBBR-Anammox investigation, there were evidence that filamentous, rod-like, and cocci-like bacteria; in addition to yeast-like cells grew on the ABDite® medium (Figures 4.9 – 15). While Domenico et al., (2015) found clusters of cocci using MFC; Zekker et al., (2012) reported the same cocci and spherical bacteria formed on a blank biocarriers in a MBBR Anammox investigations. Wang et al., (2019) detected rod, cocci, filamentous bacteria during an Anammox wastewater treatment experiment using ABBR. The reports from the previous Anammox studies, which found the same types of bacteria that were present in the EBBR system, suggest that Anammox bacteria-like cells (rod, cocci, filamentous) developed on the EBBR-Anammox ABDite growth medium.

In the EBBR-Anammox system, SEM examination detected brick red biofilms with the characteristic Anammox bacteria cauliflower structure on the EBBR ABDite growth medium. In addition, bacteria-like cells (filamentous, cocci and rods); and yeast-like cell also grew on the biomass support.

The comparative evaluation of the EBBR-Anammox SEM against data from other established Anammox systems: ABBR (Wang et al., 2019), SBR (Xu et al., 2018; Ni et al., 2018) MFC Domenico et al., (2015), MBR (Wang et al., (2018); Ni et al., (2018), FBR (Liu et al., 2017) and MBBR (Wang et al., 2018; Zekker et al., (2012) found some morphological similarities in the biofilm and organisms that developed in these bioreactors, even though the seed culture came from different sources and the growth medium different. The similarities include the following:

a) Development of Anammox bacteria biofilm with the cauliflower structure in the EBBR (Figures 4. 5 - 4.9), as was reported in MBBR (Wang et al., 2018) and FBR (Liu et al., 2017)

b) Filamentous bacterial-like cells found in the ABDite biofilm (Figure 4.10 & 4.11) was described by Wang et al., (2019) using ABBR and in SBR (Xu et al., 2018; Ni et al.,2018).

c) Rod-like bacterial cells (1-2 μm) detected on EBBR-Anammox biofilm (Figures 4.10, 4.114.&.14a) were also recovered from SBR (Xu et al., 2018) and ABBR (Wang et al., 2019).

d) Clusters of cocci-like bacteria cells (1 μm) developed in the EBBR (Figures 4.9, 4.12 & 4.13); MBBR (Wang et al., 2017) and and MFC inoculated with anaerobic digester by Domenico et al., (2015).

e) Presence of extra polymeric substances (EPS). The EBBR biofilms were supported by a network of EPS (Figures 4.6, 4.10 & 4.15) as reported by Anammox investigation by Crouzet et al., (2014); and Fernandez et al., (2008), who used UASB for to study the stages of Anammox bacteria biofilm formation.

These observed similarities in the EBBR results as previously described by other Anammox studies suggested that irrespective of the type of bioreactor or source of the seed organism, Anammox bacteria will develop in any system that provided the right growth conditions; therefore, the EBBR surely must have met these growth requirements.

Conversely, there were distinctions between the EBBR SEM biofilm images and those from the other literature compared above. The major difference in the EBBR SEM analysed biofilm data against the other bioreactors include:

a) Aggregate of cocci-like bacteria cells - while the three morphological types of Anammox bacteria - like cells (rod, cocci in cluster and filamentous bacteria) all developed both the EBBR and the other bioreactor, the cocci-like bacteria in aggregate with around 100 cells (Figure 4.13) and yeast-like cells (Figures 4.14 & 4.15) found in the EBBR were not reported to have developed in the other reactors compared.

These results suggested the EBBR-Anammox ABDite® medium might have supported a more diverse community of Anammox microorganisms; and therefore, was working well.

The research is the very first time that Anammox bacteria granules, Anammox biofilm with cauliflower structure, bioparticles with diverse suspected Anammox bacteria and yeasts-like cells have been developed on the ABDite® biomass medium used only by the EBBR technology (Akhidime and Dempsey, 2009). This result has answered the chapter research question ‘could Anammox biofilm be established on the lab scale EBBR ABDite® biomass support medium? and therefore, represents a major contribution to knowledge by increasing the number of established Anammox biomass support medium.

The implication of the development of Anammox bacteria biofilm on the EBBR ABDite medium is that the ABDite medium has the capacity to provide large surface area to develop and retain Anammox bacteria-like cells that could be useful in future Anammox wastewater treatment process.

4.5 Conclusion

Biofilm formation on EBBR-Anammox ABDite© medium detected by phase contrast microscope (Figure 4.2) was confirmed by SEM micrographs alongside with fully developed bioparticles rich in diverse bacteria – like and yeast cells.

Results from physical examination, PCM and SEM micrographs of biofilm with the cauliflower structure, bioparticles with diverse suspected Anammox bacteria which developed in the EBBR strongly suggested that the conditions within the bioreactor supported the growth of the multispecies microbial communities of the Anammox process. The biofilm and bioparticle development on the ABDite growth medium in turn lend credence to the N-removal data in chapters 2 and 3 as additional evidence that the Anammox process was established in the EBBR.

Results from this research are suggesting that the EBBR technologies could contribute to Anammox wastewater treatment; and the development of Anammox bacterial biofilm, distinct bioparticles and bacterial – like and yeast cells on the EBBR ABDite© biomass growth medium has therefore, made contribution to knowledge.

CHAPTER FIVE

Identification of the Microbial Communities and their Role in the EBBR-Anammox Process

5.0 Research question

What role did the key Anammox microbial communities play at different stages of the lab scale EBBR-Anammox wastewater treatment process with ABDite?

Sub questions

1. What was the role of the Anammox microbial community present in the EBBR?
2. What influence did the presence of each microbial community have on the EBBR-Anammox process?
3. How did the EBBR-Anammox bacterial community on the ABDite bioparticles compared to the communities in the seed granules?
4. Were there variations in the EBBR-Anammox bacterial communities present at different stages of the research; and what was the effect of the variation?
5. Was the Anammox process established in the EBBR, and what does it mean for wastewater treatment if it was?

5.1 Introduction

5.1.1 Source of, and microbial communities of Anammox bacteria seed granules

The anaerobic ammonia oxidizing (Anammox) bacteria are introduced into a bioreactor as seed granules sourced from companies that run such plants, e.g., Severn Trent Water (STW), UK. The mixed microbial communities contained in the seed granules include Anammox bacteria, which convert ammonia and nitrite to nitrogen gas, alongside ammonia oxidizing bacteria (AOB) that partially oxidize ammonia to nitrite. Others are nitrite oxidizing bacteria (NOB), which oxidize nitrite to nitrate; complete ammonia oxidation (Commamox) bacteria, that can metabolize ammonia to nitrate, heterotrophs and yeasts that support the development of stable biofilm on growth support medium (Daims et al., 2015; Guo et al., 2013; Van Niftrik et al., 2012).

5.1.2 Anammox bacteria biofilm development on growth support medium

The Anammox wastewater treatment processes are conducted by any of the five Anammox bacteria genera identified as *Anammoxoglobus, Brocadia, Jettenia, Kuenenia* and *Scalindua* (Daims et al., 2015; Wang et al., 2015). Anammox bacteria can grow either as granules suspended in liquids or as biofilms attached to growth (biomass) support medium or as both types in the same bioreactor. Biomass support materials are used to increase biomass retention because Anammox bacteria grow very slowly (Liu et al., 2021; Van Der Star et al., 2008).

Biomass growth media already used to develop Anammox organisms include volcanic rock, zeolite particles, porous ceramics (Niederdorfer et al., 2021; Dai et al., 2019; Lu et al., 2018; Sharma et al., 2018). However, in the current study, both the EBBR and the microporous ABDite® growth support medium previously used to grow ammonia oxidizing bacteria for total nitrification (Akhidime and Dempsey, 2009) will be used for the first time to develop the biomass for the Anammox synthetic wastewater treatment process. Comparing the EBBR-Anammox microbes to the bacterial communities in the supplied Anammox seed granules will be necessary to determine if the EBBR-Anammox system is able to support the same organisms.

5.1.3 Molecular biology techniques used in the study of Anammox microbial communities

The isolation of a pure culture of Anammox bacteria has been unsuccessful due to their very slow growth rate and stringent growth conditions (Kuenen, 2020; Meng et al., 2017; Wang et al., 2015). Therefore, the detection and identification of the Anammox species in samples collected from natural and engineered environments are limited to the use of molecular biology techniques, including DNA extraction, polymerase chain reaction (PCR), followed by agarose gel electrophoresis (AGE), Sanger and next Generation Sequencing (NGS) (Daims et al., 2015; Wang et al., 2015).

The use of extracted DNA for microbial identification is ideal because DNA has relatively stable characteristics as a fingerprint for specific organisms (Khan, 2015; Beneduce et al., 2007). Advancements in DNA research technology have resulted in a quick and accurate identification and classification of the Anammox microbial communities present in wastewater samples (Li and Gu, 2011; Dale et al., 2009). Molecular techniques for the detection of Anammox bacteria that are based on DNA/RNA include Fluorescence In Situ Hybridization (FISH), which uses specific probes that target either the 16S rRNA genes or the 23S rRNA gene of Anammox

bacteria. FISH is useful for studying the abundance and metabolic activity of Anammox bacteria from environmental samples. Polymerase chain reaction (PCR) using species-specific primers for amplification of sample DNA, followed by phylogenetic analysis is described as the most successful molecular method to detect Anammox bacteria 16S biomarker (Li and Gu, 2011). Species-specific primer-based PCR and quantitative PCR (Q-PCR) methods were used to identify *Brocadia, Kuenenia, Jettenia* and *Scalindua* from estuarine water samples (Dale et al., 2009).

PCR has been used to amplify DNA belonging to key organisms of the Anammox process, detected by species-specific primers. Two highly specific primer sets frequently used for the detection of the 16S rRNA genes of the different Anammox bacteria genera are Amx368F/Amx820R and Brod541F/Amx820R (Wang et al., 2015). The amplified DNA detected by specific primers can be submitted for Sanger sequencing to produce nucleotide sequences that are subsequently used to identify individual bacteria species present in an experimental sample by matching the query sequence with homologous sequences in the Basic Local Alignment Search Tool (BLAST) database (Table 5.1).

One of the challenges of using PCR to detect the DNA of Anammox bacteria is that that the primers used must have high specificity to targeted Anammox bacteria genera, if not, non-Anammox genera such as *Vibrio* could be detected when Anammox DNA is very low in the sample (Li and Guo, 2011). In addition, there is a possibility that even with highly specific primers, the predominant Anammox genera DNA present in a sample could be detected, leaving others behind. Furthermore, an Anammox wastewater treatment bioreactor usually contains genes from Anammox bacteria as well as genes from a variety of other organisms such as AOB, NOB, Commamox, heterotrophic bacteria (HB) and even yeasts (Daims et al*.,* 2015; Guo et al*.,* 2013; Van Niftrik et al*.,* 2012).

The composition and abundance of the microbial communities within an Anammox system may change over time due to several factors, which include operating conditions, competitions, and duration of operation amongst others. He et al.,

(2018) found that 55.18% of Anammox bacteria within a lab-scale Anammox up-flow anaerobic sludge blanket (UASB) reactor was Candidatus *Kuenenia* below 15 ^{o} C. Another limitation of PCR is that although using species-specific primers can produce the expected band size indicating the presence of a bacterial species, or a group, sometimes the PCR product may or may not be suitable for Sanger Sequencing. The use of PCR to amplify DNA, accompanied by Sanger, only gives very limited information of specific groups, and that is very laborious and time consuming; so, to obtain sequences for all organisms, a more robust method such as the NGS is needed.

Next Generation sequencing (NGS) gives a more complete picture of the microbial communities within the system; allowing their DNA sequences to be analysed, rather than targeting specific bacteria (Ciesielski et al., 2018). The need for NGS is not only to identify other types of bacteria, but also to accurately identify the range of Anammox bacteria and their abundance too (Li and Gue, 2011).

In this study, NGS method will be used to identify the microbial communities of the EBBR-Anammox process, and the communities present in the supplied seed Anammox bacteria granules to determine if the EBBR-Anammox system was fully functional and able to sustain the same microbial communities that came in the seed culture; with emphasis on the bacteria that developed on the EBBR ABDite medium (bioparticles).

The EBBR-Anammox investigation will look at extracting DNA from the liquid samples (to check the presence of DNA in the liquid phase), Anammox bacteria granules (AnGBE) and ABDite® bioparticles (the focus of the study) taken from the EBBR to identify all the microbes present in the EBBR, and from the seed Anammox bacteria granules (SAnBG) supplied by STW at various stages during the study. The concentration and purity of extracted sample DNA will be quantified by Nanodrop before downward applications in PCR and Sequencing (Abdel-Latif and Osman 2017; Lucena-Aguilar et al., 2016; O'neill et al., 2011).

5.1.4 Aim

The aim of the research in this chapter (chapter five) is to characterise the bacterial communities and determine their influence on the functioning of the EBBR-Anammox process.

The objectives

1. Extraction and quantification of DNA from the lab scale EBBR samples (ABDite® support medium, granules, and liquid) and the STW supplied seed granules over time
2. Detection of the 16S rRNA of the EBBR- Anammox organisms with species specific primers and DNA amplification by PCR
3. Sanger sequencing of the PCR products from objective 2 to verify the identity of detected bacteria
4. Next generation (NGS) to identify the microbial communities of the EBBR-Anammox to compare with the STW seed granules

5.2 Methods

5.2.1. Extraction and quantification of DNA from the lab scale EBBR-Anammox samples and seed granules.

Template DNA was extracted from ABDite® growth medium with attached biomass, Anammox bacteria granules (AnBGE**),** liquid samples from the EBBR; and from the seed Anammox bacteria granules (SAnBG) supplied by Severn Trent Water (STW) at different points over a period of 24 months. Genomic DNA was extracted from 0.25 g of solid and 250 µL of liquid samples using a Power Soil DNA isolation Kit (Qiagen).

The concentration and purity of the extracted DNA samples were determined using a NanodropTM spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA). The same buffer solution was used for both the DNA extraction and blank for Nanodrop to provide the same pH to maintain consistency in the spectra pattern generated by the Nanodrop analysis (Desjardins and Conklin, 2010).

5.2.2 Detection of the 16S rRNA of the lab scale EBBR- Anammox organisms and DNA amplification by PCR

5.2.1.1 Sources of species-specific oligonucleotide primers

Oligonucleotides, made up of 2-deoxyribonucleotides are the primers used in PCR. The primers for the EBBR-Anammox investigation were selected from primers sets

published in papers that they have successfully used them previously to amplify specific DNA belonging to the organisms that carry out the Anammox process; alongside the PCR reaction conditions and the expected product size (Table 5.1). For example, Amx368F/Amx820R and Brod541F/Amx820R primers are described to be effective for retrieving Anammox bacteria from different environments (Wang et al., 2015).

Table 5.1 Primers and PCR conditions for amplifying DNA extracted from bacteria present in the EBBR-Anammox wastewater treatment process

Primer name	Target	Sequence (5' to 3')	PCR Conditions (annealing temperature °C)	Number of cycles	Expected size (bp)	Reference
Amx368F / Amx820R	All Anammox bacteria *(Kuenenia, Brocadia, Jettenia, etc)*	TTCGCAATGCCCGAAAGG /AAAACCCCTCTACTTAGT GCCC	95 (3mins), 95 (45 sec) **56 (1min),** 72 (1min), **72(10min)**	30 - 32	450	Wang et al., 2015; Dale et al., 2009
Brod541F / Amx820R	Anammox *(Scalindua Brocadia)*	GAGCACGTAGGTGGGTTT GT	95 (5mins), 95(45 sec) **57 (45 sec),** 72(45 sec), **72 (10min)**	32	279	Wang et al., 2015; Han et al., 2013
amoA-1F. / amoA-2IR	Ammonia oxidizers	GGGGTTTCTACTGGTGGT /CCCCTCIGIAAAGCCTTCT TC	94(2 min), 94 (30 sec), 56 (30 sec), 72 (30 sec), **72 (10min)**	30	491	Tago et al., 2015; Shimomura et al., 2012
FGPS872 / FGPS1269	*Nitrobacter*	CTAAAACTCAAAGGAATTG A / TTTTTTGAGATTTGCTAG	94 (45 s) 94 (45s), **50** (30), 72 (120 s), **72 (10 min)**	35	397	Saija et al., 2016;
Nino_amo A_19F / Nino_amo A_252R	Nitrospira inopinata amoA gene	ATAATCAAAGCCGCCAAGT TGC / AACGGCTGACGATAATTG ACC	95 (10 min), 94 (40 s), **52 (40 s),** 72 (45 s) **72** (10min)	43	550	Daims et al.,2015; Pinto et al., 2016
ITS1/ITS2	Yeast	CTT GGT CAT TTA GAG GAA GTA A / GCT GCG TTC TTC ATC GAT GC	94 (5 min) 94 (30s), 55(30 s), 72(30 s), **72 (10 min)**	35	400 - 600	Martin and Rygiewicz, 2005
NTG200F/ NTG840R	Nitrotoga arctica	CTCGCGTTTTCGGAGCGG/ CTAAGGAAGTCTCCTCCC	96 (2 min**), 58 (50 s),** 72 (50 s), **72 (4 min)**	28	-	Alawi et al., 2007
Ar109F/Ar 912rt R	Archaeal ammonia monooxygenase gene	5′-ACK GCT CAG TAA CAC GT-3′) (5′-GTG CTC CCC CGC CAA TTC CTT TA-3′)	94(5 min), 94(30s), **52** (30s), 72 (1min), **72 (5 min)**	30	800	Beckmann et al.,2011

Invitrogen, UK supplied all primers used for this research (Table 5.1). The Basic Local Alignment Search Tool (BLAST) on the National Centre confirmed the specificity of the supplied primers on the Biotechnology Information. (NCBI) website (http://www/ncbi.nlm.nih.gov/ BLAST/) and showed 100% identity with the specific bacteria.

The preparation of the primer stock solution (PSS) followed the methods described by the manufacturer to make up a primer stock at a concentration of 100 µM. The PSS was prepared by centrifuging the pellets at 10,000x g for 10 sec; the specified volume (µL) of molecular grade water (MGW) added and the mixtures vortexed for 5 sec. The primer working stock (PWS) was made by diluting 10 µL PSS in 90 µL (1:10 dilution) MGW to give a final concentration of 10 µM. The solutions were stored at – 20 ^{o}C for subsequent use.

5.2.1.2 Polymerase chain reaction to confirm the presence of targeted bacteria using species-specific primers

Polymerase chain reaction (PCR) was performed for all the primer sets (Table 5.1) to determine the presence or absence of DNA belonging to the key bacterial groups of the EBBR-Anammox process, extracted from the 6 ABDite and 5 granules sampled at various stages of the study. A 20 µL reactions mixture was set up for each DNA/specific primer pair as follows: 10 µL Biomix (Bioline, UK – see details in Table 5.2), 1 µL forward and 1 µL reverse primers (10pmol each), 2 µL of extracted sample DNA. To make up to 20 µL, 6 µL of molecular grade water (MGW) was added.

Negative control tubes containing all the reagents with DNA replaced by MGW were also set up for each primer pair. PCR reactions were carried out using a Quanta Biotech Qcycler II. The amplification of DNA belonging to the different Anammox bacterial groups followed specific conditions for each primer set (Table 5.1).

Table 5. 2: Composition and volume of reagents used for PCR amplification of EBBR-Anammox bacteria DNA

Name of reagent	Composition
Biomix	5U µL $^{-1}$ DNA polymerase, BIOTAQ DNA Polymerase
	1x reaction buffer 10x NH4 Reaction Buffer
	25 mM dNTPs (dNTPs mix)
	1.5 mM $MgCl_2$
Reaction mix (20 µL)	10 µL Biomix
	1 µL Forward primer
	1 µL Reverse primer
	2 µL Extracted sample DNA
	6 µL Molecular grade water

5.2.1.3 Estimation of the size of PCR products using Agarose gel electrophoresis

A 1.5% agarose (Boehringer, Germany) gel was prepared by mixing 0.7 g agarose powder in 50 mL 1x TBE buffer containing 0.1 M Tris Base (Fisher Scientific, UK), 0.1 M boric acid (Fisher BioReagents, UK), and 0.02 M EDTA sodium salt (diaminoethanetetra-acetic acid, Fisher Scientific, UK) dissolved in distilled water.

The mixture was heated in a microwave oven (Hineri Lifestyle, UK) until molten; allowed to cool until hand hot and 3.0 µL of nucleic acid stain, Midori Green (ThermoFisher, UK) added.

The gel was cast in the electrophoresis tank (Jencons, BIO-RAD, UK) and once set, submerged in 1x TBE buffer. To run the gel, 5 µL of post PCR product to which 2 µL of orange G (orange G prepared by dissolving 20g of Sucrose in 40ml water into which was dissolved 100mg of Orange G) loading dye was added. Samples were loaded unto the gel well alongside 5 µL of standard DNA size ladder (HyperLadder™ 100 bp Bioline, UK) and ran in 1x TBE buffer at 80 Volts between 45 min to 1 hour until ladder bands to which 5ul of orange G was added were sufficiently resolved.

The agarose gel electrophoresis results were visualised and documented using a transilluminator (Geneflash Gel Documentation Darkroom, Bio Imaging, UK) under UV light at 490 nm. The PCR product band position on the gel compared to the 100bp ladder (Bioline) was used to estimate the DNA size.

5.2.3 Sanger sequencing of PCR product to confirm the presence of targeted organism in the EBBR- Anammox system

5.2.3.1 Cleaning of PCR products

The PCR products that yielded bands with the expected sizes based on their position on the gel (Wang et al. 2015; Tago et al., 2015; Dale et al. 2009) were cleaned before being sent for Sanger sequencing. The PCR products were cleaned using ExoSAP-IT reagent (Express PCR Product Clean-up, Applied Biosystems, UK) to remove excess primers and dNTPs not incorporated. The ExoSAP -IT protocol involved adding 5 µL of post PCR product to 2 µL ExoSAP-IT reagent in a 0.2 mL PCR tubes. The mixtures were gently vortexed, incubated in a thermal cycler (Quanta, UK) at 37 °C for 4 min; then at 80 °C for 1 min, and finally held at 4 °C.

5.2.3.2 Preparation of PCR products for Sanger sequencing

Each PCR product was prepared separately using either the forward or reverse primers set targeting specific species in the EBBR. The targeted bacteria were Anammox (Amx368F/Amx820R, Brod541F/Amx820R) bacteria, ammonia oxidizer-*Nitrosomonas* (amoA-1F/amoA-2IR); complete ammonia oxidizer- *Nitrospira inopinata* (Nino_amoA_19F/Nino_amoA_252R), nitrite oxidizing bacteria - *Nitrosomonas* (FGPS872 /FGPS1269); and yeast (ITS1 or ITS2).

Samples for Sanger sequencing were prepared by mixing 3 µL of the cleaned PCR product with1 µL of either forward or reverse of species-specific primer; and 6 µL MGW added to give a 10 µL total volume (Table 5.3).

A total of 86 PCR products consisting of EBBR-Anammox ABDite® growth medium (72), EBBR Anammox bacteria granules (10) and STW seed Anammox bacteria granules (4) were sent for Sanger sequencing (Table 5.3). The PCR products were sequenced at the DNA Sanger sequencing Facility at the University of Manchester. The sequencing was performed using BigDye v3.1 terminator run on an ABI 3730 48-well capillary DNA Analyser (Applied Biosystems, California, USA).

Table 5.3: PCR products sent for Sanger sequencing to confirm the presence of targeted bacteria in the EBBR-Anammox system. **Key: ABDite –** ABDite growth medium from EBBR; **AnBGE** - Anammox bacteria granules from EBBR; **SAnBG** seed Anammox bacteria granules supplied by SWT.

Primer	***Amx368F/Amx820R***	***Brod541F/Amx820R***	***amoA-1F/amoA-2IR***	***FGPS87 / FGPS129***	***Nino_amoA_19F/Nino_amoA_252R***	***ITS / ITS2***
ABDite 1	2	2	2	2	2	2
ABDite 2	*2*	*2*	*2*	*2*	*2*	*2*
ABDite 3	*2*	*2*	*2*	*2*	*2*	*2*
ABDite 4	*2*	*2*	*2*	*2*	*2*	*2*
ABDite 5	*2*	*2*	*2*	*2*	*2*	*2*
ABDite 6	*2*	*2*	*2*	*2*	*2*	*2*
AnBGE	*2*	*2*	*2*	*2*	*2*	-
SAnBG	*2*	*2*	-	-	-	-
Total	16	16	14	14	14	12

5.2.3.3 BLAST analysis to identify EBBR-Anammox targeted organisms

The National Centre for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) analysis was conducted to match the EBBR-Anammox nucleotide query nucleotides sequences with sequences in the data base that have the same homology (NCBI 2015; 2019). The targeted bacteria were identified from the list of the top 100 'hits' (organisms that matched the input sequence) with at least 80 % query coverage; and 85 % identity (Hall, 2013).

5.2.3.4 Nucleotide alignment and phylogenetic tree construction to compare the evolutionary relationship of identified bacteria

The nucleotide sequences were aligned using Muscle method inbuilt in the MEGA 11 software (Edgar 2004a, 2004b). The phylogenetic tree analyses to estimate the evolutionary relatedness of the sequences used to identify the EBBR-Anammox bacteria was constructed with MEGA 11; the bootstrap reliability replicates was set at 1000 alongside p-distance and complete deletion methods to complete the tree (Kumar et al., 2021).

5.2.4 Identification of EBBR-Anammox microbial communities by Next generation sequencing

5.2.4.1 EBBR-Anammox samples sent to Eurofins Genomics, Germany for NGS

To identify all the species that make up the microbial communities present in the EBBR-Anammox wastewater treatment system, Next Generation Sequencing (NGS) of DNA samples extracted over time is necessary. Next Generation Sequencing (NGS) was conducted to confirm Sanger sequencing results and identify all the microbial communities of the EBBR-Anammox process to fulfil objective 4. A total of 16 DNA samples consisting of EBBR ABDite bioparticles (6); Liquid (2); Anammox bacteria granules (6) taken from the expanded bed column and seed granules from SWT (2) were submitted for NGS analysis (Table 5.4).

Table 5.4 EBBR-Anammox samples DNA sent for NGS analysis. ***Key:*** **ABDite** – ABDite growth medium removed from the EBBR; **Liquid** - Liquid samples taken from the EBBR **SAnBG** - Seed Anammox bacteria granule supplied by STW**;**

sampling time (month)	Sample number	DNA source	Sample volume (µL)
1	1	ABDite 1	30
	2	Liquid 1	30
	3	SAnBG 2	30
5	4	ABDite 2	30
	5	AnBGE 1	30
	6	AnBGE 2	30
	7	Liquid 2	30
7	8	ABDite 3	30
	9	AnBGE 3	30
13	10	AnBGE 4	30
	11	ABDite 4	30
21	12	ABDite 5	30
	13	AnBGE 5	30
24	14	ABDite 6	30
	15	SAnBG 2	30
	16	AnBGE 6	30

AnBGE – Anammox bacteria granule that developed in the EBBR.

Each sample had a total volume of 30 µL, which contained a range (2.5 - 33.0 ng μL^{-1}) of DNA concentrations due to the different amounts extracted from the sample source. The NGS was performed by Eurofins Genomics Europe Sequencing GmbH, Jakob-Stadler-Platz 7, 78467 Konstanz, Germany under the Project number (**NG-25934**) and Report number (**2022113-1**). The EBBR-Anammox sample DNA analysis were analysed with two NGS primers standard V3V4a for bacteria; and AV3V4a for archaea and bacteria.

5.2.4.2 NGS analysis performed by Eurofins Genomics, Germany

The NGS protocols conducted by the Eurofins company were microbiome analysis including chastity testing, demultiplexing, merged reading performed using FLASH (2.2.00) software, FASTAQ Quality filtering, OTUs identification and taxonomic assignments.

At Eurofins, an operational taxonomic unit (OTU) represents a distinct cluster with significant sequence divergence to any other cluster (Blaxter *et al.,* 2005). The OTUs were identified by employing Shannon entropy MED - an identity-based clustering algorithm. The MED used only the information-rich nucleotide positions across reads to partition the large datasets and omitted stochastic variation. The MED procedure identified and filtered random "noise" in the dataset, i.e., sequences with a very low abundance (less than 0.02% of the average sample size).

To assign taxonomy to the identified OTUs, DC-MEGABLAST alignments of cluster representative sequences to the database sequence was performed by Eurofins. A most specific taxonomic assignment for each OTU was then transferred from the set of best-matching reference sequences (lowest common taxonomic unit of all best hits). A sequence identity of 70% across at least 80% of the representative sequence was the minimal requirement for considering reference sequences. The company performed further processing of OTUs and taxonomic assignments using the QIIME software package (version 1.9.1, http://qiime.org/). Abundances of bacteria and archaea taxonomic units were normalized using lineage-specific copy

numbers of the relevant marker genes to improve estimates accuracy (Angly, 2014).

5.3 Results

The molecular analysis of the EBBR-Anammox process samples was aimed at identifying the targeted Anammox bacteria and the communities of organisms present within the EBBR. The results reported in the same order as the objectives include that of DNA extraction and quantification by Nanodrop, PCR amplification using species specific primers to detect presence of targeted groups; followed by Sanger sequencing with BLAST analysis to confirm the identify of those bacteria expected to be present using specific primers sets. Finally, next generation sequencing (NGS) was conducted for the identification of entire organisms within the EBBR-Anammox system, whether they were in the Anammox process or not.

5.3.1 Nanodrop quantified concentration and quality of EBBR-Anammox bacteria DNA

Sampling for DNA extraction was carried out at specific points to detect the presence or absence of the different microbial communities within the EBBR-Anammox system, as the composition might change over time. At each sampling point, for 24 months, DNA was extracted in duplicates using 0.25 g of EBBR ABDite® growth medium, Anammox bacteria granules (AnBGE), liquid (250 µL), and from the seed Anammox bacteria granules (SAnBG) supplied by the Severn Trent Water (SWT). Extracted DNA templates were quantified by Nanodrop 2000 to determine the concentration and purity (Table 5.5).

The DNA quantification result suggested that the seed granules (SAnBG) yielded the highest amount (33.0 ng μL^{-1}) of DNA compared to the 15.6 ng μL^{-1} (AnBGE) and 6.2 ng μL^{-1} obtained from the ABDite growth support medium. The DNA extracted from the SAnBG contained about 60 % more microbial DNA compared to the total (21.8 ng μL^{-1}) DNA from the EBBR samples (AnBGE and ABDite).

Table 5.5: Concentration of DNA in EBBR-Anammox samples quantified by Nanodrop **Key: SAnBG** - Seed Anammox bacteria granule supplied by STW; **ABDite** – ABDite growth medium removed from the EBBR; A**nBGE** – Anammox bacteria granule that developed in the EBBR; **Liquid** - Liquid samples taken from the EBBR. (Each data point represents a mean of 2 replicate samples (n=2); and error bars represent +/- 1SD of the mean).

Sample ID	***Sampling time (month)***						
	1	5	7	13	21	24	Mean DNA concentration (ng μL^{-1}) ± STD
SAnBG	32.9	32.3	33.4	33.4	-	-	33.0 ±0.5
ABDite	4.9	6.1	7.2	9.8	4.6	4.4	6.2 ± 2.1
AnBGE	-	5.7	19.4	31.2	14.0	7.8	15.6 ± 10.2
Liquid	2.5	-1.1	- 0.7	1.2	-	-	1.9 ± 0.9

However, the 6.2 ng μL^{-1} DNA extracted from the EBBR-Anammox biomass attached on the ABDite medium (bioparticles) provided evidence that the EBBR ABDite growth medium supported the development of biomass, which might be from the bacteria expected in Anammox wastewater treatment (Table 5.4).

On the contrary, only small amount of DNA (1.9 ng μL^{-1}) was quantified from the liquid samples, which might be contaminant, as DNA was not detected in the other

samples. The low levels of DNA in liquid samples could also be due to the small volume (250 µL) of liquid used, which was specified by manufacturer of the kit for DNA extraction from liquid samples. The overall absence of DNA from liquid samples and presence on the EBBR granules and ABDite medium indicated that the Anammox biomass were retained on the ABDite® particles and granules, instead of floating in the liquid phase. DNA extraction from the ABDite growth medium is a confirmation of the results obtained using phase contrast (Figures 4.2) and scanning electron microscopy analysis (Figures 4. 3 – 4.10); both sets of results revealed the development of Anammox microbial biomass in the pores and surfaces of the ABDite® growth support medium.

Comparatively, there were variations in the concentration of DNA extracted from the ABDite® growth support medium over the 24-months. The result suggested that DNA concentration steadily increased from 4.5 from the 1st month to 9.4 ng µL^{-1} in 13 months. The 52 % increase in DNA concentration indicated stability for biomass development within the bioreactor that resulted in the increase. Then, there was a decrease in DNA concentration between 21 – 24 months, a suggestion that the growth medium was less colonised; possibly caused by changes in process conditions within the EBBR column, hence, the low DNA yield (Table 5.4).

The purity (quality) of the extracted DNA samples quantified by Nanodrop; and expressed as the ratio of 260/280 nm was within the range (1.2 – 2) expected of clean DNA samples (Abdel-Latif and Osman 2017; Lucena-Aguilar et al., 2016; O'neill et al., 2011).

The quantification of the extracted DNA from the EBBR-Anammox ABDite, AnBGE, liquid and SAnBG by Nanodrop technique fulfilled objective 1of the chapter.

5.3.2 Detection of targeted EBBR- Anammox bacteria 16S rRNA from PCR products

The major groups of bacteria targeted with species specific primers in the EBBR-Anammox system were the anaerobic ammonia oxidizing bacteria (AnAOB), aerobic ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Other communities targeted were ammonia oxidizing archaea (AOA), complete ammonia oxidizing bacteria (Commamox) and yeasts (Table 5.1). The polymerase chain reaction (PCR), followed by agarose gel electrophoresis (AGE) revealed that the right DNA bands with the right size expected from most of the targeted species were amplified with species specific primers (Figures 5.1 - 5.5).

5.3.2.1 Presence of anaerobic ammonia oxidizing (Anammox) bacteria DNA in the EBBR

The two primer sets, Amx368F/Amx820R and primer Brod541F/ Amx820R detected the presence of Anammox bacteria DNA from ABDite® medium sampled within a 24-month period (Figures 5.1 and 5.4). The Amx368F/Amx820R primer targeting any member of the Anammox bacteria genera (*Candidatus* - *Anammoxoglobus, Brocadia, Jettenia, Kuenenia* and *Scalindua)* detected the right size of DNA bands from AnAOB (Lanes 2, 4, 6 and 8) extracted from 4 ABDite samples between 1-13 months (Figure 5.1). The right sized DNA bands belonging either to *Candidatus Brocadia* or *Scalindua* was detected with Brod541F/ Amx820R pair from ABDite 1, 2 and 3 in lanes 3, 5 and 7 respectively (Figure 5.1).

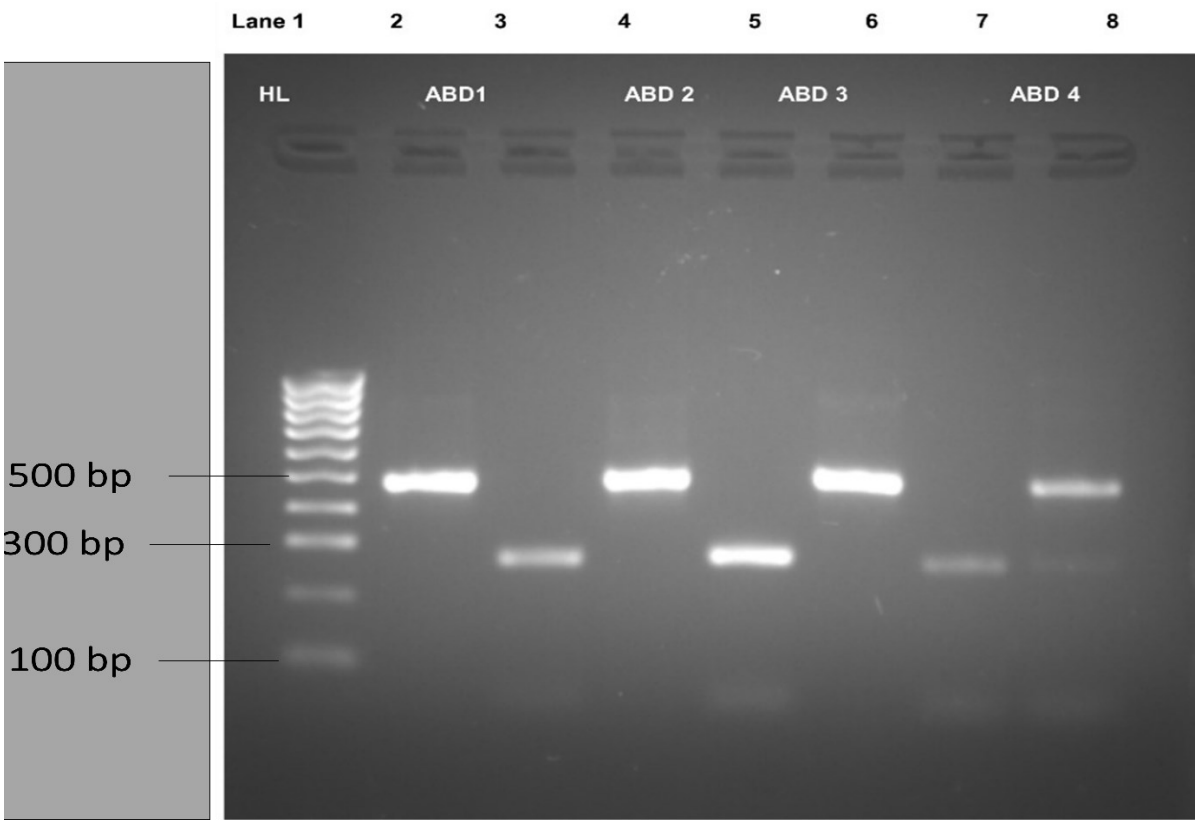

Figure 5.1: Gel image of amplified Anammox bacteria DNA extracted from ABDite® medium 1, 2, 3, and 4 sampled over a period of 13 months, and detected using two Anammox bacteria species-specific primers, Amx368F / Amx820R and Brod541F / Amx820R. **Key** ***Lane*** 1: 100 bp hyper Ladder; ABD1 to ABD 4 → ABDite medium; **Lanes** 2, 4, 6 & 8 are DNA bands detected with Amx368F/ Amx820R any Anammox bacteria (*Candidatus - Anammoxoglobus, Brocadia, Jettenia, Kuenenia* and *Scalindua*). **Lanes** 3, 5 & 7 are DNA bands detected with Brod541F/ Amx820R for either *Brocadia or Scalindua*. **Lanes** 2 and 3 are DNA from ABDite medium sampled in the 1st month; **Lanes** 4 and 5 (5th month); **Lanes** 6 and 7 (7th month) and **Lanes** 8 (13th month).

The size of the DNA estimated from the gel photographs and standard curve indicated that they were within the expected range for those targeted Anammox bacteria detected by the specific primers. The size of the DNA obtained with Amx368F/Amx820R was between 475 – 480 bp; and 280 bp for the DNA detected by Brod541F/ Amx820R primer pair (Figure 5.1 and Table 5.6).

Table 5.6 Sizes of specific Anammox bacteria DNA estimated from amplified PCR product band on agarose gel. ***Key***: **Anammox** (anaerobic ammonia oxidizing bacteria); **AOB** (ammonia oxidizing bacteria); **NOB** (Nitrite oxidizing bacteria); **Commamox** (complete ammonia oxidizing bacteria); **Ca.** (Candidatus).

Primer set	Bacterial group	Expected size of DNA (bp)	Estimated size of DNA (bp)
amoA-1F / **amoA-2R**	*Nitrosomonas* **(AOB)**	491	490
Amx368F/ **Amx820R**	Ca. *Brocadia,* Ca. *Kuenenia* **(Anammox)**	450 - 480	475 - 480
Brod541F/ **Amx820R**	Ca *Brocadia,* Ca. *Scalindua* **(Anammox)**	279	280
FGPS 872 / **FGPS 1269**	*Nitrobacter* **(NOB)**	397	395
Nino_amoA_19F /Nino_amoA_252R	*Nitrospira inopinata* **(Commamox)**	550	550
comaA244F/ **comaA-659R**	*Nitrospira inopinata* **(Commamox)**	550	555

5.3.2.2 Presence of ammonia oxidizing bacteria (AOB) DNA in the EBBR

The DNA of AOB was found in four EBBR-Anammox ABDite (ABD 1- ABD 4) medium extracted within the first 13 months; and detected using amoA-1F/ amoA-2IR, which targeted the ammonia monooxygenase gene of *Nitrosomonas* (Figure

5.2). The estimated size of AOB DNA from the lab scale a EBBR was estimated to be 490 bp, and within that expected size range (Figures 5.2 and 5.4; Table 5.6).

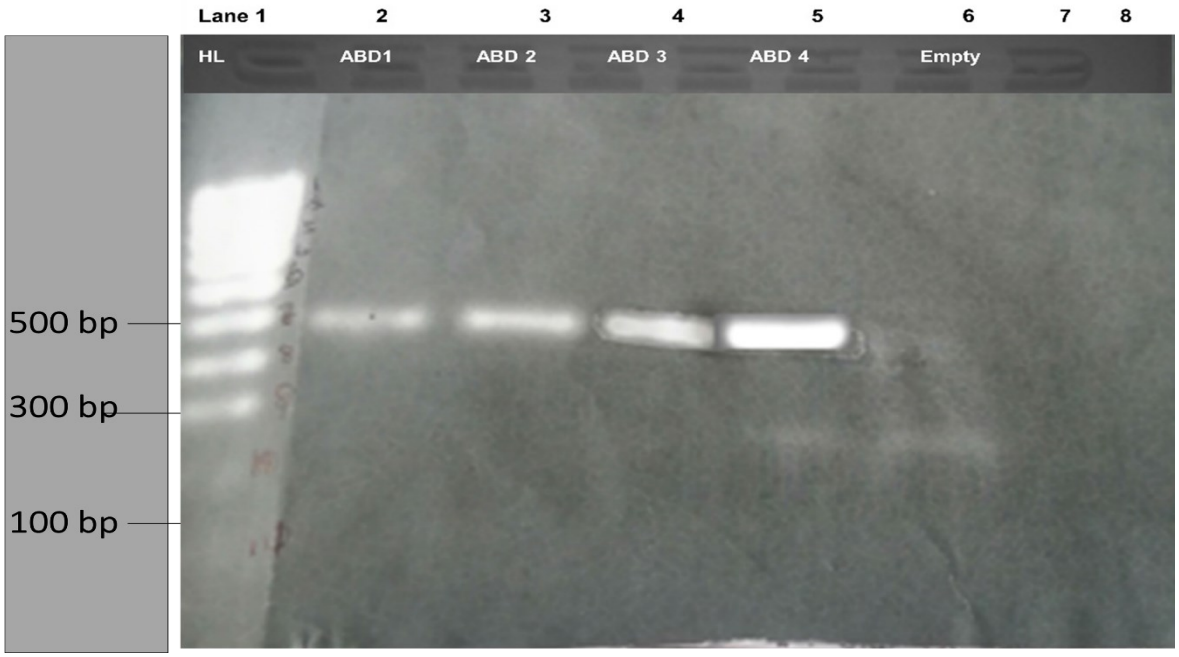

Figure 5.2: Gel image of amplified ammonia oxidizing bacteria DNA extracted from ABDite® medium 1, 2, 3, and 4 sampled over a period of 13 months, and detected using amoA-1F/ amoA-2IR. **Key:** The position of ammonia oxidizing bacteria DNA band from ABDite medium 1- 4 extracted at different sampling points are shown on gels as follow: ***Lane*** 1: 100 bp hyper Ladder; **ABD→** ABDite growth medium; ***Lanes*** 6 - 8: Empty; ***Lanes*** 2- ABDite 1 (1st month); ***Lane*** 3 - ABDite 2 (5th month); ***Lanes*** 4- ABDite 3 (7th month); ***Lanes*** 5 - ABDite 4 (13th month)

5.3.2.3 Presence of complete ammonia oxidizing bacteria (Commamox) DNA in the EBBR

Gel electrophoresis analysis of post PCR products revealed the detection of the *Nitrospira inopinata* (Commamox) DNA with two primer pairs, Nino_amoA_252R 9F and comaA-244F/ comaA-659R; extracted from ABDite® medium (1 - 6) over a period of 24 months (Figures 5.3a and 5.3b and 5.4).

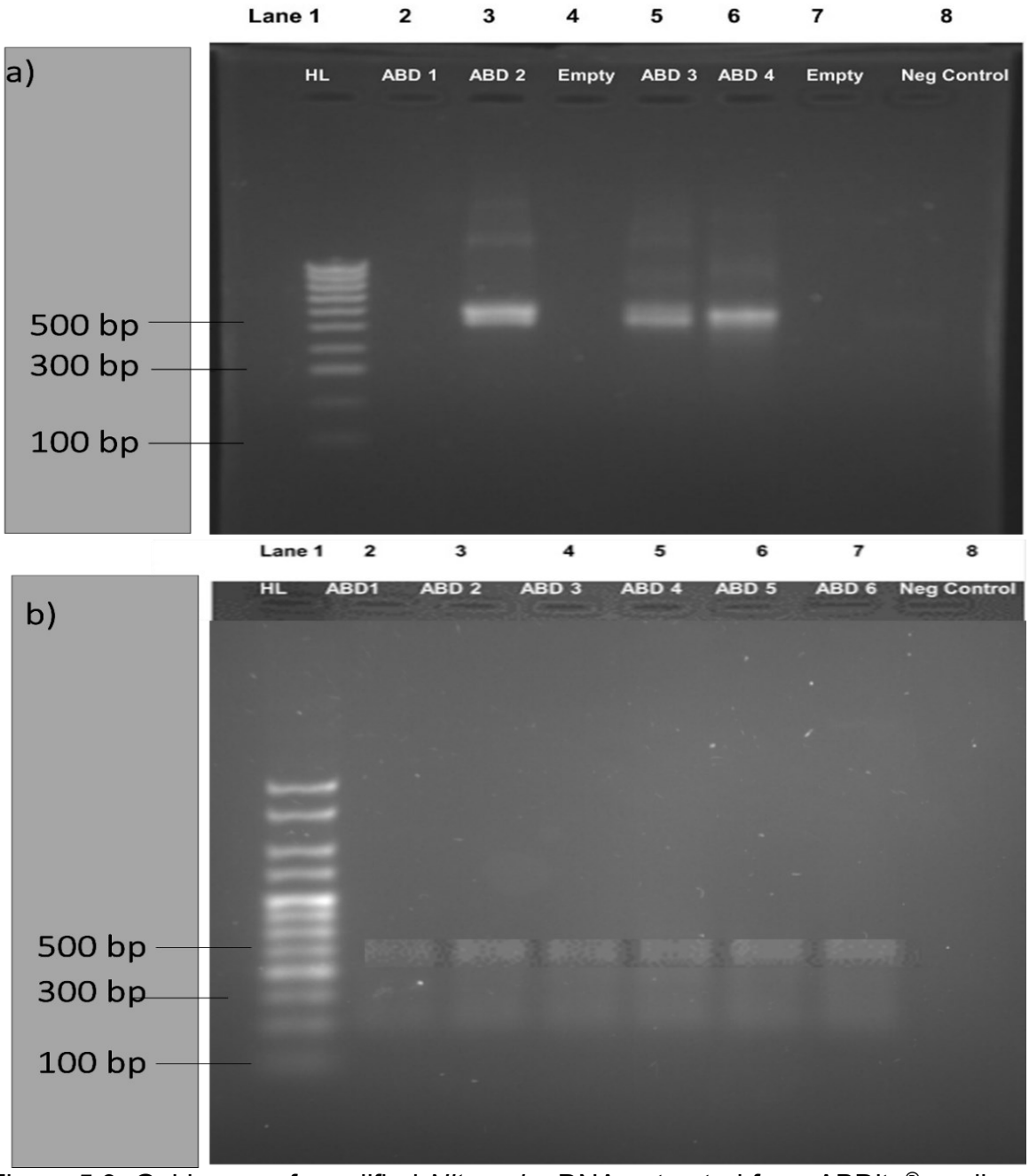

Figure 5.3: Gel image of amplified *Nitrospira* DNA extracted from ABDite® medium 1 – 6; and detected using (**a**) Nino_amoA_1 / Nino_amoA_252R 9F (1- 13 months) and (**b**) comaA-244F / comaA-659R)1- 24 months). ***Key:*** **HL** → 100bp hyper Ladder; **ABD**→ ABDite medium***; Lane*** 8: Negative control; Nitrospira DNA from ABDite medium extracted at different sampling points are shown on the gels as follow: **a) (Nino_amoA_1 / Nino_amoA_252R 9F); Lanes** 4 & 7: Empty; **Lane 2**: ABDite 1(1st month, DNA band was not detected); ***Lane*** 3: ABDite 2 (5th month); **Lanes 5** ABDite 4 (7th month); **Lanes 5:** ABDite 4 (13th month). **b) (comaA-244F / comaA-659R)** ***Lane*** 2: ABDite 1 (1st month); **Lane 3**: ABDite 2 (5th month); ***Lanes*** 4 ABDite 3 (7th month); **Lanes 5** ABDite 4 (13th month); ***Lanes*** 6 ABDite 5 (21st month); ***Lanes*** 7: ABDite 6 (24th month).

Using Nino_amoA_1 / Nino_amoA_252R 9F primer, *Nitrospira inopinata* DNA was not present in ABDite 1 sampled in the 1st month but detected in ABDite 2- 4 sampled between 5 – 13 months (Figure 5.3a) and in ABDite 5 & 6; 21- 24 months later (Figure 5.4). *Nitrospira inopinata* DNA was also recovered from the same ABDite medium 1-6 samples using comaA-244F/ comaA-659R (Figure 5.3b). The PCR gel image indicated that comaA-244F/ comaA-659R was more effective at specifying *Nitrospira inopinata* DNA from the EBBR-Anammox samples. The size of *Nitrospira* DNA was between 550 - 555 (Figures 5.2c and 5.2d, 5.3b and 5.3c) in line with the expected size (Table 5.6).

5.3.2.4 Presence of nitrite oxidizing bacteria (NOB) DNA in the EBBR

From the PCR gel image indicated that the DNA of *Nitrobacter (*NOB) was detected in ABDite 5 sampled 21 months into the EBBR-Anammox study using FGPS 872 and FGPS 1269 primer set alongside DNA for other Anammox bacteria species (Figure 5. 4; Table 5.6).

The detection of *Nitrobacter* DNA only after 21months suggested that the operating conditions within the EBBR effectively inhibited the growth of the NOB for most of the research duration. While the ABDite 5 DNA band in lane 6 was estimated to have a size of 395 bp, which is within the expected size, ABDite 6 sample ran on lane 12 did not yield a positive result. (Tables 5.1 and 5.6).

On the same gel, the presence of the DNA from other targeted bacterial groups were also detected from the same ABDite 5 and 6 samples taken at 21 and 24 months. The DNA belonging to Anammox bacteria was detected in L3 and L9, (Amx368F/ Amx820R; and L4 and L10 (Brod541F/ Amx820R); and AOB (L5 and L11, amoA-1F and amoA-2R). Both AnAOB ad AOB have been amplified previously from ABDite 1 – ABDite 4 medium sampled within 1- 13 months (Figures 5.1 and 5.2). The DNA of the same bacteria were also present in ABDite 5 & 6 sampled 21- 24 months.

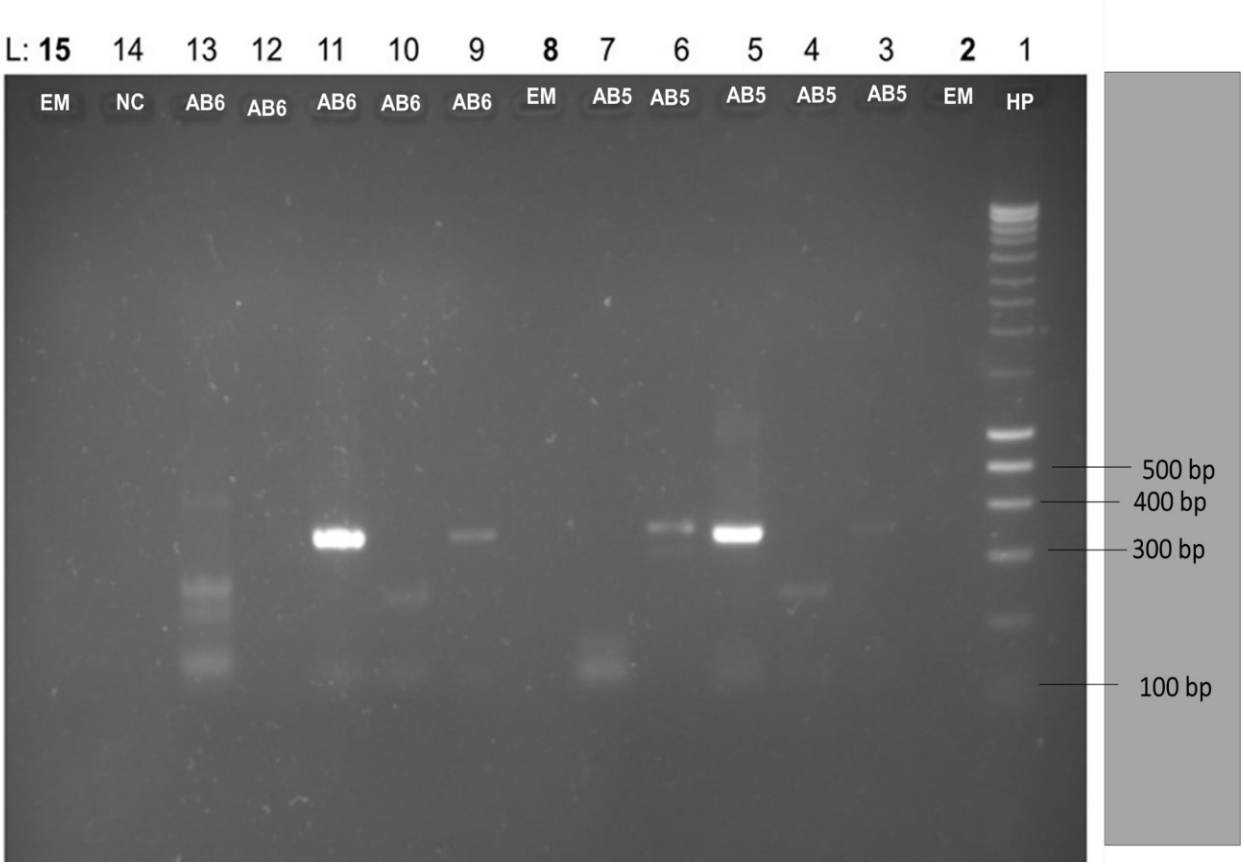

Figure 5.4: Gel image of PCR amplified DNA bands from ABDite 5 sampled at 21 month and ABDite 6 (24 month); detected using species specific primers for targeted bacterial groups of the EBBR-Anammox process. **Key: Lane 1**: HP 100 bp hyper Ladder; **L2, 8** & **15**: empty (**EM**); **L14**: Negative control (**NC**); **L3 - L7**:(ABDite **5)**; **L8 - L3** (ABDite **6**); **L3** & **9:** Anammox bacteria primer Amx368F/ Amx820R- targeting any Anammox bacteria species; **L4** & **10:** Anammox bacteria primer Brod541F/ Amx820R – targeting Anammox bacteria, (*Brocadia* & *Scalindua***); L5** & **11**: Ammonia oxidizing bacteria primer amoA-1F and amoA-2R targeting *Nitrosomonas;* **L6** & **12**: Nitrite oxidizing bacteria primer FGPS 872 /FGPS 1269 targeting -*Nitrobacte*r species**; L7** & **13**: Nino_amoA_1/ Nino_amoA_252R 9F targeting *Nitrospira inopinata*.

These results suggested that these two communities of bacteria were present in all the EBBR-Anammox samples analysed (Figure 5.1 & 5.4); and therefore, were in abundance in the EBBR-Anammox system

Also shown on the same gel (Figure 5.4) were DNA bands of *Nitrospira inopinata* in L7 (ABDite 5) and L13 (ABDite 6), amplified using amoA_1/ Nino_amoA_252R 9F primer set. Although this bacterium DNA could not be retrieved from ABDite 1 taken in the first

month of the study (Figure using the same primer (5.3a); it was subsequently detected consistently in all the 6 ABDite samples when comaA-244F / comaA-659R primers within the 24 months (Figure 5.3b). The high presence of *Nitrospira*, capable of complete oxidation of ammonia to nitrate in the reactor, could contribute to increase in nitrate concentration, which can reduce the effectiveness of the Anammox process.

In addition, the DNA of nitrite oxidizing bacteria (NOB) was detected in ABDite 5 in L6 (Figure 5.4). This result indicated that the growth of the NOB within the bioreactor was limited; and therefore, unable outcompete with the Anammox bacteria for nitrite, needed by the Anammox bacteria to convert ammonia to nitrogen gas.

Additionally, yeast amplified DNA were found in 5 EBBR-Anammox ABDite® medium sampled within the 24 months (Figure 5.5) The DNA was detected by the fungal universal primer, ITS1/ITS2 alongside *Saccharomyces cerevisiae* DNA used as the positive control (Lane 2). The post PCR product yielded two DNA bands with sizes estimated to be between 302 - 447 bp; the size was within the 300 – 450 bp, where the expected size range for yeasts DNA (Table 5.7).

Table 5.7 Size of yeast DNA estimated from amplified PCR product band on agarose

Primer set	**Microbial group**	**Expected size of DNA (bp)**	**Estimated size of DNA (bp)**
	Yeast		
ITS1 /ITS2	Saccharomyces cerevisiae	450	447
	Yeast	300 - 450	301 – 447

gel

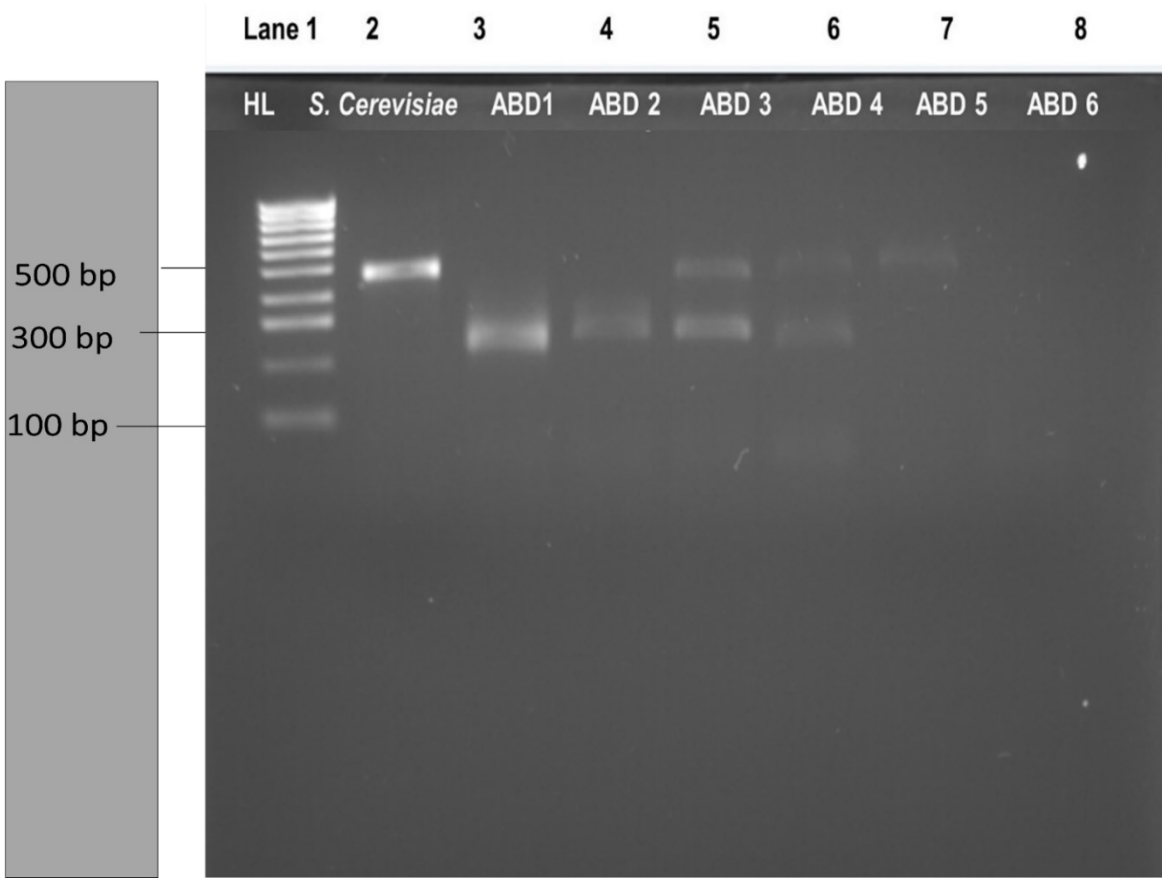

Figure 5.5: Gel image of PCR amplified yeast DNA bands from 6 ABDite the EBBR-Anammox process sampled within 24 month and detected using yeast universal primers in ITS1 /ITS2. **Key:** ***Lane* 1**: 100 bp hyper Ladder; ***L2:*** positive control (*Saccharomyces cerevisiae*); ***L*3:** ABDite 1 (1st month); ***L***4: ABDite 2 (5th month); ***L5*** ABDite 3 (7th month); ***L6***: ABDite 4 (13th month); ***L7*** ABDite 5 (21st month); ***L8***: ABDite 6 (24th month, DNA band was not detected).

The detection of yeast DNA in the EBBR-Anammox system suggested they coexistence alongside the other Anammox bacteria communities within the EBBR column; thereby making yeast is a member of the microbial communities of the EBBR-Anammox process, which is linked to objective 4 of the chapter.

The visualisation of the PCR products by gel electrophoretic analysis confirmed the presence of DNA from Anammox bacteria, AOB, Commamox, NOB; in addition to and yeast as communities within the EBBR. Unfortunately, using NTG200F/ NTG840R and (Ar109F/ Ar912rt R) respectively, the DNA of *Nitrotoga arctica* and Archaeal ammonia monooxygenase genes were not quantified from any of the 6 EBBR-Anammox ABDite samples analysed over the 24 – month period. The lack of DNA detection could be because these microbial groups were either absent or low in the abundance within the bioreactor (Figures 5.1 and 5.6; Table 5.7). This premise will be confirmed by conducting next generation sequence (NGS), which could detect organisms low in abundance within a bioreactor.

The confirmation of the presence or absence of DNA from bacterial groups in the EBBR targeted with species-specific primers for PCR amplification has fulfilled objective 2 of chapter 5.

5.3.3 Confirmation of the presence of targeted EBBR-Anammox bacterial groups from Sanger sequenced nucleotides

To fulfil objective 3, Sanger sequencing of EBBR-Anammox sample PCR products was carried out to obtain nucleotide sequences expected to belong to the targeted bacteria species in the bioreactor. The Sanger sequenced nucleotides were used to further identify the bacteria those sequences belonged to; and thereby, confirming their presence in the EBBR-Anammox system.

Out of a total of 86 samples were sent for Sanger sequencing, 72 samples came from the ABDite 1- 6 growth medium; the main focus of the research prepared using either the forward or reverse of the species-specific primers - Anammox/ Amx368F & Amx820R or Brod541F & Amx820R (Anammox); amoA-1F & amoA-2R (AOB); Nino_amoA_252R 9F (Commamox); FGPS 872 & FGPS 1269 (NOB); and yeast universal primer ITS1 & ITS2. Using the same primers, 10 AnBGE and 4 SAnBG from STW were Sanger sequenced for comparison (Table 5.3, Tables 5.8-5.12.).

5.3.3.1 Presence of anaerobic ammonia oxidizing bacteria (Anammox) confirmed in the EBBR-Anammox system

The Sanger sequencing generated nucleotide belonging to Anammox bactera using either the forward or reverse of Amx368F/Amx820R, which targeted genera (*Anammoxoglobus, Brocadia, Jettenia, Kuenenia* and *Scalindua*) of Anammox bacteria (Figure 5.8a). With the Amx368F/Amx820R primer pair, nucleotide sequences were obtained from ABDite 1- 6 growth medium, AnBGE and SAnBG PCR products. With Brod541F/ Amx820R, specific for Scalindua and *Brocadia,* sequences were generated from all sample except for ABDite 5 that yielded no results (Table 5.8b). The role of anaerobic ammonia oxidizing (Anammox) bacteria is to convert wastewater ammonia and nitrite to nitrogen gas. The generation of Anammox bacteria nucleotides from almost all the post PCR samples submitted for sequencing with both primers, suggested a high presence of the Anammox bacteria among the EBBR microbial communities

Table 5.8a EBBR Anaerobic ammonia oxidizing (Anammox) bacteria Sanger sequenced nucleotides. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW; (**F/R**) - Forward or Reverse primers.

Sample	Sequence (Amx**368F**/Amx820R)
ABDite 1 (F)	CCACCTGCCTTTTTTAANNCNNNGGGGATGACGGGGTTTTATACCTTCGGGTTAGTAACCTANNTGTCGGGAGTTAGGAAATGCATANGCGTTAATAGCTTTCTTGCTTGACTAAGG CTCCAGAGGAAGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCCTTGCAAGTCAGT TGTGAAAGCCTTCCGCTTAACGGAAGAACGGCATCTGATACTACAGGGCTTGAGTACGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAA CGCCGGCGGCGAAGGCGACTCTCTGGTCCAAAACTGACGCTGAGTGTGCGAAAGCAAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGGGCACTAA
ABDite 2 (R)	GAAATTTNCTGGATACGGGGGTATCTAATCCCGTTTGCTCCCCTAGCTTTCGCACACTCAGCGTCAGTTATGGACCAGAGAGTCGCCTTCGCCGCCGGTGTTCCTTCTGATATCTA CGCATTTCACCGCTCCACCAGAAGTTCCACTCTCCCCTCCCACACTCAAGCTATGCAGTATCGGATGCCGTTCTTCCGTTGAGCGGAAGGCTTTCACAACCGACTTACACAGCCGC CTACTGTACGCCCAATAATTCCGAACAACGCTTGCCGCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGTGGCTTCCTCCGGAGCCTTAGTCAAGCAAGAGCGCTATTA
ABDite 3 (F)	AATAACCTTTGGGGGAAAAGGCCTTCGGGTTGTAACCACTGTCGGGAGTTAGGAAATGCAGGTGCGTTAATAGCGCACTTGCTTGACTAAGGCTCCAGAGGAAGCCACGGCTAAC TCTGTGCCAGCAGCCGCGGTAATACAGAGGCGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCCTTGCAAGTCAGTTGTGAAAGCCTTCCGCTTAACGGA AGAACGGCATCTGATACTACAGGGCTTGAGTACGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACGCCGGCGGCGAAAGCGACTCTCT
ABDite4 (F)	CACCTACTAANCTTTGNGGGAGTAGGCCTTCGGGTTGTAACCACTGTCGGGAGTTANGAAATGCAGNTGCGTTAATAGCGCACTTGCTTGACTAAGGCTCCAGAGGAAGCCACGG CTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCCGTGCAAGTCAGTTGTGAAAGCCTTCCGCTTA ACGGAGAACGGCATCTGATACTACAGGGCTTGAGTACGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACGCCGGCGGCGAAAGCGAC
ABDite 5	TGGGGGAAACCNNCNNGCGATTATNGGGTTTTATNCTTCGGGTTGTAACCGCTGTCGGGGGAGTTAAGAAATGCAAGATGTTAANCCTTTACTTGCTTGACTAAGGCTCCGGAGGA AGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCNNTGTAAGTCAGTTGTGAAAGCC TTCCGCTNAACGAAAGAAACGGCATCTGATACTNCATAGCTTGAGTNNGGAAGGGGAAAAAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACACCGGCG GCGAAAGCAACTCTCTGGTCCNNAACTGACGCTAAGTGTGCGAAAGCTAGGGAAGCAAACGGGATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGGGCACTAAGTAGAAGG
ABDite 6 (F)	ACCTAGNGNCTCNGGGGAGAGGCCTTCGGGTTGTAACCACTGTCGGGAGTTAGGAAATGCAGGTGCGTTAATAGCGCACTTGCTTGACTAAGGCTCCAGAGGAAGCCACGGCTA ACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCCTTGCAAGTCAGTTGTGAAAGCCTTCCGCTTAACG GAAGAACGGCATCTGATACTACAGGGCTTGAGTACGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACGCCGGCGGCGAAAGCGACTCT CTGGTCCGAAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGGGCACTA
AnBGE (F)	CCCTAAAAACCTTTAGGTGNGAGAAGCCTTCGGGTTGTAACCAGCTGTCGGGAGTTAAGAAATGCANGTGCGTTAATAGCGCACTTGCTTGACTAAGGCTCCAGAGGAAGCCACG GCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCCGTGCAAGTCAGTTGTGAAAGCCTTCCGCT TAACGGAAGAACGGCATCTGATACTGCAGGGCTTGAGTACGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACGCCGGCGGCGAAAGC
SAnBG (R)	GCGTACGNGCAGTCGACGAAGGAGCATCCGTATCGGATGCTAACCTGGTGGCGTAGTGCTGAGTAATACATTGATCACCTACCTCCTTATGGGAATAACAACGTTCCGGAACTTG GGGCTACCAATGCGGTTGCTAATACTGGATAAAATTATTGTTGCTTCGGCAAGAATAGTCAAATACGCGGAATCGAAAGATTCCGCTTGCTTGAAAAGGGGTCAATGTCCTATCAGC TAGTTGGGGGGGTAATAGCCCACCAAGGCCAATACGGGTAACCGGCCTGACAGGGTGGTCGGCCACGCTGGGACTGAGATACTGGCCAGACGCCTACGGGAGGCTGCAATCAA GAATCTTTGGCAATGCCCGAAAGGGTGACTAAACGACCCCGCGTGAGGGAGGAAAGCCTTCGGGTTGTAAACCGCTGTCGGGAGTTAAGAAATGCAAGAATGTTAATACCGTTCT

Table 5.8b: EBBR Anaerobic ammonia oxidizing (Anammox) bacteria Sanger sequenced nucleotides. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW; (**F/R**) - Forward or Reverse primers

Sample	Sequence (Brod541F/ Amx820R)
ABDite 1 (F)	TTTAGTTNATTTAGACCGCCTCCGAATACTGCATAGCTTGAGTGNGGGAGGAGGGAGTGGANNTTCTGGTGGAGCGGTGAAATGCGTAGAT ATCAGAAGGAACACCCGGCGGCGAAGGCGACTCTTCTGGTCCATAACTGGACGCTGAGTGTGCGAACAGCAAGAGGAGCAAACGGGATTA GATACCCCGGTAGTCCTAGCCGTAAACGATGTGCACTAAGTAGAAGAGGTTCTNAAA
ABDite 2 (R)	GGAATTGGCTGANTANCGGGGTATCTAATCCCGTTTGCTCCCCTAGCTTTCGCACACTCAGCGTCAGTTATGGACCAGAGAGTCGCCTTCG CCGCCGGTGTTCCTTCTGATATCTACGCATTTCACCGCTCCACCAGAAGTTCCACTCTCCCCTCCCACACTCAAGCTATGCAGTATCGGATG CCGTTCTTCCGTTGAGCGGAAGGCTTTCACAACCGACTTACAAACCCACCTACGTGCTCA
ABDite 3 (F)	GGGGGGTCGANTCACGGAAGACGGCATCCGATACTGCATAGCTTGAGTGTGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATG CGTAGATATCAGAAGGAACACCGGCGGCGAAGGCGACTCTCTGGTCCATAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGG ATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGGGCACTAAGTAGAGGGGTTTTAA
ABDite 4 (F)	CGGNAGACGGCATCCGATACTGCATAGCTTGAGTGTGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAG GAACACCGGCGGCGAAGGCGACTCTCTGGTCCATAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGT AGTCCTAGCCGTAAACGATGGGCACTAAGTAGAGGGGTTTTAAAA
ABDite 5	None
ABDite 6 (F)	TNGACGGCATCNNGATACTGCATAGCTTGAGTGTGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGA ACACCGGCGGCGAAGGCGACTCTCTGGTCCATAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAG TCC
AnBGE (F)	CGGGANNTNTACGGGTTTTNGACGGCATCCGATACTGCATAGCTTGAGTGTGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATG CGTAGATATCAGAAGGAACACCGGCGGCGAAGGCGACTCTCTGGTCCATAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGG ATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGGGCACTAAGTAGAGGGGTTTTAAT
SAnBG (F)	CTGAGGCTCAGTCATTCTNACGCTATTAAATTCTTGCATTTCTTAAATCCCAATTTCCGTTTACACCCCCAAGGCCTTCCTCCCGCCGCGCTG TCCCTTCCTCACCCTTTCTGGCATTGCTAAAGATTCGCGACTGCGCCCTCCCGTAGGAGTCGGGGCAGTACCTCAGTCCCAGTGTGGCCG ACCACCCTCTCAGGCCGGCTACCCGTCTTCCCCTTGGTGGGCTATTACCCCACCAACTAGCTGATAGGACACTGACCCCTCTTCAACCAAG CGGAATCTTTCCACTCCGC

5.3.3.2 Presence of ammonia oxidizing bacteria (AOB) confirmed in the EBBR-Anammox system

The presence of the targeted AOB, N*itrosomonas* species in the EBBR was confirmed by the nucleotide sequences produced from EBBR ABDite and AnBGE PCR products samples prepared either with the forward or reverse of amoA-1F & amoA-2R primer (Table 5.9). Only ABDite 5 and SAnBG did not produce sequences from the post PCR products used for Sanger. The generation of nucleotide sequences belonging to ammonia oxidizing bacteria from the EBBR-Anammox samples confirmed their presence and suggested that the ammonia oxidizers, that partially convert ammonia supplied in the wastewater to nitrite was a major contributor to the microbial communities of the EBBR-Anammox process.

Table 5.9 EBBR Sanger sequenced nucleotides of the ammonia oxidizing bacteria (AOB) – *Nitrosomonas*. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW; (**F/R**)- Forward or Reverse primers.

Sample	Sequence (amoA-1F & amoA-2R)
ABDite 1 (F)	ACGAATTNGACCCGGGTNCATGCTCCCGGGTGCANTGATGTTGGACTTCACAATGTATCTGACACGTAACTGGTTGGTGACTGCCTTGGTTGGGGGTGGATTCTTTGGCCTGATGT TTTACCCAGGTAACTGGCCAATCTTTGGCCCGACCCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGTCACCTGTATGTTCGTACGGGTACACCT GAGTATGTTCGTCATATTGAACAAGGTTCATTACGTACCTTTGGTGGTCACACCACAGTTATTGCGGCATTCTTCGCTGCGTTTGTATCCATGCTGATGTTTGCAGTCTGGTGGTATC TTGGA
ABDite 2 (R)	ACTTGTANCCGGGGCNTCATGCTTCCGGGTGCACTGATGCTGGACTTCACGACTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTTGGAGGTGGATTCTTCGGTCTGCTG TTCTATCCGGGTAACTGGCCGATCTTTGGCCCAACCCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGTTCGTACAGGTACACCC GAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGTATGGTGGTATC TTGG
ABDite 3 (F)	CGGGGNNNATGCTTCCAGGTGCNCTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGG TAACTGGCCGATCTTTGGTCCAACGCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGTTCGTACAGGTACACCCGAGTATGTTCG TCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGTATGGTGGTATCTTGGAAAAGTT TACTG
ABDite 4(F)	CCCCGGGNNAAGGGCGGGGCATTATGCTTCCGGGTGCACTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTTGGAGGCGGATTCTTCGGTC TGCTGTTCTATCCGGGTAACTGGCCGATCTTTGGCCCAACGCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGTTCGTACAGGTA CACCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGTATGGTG GTAT
ABDite5	None
ABDite 6 (F)	CCGCGGGGGGGNTATTATGCTTCCGGGTGCACTGATGCTGGACTTCACGCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTTGGAGGCGGATTCTTCGGTCTGCTGTTC TATCCGGGTAACTGGCCGATCTTTGGTCCAACGCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGTTCGTACAGGTACACCCGA GTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACCGTATGGTGGTATCTT GGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTATCGTACATCGCAATGATGTTACCGCATTCGGTGAAGAAGGCTTCCCCAAGGGGAAGAAA
AnBGE	GGNNCANNAACAANGCGATGTACGATACGACCTCTTTTACCTTTAACGTAGAAAAAGGCTGTACAGTAAACTTTTCCAAGATACCACCATACGGTGAACATCAACATTGATACGAAC GCAGAGAAGAATGCTGCAATAACTGTGGTATGACCACCAAAGGTACGCAGTGAACCTTGCTCAATATGACGAACATACTCGGGTGTACCTGTACGAACATACAGATGTCCCATGTAA TCAGCCATCGACAGCAATGTGCCTTCTACAACGATTGGCAAATGGGTTGGTCCAAAAATCGGCCAGTTACCCGGATAGAACAGCAGACCGAAGAATCCACCTCCAACCAGAGCCGT CACCAGCCAGTTGCGTGTCAGATACAGCGTGAAGTCCAGCATCAGCGCACCCGGAAGCATAATGCCCGGTGTTACGAAGTTGATGGGGTAGTGTGACCACCAATAGAAACCCCAG GATCA
SAnBG	None

5.3.3.3 Presence of complete ammonia oxidizing bacteria (Commamox) - *Nitrospira* confirmed in the EBBR-Anammox system.

The Sanger sequencing analysis produced *Nitrospira inopinata* nucleotide sequences from the PCR products extracted from ABDite medium (bioparticles) and AnBGE and detected with the Nino_amoA_19F/ Nino_amoA_252R primer set (Table 5.10); thereby confirming the presence of this Commamox as a member of the microbial communities in the EBBR. The complete oxidation of ammonia by *Nitrospira inopinata* could lead to increase in the concentration of nitrite, which could inhibit Anammox bacterial activity; or increase competition NO_2-N from nitrite oxidizers by reducing their ability to effectively convert wastewater ammonia and nitrite to nitrogen gas. However, with a high presence of Anammox bacteria and AOB within the EBBR, these situations might not be the case (Tables 5.8 & 8b; 5.9).

Table 5.10: EBBR Sanger sequenced nucleotides of the complete ammonia oxidizing (Commamox) bacteria-Nitrospira inopinata. **Key: ABDite** – ABDite growth medium removed from the EBBR; **AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW; (F/R)- Forward or Reverse primers.

Sample	Sequence (Nino_amoA_1 & Nino_amoA_252R 9F)
ABDite 1 (F)	GANTANNCAGCTAGAGACGGTCAATTATCGTCAGCCGTTAGCTAGTCCGCTTATAGCGTTGGGGTTGCAGGTCAATTATCGTCAGCTGTTACCGTTATTTGTGGGTTGGGCC CGGGGGCCCGGCCTTATCCGGGNCAACCTCCGGCCGATGGAATCATATATCAAAAAGGTTGATCCATTAAGNCCTTAAACGGGGAGCAGGAAGGTACTCGAGTCAACGAAG CGGGAAGCCTGGGGAATTTCGGGAATCAGGCGAGTTGATTGTTTTTTCTTGTTGGAGCTTTTGCCCNTTTATTAAAAGGTTTCCAGAATTTAGCGCTTCTCTGGATAGCTGGA CGGGGGTTGGCCGCCCCAAAATCAACAATTATTATGGCGTAGCGGGTGACGTTGAGAGGGCGGGCGACCGGTTCGGCCCGGTAATTCGGGAAGCGTNTTAAAAAGGG
ABDite 2 (R)	ATACACGNNNCGGCGGTGATCAGTGTATGGGCATCTTGGATAGCTTTGATTATAACNTGCGGGTTGGGGGCTTGGATTATNACGAAGGGGAGCCCCTTGCGGTCTTCCCGT ACTGCCCTGGCTTTTTGCCAGGAGCGGGGTTCATACCAGCTTTGTTAATATTAAATGTTAACGCTCATCTTGAAGGATTTGATTATGCGACTGATTCCGTTTCTGGACTTGGTG GCGCTGTCTGGTCTACTAATGAATCACGCGCTTTGCAACTTGGCGGCTTTGATTATTTATTCTAGGCGGGGGGCTACGTGGGGGATTTCTGAAACGATAAGAAGTGAANGGA
ABDite 3 (F)	GGTAGGAAAATTCCGCCTATTCGANGTANAGGTTTAAAGTAATGATCAACGTAGCGATTAGGTTACCGTTAAAATCCTATTAGTCGGATATGACATCCAGCAAGAACAACCCT GCGGGTTCGTACAGGAAGATACATTCGCCAGCAAGGGTCAAAAGCTGATTTATCACGGGCGGCTCATACCGTTCGAAAAAATTCTACTGCAAAGATCCGCTGGTCCCCATGG TCAATGTATCGTCAGCCGTTATAA
ABDite4(F)	CAGCCGTTNTTGTCCGANTGAGAATGTTTGCAATTATAATCGCTACGTTATCNACTTTAAATTTTATTATTGAAATAACCGGTCCGAAAATTTCTTCCTGCGCGTTCCTCATTTC GTTTCGAACATTTATAAATGCGGTGGGTTTTATAAAAAAGCCGGTAGTCCCATAACGTTCGCCTCCTGTCATTAATTTTGCGCCTTCATCTTTTGCAATTTTAATATAGTTCAAA ACTTTGTTGAATTGT
ABDite5 (F)	GGCCTCTATAAGATNNTANNANACATGACGGTCCTATCGTCAGNTGATAGACAGACGTTACCTTTTAGTCGATTGTCGGCAGACNTANGTTAGGTAGCTAGGCCANCTGGGC CGGGAGTCTCCTAGGNACAGCCGGTAGGCCGCCTAGNNGGACNGTTAGGTTGGGGCGTTANGTTGTGTCCCTCCGAACCTAGCCTTGTTGGTACGTGTCCCTTTCCTCTCC TGAAACCGTCTGGNNAAGGAACCACTCCGTAGTTANNGAGAGGAACGTACGAGCCTGCGGGTCACTTATCGTCAGCCGTTATCATCGCGTACGACAAAAGGTTGGTCGTGT GATACCAACTCACGGAAAACACGCTGCTTGGCAATGACCAAACCCGTCTTCAGTTTTGCTTCAAGTGCACCTCGGAGCCTGAATTTTCGGTACGCAAGCTCGTCTATTG
ABDit 6 (F)	TNTACGCAGCCCTTAGAGTGATCATCGTGGTCCGTTAAGCATTGACGTGCGCAANGCCGGCCNGCGGGTAGGGAATGGGGNATGCGGGTAGGGCGGTATTATTCCGATCG CAACTTGCTTTGTTGTTGCGTTTCCCCAGGTGGCGGTTGGTAAATGATTGGCGGTATCCCGCTAGTGTTCGCCTACTGTACTTTTTTTTTTCCCATTCCCTATCAACGTGAAAA TGTGCCTCTGAACCTGGAAAACTTGGGCATCCGAATGAACGCAGCAATAACGGCCCTGCTTTAATATTAAAGCGTATTCATCGCGTACGACAAAAGGTTGGTCGTGTGCT
AnBGE (F)	CGTANNGATAGATATTAGTTAACTTAAGTCAATATTGGCATCTGTTATCCACTCATCCCCCCTTAACGACGGCCAGTACCCTGTACGTAAAGGTCTATCCTTCNAGGGGGTTAT TCATAAATGTCCAGTAGGTAGACAATGACGGAAACATATTGAAATTCGGGTCAGCCCTTGTACACATTCCTCCCGTTGCATCATACATACCTGCTCCTTTTCGTAAGCAAAAGC CAATGGGTCAATTAACGTCTCTCCTTCTGGAACATCGGTGATCTTGTGATCGCCCACATACACAATCCAGACGCTGGCGAACCTGGTCAATTATCGTCAGCCGTTCCGN
SAnBG(F)	**None**

5.3.3.4 Presence of nitrite oxidizing bacteria (NOB) confirmed in the EBBR-Anammox system

Out of the 6 PCR products prepared with either the forward or reverse of FGPS 872 & FGPS 1269 pair and sent for Sanger sequencing, 4 samples produced nucleotide sequences, thereby confirming *Nitrobacter* (NOB) DNA presence in the EBBR. ABDite 1 and ABDite 5 PCR products did not yield any nucleotide sequence (Table 5.11). Compared to Anammox, AOB and Commamox, Sanger results suggested that the presence of the NOB in the EBBR-Anammox system was low, as a smaller number of nucleotides were sequenced form their PCR products. The reduced presence of the NOB in the EBBR is favourable to Anammox bacteria activity; as the NOB will not outcompete the Anammox bacteria for nitrite, the electron acceptor for the Anammox process.

Table 5.11: EBBR Sanger sequenced nucleotides of the nitrite oxidizing bacteria (NOB) -*Nitrobacter.* **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW; (**F/R**) - Forward or Reverse primers.

Sample	Sequence (FGPS 872 & FGPS 1269)
ABDite 1	**None**
ABDite 2 (R)	GTAACAGTGTGTAGACTAGNCCGTAGGGCCATGAGGACTTGACGTTCTNCCTCGCGGCTTATCACCGGCAGTCTCCTTACANNNNTAANANANATG AATGGTAGCAACTAAGGACGGGGGTCGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACTTTCTGACGACAGCCATGCAGCACCTGTCTC CGGTCCTCCCAAAATGAAGGAATACCTCTCTGGAAACCGCGGGCCATGTGTCAACTGCTGGTAAGGTTCCGCGTGTTGCCTAAAATTAAACCATGT TCTCCCCTTTTGTGG
ABDite 3 (F)	GGGGGCCACNATGCTACNTGTGGTTTATTCGACGCACGCGCAGAACCTTACCAGCCCTTGACATGTCCAGGACTTTTCGCAGAGATGTGACCCTC TCTTCGGAGCCTGGAGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGTCCT TAGTTGCTACCATTCAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGG GCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGATGCAAAAGGGCAACCTCCTAGCAAATCTCAAAAAAAA
ABDite 4 (F)	TTTNTTCGACGCAACGCGCAGAACCTTACCAGCCCTTGACATGCCCGGGACGGGTTCCAGAGATGGATTCCTCTCTTCCGAGCGGGGAGCAGACG TGCTGCATGGGTGTCGTCAGCTCTCGTGTCGAGATGTGGGGTTAAGTCCCCCAACGAGCGCAACCCCCCCCCTTATTTGCGCTCATTTTTTTGGGC ACTCTCTGGGGACTGTCCGTGATAAACCCCGAGGAAAGTGGGGATAACATCAAGTCCTCTTGGGCCTTATAGGGTGGGCTACACACGTGCTACAA TGTGGGTGACAATGGGATGCAAAAGGGCGACCCCTATAAAATCTCA
ABDite 5	**None**
ABDite 6 (F)	GANATGTGGTTTAATTCGACGCAACGCGCAGAACCTTACCAGCCCTTGACATGTCCATGANNGACGCAGAGATGTGACCTTCTCTTCGGAGCATGG AGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGTCCTTAGTTGCTACCATT TAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACA CG
AnBGE	CNCGCCATTGTAGCACGTGTGTAGCCCAGCCTGTAAGGGCCATGAGGACTTGACGTCNGNNNANCTTCCTCGCGGCTTATCACCGGCAGTCCCCT TACAGTGCCCAACTGAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCA TGCAGCACCTGTGTCCCGAGCTCCGAAAATAAGGGAACATCTCTGGGAAAAGTCCAGGCGATGTCAAAAGGGGGGAAGGTTCTGCGCGTTGTGCC TAATTAAACCACATGCTCCACCCCTTGTGCGGGCCCCCCCCAATT
SAnBG	**None**

5.3.3.4 Presence of yeast confirmed in the EBBR-Anammox system

To confirm the presence of yeasts DNA in the EBBR-Anammox system, PCR product prepared with the forward of reverse yeast (ITS1/ITS2) were Sanger sequenced. Except for ABDite 2, nucleotide sequences were generated from the 5 ABDite samples (Table 5:12); this confirms that the PCR product were from yeasts, that inhabited the EBBR alongside other microbial communities. The presence of yeast within the reactor EBBR suggests the yeasts might have supported the growth of the EBBR-Anammox bacteria on the ABDite bioparticles.

Sanger analysis has confirmed the presence of DNA from key bacterial (AOB, Anammox, Commamox and NOB) communities in addition to yeast on the EBBR ABDite growth medium and granules (AnBGE). These results are evidence suggesting that the microbial communities which originally came from the seed Anammox bacteria granules (SAnBG) were supported by the EBBR-Anammox system, thereby, partly fulfilling objective 3 (Sanger sequencing).

Table 5.12 EBBR Sanger sequenced nucleotides of yeasts. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW; (**F/R**) - Forward or Reverse primers.

ABDite	Sequence (ITS1 / ITS2)
1(F)	GGGTTTNCNTAGGTGACCTGTCGGACGGATGGAATAGAGCTTGCTCTTATGAAGTTAGGGCGGATGGTTGAGTAACACGTGGGTA AGCTGCCCATAAGACTGGGATAACTCCNGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTANAAATTGAAAGGC GGCTTCAGCTGTCACTTATGGATGGACCCNCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCTACAATGCNTAGCC GACCTGAGAGGGTGATCGGCCACACTGGGACTGATACACGGCCCAGACTCCTACGGGAGGCAGCANTAGGGAATCCTCCGCAAT GGACGAAAGTCTGACGGACAACGCCNCGTGAGTGATGATAGCTTTCG
3 (R)	AGANCTGCTGAGACATTATTGAATTTAGTTGCTGGGTTCAGCCGACGACNATATCATTATCCTAACACCTGTGCACTGTTGGATGTT TAATACATCCGTTTTACACTAAACAATATTGTTACAAATGTAGTCTTATTATAACATAATAAAACTTTCAACAACGGATCTCTTGGCTC TCGCATCGATGAAGAACGCAGCA
4 (F)	AGANCTGCTGAGACATTATTGAATTTAGTTGCTGGGTTCAGCCGACGACNATATCATTATCCTAACACCTGTGCACTGTTGGATGTT TAATACATCCGTTTTACACTAAACAATATTGTTACAAATGTAGTCTTATTATAACATAATAAAACTTTCAACAACGGATCTCTTGGCTC TCGCATCGATGAAGAACGCAGCA
5 (R)	CANCAGACCTGCGGAGGATCATTATTGAATTTAGTTGTCTGGCTTTCGGCTTCTACTTTTTCATTATCCATAACACCTGTGCACTGTT GGATGTTTAATACATCCGTTTTACACTAACCAATATTGCTACCAATGTAGTCTTATTATAACATAATAAAACTTTCAACAACGGATCTC TTGCCTCTCGCATCGATGAATAACGCTTCAGTTTTAGGACAATTAAAACAGCTTCATTACACCCCCCTGGGGGGTTTTCTTATCTTTG CACCTTTTTCTTTGGGCATTCAAGCAACCGGGGCCCCCAGGGAACAACCCCCACCAATTTTTTTTTTTCTTTAAATTTTTTTCAAAAA CCAAAATTTTCCTCACTGGGGATTTTTAAAAATTAAAAACTTCTTCCAACGGATCTCCTGGTTCTCCCCCCCACAAAAAACACCCCAA
6 (F)	GCAGNAGACCTGCGGAGGATCATTATTGAATTTAGTTGTCTGGCTTTCGCCGACGACGATATCATTATCCATAACACCTGTGCACTG TTGGATGTTTAATACATCCGTTTTACACTAAACAATATTGTTACAAATGTAGTCTTATTATAACATAATAAAACTTTCAACAACGGATCT CTTGGCTCTCGCATCGATGAAGAACGCAGCACGGCGTTGATGAACACCGCAGCAATGGTTAGTATGAACGGGGGAACTTTGGTTG GACCGTCACTGGCCAACGGGGTATACACAACTTTCGGGGACGGATCTCTTGGTTCTCGCATCGTTGAATTTATGCGTCAATTTTTTG GAAAAAACAAAAAACTTCTTGCACTGGG

5.3.3.5 Targeted bacteria identified in the EBBR-Anammox system by BLAST analysis

The Sanger sequenced nucleotides were submitted as input (query) into the Basic Local Alignment Search Tool (BLAST) to identify the bacteria communities targeted by species-specific primers in the lab scale EBBR. BLAST search matched the query nucleotides with homologous sequences on the database thereby identifying the bacteria. Identification was necessary to confirm that the nucleotides belonged to the expected bacteria targeted by using species-specific primers; and to compare the Anammox bacteria that colonized the ABDite growth medium at different stages of the research. The criteria used for bacteria identification from the best 100 hits were at least 80 % coverage of the query sequence and 85 % identity (Hall, 2013).

5.3.3.5 .1 Anaerobic ammonia oxidizing (Anammox) bacteria identified in the EBBR

The Anammox bacteria identified using 90 % of the Amx368F/ Amx820R primer generated query sequence had 90 – 99 % identity. The identified Anammox bacteria include 6 strains of *Candidatus Kuenenia stuttgartiensis (*CSTR1, mbr1-rui, MBR1, KUST-E, E14-1 and RAS-Val-1); *2* strains of *Candidatus Jettenia* sp. (Kumadai – 4 and kumadai-3); and several species of *Candidatus Brocadia, which include Brocadia* sp. TKU-1, *Brocadia fulgida, Brocadia sapporoensis, Brocadia caroliniensis, Brocadia pituitae (*Table 5.13a).

With the Brod541F/ Amx820R primer set, which targeted Candidatu*s Brocadia and* Candidatus *Scalindua*, only the same *Candidatus Brocadia* species previously detected with Amx368F/ Amx820R were identified using 95 % query sequence with 88 - 97 % identity (Table 5.13b).

The BLAST results found that *Candidatus Brocadia* species more in abundance than *Kuenenia stuttgartiensis* using both Anammox bacteria primers (Amx368F/ Amx820R and Brod541F/ Amx820R; suggesting the EBBR conditions might have supported the growth of Candidatus *Brocadia* species more compared to

Candidatus *Kuenenia stuttgartiensis* and *Candidatus Jettenia* (Tables 5.13a & 5.13b).

The same three Anammox bacteria genera (Candidatus *Brocadia,* Candidatus *Kuenenia stuttgartiensis and* Candidatus *Jettenia)* were enumerated from the nucleotide sequences from the EBBR-Anammox ABDite bioparticles and granules (AnBGE); and the SWT seed granules (SAnBG), except for Candidatus *Kuenenia* sp. clone RAS-Val-1, which was only found in AnBGE and SAnBG, but not the ABDite medium. In addition, Candidatus *Brocadia brasiliensis* was isolated only from SAnBG but absent from the EBBR samples (Table 5.13b); indicating this species might not have survived in the reactor.

Candidatus *Scalindua* and Candidatus *Anammoxoglobus* were not identified from any of the EBBR-Anammox samples, possibly because they were outcompeted by the other Anammox bacteria genera identified, or completely absent as they were not found even in the seed granules (Tables 5.13a & 5.13b).

The identification of three genera of Anammox bacteria from the EBBR ABDite and granules for the first time of deploying the EBBR for the Anammox process, suggested the system was established to support these bacteria for effective removal of the ammonia in the synthetic wastewater.

Table 5.13a Anammox bacteria species identified in EBBR-Anammox samples by BLAST analysis using 90 % of the query nucleotides sequence. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW**.** Candidatus – all Anammox bacteria genera are described as Candidatus (**Ca**.)

Primer set used	Sample	Anammox Expected Bacteria	Bacteria Identified & Accession number	E-Value	(%) identity
Amx368F/ Amx820R	ABDite 1	*Kuenenia Brocadia, Jettenia Scalindua*	Ca. *Kuenenia stuttgartiensis* - CSTR1 - P049055; Kuenenia stuttgartiensis _mbr1-rui - LT934425 Ca. Kuenenia stuttgartiensis _MBR1 - CT573071; Kuenenia stuttgartiensis – KUST-E - KF442618 Ca. Jettenia sp. Kumadai - KX027318; Jettenia sp. kumadai-3 - KX027317 Ca. Brocadia sp. TKU-1 - KX020503; Brocadia sapporoensis HKD_MBR4 - LC385795; Brocadia pituitae- AP021856*;* Brocadia sp. clone KTH11 - KM884879	**0.0**	**96**
	ABDite 3		Ca. *Kuenenia stuttgartiensis* - E14-1- HM769655; *Kuenenia stuttgartiensis* – KUST-E - KF442618 Ca. *Jettenia* sp. Kumadai - KX027318; *Jettenia* sp. kumadai-3 - KX027317 *Brocadia sp. clone ANA20007* - KX020503; *Brocadia sp*. TKU-1 - KX020503; Ca. *Brocadia fulgida* - EU478693; *Brocadia* sp. clone 40 -- AM285341	**0.0**	**90**
	ABDite 6		Ca. *Kuenenia stuttgartiensis* - E14-1- HM769655; *Kuenenia stuttgartiensis* _MBR1 - LT934425 Ca. *Jettenia* sp. Kumadai - KX027318; *Jettenia* sp. kumadai-3 - KX027317 *Brocadia* sp. TKU-1 - KX020503; *Brocadia sapporoensis* HKD_MBR4 - LC385795; *Brocadia* sp. clone KTH11 - KM884879; KY659581; *Brocadia caroliniensis* clone 20b_19 - KX020518; *Brocadia* sp. clone RAS-Ina-1- KF606756.	**0.0**	**98 – 99**
	AnBGE		Ca. *Kuenenia stuttgartiensis - CSTR1 - P049055;* Ca. *Kuenenia* sp. clone RAS-Val-1 Ca. *Jettenia* sp. Kumadai - KX027318; *Jettenia* sp. kumadai-3 - KX027317; Jettenia sp. kumadai-4 - KX027318; Ca. Jettenia sp. kumadai-3 - KX027317; *Brocadia* sp. clone 40 - KF606757 Ca. *Brocadia fulgida* - EU478693	**0.0**	**90 – 99**
	SAnBG		Ca. *Kuenenia stuttgartiensis* _MBR1 - LT934425*; Kuenenia* sp. clone RAS-Val-1 Ca. *Jettenia* sp. Kumadai - KX027318; *Jettenia* sp. kumadai-3 - KX027317 Ca. *Brocadia* sp. clone 40 -- AM285341; *Brocadia pituitae*- AP021856*; Brocadia caroliniensis* clone W-DXG-33 - JQ889546; *Brocadia fulgida* - EU478693; *Brocadia sapporoensis* HKD_MBR4 - LC385795; *Brocadia sp.*LK-1 - LC385795 Ca. *Brocadia* sp. TKU-1 - KX020503	**0.0**	**90 – 99**

Table 5.13b Anammox bacteria species identified in EBBR-Anammox samples by BLAST analysis using 90 % of the query nucleotides sequence. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granules supplied by STW.

Primer set used	Sample	Expected Bacteria group	Bacteria Identified & Accession number	E-Value	(%) identity
Brod541F/ Amx820R	**ABDite 1**	***Brocadia & Scalindua***	Ca. *Brocadia* sp. clone ANA23064 - KX020520; *Brocadia sp.*LK-1- *LC385795 Brocadia* sp. TKU-1- KM884879; *Brocadia* sapporoensis HKD_MBR4 - LC385795; Brocadia *pituitae- AP021856*; *Brocadia* sp. clone ANA20005; *Candida Brocadia*	**1e-71**	**94**
	ABDite 3		sp. clone ANA20007 - KX020503; *Brocadia* sp. clone ANA19042 - KX020498; *Brocadia sp.*LK-1- LC385795; *Brocadia* sp. clone A19 - MF000737; *Brocadia sp. clone ANA19042*; *Brocadia* sp. clone 40 - AM285341;	**2e-113**	**97**
	ABDite 6		*Brocadia* sp. SNAD 2; *Brocadia* sp. SNAD 1 - HM769652; *Brocadia* sp. clone 40 - AM285341; *Brocadia* sp. clone ANA23064; *Brocadia* sp. TKU-1- KX020503; *Brocadia* sp. clone ANA20005 - KX020501; *Brocadia* sapporoensis HKD_MBR4-LC385795	**6e-110**	**97**
	AnBGE		Ca. *Brocadia* sp. clone ANA23064; *Brocadia* sp. TKU-1- KX020503 *Brocadia* sapporoensis HKD_MBR4 - LC385795; *Brocadia fulgida* - KP663625; *Brocadia* sp. clone ANA20005 - KX020501; *Brocadia sp. clone ANA19042*; *Brocadia* sp. clone 40 - AM285341; *Brocadia sp. clone ANA19042Brocadia* sp. TKU-5 - KM884883; *Brocadia* sp. TKU-4 - KM884882;	**6e-110**	**97**
	SAnBG		*Brocadia* sp. clone ANA23064 - KX020520; *Brocadia* sp. clone KTH11- KM884879; *Brocadia* sapporoensis HKD_MBR4 - LC385795; *Brocadia sp.LK-1- LC385795; Brocadia* sp. clone ANA20005 - KX020501; *Brocadia* sp. clone 40 - AM285341; *Brocadia* brasiliensis clone JCA11 - GQ896513; *Brocadia* caroliniensis clone W-DXG-33 - JQ889546; *Brocadia* sp. clone Br02 - KR003826; *Brocadia* sp. clone A19; *Brocadia* fulgida - KP663625; *Brocadia* fulgida - EU478693	**2e-144**	**88**

5.3.3.5 .2 Ammonia oxidizing (AOB) bacteria identified in the EBBR

BLAST analysis identified several strains of *Nitrosomonas* (AOB) targeted with the amoA-1F & amoA-2R primer pair by using 95 - 96 % of the query sequences with 99 % identity (Table 5.14). There was a high presence of *Nitrosomonas eutropha* strains such as *Nitrosomonas eutropha amoB1, Nitrosomonas eutropha Nm, Nitrosomonas europaea22 and Nitrosomonas europaea* ATCC 19178 in both the ABDite and AnBGE analysed. Other *Nitrosomonas* identified include *Nitrosomonas* sp. clone 15, *Nitrosomonas* sp. GH22, *Nitrosomonas* sp. CNS332, *Nitrosomonas* sp. K1, *Nitrosomonas* sp. TK794 amongst others (Table 5.14).

Table 5.14 Ammonia oxidizing bacteria -*Nitrosomonas* identified from the EBBR-Anammox samples by BLAST analysis using 95 -96 % of the query nucleotides sequence. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** -Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW**.**

Primer set used	Sample	Expected Bacteria group	Bacteria Identified & Accession number	E-Value	(%) identity
amoA-1F & amoA-2R	**ABDite** 1	**Nitrosomonas sp.**	*Nitrosomonas* sp. GH22 - AF327917; *Nitrosomonas* sp. clone M11- MG831218; *Nitrosomonas* sp. clone A45 - MG846257; *Nitrosomonas* sp. CNS332 -DQ228468; *Nitrosomonas* sp. K1 - AB900137*;* *Nitrosomonas* sp. TK794 - AB031869; *Nitrosomonas* sp. strain Nm175 - KU747140; *Nitrosomonas* sp. IWT310 - DQ228467; *Nitrosomonas eutropha* (amoA) gene -AY177932; *Nitrosomonas* sp. clone 15 - HM149333; *Nitrosomonas eutropha* (amoA2) - U72670; *Nitrosomonas* sp. clone NCAAH 15 - KT429266; *Nitrosomonas eutropha* amoB1 - U51630; *Nitrosomonas* sp. IWT202 - AB900136; *Nitrosomonas* sp. clone NCAAH 15 A16 - KT429261	**0.0**	**99**
	ABDite 3		*Nitrosomonas* sp. clone M20 - MG831262; Nitrosomonas eutropha strain Nm 57 - KU747123 Nitrosomonas sp. clone NCAAH 30 - KT429255; *Nitrosomonas sp.* clone M15 - MG831230; *Nitrosomonas* sp. clone B39 - MG846303; *Nitrosomonas sp.* clone A3 - MG846254; Nitrosomonas sp. clone A2 - MG846253; *Nitrosomonas sp.* clone A38 - MG846237; *Nitrosomonas sp.* clone A55 - MG846234; *Nitrosomonas sp.* clone A42 - MG846232; *Nitrosomonas sp.* clone M9 - MG831275;	**0.0**	**99**
	ABDite 6		*Nitrosomonas sp.* clone A3 - MG846254; *Nitrosomonas* sp. clone M14 - MG831276; Nitrosomonas sp. clone A45 - MG846257; Nitrosomonas sp. clone M11- MG831218; Nitrosomonas sp. clone A28 - MG846255; *Nitrosomonas sp.* clone M13 - MG831208; Nitrosomonas sp. clone A28 - MG846255; *Nitrosomonas sp.* clone M20 - MG831262; Nitrosomonas sp. clone He;	**0.0**	**99**
	AnBGE		*Nitrosomonas europaea* strain Nm50 -KU7471, *Nitrosomonas* sp. clone NCAAH 15 A16 - KT429261; *Nitrosomonas* sp. TK794 - AB031869; *Nitrosomonas europaea22* - AF058692 *Nitrosomonas europaea* ATCC 19178 - JN099309; *Nitrosomonas* sp. clone A45 - MG846257 *Nitrosomonas* sp. clone M11- MG831218; *Nitrosomonas* sp. K1 - AB900137; *Nitrosomonas* sp. clone M13 - MG831208*; Nitrosomonas* sp. clone NCAAH 30 A21 - KT429256;	**0.0**	**99**

5.3.3.5.3 Complete ammonia oxidizing (Commamox) bacteria not identified in the EBBR

The nucleotides sequences suspected to come from the *Nitrospira*, a Commamox (Table 5.10) submitted for BLAST analysis did not match with *Nitrospira inopinata* sequence in the database; even when the Algorithm parameter was changed from 100 to 5000, to widely search the database. The result indicated an error could have occurred during the sequencing; and highlighted the limitation of Sanger sequencing and the need for NGS to fully identify the microbial communities of the EBBR-Anammox process.

5.3.3.5.4 Nitrite oxidizing bacteria (NOB) identified in the EBBR

Using 92 – 94 % of the NOB query nucleotide sequences, strains of *Nitrobacter* targeted with FGPS 872 & FGPS1269 primer were identified from ABDite biomass support medium with 83 – 99 % identity *(*Table 5.15). *Nitrobacter* species enumerated include *Nitrobacter hamburgensis* (more prominent), *Nitrobacter vulgaris* and *Nitrobacter winogradskyi.* Compared to ABDite 2 sampled from EBBR, 5 months from the setup of the EBBR-Anammox investigation; BLAST results suggested an increased presence of *Nitrobacter* in the EBBR over time; as a result, the number identified from ABDite 3 and ABDite 6 sampled in the 7^{th} and 24^{th} months respectively were more (Table 5.15).

Table 5.15 Nitrite oxidizing bacteria -*Nitrobacter* identified from the EBBR-Anammox samples by BLAST analysis using 92 -94 % of the query nucleotides sequence. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW**.**

Primer set used	Sample	Expected Bacteria group	Bacteria Identified & Accession number	E-Value	(%) identity
FGPS 872 & FGPS 1269	ABDite 2	Nitrobacter	*Nitrobacter* sp. clone BR_02 - KP337440; *Nitrobacter* sp. clone 64 - HQ424506 *Nitrobacte*r sp. - HQ424483; *Nitrobacter* sp. clone 74 clone 8 - HQ424516 *Nitrobacter* sp. clone 23 - HQ424491	2e-63	83
	ABDite 3		*Nitrobacter* sp. *NS5-5* - JQ806744; *Nitrobacter* sp. 263 - AM286383 *Nitrobacter* sp. *A67* - LC702421*; Nitrobacter* sp. 219 - AM286375 *Nitrobacter* sp. *clone 100* - HQ424528; N*itrobacter* sp. clone 77 - HQ424518 *Nitrobacter* sp. *clone 72* - HQ424514; *Nitrobacter* sp. clone 18 - HQ424489 *Nitrobacter hamburgensis strain IFP5* - KM047477; *Nitrobacter* sp. strain TS12 - MZ413963; *Nitrobacter* sp. *BS5/19* - AM286387*; Nitrobacter* sp. clone: NBAC-*2* - AB500056; *Nitrobacter* sp. clone DM4-28 - KC172240; *Nitrobacter* sp. clone: MnDHS-C03. - AB980134	1e-160	97- 98
	ABDite 6		*Nitrobacter* sp. clone Ntb_2 - KP337429; *Nitrobacter* sp. clone SBL19 - KM108698; *Nitrobacter hamburgensis* strain IFP5 - KM047477; *Nitrobacter* sp. NKU - KM061381; *Nitrobacter* sp. strain N1 - ON117826; *Nitrobacter* sp. clone 26-31 - KJ627744; *Nitrobacter* sp. clone D-05Biofilm069 - KJ600490; *Nitrobacter vulgaris* strain NBW3 - KF618622; *Nitrobacter* sp. NBW1 - KF618620; *Nitrobacter* sp. strain TS12 - MZ413963; *Nitrobacter* sp. clone DM4-28 - KC172240; *Nitrobacter winogradskyi* strain Nb-255 - NR_074324; *Nitrobacter hamburgensis* X14 - NR_074313; *Nitrobacter* sp. clone TH0909362_3-3N-28 - JQ595619	9e-150	99

5.3.3.5.5 Yeast species identified in the EBBR

Two yeasts, *Filobasidium uniguttulatum* and *Cryptococcus* sp. were identified in the EBBR-Anammox system with 92 % of query nucleotide sequence used at 97 % identity (Table 5.16). BLAST result showed that the same yeasts were present in the EBBR samples analysed over time; *Filobasidium uniguttulatum* strains were present in all the samples; and therefore, more abundant than *Cryptococcus* sp. (Table 5.16). These human pathogenic yeasts that coexisted with both the seed Anammox and EBBR-Anammox bacteria communities, has highlighted the need for safety measures while dealing with biohazards.

The identification of the key bacterial groups of the Anammox process (AnAOB, AOB and NOB) in addition to yeast targeted by species-specific primers, as confirmed by BLAST results infer that the EBBR-Anammox system was effective at supporting these microbial communities. The identification data also suggested that the previous molecular processes (DNA extraction, PCR and Sanger sequencing) were effective, leading to the identification of the targeted bacteria in the lab scale EBBR.

Table 5.16 Yeast species identified from the EBBR-Anammox samples by BLAST analysis using 92 % of the query nucleotides sequence. **Key: ABDite** – ABDite growth medium removed from the EBBR**; AnBGE** – Anammox bacteria granule that developed in the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW.

Primer set used	Sample	Expected Bacteria group	Bacteria Identified & Accession number	E-Value		(%) identity
ITS1 / ITS2	ABDite 3	Yeast	*Filobasidium uniguttulatum* strain OWF01- MK088094; *Filobasidium uniguttulatum* isolate 23A - MK802118; Filobasidium uniguttulatum isolate R96754_ITS - MK267938; *Filobasidium uniguttulatum* isolate R96722_ITS- MK267843; *Filobasidium uniguttulatum* strain CBS 2770 - MH084827; *Cryptococcus* sp. - KU961663	**2e-79**		**97**
	ABDite 4		*Filobasidium uniguttulatum* CBS:4257 - KY103443; *Filobasidium uniguttulatum* isolate R96754_ITS - MK267938; *Filobasidium uniguttulatum* isolate R96722_ITS- MK267843; *Filobasidium uniguttulatum* strain CBS 2770 - MH084827; *Filobasidium uniguttulatum* strain OWF01- MK088094; *Filobasidium uniguttulatum* isolate 23A - MK802118*;* *Cryptococcus* sp. - KU961663; *Filobasidium sp. BF9 ITS - AM901672*	**2e-79**		**97**
	ABDite 6		*Filobasidium uniguttulatum* strain WM 13.160 -KP132189; *Filobasidium uniguttulatum* isolate R96722_ITS- MK267843*;* *Filobasidium uniguttulatum* strain CBS 2770 - MH084827; *Filobasidium uniguttulatum* strain OWF01- MK088094; *Filobasidium uniguttulatum* isolate 23A - MK802118	**2e-79**		**97**

5.3.3.6 Phylogenetic Trees Construction

The phylogenetic trees of the identified bacterial groups (Anammox, AOB, NOB) and yeast were constructed with MEGA 11 (Kumar *et al.,* 2021) to visualize the taxa relatedness of the microbial communities that played specific roles in the EBBR-Anammox process. The bootstrap for the consensus tree constructed used 1000 replicates for test of reliability; using the bacteria species or strains identified from all the EBBR samples (ABDite & AnBGE) and SAnBG for each group, once

5.3.3.6.1 Anaerobic ammonia oxidizing (Anammox) bacteria phylogenetic tree

Anammox phylogenetic tree was constructed using the bacteria identified from ABDite 1, 3 & 6, AnBGE and SAnBG samples targeted by Amx368F/ Amx820R and Brod541F/ Amx820R primer sets (Table 5.13a & 5.13b; Figure 5.6). The Anammox tree was constructed using 33 nucleotide sequences, suggested that 76 % of the Anammox bacteria were *Candidatus Brocadia*, 18 % *Kuenenia stuttgartiensis* and 6 % *Candidatus Jettenia* (Figure 5.6); indicating a high presence of *Candidatus Brocadia* in the EBBR (Figure 5.6). With a bootstrap reliability of mostly 99 %, the tree indicated that clusters of *Kuenenia stuttgartiensis* and *Candidatus Jettenia* descended from the specified nodes (Hall 2013). The tree suggested that the strains of *Candidatus Brocadia* although distantly related, were better adapted to the EBBR-Anammox system; and could have contributed more to the Anammox process.

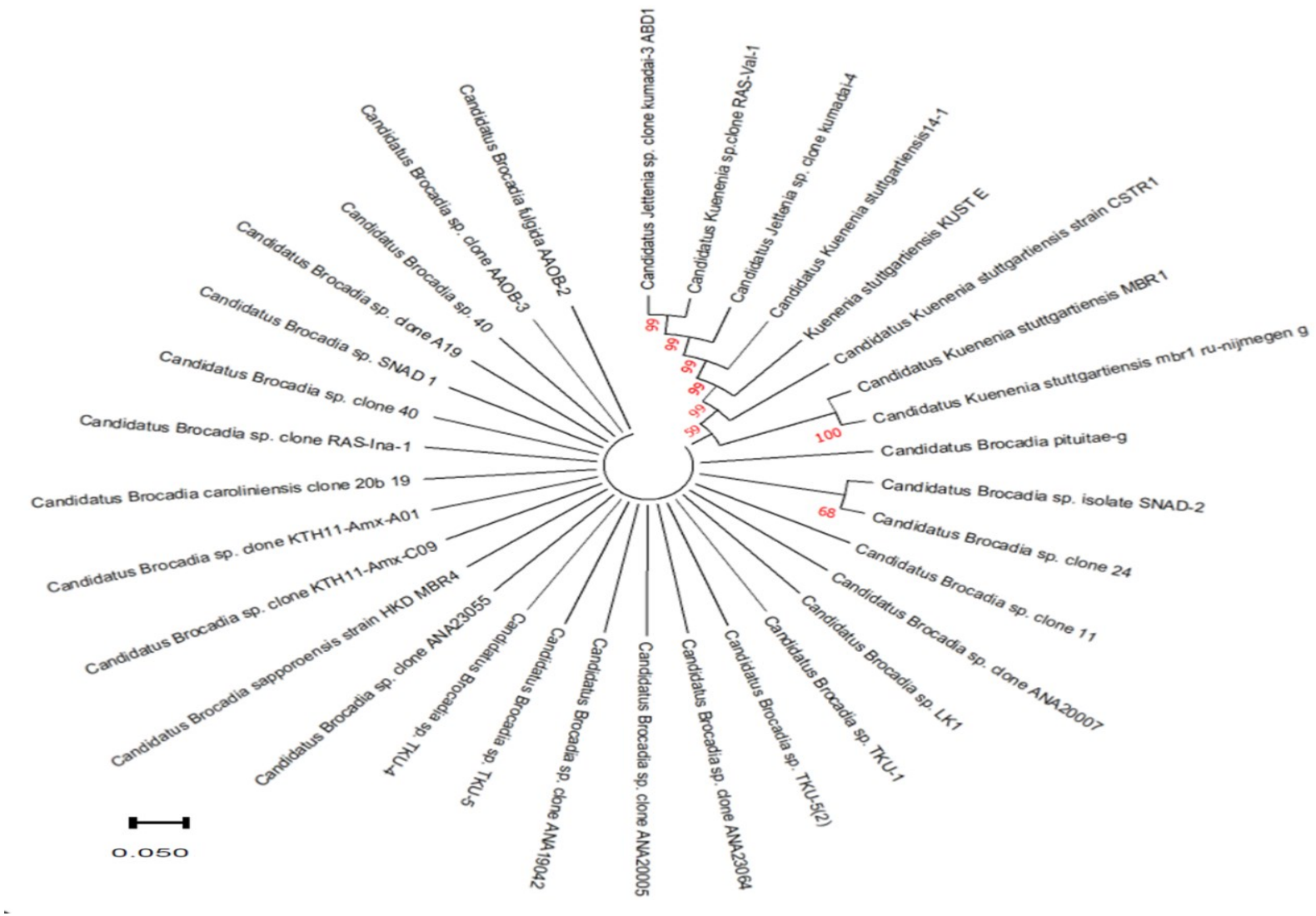

Figure 5.6 Unrooted EBBR-Anammox bacteria phylogenetic tree based on 33 nucleotide sequence from targeted Anammox bacteria. The scale bar represents 5 % substitutions per 1000 bootstrap nucleotides replicates.

Candidatus Brocadia (76 %) was dominant over *Kuenenia stuttgartiensis* (18 %) and *Candidatus Jettenia* (6 %) in the EBBR.

5.3.3.6.2 Ammonia oxidizing (AOB) bacteria phylogenetic tree

The AOB phylogenetic tree was constructed using *Nitrosomonas* species identified from ABDite 1, 3 & 6, AnBGE and SAnBG samples (Table 5.14; Figure 5.7). Out of the 31 AOB used, with bootstrap reliability mostly between 60 – 100 % *Nitrosomonas eutropha* made up 13 %; the 27 other species contributed 87 %; the result indicated that *Nitrosomonas eutropha* has a high presence among the AOB. The tree suggested that 42 % of the AOB on separate nodes were distantly related to the other 18 taxa (Figure 5.7).

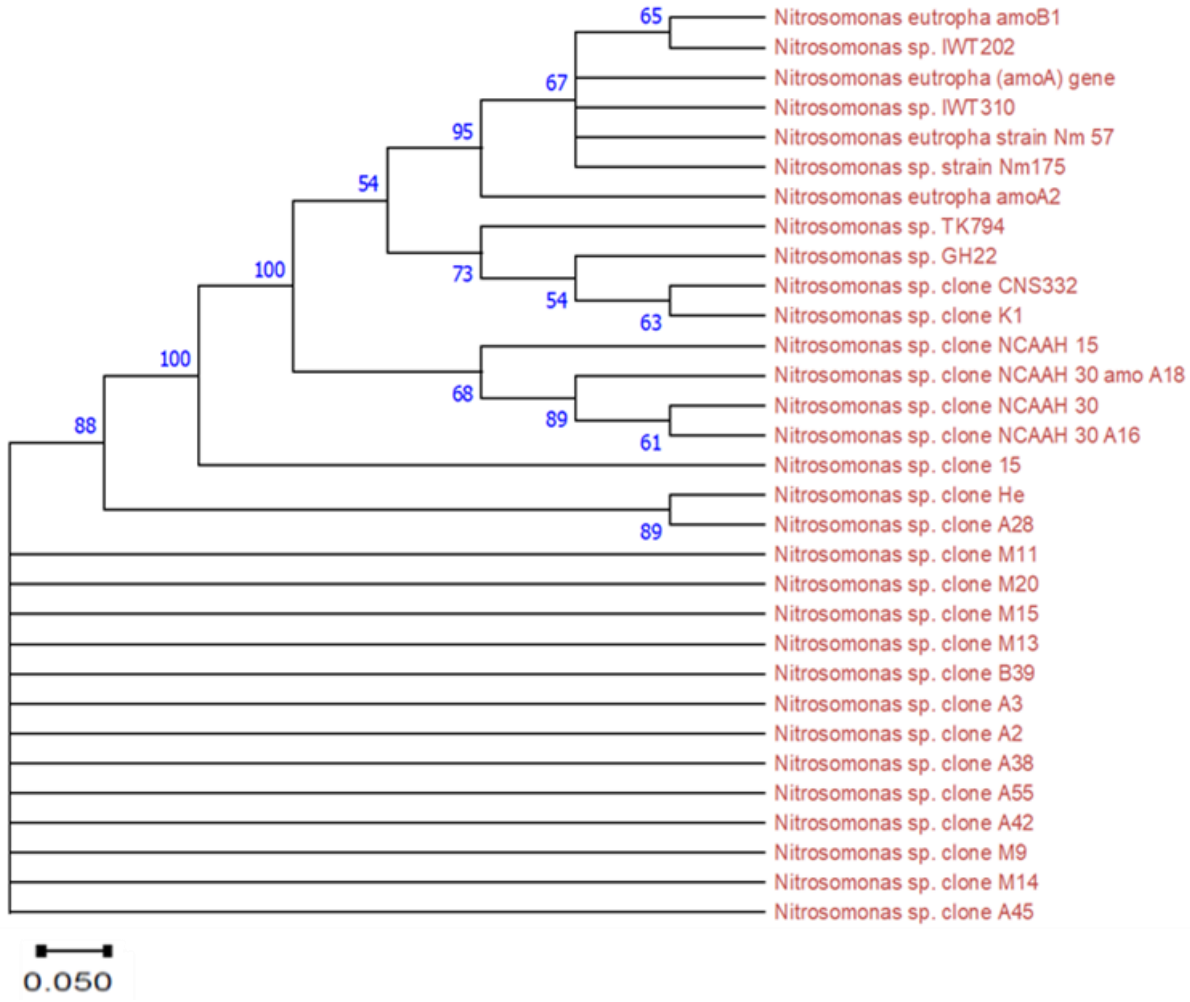

Figure 5.7 Unrooted Ammonia oxidizing bacteria phylogenetic tree based on 31 nucleotide sequence from EBBR *Nitrosomonas species.* The scale bar represents 5 % substitutions per 1000 bootstrap nucleotides replicates. AOB in the EBBR was *Nitrosomonas eutropha* (12 %); and other species (87 %).

5.3.3.6.3 Ammonia oxidizing (NOB) bacteria phylogenetic tree

The NOB evolutionary relatedness was estimated using 30 *Nitrobacter* species recovered from ABDite growth medium 2, 3 & 6 (Table 5.14; Figure 5.8). *Nitrobacter hamburgensis* contributed 7 %; *Nitrobacter winogradskyi* and *Nitrobacter vulgaris*, another 7 % and the other NOB, 86 %. The bootstrap reliability test ranged between 15 – 100 %; an indication *that most of the Nitrobacter* species in the EBBR were different, except for the few with scores above 70 %.

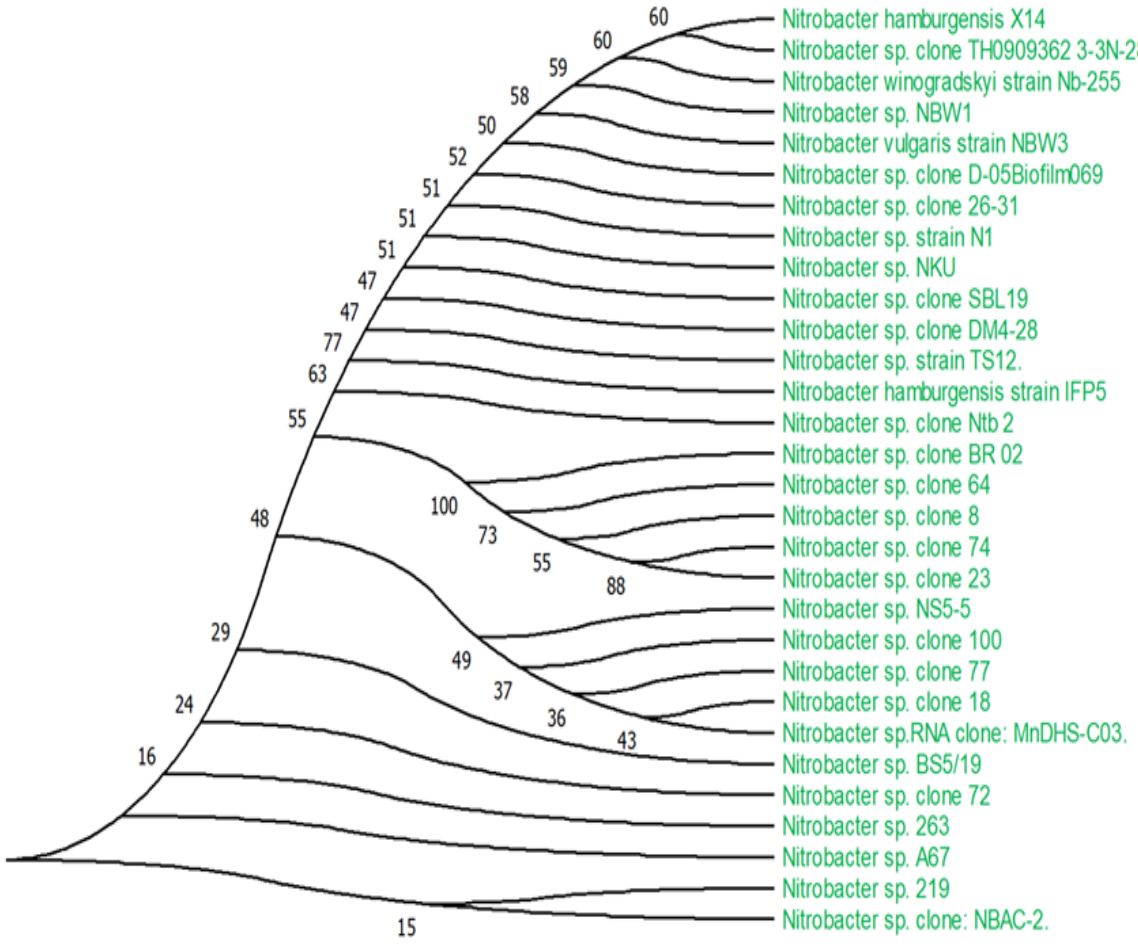

Figure 5.8 Unrooted nitrite oxidizing bacteria phylogenetic tree based on 30 nucleotide sequences from *Nitrobacter species; constructed with* 1000 bootstrap nucleotides replicates. NOB in the EBBR had *N. hamburgensis* (7%); *N. winogradskyi* and *N. vulgaris* (7 % and other NOB species (86 %).

5.3.3.6.4 Yeast phylogenetic tree

Yeast phylogenetic tree was constructed estimated using 8 nucleotide sequences from two 2 yeast genera (*Filobasidium* and *Cryptococcus*) from EBBR ABDite bioparticles 2, 4 & 6 (Table 5.15; Figure 5.9). While the dominant *Filobasidium uniguttulatum made up 8*7 %, *Cryptococcus* was 13 % of the yeast community in the EBBR. The bootstrap reliability is 94 %, which indicated that *Filobasidium uniguttulatum* taxa descended from the nodes specified; but distantly related to the *Cryptococcus* species.

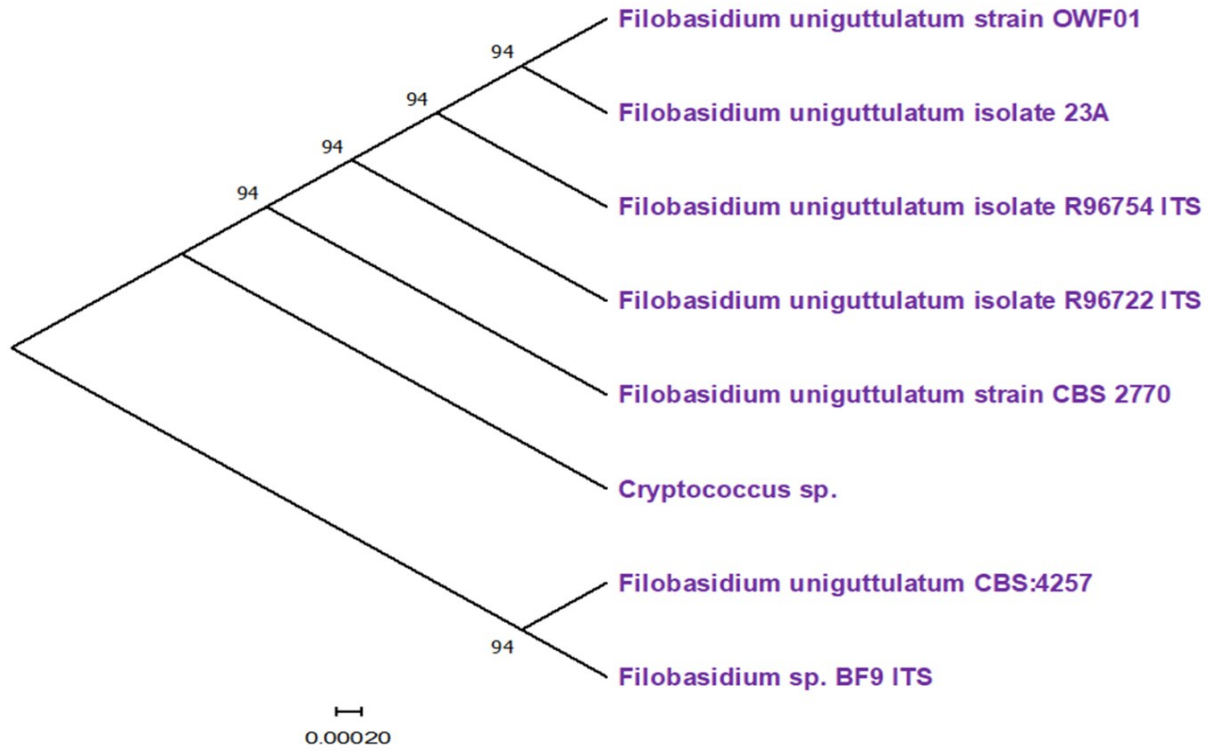

Figure 5.9 Unrooted yeast phylogenetic tree based on 8-nucleotide sequences from EBBR *yeasts;* The scale bar represents 0.02 % substitutions per 1000 bootstrap nucleotides replicates. EBBR yeast community contained 87 % of *Filobasidium uniguttulatum* and 13 % of *Cryptococcus* species.

The presence of different yeast species in the EBBR indicated that other than the targeted bacteria groups, other communities were present in the EBBR; making NGS analysis a necessity to identify these communities that might have influenced the EBBR-Anammox process, which is linked to objective 4.

5.3.4 EBBR-Anammox microbial communities identified by NGS

Next generation sequencing (NGS) of the EBBR-Anammox DNA samples was carried out to identify the microbial communities present in the lab scale EBBR, whether they contributed to the Anammox process or not; and in addition, confirm Sanger results, which identified only targeted bacteria with species specific primer sets (Figures 5.6- 5.9).

5.3.4.1 Concentration of DNA in EBBR-Anammox samples quantified by Eurofins for NGS

Eurofin (NG-25934 and Report number 2022113-1) measurements found variation in the concentration of the 16 DNA samples from EBBR ABDite bioparticles, liquid, Anammox bacteria granules (AnBGE) and seed Anammox bacteria granule (SAnBG) sent for NGS analysis (Table 5.17). The SAnBG contained the highest amount of DNA (30 ng μL^{-1}); and liquid samples the least (2 ng µL-1) form the same volume (30 µL). In addition, the 6.7 ng µL-1 of DNA from the ABDite quantified by Eurofins was like the 6.2 ng µL-1 of DNA measured by Nanodrop 20 (Table 5.5). Eurofins confirmation of EBBR-Anammox DNA concentration suggested that both Nanodrop and NGS DNA quantification were reliable (Table 5:17).

Table 5:17 Concentration of EBBR-Anammox bacteria DNA determined by NGS analysis by Eurofins. **Key**: **ABDite** – ABDite growth medium removed from the EBBR; **AnBGE** – Anammox bacteria granule that developed in the EBBR; **Liquid** - Liquid samples taken from the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW

EBBR sample Number	EBBR Sample source	Eurofin sample Barcode	Eurofin determined DNA concentration (ng μL^{-1})
1	ABDite 1	A26096	6
2	Liquid 1	A26097	2
3	SAnBG 1	A26098	30
4	ABDite 2	A26099	7
5	AnBGE 1	A26100	11
6	AnBGE 2	A26101	16
7	Liquid 2	A26102	2
8	ABDite 3	A26103	10
9	AnBGE 3	A26104	23
10	AnBGE 4	A26105	8
11	ABDite 4	A26106	8
12	ABDite 5	A26107	5
13	AnBGE 5	A26108	6
14	ABDite 6	A26109	6
15	SAnBG 2	A26110	29
16	AnBGE 6	A26111	4

5.3.4.2 Number of EBBR-Anammox nucleotide sequences assigned operational taxonomic units (OTUs)

Eurofins used two NGS primers V3V4a and AV3V4a in the analysis of the EBBR-Anammox samples to compare the microbial communities identified with each primer. From the NGS, results, 12 nucleotide sequences were obtained using the V3V4a, and 13 with AV3V4a primer out the16 DNA samples submitted (Table 5:18). Nucleotide sequences were obtained from DNA extracted from the 6 EBBR ABDite medium; 4 AnBGE; 2 SAnBG; but none form AnBGE 3 and liquid samples. The results suggested that the bacteria communities were retained within the EBBR ABDite and granules instead of floating in the liquid and washed out of the expanded bed; and were therefore, able to conduct the Anammox process.

Approximately 70 – 77 % of sequences generated with V3V4a, and 50 – 82 % of AV3V4a primers were assigned operational taxonomic units (OTUs) for the identification of the different microbial communities present within the ABBR-Anammox system (Table 5.18).

Table 5.18 EBBR-Anammox sample sequences assigned to operational taxonomic unit (OUT) using the NGS **V3V4a** and AV3V4a **p**rimers. ***Key:*** **1)** Input sequences. **2) % of** sequences assigned to OTUs; ABDite – ABDite growth medium removed from the EBBR; **AnBGE** – Anammox bacteria granule that developed in the EBBR; **Liquid** - Liquid samples taken from the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW.

EBBR sample DNA source	Eurofin sample Barcode	V3V4a primer			AV3V4a primer		
		Number	1)	2)	Number	1)	2)
1. ABDite 1	A26096	11.V3V4a	178038	77.7%	11.V3V4a	112 677	72.4%
2. Liquid 1	A26097	-	-	-	-	-	-
3. SAnBG 1	A26098	3.V3V4a	194594	74.3%	3.AV3V4a	110 473	76.4%
4. ABDite 2	A26099	8.V3V4a	165347	71.3%	8.AV3V4a	113 633	76.3%
5. AnBGE 1	A26100	5.V3V4a	208591	73.6%	5.AV3V4a	135 961	74.6%
6. AnBGE 2	A26101	6.V3V4a	182891	70.2%	6.AV3V4a	85 311	75.5%
7. Liquid 2	A26102	-	-	-	-	-	-
8. ABDite 3	A26103	4.V3V4a	208080	74.6%	4.AV3V4a	134 276	76.4%
9. AnBGE 3	A26104	-	-	-	-	-	-
10. AnBGE 4	A26105	10.V3V4a	191900	74.5%	10.AV3V4a	130 646	75.5%
11. ABDite 4	A26106	1.V3V4a	235513	72.9%	1.AV3V4a	143 456	75.1%
12. ABDite 5	A26107	12.V3V4a	178038	77.7%	12.AV3V4a	149 463	82.9%
13. AnBGE 5 f	A26108	13.V3V4a	184715	76.4%	13.AV3V4a	50 120	74.6%
14. ABDite 6	A26109	14.V3V4a	153681	73.9%	14.AV3V4a	88 597	50.5%
15. SAnBG 2	A26110	15.V3V4a	226611	72.0%	15.AV3V4a	114 452	75.0%
16. AnBGE 6	A26111	-	-	-	9.AV3V4a	145 265	76.0%

5.3.4.3 Communities of EBBR-Anammox bacteria identified from NGS analysis

The NGS microbiome analysis identified communities of bacteria that were/not directly involved with the EBBR-Anammox process with both primers (V3V4a & AV3V4a); and the proportion contributed to the community, reported as % OTU. The bacteria communities with specific roles in the Anammox wastewater treatment process identified from the EBBR samples were anaerobic ammonia oxidizing (Anammox) bacteria, ammonia oxidizing bacteria (AOB), complete ammonia oxidizing bacteria (Commamox) and nitrite oxidizing bacteria (NOB) (Figures 10 -12). Each bacteria group contributed different % of operational taxonomic unit (OTU) to the entire communities that developed in the EBBR-Anammox system.

The % OTU contributed to the EBBR-Anammox microbial communities by groups with specific role in the Anammox process identified by NGS V3V4a and AV34a primers were as follows:

Anaerobic ammonia oxidizing (Anammox) bacteria community

Two Anammox bacteria (Candidatus *Brocadia* and Candidatus *Kuenenia)* were identified by NGS analysis (Figure 5.10 - 5.12). NGS analysis found one strain of Candidatus *Brocadia* sp. TKU.1 (Figure 5.12), which was in contrast with Sanger result that identified several species Candidatus *Brocadia* (Figures 5.6).

The total OTU contribution made by Candidatus *Kuenenia* to the microbial communities identified with V3V4a was 14.10 %; ABDite bioparticle contributed 6.5%, AnBGE (6.2 %) and SAnBG (1.4 %). Using the AV34a primer, out of the total 7.9 % OTU, 2.6 % came from ABDite sample, AnBGE (3.8 %) and SAnBG (0.6 %). OTUs contributions from ABDite bioparticle were higher compared to AnBGE and SAnBG; the V3V4a ABDite OTUs (6.5%) was 60 % higher than AV34a ABDite OTUs (2.6 %), suggesting that the ABDite bioparticles provided a better growth condition. Candidatus *Kuenenia* had a 19.1 % combined OTUs from EBBR ABDite bioparticles and AnBGE samples from both primers; this result is approximately 17 times higher the 2.0 % OTUs from SAnBG

samples; indicating that the growth conditions within the EBBR supported Candidatus *Kuenenia* more compared to the storage condition. The OTU results indicated that Candidatus *Kuenenia* was established in the EBBR; as the % OTU increased form 0.3 % (ABDite 1 - 1st month) to 2.6 % (ABDite 4 - 13th month), which is about 88 % increment within 12 months (Figures 5.10 & 5.11).

Candidatus *Kuenenia* was absent from ABDite 5 & 6, and AnBGE 5 analysed by both primers, which coincided with the period of insatiability due to temporary repair; confirming Sanger sequencing result that did not generate nucleotide sequence from ABDite 5 (Table 5.8b).

The identification of Candidatus Kuenenia and Candidatus Brocadia by NGS analysis is a confirmation of the Sanger sequencing results, which reported that these Anammox bacteria were present in the EBBR in abundance for effective removal of wastewater ammonia; the NGS finding has increased the reliability of the EBBR-Anammox investigation results.

Ammonia oxidizing bacteria (AOB) community

The NGS analysis also identified *Nitrosomonas, Nitrosospira* and other AOB species from EBBR ABDite bioparticle, AnBGE and SAnBG samples (Figures 5.10 - 5.12), which confirmed Sanger and BLAST *identification* of *Nitrosomonas* in the EBBR *(*Figure 5.7); and satisfied objective 4 that NGS has capacity to identify more bacteria than Sanger, hence other AOB e.g., *Nitrosospira* not targeted by species specific primer were enumerated by NGS.

Nitrosomonas eutropha contributed 88 % OTUs from ABDite bioparticle samples, 33.2% form AnBGE and SAnBG 61 % from V3V4a and AV34a combined; for *Nitrosospira, it was* 2.6 % (ABDite); 10.8 % and SAnBG, 5.9 %. Using the primers separately, the total OTUs for *Nitrosomonas eutropha was* 133.2 % (V3V4a) and 48.9% (AV34a); and *Nitrosospira,* 12.2 % (V3V4a), and 7.2 % (AV34a); *Nitrosomonas eutropha* had 159 % more OTUs than *Nitrosospira;* making it the dominant AOB in the EBBR-Anammox

system; another confirmation of Sanger result that *Nitrosomonas eutropha* alone contributed 12 % of the total AOB in the EBBR (Figure 5.7).

Nitrosomonas was identified from all the EBBR-Anammox samples analysed with both primes, except from ABDite 1,2 & 6 using A3V4a (Figure 5.11). The observed absence in those samples could have come from competitions from other microbial communities such as the *Comamonas, a* nitrifying proteobacteria *(*NPB) that also uses ammonia as substrate; and was dominant in those samples (Figure 5.11).

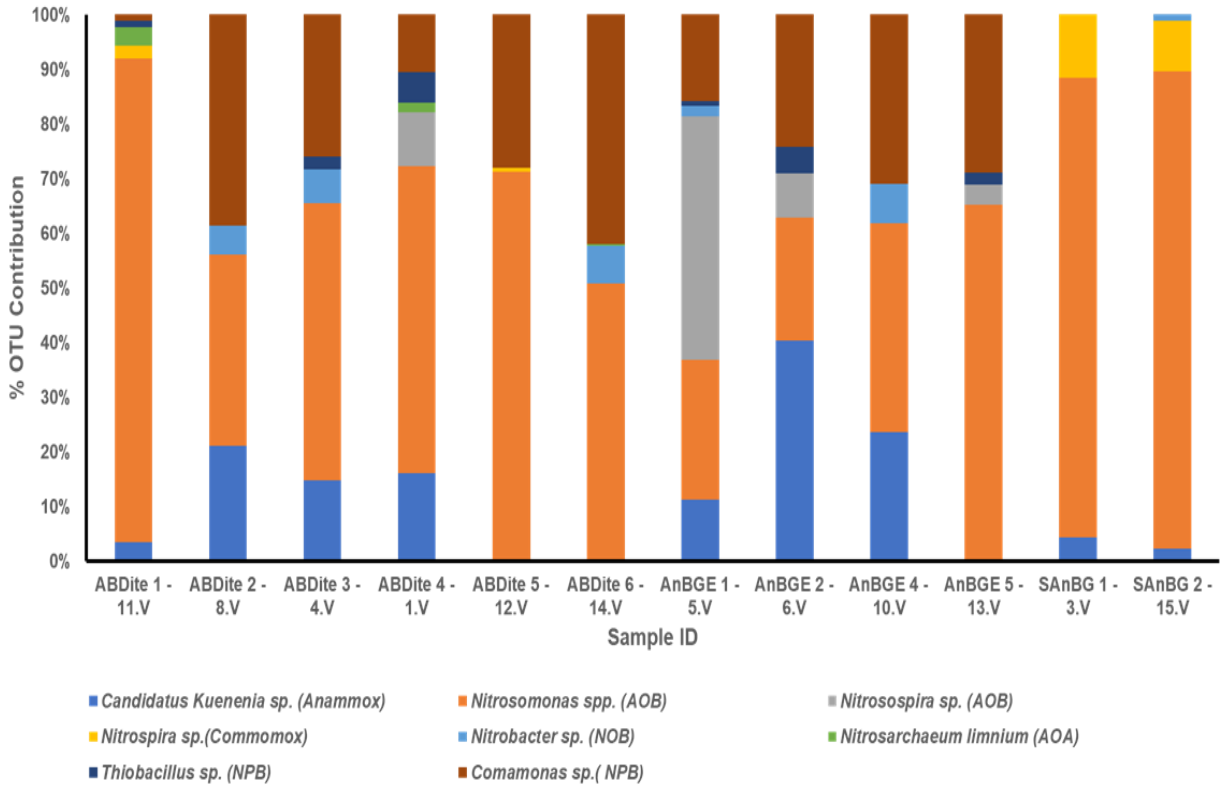

Figure 5.10 Percentage OTUs contributed to the EBBR-Anammox microbial communities identified using **V3V4a NGS** primer**.** **Key:** **ABDite** - ABDite medium sampled from the EBBR at different stages: **ABDite 1** (1st month); **ABDite 2** (5th month); **ABDite 3** (7th month); **ABDite 4** (13th month); **ABDite 5** (21st month); **ABDite 6** (24th month). **AnBGE** – Anammox bacteria granule sampled from the EBBR at different stages: **AnBGE** 1 & 2 (5th month); **AnBGE** (7th month); **AnBGE** 4 (13th month); **AnBGE** 5 (21st month); **AnBGE** 6 (24th month). **SAnBG** – Anammox bacteria granule supplied by STW; **Anammox** - anaerobic ammonia oxidizing bacteria; **AOB** - ammonia oxidizing bacteria; **Commamox** – complete ammonia oxidizing bacteria; **NOB** – nitrite oxidizing bacteria; **NPB** – nitrifying proteobacteria. % OTU contributed to the EBBR-Anammox

communities by Anammox bacteria (14 %); AOB (145 %); Commamox (5.2); NOB (6 %); **AOA** (0.7 %) **NPB** (51 %).

Complete ammonia oxidizing (Commamox) bacteria community

Nitrospira (Commamox) species contributed 7.1% OTUs to the EBBR-Anammox microbial communities; mainly form SAnBG samples with 6.9 % OTUs. Only 0.4% came from ABDite bioparticles and none from AnBGE. The NGS result is a confirmation of PCR detection of *Nitrospira* DNA, *Nitrospira*; the only Commamox within the EBBR was very low in abundance; suggesting it had minimal influence in the EBBR-Anammox process (Figures 5. 8, 5.10 & 5.12).

Nitrite **oxidizing bacteria (NOB) community**

Furthermore, *Nitrobacter* sp. LAMK1242 *was* the most dominant NOB identified as a member of EBBR-Anammox communities; *Nitrobacter* contributed 6 .0% OTUs from ABDite bioparticles AnBGE and SAnBG 2 using V3V4a and 0 % with AV34a (Figures 5.10 & 5.12). The highest OTUs (3.5 %), contribution was from ABDite 6, when the EBBR was dominated by nitrifying *Nitrosomonas* (AOB) and *Comamonas* (NPB); hence, no Anammox bacteria detected, suggesting the Anammox bacteria were partially inhibited

The NGS data is a confirmation of PCR and Sanger reports, which found low presence of NOB in the EBBR, compared to Anammox bacteria and AOB; and therefore, had a low influence on the Anammox process (Figures 5.4 & 5.10; Table 5.11).

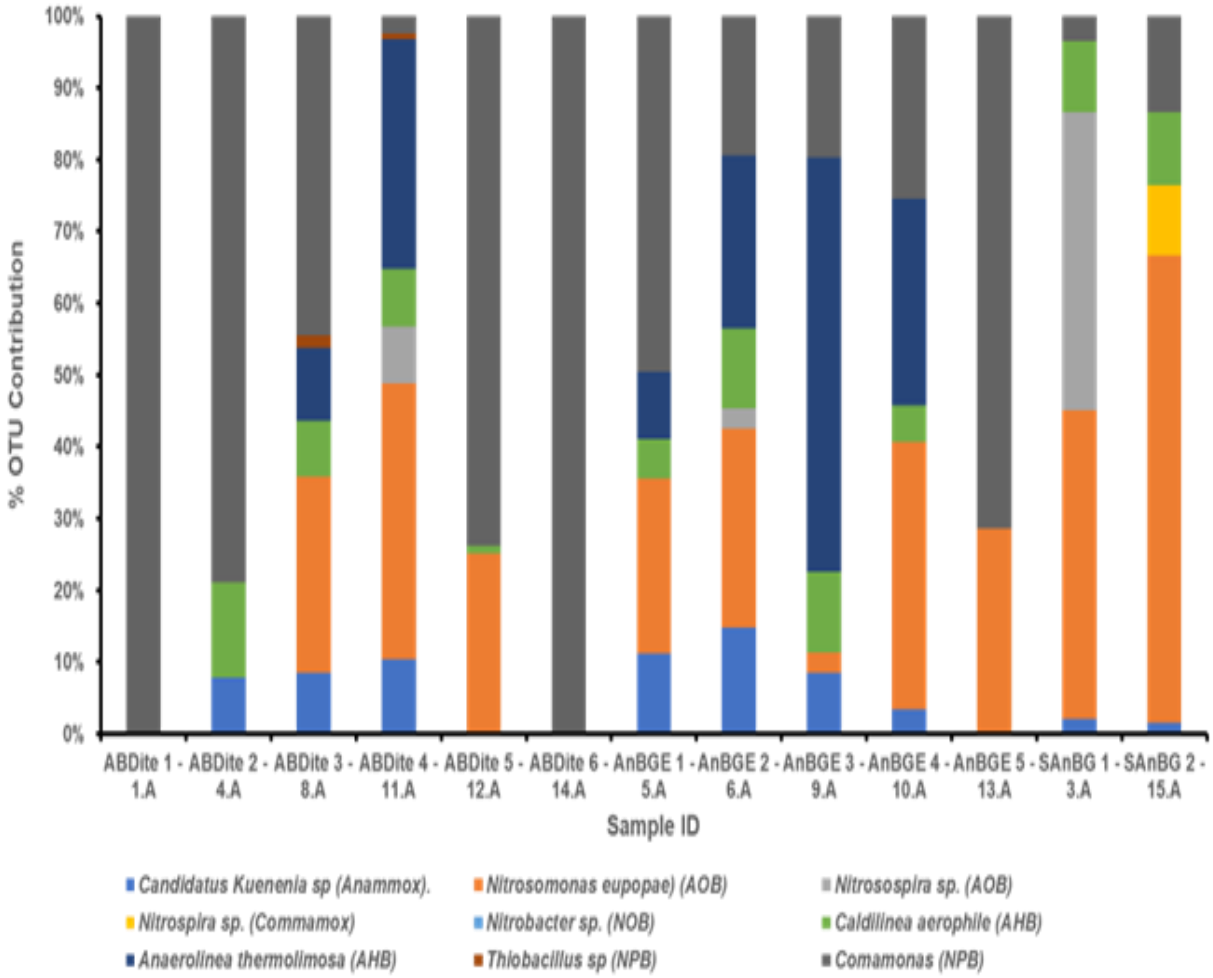

Figure 5.11 Percentage OTUs contributed to the EBBR-Anammox microbial communities identified using **A3V4a NGS** primer **Key: ABDite** - ABDite bioparticles sampled from the EBBR at different stages: **ABDite 1** (1st month); **ABDite 2** (5th month); **ABDite 3** (7th month); **ABDite 4** (13th month); **ABDite 5** (21st month); **ABDite 6** (24th month). **AnBGE** – Anammox bacteria granule sampled from the EBBR at different stages: **AnBGE** 1 & 2 (5th month); **AnBGE** (7th month); **AnBGE** 4 (13th month); **AnBGE** 5 (21st month); **AnBGE** 6 (24th month). **SAnBG** – Anammox bacteria granule supplied by STW; **Anammox** - anaerobic ammonia oxidizing bacteria; **AOB** - ammonia oxidizing bacteria; **Commamox** – complete ammonia oxidizing bacteria; **NOB** – nitrite oxidizing bacteria; **NPB** – nitrifying proteobacteria. **%** OTU contributed to the EBBR-Anammox communities by Anammox bacteria (7 %); AOB (56 %); Commamox (1.9 %); NOB (0 %); **AHB** (25 %); **NPB** (68 %)

Other EBBR-Anammox microbial communities

Other microbial communities identified by NGS that could have influenced the EBBR-Anammox process include ammonia oxidizing Archaea (AOA); nitrifying proteobacteria (NPB), nitrate reducing bacteria (NRB) amongst others (Figure 5.11 & 5.12).

One species of ammonia oxidizing Archaea (AOA) *Nitrosarchaeum limnium* was identified from ABDite bioparticles 1 & 4 with 0.6 % total OTU contribution using the V3V4a primer; AOA was not identified in samples analysed with A3V4a. The NGS result confirmed that the presence of *Nitrosarchaeum limnium*, which oxidizes ammonia to nitrite, was low in the EBBR. The low presence could explain why AOA DNA was not detected with the Ar109F/Ar912rt primer set (Table 5.1); suggesting AOA had reduced presence and influence on the EBBR-Anammox process.

Two nitrifying proteobacteria (NPB), *Thiobacillus and* Comamonas were identified from the NGS analysis (Figure 5.11 & 5.12). *Thiobacillus* had more OTU (2.2 %) from ABDite bioparticle and AnBGE samples with V3V4a primer, compared to the 0.3 % OTU using A3V4a primer, the V3V4a OTUs value was 1.9 % more. The more abundant NPB was *Comamonas* species found in all EBBR ABDite bioparticle & AnBGE samples, which contributed with 47.90 % total OTU using V3V4a; this value was 12 % less than the 60.3 % obtained with AV34a primer. This result indicated that *Comamonas* dominated over *Thiobacillus* in the EBBR-Anammox system; and confirmed the importance of NGS in enumerating the different communities in the EBBR, which Sanger sequencing could not accomplish. The high presence of *Comamonas* in ABDite bioparticles 1, 5 & 6 could have led to accumulation of nitrite to a level that inhibited the activity of the Anammox bacteria, which explains the absence of Candidatus *Kuenenia* from these samples (Figures 5.10 & 5.11).

The NGS analysis results had satisfied objective 4 by confirming Sanger sequencing report that identified the Anammox, AOB, NOB and yeast communities

of the EBBR-Anammox process. In addition, NGS identified the AOA, nitrate reducing bacteria (NRB) *Nitratireductor* sp., species of anaerobic heterotrophic bacteria (AHB) - *Caldilinea aerophile* and *Anaerolinea thermolimosa* that contributed 25 % OTUs to the EBBR-Anammox communities alongside others not described (Figure 5.11 & 5.12).

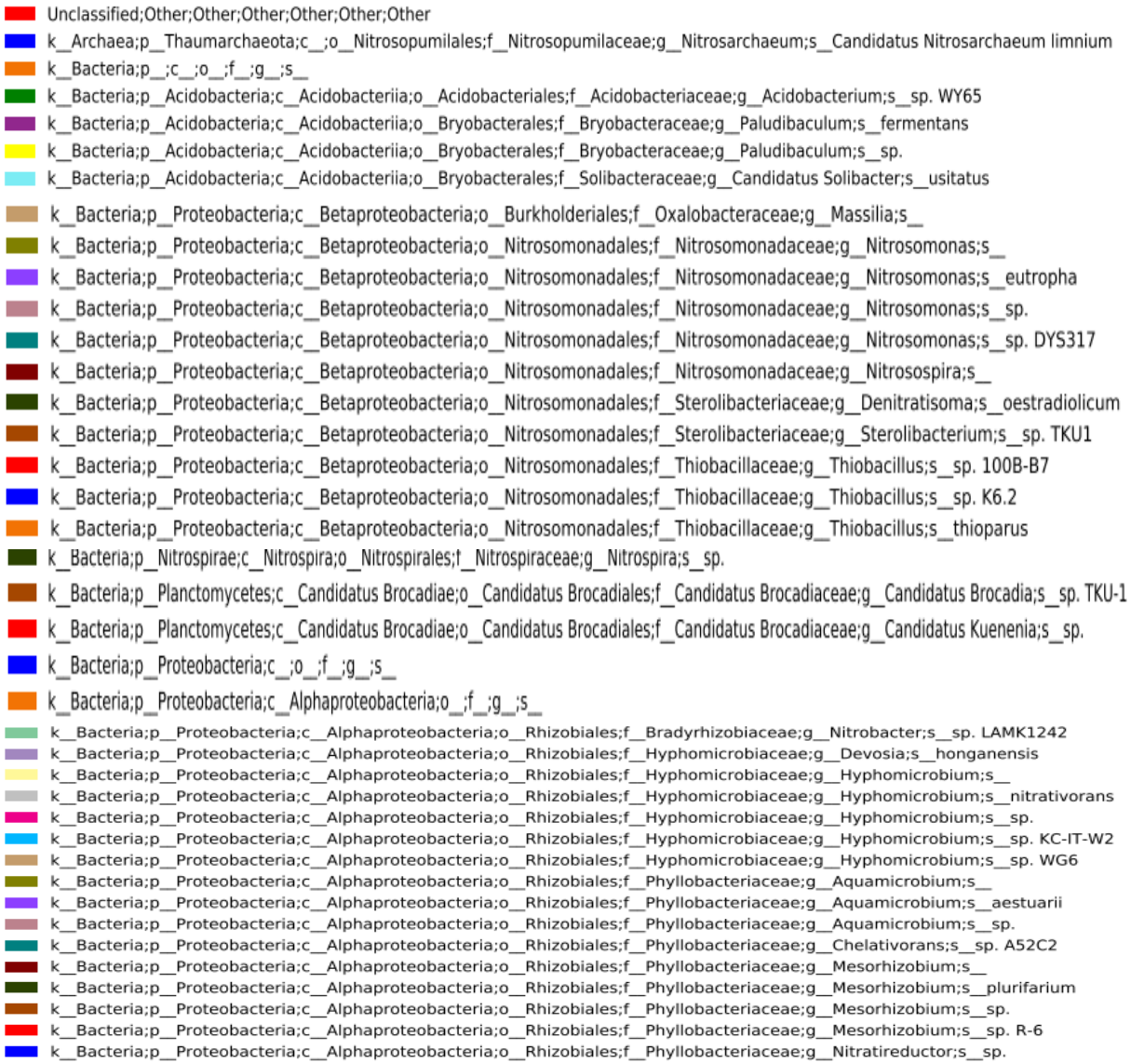

Figure 5.12 Mixed microbial communities of EBBR-Anammox process identified by NGS.

The NGS result of the microbial communities that came in the seed Anammox bacteria granules (SAnBG) from STW were compared with the communities identified from the EBBR ABDite bioparticles and granules (AnBGE) (Figures 5.10 - 10.12). The results indicated that the microbial communities were very similar as all the communities (Anammox, AOB, Commamox, NOB, NPB, AHB, NRB) except that the AOA, *Nitrosarchaeum limnium* identified only from ABDite bioparticle samples were present in the SAnBG (Figure 5.10). In addition, the NGS results suggested that all key bacterial communities of the EBBR-Anammox process were better established on the ABDite bioparticle, hence the highest % OTU contributions made to EBBR-Anammox communities by the Anammox bacteria Candidatus *Kuenenia* (6.5%), AOB, *Nitrosomonas eutropha* (88 %) NOB, *Nitrobacter* (3.5 %) came from DNA samples extracted from the EBBR ABDite bioparticle; and for DNA from SAnBG, the % OTU contributed were 1.4 % 61 % and 0.4 % for the same bacteria respectively

The NGS results has provided strong evidence that the EBBR-Anammox system, particularly the ABDite bioparticles, supported the growth of all the microbial communities responsible for the Anammox wastewater treatment process and others not directly involved; and therefore, effective.

5.4 Discussion

The microbial communities identified in the lab scale EBBR-Anammox wastewater treatment system were Anammox bacteria, AOB, AOA, NOB, NRB and NPB. The evidence for the presence of these organisms in the EBBR came from NGS microbiome analysis (Figures 5.10 - 5.12). Sanger sequencing BLAST analysis identified the same organisms targeted by species-specific primer sets (Tables 5.13 – 5.15 and 5.6 – 5. 9, Figure 5.4). In addition, the NGS reports (Figure 5.10 - 5.12) agreed with the phase contrast and SEM microscopy results that revealed the development of Anammox biomass on the ABDite® bioparticles (Figures 4. 3 - 4.10). The EBBR-Anammox data positively aligned with the reports that Anammox bacteria (AnAOB) prefer communal life in which each member of the microbial community play different roles. For example, Niederdorfer et al., (2021) in his study of stages of biofilm development reported that heterotrophic bacteria initiate the formation of the first layer of biofilm, which provides stability for AnAOB colonisation. Living as community provide AnAOB with other benefits such as protection from grazing protozoa (Mozumder et al., 2014). A study involving a membrane bioreactor by Botchkova et al., (2015) found that the coexistence of AnAOB with other communities including AOB and yeast shielded the AnAOB from molecular oxygen; and increased AnAOB ability to resist inhibition by high nitrite concentration of up to 400 mg L^{-1}. The identification of these communities reported by other studies in the EBBR-Anammox system suggests that the AnAOB in my bioreactor must have had similar benefits from the presence of other communities for Anammox effective activity.

The EBBR contained 3 genera (Candidatus *Brocadia, Kuenenia and Jettenia*) of Anammox bacteria that convert wastewater ammonia (NH_3) and nitrite to nitrogen gas (Kuenen, 2020; Rodriguez-Sanchez *et al*., 2014). The nitrite for the Anammox process was made available by ammonia oxidizing bacteria (AOB), which partially oxidize ammonia to nitrite (NO_2). Nitrite oxidizing bacteria (NOB) convert NO_2 to nitrate (NO_3); this completes the nitrification process. The presence of

Nitrosomonas and Nitrosospira (AOB), and *Nitrobacter* (NOB) in the EBBR confirms that nitrification is a two-step oxidation of NH_3 to NO_2, and then NO_2 to NO_3 occurred in in the bioreactor (Roy et al., 2017). Other microbial communities involved in NH_3 oxidation that were present in the EBBR were NH_3 oxidizing Archaea (AOA) - *Nitrosarchaeum limnium* that oxidize NH_3 to NO_2 (Wang et al., 2017). In addition, was *Nitrospira, a* complete ammonia oxidizing (Commamox) bacterium, that oxidize NH_3 to NO_3 (Koch et al., 2019; Daims et al., 2015), nitrifying proteobacteria - *Comamonas and Thiobacillus,* which convert ammonia to nitrate (Wuchter et al.; 2006). The presence of these bacteria has important implications in the EBBR-Anammox process (see below).

Additionally, present among the EBBR-Anammox communities were nitrate reducing bacteria (NRB), e.g., *Nitratireductor* sp. that can reduce nitrate to ammonia (Holmes et al., 2019); Denitrifying heterotrophic bacteria (DHB) such as *Caldilinea aerophile* and *Anaerolinea thermolimosa, which* anaerobically convert nitrate to nitrogen gas (Fajardo et al., 2014). Denitrification process by Anammox bacteria and denitrification of nitrate and nitrite to nitrogen gas conducted by heterotrophic denitrifying bacteria was reported in a SBR by Langone et al., (2014), in which nitrifying bacteria was found to support the Anammox process, suggesting that the similar presence of these groups in the EBBR system could have also benefited the AnAOB from the denitrification processes by the DHB described.

Finally, yeasts, which support the development of stable biofilm on growth support medium were also present in the EBBR. The coexistence of Anammox bacteria and yeast is widely reported in literature, since the relationship between yeasts and Anammox bacteria was discovered first in the 1990 in a yeast factory (Kuenen, 2020; Zekker et al., 2014; Van De Vossenberg et al., 2008). Yeasts do not contribute directly to the Anammox process, but they are thought to provide micro anoxic environment to protect Anammox bacteria from molecular oxygen AOB use to oxidize ammonia to nitrite as reported by Crouzet et al., (2014); Van Niftrik et al., (2012). The presence of yeasts in the EBBR-Anammox system suggest that these yeasts could have provided the same protection for the AnAOB in this study.

Additionally, the yeasts (*Filobasidium uniguttulatum and Cryptococcus*) species, found in the EBBR (Table 5.15 and Figure 5.9) are human pathogens that cause cryptococcal meningitis according to Hagen et al., (2016). The coexixtence of pathogenic yeast, *Candida tropicalis* in Anammox wastewater treament was reported by in a study by Sun et al., (2021); and Molly (2006) found pathogenic yeast among a complex microbial commuities dominated by AnAOB. Although these pathogenic yeasts could have supported the Anammox bacteria as stated earlier, their presence in EBBR and other Anammox wastewater have important health and environmental implications. These include the need for tertiary wastewater treatment (WWT) using methods such as the Anammox process to removal nitrogenous compounds and pathogens by chlorination (not the focus for this study), before the treated wastewater is discharged into natural environment to reduce the risk of human infection and death of aquatic lives described by Blaettle, (2018) and Fatta-Kassinos et al., (2011).

The presence of the different Anammox microbial communities isolated from the EBBR had different levels of influence on the EBBR-Anammox process. Representing the Anammox (AnAOB) bacteria community were Candidatus *Brocadia*, Candidatus *Kuenenia* and Candidatus *Jettenia* identified by Sanger sequencing (Table 5.13, Figure 5.6) and NGS analysis (Figure 5.10 -5.12). Of the three genera of AnAOB in the EBBR, there were many species of Candidatus *Brocadia (e.g., Brocadia* sp. TKU-1, *Brocadia fulgida, Brocadia sapporoensis, Brocadia caroliniensis, Brocadia pituitae* etc. There were six strains of *Candidatus Kuenenia stuttgartiensis* e.g., *Kuenenia stuttgartiensis* - CSTR1, *Kuenenia stuttgartiensis* _MBR1 and Kuenenia sp. clone RAS-Val-1). In addition, were two strains of Candidatus *Jettenia* sp. (*Jettenia* sp. Kumadai – 4 and *Jettenia* sp. kumadai-3). Candidatus *Brocadia* contributed 76 %; Candidatus *Kuenenia stuttgartiensis (18 %) and* Candidatus *Jettenia* (6 %) of the total AnAOB in the EBBR (Figure 5.6). The results suggests that Candidatus *Brocadia* had more influence over *Kuenenia* and *Jettenia*; and therefore, could have contributed more to the Anammox process (conversion of ammonia NH_3-N and NO_2-N to N_2 gas) according to Sanger-BLAST reports (Figure 5.6).

The prevalence of Candidatus *Brocadia* in the current research supported the report that Candidatus *Brocadia* dominated over other Anammox bacteria genera in sludge granules collected from a wastewater treatment plant (Han et al., 2013) ; and a in sequencing batch bioreactor investigation that studied the performance of the microbial communities of a simultaneous Anammox and denitrification by Li et al., (2016), and found that Candidatus *Brocadia* was not inhibited by high nitrite concentration. The implication of the above reports and the EBBR results is that *Brocadia* plays a vital role in Anammox process irrespective of the bioreactor ; and that the EBBR-Anammox process was working well and therefore, *Brocadia* will be considered in future Anammox wastewater treatment using the EBBR technology.

Candidatus *Brocadia* sp. TKU.1 (Figure 5.12), and Candidatus *Kuenenia* identified by NGS contributed 21% OTUs to the EBBR-Anammox bacterial communities (Figure 5.10 -5.12). The NGS findings was in contrast with Sanger result that identified several species of Candidatus *Brocadia* (Figures 5.6). The reason for the low presence of Candidatus *Brocadia* according to NGS analysis could be that the primers used were more effective at detection Candidatus *Kuenenia.* In addition, the decline in Candidatus *Brocadia* enumerated by NGS could be linked to DNA damage caused by low storage temperature as reported by Gamon et al., (2019); Grubwieser et al., (2006) over time.

However, the dominance of Candidatus *Kuenenia* over other AnAOB bacteria in the EBBR study agrees with NGS findings from Anammox sequencing biofilm batch reactor (SBBR) experiment that treated landfill leachate (He et al., 2018). EBBR results also agreed with the report from an up-flow anaerobic sludge blanket reactor (UASBR), which investigated the long-term effect of MnO_2 on Anammox process performance (Miao et al., 2018). These results seem to suggest that NGS analysis is tailored toward detecting Candidatus *Kuenenia* than other genera of Anammox bacteria.

The high presence of both Candidatus *Brocadia* and *Candidatus Kuenenia stuttgartiensis* in the EBBR-Anammox research has demonstrated that both bacteria had positive influence and contributed to the Anammox bacteria activity in

the EBBR. However, with more than five species of Candidatus *Brocadia* versus *Candidatus Kuenenia stuttgartiensis*, the former should have had more influence in the current Anammox study, and therefore will be useful for in EBBR-Anammox future investigations.

The influence exerted by the ammonia oxidizing communities in the EBBR-Anammox process varied. Two AOBs, *(Nitrosomonas and Nitrosospira sp.)* had a positive influence by producing the nitrite for the Anammox process. *Nitrosomonas eutropha* alone contributed more; making up 18 % of AOB identified by Sanger and % 182 OTUs of communities identified by NGS (Figures 5.11 -5.12(Figure 5.7). The positive influence of the AOB was demonstrated by NGS results, which indicated that Anammox bacteria Candidatus *Kuenenia stuttgartiensis* had more OTUs, an indication of Anammox reaction occurred only when the % OTUs of the AOB in the EBBR was < 10 %; when OTU was > 20 %, Anammox bacterial activity was inhibited (Figures 5.10 & 5.11). For example, in ABDite 5 & ABDite and AnBGE, where Nitrosomonas % OTUs were 8.2 and 9.1 respectively, Candidatus Kuenenia stuttgartiensis had % OTUs of 2.4 and 2.6 %; but when the OTUs contributed by *Nitrosomonas* rose to 20.6 and 25.5 %; Candidatus *Kuenenia stuttgartiensis* had 0%. This Indicated partial loss of Anammox activity, possibly because of increase in nitrite concentration (Figure 5.10). Inhibition of the EBBR-Anammox process due to nitrite accumulation confirms similar reports by other Anammox technologies (Bettazzi et al., 2010; Langone et al., 2014), described in detail in chapter 2.

The nitrifying proteobacteria (NPB), *Comamonas* had a similar pattern of influence on the EBBR-Anammox process as the AOB; it contributed 116 OTUs to the EBBR microbial communities. The high influence of *Nitrosomonas* and *Comamonas* had on the EBBR-Anammox process corroborated with the result of an Anammox investigation using anaerobic granular reactor, which found that *Comamonas* and *Nitrosomonas* were the dominant ammonia oxidizers (Ramos et al., 2016). While the presence of these two groups of bacteria was needed for partial nitrification,

their activity should be controlled to avoid excess production of nitrite that reduces the efficiency of the Anammox process as reported by Jin et al., (2012).

The ammonia oxidizing archaea (AOA), *Nitrosarchaeum limnium*, contributed < 1.0 % OTU to the EBBR communities; and therefore, had minimal influence on the EBBR-Anammox process. The reduced presence of AOA implied less activity and competition with AOB for ammonia; this favours the Anammox bacteria because it will keep nitrite accumulation below inhibitory level. Erguder, et al., (2009) showed that low levels of AOA favoured Anammox bacteria as it reduced nitrite accumulation, which suggests the low levels seen in this study had similarly favoured the efficient running of the EBBR-Anammox process. The EBBR-Anammox finding was different from the report of an Anammox bioreactor investigation by Yang et al., (2020) in which *Thaumarchaeota* (AOA), outcompeted *Nitrosomonas (*AOB), thereby reducing the efficiency of the Anammox operation.

Another microbial community that could have negatively influenced the Anammox process is the complete ammonia oxidizing (Commamox) bacteria because it produces both nitrite and nitrate (Koch et al., 2019; Daims et al., 2015). *Nitrosospira*, the only Commamox identified in the EBBR investigation contributed about 7.1 % OTUs, of which only 0.4 % OTU came from ABDite bioparticles and the rest from SAnBG samples EBBR-Anammox process. If the number of *Nitrosospira* increases within a system, they can outnumber the AOB and completely oxidizes NH_3 to NO_3. The negative impacts include non-availability of NO_2 and increase in NO_3 concentration, which reduces the activity of the Anammox bacteria. However, with a reduced presence in the EBBR-Anammox investigation, the influence of *Nitrosospira* was minimal. The low impact of *Nitrosospira* to EBBR-Anammox process aligned with the result from a partial nitrification- Anammox bioreactor experiment conducted by Shao et al., (2021), which presented evidence that effective Anammox process can only be achieved if the activity of *Nitrospira* is reduced. These two reports suggests that the activity of *Nitrospira* in the EBBR was limited, thereby suggesting that the EBBR-Anammox process was effective.

The NGS analysis result revealed that *Nitrobacter* (NOB), made the highest OTUs (3.5 %) contribution to the EBBR-Anammox system when the presence of the AOB was high, with 25.5% OTU (Figure 5.10) resulting in the absence of Anammox bacteria in ABDite 5 and 6 using both primers (Figure 5.10 & 5.11). The EBBR-Anammox data positively agreed with the report that NOB grow when oxygen and nitrite levels are high as reported by (Rodriguez-Sanchez et al., 2014; Munz, et al., 2011). The identification of *Nitrobacter* species, within the EBBR-Anammox system at that stage of the study indicated that this bacterium might have contributed to the oxidation NO_2 to NO_3, thereby inhibiting the Anammox process. Increase in the activity of NOB reduces the efficiency of the Anammox process because NOB compete with Anammox bacteria for nitrite. Li and Sung (2015) investigated the Anammox process using a lab scale upflow anaerobic sludge blanket (UASB) reactor; and presented evidence that Anammox was only established in the bioreactor without NO_2 or NO_3 accumulation. The results suggest that apart from the short (21- 24 months) period in which the activities of the AOB and NOB were high, there was no accumulation of NO_2 or NO_3 in the EBBR; and therefore, the Anammox process was effective at removing the wastewater ammonia (Figures 5.10 – 5.11).

*Anaerolinea thermolimosa and Caldilinea aerophile (*AHB) contributed of 9 – 16 % OTUs to the EBBR-Anammox process; The denitrifying activity of the AHB could have benefited the EBBR-Anammox process by reducing NO_3 concentration within the bioreactor; and by increasing the stability of the Anammox biofilm formed on the ABDite biomass medium. The importance of the heterotrophs in dentification and in maintaining an active biofilm has been reported in a study involving nitritation-anammox granular sludge reactors (Jia et al., 2018; Hubaux et al. 2015). Denitrification by Anammox bacteria process and denitrification by HDB reduced the activity of NOB in a was reported from a SBR experiment (Langone et al., 2014). These findings suggest that the EBBR-Anammox operation benefited in a similar way from the activity of the AHB community as reported these studies.

Comparing the microbial communities that came in the seed Anammox bacteria granules (SAnBG) supplied by STW with the communities identified from the EBBR

ABDite bioparticles and granules (AnBGE) by Sanger sequencing (Tables 5.13b & 5.13b) and NGS (Figures 5.10 -10.12), both methods revealed that the compositions of the communities were the same. Sanger results that targeted specific communities were confirmed by NGS identified all the microbial communities (Anammox, AOB, Commamox, NOB, NPB, AHB, NRB) expected in any Anammox bioreactor were mainly form the EBBR ABDite bioparticles; AnBGE and SAnBG samples. Niederdorfer et al., (2021) identified these same microbial communities in his study of stages of Anammox biofilm development in a SBR. Additionally, Pereira et la., (2017) in a study of microbial communities in Anammox reactors using a rotating biological contactor reactor found the same communities that were present in the EBBR. The findings from these studies indicated that the EBBR ABDite bioparticles supported the growth of the same key communities, but with higher % OTU (Figures 5.10 & 5.11) as previously discussed; and therefore, was operational.

The only group missing from STW SAnBG was Nitrosarchaeum limnium (AOA), identified only from ABDite bioparticle with 0.7 % OTU. (Figure 5.10) The very low presence of the AOA reported by NGS, explained why Sanger sequencing using Ar109F / Ar912rt R primer set did not detect AOA. Sanger and NGS results have provided strong evidence that the EBBR-Anammox system supported the growth of all the microbial communities responsible for the Anammox wastewater treatment process reported by other Anammox investigations (Pjevac et al., 2017; Person et al., 2016). A suggestion that the EBBR supported the growth of the same communities of Anammox bacteria, and so was working well.

Evaluation of the key Anammox communities (AnAOB, AOB and NOB) identified from EBBR samples analysed at different stages of the Anammox investigation found that the compositions of organism were the same; but there were variations in the abundance of bacteria identified (Figures 5.6 -9 & 5.10 -10.12). Meng et al., (2017) presented evidence that the composition of bacteria community was similar but there was a variation in the abundance of Candidatus *Brocadia* in his Anammox wastewater treatment investigation. At the start-up of the investigation, the variation in the EBBR Anammox bacteria (AnAOB) identified from ABDite

bioparticles revealed variation in abundance over the study period. For example, ABDite 1 sampled in the first month had 0.3 % OTU; partly because Anammox bacteria (AnAOB) have slow growth rate and needed time to adjust to the new process conditions within the EBBR. Kartal et al., et al., *(*2010) found that within eight Anammox bioreactors, the bacteria did not become established until long after inoculation due to Anammox bacteria slow growth. The abundance of the AnAOB steady increased to 2.6 % OTUs (ABDite 4) 13 months later; this suggested stability within the bioreactor. Between 21 - 24 months (ABDite 5 and 6), the AnAOB contributed 0 % OTU from ABDite bioparticle samples (Figure 5.10); the insatiability possibly caused by fault in the air flow meter that could have increased oxygen and nitrite concentrations to levels that inhibited the activity of AnAOB. Liu et al., (2020) investigated shifts in microbial communities using an Anammox biofilm reactor; and found that the abundance of Candidatus *Kuenenia* to Candidatus *Brocadia* fell below 25 % with increase in nitrogen loading rate, like the EBBR report.

The AOB also showed variation in abundance; % OTUs was about 10 % (ABDite bioparticles 1 – 4) in the first 13 months from start-up of EBBR-Anammox operation and was > 20% in (ABDite 5 & 6). The EBBR results highlighted that at both the start- up (1st month) and end of the investigation (21- 24 months) that the abundance of the AOB was 7.5 and 25 % OTUs compared to the 0.3 & 0 % contributed by AnAOB respectively at the same period. These results suggest that the presence and absence the AnAOB was determined by AOB. Additionally, while the NOB was the least abundant compared to the AnAOB and AOB, variation was also found in their OTUs contributed. The NOB, which was absent in ABDite 1 (1st month) rose to 3.5 % (ABDite 6) when the OTUs of the AOB (25 %) and NPB (21 %) were highest and AnAOB absent. The EBBR-Anammox findings suggest that instability in EBBR due to possible increase in oxygen, nitrite and nitrate concentrations inhibited Anammox activity is a confirmation that these conditions should be maintained below the level that inhibits AnAOB, but supports AOB, and limit NOB activity for success Anammox process. These conditions have been

shown in other systems where Anammox bacteria activity have been inhibited, and discussed previously (Carvajal-Arroyo et al., 2013; Langone et al., 2014).

The results of the EBBR-Anammox study presented were obtained using molecular biology identification tools; and came from DNA extraction/Nanodrop quantification (Table 5.5); PCR/Agarose gel (Figures 5.1 – 5.5); Sanger sequencing/BLAST analysis (Figures 5.6 – 5.9) and NGS (Figures 5.10 – 5.12). The EBBR-Anammox data have provided evidence suggesting that while the Anammox process was slow at the start and partially inhibited at the end, the Anammox wastewater treatment process was established within the EBBR, and therefore, effective. The establishment of the Anammox process within the EBBR has implication for wastewater treatment. Apart from adding to the body of knowledge, this research has shown that the Anammox wastewater treatment can be expanded using the EBBR technology; and finally, it highlights the need for more studies to overcome to challenges posed by operating process conditions; to maximize the potentials of the Anammox wastewater treatment process using the EBBR.

5.5 Conclusion

The molecular biology techniques (DNA extraction, PCR Sanger, BLAST and NGS) were effective for the identification of the microbial communities present in the EBBR-Anammox System. By identifying all key groups of bacteria (Anammox, AOB and NOB) that participate in the Anammox process alongside others, these results suggest that the prevailing operation conditions within the bioreactor supported their growth. Even though Anammox bacteria have very slow growth rates and demanding growth conditions, the EBBR ABDite bioparticle seemed to have met the cultivation conditions; hence the % OTU contributions made to EBBR-Anammox communities by the Anammox bacteria increased from 0.3 % at the start-up of operation to 2.6 % (Figures 5.10 – 12). NGS results also supported the conclusion that the extracted DNA from the EBBR-Anammox ABDit belonged to the same key bactria (AnAOB, AOB and NOB, etc) isolated in other established Anammox wastewater bioreactors, leading to the identification of the same bacteria. Therefore, the indication of the EBBR-Anammox NGS results is that the Anammox wastewater treatment process was established in the EBBR; this has created the opportunity for potential adaptation of the EBBR technology for future Anammox wastewater treatment.

CHAPTER SIX

Discussion and Conclusion of Research

6.0 Review of research aim, summary, limitations, and future work

Chapter six reviewed the original aim of the EBBR-Anammox synthetic wastewater treatment research and presents the key findings from each chapter. It finally, identifies the limitations of the study and provides recommendations for future work.

6.1 Review of research aim

The original aim of the research was to determine whether the anaerobic ammonia oxidation (Anammox) wastewater treatment process could be developed using a lab scale expanded bed biofilm reactor (EBBR).

Chapter 1 was the general introduction and review of the Anammox process in literature.

In chapter 2, the EBBR was designed, inoculated with the STW Anammox seed granules and maintained in continuous culture with synthetic wastewater for 560 days. Physical observation for evidence of Anammox process (bed hight increase due to biomass development on the ABDite medium (bioparticles), presence of granules and nitrogen gas production) was conducted; nitrogen removal rate (NRR) and nitrogen removal efficiency (NRE) were calculated from influent and effluent ammonia-nitrogen (NH_3-N) and nitrite-nitrogen (NO_2-N) concentrations.

Chapter 3 dealt with the optimization of the EBBR-Anammox process conditions of pH and temperature combinations, to determine the optimum pH and temperature set to yield the highest nitrogen removal rate.

In chapter 4, physical observation EBBR for development of Anammox bacteria granules and biofilms formation on the ABDite® growth medium was coupled with microscopy techniques examination the ABDite bioparticles.

Finally, in chapter 5, molecular biology techniques were used for DNA extraction, PCR amplification, Sanger (species specific) and NGS (non – specific) sequencing techniques were used to identify targeted Anammox bacteria and other microbial communities present within the EBBR-Anammox system.

The performance of the EBBR-Anammox synthetic wastewater treatment (WWT) process was compared with data from established Anammox bioreactors in literature.

6.2 Summary of findings in each chapter

Chapter two of the study provides the first quantitative data obtained from physical measurements and chemical analysis suggesting the establishment of the Anammox wastewater treatment process using the EBBR. The main findings include the following:

- The EBBR was in continuous culture operation for over 560 days with brick red bioparticles and Anammox granules developed (Figures 2.1 & 2.14).
- Sparging the EBBR-Anammox system with nitrogen gas was effective for Anammox bacteria activity; without inhibiting the AOB activity.
- Gas suspected to be N_2 gas, but unconfirmed was produced (Figure 2.5).
- Biomass development on the ABDite® growth medium resulted in a 63 % (expanded) and 54 % (static) bed heights increases (Figure 2.6).
- Adding 5 cm of sand was effectively stopped ABDite medium from grinding and leaving the EBBR expanded column.
- Washing with deionized water and replacement with SWW every three months maintained the viability of the stored Anammox seed granules.
- Approximately, a maximum of 300 mg L^{-1} (NH_3-N) and 400 mg L^{-1} (NO_2-N) were removed from the EBBR-Anammox synthetic wastewater at a ratio of 1:1.3 (Figures 2.6- 2.8).
- Maximum NRR achieved in this research was 6.1 Kg N m^{-3} d^{-1} (Figures 2.9 – 2.11)
- Maximum NRE efficiency of 80 – 100 % (Figure 2.12 – 2.14).

In chapter 3, the optimization of the EBBR-Anammox process centred on investigating the effect of different pH and temperature combinations on NRR. The experiments determined the temperature and pH set at which the lowest and highest NRR were achieved. The findings, which added more evidence that the Anammox process was being established within the EBBR column were that:

- The EBBR reached a steady state operation between 1.5 – 2 h during the optimization process (Figures 3.2 – 3.5).
- Changes in temperatures (28, 30 °C) and pH (pH 7.7 - 8.3) affected NRE of the EBBR-Anammox process; and obtained NRR between 0.46 to 2.23 Kg N m^{-3} d^{-1} (Table 3.3; Figures 3.3 - 3.4).

- The EBBR-Anammox process conducted at 28 °C and pH 8.2 produced the highest rate of nitrogen removal (2.23 Kg N m^{-3} d^{-1}); and was therefore, reported as the optimum process condition for removing nitrogen from the EBBR-Anammox synthetic wastewater at ($p <= 0.0001$).
- The lowest NRR achieved was 0.46 ± 0.06 kg N m^{-3} d^{-1} when the bioreactor was operated at pH 7.7, T 30 ºC ($P <= 0.005$).

In chapter 4, from physical (visual inspection) and microscopy (phase-contrast and scanning electron) examination of the EBBR expanded bed column' and the biofilm / bioparticles, the following findings were made:

- Brick red granules and biofilm consistent with Anammox bacteria developed in the EBBR column (Figure 4.1). This red colour indicated the presence of an actively growing Anammox bacteria capable of converting ammonia and reducing nitrite to nitrogen gas (Lage et al., 2019; Lu et al., 2018; Ni and Zhang, 2013).
- Biofilm formation on the ABDite® bioparticles was confirmed by both phase contrast and SEM microscopes (Figures 4.2 & 4. 5).
- The EBBR-Anammox ABDite biofilms had the cauliflower-like morphology (Figures 4.6 - 4.10) reported previously by other established Anammox bioreactors in literature (Miller et al., 2012; Fernandez et al., 2008).
- Suspected bacterial cells including filamentous rods-like, cocci-like bacteria; and yeast-like cells developed on the EBBR ABDite bioparticles (Figures 4.11 - 4.15). The bacterial cells types found in the EBBR were similar to those reported by other bioreactors such as the ABBR (Wang et al., 2019); MBBR (Zekker et al., 2012); MFCR (Domienico et al., 2015); MBR (Wang et al., 2018) and SBR (Xu et al., 2018) amongst others published in literature

Chapter 5, which focused on the use of molecular biology techniques for the identification of targeted and all the microbial communities that inhabited the EBBR-Anammox system, had these findings:

- Sample DNA was successfully extracted from EBBR ABDite bioparticles and Anammox granules; and STW seed Anammox bacteria with the Power Soil DNA isolation Kit.

- Nanodrop quantified highest DNA concentrations were STW seed granules (33.0 ± 0.5); EBBR granules (15.6 ± 10.2) and ABDite medium (6 .2± 2.1 (Table 5.5).

- PCR amplified DNA with the right band size using species specific primers that targeted different Anammox bacteria visualized on 1.5 % agarose gel electrophoresis (Figure 5.1 – 5.5; Tables 5.1 & 5.5); estimated sizes were AOB (490 bp) with amoA-1F/amoA-2R); AnAOB ((480 bp, Amx368F/Amx820R) and (280 bp Brod541F/Amx820R)); Commamox - *Nitrospira inopinata* (550 bp, Nino_amoA_19F/Nino_amoA_252R) and NOB (395 bp, FGPS 872 /FGPS 1269)

- Sanger sequencing and BLAST analysis identified Anammox bacteria - Candidatus *Brocadia,* Candidatus *Kuenenia* and Candidatus *Jettenia* with Brocadia being the predominant; *Nitrosomonas* species (AOB) and nitrite oxidizer and *Nitrobacter* species (NOB) and yeasts (e. g. *Cryptococcus* sp.) were also identified with 98 - 100 % (Table 5.13 – 5.16; Figures 5.6 – 5.9).
- NGS microbiome analysis confirmed the presence of Candidatus *Brocadia and* Candidatus *Kuenenia (*Anammox) bacteria, *Nitrosomonas* and *Nitrosospira spp.* (AOB) *Nitrobacter* spp., (NOB). NGS also identified *Nitrospira spp.* (Commamox), ammonia oxidizing Archaea (AOA) *Nitrosarchaeum limnium;* nitrate reducing bacteria (NRB) (e. g. *Nitratireductor sp.*), anaerobic heterotrophic bacteria (AHB) e. g. *Anaerolinea thermolimosa)* amongst others (Figure 5.10 – 5.12).

- The bacteria isolated from the EBBR-Anammox process are well documented in literature to be present in other Anammox wastewater treatment bioreactors (Daims *et al.,* 2015; Guo *et al.,* 2013; Van Niftrik *et al.,* 2012).
- The compositions of the microbial communities from the STW seed granules and EBBR ABDite bioparticles were very similar in terms of the key metabolic Anammox bacteria and other organisms, but the bioparticles DNA contributed more % OTU; and effectively supported the growth of the organisms that came from the active Anammox wastewater treatment plant (Figure 5.10 -5.12).
- The molecular analysis results confirmed the identity of the suspected Anammox bacteria-like cells (rods, cocci and filamentous detected from SEM examination. Ali et al., (2013), Wang et al., (2019) and Winkler et al., (2012) reported that Anammox bacteria are rod, cocci, and filamentous bacteria.

6.3 Limitations and future work

Although the EBBR-Anammox study was conducted thoroughly, there were certain limitations as the aim of the study was explored. It is therefore important that these areas are highlighted to enable future researchers take the necessary steps to improve their work.

- Measurements of nitrogen species (NH_3-N, NO_2-N and NO_3-N) within the EBBR-Anammox system was by spectrophotometric method. This method was chosen because it was reported in literature as the method of choice

for the analysis of nitrogen concentration in wastewater because of limited online sensor equipment for the Anammox process (Lackner *et al.*, 2014).

- Future EBBR-Anammox study would benefit from an online sensor connected to data logger with sufficient data storage, for N-species, temperature, pH, and dissolved oxygen (DO) measurements in real-time (Pedrousa *et al.,* 2018; Lackner *et al.*, 2014; Lackner and Horn, 2013). This is recommended to save time, provide additional data which could be compared with the spectrophotometric data.

- In the EBBR-Anammox investigation, nitrogen gas was suspected to have been produced, but not confirmed.
  - The need to confirm the identity of the gas to be molecular nitrogen or not in suggested for future EBBR-Anammox work. The advancement of research work in this area is necessary because if the gas is confirmed as nitrogen, this will further reduce the overall cost of the process. The gas could be used to deoxygenate the rig or collected and sold; gas collection will, however, require the modification of the EBBR at a cost.

- While conducting the research, it was determined that the optimization process will investigate at least a minimum of 10 different pH (from 7.5 to 8.5) and temperature (25 to 35 °C) combinations. However, after 6 sets of conditions were tested, a major breakdown in the expanded bed column meant that the data optimization experiment was not fully completed. The break down might be because the EBBR has been in operation for many years before it was adapted for the current study. Therefore, two recommendations are made herewith for future studies.
  - The design of EBBR for lab scale Anammox wastewater treatment from of old borosilicate glass should be given adequate consideration to ensure the bioreactor will last throughout the duration of the research; and if not, a new bioreactor should be installed to avoid mishaps.

- Extension of the optimization experiment to collect more robust data to further strengthen that evidence that pH 8.2 at a temperature of 28 ° C process condition established in the current study is the optimum condition to achieve the highest NRR for the EBBR-Anammox wastewater treatment operation.

- The molecular analysis found that by using two 16S rRNA gene-based PCR primer sets (Brod541F/Amx820R and Amx368F/ Amx820R) could only retrieve 3 (Candidatus *Brocadia*, Candidatus *Kuenenia* and Candidatus *Jettenia*) from the EBBR-Anammox ABDite® bioparticle out of the 5 identified Anammox bacteria genera (*Brocadia, Anammoxoglobus Kuenenia, Jettenia* and *Scalindua)*. Although Candidatus *Anammoxoglobus* was successfully detected with Brod541F/Amx820R primers in another study (Hsu et al., 2014), this was not the case when the same primer was used in the current research. The distribution of Anammox bacteria is specific to the sampling source; and no single 16S rRNA gene-based primers can enumerate all Anammox bacteria from a sample under investigation (Han et al., 2017).
  - In the future screening of EBBR-Anammox bacteria, other 16S rRNA primer sets such as A438f/A684r, that can detect DNA from more Anammox bacteria from all environmental samples is recommended (Zhou et al., 2018; Han et al., 2013).

6.4. Conclusion

The effectiveness of the Anammox process to treat a wide range of wastewater rich in ammonia using different bioreactors have been reported by several Laboratory, pilot, and industrial-scales studies. Results from the current study have provided strong evidence of the establishment of the Anammox treatment

process using the lab scale expanded bed biofilm reactor (EBBR) after 560 days of continuous bioreactor operation, achieving nitrogen removal rate of 6.1 Kg N m-3 d-1 at 80 – 100 % nitrogen removal efficiency and supporting Anammox species like Candidatus Brocadia, Candidatus Kuenenia and Candidatus Jettenia in the expanded bed ABDIte bioparticles. Therefore, the original aim of the research, which was 'the establishment of the Anammox wastewater treatment process using the EBBR' is hereby fulfilled. The Anammox-EBBR adapted from EBBR for wastewater nitrification process is therefore a viable option for Anammox wastewater treatment.

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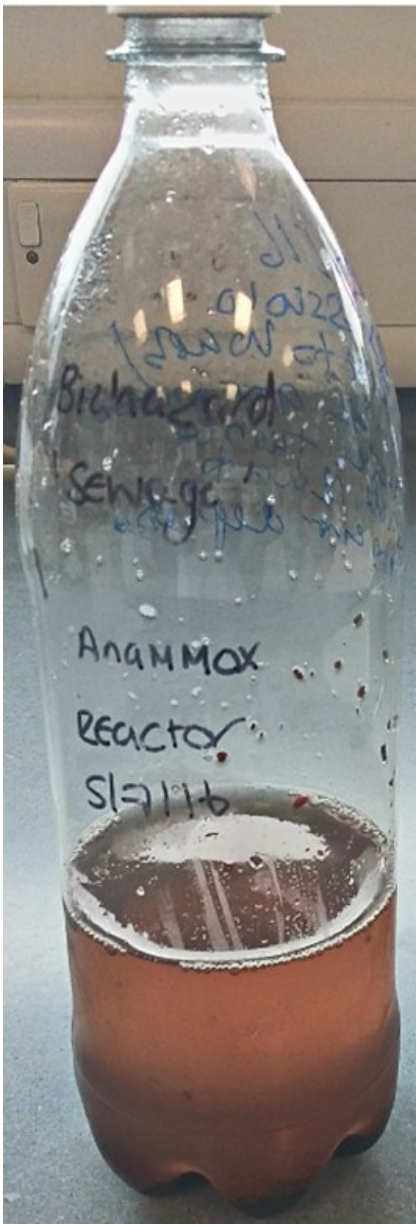
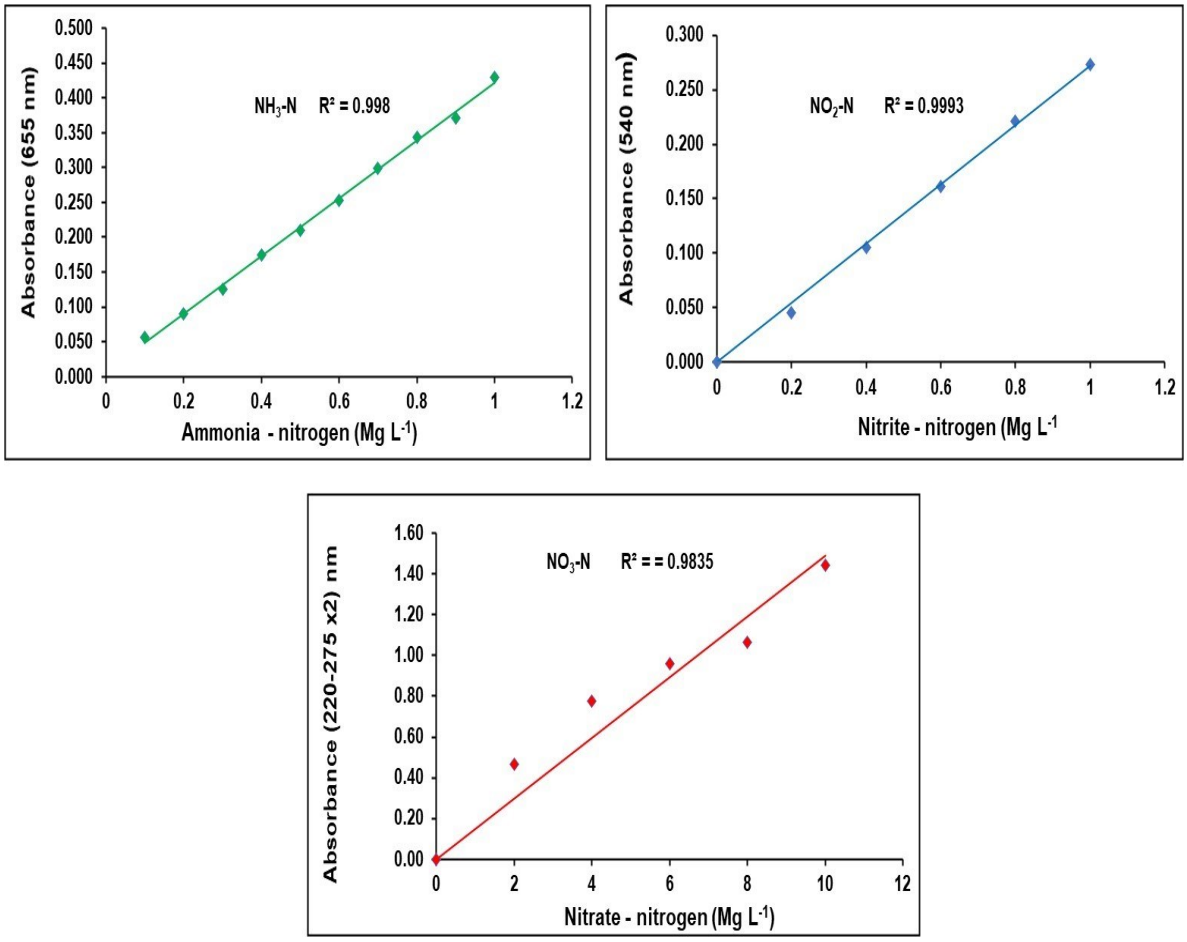
Appendix



SI 2.1: Seed Anammox granules supplied by Severn Trent Waters (STW), UK

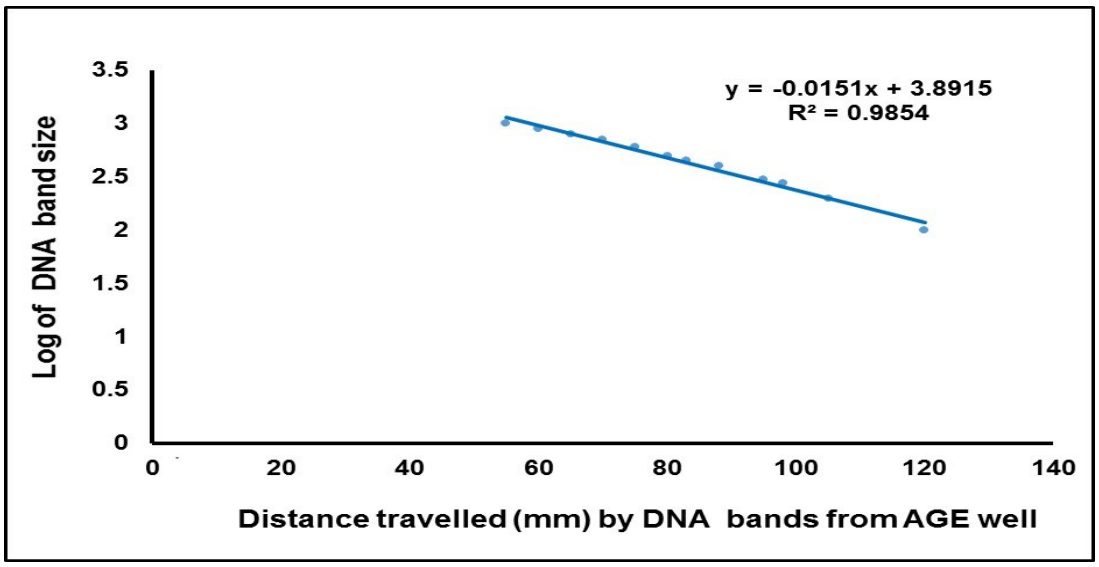


SI 2.2: Standard calibration nitrogen species in synthetic wastewater water using spectrophotometer. Ammonia- nitrogen (R^2 = 0.998), nitrite nitrogen (R^2 = 0.9993), nitrate nitrogen (R2 = 0.9835).

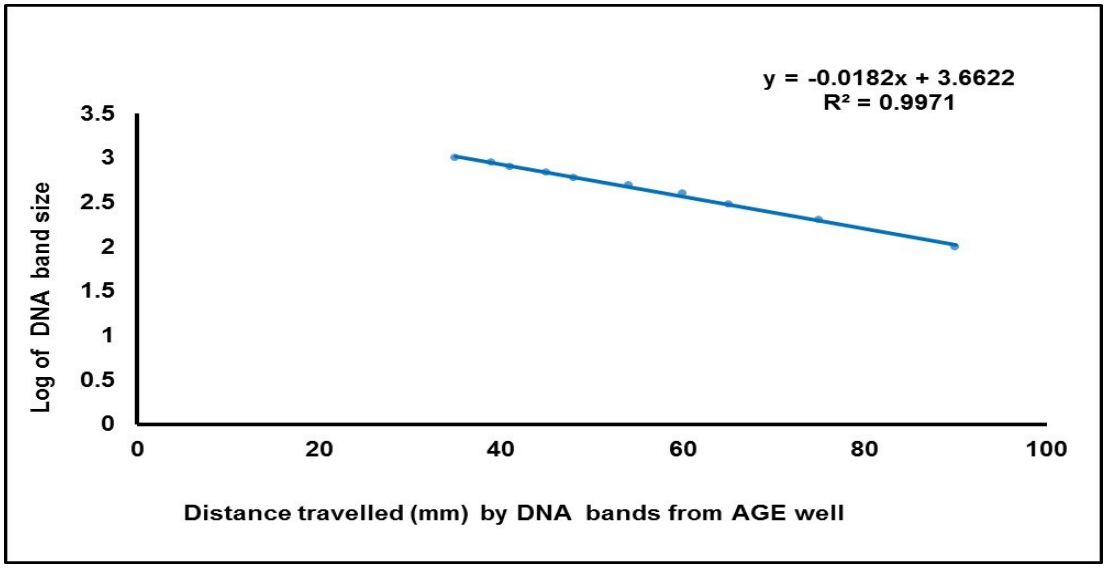
SI 4.1: ABDite® bioparticles coated with thin layer of gold mounted on studs for SEM analysis.

SI 5.1 DNA purity value quantified by Nanodrop ***Key:*** **ABDite** – ABDite bioparticles removed from the EBBR; **Liquid** - Liquid samples taken from the EBBR; **SAnBG** - Seed Anammox bacteria granule supplied by STW; **AnBGE** – Anammox bacteria granule that developed in the EBBR.

Sampling time (months)	Sample ID	Mean (260/280 nm) ratio + STDV
1	ABDite	1.3 ± 0.1
	Liquid	-
	SAnBG	2.0 ± 0.0
5	ABDite	1.5 ± 0.0
	Liquid	2.0 ± 0.5
	SAnBG	1.8 ± 0.0
7	ABDite	1.8 ± 0.00
	AnBGE	1.7 ± 0.00
	Liquid	-1.6 ± 1. 0
13	ABDite	1.6 ± 0.0
	Liquid	-10.91 ± 0.5
	AnBGE	1.9 ± 0.0
21	ABDite	1.70 ± 0.2
	AnBGE	1.8 ± 0.0
24	ABDite	2.8 ± 0.2
	AnBGE	1.6 ± 0.1



SI 5.2a: Calibration curve for estimation of Anammox bacteria DNA size detected with Amx368F/Amx820R and Brod541F/ Amx820R primer sets.



SI 5.2b: Calibration curve for estimation of yeast DNA size detected with detected by ITS1/ITS2

SI 5.3: EBBR-Anammox sample sequences assigned to OUT and taxa. ***Key*** **1)** Input sequences. **2**) Sequences after pre-processing and chimera removal. **3**) Sequences assigned to OTUs. **4**) Sequences assigned to taxa. **5**) Count after lineage-specific copy-number correction. **6**) Median sequence length after pre-processing.

Eurofin Sample Barcode	Eurofin Sample ID	EBBR sample source	1)	2)	3)	4)	5	6)
A26096	1.V3V4a	ABDite 1	235513	99.8%	72.9%	72.9%	101520	422
A26097	-	Liquid 1	-	-	-	-	-	-
A26098	3.V3V4a	SAnBG 1	194594	99.9%	74.3%	74.2%	86 437	422
A26099	4.V3V4a	ABDite 2	208080	99.8%	74.6%	74.6%	81 583	422
A26100	5.V3V4a	AnBGE 1	208591	99.7%	73.6%	73.6%	81 763	422
A26101	6.V3V4a	AnBGE 2	182891	99.9%	70.2%	70.2%	66 843	422
A26102	-	Liquid 2	-	-	-	-	-	-
A26103	8.V3V4a	ABDite 3	165347	99.9%	71.3%	71.3%	61 780	422
A26104	-	AnBGE 3	-	-	-	-	-	-
A26105	10.V3V4a	AnBGE 4	191900	99.99%	74.5%	74.5%	76 794	422
A26106	11.V3V4a	ABDite 4	178038	100.0%	77.7%	77.7%	74 936	422
A26107	12.V3V4a	ABDite 5	178038	100.0%	77.7%	77.7%	74 936	422
A26108	13.V3V4a	AnBGE 5	184715	99.9%	76.4%	76.4%	80 602	424
A26109	14.V3V4a	ABDite 6	153681	100.0%	73.9%	73.9%	60 501	424
A26110	15.V3V4a	SAnBG 2	226611	100.0%	72.0%	72.0%	94 495	422
A26111	-	AnBGE 6	-	-	-	-	-	-

Sample Name (copy-number corrected read counts)

Taxonomic Level	Taxonomic Unit	Normalized Fraction	Raw Fraction
1.V3V4a (101 520 reads)			
s	**Denitratisoma oestradiolicum** (4 OTUs with 97-98% identity in 424bp to: Denitratisoma oestradiolicum)	**26.3%**	15.6%
s	**Ignavibacterium album** (27 OTUs with 99-100% identity in 419-422bp to: Ignavibacterium album)	**16.8%**	23.9%
s	**Paludibaculum sp.** (3 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**13.9%**	9.6%
g	**Nitrosomonas** (3 OTUs with 97-100% identity in 424bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**8.7%**	5.1%
s	**Steroidobacter sp.** (6 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**3.9%**	4.8%
f	**Comamonadaceae** (1 OTU with 79% identity in 428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Malaminivora alkalimesophila)	**2.7%**	3.1%
s	**Candidatus Kuenenia sp.** (2 OTUs with 95-97% identity in 424bp to: Candidatus Kuenenia sp.)	**2.6%**	4.5%
f	**Chitinophagaceae** (1 OTU with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**2.4%**	3.8%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**2.2%**	1.3%
s	**Ralstonia sp. P-4CB2** (5 OTUs with 96-97% identity in 425bp to: Ralstonia sp. P-4CB2)	**2.2%**	6.8%
g	**Nitrosospira** (1 OTU with 98% identity in 424bp to: 3 unclassified Nitrosospira strains)	**1.6%**	0.9%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**1.4%**	2.2%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**1.1%**	2.3%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 424bp to: Sterolibacterium sp. TKU1)	**1.0%**	0.6%
s	**Candidatus Solibacter usitatus** (2 OTUs with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**1.0%**	0.7%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**0.9%**	1.1%
s	**Racemicystis persica** (1 OTU with 92% identity in 423bp to: Racemicystis persica)	**0.8%**	1.9%
s	**Acidobacterium sp. WY65** (1 OTU with 95% identity in 399bp to: Acidobacterium sp. WY65)	**0.7%**	0.5%
s	**Lentimicrobium saccharophilum** (2 OTUs with 88% identity in 420-422bp to: Lentimicrobium saccharophilum)	**0.6%**	1.0%
s	**Parvibaculum sp.** (1 OTU with 93% identity in 399bp to: Parvibaculum sp.)	**0.5%**	0.7%
s	**Hyphomicrobium sp.** (2 OTUs with 99% identity in 399bp to: Hyphomicrobium sp.)	**0.5%**	0.4%
s	**Haliea sp. SAOS-164** (2 OTUs with 90% identity in 425bp to: Haliea sp. SAOS-164)	**0.5%**	0.6%
s	**Hyphomicrobium sp. KC-IT-W2** (2 OTUs with 97-99% identity in 399bp to: Hyphomicrobium sp. KC-IT-W2)	**0.5%**	0.4%

g	**Hyphomicrobium** (2 OTUs with 95-100% identity in 399bp to: Hyphomicrobium aestuarii, Hyphomicrobium sp., Hyphomicrobium vulgare, Hyphomicrobium zavarzinii)	**0.5%**	0.4%
s	**Gemmatimonas aurantiaca** (1 OTU with 94% identity in 402bp to: Gemmatimonas aurantiaca)	**0.5%**	0.3%
s	**Aciditerrimonas ferrireducens** (2 OTUs with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.4%**	0.5%
s	**Nitrosomonas sp.** (2 OTUs with 96-97% identity in 424-425bp to: Nitrosomonas sp.)	**0.4%**	0.3%
s	**Turneriella parva** (2 OTUs with 87-95% identity in 424-425bp to: Turneriella parva)	**0.4%**	0.4%
g	**Lacibacter** (1 OTU with 95% identity in 422bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**0.4%**	0.6%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	**0.3%**	0.3%
s	**Hyphomicrobium nitrativorans** (1 OTU with 100% identity in 399bp to: Hyphomicrobium nitrativorans)	**0.3%**	0.2%
s	**Holophaga sp. WY42** (1 OTU with 91% identity in 426bp to: Holophaga sp. WY42)	**0.3%**	0.2%
s	**Chelativorans sp. A52C2** (1 OTU with 88% identity in 402bp to: Chelativorans sp. A52C2)	**0.3%**	0.3%
s	**Longimicrobium terrae** (1 OTU with 88% identity in 426bp to: Longimicrobium terrae)	**0.3%**	0.1%
o	**Burkholderiales** (2 OTUs with 79-96% identity in 424-430bp to: 4 unclassified Roseateles strains, Aquincola sp., Aquincola tertiaricarbonis, Idonella sp., Paucibacter sp.)	**0.2%**	0.4%
g	**Desulfonatronum** (1 OTU with 90% identity in 426bp to: Desulfonatronum parangueonense, Desulfonatronum thiosulfatophilum)	**0.2%**	0.3%
g	**Rhodovibrio** (1 OTU with 92% identity in 402bp to: 3 unclassified Rhodovibrio strains)	**0.2%**	0.1%
s	**Thiobacillus sp. K6.2** (1 OTU with 98% identity in 424bp to: Thiobacillus sp. K6.2)	**0.2%**	0.1%
s	**Anaeromyxobacter dehalogenans** (1 OTU with 82% identity in 429bp to: Anaeromyxobacter dehalogenans)	**0.2%**	0.3%
s	**Iamia majanohamensis** (1 OTU with 87% identity in 400bp to: Iamia majanohamensis)	**0.2%**	0.2%
f	**Leptospiraceae** (1 OTU with 95% identity in 403bp to: Leptonema illini, Leptospira sp.)	**0.2%**	0.2%
s	**Pandoraea sp.** (1 OTU with 94% identity in 424bp to: Pandoraea sp.)	**0.2%**	0.5%
s	**Thiobacillus thioparus** (1 OTU with 98% identity in 424bp to: Thiobacillus thioparus)	**0.1%**	0.1%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	**0.1%**	0.2%
s	**Acinetobacter radioresistens** (1 OTU with 92% identity in 424bp to: Acinetobacter radioresistens)	**0.1%**	0.4%
g	**Haliscomenobacter** (1 OTU with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**0.1%**	0.2%
f	**Rhodobacteraceae** (1 OTU with 99% identity in 399bp to: Defluviimonas aquaemixtae, Rhodobacter sp. 13630K)	**0.1%**	0.2%
s	**Rhodovastum atsumiense** (1 OTU with 96% identity in 399bp to: Rhodovastum atsumiense)	**0.1%**	0.2%
	Other	**0.9%**	1.4%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.4: EBBR-Anammox microbial communities identified by NGS **1.V3V4a**

3.V3V4a (86 437 reads)

s	**Paludibaculum sp.** (3 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**23.4%**	16.4%
g	**Nitrosomonas** (3 OTUs with 97-100% identity in 424bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**11.4%**	6.8%
s	**Ignavibacterium album** (9 OTUs with 99-100% identity in 422bp to: Ignavibacterium album)	**10.6%**	15.2%
g	**Nitrosospira** (1 OTU with 98% identity in 424bp to: 3 unclassified Nitrosospira strains)	**9.4%**	5.6%
s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 424bp to: Denitratisoma oestradiolicum)	**4.4%**	2.6%
s	**Steroidobacter sp.** (7 OTUs with 92-94% identity in 424bp to: Steroidobacter sp.)	**4.3%**	5.4%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**2.5%**	1.5%
s	**Acidobacterium sp. WY65** (1 OTU with 95% identity in 399bp to: Acidobacterium sp. WY65)	**2.2%**	1.5%
s	**Ralstonia sp. P-4CB2** (5 OTUs with 96-97% identity in 425bp to: Ralstonia sp. P-4CB2)	**2.2%**	6.9%
s	**Herbaspirillum sp. AKB-2008-TE24** (1 OTU with 97% identity in 424bp to: Herbaspirillum sp. AKB-2008-TE24)	**2.0%**	3.6%
f	**Chitinophagaceae** (1 OTU with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**1.7%**	2.6%
f	**Comamonadaceae** (1 OTU with 79% identity in 428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Malaminivora alkalimesophila)	**1.7%**	2.0%
s	**Haliea sp. SAOS-164** (2 OTUs with 90% identity in 425bp to: Haliea sp. SAOS-164)	**1.6%**	2.1%
s	**Candidatus Solibacter usitatus** (3 OTUs with 92-94% identity in 399-401bp to: Candidatus Solibacter usitatus)	**1.2%**	0.8%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 424bp to: Sterolibacterium sp. TKU1)	**1.2%**	0.7%
s	**Thiobacillus thioparus** (4 OTUs with 97-99% identity in 424bp to: Thiobacillus thioparus)	**0.9%**	0.6%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.8%**	1.0%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**0.8%**	1.2%
g	**Lacibacter** (1 OTU with 95% identity in 422bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**0.7%**	1.2%
s	**Chelativorans sp. A52C2** (1 OTU with 88% identity in 402bp to: Chelativorans sp. A52C2)	**0.7%**	0.7%
s	**Lentimicrobium saccharophilum** (2 OTUs with 88% identity in 420-422bp to: Lentimicrobium saccharophilum)	**0.7%**	1.2%
s	**Thiobacillus sp. K6.2** (1 OTU with 98% identity in 424bp to: Thiobacillus sp. K6.2)	**0.7%**	0.4%
s	**Gemmatimonas aurantiaca** (1 OTU with 94% identity in 402bp to: Gemmatimonas aurantiaca)	**0.7%**	0.4%
s	**Holophaga sp. WY42** (1 OTU with 91% identity in 426bp to: Holophaga sp. WY42)	**0.7%**	0.4%
s	**Parvibaculum sp.** (1 OTU with 93% identity in 399bp to: Parvibaculum sp.)	**0.6%**	0.9%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	**0.6%**	0.4%
g	**Rheinheimera** (1 OTU with 100% identity in 424bp to: 2 unclassified Rheinheimera strains)	**0.6%**	0.6%
s	**Hyphomicrobium sp. KC-IT-W2** (1 OTU with 99% identity in 399bp to: Hyphomicrobium sp. KC-IT-W2)	**0.6%**	0.5%
s	**Candidatus Kuenenia sp.** (2 OTUs with 95-97% identity in 424bp to: Candidatus Kuenenia sp.)	**0.6%**	1.0%
f	**Sphingomonadaceae** (4 OTUs with 98-100% identity in 399-402bp to: 11 unclassified Sphingobium strains, 2 unclassified Sphingosinicella strains, 3 unclassified Novosphingobium strains, 32 unclassified Sphingomonas strains, 9 unclassified Sphingopyxis strains, Novosphingobium lindaniclasticum, Novosphingobium panipatense, Sphingobium hydrophobicum, Sphingobium xenophagum, Sphingomonas bosoensis, Sphingopyxis granuli, Sphingosinicella microcystinivorans, Sphingosinicella xenopeptidilytica)	**0.5%**	0.7%

s	**Longimicrobium terrae** (1 OTU with 88% identity in 426bp to: Longimicrobium terrae)	**0.5%**	0.3%
s	**Hyphomicrobium sp.** (2 OTUs with 99% identity in 399bp to: Hyphomicrobium sp.)	**0.5%**	0.4%
s	**Novosphingobium arabidopsis** (1 OTU with 99% identity in 399bp to: Novosphingobium arabidopsis)	**0.4%**	0.5%
s	**Lewinella cohaerens** (1 OTU with 88% identity in 424bp to: Lewinella cohaerens)	**0.4%**	0.7%
g	**Flavobacterium** (2 OTUs with 100% identity in 422bp to: 9 unclassified Flavobacterium strains, Flavobacterium gelidilacus)	**0.4%**	0.9%
g	**Haliscomenobacter** (1 OTU with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**0.4%**	0.6%
g	**Hyphomicrobium** (2 OTUs with 95-100% identity in 399bp to: Hyphomicrobium aestuarii, Hyphomicrobium sp., Hyphomicrobium vulgare, Hyphomicrobium zavarzinii)	**0.4%**	0.3%
g	**Pseudomonas** (3 OTUs with 96-100% identity in 424bp to: 32 unclassified Pseudomonas strains, Pseudomonas anguilliseptica, Pseudomonas fluorescens, Pseudomonas oryzihabitans, Pseudomonas poli)	**0.4%**	1.0%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**0.3%**	0.7%
g	**Thioprofundum** (1 OTU with 92% identity in 424bp to: Thioprofundum hispidum, Thioprofundum lithotrophicum)	**0.3%**	0.3%
f	**Micrococcaceae** (1 OTU with 100% identity in 404bp to: 5 unclassified Arthrobacter strains, Arthrobacter globiformis, Arthrobacter humicola, Arthrobacter nitrophenolicus, Arthrobacter oryzae, Arthrobacter pascens, Pseudarthrobacter defluvii, Pseudarthrobacter enclensis, Pseudarthrobacter oxydans, Pseudarthrobacter phenanthrenivorans, Pseudarthrobacter polychromogenes, Pseudarthrobacter siccitolerans, Pseudarthrobacter sp.)	**0.3%**	0.4%
g	**Rhodovibrio** (1 OTU with 92% identity in 402bp to: 3 unclassified Rhodovibrio strains)	**0.3%**	0.2%
g	**Sulfurimonas** (1 OTU with 99% identity in 402bp to: 3 unclassified Sulfurimonas strains)	**0.3%**	0.7%
s	**Williamwhitmania taraxaci** (1 OTU with 95% identity in 423bp to: Williamwhitmania taraxaci)	**0.3%**	0.5%
s	**Rhodovastum atsumiense** (1 OTU with 96% identity in 399bp to: Rhodovastum atsumiense)	**0.3%**	0.5%
s	**Acinetobacter radioresistens** (1 OTU with 92% identity in 424bp to: Acinetobacter radioresistens)	**0.2%**	0.7%
s	**Hyphomicrobium nitrativorans** (1 OTU with 100% identity in 399bp to: Hyphomicrobium nitrativorans)	**0.2%**	0.2%
s	**Flavilitoribacter nigricans** (1 OTU with 87% identity in 424bp to: Flavilitoribacter nigricans)	**0.2%**	0.3%
s	**Roseomonas sp.** (1 OTU with 95% identity in 399bp to: Roseomonas sp.)	**0.2%**	0.4%
s	**Desulfobulbus sp. IS6** (1 OTU with 85% identity in 416bp to: Desulfobulbus sp. IS6)	**0.2%**	0.2%
s	**Paludibaculum fermentans** (1 OTU with 94% identity in 399bp to: Paludibaculum fermentans)	**0.2%**	0.1%
s	**Ilumatobacter fluminis** (1 OTU with 96% identity in 400bp to: Ilumatobacter fluminis)	**0.2%**	0.2%
s	**Nitrosomonas sp.** (1 OTU with 96% identity in 425bp to: Nitrosomonas sp.)	**0.2%**	0.1%
c	**Alphaproteobacteria** (1 OTU with 92% identity in 400bp to: Albimonas pacifica, Neomegalonema perideroedes)	**0.2%**	0.2%
s	**Terrimonas sp. YJ03** (1 OTU with 96% identity in 422bp to: Terrimonas sp. YJ03)	**0.2%**	0.3%
g	**Acidiferrimonas** (1 OTU with 91% identity in 401bp to: Acidiferrimonas ferrireducens, Acidiferrimonas sp.)	**0.2%**	0.2%
g	**Simplicispira** (1 OTU with 100% identity in 424bp to: Simplicispira piscis, Simplicispira sp. b29)	**0.2%**	0.2%
s	**Tepidiforma bonchosmolovskayae** (1 OTU with 95% identity in 404bp to: Tepidiforma bonchosmolovskayae)	**0.2%**	0.2%
s	**Pandoraea sp.** (1 OTU with 94% identity in 424bp to: Pandoraea sp.)	**0.1%**	0.5%
o	**Burkholderiales** (1 OTU with 96% identity in 424bp to: Aquincola sp., Aquincola tertiaricarbonis, Ideonella sp.)	**0.1%**	0.3%
s	**Nitrosomonas sp. DYS317** (1 OTU with 95% identity in 424bp to: Nitrosomonas sp. DYS317)	**0.1%**	0.1%
g	**Comamonas** (1 OTU with 96% identity in 424bp to: 23 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**0.1%**	0.2%
s	**Novosphingobium sp.** (1 OTU with 99% identity in 399bp to: Novosphingobium sp.)	**0.1%**	0.2%
s	**Iamia majanohamensis** (1 OTU with 87% identity in 400bp to: Iamia majanohamensis)	**0.1%**	0.2%
g	**Lutibacter** (1 OTU with 97% identity in 422bp to: Lutibacter maritimus, Lutibacter sp.)	**0.1%**	0.2%
g	**Chondromyces** (1 OTU with 96% identity in 423bp to: Chondromyces apiculatus, Chondromyces pediculatus, Chondromyces robustus)	**0.1%**	0.3%
g	**Steroidobacter** (1 OTU with 91% identity in 424bp to: 2 unclassified Steroidobacter strains)	**0.1%**	0.2%
s	**Simplicispira sp. L1R1.2** (1 OTU with 99% identity in 424bp to: Simplicispira sp. L1R1.2)	**0.1%**	0.1%

SI 5.5: EBBR-Anammox microbial communities identified by NGS **3.V3V4a**

4.V3V4a (81 583 reads)

s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 424bp to: Denitratisoma oestradiolicum)	**20.7%**	10.9%
f	**Chitinophagaceae** (18 OTUs with 79% identity in 423-424bp to: Arachidicoccus sp., Haoranjiania flava)	**14.4%**	19.8%
s	**Ignavibacterium album** (8 OTUs with 99-100% identity in 422bp to: Ignavibacterium album)	**9.7%**	12.3%
s	**Nitrosomonas sp.** (5 OTUs with 96-99% identity in 424-425bp to: Nitrosomonas sp.)	**7.0%**	3.7%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**6.5%**	3.4%
f	**Comamonadaceae** (3 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**5.7%**	5.9%
g	**Comamonas** (5 OTUs with 96-100% identity in 424bp to: 29 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**4.0%**	4.2%
s	**Paludibaculum sp.** (2 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**3.5%**	2.1%
g	**Haliscomenobacter** (1 OTU with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**3.0%**	4.1%
s	**Candidatus Kuenenia sp.** (2 OTUs with 95-97% identity in 424bp to: Candidatus Kuenenia sp.)	**2.3%**	3.6%
s	**Ralstonia sp. P-4CB2** (6 OTUs with 96-98% identity in 425bp to: Ralstonia sp. P-4CB2)	**2.1%**	5.8%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**1.7%**	2.4%
s	**Steroidobacter sp.** (4 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**1.4%**	1.5%
g	**Nitrosomonas** (2 OTUs with 97-100% identity in 424bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**1.2%**	0.7%
s	**Nitrobacter sp. LAMK1242** (2 OTUs with 99% identity in 399bp to: Nitrobacter sp. LAMK1242)	**1.0%**	0.8%
o	**Burkholderiales** (3 OTUs with 79-97% identity in 424-430bp to: 2 unclassified Ideonella strains, 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Aquincola sp., Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Paucibacter sp., Rubrivivax sp. JA309)	**0.9%**	1.6%
s	**Gaiella occulta** (1 OTU with 99% identity in 423bp to: Gaiella occulta)	**0.8%**	0.4%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**0.8%**	1.4%
s	**Thermomonas fusca** (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	**0.8%**	1.1%
s	**Denitratimonas tolerans** (2 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	**0.7%**	1.0%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.6%**	0.7%
k	**Bacteria** (4 OTUs with 78-100% identity in 424-425bp to: 33 unclassified Delftia strains, Alcaligenes faecalis, Arachidicoccus sp., Clostridium sporogenes, Delftia acidovorans, Delftia lacustris, Delftia tsuruhatensis, Eisenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava, Lactococcus raffinolactis, Pseudomonas sp. MS8_2H3, Sutterella faecalis, Sutterella sp.)	**0.5%**	0.6%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 417bp to: Gemmatimonas sp. URHD0086)	**0.5%**	0.2%
s	**Ferruginibacter sp.** (1 OTU with 94% identity in 422bp to: Ferruginibacter sp.)	**0.5%**	0.6%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 399bp to: 2 unclassified Rhodopseudomonas strains, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	**0.4%**	0.4%

g	**Lacibacter** (1 OTU with 95% identity in 422bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**0.4%**	0.6%
s	**Terrimonas sp. YJ03** (1 OTU with 96% identity in 422bp to: Terrimonas sp. YJ03)	**0.4%**	0.6%
s	**Conexibacter sp.** (1 OTU with 92% identity in 426bp to: Conexibacter sp.)	**0.4%**	0.2%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**0.4%**	0.4%
p	**Proteobacteria** (1 OTU with 100% identity in 399bp to: 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, Aquamicrobium terrae, Thiobacillus sp. BHF001)	**0.4%**	0.6%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**0.4%**	0.2%
s	**Aquamicrobium aestuarii** (1 OTU with 99% identity in 399bp to: Aquamicrobium aestuarii)	**0.3%**	0.3%
s	**Geobacter sp. CLFeRB** (1 OTU with 86% identity in 425bp to: Geobacter sp. CLFeRB)	**0.3%**	0.5%

g	**Hyphomicrobium** (2 OTUs with 95-100% identity in 399bp to: Hyphomicrobium aestuarii, Hyphomicrobium sp., Hyphomicrobium vulgare, Hyphomicrobium zavarzinii)	**0.3%**	0.2%
s	**Hyphomicrobium sp.** (1 OTU with 99% identity in 399bp to: Hyphomicrobium sp.)	**0.3%**	0.2%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**0.3%**	0.2%
s	**Racemicystis persica** (3 OTUs with 92-93% identity in 423-424bp to: Racemicystis persica)	**0.3%**	0.6%
s	**Turneriella parva** (1 OTU with 95% identity in 424bp to: Turneriella parva)	**0.3%**	0.2%
s	**Pseudorhodoplanes sinuspersici** (2 OTUs with 96-100% identity in 399bp to: Pseudorhodoplanes sinuspersici)	**0.3%**	0.3%
f	**Sphingomonadaceae** (1 OTU with 100% identity in 399bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	**0.3%**	0.3%
g	**Aquamicrobium** (1 OTU with 100% identity in 399bp to: 2 unclassified Aquamicrobium strains, Aquamicrobium ahrensii, Aquamicrobium defluvii, Aquamicrobium lusatiense)	**0.2%**	0.2%
s	**Ilumatobacter fluminis** (1 OTU with 96% identity in 400bp to: Ilumatobacter fluminis)	**0.2%**	0.2%
s	**Tepidiforma bonchosmolovskayae** (1 OTU with 95% identity in 404bp to: Tepidiforma bonchosmolovskayae)	**0.2%**	0.2%
s	**Acidobacterium sp. WY65** (1 OTU with 95% identity in 399bp to: Acidobacterium sp. WY65)	**0.2%**	0.1%
g	**Desulfonatronum** (1 OTU with 90% identity in 426bp to: Desulfonatronum parangueonense, Desulfonatronum thiosulfatophilum)	**0.2%**	0.2%
s	**Parvibaculum sp. MBNA2** (1 OTU with 98% identity in 402bp to: Parvibaculum sp. MBNA2)	**0.2%**	0.2%
s	**Gordonia iterans** (1 OTU with 100% identity in 404bp to: Gordonia iterans)	**0.2%**	0.2%
s	**Truepera sp.** (2 OTUs with 96% identity in 400bp to: Truepera sp.)	**0.2%**	0.2%
g	**Geobacter** (1 OTU with 88% identity in 425bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	**0.2%**	0.2%
s	**Roseomonas sp.** (1 OTU with 95% identity in 399bp to: Roseomonas sp.)	**0.2%**	0.2%
s	**Azospira restricta** (1 OTU with 99% identity in 424bp to: Azospira restricta)	**0.1%**	0.1%
c	**Alphaproteobacteria** (1 OTU with 100% identity in 399bp to: 15 unclassified Bosea strains, Afipia genosp. 7, Bosea robiniae, Sphingopyxis sp. YIM102)	**0.1%**	0.2%
s	**Quisquiliibacterium transsilvanicum** (1 OTU with 98% identity in 424bp to: Quisquiliibacterium transsilvanicum)	**0.1%**	0.4%
s	**Chryseolinea soli** (1 OTU with 91% identity in 422bp to: Chryseolinea soli)	**0.1%**	0.2%
s	**Aquamicrobium sp.** (1 OTU with 99% identity in 399bp to: Aquamicrobium sp.)	**0.1%**	0.1%
s	**Iamia majanohamensis** (1 OTU with 87% identity in 400bp to: Iamia majanohamensis)	**0.1%**	0.1%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	**0.1%**	0.1%
p	**Bacteroidetes** (1 OTU with 86% identity in 422bp to: 2 unclassified Mucilaginibacter strains, Owenweeksia hongkongensis, Solitalea canadensis)	**0.1%**	0.1%
	Other	**1.1%**	1.7%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.6: EBBR-Anammox microbial communities identified by NGS **4.V3V4a**

5.V3V4a (81 763 reads)

s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 424bp to: Denitratisoma oestradiolicum)	**26.2%**	13.9%
f	**Chitinophagaceae** (12 OTUs with 79% identity in 423-424bp to: Arachidicoccus sp., Haoranjiania flava)	**14.3%**	19.9%
s	**Ignavibacterium album** (9 OTUs with 99-100% identity in 419-422bp to: Ignavibacterium album)	**9.9%**	12.6%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**7.2%**	3.8%
f	**Comamonadaceae** (3 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**5.9%**	6.2%
s	**Paludibaculum sp.** (2 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**5.0%**	3.1%
s	**Nitrosomonas sp.** (4 OTUs with 96-99% identity in 424-425bp to: Nitrosomonas sp.)	**4.8%**	2.6%
g	**Haliscomenobacter** (1 OTU with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**3.8%**	5.3%
g	**Comamonas** (5 OTUs with 96-100% identity in 424bp to: 29 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**3.4%**	3.6%
s	**Candidatus Kuenenia sp.** (2 OTUs with 95-97% identity in 424bp to: Candidatus Kuenenia sp.)	**2.4%**	3.8%
s	**Ralstonia sp. P-4CB2** (4 OTUs with 96-97% identity in 425bp to: Ralstonia sp. P-4CB2)	**2.2%**	6.3%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**1.4%**	2.0%
s	**Steroidobacter sp.** (3 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**1.2%**	1.3%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**1.0%**	1.8%
o	**Burkholderiales** (3 OTUs with 79-97% identity in 424-430bp to: 2 unclassified Ideonella strains, 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Aquincola sp., Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Paucibacter sp., Rubrivivax sp. JA309)	**0.9%**	1.6%
g	**Nitrosomonas** (1 OTU with 100% identity in 424bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**0.7%**	0.4%
s	**Denitratimonas tolerans** (2 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	**0.7%**	1.0%
s	**Thermomonas fusca** (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	**0.7%**	0.9%
s	**Candidatus Solibacter usitatus** (2 OTUs with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**0.6%**	0.4%
g	**Lacibacter** (1 OTU with 95% identity in 422bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**0.5%**	0.7%
s	**Terrimonas sp. YJ03** (2 OTUs with 96% identity in 422bp to: Terrimonas sp. YJ03)	**0.5%**	0.6%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**0.4%**	0.5%
k	**Bacteria** (3 OTUs with 78% identity in 425bp to: Arachidicoccus sp., Eisenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava, Sutterella faecalis, Sutterella sp.)	**0.4%**	0.5%
s	**Geobacter sp. CLFeRB** (1 OTU with 86% identity in 425bp to: Geobacter sp. CLFeRB)	**0.4%**	0.6%
g	**Geobacter** (1 OTU with 88% identity in 425bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	**0.3%**	0.5%
s	**Acidobacterium sp. WY65** (1 OTU with 95% identity in 399bp to: Acidobacterium sp. WY65)	**0.3%**	0.2%
s	**Turneriella parva** (1 OTU with 95% identity in 424bp to: Turneriella parva)	**0.3%**	0.3%
s	**Ferruginibacter sp.** (2 OTUs with 92-94% identity in 422bp to: Ferruginibacter sp.)	**0.3%**	0.4%
g	**Desulfonatronum** (1 OTU with 90% identity in 426bp to: Desulfonatronum paranguaonense, Desulfonatronum thiosulfatophilum)	**0.3%**	0.3%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 417bp to: Gemmatimonas sp. URHD0086)	**0.3%**	0.2%
s	**Aquamicrobium aestuarii** (1 OTU with 99% identity in 399bp to: Aquamicrobium aestuarii)	**0.3%**	0.2%
f	**Sphingomonadaceae** (1 OTU with 100% identity in 399bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	**0.3%**	0.3%
s	**Gaiella occulta** (1 OTU with 99% identity in 423bp to: Gaiella occulta)	**0.2%**	0.1%
s	**Nitrobacter sp. LAMK1242** (1 OTU with 99% identity in 399bp to: Nitrobacter sp. LAMK1242)	**0.2%**	0.2%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**0.2%**	0.1%

s	Aquamicrobium aestuarii (1 OTU with 99% identity in 399bp to: Aquamicrobium aestuarii)	0.3%	0.2%
f	Sphingomonadaceae (1 OTU with 100% identity in 399bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	0.3%	0.3%
s	Gaiella occulta (1 OTU with 99% identity in 423bp to: Gaiella occulta)	0.2%	0.1%
s	Nitrobacter sp. LAMK1242 (1 OTU with 99% identity in 399bp to: Nitrobacter sp. LAMK1242)	0.2%	0.2%
s	Gemmatimonas phototrophica (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	0.2%	0.1%
s	Aciditerrimonas ferrireducens (1 OTU with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	0.2%	0.2%
f	Bradyrhizobiaceae (1 OTU with 100% identity in 399bp to: 2 unclassified Rhodopseudomonas strains, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	0.2%	0.2%
s	Racemicystis persica (2 OTUs with 92% identity in 423-424bp to: Racemicystis persica)	0.2%	0.4%
s	Parvibaculum sp. MBNA2 (1 OTU with 98% identity in 402bp to: Parvibaculum sp. MBNA2)	0.2%	0.2%
s	Quisquiliibacterium transsilvanicum (1 OTU with 98% identity in 424bp to: Quisquiliibacterium transsilvanicum)	0.2%	0.4%
s	Fimbriimonas ginsengisoli (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	0.2%	0.2%
s	Truepera sp. (2 OTUs with 96% identity in 400bp to: Truepera sp.)	0.1%	0.2%
s	Tepidiforma bonchosmolovskayae (1 OTU with 95% identity in 404bp to: Tepidiforma bonchosmolovskayae)	0.1%	0.2%
s	Roseomonas sp. (1 OTU with 95% identity in 399bp to: Roseomonas sp.)	0.1%	0.2%
s	Hyphomicrobium sp. (1 OTU with 99% identity in 399bp to: Hyphomicrobium sp.)	0.1%	0.1%
p	Proteobacteria (1 OTU with 100% identity in 399bp to: 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, Aquamicrobium terrae, Thiobacillus sp. BHF001)	0.1%	0.2%
f	Rhodobacteraceae (1 OTU with 99% identity in 399bp to: Defluviimonas aquaemixtae, Rhodobacter sp. 13630K)	0.1%	0.2%
s	Azospira restricta (1 OTU with 99% identity in 424bp to: Azospira restricta)	0.1%	0.1%
	Other	0.6%	1.0%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.7: EBBR-Anammox microbial communities identified by NGS **5.V3V4a**

6.V3V4a (66 843 reads)

s	**Denitratisoma oestradiolicum** (4 OTUs with 96-98% identity in 424bp to: Denitratisoma oestradiolicum)	**22.1%**	11.5%
s	**Ignavibacterium album** (10 OTUs with 99-100% identity in 422bp to: Ignavibacterium album)	**12.4%**	15.5%
f	**Chitinophagaceae** (8 OTUs with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**12.0%**	16.4%
s	**Paludibaculum sp.** (2 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**8.3%**	5.1%
f	**Comamonadaceae** (3 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**6.9%**	7.0%
g	**Nitrosomonas** (3 OTUs with 97-100% identity in 424bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**4.1%**	2.1%
s	**Steroidobacter sp.** (6 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**3.1%**	3.3%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**3.0%**	1.6%
s	**Ralstonia sp. P-4CB2** (5 OTUs with 96-97% identity in 425bp to: Ralstonia sp. P-4CB2)	**2.6%**	7.3%
s	**Candidatus Kuenenia sp.** (2 OTUs with 95-97% identity in 424bp to: Candidatus Kuenenia sp.)	**2.5%**	3.9%
g	**Comamonas** (3 OTUs with 96-100% identity in 424bp to: 26 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**1.5%**	1.6%
s	**Nitrosomonas sp.** (3 OTUs with 96-99% identity in 424-425bp to: Nitrosomonas sp.)	**1.4%**	0.7%
g	**Haliscomenobacter** (1 OTU with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**1.1%**	1.5%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**0.9%**	1.6%
g	**Desulfonatronum** (1 OTU with 90% identity in 426bp to: Desulfonatronum paranguaonense, Desulfonatronum thiosulfatophilum)	**0.9%**	0.9%
s	**Denitratimonas tolerans** (3 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	**0.8%**	1.2%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**0.8%**	1.1%
g	**Lacibacter** (1 OTU with 95% identity in 422bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**0.7%**	1.0%
s	**Candidatus Solibacter usitatus** (2 OTUs with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**0.7%**	0.4%
s	**Turneriella parva** (2 OTUs with 95-97% identity in 424bp to: Turneriella parva)	**0.7%**	0.6%
s	**Thermomonas brevis** (1 OTU with 99% identity in 427bp to: Thermomonas brevis)	**0.6%**	0.9%
s	**Holophaga sp. WY42** (1 OTU with 91% identity in 426bp to: Holophaga sp. WY42)	**0.6%**	0.3%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**0.6%**	0.6%
s	**Thermomonas fusca** (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	**0.5%**	0.7%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 424bp to: Sterolibacterium sp. TKU1)	**0.5%**	0.3%
g	**Nitrosospira** (1 OTU with 98% identity in 424bp to: 3 unclassified Nitrosospira strains)	**0.5%**	0.3%
s	**Aciditerrimonas ferrireducens** (2 OTUs with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.5%**	0.5%
s	**Acidobacterium sp. WY65** (1 OTU with 95% identity in 399bp to: Acidobacterium sp. WY65)	**0.5%**	0.3%
s	**Hyphomicrobium sp.** (2 OTUs with 99% identity in 399bp to: Hyphomicrobium sp.)	**0.5%**	0.3%

s	**Lentimicrobium saccharophilum** (1 OTU with 88% identity in 422bp to: Lentimicrobium saccharophilum)	**0.4%**	0.6%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**0.4%**	0.2%
s	**Parvibaculum sp.** (1 OTU with 93% identity in 399bp to: Parvibaculum sp.)	**0.4%**	0.5%
o	**Burkholderiales** (1 OTU with 79% identity in 430bp to: 4 unclassified Roseateles strains, Paucibacter sp.)	**0.4%**	0.7%
s	**Hyphomicrobium sp. KC-IT-W2** (1 OTU with 99% identity in 399bp to: Hyphomicrobium sp. KC-IT-W2)	**0.4%**	0.3%
s	**Racemicystis persica** (2 OTUs with 92% identity in 423-424bp to: Racemicystis persica)	**0.4%**	0.7%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	**0.3%**	0.4%
s	**Fluviicola kyonggii** (2 OTUs with 94-95% identity in 423bp to: Fluviicola kyonggii)	**0.3%**	0.4%
g	**Hyphomicrobium** (2 OTUs with 95-100% identity in 399bp to: Hyphomicrobium aestuarii, Hyphomicrobium sp., Hyphomicrobium vulgare, Hyphomicrobium zavarzinii)	**0.3%**	0.2%
g	**Pseudomonas** (2 OTUs with 100% identity in 424bp to: 9 unclassified Pseudomonas strains, Pseudomonas caeni)	**0.3%**	0.7%
k	**Bacteria** (2 OTUs with 78% identity in 425bp to: Arachidicoccus sp., Eisenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava, Sutterella faecalis, Sutterella sp.)	**0.3%**	0.4%
s	**Gemmatimonas aurantiaca** (1 OTU with 94% identity in 402bp to: Gemmatimonas aurantiaca)	**0.3%**	0.1%
g	**Simplicispira** (1 OTU with 100% identity in 424bp to: Simplicispira piscis, Simplicispira sp. b29)	**0.3%**	0.3%
s	**Pseudomonas sp. ARMY** (1 OTU with 97% identity in 424bp to: Pseudomonas sp. ARMY)	**0.2%**	0.6%
s	**Novosphingobium arabidopsis** (1 OTU with 99% identity in 399bp to: Novosphingobium arabidopsis)	**0.2%**	0.2%
s	**Herbaspirillum sp. AKB-2008-TE24** (1 OTU with 97% identity in 424bp to: Herbaspirillum sp. AKB-2008-TE24)	**0.2%**	0.4%
g	**Flavobacterium** (1 OTU with 100% identity in 422bp to: 4 unclassified Flavobacterium strains)	**0.2%**	0.4%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	**0.2%**	0.1%
g	**Rhodovibrio** (1 OTU with 92% identity in 402bp to: 3 unclassified Rhodovibrio strains)	**0.2%**	0.1%
s	**Terrimonas sp. YJ03** (1 OTU with 96% identity in 422bp to: Terrimonas sp. YJ03)	**0.2%**	0.3%
f	**Rhodobacteraceae** (1 OTU with 99% identity in 399bp to: Defluviimonas aquaemixtae, Rhodobacter sp. 13630K)	**0.2%**	0.3%
g	**Rheinheimera** (1 OTU with 100% identity in 424bp to: 2 unclassified Rheinheimera strains)	**0.2%**	0.2%
s	**Simplicispira suum** (1 OTU with 100% identity in 424bp to: Simplicispira suum)	**0.2%**	0.2%
g	**Geobacter** (1 OTU with 88% identity in 425bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	**0.2%**	0.3%
s	**Chelativorans sp. A52C2** (1 OTU with 88% identity in 402bp to: Chelativorans sp. A52C2)	**0.2%**	0.1%
s	**Lewinella cohaerens** (1 OTU with 88% identity in 424bp to: Lewinella cohaerens)	**0.2%**	0.2%
p	**Proteobacteria** (1 OTU with 98% identity in 424bp to: Rhodanobacter sp. 7B-393, Simplicispira sp.)	**0.2%**	0.2%
s	**Geobacter sp. CLFeRB** (1 OTU with 86% identity in 425bp to: Geobacter sp. CLFeRB)	**0.1%**	0.2%
s	**Iamia majanohamensis** (1 OTU with 87% identity in 400bp to: Iamia majanohamensis)	**0.1%**	0.1%
s	**Desulfobulbus sp. IS6** (1 OTU with 85% identity in 416bp to: Desulfobulbus sp. IS6)	**0.1%**	0.1%
s	**Bdellovibrio sp. MPA** (1 OTU with 88% identity in 405bp to: Bdellovibrio sp. MPA)	**0.1%**	0.2%
s	**Pandoraea sp.** (1 OTU with 94% identity in 424bp to: Pandoraea sp.)	**0.1%**	0.3%
s	**Chryseolinea soli** (1 OTU with 91% identity in 422bp to: Chryseolinea soli)	**0.1%**	0.1%
g	**Thermomonas** (1 OTU with 99% identity in 427bp to: Thermomonas fusca, Thermomonas haemolytica, Thermomonas koreensis, Thermomonas sp. ZY008)	**0.1%**	0.1%
	Other	**0.5%**	1.0%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.7: EBBR-Anammox microbial communities identified by NGS **7.V3V4a**

8.V3V4a (61 780 reads)

s	**Denitratisoma oestradiolicum** (6 OTUs with 95-98% identity in 424bp to: Denitratisoma oestradiolicum)	**24.7%**	13.0%
g	**Haliscomenobacter** (5 OTUs with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**9.2%**	12.6%
s	**Thermomonas fusca** (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	**6.2%**	8.5%
s	**Ignavibacterium album** (2 OTUs with 99-100% identity in 422bp to: Ignavibacterium album)	**4.5%**	5.6%
s	**Gemmatimonas aurantiaca** (2 OTUs with 94-95% identity in 418bp to: Gemmatimonas aurantiaca)	**4.5%**	2.3%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**4.2%**	4.4%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**4.2%**	2.2%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**3.3%**	3.5%
s	**Ralstonia sp. P-4CB2** (6 OTUs with 96-98% identity in 425bp to: Ralstonia sp. P-4CB2)	**2.9%**	8.2%
f	**Chitinophagaceae** (1 OTU with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**2.6%**	3.6%
g	**Comamonas** (4 OTUs with 96-99% identity in 424bp to: 32 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**2.2%**	2.3%
s	**Candidatus Solibacter usitatus** (3 OTUs with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**2.1%**	1.3%
s	**Ferruginibacter sp.** (2 OTUs with 92-94% identity in 422bp to: Ferruginibacter sp.)	**2.0%**	2.7%
o	**Burkholderiales** (5 OTUs with 79-98% identity in 424-430bp to: 2 unclassified Aquincola strains, 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Aquincola tertiaricarbonis, Caldimonas hydrothermale, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Ideonella sp., Paucibacter sp., Rubrivivax sp. JA309)	**1.8%**	3.0%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 417bp to: Gemmatimonas sp. URHD0086)	**1.7%**	0.9%
s	**Chryseotalea sanaruensis** (2 OTUs with 91% identity in 421bp to: Chryseotalea sanaruensis)	**1.4%**	2.0%
s	**Paludibaculum sp.** (2 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**1.4%**	0.8%
s	**Nitrosomonas sp.** (2 OTUs with 97% identity in 424-425bp to: Nitrosomonas sp.)	**1.3%**	0.7%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 424bp to: Candidatus Kuenenia sp.)	**1.2%**	1.8%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**1.1%**	0.6%
s	**Denitratimonas tolerans** (3 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	**1.0%**	1.5%
s	**Conexibacter sp.** (2 OTUs with 92-93% identity in 424-426bp to: Conexibacter sp.)	**1.0%**	0.5%
g	**Ferruginibacter** (1 OTU with 95% identity in 422bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	**0.9%**	1.2%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 424bp to: Thioprofundum hispidum)	**0.8%**	0.8%
s	**Terrimonas sp. YJ03** (2 OTUs with 96% identity in 422bp to: Terrimonas sp. YJ03)	**0.8%**	1.1%
s	**Steroidobacter sp.** (3 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**0.8%**	0.9%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**0.8%**	1.1%
g	**Nitrosomonas** (1 OTU with 100% identity in 424bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**0.8%**	0.4%
s	**Roseisolibacter agri** (1 OTU with 91% identity in 416bp to: Roseisolibacter agri)	**0.7%**	0.4%
s	**Chryseolinea soli** (1 OTU with 91% identity in 422bp to: Chryseolinea soli)	**0.7%**	1.0%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 424bp to: Aquincola sp. HME6814)	**0.5%**	0.9%
f	**Myxococcaceae** (1 OTU with 93% identity in 423bp to: Corallococcus macrosporus, Pyxidicoccus fallax)	**0.5%**	0.8%

f	**Myxococcaceae** (1 OTU with 93% identity in 423bp to: Corallococcus macrosporus, Pyxidicoccus fallax)	**0.5%**	0.8%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	**0.5%**	0.3%
s	**Sediminibacterium sp.** (1 OTU with 95% identity in 422bp to: Sediminibacterium sp.)	**0.5%**	0.7%
s	**Turneriella parva** (2 OTUs with 87-95% identity in 424-425bp to: Turneriella parva)	**0.5%**	0.4%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**0.4%**	0.7%
s	**Aciditerrimonas ferrireducens** (2 OTUs with 91-93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.4%**	0.4%
s	**Woodsholea maritima** (1 OTU with 95% identity in 399bp to: Woodsholea maritima)	**0.4%**	0.3%
p	**Proteobacteria** (1 OTU with 100% identity in 399bp to: 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, Aquamicrobium terrae, Thiobacillus sp. BHF001)	**0.4%**	0.5%
s	**Nitrobacter sp. LAMK1242** (1 OTU with 99% identity in 399bp to: Nitrobacter sp. LAMK1242)	**0.3%**	0.3%
c	**Gammaproteobacteria** (1 OTU with 90% identity in 424bp to: Acidibacter ferrireducens, Coxiella endosymbiont of Ornithodoros marocanus, Steroidobacter sp., Thioprofundum hispidum)	**0.3%**	0.4%
s	**Parasediminibacterium paludis** (1 OTU with 94% identity in 422bp to: Parasediminibacterium paludis)	**0.3%**	0.4%
s	**Gaiella occulta** (1 OTU with 99% identity in 423bp to: Gaiella occulta)	**0.3%**	0.2%
o	**Myxococcales** (1 OTU with 91% identity in 423bp to: Phaselicystis flava, Racemicystis crocea, Racemicystis persica)	**0.3%**	0.3%
o	**Rhodocyclales** (1 OTU with 92% identity in 424bp to: 3 unclassified Thauera strains, Azonexus caeni)	**0.3%**	0.4%
s	**Phenylobacterium falsum** (1 OTU with 100% identity in 402bp to: Phenylobacterium falsum)	**0.2%**	0.1%
s	**Fluviicola kyonggii** (2 OTUs with 94-95% identity in 423bp to: Fluviicola kyonggii)	**0.2%**	0.3%
s	**Tangfeifania diversioriginum** (2 OTUs with 92% identity in 422bp to: Tangfeifania diversioriginum)	**0.2%**	0.3%
f	**Sphingomonadaceae** (1 OTU with 100% identity in 399bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	**0.2%**	0.2%
s	**Parvibaculum sp.** (1 OTU with 98% identity in 399bp to: Parvibaculum sp.)	**0.2%**	0.2%
s	**Truepera sp.** (1 OTU with 96% identity in 400bp to: Truepera sp.)	**0.2%**	0.2%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	**0.2%**	0.2%
s	**Ferruginibacter alkalilentus** (1 OTU with 95% identity in 422bp to: Ferruginibacter alkalilentus)	**0.2%**	0.2%
s	**Tepidiforma bonchosmolovskayae** (1 OTU with 95% identity in 404bp to: Tepidiforma bonchosmolovskayae)	**0.2%**	0.2%
g	**Hyphomicrobium** (1 OTU with 97% identity in 399bp to: 2 unclassified Hyphomicrobium strains)	**0.2%**	0.1%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 423bp to: Rhodohalobacter sp. SW132)	**0.2%**	0.2%
s	**Desulfuromonas sp. AOP6** (1 OTU with 82% identity in 426bp to: Desulfuromonas sp. AOP6)	**0.2%**	0.1%
s	**Pseudorhodoplanes sinuspersici** (1 OTU with 96% identity in 399bp to: Pseudorhodoplanes sinuspersici)	**0.2%**	0.2%
s	**Ideonella sp.** (1 OTU with 97% identity in 424bp to: Ideonella sp.)	**0.2%**	0.3%
s	**Bradymonas sediminis** (1 OTU with 81% identity in 416bp to: Bradymonas sediminis)	**0.1%**	0.1%
s	**Desulfonatronum parangueonense** (1 OTU with 86% identity in 425bp to: Desulfonatronum parangueonense)	**0.1%**	0.1%
s	**Racemicystis persica** (1 OTU with 93% identity in 424bp to: Racemicystis persica)	**0.1%**	0.2%
	Other	**0.5%**	0.9%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.8: EBBR-Anammox microbial communities identified by NGS **8.V3V4a**

10.V3V4a (76 794 reads)

s	**Denitratisoma oestradiolicum** (9 OTUs with 95-98% identity in 424bp to: Denitratisoma oestradiolicum)	**29.3%**	15.8%
g	**Haliscomenobacter** (1 OTU with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**7.8%**	11.0%
s	**Thermomonas fusca** (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	**6.0%**	8.5%
s	**Ignavibacterium album** (4 OTUs with 99-100% identity in 422bp to: Ignavibacterium album)	**5.2%**	6.7%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**4.4%**	4.7%
s	**Gemmatimonas aurantiaca** (2 OTUs with 94-95% identity in 418bp to: Gemmatimonas aurantiaca)	**4.1%**	2.2%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**3.0%**	3.2%
s	**Ralstonia sp. P-4CB2** (6 OTUs with 96-98% identity in 425bp to: Ralstonia sp. P-4CB2)	**3.0%**	8.6%
s	**Chryseotalea sanaruensis** (2 OTUs with 91% identity in 421bp to: Chryseotalea sanaruensis)	**2.3%**	3.2%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**2.2%**	1.2%
f	**Chitinophagaceae** (1 OTU with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**2.2%**	3.1%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**2.1%**	1.1%
o	**Burkholderiales** (4 OTUs with 79-98% identity in 424-430bp to: 2 unclassified Aquincola strains, 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Aquincola tertiaricarbonis, Caldimonas hydrothermale, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Ideonella sp., Paucibacter sp., Rubrivivax sp. JA309)	**1.7%**	3.0%
g	**Comamonas** (4 OTUs with 96-99% identity in 424bp to: 32 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**1.7%**	1.8%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 417bp to: Gemmatimonas sp. URHD0086)	**1.6%**	0.8%
s	**Paludibaculum sp.** (2 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**1.5%**	0.9%
s	**Candidatus Solibacter usitatus** (2 OTUs with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**1.5%**	0.9%
s	**Nitrosomonas sp.** (2 OTUs with 97% identity in 424-425bp to: Nitrosomonas sp.)	**1.5%**	0.8%
s	**Ferruginibacter sp.** (2 OTUs with 92-94% identity in 422bp to: Ferruginibacter sp.)	**1.4%**	2.0%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 424bp to: Candidatus Kuenenia sp.)	**1.3%**	2.0%
s	**Conexibacter sp.** (3 OTUs with 92-93% identity in 424-426bp to: Conexibacter sp.)	**1.3%**	0.7%
s	**Denitratimonas tolerans** (3 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	**1.2%**	1.8%
s	**Roseisolibacter agri** (1 OTU with 91% identity in 416bp to: Roseisolibacter agri)	**0.7%**	0.4%
s	**Turneriella parva** (2 OTUs with 87-95% identity in 424-425bp to: Turneriella parva)	**0.7%**	0.6%
s	**Steroidobacter sp.** (3 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**0.7%**	0.7%
g	**Nitrosomonas** (1 OTU with 100% identity in 424bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**0.6%**	0.3%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 424bp to: Thioprofundum hispidum)	**0.6%**	0.6%
s	**Chryseolinea soli** (1 OTU with 91% identity in 422bp to: Chryseolinea soli)	**0.6%**	0.8%
s	**Woodsholea maritima** (1 OTU with 95% identity in 399bp to: Woodsholea maritima)	**0.6%**	0.4%
f	**Myxococcaceae** (1 OTU with 93% identity in 423bp to: Corallococcus macrosporus, Pyxidicoccus fallax)	**0.6%**	0.9%
s	**Aciditerrimonas ferrireducens** (2 OTUs with 91-93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.5%**	0.6%
s	**Terrimonas sp. YJ03** (2 OTUs with 96% identity in 422bp to: Terrimonas sp. YJ03)	**0.5%**	0.7%
s	**Gaiella occulta** (1 OTU with 99% identity in 423bp to: Gaiella occulta)	**0.5%**	0.2%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 424bp to: Aquincola sp. HME6814)	**0.4%**	0.7%

g	**Ferruginibacter** (1 OTU with 95% identity in 422bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	**0.4%**	0.6%
o	**Myxococcales** (1 OTU with 91% identity in 423bp to: Phaselicystis flava, Racemicystis crocea, Racemicystis persica)	**0.4%**	0.5%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	**0.4%**	0.3%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**0.4%**	0.5%
s	**Nitrobacter sp. LAMK1242** (1 OTU with 99% identity in 399bp to: Nitrobacter sp. LAMK1242)	**0.4%**	0.3%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**0.3%**	0.5%
c	**Gammaproteobacteria** (1 OTU with 90% identity in 424bp to: Acidibacter ferrireducens, Coxiella endosymbiont of Ornithodoros marocanus, Steroidobacter sp., Thioprofundum hispidum)	**0.3%**	0.3%
p	**Proteobacteria** (1 OTU with 100% identity in 399bp to: 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, Aquamicrobium terrae, Thiobacillus sp. BHF001)	**0.3%**	0.4%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 426bp to: Sterolibacterium sp. TKU1)	**0.3%**	0.1%
s	**Sediminibacterium sp.** (1 OTU with 95% identity in 422bp to: Sediminibacterium sp.)	**0.3%**	0.4%
s	**Fluviicola kyonggii** (2 OTUs with 94-95% identity in 423bp to: Fluviicola kyonggii)	**0.2%**	0.4%
s	**Parasediminibacterium paludis** (1 OTU with 94% identity in 422bp to: Parasediminibacterium paludis)	**0.2%**	0.3%
s	**Lentimicrobium saccharophilum** (1 OTU with 88% identity in 422bp to: Lentimicrobium saccharophilum)	**0.2%**	0.3%
s	**Racemicystis persica** (2 OTUs with 92-93% identity in 424bp to: Racemicystis persica)	**0.2%**	0.4%
s	**Ideonella sp.** (1 OTU with 97% identity in 424bp to: Ideonella sp.)	**0.2%**	0.3%
o	**Rhodocyclales** (1 OTU with 92% identity in 424bp to: 3 unclassified Thauera strains, Azonexus caeni)	**0.2%**	0.3%
s	**Hyphomicrobium sp.** (1 OTU with 99% identity in 399bp to: Hyphomicrobium sp.)	**0.2%**	0.1%
s	**Desulfuromonas sp. AOP6** (1 OTU with 82% identity in 426bp to: Desulfuromonas sp. AOP6)	**0.2%**	0.1%
s	**Truepera sp.** (2 OTUs with 96% identity in 400bp to: Truepera sp.)	**0.1%**	0.2%
s	**Tepidiforma bonchosmolovskayae** (1 OTU with 95% identity in 404bp to: Tepidiforma bonchosmolovskayae)	**0.1%**	0.2%
s	**Pseudorhodoplanes sinuspersici** (1 OTU with 96% identity in 399bp to: Pseudorhodoplanes sinuspersici)	**0.1%**	0.2%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	**0.1%**	0.2%
s	**Tangfeifania diversioriginum** (2 OTUs with 92% identity in 422bp to: Tangfeifania diversioriginum)	**0.1%**	0.2%
g	**Geobacter** (1 OTU with 88% identity in 425bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	**0.1%**	0.2%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 423bp to: Rhodohalobacter sp. SW132)	**0.1%**	0.2%
f	**Sphingomonadaceae** (1 OTU with 100% identity in 399bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	**0.1%**	0.1%
o	**Rhizobiales** (1 OTU with 92% identity in 399bp to: Breoghania corrubedonensis, Breoghania sp. BR-5, Mycoplana sp. G110)	**0.1%**	0.1%
s	**Parvibaculum sp.** (1 OTU with 98% identity in 399bp to: Parvibaculum sp.)	**0.1%**	0.1%
g	**Desulfonatronum** (1 OTU with 90% identity in 426bp to: Desulfonatronum parangueonense, Desulfonatronum thiosulfatophilum)	**0.1%**	0.1%
	Other	**0.4%**	0.7%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.9: EBBR-Anammox microbial communities identified by NGS **10.V3V4a**

11.V3V4a (68 128 reads)

s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 424bp to: Denitratisoma oestradiolicum)	**12.6%**	5.9%
c	**Alphaproteobacteria** (1 OTU with 100% identity in 399bp to: 4 unclassified Bravundimonas strains, 4 unclassified Mycoplana strains, Bravundimonas subvibrioides)	**5.9%**	6.0%
s	**Thermomonas carbonis** (2 OTUs with 98-99% identity in 427bp to: Thermomonas carbonis)	**5.4%**	7.1%
s	**Chryseotalea sanaruensis** (2 OTUs with 91% identity in 421bp to: Chryseotalea sanaruensis)	**5.1%**	6.3%
f	**Chitinophagaceae** (1 OTU with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**4.1%**	5.0%
g	**Haliscomenobacter** (1 OTU with 91% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**4.1%**	5.0%
s	**Nitrosomonas sp.** (2 OTUs with 97-98% identity in 424-425bp to: Nitrosomonas sp.)	**4.1%**	1.9%
g	**Nitrosomonas** (2 OTUs with 97-100% identity in 424bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**3.5%**	1.6%
s	**Ralstonia sp. P-4CB2** (5 OTUs with 96-98% identity in 425bp to: Ralstonia sp. P-4CB2)	**3.0%**	7.5%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**2.6%**	1.2%
s	**Ignavibacterium album** (3 OTUs with 87-100% identity in 422-425bp to: Ignavibacterium album)	**2.0%**	2.3%
c	**Betaproteobacteria** (3 OTUs with 98-100% identity in 424bp to: 17 unclassified Simplicispira strains, 2 unclassified Aquaspirillum strains, Simplicispira limi)	**2.0%**	2.1%
s	**Vicingus serpentipes** (1 OTU with 93% identity in 424bp to: Vicingus serpentipes)	**1.9%**	1.8%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 426bp to: Sterolibacterium sp. TKU1)	**1.9%**	0.9%
g	**Thermomonas** (3 OTUs with 98-100% identity in 427bp to: 3 unclassified Thermomonas strains, Thermomonas fusca, Thermomonas haemolytica, Thermomonas koreensis)	**1.9%**	2.5%
g	**Pseudomonas** (3 OTUs with 100% identity in 424bp to: 35 unclassified Pseudomonas strains, Pseudomonas baetica, Pseudomonas caeni, Pseudomonas jessenii, Pseudomonas laurylsulfativorans, Pseudomonas migulae, Pseudomonas mohnii, Pseudomonas moorei, Pseudomonas putida, Pseudomonas umsongensis)	**1.8%**	3.8%
g	**Comamonas** (4 OTUs with 96-99% identity in 424bp to: 33 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**1.7%**	1.6%
s	**Wandonia haliotis** (1 OTU with 86% identity in 422bp to: Wandonia haliotis)	**1.6%**	1.9%
s	**Ferruginibacter alkalilentus** (1 OTU with 95% identity in 422bp to: Ferruginibacter alkalilentus)	**1.4%**	1.7%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**1.3%**	0.6%
s	**Prochlorococcus sp. SCGC AAA298-C17** (1 OTU with 85% identity in 422bp to: Prochlorococcus sp. SCGC AAA298-C17)	**1.2%**	0.9%
)			
s	**Curvibacter sp. R-36930** (1 OTU with 99% identity in 424bp to: Curvibacter sp. R-36930)	**1.2%**	1.1%
s	**Gemmatimonas aurantiaca** (2 OTUs with 94-95% identity in 418bp to: Gemmatimonas aurantiaca)	**1.2%**	0.5%
s	**Thermomonas fusca** (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	**1.1%**	1.4%
s	**Steroidobacter sp.** (3 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**1.1%**	1.0%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**1.0%**	1.0%
s	**Williamwhitmania taraxaci** (2 OTUs with 86-87% identity in 423bp to: Williamwhitmania taraxaci)	**0.9%**	1.3%
s	**Thiobacillus sp. 100B-B7** (1 OTU with 99% identity in 424bp to: Thiobacillus sp. 100B-B7)	**0.9%**	0.4%
s	**Thermomonas brevis** (1 OTU with 99% identity in 427bp to: Thermomonas brevis)	**0.9%**	1.1%
s	**Tangfeifania diversioriginum** (2 OTUs with 92% identity in 422bp to: Tangfeifania diversioriginum)	**0.9%**	1.2%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 423bp to: Rhodohalobacter sp. SW132)	**0.8%**	0.9%
s	**Paludibaculum sp.** (2 OTUs with 92-93% identity in 399bp to: Paludibaculum sp.)	**0.8%**	0.5%
s	**Ferruginibacter sp.** (1 OTU with 92% identity in 422bp to: Ferruginibacter sp.)	**0.8%**	1.0%
s	**Pedobacter sp.** (1 OTU with 99% identity in 422bp to: Pedobacter sp.)	**0.7%**	1.1%
s	**Simplicispira suum** (1 OTU with 100% identity in 424bp to: Simplicispira suum)	**0.7%**	0.7%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**0.7%**	0.6%

g	**Massilia** (2 OTUs with 99% identity in 424bp to: Massilia brevitalea, Massilia sp. TA_JX)	**0.7%**	0.8%
g	**Rhodococcus** (1 OTU with 100% identity in 404bp to: 5 unclassified Rhodococcus strains, Rhodococcus degradans, Rhodococcus erythropolis, Rhodococcus qingshengii)	**0.5%**	0.5%
s	**Chryseolinea soli** (1 OTU with 91% identity in 422bp to: Chryseolinea soli)	**0.5%**	0.6%
s	**Thauera terpenica** (2 OTUs with 99-100% identity in 424bp to: Thauera terpenica)	**0.5%**	0.7%
s	**Denitratimonas tolerans** (3 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	**0.5%**	0.7%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 417bp to: Gemmatimonas sp. URHD0086)	**0.5%**	0.2%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**0.5%**	0.3%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 399bp to: 2 unclassified Bradyrhizobium strains, 3 unclassified Afipia strains, Afipia massiliensis)	**0.5%**	0.4%
s	**Woodsholea maritima** (1 OTU with 95% identity in 399bp to: Woodsholea maritima)	**0.4%**	0.3%
g	**Ferruginibacter** (1 OTU with 95% identity in 422bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	**0.4%**	0.5%
s	**Gaiella occulta** (1 OTU with 93% identity in 423bp to: Gaiella occulta)	**0.4%**	0.2%
s	**Mucibacter soli** (1 OTU with 96% identity in 422bp to: Mucibacter soli)	**0.4%**	0.5%
s	**Hypericibacter adhaerens** (1 OTU with 94% identity in 400bp to: Hypericibacter adhaerens)	**0.4%**	0.6%
s	**Roseisolibacter agri** (1 OTU with 91% identity in 416bp to: Roseisolibacter agri)	**0.4%**	0.2%
g	**Rhodanobacter** (2 OTUs with 99-100% identity in 424bp to: 3 unclassified Rhodanobacter strains, Rhodanobacter lindaniclasticus, Rhodanobacter spathiphylli, Rhodanobacter xiangquanii)	**0.4%**	0.6%
g	**Simplicispira** (1 OTU with 100% identity in 424bp to: Simplicispira piscis, Simplicispira sp. b29)	**0.4%**	0.3%
s	**Pseudoxanthomonas sp. T3-YC6797** (1 OTU with 99% identity in 424bp to: Pseudoxanthomonas sp. T3-YC6797)	**0.4%**	0.2%
o	**Myxococcales** (1 OTU with 91% identity in 423bp to: Phaselicystis flava, Racemicystis crocea, Racemicystis persica)	**0.3%**	0.4%
s	**Thauera sp. O** (1 OTU with 100% identity in 424bp to: Thauera sp. O)	**0.3%**	0.5%
s	**Conexibacter sp.** (1 OTU with 93% identity in 426bp to: Conexibacter sp.)	**0.3%**	0.2%
s	**Treponema stenostreptum** (1 OTU with 96% identity in 427bp to: Treponema stenostreptum)	**0.3%**	0.3%
s	**Racemicystis persica** (1 OTU with 92% identity in 423bp to: Racemicystis persica)	**0.3%**	0.6%
g	**Lacibacter** (1 OTU with 95% identity in 422bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**0.3%**	0.4%
s	**Candidatus Nitrosarchaeum limnium** (1 OTU with 94% identity in 400bp to: Candidatus Nitrosarchaeum limnium)	**0.3%**	0.2%
p	**Proteobacteria** (2 OTUs with 98-100% identity in 399-424bp to: 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, Aquamicrobium terrae, Rhodanobacter sp. 7B-393, Simplicispira sp., Thiobacillus sp. BHF001)	**0.3%**	0.4%
s	**Hyphomicrobium sp.** (1 OTU with 99% identity in 399bp to: Hyphomicrobium sp.)	**0.3%**	0.2%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 424bp to: Candidatus Kuenenia sp.)	**0.3%**	0.4%
f	**Microbacteriaceae** (1 OTU with 100% identity in 405bp to: 30 unclassified Cryobacterium strains, 5 unclassified Frigoribacterium strains, Cryobacterium arcticum, Cryobacterium psychrotolerans, Cryobacterium soli, Cryobacterium zongtaii, Leifsonia sp. 7PE5.6, Salinibacterium sp. Hb10)	**0.3%**	0.3%
s	**Pseudorhodobacter ponti** (1 OTU with 100% identity in 399bp to: Pseudorhodobacter ponti)	**0.2%**	0.3%
s	**Parvibaculum sp.** (1 OTU with 98% identity in 399bp to: Parvibaculum sp.)	**0.2%**	0.3%
s	**Nitrospira sp.** (1 OTU with 98% identity in 417bp to: Nitrospira sp.)	**0.2%**	0.1%
g	**Afipia** (1 OTU with 99% identity in 399bp to: 2 unclassified Afipia strains, Afipia clevelandensis)	**0.2%**	0.2%
g	**Hyphomicrobium** (1 OTU with 95% identity in 399bp to: Hyphomicrobium sp., Hyphomicrobium zavarzinii)	**0.2%**	0.2%
g	**Desulfonatronum** (1 OTU with 90% identity in 426bp to: Desulfonatronum paranguaonense, Desulfonatronum thiosulfatophilum)	**0.2%**	0.2%

f	**Rhodobacteraceae** (1 OTU with 97% identity in 399bp to: 3 unclassified Pseudorhodobacter strains, 6 unclassified Rhodobacter strains, Pseudorhodobacter antarcticus, Pseudorhodobacter ferrugineus)	**0.2%**	0.3%
g	**Lutibacter** (1 OTU with 96% identity in 422bp to: Lutibacter litorisediminis, Lutibacter sp. 16SWWE-6)	**0.2%**	0.3%
o	**Burkholderiales** (1 OTU with 79% identity in 430bp to: 4 unclassified Roseateles strains, Paucibacter sp.)	**0.2%**	0.3%
g	**Rhodovibrio** (1 OTU with 92% identity in 402bp to: 3 unclassified Rhodovibrio strains)	**0.2%**	0.1%
k	**Bacteria** (1 OTU with 100% identity in 424bp to: 33 unclassified Delftia strains, Alcaligenes faecalis, Clostridium sporogenes, Delftia acidovorans, Delftia lacustris, Delftia tsuruhatensis, Lactococcus raffinolactis, Pseudomonas sp. MS8_2H3)	**0.2%**	0.2%
f	**Xanthomonadaceae** (1 OTU with 100% identity in 424bp to: Pseudoxanthomonas sp. BZ29r, Pseudoxanthomonas yeongjuensis, Stenotrophomonas sp. FS-YC6686)	**0.2%**	0.3%
s	**Thermosulfidibacter takaii** (1 OTU with 80% identity in 429bp to: Thermosulfidibacter takaii)	**0.2%**	0.2%
s	**Ideonella sp.** (1 OTU with 97% identity in 424bp to: Ideonella sp.)	**0.2%**	0.3%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	**0.2%**	0.1%
g	**Variovorax** (1 OTU with 100% identity in 424bp to: 11 unclassified Variovorax strains, Variovorax paradoxus)	**0.2%**	0.2%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**0.2%**	0.3%
g	**Rhodobacter** (1 OTU with 100% identity in 399bp to: 4 unclassified Rhodobacter strains, Rhodobacter gluconicum)	**0.2%**	0.3%
s	**Chryseobacterium sp. Iso-52** (1 OTU with 96% identity in 422bp to: Chryseobacterium sp. Iso-52)	**0.1%**	0.2%
s	**Lewinella aquimaris** (1 OTU with 85% identity in 420bp to: Lewinella aquimaris)	**0.1%**	0.2%
s	**Simplicispira sp.** (1 OTU with 99% identity in 425bp to: Simplicispira sp.)	**0.1%**	0.1%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 424bp to: Thioprofundum hispidum)	**0.1%**	0.1%
s	**Simplicispira sp. L1R1.2** (1 OTU with 99% identity in 424bp to: Simplicispira sp. L1R1.2)	**0.1%**	0.1%
g	**Alicycliphilus** (1 OTU with 100% identity in 424bp to: 5 unclassified Alicycliphilus strains, Alicycliphilus denitrificans)	**0.1%**	0.2%
g	**Paracoccus** (1 OTU with 100% identity in 399bp to: 41 unclassified Paracoccus strains, Paracoccus aminophilus, Paracoccus aminovorans, Paracoccus caeni, Paracoccus chinensis, Paracoccus denitrificans, Paracoccus fontiphilus, Paracoccus huijuniae, Paracoccus kondratievae, Paracoccus sediminis, Paracoccus subflavus, Paracoccus tibetensis)	**0.1%**	0.2%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	**0.1%**	0.1%
s	**Dokdonella sp.** (1 OTU with 96% identity in 425bp to: Dokdonella sp.)	**0.1%**	0.2%
s	**Afipia sp. THI201** (1 OTU with 100% identity in 399bp to: Afipia sp. THI201)	**0.1%**	0.1%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.1%**	0.1%
g	**Desulfovibrio** (1 OTU with 99% identity in 424bp to: 10 unclassified Desulfovibrio strains, Desulfovibrio oryzae, Desulfovibrio oxamicus, Desulfovibrio vulgaris)	**0.1%**	0.2%
	Other	**0.8%**	1.6%
	Unclassified (0 reads)		

SI 5.10: EBBR-Anammox microbial communities identified by NGS **11.V3V4a**

Filtered (0 reads)

12.V3V4a (74 936 reads)

s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**11.9%**	6.4%
s	**Nitrosomonas sp.** (5 OTUs with 96-99% identity in 424-425bp to: Nitrosomonas sp.)	**10.5%**	5.7%
g	**Nitrosomonas** (2 OTUs with 97-100% identity in 424bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**9.7%**	5.2%
g	**Comamonas** (5 OTUs with 96-99% identity in 424bp to: 34 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**8.1%**	8.8%
f	**Chitinophagaceae** (1 OTU with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**7.3%**	10.4%
s	**Ferruginibacter sp.** (4 OTUs with 92-96% identity in 422bp to: Ferruginibacter sp.)	**6.3%**	8.9%
f	**Comamonadaceae** (4 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 6 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Acidovorax caeni, Alicycliphilus denitrificans, Comamonas granuli, Comamonas sp. RS-M7Pp2013, Melaminivora alkalimesophila, Pseudacidovorax sp. A14(2010), Simplicispira sp. YIM219, Variovorax paradoxus)	**5.7%**	6.1%
g	**Thermomonas** (3 OTUs with 97-99% identity in 427bp to: 3 unclassified Thermomonas strains, Thermomonas brevis)	**3.0%**	4.6%
s	**Gemmatimonas aurantiaca** (1 OTU with 95% identity in 418bp to: Gemmatimonas aurantiaca)	**2.5%**	1.4%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**2.3%**	2.5%
s	**Lysobacter tongrenensis** (1 OTU with 97% identity in 425bp to: Lysobacter tongrenensis)	**2.1%**	3.2%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 399bp to: 2 unclassified Rhodopseudomonas strains, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	**2.0%**	1.7%
s	**Denitratisoma oestradiolicum** (3 OTUs with 95-98% identity in 424bp to: Denitratisoma oestradiolicum)	**1.6%**	0.9%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**1.3%**	0.7%
s	**Roseisolibacter agri** (1 OTU with 91% identity in 416bp to: Roseisolibacter agri)	**1.2%**	0.7%
s	**Ralstonia sp. P-4CB2** (3 OTUs with 97-98% identity in 425bp to: Ralstonia sp. P-4CB2)	**1.1%**	3.3%
o	**Burkholderiales** (4 OTUs with 79-97% identity in 424-430bp to: 2 unclassified Aquincola strains, 2 unclassified Ideonella strains, 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Caldimonas hydrothermale, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Paucibacter sp., Rubrivivax sp. JA309)	**1.1%**	2.0%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**1.0%**	0.6%
g	**Hyphomicrobium** (5 OTUs with 95-100% identity in 399bp to: 7 unclassified Hyphomicrobium strains, Hyphomicrobium aestuarii, Hyphomicrobium denitrificans, Hyphomicrobium nitrativorans, Hyphomicrobium vulgare, Hyphomicrobium zavarzinii)	**0.9%**	0.7%
s	**Terrimonas sp. YJ03** (4 OTUs with 95-98% identity in 422bp to: Terrimonas sp. YJ03)	**0.9%**	1.3%
s	**Thermomonas carbonis** (1 OTU with 98% identity in 427bp to: Thermomonas carbonis)	**0.9%**	1.4%
s	**Racemicystis persica** (3 OTUs with 92-93% identity in 423-424bp to: Racemicystis persica)	**0.7%**	1.4%

g	**Hyphomicrobium** (5 OTUs with 95-100% identity in 399bp to: 7 unclassified Hyphomicrobium strains, Hyphomicrobium aestuarii, Hyphomicrobium denitrificans, Hyphomicrobium nitrativorans, Hyphomicrobium vulgare, Hyphomicrobium zavarzinii)	**0.9%**	0.7%
s	**Terrimonas sp. YJ03** (4 OTUs with 95-98% identity in 422bp to: Terrimonas sp. YJ03)	**0.9%**	1.3%
s	**Thermomonas carbonis** (1 OTU with 98% identity in 427bp to: Thermomonas carbonis)	**0.9%**	1.4%
s	**Racemicystis persica** (3 OTUs with 92-93% identity in 423-424bp to: Racemicystis persica)	**0.7%**	1.4%

g	**Haliscomenobacter** (1 OTU with 92% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**0.6%**	0.9%
c	**Betaproteobacteria** (2 OTUs with 98% identity in 424bp to: 3 unclassified Simplicispira strains, Aquaspirillum sp. R-22832)	**0.6%**	0.7%
o	**Rhodocyclales** (1 OTU with 92% identity in 424bp to: 3 unclassified Thauera strains, Azonexus caeni)	**0.6%**	0.9%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 424bp to: Aquincola sp. HME6814)	**0.6%**	1.0%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**0.6%**	1.0%
s	**Labilithrix luteola** (3 OTUs with 88% identity in 426bp to: Labilithrix luteola)	**0.6%**	0.7%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 424bp to: Thioprofundum hispidum)	**0.6%**	0.5%
f	**Microbacteriaceae** (1 OTU with 99% identity in 404bp to: 2 unclassified Chryseoglobus strains, 3 unclassified Salinibacterium strains, 8 unclassified Microcella strains, Chryseoglobus frigidaquae, Conyzicola lurida, Conyzicola nivalis, Conyzicola sp. B33-1, Cryobacterium mesophilum, Cryobacterium sp., Cryobacterium tepidiphilum, Diaminobutyricimonas massiliensis, Leifsonia sp. MSL 02, Lysinimonas sp. LM-2018, Microcella alkaliphila, Salinibacterium xinjiangense)	**0.5%**	0.6%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.5%**	0.6%
s	**Sediminibacterium sp.** (1 OTU with 95% identity in 422bp to: Sediminibacterium sp.)	**0.5%**	0.7%

s	Acidobacterium sp. WY65 (2 OTUs with 92-95% identity in 399bp to: Acidobacterium sp. WY65)	0.5%	0.3%
s	Denitratimonas tolerans (2 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	0.5%	0.7%
s	Gaiella occulta (2 OTUs with 94-99% identity in 423bp to: Gaiella occulta)	0.5%	0.2%
s	Chryseotalea sanaruensis (1 OTU with 91% identity in 421bp to: Chryseotalea sanaruensis)	0.4%	0.6%
s	Stenotrophobacter terrae (1 OTU with 96% identity in 402bp to: Stenotrophobacter terrae)	0.4%	0.3%
s	Nitrosomonas eutropha (1 OTU with 100% identity in 424bp to: Nitrosomonas eutropha)	0.4%	0.2%
s	Parvibaculum sp. MBNA2 (1 OTU with 98% identity in 402bp to: Parvibaculum sp. MBNA2)	0.4%	0.5%
p	Proteobacteria (3 OTUs with 97-100% identity in 399-424bp to: 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, 3 unclassified Shinella strains, 8 unclassified Rhizobium strains, Aquamicrobium terrae, Aquincola sp., Bradyrhizobium sp., Ochrobactrum anthropi, Ochrobactrum sp., Rhizobium gallicum, Rhizobium yanglingense, Shinella curvata, Shinella kummerowiae, Shinella yambaruensis, Shinella zoogloeoides, Thiobacillus sp. BHF001, Variovorax sp. 4LI(+)_OTU30)	0.4%	0.6%
s	Conexibacter sp. (2 OTUs with 92% identity in 424-426bp to: Conexibacter sp.)	0.3%	0.2%
s	Lewinella aquimaris (1 OTU with 85% identity in 420bp to: Lewinella aquimaris)	0.3%	0.5%
s	Steroidobacter sp. (1 OTU with 93% identity in 424bp to: Steroidobacter sp.)	0.3%	0.4%
s	Thermomonas fusca (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	0.3%	0.5%
f	Sphingomonadaceae (1 OTU with 100% identity in 399bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	0.3%	0.4%
o	Rhizobiales (1 OTU with 92% identity in 399bp to: Breoghania corrubedonensis, Breoghania sp. BR-5, Mycoplana sp. G110)	0.3%	0.4%
s	Hyphomicrobium sp. WG6 (1 OTU with 98% identity in 400bp to: Hyphomicrobium sp. WG6)	0.3%	0.2%
g	Ferruginibacter (1 OTU with 95% identity in 422bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	0.3%	0.4%
g	Aquamicrobium (1 OTU with 97% identity in 399bp to: 2 unclassified Aquamicrobium strains, Aquamicrobium aerolatum, Aquamicrobium ahrensii, Aquamicrobium defluvii, Aquamicrobium lusatiense, Aquamicrobium soli)	0.3%	0.2%
s	Thermomonas brevis (1 OTU with 99% identity in 427bp to: Thermomonas brevis)	0.3%	0.4%
s	Diaphorobacter sp. (1 OTU with 98% identity in 424bp to: Diaphorobacter sp.)	0.3%	0.4%
s	Daejeonella rubra (1 OTU with 86% identity in 422bp to: Daejeonella rubra)	0.2%	0.5%
g	Brevundimonas (1 OTU with 100% identity in 399bp to: 29 unclassified Brevundimonas strains, Brevundimonas diminuta, Brevundimonas intermedia, Brevundimonas naejangsanensis, Brevundimonas olei, Brevundimonas vesicularis)	0.2%	0.1%
s	Nitrospira sp. (1 OTU with 98% identity in 417bp to: Nitrospira sp.)	0.2%	0.1%

s	**Rhodovastum atsumiense** (1 OTU with 96% identity in 399bp to: Rhodovastum atsumiense)	**0.2%**	0.4%
g	**Gaiella** (1 OTU with 98% identity in 423bp to: 2 unclassified Gaiella strains)	**0.2%**	0.1%
g	**Bdellovibrio** (1 OTU with 96% identity in 405bp to: Bdellovibrio bacteriovorus, Bdellovibrio sp. R4)	**0.2%**	0.3%
s	**Mesorhizobium sp.** (1 OTU with 100% identity in 399bp to: Mesorhizobium sp.)	**0.2%**	0.2%
f	**Mycobacteriaceae** (1 OTU with 100% identity in 404bp to: 2 unclassified Mycobacterium strains, Mycolicibacterium conceptionense, Mycolicibacterium fortuitum, Mycolicibacterium montmartrense, Mycolicibacterium peregrinum, Mycolicibacterium porcinum, Mycolicibacterium septicum, Mycolicibacterium sp.)	**0.2%**	0.2%
s	**Methylocapsa aurea** (1 OTU with 96% identity in 400bp to: Methylocapsa aurea)	**0.2%**	0.2%
s	**Roseomonas sp.** (2 OTUs with 95-98% identity in 399bp to: Roseomonas sp.)	**0.2%**	0.3%
s	**Polyangium thaxteri** (1 OTU with 96% identity in 423bp to: Polyangium thaxteri)	**0.2%**	0.4%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	**0.2%**	0.1%
s	**Bdellovibrio sp. MPA** (1 OTU with 88% identity in 405bp to: Bdellovibrio sp. MPA)	**0.2%**	0.3%
s	**Terrimonas sp.** (1 OTU with 97% identity in 422bp to: Terrimonas sp.)	**0.2%**	0.3%
g	**Geobacter** (1 OTU with 88% identity in 425bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	**0.2%**	0.3%
s	**Curvibacter sp. R-36930** (1 OTU with 99% identity in 424bp to: Curvibacter sp. R-36930)	**0.2%**	0.2%
s	**Pseudorhodoplanes sinuspersici** (1 OTU with 96% identity in 399bp to: Pseudorhodoplanes sinuspersici)	**0.2%**	0.2%
s	**Casimicrobium huifangae** (1 OTU with 99% identity in 425bp to: Casimicrobium huifangae)	**0.2%**	0.2%
g	**Niveispirillum** (1 OTU with 89% identity in 402bp to: 2 unclassified Niveispirillum strains)	**0.1%**	0.3%
s	**Thauera sp. O** (1 OTU with 100% identity in 424bp to: Thauera sp. O)	**0.1%**	0.2%
s	**Bdellovibrio sp.** (1 OTU with 93% identity in 405bp to: Bdellovibrio sp.)	**0.1%**	0.2%
s	**Granulosicoccus sp. SS10.26** (1 OTU with 92% identity in 424bp to: Granulosicoccus sp. SS10.26)	**0.1%**	0.1%
g	**Mesorhizobium** (1 OTU with 100% identity in 399bp to: 2 unclassified Mesorhizobium strains, alpha proteobacterium WG1)	**0.1%**	0.1%
g	**Nakamurella** (1 OTU with 99% identity in 404bp to: Nakamurella flava, Nakamurella sp.)	**0.1%**	0.1%
s	**Tepidiforma bonchosmolovskayae** (1 OTU with 95% identity in 404bp to: Tepidiforma bonchosmolovskayae)	**0.1%**	0.1%
c	**Alphaproteobacteria** (1 OTU with 100% identity in 399bp to: 15 unclassified Bosea strains, Afipia genosp. 7, Bosea robiniae, Sphingopyxis sp. YIM102)	**0.1%**	0.1%
c	**Actinobacteria** (1 OTU with 95% identity in 404bp to: Jatrophihabitans sp., Sporichthya brevicatena)	**0.1%**	0.1%
g	**Paracoccus** (1 OTU with 100% identity in 399bp to: 41 unclassified Paracoccus strains, Paracoccus aminophilus, Paracoccus aminovorans, Paracoccus caeni, Paracoccus chinensis, Paracoccus denitrificans, Paracoccus fontiphilus, Paracoccus huijuniae, Paracoccus kondratievae, Paracoccus sediminis, Paracoccus subflavus, Paracoccus tibetensis)	**0.1%**	0.1%
	Other	**0.5%**	1.1%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.11: EBBR-Anammox microbial communities identified by NGS **12. V3V4a**

13.V3V4a (80 602 reads)

s	**Gemmatimonas phototrophica** (7 OTUs with 92% identity in 416bp to: Gemmatimonas phototrophica)	**20.5%**	11.7%
s	**Denitratisoma oestradiolicum** (6 OTUs with 95-98% identity in 424bp to: Denitratisoma oestradiolicum)	**18.5%**	10.6%
s	**Thermomonas carbonis** (2 OTUs with 98% identity in 424-427bp to: Thermomonas carbonis)	**7.6%**	12.2%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**4.5%**	5.1%
s	**Denitratimonas tolerans** (3 OTUs with 97-100% identity in 424bp to: Denitratimonas tolerans)	**4.4%**	7.0%
g	**Nitrosomonas** (3 OTUs with 97-100% identity in 424bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**4.2%**	2.4%
g	**Comamonas** (6 OTUs with 96-100% identity in 424bp to: 34 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**3.9%**	4.5%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**3.6%**	2.1%
s	**Nitrosomonas sp.** (3 OTUs with 97-98% identity in 424-425bp to: Nitrosomonas sp.)	**2.3%**	1.3%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**2.0%**	2.3%
f	**Alcaligenaceae** (1 OTU with 98% identity in 424bp to: 2 unclassified Pusillimonas strains, Candidimonas bauzanensis)	**1.7%**	1.7%
s	**Parvibaculum sp. MBNA2** (1 OTU with 100% identity in 402bp to: Parvibaculum sp. MBNA2)	**1.6%**	2.1%
s	**Lysobacter tongrenensis** (1 OTU with 97% identity in 425bp to: Lysobacter tongrenensis)	**1.4%**	2.2%
s	**Curvibacter sp. R-36930** (1 OTU with 99% identity in 424bp to: Curvibacter sp. R-36930)	**1.4%**	1.6%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	**1.4%**	2.6%
s	**Ferruginibacter sp.** (1 OTU with 92% identity in 422bp to: Ferruginibacter sp.)	**1.3%**	1.9%
s	**Lysobacter concretionis** (3 OTUs with 99-100% identity in 424bp to: Lysobacter concretionis)	**1.2%**	1.9%
g	**Thermomonas** (4 OTUs with 97-99% identity in 427bp to: 3 unclassified Thermomonas strains, Thermomonas brevis)	**1.2%**	1.9%
s	**Gemmatimonas aurantiaca** (1 OTU with 95% identity in 418bp to: Gemmatimonas aurantiaca)	**1.1%**	0.7%
s	**Lysobacter sp.** (1 OTU with 99% identity in 424bp to: Lysobacter sp.)	**1.1%**	1.8%
s	**Chryseotalea sanaruensis** (2 OTUs with 91% identity in 421bp to: Chryseotalea sanaruensis)	**1.0%**	1.6%
s	**Ralstonia sp. P-4CB2** (3 OTUs with 97-98% identity in 425bp to: Ralstonia sp. P-4CB2)	**1.0%**	2.9%
s	**Turneriella parva** (2 OTUs with 95-97% identity in 424bp to: Turneriella parva)	**0.9%**	0.9%
s	**Racemicystis persica** (3 OTUs with 92-93% identity in 423-424bp to: Racemicystis persica)	**0.9%**	2.0%

f	**Chitinophagaceae** (1 OTU with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**0.8%**	1.2%
g	**Desulfonatronum** (1 OTU with 90% identity in 426bp to: Desulfonatronum paranguiense, Desulfonatronum thiosulfatophilum)	**0.8%**	0.9%
g	**Gelidibacter** (1 OTU with 99% identity in 421bp to: 4 unclassified Gelidibacter strains, Gelidibacter flavus, Gelidibacter gilvus, Gelidibacter japonicus)	**0.8%**	1.2%
s	**Casimicrobium huifangae** (1 OTU with 99% identity in 425bp to: Casimicrobium huifangae)	**0.6%**	0.7%
s	**Terrimonas sp. YJ03** (4 OTUs with 96-98% identity in 422bp to: Terrimonas sp. YJ03)	**0.5%**	0.8%
s	**Thiobacillus sp. 100B-B7** (1 OTU with 99% identity in 424bp to: Thiobacillus sp. 100B-B7)	**0.5%**	0.3%
p	**Proteobacteria** (2 OTUs with 97-98% identity in 424bp to: Aquincola sp., Bradyrhizobium sp., Rhodanobacter sp. 7B-393, Simplicispira sp.)	**0.5%**	0.8%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**0.5%**	0.3%
f	**Myxococcaceae** (1 OTU with 93% identity in 423bp to: Corallococcus macrosporus, Pyxidicoccus fallax)	**0.4%**	0.7%
f	**Bradyrhizobiaceae** (2 OTUs with 100% identity in 399bp to: 2 unclassified Nitrobacter strains, 2 unclassified Rhodopseudomonas strains, 3 unclassified Afipia strains, Afipia birgiae, Afipia broomeae, Afipia genosp. 10, Afipia lausannensis, Afipia massiliensis, Bradyrhizobium jicamae, Bradyrhizobium sp., Rhodopseudomonas faecalis)	**0.4%**	0.4%
o	**Burkholderiales** (1 OTU with 79% identity in 430bp to: 4 unclassified Rosatales strains, Paucibacter sp.)	**0.4%**	0.7%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 424bp to: Aquincola sp. HME6814)	**0.4%**	0.7%
s	**Aciditerrimonas ferrireducens** (2 OTUs with 93% identity in 401bp to: Aciditerrimonas ferrireducens)	**0.4%**	0.4%
s	**Thermomonas fusca** (1 OTU with 98% identity in 427bp to: Thermomonas fusca)	**0.3%**	0.5%
s	**Roseisolibacter agri** (1 OTU with 91% identity in 416bp to: Roseisolibacter agri)	**0.3%**	0.2%
s	**Hyphomicrobium sp. KC-IT-W2** (1 OTU with 99% identity in 399bp to: Hyphomicrobium sp. KC-IT-W2)	**0.3%**	0.2%
s	**Lentimicrobium saccharophilum** (1 OTU with 98% identity in 422bp to: Lentimicrobium saccharophilum)	**0.3%**	0.5%
f	**Microbacteriaceae** (1 OTU with 99% identity in 404bp to: 2 unclassified Chryseoglobus strains, 3 unclassified Salinibacterium strains, 8 unclassified Microcella strains, Chryseoglobus frigidaquae, Conyzicola lurida, Conyzicola nivalis, Conyzicola sp. B33-1, Cryobacterium mesophilum, Cryobacterium sp., Cryobacterium sapidiphilum, Diaminobutyricimonas massiliensis, Leifsonia sp. MSL 02, Lysinimonas sp. LM-2018, Microcella alkaliphila, Salinibacterium xinjiangense)	**0.3%**	0.3%
g	**Dokdonella** (1 OTU with 97% identity in 424bp to: Dokdonella immobilis, Dokdonella sp.)	**0.3%**	0.5%
s	**Polyangium thaxteri** (1 OTU with 96% identity in 423bp to: Polyangium thaxteri)	**0.3%**	0.6%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 424bp to: Thioprofundum hispidum)	**0.3%**	0.2%
s	**Arhodomonas recens** (1 OTU with 91% identity in 424bp to: Arhodomonas recens)	**0.3%**	0.2%
s	**Simplicispira sp. L1R1.2** (1 OTU with 98% identity in 424bp to: Simplicispira sp. L1R1.2)	**0.2%**	0.3%
s	**Steroidobacter sp.** (2 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**0.2%**	0.3%
s	**Azoarcus taiwanensis** (1 OTU with 98% identity in 424bp to: Azoarcus taiwanensis)	**0.2%**	0.4%
g	**Haliscomenobacter** (1 OTU with 92% identity in 422bp to: 2 unclassified Haliscomenobacter strains)	**0.1%**	0.2%
s	**Labilithrix luteola** (1 OTU with 88% identity in 426bp to: Labilithrix luteola)	**0.1%**	0.1%
	Other	**0.8%**	1.2%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.12: EBBR-Anammox microbial communities identified by NGS **13. V3V4a**

14.V3V4a (60 501 reads)

s	**Nitrosomonas eutropha** (1 OTU with 100% identity in 424bp to: Nitrosomonas eutropha)	**20.2%**	10.8%
s	**Comamonas sp. NLF-7-7** (4 OTUs with 98-100% identity in 424bp to: Comamonas sp. NLF-7-7)	**17.6%**	18.7%
c	**Alphaproteobacteria** (1 OTU with 100% identity in 399bp to: 12 unclassified Brevundimonas strains, 5 unclassified Mycoplana strains, Brevundimonas subvibrioides)	**9.6%**	11.2%
f	**Xanthomonadaceae** (2 OTUs with 99-100% identity in 424bp to: 22 unclassified Stenotrophomonas strains, Stenotrophomonas acidaminiphila, Stenotrophomonas maltophilia, Stenotrophomonas nitritireducens, Xanthomonas sp. HH69)	**5.8%**	8.6%
g	**Pseudomonas** (12 OTUs with 99-100% identity in 424bp to: 2 unclassified Pseudomonas strains, Pseudomonas caeni, sulfide-oxidizing bacterium ISW_10)	**5.6%**	13.4%
s	**Nitrosomonas sp.** (1 OTU with 98% identity in 425bp to: Nitrosomonas sp.)	**4.8%**	2.5%
s	**Nitrobacter sp. LAMK1242** (4 OTUs with 99% identity in 399bp to: Nitrobacter sp. LAMK1242)	**3.5%**	2.9%
g	**Comamonas** (5 OTUs with 97-100% identity in 424bp to: 7 unclassified Comamonas strains, Comamonas faecalis)	**3.5%**	3.7%
g	**Pseudoxanthomonas** (1 OTU with 99% identity in 424bp to: Pseudoxanthomonas kaohsiungensis, Pseudoxanthomonas koreensis, Pseudoxanthomonas sp. ROi44, Pseudoxanthomonas suwonensis)	**3.2%**	1.7%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	**3.0%**	1.6%
p	**Proteobacteria** (4 OTUs with 100% identity in 399-424bp to: 13 unclassified Alcaligenes strains, 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, 3 unclassified Methylobacterium strains, 5 unclassified Blastomonas strains, 6 unclassified Sphingomonas strains, Alcaligenes faecalis, Aquamicrobium terrae, Blastobacter sp. 'SMCC B0477', Blastomonas natatoria, Bordetella sp. AC3, Brevundimonas sp., Cupriavidus sp., Methylobacterium brachiatum, Methylobacterium fujisawaense, Methylobacterium mesophilicum, Methylobacterium oryzae, Methylobacterium phyllostachyos, Methylobacterium radiotolerans, Methylobacterium tardum, Pusillimonas sp. N12, Rhodoferax ferrireducens, Sphingomonas ursincola, Thiobacillus sp. BHF001)	**2.9%**	4.3%
g	**Aquamicrobium** (3 OTUs with 97-100% identity in 399bp to: 8 unclassified Aquamicrobium strains, Aquamicrobium aerolatum, Aquamicrobium ahrensii, Aquamicrobium defluvii, Aquamicrobium lusatiense, Aquamicrobium soli, Aquamicrobium terrae)	**2.9%**	2.6%
s	**Empedobacter sp. B202** (1 OTU with 95% identity in 422bp to: Empedobacter sp. B202)	**1.6%**	2.2%
g	**Cellulosimicrobium** (1 OTU with 100% identity in 404bp to: 13 unclassified Cellulosimicrobium strains, Cellulosimicrobium aquatile, Cellulosimicrobium cellulans, Cellulosimicrobium funkei)	**1.5%**	1.7%
s	**Aquamicrobium sp.** (1 OTU with 99% identity in 399bp to: Aquamicrobium sp.)	**1.5%**	1.3%
g	**Phenylobacterium** (1 OTU with 100% identity in 399bp to: Phenylobacterium koreense, Phenylobacterium sp. S140)	**1.4%**	0.8%
f	**Bradyrhizobiaceae** (2 OTUs with 99-100% identity in 399bp to: 2 unclassified Rhodopseudomonas strains, 3 unclassified Bradyrhizobium strains, Nitrobacter hamburgensis, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	**0.9%**	0.7%
s	**Parvibaculum sedimenti** (1 OTU with 98% identity in 403bp to: Parvibaculum sedimenti)	**0.9%**	1.0%
s	**Gordonia iterans** (1 OTU with 100% identity in 404bp to: Gordonia iterans)	**0.8%**	0.9%
g	**Gordonia** (1 OTU with 100% identity in 404bp to: Gordonia iterans, Gordonia sp. CCUG 60728)	**0.7%**	0.8%
g	**Gaiella** (1 OTU with 99% identity in 423bp to: 2 unclassified Gaiella strains)	**0.7%**	0.4%
s	**Chryseobacterium lacus** (1 OTU with 100% identity in 422bp to: Chryseobacterium lacus)	**0.6%**	0.8%
s	**Mesorhizobium plurifarium** (1 OTU with 100% identity in 399bp to: Mesorhizobium plurifarium)	**0.6%**	0.5%
s	**Alcaligenes sp. C4M17** (2 OTUs with 98% identity in 424bp to: Alcaligenes sp. C4M17)	**0.6%**	0.5%
f	**Alcaligenaceae** (3 OTUs with 98% identity in 424bp to: 2 unclassified Pusillimonas strains, 3 unclassified Alcaligenes strains, Candidimonas bauzanensis, Eoetvoesia caeni)	**0.5%**	0.5%
g	**Nitrosomonas** (2 OTUs with 98-100% identity in 424bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**0.5%**	0.3%
g	**Rhodococcus** (1 OTU with 100% identity in 404bp to: 5 unclassified Rhodococcus strains, Rhodococcus degradans, Rhodococcus erythropolis, Rhodococcus qingshengii)	**0.5%**	0.6%

s	**Aquamicrobium aestuarii** (2 OTUs with 98-100% identity in 399bp to: Aquamicrobium aestuarii)	**0.4%**	0.3%
s	**Pseudolabrys sp.** (1 OTU with 96% identity in 399bp to: Pseudolabrys sp.)	**0.3%**	0.4%
s	**Devosia honganensis** (1 OTU with 99% identity in 399bp to: Devosia honganensis)	**0.3%**	0.3%
g	**Afipia** (1 OTU with 99% identity in 399bp to: 2 unclassified Afipia strains, Afipia clevelandensis)	**0.3%**	0.3%
s	**Mesorhizobium sp. R-6** (1 OTU with 100% identity in 399bp to: Mesorhizobium sp. R-6)	**0.3%**	0.3%
f	**Mycobacteriaceae** (1 OTU with 100% identity in 404bp to: 2 unclassified Mycobacterium strains, Mycolicibacterium conceptionense, Mycolicibacterium fortuitum, Mycolicibacterium montmartrense, Mycolicibacterium peregrinum, Mycolicibacterium porcinum, Mycolicibacterium septicum, Mycolicibacterium sp.)	**0.3%**	0.3%
f	**Microbacteriaceae** (2 OTUs with 99-100% identity in 404-405bp to: 2 unclassified Chryseoglobus strains, 2 unclassified Leifsonia strains, 30 unclassified Cryobacterium strains, 4 unclassified Salinibacterium strains, 5 unclassified Frigoribacterium strains, 8 unclassified Microcella strains, Chryseoglobus frigidaquae, Conyzicola lurida, Conyzicola nivalis, Conyzicola sp. B33-1, Cryobacterium arcticum, Cryobacterium mesophilum, Cryobacterium psychrotolerans, Cryobacterium soli, Cryobacterium tepidiphilum, Cryobacterium zongtaii, Diaminobutyricimonas massiliensis, Lysinimonas sp. LM-2018, Microcella alkaliphila, Salinibacterium xinjiangense)	**0.2%**	0.3%
g	**Arenibacter** (1 OTU with 95% identity in 422bp to: 2 unclassified Arenibacter strains)	**0.2%**	0.3%
s	**Hyphomicrobium nitrativorans** (1 OTU with 99% identity in 399bp to: Hyphomicrobium nitrativorans)	**0.2%**	0.1%
s	**Perlucidibaca sp. RIPI-A18** (1 OTU with 94% identity in 424bp to: Perlucidibaca sp. RIPI-A18)	**0.2%**	0.4%
s	**Aquihabitans daechungensis** (1 OTU with 93% identity in 402bp to: Aquihabitans daechungensis)	**0.2%**	0.2%
s	**Parvibaculum sp. MBNA2** (1 OTU with 98% identity in 402bp to: Parvibaculum sp. MBNA2)	**0.1%**	0.2%
f	**Nocardioidaceae** (1 OTU with 99% identity in 404bp to: 2 unclassified Aeromicrobium strains, Nocardioides sp. COL-46)	**0.1%**	0.1%
s	**Ruegeria sp.** (1 OTU with 95% identity in 399bp to: Ruegeria sp.)	**0.1%**	0.2%
s	**Ferruginibacter sp.** (1 OTU with 94% identity in 422bp to: Ferruginibacter sp.)	**0.1%**	0.2%
g	**Chryseobacterium** (1 OTU with 96% identity in 422bp to: 2 unclassified Chryseobacterium strains, Chryseobacterium hominis)	**0.1%**	0.1%
s	**Nitratireductor sp.** (1 OTU with 99% identity in 399bp to: Nitratireductor sp.)	**0.1%**	0.1%
	Other	**0.5%**	0.7%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.13: EBBR-Anammox microbial communities identified by NGS **14. V3V4a**

15.V3V4a (94 495 reads)

s	**Nitrosomonas sp.** (11 OTUs with 96-99% identity in 424-425bp to: Nitrosomonas sp.)	**28.6%**	16.5%
f	**Chitinophagaceae** (36 OTUs with 79% identity in 424bp to: Arachidicoccus sp., Haoranjiania flava)	**17.5%**	26.5%
s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 424bp to: Denitratisoma oestradiolicum)	**10.7%**	6.2%
f	**Comamonadaceae** (3 OTUs with 79-100% identity in 424-428bp to: 2 unclassified Alicycliphilus strains, 6 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Acidovorax caeni, Alicycliphilus denitrificans, Comamonas granuli, Comamonas sp. RS-M7Pp2013, Melaminivora alkalimesophila, Pseudacidovorax sp. A14(2010), Simplicispira sp. YIM219, Variovorax paradoxus)	**3.3%**	3.8%
s	**Nitrospira sp.** (1 OTU with 98% identity in 417bp to: Nitrospira sp.)	**3.2%**	1.9%
s	**Ideonella sp.** (1 OTU with 97% identity in 424bp to: Ideonella sp.)	**2.0%**	3.8%
s	**Acidobacterium sp. WY65** (2 OTUs with 92-95% identity in 399bp to: Acidobacterium sp. WY65)	**1.9%**	1.3%
g	**Nitrosomonas** (1 OTU with 100% identity in 424bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**1.9%**	1.1%
s	**Chryseotalea sanaruensis** (2 OTUs with 91% identity in 421bp to: Chryseotalea sanaruensis)	**1.8%**	2.8%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 426bp to: Sterolibacterium sp. TKU1)	**1.8%**	1.1%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 422bp to: Haematococcus lacustris)	**1.5%**	0.9%
s	**Chryseolinea soli** (2 OTUs with 91% identity in 422bp to: Chryseolinea soli)	**1.3%**	1.9%
s	**Steroidobacter sp.** (2 OTUs with 93-94% identity in 424bp to: Steroidobacter sp.)	**1.2%**	1.5%
s	**Terrimonas sp.** (2 OTUs with 96-97% identity in 422bp to: Terrimonas sp.)	**1.2%**	1.8%
g	**Comamonas** (4 OTUs with 96-99% identity in 424bp to: 32 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**1.2%**	1.4%
s	**Ralstonia sp. P-4CB2** (3 OTUs with 97-98% identity in 425bp to: Ralstonia sp. P-4CB2)	**1.1%**	3.5%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 399bp to: Candidatus Solibacter usitatus)	**1.1%**	0.8%
s	**Pseudorhodoplanes sinuspersici** (1 OTU with 96% identity in 399bp to: Pseudorhodoplanes sinuspersici)	**1.0%**	1.3%
s	**Hyphomicrobium sp. WG6** (1 OTU with 98% identity in 400bp to: Hyphomicrobium sp. WG6)	**0.9%**	0.7%
s	**Stenotrophobacter terrae** (1 OTU with 96% identity in 402bp to: Stenotrophobacter terrae)	**0.9%**	0.6%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 424bp to: Candidatus Kuenenia sp.)	**0.8%**	1.4%
s	**Conexibacter sp.** (3 OTUs with 92-93% identity in 424-426bp to: Conexibacter sp.)	**0.8%**	0.4%
g	**Hyphomicrobium** (4 OTUs with 95-100% identity in 399bp to: 3 unclassified Hyphomicrobium strains, Hyphomicrobium aestuarii, Hyphomicrobium nitrativorans, Hyphomicrobium vulgare, Hyphomicrobium zavarzinii)	**0.8%**	0.6%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 399bp to: 2 unclassified Rhodopseudomonas strains, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	**0.7%**	0.6%

f	**Sphingomonadaceae** (1 OTU with 100% identity in 399bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	**0.7%**	0.9%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 422bp to: Owenweeksia hongkongensis)	**0.7%**	0.8%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 424bp to: Aquincola sp. HME6814)	**0.6%**	1.2%
s	**Gemmatimonas aurantiaca** (1 OTU with 95% identity in 418bp to: Gemmatimonas aurantiaca)	**0.6%**	0.4%
s	**Labilithrix luteola** (3 OTUs with 88% identity in 426bp to: Labilithrix luteola)	**0.6%**	0.8%
s	**Ignavibacterium album** (3 OTUs with 87-100% identity in 422-425bp to: Ignavibacterium album)	**0.5%**	0.8%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 424bp to: Thioprofundum hispidum)	**0.5%**	0.5%
s	**Tepidiforma bonchosmolovskayae** (3 OTUs with 95% identity in 404bp to: Tepidiforma bonchosmolovskayae)	**0.4%**	0.5%

s	Gemmatimonas phototrophica (1 OTU with 92% identity in 416bp to: Gemmatimonas phototrophica)	0.4%	0.2%
s	Nitrobacter sp. LAMK1242 (1 OTU with 99% identity in 399bp to: Nitrobacter sp. LAMK1242)	0.4%	0.3%
s	Pandoraea sp. (1 OTU with 94% identity in 424bp to: Pandoraea sp.)	0.3%	1.0%
g	Mesorhizobium (1 OTU with 100% identity in 399bp to: 2 unclassified Mesorhizobium strains)	0.3%	0.3%
g	Azoarcus (1 OTU with 93% identity in 424bp to: 3 unclassified Azoarcus strains, Azoarcus olearius)	0.3%	0.6%
o	Burkholderiales (3 OTUs with 79-97% identity in 424-430bp to: 4 unclassified Rosearales strains, Aquabacterium sp. AKB-2008-KU6, Aquincola sp. HME6814, Caldimonas hydrothermalis, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Ideonella sp., Paucibacter sp., Rubrivivax sp. JA309)	0.3%	0.6%
s	Roseisolibacter agri (1 OTU with 91% identity in 416bp to: Roseisolibacter agri)	0.3%	0.2%
o	Rhodocyclales (1 OTU with 92% identity in 424bp to: 3 unclassified Thauera strains, Azonexus caeni)	0.3%	0.5%
s	Steroidobacter denitrificans (1 OTU with 97% identity in 424bp to: Steroidobacter denitrificans)	0.3%	0.4%
s	Racemicystis persica (1 OTU with 92% identity in 423bp to: Racemicystis persica)	0.3%	0.7%
s	Tangfeifania diversioriginum (2 OTUs with 92% identity in 422bp to: Tangfeifania diversioriginum)	0.3%	0.5%
g	Thermomonas (2 OTUs with 98-99% identity in 427bp to: 3 unclassified Thermomonas strains, Thermomonas fusca, Thermomonas haemolytica, Thermomonas koreensis)	0.3%	0.5%
s	Terrimonas sp. YJ03 (1 OTU with 98% identity in 422bp to: Terrimonas sp. YJ03)	0.3%	0.4%
s	Ferruginibacter sp. (1 OTU with 92% identity in 422bp to: Ferruginibacter sp.)	0.3%	0.4%
k	Bacteria (2 OTUs with 78% identity in 425bp to: Arachidicoccus sp., Haoranjiania flava, Sutterella faecalis, Sutterella sp.)	0.3%	0.4%
p	Bacteroidetes (2 OTUs with 79-86% identity in 422-424bp to: 2 unclassified Mucilaginibacter strains, Arachidicoccus sp., Elsenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava, Owenweeksia hongkongensis, Solitalea canadensis)	0.2%	0.4%
s	Quisquiliibacterium transsilvanicum (2 OTUs with 95-98% identity in 424bp to: Quisquiliibacterium transsilvanicum)	0.2%	0.6%
s	Methylocapsa aurea (1 OTU with 96% identity in 400bp to: Methylocapsa aurea)	0.2%	0.2%
s	Thioalkalivibrio paradoxus (1 OTU with 91% identity in 424bp to: Thioalkalivibrio paradoxus)	0.2%	0.1%
s	Fimbriimonas ginsengisoli (1 OTU with 87% identity in 404bp to: Fimbriimonas ginsengisoli)	0.2%	0.3%
c	Betaproteobacteria (2 OTUs with 98% identity in 424bp to: 3 unclassified Simplicispira strains, Aquaspirillum sp. R-22832)	0.2%	0.3%
f	Streptomycetaceae (1 OTU with 97% identity in 404bp to: 6 unclassified Streptomyces strains, Embleya scabrispora)	0.2%	0.2%
s	Dokdonella ginsengisoli (1 OTU with 99% identity in 424bp to: Dokdonella ginsengisoli)	0.2%	0.4%
g	Geobacter (1 OTU with 88% identity in 425bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	0.2%	0.3%
s	Phenylobacterium falsum (1 OTU with 100% identity in 402bp to: Phenylobacterium falsum)	0.2%	0.1%
s	Rhodovastum atsumiense (1 OTU with 96% identity in 399bp to: Rhodovastum atsumiense)	0.1%	0.2%
s	Denitratimonas tolerans (1 OTU with 97% identity in 424bp to: Denitratimonas tolerans)	0.1%	0.2%
s	Turneriella parva (1 OTU with 95% identity in 424bp to: Turneriella parva)	0.1%	0.1%
s	Ilumatobacter fluminis (1 OTU with 96% identity in 400bp to: Ilumatobacter fluminis)	0.1%	0.1%
s	Vicingus serpentipes (1 OTU with 93% identity in 424bp to: Vicingus serpentipes)	0.1%	0.1%
s	Aciditerrimonas ferrireducens (1 OTU with 91% identity in 401bp to: Aciditerrimonas ferrireducens)	0.1%	0.1%
f	Mycobacteriaceae (1 OTU with 100% identity in 404bp to: 2 unclassified Mycobacterium strains, Mycolicibacterium conceptionense, Mycolicibacterium fortuitum, Mycolicibacterium montmartrense, Mycolicibacterium peregrinum, Mycolicibacterium porcinum, Mycolicibacterium septicum, Mycolicibacterium sp.)	0.1%	0.1%
c	Actinobacteria (1 OTU with 95% identity in 404bp to: Jatrophihabitans sp., Sporichthya brevicatena)	0.1%	0.1%
f	Xanthomonadaceae (1 OTU with 100% identity in 424bp to: 20 unclassified Pseudoxanthomonas strains, Pseudoxanthomonas mexicana, Xanthomonas campestris)	0.1%	0.2%
	Other	0.4%	0.6%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.14: EBBR-Anammox microbial communities identified by NGS **15. V3V4a**

Sample Name (copy-number corrected read counts)

Taxonomic Level	Taxonomic Unit	Normalized Fraction	Raw Fraction
1.AV3V4a (52 597 reads)			
s	**Denitratisoma oestradiolicum** (5 OTUs with 97-98% identity in 429bp to: Denitratisoma oestradiolicum)	**12.5%**	6.1%
s	**Litorilinea aerophila** (15 OTUs with 88-93% identity in 405-406bp to: Litorilinea aerophila)	**10.6%**	10.9%
s	**Brevefilum fermentans** (8 OTUs with 83% identity in 402bp to: Brevefilum fermentans)	**10.0%**	9.7%
s	**Ignavibacterium album** (7 OTUs with 99-100% identity in 424bp to: Ignavibacterium album)	**9.7%**	11.4%
s	**Thermomarinilinea lacunifontana** (6 OTUs with 84-88% identity in 396-402bp to: Thermomarinilinea lacunifontana)	**6.3%**	6.1%
s	**Paludibaculum sp.** (2 OTUs with 93% identity in 404bp to: Paludibaculum sp.)	**5.0%**	2.8%
g	**Nitrosomonas** (3 OTUs with 97-100% identity in 429bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**4.8%**	2.3%
s	**Thermanaerothrix daxensis** (4 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**4.5%**	4.3%
f	**Anaerolineaceae** (4 OTUs with 83-86% identity in 402-407bp to: Anaerolinea thermolimosa, Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	**4.0%**	3.9%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**3.4%**	3.3%
s	**Tepidiforma bonchosmolovskayae** (7 OTUs with 91-95% identity in 406-407bp to: Tepidiforma bonchosmolovskayae)	**3.1%**	3.2%
s	**Steroidobacter sp.** (4 OTUs with 93-94% identity in 429bp to: Steroidobacter sp.)	**2.0%**	2.0%
s	**Desulfotalea psychrophila** (6 OTUs with 79-80% identity in 418bp to: Desulfotalea psychrophila)	**1.7%**	5.7%
f	**Comamonadaceae** (1 OTU with 79% identity in 430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Melaminivora alkalimesophila)	**1.6%**	1.5%
g	**Lacibacter** (1 OTU with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**1.5%**	2.0%
s	**Sphaerobacter thermophilus** (5 OTUs with 88-94% identity in 407-408bp to: Sphaerobacter thermophilus)	**1.4%**	1.4%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**1.4%**	4.0%
s	**Candidatus Kuenenia sp.** (2 OTUs with 95-97% identity in 429bp to: Candidatus Kuenenia sp.)	**1.3%**	1.9%
f	**Chitinophagaceae** (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**1.3%**	1.6%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**1.1%**	0.6%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97% identity in 430bp to: Ralstonia sp. P-4CB2)	**1.0%**	2.6%
g	**Nitrosospira** (1 OTU with 98% identity in 429bp to: 3 unclassified Nitrosospira strains)	**1.0%**	0.5%
s	**Caldilinea aerophila** (2 OTUs with 88-90% identity in 405bp to: Caldilinea aerophila)	**1.0%**	1.0%

SI 5.15: EBBR-Anammox microbial communities identified by NGS **1.A3V4a**

s	**Longilinea sp.** (4 OTUs with 82-88% identity in 404-406bp to: Longilinea sp.)	**0.8%**	0.7%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**0.7%**	1.2%
s	**Wandonia haliotis** (1 OTU with 86% identity in 424bp to: Wandonia haliotis)	**0.6%**	0.8%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 429bp to: Sterolibacterium sp. TKU1)	**0.5%**	0.3%
g	**Haliscomenobacter** (1 OTU with 91% identity in 424bp to: 2 unclassified Haliscomenobacter strains)	**0.5%**	0.7%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**0.5%**	0.5%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**0.5%**	0.5%
s	**Lewinella cohaerens** (1 OTU with 88% identity in 426bp to: Lewinella cohaerens)	**0.5%**	0.6%
s	**Terrimonas sp. YJ03** (1 OTU with 96% identity in 424bp to: Terrimonas sp. YJ03)	**0.4%**	0.6%
s	**Candidatus Solibacter usitatus** (2 OTUs with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.4%**	0.2%
s	**Dehalococcoides sp. BHI80-15** (1 OTU with 82% identity in 405bp to: Dehalococcoides sp. BHI80-15)	**0.4%**	0.4%
s	**Racemicystis persica** (1 OTU with 92% identity in 428bp to: Racemicystis persica)	**0.4%**	0.7%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.3%**	0.3%
g	**Comamonas** (1 OTU with 96% identity in 429bp to: 23 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**0.3%**	0.3%
s	**Haliea sp. SAOS-164** (2 OTUs with 90% identity in 430bp to: Haliea sp. SAOS-164)	**0.3%**	0.3%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	**0.3%**	0.3%
s	**Lentimicrobium saccharophilum** (2 OTUs with 88% identity in 422-424bp to: Lentimicrobium saccharophilum)	**0.3%**	0.4%
s	**Vicinamibacter silvestris** (1 OTU with 91% identity in 430bp to: Vicinamibacter silvestris)	**0.3%**	0.1%
s	**Acidobacterium sp. WY65** (1 OTU with 96% identity in 404bp to: Acidobacterium sp. WY65)	**0.3%**	0.1%
s	**Parvibaculum sp.** (1 OTU with 93% identity in 404bp to: Parvibaculum sp.)	**0.2%**	0.3%
f	**Caldilineaceae** (1 OTU with 88% identity in 405bp to: Caldilinea aerophila, Litorilinea aerophila)	**0.2%**	0.2%
s	**Methanolinea mesophila** (1 OTU with 96% identity in 384bp to: Methanolinea mesophila)	**0.2%**	0.1%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 406bp to: Aciditerrimonas ferrireducens)	**0.2%**	0.2%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.1%**	0.2%
s	**Methanothrix soehngenii** (1 OTU with 100% identity in 388bp to: Methanothrix soehngenii)	**0.1%**	0.1%
s	**Iamia majanohamensis** (1 OTU with 87% identity in 402bp to: Iamia majanohamensis)	**0.1%**	0.1%
s	**Ornatilinea apprima** (1 OTU with 89% identity in 397bp to: Ornatilinea apprima)	**0.1%**	0.1%
	Other	**0.3%**	0.6%
	Unclassified (0 reads)		

SI 5.16: EBBR-Anammox microbial communities identified by NGS **2.A3V4a**

3.AV3V4a (40 649 reads)

s	**Paludibaculum sp.** (4 OTUs with 92-93% identity in 404bp to: Paludibaculum sp.)	**12.3%**	7.2%
s	**Tepidiforma bonchosmolovskayae** (12 OTUs with 91-96% identity in 406-407bp to: Tepidiforma bonchosmolovskayae)	**9.5%**	10.0%
s	**Litorilinea aerophila** (13 OTUs with 88-93% identity in 405-406bp to: Litorilinea aerophila)	**7.7%**	8.1%
g	**Nitrosomonas** (3 OTUs with 97-100% identity in 429bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**6.1%**	3.1%
g	**Nitrosospira** (1 OTU with 98% identity in 429bp to: 3 unclassified Nitrosospira strains)	**5.9%**	3.0%
s	**Ignavibacterium album** (5 OTUs with 99-100% identity in 424bp to: Ignavibacterium album)	**5.8%**	7.0%
g	**Lacibacter** (2 OTUs with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**3.3%**	4.3%
s	**Thermomarinilinea lacunifontana** (4 OTUs with 84-88% identity in 397-402bp to: Thermomarinilinea lacunifontana)	**3.0%**	3.0%
s	**Steroidobacter sp.** (4 OTUs with 93-94% identity in 429bp to: Steroidobacter sp.)	**2.7%**	2.8%
s	**Lewinella cohaerens** (1 OTU with 88% identity in 426bp to: Lewinella cohaerens)	**2.5%**	3.3%
g	**Haliscomenobacter** (1 OTU with 91% identity in 424bp to: 2 unclassified Haliscomenobacter strains)	**2.4%**	3.2%
s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 429bp to: Denitratisoma oestradiolicum)	**2.3%**	1.2%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**1.9%**	0.9%
s	**Thermanaerothrix daxensis** (4 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**1.8%**	1.8%
s	**Vibrio sp. Gp-3-5.1** (4 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**1.7%**	5.0%
s	**Herbaspirillum sp. AKB-2008-TE24** (1 OTU with 97% identity in 429bp to: Herbaspirillum sp. AKB-2008-TE24)	**1.4%**	2.1%
s	**Caldilinea aerophila** (1 OTU with 88% identity in 405bp to: Caldilinea aerophila)	**1.2%**	1.3%
s	**Vicinamibacter silvestris** (2 OTUs with 90-91% identity in 430bp to: Vicinamibacter silvestris)	**1.2%**	0.7%
s	**Halobacterium sp. GoM2** (1 OTU with 73% identity in 326bp to: Halobacterium sp. GoM2)	**1.2%**	0.6%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97% identity in 430bp to: Ralstonia sp. P-4CB2)	**1.2%**	3.2%
s	**Haliea sp. SAOS-164** (2 OTUs with 90% identity in 430bp to: Haliea sp. SAOS-164)	**1.2%**	1.2%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**1.1%**	1.2%
s	**Flavilitoribacter nigricans** (1 OTU with 87% identity in 426bp to: Flavilitoribacter nigricans)	**1.1%**	1.5%
s	**Desulfotalea psychrophila** (2 OTUs with 79-80% identity in 418bp to: Desulfotalea psychrophila)	**1.1%**	3.9%
f	**Chitinophagaceae** (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**1.1%**	1.5%
s	**Brevefilum fermentans** (1 OTU with 83% identity in 402bp to: Brevefilum fermentans)	**1.0%**	1.0%
s	**Sphaerobacter thermophilus** (3 OTUs with 88-90% identity in 407-408bp to: Sphaerobacter thermophilus)	**1.0%**	1.0%

s	**Thiobacillus sp. K6.2** (1 OTU with 98% identity in 429bp to: Thiobacillus sp. K6.2)	**0.9%**	0.5%
s	**Acidobacterium sp. WY65** (1 OTU with 96% identity in 404bp to: Acidobacterium sp. WY65)	**0.9%**	0.5%
f	**Comamonadaceae** (1 OTU with 79% identity in 430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Melaminivora alkalimesophila)	**0.9%**	0.9%
s	**Terrimonas sp. YJ03** (2 OTUs with 96% identity in 424bp to: Terrimonas sp. YJ03)	**0.8%**	1.1%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 429bp to: Sterolibacterium sp. TKU1)	**0.8%**	0.4%
f	**Anaerolineaceae** (2 OTUs with 85% identity in 407bp to: Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	**0.8%**	0.8%
s	**Candidatus Solibacter usitatus** (2 OTUs with 92-94% identity in 404-406bp to: Candidatus Solibacter usitatus)	**0.6%**	0.4%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.5%**	0.5%
g	**Comamonas** (1 OTU with 96% identity in 429bp to: 23 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**0.5%**	0.5%
s	**Wandonia haliotis** (1 OTU with 86% identity in 424bp to: Wandonia haliotis)	**0.5%**	0.6%
s	**Holophaga sp. WY42** (1 OTU with 91% identity in 431bp to: Holophaga sp. WY42)	**0.4%**	0.2%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 406bp to: Aciditerrimonas ferrireducens)	**0.4%**	0.4%
s	**Gemmatimonas aurantiaca** (1 OTU with 94% identity in 407bp to: Gemmatimonas aurantiaca)	**0.4%**	0.2%
s	**Parvibaculum sp.** (1 OTU with 93% identity in 404bp to: Parvibaculum sp.)	**0.4%**	0.4%
s	**Methanolinea mesophila** (1 OTU with 96% identity in 384bp to: Methanolinea mesophila)	**0.4%**	0.2%
f	**Caldilineaceae** (1 OTU with 88% identity in 405bp to: Caldilinea aerophila, Litorilinea aerophila)	**0.4%**	0.4%
s	**Hyphomicrobium sp. KC-IT-W2** (1 OTU with 99% identity in 404bp to: Hyphomicrobium sp. KC-IT-W2)	**0.4%**	0.3%
g	**Rheinheimera** (1 OTU with 100% identity in 429bp to: 2 unclassified Rheinheimera strains)	**0.4%**	0.3%
s	**Lentimicrobium saccharophilum** (2 OTUs with 88% identity in 422-424bp to: Lentimicrobium saccharophilum)	**0.4%**	0.5%
s	**Novosphingobium arabidopsis** (1 OTU with 99% identity in 404bp to: Novosphingobium arabidopsis)	**0.4%**	0.3%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**0.3%**	0.5%
s	**Candidatus Methanomassiliicoccus intestinalis** (1 OTU with 96% identity in 386bp to: Candidatus Methanomassiliicoccus intestinalis)	**0.3%**	0.2%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 429bp to: Thioalkalivibrio paradoxus)	**0.3%**	0.2%
s	**Glaucocystis sp. BBH** (1 OTU with 77% identity in 409bp to: Glaucocystis sp. BBH)	**0.3%**	0.2%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**0.3%**	0.3%
s	**Longimicrobium terrae** (1 OTU with 88% identity in 431bp to: Longimicrobium terrae)	**0.3%**	0.1%
s	**Chelativorans sp. A52C2** (1 OTU with 88% identity in 404bp to: Chelativorans sp. A52C2)	**0.3%**	0.2%
s	**Hyphomicrobium sp.** (1 OTU with 99% identity in 404bp to: Hyphomicrobium sp.)	**0.2%**	0.2%
s	**Longilinea sp.** (2 OTUs with 87-88% identity in 406bp to: Longilinea sp.)	**0.2%**	0.2%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.2%**	0.5%
s	**Methanothrix soehngenii** (1 OTU with 100% identity in 388bp to: Methanothrix soehngenii)	**0.2%**	0.2%
s	**Chitinophaga arvensicola** (1 OTU with 92% identity in 424bp to: Chitinophaga arvensicola)	**0.2%**	0.3%
g	**Thioprofundum** (1 OTU with 93% identity in 429bp to: Thioprofundum hispidum, Thioprofundum lithotrophicum)	**0.2%**	0.2%
g	**Flavobacterium** (1 OTU with 100% identity in 424bp to: 4 unclassified Flavobacterium strains)	**0.2%**	0.4%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**0.2%**	0.4%
s	**Caldilinea sp.** (1 OTU with 85% identity in 404bp to: Caldilinea sp.)	**0.2%**	0.2%
g	**Pseudomonas** (2 OTUs with 100% identity in 429bp to: 30 unclassified Pseudomonas strains, Pseudomonas anguiliseptica, Pseudomonas fluorescens, Pseudomonas oryzihabitans, Pseudomonas peli)	**0.2%**	0.5%
s	**Portibacter lacus** (1 OTU with 88% identity in 425bp to: Portibacter lacus)	**0.2%**	0.3%
s	**Iamia majanohamensis** (1 OTU with 87% identity in 402bp to: Iamia majanohamensis)	**0.2%**	0.2%

f	Micrococcaceae (1 OTU with 100% identity in 409bp to: 5 unclassified Arthrobacter strains, Arthrobacter globiformis, Arthrobacter humicola, Arthrobacter nitrophenolicus, Arthrobacter oryzae, Arthrobacter pascens, Pseudarthrobacter defluvii, Pseudarthrobacter enclensis, Pseudarthrobacter oxydans, Pseudarthrobacter phenanthrenivorans, Pseudarthrobacter polychromogenes, Pseudarthrobacter siccitolerans, Pseudarthrobacter sp.)	0.2%	0.2%
s	Acinetobacter radioresistens (1 OTU with 92% identity in 427bp to: Acinetobacter radioresistens)	0.2%	0.4%
g	Sulfurimonas (1 OTU with 99% identity in 404bp to: 3 unclassified Sulfurimonas strains)	0.1%	0.3%
s	Sulfurihydrogenibium rodmanii (1 OTU with 77% identity in 405bp to: Sulfurihydrogenibium rodmanii)	0.1%	0.1%
s	Pandoraea sp. (1 OTU with 94% identity in 429bp to: Pandoraea sp.)	0.1%	0.3%
s	Williamwhitmania taraxaci (1 OTU with 96% identity in 425bp to: Williamwhitmania taraxaci)	0.1%	0.2%
s	Membranicola marinus (1 OTU with 94% identity in 424bp to: Membranicola marinus)	0.1%	0.2%
s	Desulfobulbus sp. IS6 (1 OTU with 85% identity in 418bp to: Desulfobulbus sp. IS6)	0.1%	0.1%
s	Ilumatobacter fluminis (1 OTU with 96% identity in 405bp to: Ilumatobacter fluminis)	0.1%	0.1%
s	Rhodovastum atsumiense (1 OTU with 97% identity in 404bp to: Rhodovastum atsumiense)	0.1%	0.2%
	Other	0.1%	0.2%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.17: EBBR-Anammox microbial communities identified by NGS 3.**A3V4a**

4.AV3V4a (46 765 reads)

g	**Haliscomenobacter** (12 OTUs with 91-92% identity in 421-424bp to: 2 unclassified Haliscomenobacter strains)	**12.4%**	14.8%
s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 429bp to: Denitratisoma oestradiolicum)	**10.1%**	4.6%
s	**Tepidiforma bonchosmolovskayae** (10 OTUs with 92-95% identity in 406-407bp to: Tepidiforma bonchosmolovskayae)	**10.0%**	9.6%
s	**Thermomarinilinea lacunifontana** (5 OTUs with 84-88% identity in 397-402bp to: Thermomarinilinea lacunifontana)	**8.2%**	7.5%
f	**Chitinophagaceae** (8 OTUs with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**8.0%**	9.6%
g	**Comamonas** (9 OTUs with 96-100% identity in 429bp to: 33 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**5.4%**	4.9%
s	**Litorilinea aerophila** (9 OTUs with 90-93% identity in 405-406bp to: Litorilinea aerophila)	**5.1%**	4.9%
s	**Thermomarinilinea sp.** (3 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**4.7%**	4.3%
s	**Ignavibacterium album** (2 OTUs with 99-100% identity in 424bp to: Ignavibacterium album)	**3.9%**	4.3%
s	**Nitrosomonas sp.** (2 OTUs with 97% identity in 429-430bp to: Nitrosomonas sp.)	**2.8%**	1.3%
s	**Brevefilum fermentans** (1 OTU with 83% identity in 402bp to: Brevefilum fermentans)	**2.7%**	2.4%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**2.4%**	1.1%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**2.2%**	1.9%
s	**Desulfotalea psychrophila** (4 OTUs with 79-80% identity in 418bp to: Desulfotalea psychrophila)	**1.8%**	5.6%
s	**Terrimonas sp. YJ03** (4 OTUs with 95-96% identity in 424bp to: Terrimonas sp. YJ03)	**1.7%**	2.0%
s	**Sphaerobacter thermophilus** (4 OTUs with 90-91% identity in 406-407bp to: Sphaerobacter thermophilus)	**1.6%**	1.5%
g	**Lacibacter** (1 OTU with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**1.4%**	1.7%
s	**Paludibaculum sp.** (2 OTUs with 93% identity in 404bp to: Paludibaculum sp.)	**1.2%**	0.6%
f	**Anaerolineaceae** (3 OTUs with 83-85% identity in 402-407bp to: Anaerolinea thermolimosa, Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	**1.2%**	1.1%
s	**Ferruginibacter sp.** (2 OTUs with 92-94% identity in 424bp to: Ferruginibacter sp.)	**1.2%**	1.4%
s	**Thermanaerothrix daxensis** (4 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**1.1%**	1.0%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**1.0%**	1.4%
s	**Ralstonia sp. P-4CB2** (3 OTUs with 96-97% identity in 430bp to: Ralstonia sp. P-4CB2)	**1.0%**	2.4%
s	**Caldilinea aerophila** (2 OTUs with 88-90% identity in 405bp to: Caldilinea aerophila)	**0.9%**	0.9%

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s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.9%**	0.8%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.6%**	1.0%
s	**Steroidobacter sp.** (2 OTUs with 94% identity in 429bp to: Steroidobacter sp.)	**0.6%**	0.6%
s	**Wandonia haliotis** (1 OTU with 86% identity in 424bp to: Wandonia haliotis)	**0.5%**	0.6%
s	**Truepera sp.** (2 OTUs with 96% identity in 402bp to: Truepera sp.)	**0.5%**	0.5%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**0.4%**	0.7%
g	**Nitrosomonas** (1 OTU with 100% identity in 429bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**0.4%**	0.2%
s	**Nitrobacter sp. LAMK1242** (1 OTU with 99% identity in 404bp to: Nitrobacter sp. LAMK1242)	**0.4%**	0.3%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.4%**	0.9%
s	**Thermomonas fusca** (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	**0.3%**	0.4%
o	**Burkholderiales** (2 OTUs with 79-96% identity in 429-432bp to: 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Ideonella sp., Paucibacter sp., Rubrivivax sp. JA309)	**0.3%**	0.5%
s	**Stenotrophobacter terrae** (1 OTU with 96% identity in 404bp to: Stenotrophobacter terrae)	**0.3%**	0.1%
s	**Candidatus Saccharimonas aalborgensis** (1 OTU with 85% identity in 397bp to: Candidatus Saccharimonas aalborgensis)	**0.3%**	0.3%
p	**Bacteroidetes** (1 OTU with 79% identity in 426bp to: Arachidiccoccus sp., Eisenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava)	**0.2%**	0.3%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 406bp to: Aciditerrimonas ferrireducens)	**0.2%**	0.2%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**0.2%**	0.2%
s	**Denitratimonas tolerans** (1 OTU with 100% identity in 429bp to: Denitratimonas tolerans)	**0.2%**	0.3%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.2%**	0.1%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 404bp to: 2 unclassified Rhodopseudomonas strains, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	**0.2%**	0.1%
p	**Proteobacteria** (1 OTU with 100% identity in 404bp to: 2 unclassified Mesorhizobium strains, 3 unclassified Aquamicrobium strains, Aquamicrobium terrae, Thiobacillus sp. BHF001)	**0.2%**	0.2%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	**0.2%**	0.2%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**0.2%**	0.1%
s	**Aquamicrobium aestuarii** (1 OTU with 99% identity in 404bp to: Aquamicrobium aestuarii)	**0.1%**	0.1%
s	**Geobacter sp. CLFeRB** (1 OTU with 86% identity in 430bp to: Geobacter sp. CLFeRB)	**0.1%**	0.2%
s	**Lewinella cohaerens** (1 OTU with 88% identity in 426bp to: Lewinella cohaerens)	**0.1%**	0.1%
s	**Chitinophaga pinensis** (1 OTU with 92% identity in 422bp to: Chitinophaga pinensis)	**0.1%**	0.1%
	Other	**0.1%**	0.2%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.18: EBBR-Anammox microbial communities identified by NGS **4.A3V4a**

5.AV3V4a (46 437 reads)

s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 429bp to: Denitratisoma oestradiolicum)	**13.8%**	6.3%
g	**Haliscomenobacter** (14 OTUs with 91-92% identity in 421-424bp to: 2 unclassified Haliscomenobacter strains)	**12.9%**	15.5%
f	**Chitinophagaceae** (8 OTUs with 79% identity in 424-426bp to: Arachidicoccus sp., Haoranjiania flava)	**11.4%**	13.7%
s	**Thermomarinilinea lacunifontana** (5 OTUs with 84-88% identity in 396-402bp to: Thermomarinilinea lacunifontana)	**7.1%**	6.5%
s	**Tepidiforma bonchosmolovskayae** (7 OTUs with 95% identity in 406bp to: Tepidiforma bonchosmolovskayae)	**7.0%**	6.8%
g	**Comamonas** (6 OTUs with 96-100% identity in 429bp to: 29 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**5.3%**	4.8%
s	**Ignavibacterium album** (4 OTUs with 99-100% identity in 424bp to: Ignavibacterium album)	**4.1%**	4.5%
s	**Brevefilum fermentans** (1 OTU with 83% identity in 402bp to: Brevefilum fermentans)	**3.9%**	3.6%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**3.7%**	1.7%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**3.1%**	2.8%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**2.6%**	2.3%
s	**Terrimonas sp. YJ03** (4 OTUs with 95-96% identity in 424bp to: Terrimonas sp. YJ03)	**2.4%**	2.9%
g	**Lacibacter** (1 OTU with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**2.1%**	2.5%
s	**Nitrosomonas sp.** (1 OTU with 97% identity in 429bp to: Nitrosomonas sp.)	**2.0%**	0.9%
s	**Paludibaculum sp.** (2 OTUs with 93% identity in 404bp to: Paludibaculum sp.)	**1.9%**	1.0%
s	**Desulfotalea psychrophila** (4 OTUs with 79-80% identity in 418bp to: Desulfotalea psychrophila)	**1.8%**	5.7%
s	**Litorilinea aerophila** (5 OTUs with 90-93% identity in 405-406bp to: Litorilinea aerophila)	**1.7%**	1.6%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**1.2%**	1.6%
s	**Ralstonia sp. P-4CB2** (3 OTUs with 96-97% identity in 430bp to: Ralstonia sp. P-4CB2)	**1.1%**	2.8%
f	**Anaerolineaceae** (3 OTUs with 83-85% identity in 402-407bp to: Anaerolinea thermolimosa, Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	**1.0%**	1.0%
s	**Ferruginibacter sp.** (2 OTUs with 92-94% identity in 424bp to: Ferruginibacter sp.)	**0.9%**	1.0%
s	**Wandonia haliotis** (1 OTU with 86% identity in 424bp to: Wandonia haliotis)	**0.7%**	0.8%
s	**Thermanaerothrix daxensis** (3 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**0.7%**	0.6%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**0.6%**	0.9%
s	**Truepera sp.** (2 OTUs with 96% identity in 402bp to: Truepera sp.)	**0.6%**	0.7%
s	**Caldilinea aerophila** (2 OTUs with 88-90% identity in 405bp to: Caldilinea aerophila)	**0.6%**	0.6%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.6%**	1.0%
s	**Steroidobacter sp.** (3 OTUs with 93-94% identity in 429bp to: Steroidobacter sp.)	**0.6%**	0.5%
g	**Nitrosomonas** (1 OTU with 100% identity in 429bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**0.5%**	0.2%
s	**Sphaerobacter thermophilus** (2 OTUs with 90% identity in 407bp to: Sphaerobacter thermophilus)	**0.4%**	0.4%
s	**Thermomonas fusca** (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	**0.4%**	0.4%
o	**Burkholderiales** (2 OTUs with 79-96% identity in 429-432bp to: 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanen-sis, Hylemella sp., Pauljensenia sp., Rubrivivax sp. JA209)	**0.4%**	0.5%

s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	**0.3%**	0.4%
s	**Denitratimonas tolerans** (2 OTUs with 97-100% identity in 429bp to: Denitratimonas tolerans)	**0.3%**	0.4%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.3%**	0.1%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.3%**	0.2%
p	**Bacteroidetes** (1 OTU with 79% identity in 426bp to: Arachidiccoccus sp., Eisenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava)	**0.2%**	0.3%
s	**Geobacter sp. CLFeRB** (1 OTU with 86% identity in 430bp to: Geobacter sp. CLFeRB)	**0.2%**	0.2%
s	**Thermodesulforhabdus n. sp. M40/2 CIV-3.2** (1 OTU with 82% identity in 421bp to: Thermodesulforhabdus n. sp. M40/2 CIV-3.2)	**0.2%**	0.2%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**0.2%**	0.2%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.2%**	0.4%
s	**Lewinella cohaerens** (1 OTU with 88% identity in 426bp to: Lewinella cohaerens)	**0.2%**	0.2%
s	**Candidatus Saccharimonas aalborgensis** (1 OTU with 85% identity in 397bp to: Candidatus Saccharimonas aalborgensis)	**0.2%**	0.2%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**0.2%**	0.1%
s	**Acidobacterium sp. WY65** (1 OTU with 96% identity in 404bp to: Acidobacterium sp. WY65)	**0.1%**	0.1%
s	**Chitinophaga pinensis** (1 OTU with 92% identity in 422bp to: Chitinophaga pinensis)	**0.1%**	0.1%
g	**Geobacter** (1 OTU with 89% identity in 430bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	**0.1%**	0.1%
	Other	**0.2%**	0.6%
	Unclassified (0 reads)		
	Filtered (0 reads)		

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s	**Denitratisoma oestradiolicum** (4 OTUs with 97-98% identity in 429bp to: Denitratisoma oestradiolicum)	**10.9%**	4.9%
f	**Chitinophagaceae** (7 OTUs with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**10.6%**	12.6%
s	**Brevefilum fermentans** (5 OTUs with 83% identity in 402bp to: Brevefilum fermentans)	**7.0%**	6.3%
s	**Ignavibacterium album** (5 OTUs with 99-100% identity in 424bp to: Ignavibacterium album)	**5.7%**	6.2%
g	**Haliscomenobacter** (7 OTUs with 91% identity in 424bp to: 2 unclassified Haliscomenobacter strains)	**5.7%**	6.7%
s	**Thermomarinilinea lacunifontana** (4 OTUs with 84-88% identity in 397-402bp to: Thermomarinilinea lacunifontana)	**4.7%**	4.3%
s	**Tepidiforma bonchosmolovskayae** (7 OTUs with 93-95% identity in 406-407bp to: Tepidiforma bonchosmolovskayae)	**4.0%**	3.8%
s	**Paludibaculum sp.** (2 OTUs with 93% identity in 404bp to: Paludibaculum sp.)	**3.8%**	2.0%
g	**Lacibacter** (1 OTU with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**3.8%**	4.5%
s	**Litorilinea aerophila** (11 OTUs with 88-93% identity in 405-406bp to: Litorilinea aerophila)	**3.7%**	3.5%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**3.5%**	3.1%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**3.4%**	3.0%
s	**Thermanaerothrix daxensis** (4 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**3.0%**	2.7%
f	**Anaerolineaceae** (4 OTUs with 83-86% identity in 402-407bp to: Anaerolinea thermolimosa, Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	**2.6%**	2.4%
g	**Nitrosomonas** (2 OTUs with 99-100% identity in 429bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**2.4%**	1.1%
s	**Steroidobacter sp.** (3 OTUs with 93-94% identity in 429bp to: Steroidobacter sp.)	**2.1%**	2.0%
g	**Comamonas** (3 OTUs with 96-99% identity in 429bp to: 29 unclassified Comamonas strains, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati)	**2.1%**	1.9%
s	**Desulfotalea psychrophila** (8 OTUs with 79-80% identity in 418bp to: Desulfotalea psychrophila)	**2.0%**	6.4%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**1.8%**	0.8%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**1.6%**	2.2%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97% identity in 430bp to: Ralstonia sp. P-4CB2)	**1.4%**	3.3%
s	**Caldilinea aerophila** (2 OTUs with 88-90% identity in 405bp to: Caldilinea aerophila)	**1.2%**	1.1%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	**1.2%**	1.3%
s	**Lewinella cohaerens** (1 OTU with 88% identity in 426bp to: Lewinella cohaerens)	**0.8%**	0.9%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.7%**	1.8%
s	**Terrimonas sp. YJ03** (1 OTU with 96% identity in 424bp to: Terrimonas sp. YJ03)	**0.7%**	0.8%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**0.7%**	1.0%
s	**Sphaerobacter thermophilus** (2 OTUs with 90-91% identity in 406-407bp to: Sphaerobacter thermophilus)	**0.6%**	0.5%
s	**Nitrosomonas sp.** (1 OTU with 97% identity in 429bp to: Nitrosomonas sp.)	**0.6%**	0.3%
s	**Wandonia haliotis** (1 OTU with 86% identity in 424bp to: Wandonia haliotis)	**0.5%**	0.6%
s	**Denitratimonas tolerans** (1 OTU with 100% identity in 429bp to: Denitratimonas tolerans)	**0.5%**	0.6%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**0.4%**	0.4%
s	**Longilinea sp.** (2 OTUs with 87-88% identity in 406bp to: Longilinea sp.)	**0.4%**	0.4%
s	**Truepera sp.** (2 OTUs with 96% identity in 402bp to: Truepera sp.)	**0.4%**	0.4%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**0.4%**	0.3%
g	**Desulfonatronum** (1 OTU with 90% identity in 428bp to: Desulfonatronum paranguaonense, Desulfonatronum thiosulfatophilum)	**0.4%**	0.3%
s	**Thermomonas fusca** (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	**0.3%**	0.4%
g	**Nitrosospira** (1 OTU with 98% identity in 429bp to: 3 unclassified Nitrosospira strains)	**0.3%**	0.1%
s	**Thermomonas brevis** (1 OTU with 99% identity in 421bp to: Thermomonas brevis)	**0.3%**	0.4%
p	**Bacteroidetes** (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Eisenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava)	**0.3%**	0.3%

Filtered (0 reads)

6.AV3V4a (28 990 reads)

s	Vicinamibacter silvestris (1 OTU with 91% identity in 430bp to: Vicinamibacter silvestris)	0.2%	0.1%
o	Burkholderiales (1 OTU with 79% identity in 432bp to: 4 unclassified Roseateles strains, Paucibacter sp.)	0.2%	0.3%
f	Caldilineaceae (1 OTU with 88% identity in 405bp to: Caldilinea aerophila, Litorilinea aerophila)	0.2%	0.2%
s	Holophaga sp. WY42 (1 OTU with 91% identity in 431bp to: Holophaga sp. WY42)	0.2%	0.1%
s	Aciditerrimonas ferrireducens (1 OTU with 93% identity in 406bp to: Aciditerrimonas ferrireducens)	0.2%	0.2%
s	Thermomicrobium roseum (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	0.2%	0.2%
s	Lentimicrobium saccharophilum (1 OTU with 88% identity in 424bp to: Lentimicrobium saccharophilum)	0.2%	0.3%
g	Pseudomonas (1 OTU with 100% identity in 429bp to: Pseudomonas caeni, Pseudomonas sp.)	0.2%	0.4%
s	Acidobacterium sp. WY65 (1 OTU with 96% identity in 404bp to: Acidobacterium sp. WY65)	0.2%	0.1%
s	Parvibaculum sp. (1 OTU with 93% identity in 404bp to: Parvibaculum sp.)	0.2%	0.2%
s	Flavilitoribacter nigricans (1 OTU with 87% identity in 426bp to: Flavilitoribacter nigricans)	0.2%	0.2%
s	Candidatus Solibacter usitatus (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	0.2%	0.1%
s	Pseudomonas sp. ARMY (1 OTU with 97% identity in 429bp to: Pseudomonas sp. ARMY)	0.2%	0.3%
s	Racemicystis persica (1 OTU with 92% identity in 428bp to: Racemicystis persica)	0.2%	0.3%
s	Limisphaera ngatamarikiensis (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	0.1%	0.2%
s	Dehalococcoides sp. BHI80-15 (1 OTU with 82% identity in 406bp to: Dehalococcoides sp. BHI80-15)	0.1%	0.1%
s	Herbaspirillum sp. AKB-2008-TE24 (1 OTU with 97% identity in 429bp to: Herbaspirillum sp. AKB-2008-TE24)	0.1%	0.2%
s	Membranicola marinus (1 OTU with 94% identity in 424bp to: Membranicola marinus)	0.1%	0.1%
s	Iamia majanohamensis (1 OTU with 87% identity in 402bp to: Iamia majanohamensis)	0.1%	0.1%
g	Flavobacterium (1 OTU with 100% identity in 424bp to: 4 unclassified Flavobacterium strains)	0.1%	0.2%
g	Geobacter (1 OTU with 89% identity in 430bp to: 4 unclassified Geobacter strains, Geobacter sulfurreducens)	0.1%	0.1%
s	Methanothrix soehngenii (1 OTU with 100% identity in 388bp to: Methanothrix soehngenii)	0.1%	0.1%
	Other	0.3%	0.5%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.19: EBBR-Anammox microbial communities identified by NGS **6.A3V4a**

8.AV3V4a (37 621 reads)

g	Haliscomenobacter (89 OTUs with 91-92% identity in 280-424bp to: 2 unclassified Haliscomenobacter strains)	41.1%	46.7%
s	Denitratisoma oestradiolicum (4 OTUs with 96-98% identity in 429bp to: Denitratisoma oestradiolicum)	6.9%	3.0%
s	Tepidiforma bonchosmolovskayae (5 OTUs with 95% identity in 406bp to: Tepidiforma bonchosmolovskayae)	5.2%	4.8%
s	Thermomarinilinea lacunifontana (2 OTUs with 84-88% identity in 397-402bp to: Thermomarinilinea lacunifontana)	4.7%	4.1%
s	Brevefilum fermentans (3 OTUs with 83% identity in 402bp to: Brevefilum fermentans)	4.7%	4.0%
s	Ferruginibacter sp. (3 OTUs with 92-94% identity in 424bp to: Ferruginibacter sp.)	3.6%	4.1%
g	Comamonas (4 OTUs with 96-99% identity in 429bp to: 32 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	3.0%	2.6%
s	Thermomarinilinea sp. (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	2.9%	2.5%
f	Anaerolineaceae (3 OTUs with 83-85% identity in 402-407bp to: Anaerolinea thermolimosa, Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	2.1%	1.8%
s	Litorilinea aerophila (5 OTUs with 90-93% identity in 405-406bp to: Litorilinea aerophila)	2.1%	1.9%
s	Terrimonas sp. YJ03 (3 OTUs with 96-98% identity in 424bp to: Terrimonas sp. YJ03)	1.9%	2.2%
g	Ferruginibacter (1 OTU with 95% identity in 424bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	1.7%	1.9%
s	Thermomonas fusca (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	1.6%	1.8%
s	Sediminibacterium sp. (1 OTU with 95% identity in 424bp to: Sediminibacterium sp.)	1.2%	1.3%

f	**Comamonadaceae** (2 OTUs with 79-96% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 2 unclassified Ramlibacter strains, 24 unclassified Comamonas strains, 8 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Curvibacter sp. PL21, Melaminivora alkalimesophila, Tibeticola sp.)	**1.1%**	1.0%
s	**Ignavibacterium album** (1 OTU with 100% identity in 424bp to: Ignavibacterium album)	**1.1%**	1.1%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**1.1%**	0.5%
s	**Gemmatimonas aurantiaca** (1 OTU with 95% identity in 423bp to: Gemmatimonas aurantiaca)	**1.0%**	0.4%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**1.0%**	0.8%
f	**Chitinophagaceae** (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**0.8%**	0.9%
s	**Sphaerobacter thermophilus** (2 OTUs with 90% identity in 407bp to: Sphaerobacter thermophilus)	**0.8%**	0.7%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.7%**	0.4%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97% identity in 430bp to: Ralstonia sp. P-4CB2)	**0.7%**	1.7%
s	**Parasediminibacterium paludis** (1 OTU with 94% identity in 424bp to: Parasediminibacterium paludis)	**0.6%**	0.7%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.6%**	1.0%
s	**Syntrophorhabdus sp. TB** (1 OTU with 79% identity in 422bp to: Syntrophorhabdus sp. TB)	**0.6%**	0.3%
s	**Thermanaerothrix daxensis** (2 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**0.6%**	0.5%
s	**Caldilinea aerophila** (2 OTUs with 88-90% identity in 405bp to: Caldilinea aerophila)	**0.5%**	0.4%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 422bp to: Gemmatimonas sp. URHD0086)	**0.5%**	0.2%
s	**Cephaloticoccus capnophilus** (1 OTU with 96% identity in 428bp to: Cephaloticoccus capnophilus)	**0.4%**	0.2%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.4%**	0.4%
s	**Chitinophaga pinensis** (1 OTU with 92% identity in 422bp to: Chitinophaga pinensis)	**0.4%**	0.4%
o	**Burkholderiales** (2 OTUs with 79-96% identity in 429-432bp to: 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Ideonella sp., Paucibacter sp., Rubrivivax sp. JA309)	**0.4%**	0.5%
s	**Desulfotalea psychrophila** (1 OTU with 80% identity in 418bp to: Desulfotalea psychrophila)	**0.4%**	1.1%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 425bp to: Rhodohalobacter sp. SW132)	**0.3%**	0.4%
s	**Ferruginibacter alkalilentus** (1 OTU with 95% identity in 424bp to: Ferruginibacter alkalilentus)	**0.3%**	0.4%
s	**Chryseotalea sanaruensis** (1 OTU with 91% identity in 415bp to: Chryseotalea sanaruensis)	**0.3%**	0.4%
s	**Truepera sp.** (2 OTUs with 96% identity in 402bp to: Truepera sp.)	**0.3%**	0.3%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**0.3%**	0.4%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 421bp to: Gemmatimonas phototrophica)	**0.3%**	0.1%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 429bp to: Thioprofundum hispidum)	**0.3%**	0.2%
s	**Denitratimonas tolerans** (2 OTUs with 99-100% identity in 429bp to: Denitratimonas tolerans)	**0.2%**	0.3%
s	**Wandonia haliotis** (1 OTU with 86% identity in 424bp to: Wandonia haliotis)	**0.2%**	0.2%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	**0.2%**	0.2%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 429bp to: Aquincola sp. HME6814)	**0.2%**	0.3%
s	**Dehalococcoides sp. BHI80-52** (1 OTU with 82% identity in 406bp to: Dehalococcoides sp. BHI80-52)	**0.2%**	0.2%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**0.2%**	0.2%
s	**Diaphorobacter sp.** (1 OTU with 98% identity in 429bp to: Diaphorobacter sp.)	**0.2%**	0.2%
s	**Chryseolinea soli** (1 OTU with 91% identity in 416bp to: Chryseolinea soli)	**0.2%**	0.2%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**0.1%**	0.2%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.1%**	0.3%
f	**Myxococcaceae** (1 OTU with 93% identity in 428bp to: Corallococcus macrosporus, Pyxidicoccus fallax)	**0.1%**	0.1%
	Other	**0.0%**	0.0%

SI 5.20: EBBR-Anammox microbial communities identified by NGS **8.A3V4a**

Filtered (0 reads)

9.AV3V4a (55 194 reads)

s	**Denitratisoma oestradiolicum** (11 OTUs with 95-98% identity in 429bp to: Denitratisoma oestradiolicum)	**21.0%**	10.5%
s	**Brevefilum fermentans** (10 OTUs with 83% identity in 402bp to: Brevefilum fermentans)	**11.4%**	11.4%
s	**Thermomarinilinea lacunifontana** (5 OTUs with 84-88% identity in 396-402bp to: Thermomarinilinea lacunifontana)	**5.2%**	5.2%
s	**Litorilinea aerophila** (13 OTUs with 88-93% identity in 405-406bp to: Litorilinea aerophila)	**4.9%**	5.2%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**4.2%**	4.2%
s	**Ignavibacterium album** (3 OTUs with 99-100% identity in 424bp to: Ignavibacterium album)	**4.1%**	4.9%
f	**Anaerolineaceae** (3 OTUs with 83-85% identity in 402-407bp to: Anaerolinea thermolimosa, Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	**4.1%**	4.1%
s	**Chryseotalea sanaruensis** (3 OTUs with 91% identity in 414-415bp to: Chryseotalea sanaruensis)	**3.8%**	5.0%
g	**Haliscomenobacter** (3 OTUs with 91% identity in 424bp to: 2 unclassified Haliscomenobacter strains)	**3.6%**	4.7%
s	**Thermanaerothrix daxensis** (4 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**3.3%**	3.3%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**3.1%**	3.1%
s	**Gemmatimonas phototrophica** (2 OTUs with 92% identity in 421bp to: Gemmatimonas phototrophica)	**2.2%**	1.1%
s	**Ferruginibacter sp.** (2 OTUs with 92-94% identity in 424bp to: Ferruginibacter sp.)	**2.1%**	2.7%
s	**Gemmatimonas aurantiaca** (2 OTUs with 95% identity in 423bp to: Gemmatimonas aurantiaca)	**1.8%**	0.9%
s	**Tepidiforma bonchosmolovskayae** (5 OTUs with 95% identity in 406bp to: Tepidiforma bonchosmolovskayae)	**1.6%**	1.7%
f	**Chitinophagaceae** (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**1.5%**	1.9%
s	**Terrimonas sp. YJ03** (3 OTUs with 96-98% identity in 424bp to: Terrimonas sp. YJ03)	**1.5%**	1.9%
g	**Comamonas** (3 OTUs with 96-99% identity in 429bp to: 30 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**1.4%**	1.4%
s	**Sphaerobacter thermophilus** (4 OTUs with 90-91% identity in 406-407bp to: Sphaerobacter thermophilus)	**1.3%**	1.3%
s	**Denitratimonas tolerans** (3 OTUs with 97-100% identity in 429bp to: Denitratimonas tolerans)	**1.3%**	1.8%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97% identity in 430bp to: Ralstonia sp. P-4CB2)	**1.3%**	3.4%
s	**Thermomonas fusca** (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	**1.2%**	1.6%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**1.0%**	1.0%
s	**Steroidobacter sp.** (3 OTUs with 93-94% identity in 429bp to: Steroidobacter sp.)	**1.0%**	1.0%
s	**Desulfotalea psychrophila** (1 OTU with 80% identity in 418bp to: Desulfotalea psychrophila)	**1.0%**	3.4%
s	**Paludibaculum sp.** (2 OTUs with 93% identity in 404bp to: Paludibaculum sp.)	**0.8%**	0.5%
s	**Syntrophorhabdus sp. TB** (1 OTU with 79% identity in 422bp to: Syntrophorhabdus sp. TB)	**0.7%**	0.4%
s	**Caldilinea aerophila** (1 OTU with 88% identity in 405bp to: Caldilinea aerophila)	**0.7%**	0.7%
s	**Sediminibacterium sp.** (1 OTU with 95% identity in 424bp to: Sediminibacterium sp.)	**0.7%**	0.9%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.6%**	1.8%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**0.6%**	0.9%
g	**Lacibacter** (1 OTU with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**0.5%**	0.7%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.5%**	0.5%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.5%**	0.9%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 425bp to: Rhodohalobacter sp. SW132)	**0.4%**	0.5%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.4%**	0.2%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 429bp to: Thioprofundum hispidum)	**0.4%**	0.3%

s	Gemmatimonas sp. URHD0086 (1 OTU with 91% identity in 422bp to: Gemmatimonas sp. URHD0086)	0.3%	0.2%
s	Woodsholea maritima (1 OTU with 95% identity in 404bp to: Woodsholea maritima)	0.3%	0.2%
s	Fimbriimonas ginsengisoli (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	0.3%	0.3%
s	Chitinophaga pinensis (1 OTU with 92% identity in 422bp to: Chitinophaga pinensis)	0.2%	0.3%
o	Myxococcales (1 OTU with 92% identity in 428bp to: Phaselicystis flava, Racemicystis crocea, Racemicystis persica)	0.2%	0.3%
s	Haematococcus lacustris (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	0.2%	0.1%
c	Gammaproteobacteria (1 OTU with 90% identity in 429bp to: Acidibacter ferrireducens, Coxiella endosymbiont of Ornithodoros marocanus, Steroidobacter sp., Thioprofundum hispidum)	0.2%	0.2%
s	Bellilinea caldifistulae (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	0.2%	0.2%
s	Saccharicrinis carchari (1 OTU with 96% identity in 424bp to: Saccharicrinis carchari)	0.2%	0.3%
s	Dokdonella ginsengisoli (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	0.2%	0.3%
o	Burkholderiales (1 OTU with 79% identity in 432bp to: 4 unclassified Roseateles strains, Paucibacter sp.)	0.2%	0.3%
s	Chryseolinea soli (1 OTU with 91% identity in 416bp to: Chryseolinea soli)	0.2%	0.2%
s	Nitrosomonas sp. (1 OTU with 97% identity in 429bp to: Nitrosomonas sp.)	0.2%	0.1%
s	Parasediminibacterium paludis (1 OTU with 94% identity in 424bp to: Parasediminibacterium paludis)	0.2%	0.2%
s	Methanothrix soehngenii (1 OTU with 100% identity in 388bp to: Methanothrix soehngenii)	0.2%	0.1%
s	Aquincola sp. HME6814 (1 OTU with 97% identity in 429bp to: Aquincola sp. HME6814)	0.2%	0.3%
g	Desulfonatronum (1 OTU with 90% identity in 428bp to: Desulfonatronum paranguaonense, Desulfonatronum thiosulfatophilum)	0.2%	0.2%
s	Daejeonella rubra (1 OTU with 86% identity in 416bp to: Daejeonella rubra)	0.1%	0.2%
f	Myxococcaceae (1 OTU with 93% identity in 428bp to: Corallococcus macrosporus, Pyxidicoccus fallax)	0.1%	0.2%
f	Caldilineaceae (1 OTU with 88% identity in 405bp to: Caldilinea aerophila, Litorilinea aerophila)	0.1%	0.1%
s	Cytophaga hutchinsonii (1 OTU with 81% identity in 416bp to: Cytophaga hutchinsonii)	0.1%	0.1%
	Other	0.3%	0.4%
	Unclassified (0 reads)		
	Filtered (0 reads)		

Table 6: Condensed overview of the taxonomic composition of samples.

SI 5.21: EBBR-Anammox microbial communities identified by NGS **9.A3V4a**

Filtered (0 reads)

10.AV3V4a (45 435 reads)

g	**Haliscomenobacter** (60 OTUs with 91-92% identity in 421-424bp to: 2 unclassified Haliscomenobacter strains)	**25.7%**	30.9%
s	**Denitratisoma oestradiolicum** (5 OTUs with 96-98% identity in 429bp to: Denitratisoma oestradiolicum)	**10.1%**	4.6%
s	**Thermomarinilinea lacunifontana** (2 OTUs with 84-88% identity in 397-402bp to: Thermomarinilinea lacunifontana)	**6.5%**	5.9%
s	**Brevefilum fermentans** (5 OTUs with 83% identity in 402bp to: Brevefilum fermentans)	**5.9%**	5.4%
s	**Tepidiforma bonchosmolovskayae** (6 OTUs with 95% identity in 406bp to: Tepidiforma bonchosmolovskayae)	**4.9%**	4.7%
s	**Thermomarinilinea sp.** (3 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**4.6%**	4.2%
s	**Litorilinea aerophila** (11 OTUs with 88-93% identity in 405-406bp to: Litorilinea aerophila)	**4.1%**	4.0%
s	**Ferruginibacter sp.** (2 OTUs with 92-94% identity in 424bp to: Ferruginibacter sp.)	**3.7%**	4.5%
f	**Anaerolineaceae** (4 OTUs with 83-85% identity in 402-407bp to: Anaerolinea thermolimosa, Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	**3.4%**	3.2%
g	**Comamonas** (4 OTUs with 96-99% identity in 429bp to: 32 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**3.0%**	2.8%

s	**Thermomonas fusca** (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	**1.9%**	2.3%
s	**Thermanaerothrix daxensis** (5 OTUs with 85-86% identity in 406-407bp to: Thermanaerothrix daxensis)	**1.9%**	1.7%
s	**Terrimonas sp. YJ03** (3 OTUs with 96-98% identity in 424bp to: Terrimonas sp. YJ03)	**1.7%**	2.0%
s	**Ignavibacterium album** (1 OTU with 100% identity in 424bp to: Ignavibacterium album)	**1.5%**	1.7%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**1.3%**	1.2%
s	**Gemmatimonas aurantiaca** (1 OTU with 95% identity in 423bp to: Gemmatimonas aurantiaca)	**1.2%**	0.5%
s	**Syntrophorhabdus sp. TB** (1 OTU with 79% identity in 422bp to: Syntrophorhabdus sp. TB)	**1.2%**	0.5%
f	**Comamonadaceae** (2 OTUs with 79-96% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 2 unclassified Ramlibacter strains, 24 unclassified Comamonas strains, 8 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Curvibacter sp. PL21, Melaminivora alkalimesophila, Tibeticola sp.)	**1.1%**	1.0%
s	**Sphaerobacter thermophilus** (4 OTUs with 90-91% identity in 406-407bp to: Sphaerobacter thermophilus)	**1.1%**	1.0%
g	**Ferruginibacter** (1 OTU with 95% identity in 424bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	**1.0%**	1.2%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.9%**	0.8%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97% identity in 430bp to: Ralstonia sp. P-4CB2)	**0.9%**	2.1%
s	**Sediminibacterium sp.** (1 OTU with 95% identity in 424bp to: Sediminibacterium sp.)	**0.8%**	0.9%
f	**Chitinophagaceae** (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**0.8%**	0.9%
s	**Parasediminibacterium paludis** (1 OTU with 94% identity in 424bp to: Parasediminibacterium paludis)	**0.7%**	0.8%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**0.7%**	0.3%
s	**Chryseotalea sanaruensis** (1 OTU with 91% identity in 415bp to: Chryseotalea sanaruensis)	**0.6%**	0.8%
s	**Caldilinea aerophila** (2 OTUs with 88-90% identity in 405bp to: Caldilinea aerophila)	**0.6%**	0.6%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.5%**	0.9%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 421bp to: Gemmatimonas phototrophica)	**0.5%**	0.2%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 422bp to: Gemmatimonas sp. URHD0086)	**0.5%**	0.2%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.5%**	0.3%
s	**Paludibaculum sp.** (1 OTU with 93% identity in 404bp to: Paludibaculum sp.)	**0.5%**	0.2%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 425bp to: Rhodohalobacter sp. SW132)	**0.4%**	0.5%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**0.4%**	0.6%
o	**Burkholderiales** (2 OTUs with 79-96% identity in 429-432bp to: 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Ideonella sp., Paucibacter sp., Rubrivivax sp. JA309)	**0.4%**	0.6%

s	Truepera sp. (2 OTUs with 96% identity in 402bp to: Truepera sp.)	0.4%	0.4%
s	Desulfotalea psychrophila (1 OTU with 80% identity in 418bp to: Desulfotalea psychrophila)	0.4%	1.1%
s	Cephaloticoccus capnophilus (1 OTU with 96% identity in 428bp to: Cephaloticoccus capnophilus)	0.3%	0.2%
s	Nitrosomonas sp. (1 OTU with 97% identity in 429bp to: Nitrosomonas sp.)	0.3%	0.1%
s	Denitratimonas tolerans (2 OTUs with 99-100% identity in 429bp to: Denitratimonas tolerans)	0.3%	0.4%
s	Bellilinea caldifistulae (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	0.3%	0.3%
s	Chitinophaga pinensis (1 OTU with 92% identity in 422bp to: Chitinophaga pinensis)	0.2%	0.3%
s	Vibrio sp. Gp-3-5.1 (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	0.2%	0.6%
s	Fimbriimonas ginsengisoli (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	0.2%	0.3%
s	Opitutus sp. VeSm13 (1 OTU with 97% identity in 428bp to: Opitutus sp. VeSm13)	0.2%	0.1%
g	Nitrosomonas (1 OTU with 100% identity in 429bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	0.2%	0.1%
s	Dehalococcoides sp. BHI80-52 (1 OTU with 82% identity in 406bp to: Dehalococcoides sp. BHI80-52)	0.2%	0.2%
s	Thioprofundum hispidum (1 OTU with 91% identity in 429bp to: Thioprofundum hispidum)	0.2%	0.2%
s	Ferruginibacter alkalilentus (1 OTU with 95% identity in 424bp to: Ferruginibacter alkalilentus)	0.2%	0.2%
s	Dokdonella ginsengisoli (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	0.2%	0.2%
s	Chryseolinea soli (1 OTU with 91% identity in 416bp to: Chryseolinea soli)	0.2%	0.2%
c	Gammaproteobacteria (1 OTU with 90% identity in 429bp to: Acidibacter ferrireducens, Coxiella endosymbiont of Ornithodoros marocanus, Steroidobacter sp., Thioprofundum hispidum)	0.2%	0.1%
o	Myxococcales (1 OTU with 92% identity in 428bp to: Phaselicystis flava, Racemicystis crocea, Racemicystis persica)	0.1%	0.2%
s	Aquincola sp. HME6814 (1 OTU with 97% identity in 429bp to: Aquincola sp. HME6814)	0.1%	0.2%
s	Steroidobacter sp. (1 OTU with 94% identity in 429bp to: Steroidobacter sp.)	0.1%	0.1%
	Other	0.3%	0.3%
	Unclassified (0 reads)		
	Filtered (0 reads)		

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11.AV3V4a (35 691 reads)

g	**Haliscomenobacter** (20 OTUs with 91-92% identity in 421-424bp to: 2 unclassified Haliscomenobacter strains)	**13.9%**	15.9%
s	**Denitratisoma oestradiolicum** (2 OTUs with 98% identity in 429bp to: Denitratisoma oestradiolicum)	**7.5%**	3.3%
s	**Ferruginibacter alkalilentus** (3 OTUs with 95% identity in 424bp to: Ferruginibacter alkalilentus)	**5.0%**	5.8%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 425bp to: Rhodohalobacter sp. SW132)	**4.9%**	5.1%
s	**Tepidiforma bonchosmolovskayae** (4 OTUs with 95% identity in 406bp to: Tepidiforma bonchosmolovskayae)	**4.0%**	3.7%
g	**Comamonas** (5 OTUs with 96-99% identity in 429bp to: 33 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**3.6%**	3.1%
s	**Litorilinea aerophila** (7 OTUs with 90-93% identity in 405-406bp to: Litorilinea aerophila)	**3.3%**	3.0%
s	**Ferruginibacter sp.** (1 OTU with 92% identity in 424bp to: Ferruginibacter sp.)	**3.1%**	3.5%
s	**Thermomonas carbonis** (2 OTUs with 98-99% identity in 421bp to: Thermomonas carbonis)	**2.7%**	3.4%
s	**Chryseotalea sanaruensis** (2 OTUs with 91% identity in 415bp to: Chryseotalea sanaruensis)	**2.7%**	3.1%
f	**Chitinophagaceae** (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**2.4%**	2.7%
c	**Alphaproteobacteria** (3 OTUs with 99-100% identity in 404bp to: 4 unclassified Brevundimonas strains, 4 unclassified Mycoplana strains, Brevundimonas subvibrioides)	**2.4%**	2.3%
s	**Nitrosomonas sp.** (1 OTU with 97% identity in 429bp to: Nitrosomonas sp.)	**2.3%**	1.0%
g	**Nitrosomonas** (2 OTUs with 97-100% identity in 429bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**2.1%**	0.9%
s	**Sphaerobacter thermophilus** (4 OTUs with 90-91% identity in 406-407bp to: Sphaerobacter thermophilus)	**2.0%**	1.7%
s	**Ralstonia sp. P-4CB2** (3 OTUs with 97-98% identity in 430bp to: Ralstonia sp. P-4CB2)	**1.7%**	3.9%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**1.6%**	0.7%
g	**Ferruginibacter** (1 OTU with 95% identity in 424bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	**1.5%**	1.7%
g	**Lacibacter** (1 OTU with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	**1.3%**	1.5%
s	**Vicingus serpentipes** (1 OTU with 93% identity in 426bp to: Vicingus serpentipes)	**1.2%**	1.0%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**1.1%**	1.0%
c	**Betaproteobacteria** (2 OTUs with 98-100% identity in 429bp to: 17 unclassified Simplicispira strains, 2 unclassified Aquaspirillum strains, Simplicispira limi)	**1.1%**	1.1%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**1.1%**	1.0%
s	**Mucibacter soli** (1 OTU with 96% identity in 424bp to: Mucibacter soli)	**1.1%**	1.3%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 431bp to: Sterolibacterium sp. TKU1)	**1.1%**	0.5%
s	**Ignavibacterium album** (3 OTUs with 87-100% identity in 424-427bp to: Ignavibacterium album)	**1.1%**	1.1%
s	**Thermanaerothrix daxensis** (3 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**1.1%**	0.9%
g	**Pseudomonas** (3 OTUs with 100% identity in 429bp to: 35 unclassified Pseudomonas strains, Pseudomonas baetica, Pseudomonas caeni, Pseudomonas jessenii, Pseudomonas laurylsulfativorans, Pseudomonas migulae, Pseudomonas mohnii, Pseudomonas moorei, Pseudomonas putida, Pseudomonas umsongensis)	**1.0%**	2.0%

s	**Thermomarinilinea lacunifontana** (3 OTUs with 82-88% identity in 397-406bp to: Thermomarinilinea lacunifontana)	**1.0%**	0.9%
s	**Syntrophorhabdus sp. TB** (1 OTU with 79% identity in 422bp to: Syntrophorhabdus sp. TB)	**0.9%**	0.4%
g	**Thermomonas** (2 OTUs with 99-100% identity in 421bp to: Thermomonas fusca, Thermomonas haemolytica, Thermomonas koreensis, Thermomonas sp. ZY008)	**0.9%**	1.1%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.8%**	2.1%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**0.8%**	0.7%
s	**Gemmatimonas aurantiaca** (1 OTU with 95% identity in 423bp to: Gemmatimonas aurantiaca)	**0.7%**	0.3%
s	**Prochlorococcus sp. SCGC AAA298-C17** (1 OTU with 85% identity in 424bp to: Prochlorococcus sp. SCGC AAA298-C17)	**0.7%**	0.4%
s	**Tangfeifania diversioriginum** (2 OTUs with 92% identity in 424bp to: Tangfeifania diversioriginum)	**0.7%**	0.9%
s	**Stenotrophobacter terrae** (1 OTU with 97% identity in 404bp to: Stenotrophobacter terrae)	**0.7%**	0.3%
s	**Steroidobacter sp.** (2 OTUs with 94% identity in 429bp to: Steroidobacter sp.)	**0.7%**	0.6%
s	**Wandonia haliotis** (1 OTU with 86% identity in 424bp to: Wandonia haliotis)	**0.6%**	0.8%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 421bp to: Gemmatimonas phototrophica)	**0.6%**	0.3%
s	**Terrimonas sp. YJ03** (3 OTUs with 96-98% identity in 424bp to: Terrimonas sp. YJ03)	**0.6%**	0.7%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**0.6%**	0.5%
s	**Thermomonas fusca** (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	**0.5%**	0.6%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 404bp to: 2 unclassified Bradyrhizobium strains, 3 unclassified Afipia strains, Afipia massiliensis)	**0.5%**	0.3%
s	**Thermomonas brevis** (1 OTU with 99% identity in 421bp to: Thermomonas brevis)	**0.5%**	0.6%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 429bp to: Aquincola sp. HME6814)	**0.5%**	0.7%
s	**Pedobacter sp.** (1 OTU with 99% identity in 424bp to: Pedobacter sp.)	**0.5%**	0.6%
s	**Simplicispira suum** (1 OTU with 100% identity in 429bp to: Simplicispira suum)	**0.5%**	0.4%
s	**Williamwhitmania taraxaci** (2 OTUs with 87% identity in 425bp to: Williamwhitmania taraxaci)	**0.4%**	0.6%
s	**Curvibacter sp. R-36930** (1 OTU with 99% identity in 429bp to: Curvibacter sp. R-36930)	**0.4%**	0.4%
g	**Simplicispira** (1 OTU with 100% identity in 429bp to: Simplicispira piscis, Simplicispira sp. b29)	**0.4%**	0.3%
s	**Chryseolinea soli** (1 OTU with 91% identity in 416bp to: Chryseolinea soli)	**0.4%**	0.4%
g	**Rhodococcus** (1 OTU with 100% identity in 409bp to: 5 unclassified Rhodococcus strains, Rhodococcus degradans, Rhodococcus erythropolis, Rhodococcus qingshengii)	**0.4%**	0.4%
s	**Denitratimonas tolerans** (1 OTU with 100% identity in 429bp to: Denitratimonas tolerans)	**0.4%**	0.5%
s	**Thauera terpenica** (1 OTU with 100% identity in 429bp to: Thauera terpenica)	**0.3%**	0.4%
o	**Myxococcales** (1 OTU with 92% identity in 428bp to: Phaselicystis flava, Racemicystis crocea, Racemicystis persica)	**0.3%**	0.3%
g	**Massilia** (2 OTUs with 99% identity in 429bp to: Massilia brevitalea, Massilia sp. TA_JX)	**0.3%**	0.3%
s	**Gemmatimonas sp. URHD0086** (1 OTU with 91% identity in 422bp to: Gemmatimonas sp. URHD0086)	**0.3%**	0.1%
s	**Desulfotalea psychrophila** (1 OTU with 80% identity in 418bp to: Desulfotalea psychrophila)	**0.3%**	0.8%
s	**Sediminibacterium sp.** (1 OTU with 95% identity in 424bp to: Sediminibacterium sp.)	**0.3%**	0.3%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.3%**	0.1%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.3%**	0.4%
s	**Fimbriimonas ginsengisoli** (1 OTU with 87% identity in 406bp to: Fimbriimonas ginsengisoli)	**0.2%**	0.3%
s	**Hypericibacter adhaerens** (1 OTU with 94% identity in 405bp to: Hypericibacter adhaerens)	**0.2%**	0.4%
s	**Treponema stenostreptum** (1 OTU with 96% identity in 429bp to: Treponema stenostreptum)	**0.2%**	0.2%
s	**Pseudorhodobacter ponti** (1 OTU with 100% identity in 404bp to: Pseudorhodobacter ponti)	**0.2%**	0.2%
g	**Rhodanobacter** (2 OTUs with 99-100% identity in 429bp to: 3 unclassified Rhodanobacter strains, Rhodanobacter lindaniclasticus, Rhodanobacter spathiphylli, Rhodanobacter xiangquanii)	**0.2%**	0.3%

s	Woodsholea maritima (1 OTU with 95% identity in 404bp to: Woodsholea maritima)	0.2%	0.1%
s	Truepera sp. (1 OTU with 96% identity in 402bp to: Truepera sp.)	0.2%	0.2%
f	Xanthomonadaceae (1 OTU with 100% identity in 429bp to: Pseudoxanthomonas sp. BZ29r, Pseudoxanthomonas yeongjuensis, Stenotrophomonas sp. FS-YC6686)	0.2%	0.2%
s	Bellilinea caldifistulae (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	0.2%	0.1%
s	Thauera sp. O (1 OTU with 100% identity in 429bp to: Thauera sp. O)	0.2%	0.2%
s	Racemicystis persica (1 OTU with 92% identity in 428bp to: Racemicystis persica)	0.2%	0.3%
s	Candidatus Cyclonatronum proteinivorum (1 OTU with 87% identity in 424bp to: Candidatus Cyclonatronum proteinivorum)	0.2%	0.2%
s	Ideonella sp. (1 OTU with 97% identity in 429bp to: Ideonella sp.)	0.2%	0.2%
f	Anaerolineaceae (1 OTU with 85% identity in 407bp to: Thermanaerothrix daxensis, Thermomarinilinea lacunifontana)	0.2%	0.1%
s	Diaphorobacter sp. (1 OTU with 98% identity in 429bp to: Diaphorobacter sp.)	0.2%	0.2%
k	Bacteria (1 OTU with 100% identity in 429bp to: 33 unclassified Delftia strains, Alcaligenes faecalis, Clostridium sporogenes, Delftia acidovorans, Delftia lacustris, Delftia tsuruhatensis, Lactococcus raffinolactis, Pseudomonas sp. MS8_2H3)	0.1%	0.2%
g	Desulfonatronum (1 OTU with 90% identity in 428bp to: Desulfonatronum parangueonense, Desulfonatronum thiosulfatophilum)	0.1%	0.1%
g	Lutibacter (1 OTU with 96% identity in 424bp to: Lutibacter litorisediminis, Lutibacter sp. 16SWWE-6)	0.1%	0.2%
s	Dehalococcoides sp. BHI80-52 (1 OTU with 82% identity in 406bp to: Dehalococcoides sp. BHI80-52)	0.1%	0.1%
f	Rhodobacteraceae (1 OTU with 97% identity in 404bp to: 3 unclassified Pseudorhodobacter strains, 6 unclassified Rhodobacter strains, Pseudorhodobacter antarcticus, Pseudorhodobacter ferrugineus)	0.1%	0.2%
s	Aciditerrimonas ferrireducens (1 OTU with 93% identity in 406bp to: Aciditerrimonas ferrireducens)	0.1%	0.1%
p	Proteobacteria (1 OTU with 98% identity in 429bp to: Rhodanobacter sp. 7B-393, Simplicispira sp.)	0.1%	0.1%
g	Rhodobacter (1 OTU with 100% identity in 404bp to: 4 unclassified Rhodobacter strains, Rhodobacter gluconicum)	0.1%	0.2%
s	Dokdonella ginsengisoli (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	0.1%	0.2%
s	Chryseobacterium sp. Iso-52 (1 OTU with 96% identity in 424bp to: Chryseobacterium sp. Iso-52)	0.1%	0.1%
	Other	0.3%	0.6%
	Unclassified (0 reads)		
	Filtered (0 reads)		

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Filtered (0 reads)

12.AV3V4a (58 893 reads)

g	**Comamonas** (16 OTUs with 96-100% identity in 429bp to: 34 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**20.0%**	19.1%
s	**Ferruginibacter sp.** (28 OTUs with 92-96% identity in 422-424bp to: Ferruginibacter sp.)	**14.9%**	18.5%
s	**Tepidiforma bonchosmolovskayae** (8 OTUs with 93-95% identity in 406-407bp to: Tepidiforma bonchosmolovskayae)	**7.7%**	7.7%
s	**Stenotrophobacter terrae** (3 OTUs with 96-97% identity in 404bp to: Stenotrophobacter terrae)	**4.3%**	2.4%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**4.1%**	2.0%
g	**Nitrosomonas** (2 OTUs with 97-100% identity in 429bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**3.6%**	1.7%
s	**Litorilinea aerophila** (8 OTUs with 90-94% identity in 405bp to: Litorilinea aerophila)	**3.4%**	3.4%
s	**Terrimonas sp. YJ03** (5 OTUs with 95-98% identity in 424bp to: Terrimonas sp. YJ03)	**3.4%**	4.2%
s	**Nitrosomonas sp.** (3 OTUs with 96-97% identity in 429-430bp to: Nitrosomonas sp.)	**3.2%**	1.5%
f	**Chitinophagaceae** (3 OTUs with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	**2.9%**	3.6%
g	**Haliscomenobacter** (1 OTU with 92% identity in 424bp to: 2 unclassified Haliscomenobacter strains)	**2.7%**	3.3%
f	**Comamonadaceae** (3 OTUs with 79-96% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 2 unclassified Ramlibacter strains, 24 unclassified Comamonas strains, 8 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Curvibacter sp. PL21, Melaminivora alkalimesophila, Tibeticola sp.)	**2.5%**	2.3%
s	**Sphaerobacter thermophilus** (5 OTUs with 87-91% identity in 406-407bp to: Sphaerobacter thermophilus)	**2.0%**	1.9%
s	**Sediminibacterium sp.** (1 OTU with 95% identity in 424bp to: Sediminibacterium sp.)	**1.9%**	2.3%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**1.9%**	1.8%
s	**Thermomarinilinea lacunifontana** (2 OTUs with 82-84% identity in 402bp to: Thermomarinilinea lacunifontana)	**1.6%**	1.5%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**1.5%**	1.4%
g	**Thermomonas** (3 OTUs with 97-99% identity in 421bp to: 3 unclassified Thermomonas strains, Thermomonas brevis)	**1.3%**	1.8%
s	**Gemmatimonas aurantiaca** (2 OTUs with 95% identity in 423bp to: Gemmatimonas aurantiaca)	**0.9%**	0.4%
s	**Lysobacter tongrenensis** (1 OTU with 97% identity in 421bp to: Lysobacter tongrenensis)	**0.9%**	1.2%
g	**Ferruginibacter** (1 OTU with 95% identity in 424bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	**0.9%**	1.1%
s	**Denitratisoma oestradiolicum** (2 OTUs with 95-96% identity in 429bp to: Denitratisoma oestradiolicum)	**0.8%**	0.4%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**0.8%**	0.7%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 404bp to: 2 unclassified Rhodopseudomonas strains, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	**0.8%**	0.6%
s	**Limisphaera ngatamarikiensis** (3 OTUs with 87-88% identity in 428-430bp to: Limisphaera ngatamarikiensis)	**0.7%**	1.3%
s	**Gemmatimonas phototrophica** (1 OTU with 92% identity in 421bp to: Gemmatimonas phototrophica)	**0.6%**	0.3%
s	**Terrimonas sp.** (1 OTU with 97% identity in 424bp to: Terrimonas sp.)	**0.6%**	0.7%
s	**Diaphorobacter sp.** (1 OTU with 98% identity in 429bp to: Diaphorobacter sp.)	**0.6%**	0.8%
o	**Burkholderiales** (3 OTUs with 79-97% identity in 429-432bp to: 2 unclassified Ideonella strains, 4 unclassified Roseateles strains, Aquabacterium sp. AKB-2008-KU6, Aquincola sp., Caldimonas manganoxidans, Caldimonas sp. B15, Caldimonas taiwanensis, Paucibacter sp., Rubrivivax sp. JA309)	**0.5%**	0.7%
s	**Thermoflexus hugenholtzii** (1 OTU with 82% identity in 405bp to: Thermoflexus hugenholtzii)	**0.4%**	0.4%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.4%**	1.2%
s	**Roseisolibacter agri** (1 OTU with 91% identity in 421bp to: Roseisolibacter agri)	**0.4%**	0.2%
g	**Hyphomicrobium** (2 OTUs with 96-97% identity in 404bp to: 3 unclassified Hyphomicrobium strains, Hyphomicrobium zavarzinii)	**0.4%**	0.3%
s	**Dehalococcoides sp. BHI80-52** (1 OTU with 82% identity in 406bp to: Dehalococcoides sp. BHI80-52)	**0.4%**	0.4%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97-98% identity in 430bp to: Ralstonia sp. P-4CB2)	**0.4%**	1.0%

s	**Bdellovibrio sp.** (2 OTUs with 96-99% identity in 406bp to: Bdellovibrio sp.)	**0.4%**	0.5%
s	**Cephaloticoccus capnophilus** (1 OTU with 96% identity in 428bp to: Cephaloticoccus capnophilus)	**0.3%**	0.2%
s	**Caldilinea aerophila** (2 OTUs with 90-92% identity in 405bp to: Caldilinea aerophila)	**0.3%**	0.3%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**0.3%**	0.3%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.3%**	0.2%
s	**Thermomonas carbonis** (1 OTU with 98% identity in 421bp to: Thermomonas carbonis)	**0.3%**	0.4%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 429bp to: Aquincola sp. HME6814)	**0.3%**	0.5%
c	**Betaproteobacteria** (2 OTUs with 98% identity in 429bp to: 3 unclassified Simplicispira strains, Aquaspirillum sp. R-22832)	**0.3%**	0.3%
s	**Ferruginibacter alkalilentus** (1 OTU with 95% identity in 424bp to: Ferruginibacter alkalilentus)	**0.3%**	0.3%
o	**Rhodocyclales** (1 OTU with 92% identity in 429bp to: 3 unclassified Thauera strains, Azonexus caeni)	**0.2%**	0.3%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 429bp to: Thioprofundum hispidum)	**0.2%**	0.2%
s	**Chitinophaga pinensis** (1 OTU with 92% identity in 422bp to: Chitinophaga pinensis)	**0.2%**	0.3%
s	**Chryseotalea sanaruensis** (1 OTU with 91% identity in 415bp to: Chryseotalea sanaruensis)	**0.2%**	0.3%
s	**Longilinea sp.** (1 OTU with 83% identity in 407bp to: Longilinea sp.)	**0.2%**	0.2%
s	**Racemicystis persica** (2 OTUs with 92-93% identity in 428-429bp to: Racemicystis persica)	**0.2%**	0.4%
s	**Truepera sp.** (1 OTU with 96% identity in 402bp to: Truepera sp.)	**0.2%**	0.2%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**0.2%**	0.3%

f	**Microbacteriaceae** (1 OTU with 99% identity in 409bp to: 2 unclassified Chryseoglobus strains, 3 unclassified Salinibacterium strains, 8 unclassified Microcella strains, Chryseoglobus frigidaquae, Conyzicola lurida, Conyzicola nivalis, Conyzicola sp. B33-1, Cryobacterium sp., Cryobacterium tepidiphilum, Diaminobutyricimonas massiliensis, Leifsonia sp. MSL 02, Microcella alkaliphila, Salinibacterium xinjiangense)	0.2%	0.2%
s	**Lewinella aquimaris** (1 OTU with 85% identity in 422bp to: Lewinella aquimaris)	0.2%	0.2%
g	**Afipia** (1 OTU with 99% identity in 404bp to: 2 unclassified Afipia strains, Afipia clevelandensis)	0.2%	0.1%
s	**Thermomonas fusca** (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	0.2%	0.2%
s	**Rhodohalobacter sp. SW132** (1 OTU with 88% identity in 425bp to: Rhodohalobacter sp. SW132)	0.2%	0.2%
f	**Sphingomonadaceae** (1 OTU with 100% identity in 404bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	0.1%	0.1%
s	**Labilithrix luteola** (1 OTU with 88% identity in 431bp to: Labilithrix luteola)	0.1%	0.2%
o	**Rhizobiales** (1 OTU with 93% identity in 404bp to: Breoghania corrubedonensis, Breoghania sp. BR-5, Mycoplana sp. G110)	0.1%	0.1%
s	**Aciditerrimonas ferrireducens** (1 OTU with 93% identity in 406bp to: Aciditerrimonas ferrireducens)	0.1%	0.1%
s	**Steroidobacter sp.** (1 OTU with 93% identity in 429bp to: Steroidobacter sp.)	0.1%	0.1%
	Other	0.9%	1.4%
	Unclassified (0 reads)		
	Filtered (0 reads)		

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13.AV3V4a (18 442 reads)

g	**Comamonas** (9 OTUs with 96-100% identity in 429bp to: 34 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**12.7%**	12.5%
s	**Gemmatimonas phototrophica** (6 OTUs with 92% identity in 421bp to: Gemmatimonas phototrophica)	**12.1%**	6.0%
s	**Denitratisoma oestradiolicum** (3 OTUs with 95-98% identity in 429bp to: Denitratisoma oestradiolicum)	**10.5%**	5.2%
s	**Thermomarinilinea sp.** (4 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**5.8%**	5.7%
s	**Ferruginibacter sp.** (5 OTUs with 92-96% identity in 424bp to: Ferruginibacter sp.)	**4.8%**	6.2%
s	**Thermomonas carbonis** (3 OTUs with 98% identity in 421bp to: Thermomonas carbonis)	**4.0%**	5.5%
s	**Litorilinea aerophila** (8 OTUs with 90-93% identity in 405-406bp to: Litorilinea aerophila)	**3.7%**	3.8%
s	**Tepidiforma bonchosmolovskayae** (6 OTUs with 93-95% identity in 406-407bp to: Tepidiforma bonchosmolovskayae)	**3.5%**	3.6%
s	**Terrimonas sp. YJ03** (5 OTUs with 95-98% identity in 424bp to: Terrimonas sp. YJ03)	**2.9%**	3.8%
s	**Denitratimonas tolerans** (2 OTUs with 97-100% identity in 429bp to: Denitratimonas tolerans)	**2.9%**	4.0%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**2.5%**	2.4%
s	**Thermanaerothrix daxensis** (4 OTUs with 86% identity in 406bp to: Thermanaerothrix daxensis)	**2.2%**	2.2%
s	**Sphaerobacter thermophilus** (5 OTUs with 90-93% identity in 406-407bp to: Sphaerobacter thermophilus)	**2.2%**	2.2%
g	**Nitrosomonas** (2 OTUs with 97-100% identity in 429bp to: 6 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**2.1%**	1.0%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**2.0%**	1.0%
s	**Vibrio sp. Gp-3-5.1** (5 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**1.9%**	5.3%
s	**Stenotrophobacter terrae** (2 OTUs with 96-97% identity in 404bp to: Stenotrophobacter terrae)	**1.8%**	1.0%
s	**Nitrosomonas sp.** (2 OTUs with 97-98% identity in 429-430bp to: Nitrosomonas sp.)	**1.7%**	0.8%
f	**Comamonadaceae** (2 OTUs with 79-96% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 23 unclassified Comamonas strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas aquatica, Comamonas jiangduensis, Comamonas phosphati, Melaminivora alkalimesophila, Melaminivora jejuensis)	**1.3%**	1.3%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**1.2%**	1.2%
s	**Dokdonella ginsengisoli** (1 OTU with 99% identity in 429bp to: Dokdonella ginsengisoli)	**1.1%**	1.8%
s	**Desulfotalea psychrophila** (1 OTU with 80% identity in 418bp to: Desulfotalea psychrophila)	**0.9%**	2.9%
s	**Lysobacter sp.** (1 OTU with 99% identity in 429bp to: Lysobacter sp.)	**0.8%**	1.1%
f	**Alcaligenaceae** (1 OTU with 98% identity in 429bp to: 2 unclassified Pusillimonas strains, Candidimonas bauzanensis)	**0.8%**	0.7%

s	Curvibacter sp. R-36930 (1 OTU with 99% identity in 429bp to: Curvibacter sp. R-36930)	0.8%	0.7%
g	Gelidibacter (2 OTUs with 99% identity in 424bp to: 4 unclassified Gelidibacter strains, Gelidibacter flavus, Gelidibacter gilvus, Gelidibacter japonicus)	0.8%	1.1%
g	Haliscomenobacter (1 OTU with 92% identity in 424bp to: 2 unclassified Haliscomenobacter strains)	0.7%	1.0%
s	Parvibaculum sp. MBNA2 (1 OTU with 100% identity in 396bp to: Parvibaculum sp. MBNA2)	0.7%	0.8%
s	Chryseotalea sanaruensis (1 OTU with 91% identity in 415bp to: Chryseotalea sanaruensis)	0.7%	0.9%
g	Thermomonas (2 OTUs with 98-99% identity in 421bp to: 2 unclassified Thermomonas strains)	0.6%	0.9%
s	Ralstonia sp. P-4CB2 (2 OTUs with 97% identity in 430bp to: Ralstonia sp. P-4CB2)	0.6%	1.5%
s	Racemicystis persica (3 OTUs with 92-93% identity in 428-429bp to: Racemicystis persica)	0.6%	1.1%
f	Chitinophagaceae (1 OTU with 79% identity in 426bp to: Arachidicoccus sp., Haoranjiania flava)	0.5%	0.7%
s	Lysobacter concretionis (2 OTUs with 99-100% identity in 429bp to: Lysobacter concretionis)	0.5%	0.7%
s	Terrimonas sp. (1 OTU with 97% identity in 424bp to: Terrimonas sp.)	0.4%	0.6%
s	Casimicrobium huifangae (1 OTU with 99% identity in 430bp to: Casimicrobium huifangae)	0.4%	0.5%
s	Ornatilinea apprima (2 OTUs with 89% identity in 397-399bp to: Ornatilinea apprima)	0.4%	0.4%
s	Thiobacillus sp. 100B-B7 (1 OTU with 99% identity in 429bp to: Thiobacillus sp. 100B-B7)	0.4%	0.2%
s	Gemmatimonas aurantiaca (1 OTU with 95% identity in 423bp to: Gemmatimonas aurantiaca)	0.4%	0.2%
s	Truepera sp. (2 OTUs with 96% identity in 402bp to: Truepera sp.)	0.4%	0.4%
g	Ferruginibacter (1 OTU with 95% identity in 424bp to: 4 unclassified Ferruginibacter strains, Ferruginibacter profundus)	0.3%	0.4%
p	Proteobacteria (2 OTUs with 97-98% identity in 429bp to: Aquincola sp., Bradyrhizobium sp., Rhodanobacter sp. 7B-393, Simplicispira sp.)	0.3%	0.5%
s	Thioprofundum hispidum (1 OTU with 91% identity in 429bp to: Thioprofundum hispidum)	0.3%	0.3%
f	Bradyrhizobiaceae (2 OTUs with 100% identity in 404bp to: 2 unclassified Nitrobacter strains, 2 unclassified Rhodopseudomonas strains, 3 unclassified Afipia strains, Afipia birgiae, Afipia broomeae, Afipia genosp. 10, Afipia lausannensis, Afipia massiliensis, Bradyrhizobium jicamae, Bradyrhizobium sp., Rhodopseudomonas faecalis)	0.3%	0.2%
s	Sediminibacterium sp. (1 OTU with 95% identity in 424bp to: Sediminibacterium sp.)	0.3%	0.4%
g	Lacibacter (1 OTU with 95% identity in 424bp to: Lacibacter nakdongensis, Lacibacter sp. JJ009)	0.3%	0.4%
s	Aquincola sp. HME6814 (1 OTU with 97% identity in 429bp to: Aquincola sp. HME6814)	0.3%	0.5%
o	Burkholderiales (1 OTU with 79% identity in 432bp to: 4 unclassified Roseateles strains, Paucibacter sp.)	0.3%	0.4%
s	Limisphaera ngatamarikiensis (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	0.3%	0.5%
s	Turneriella parva (1 OTU with 95% identity in 426bp to: Turneriella parva)	0.3%	0.2%
s	Diaphorobacter sp. (1 OTU with 98% identity in 429bp to: Diaphorobacter sp.)	0.2%	0.4%
f	Microbacteriaceae (1 OTU with 99% identity in 409bp to: 2 unclassified Chryseoglobus strains, 3 unclassified Salinibacterium strains, 8 unclassified Microcella strains, Chryseoglobus frigidaquae, Conyzicola lurida, Conyzicola nivalis, Conyzicola sp. B33-1, Cryobacterium sp., Cryobacterium tepidiphilum, Diaminobutyricimonas massiliensis, Leifsonia sp. MSL 02, Microcella alkaliphila, Salinibacterium xinjiangense)	0.2%	0.2%
g	Desulfonatronum (1 OTU with 90% identity in 428bp to: Desulfonatronum paranguaonense, Desulfonatronum thiosulfatophilum)	0.2%	0.2%
s	Lentimicrobium saccharophilum (1 OTU with 98% identity in 424bp to: Lentimicrobium saccharophilum)	0.2%	0.3%
s	Simplicispira sp. L1R1.2 (1 OTU with 98% identity in 429bp to: Simplicispira sp. L1R1.2)	0.2%	0.2%
f	Myxococcaceae (1 OTU with 93% identity in 428bp to: Corallococcus macrosporus, Pyxidicoccus fallax)	0.2%	0.3%
s	Thermomonas fusca (1 OTU with 98% identity in 421bp to: Thermomonas fusca)	0.2%	0.2%
g	Dokdonella (1 OTU with 97% identity in 429bp to: Dokdonella immobilis, Dokdonella sp.)	0.2%	0.3%
s	Acidobacterium sp. WY65 (1 OTU with 96% identity in 404bp to: Acidobacterium sp. WY65)	0.2%	0.1%
s	Bellilinea caldifistulae (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	0.2%	0.2%
s	Polyangium thaxteri (1 OTU with 96% identity in 428bp to: Polyangium thaxteri)	0.2%	0.3%
s	Aciditerrimonas ferrireducens (1 OTU with 93% identity in 406bp to: Aciditerrimonas ferrireducens)	0.1%	0.1%

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s	Chitinophaga pinensis (1 OTU with 92% identity in 422bp to: Chitinophaga pinensis)	0.1%	0.1%
s	Leptospira interrogans (1 OTU with 92% identity in 415bp to: Leptospira interrogans)	0.1%	0.1%
	Other	0.1%	0.1%
	Unclassified (0 reads)		
	Filtered (0 reads)		

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Filtered (0 reads)

14.AV3V4a (23 110 reads)

g	**Bifidobacterium** (20 OTUs with 99-100% identity in 411-413bp to: 15 unclassified Bifidobacterium strains, Bifidobacterium adolescentis, Bifidobacterium catenulatum, Bifidobacterium faecale, Bifidobacterium pseudocatenulatum)	**12.9%**	16.6%
g	**Collinsella** (8 OTUs with 99-100% identity in 405bp to: 2 unclassified Collinsella strains, Collinsella aerofaciens)	**10.3%**	6.6%
f	**Enterobacteriaceae** (10 OTUs with 99-100% identity in 429bp to: Escherichia coli, Escherichia fergusonii, Escherichia marmotae, Escherichia sp., Salmonella sp. S13, Shigella boydii, Shigella flexneri, Shigella sonnei, Shigella sp.)	**9.6%**	10.3%
g	**Faecalibacterium** (3 OTUs with 100% identity in 404bp to: 4 unclassified Faecalibacterium strains, Faecalibacterium prausnitzii)	**7.4%**	3.8%
s	**Comamonas sp. NLF-7-7** (11 OTUs with 98-100% identity in 429bp to: Comamonas sp. NLF-7-7)	**7.3%**	7.5%
s	**Acidobacterium forest soil bacterium YC04** (6 OTUs with 91-92% identity in 316bp to: forest soil bacterium YC04)	**6.5%**	3.9%
g	**Bacteroides** (7 OTUs with 99-100% identity in 424bp to: 12 unclassified Bacteroides strains, Bacteroides cellulosilyticus, Bacteroides thetaiotaomicron, Bacteroides uniformis)	**5.9%**	9.1%
g	**Coprococcus** (1 OTU with 100% identity in 404bp to: Coprococcus comes, Coprococcus sp.)	**3.8%**	2.0%
s	**Homo sapiens** (1 OTU with 100% identity in 395bp to: Homo sapiens)	**3.3%**	1.7%
o	**Bacteroidales** (3 OTUs with 99-100% identity in 424bp to: 16 unclassified Bacteroides strains, Phocaeicola dorei, Phocaeicola vulgatus)	**2.3%**	3.5%
s	**Candidatus Koribacter versatilis** (1 OTU with 93% identity in 404bp to: Candidatus Koribacter versatilis)	**1.7%**	1.1%
s	**Faecalibacterium prausnitzii** (1 OTU with 100% identity in 404bp to: Faecalibacterium prausnitzii)	**1.5%**	0.8%
g	**Solirubrobacter** (1 OTU with 94% identity in 421bp to: 4 unclassified Solirubrobacter strains)	**1.5%**	0.8%
s	**Gemmiger formicilis** (2 OTUs with 99-100% identity in 404bp to: Gemmiger formicilis)	**1.4%**	1.4%
s	**Ruminococcus bromii** (1 OTU with 100% identity in 405bp to: Ruminococcus bromii)	**1.3%**	0.7%
g	**Comamonas** (2 OTUs with 98-99% identity in 429bp to: 3 unclassified Comamonas strains)	**1.3%**	1.4%
g	**Ruminococcus** (1 OTU with 100% identity in 405bp to: Ruminococcus bromii, Ruminococcus sp.)	**1.3%**	1.4%
s	**Nitrosomonas eutropha** (1 OTU with 100% identity in 429bp to: Nitrosomonas eutropha)	**1.3%**	0.6%
s	**Methylovirgula ligni** (1 OTU with 98% identity in 404bp to: Methylovirgula ligni)	**1.1%**	1.0%
s	**Candidatus Solibacter usitatus** (1 OTU with 91% identity in 404bp to: Candidatus Solibacter usitatus)	**1.1%**	0.7%
s	**Oscillibacter sp.** (1 OTU with 95% identity in 406bp to: Oscillibacter sp.)	**1.1%**	2.4%
s	**Dorea longicatena** (1 OTU with 100% identity in 404bp to: Dorea longicatena)	**1.0%**	0.5%
o	**Clostridiales** (2 OTUs with 100% identity in 403-404bp to: 2 unclassified Blautia strains, 9 unclassified Clostridium strains, Blautia massiliensis, Clostridioides difficile, Clostridium sordelli, Paeniclostridium ghonii, Paeniclostridium sordellii, Ruminococcus sp. SR1/5, Sporacetigenium sp. HGFM123, [Eubacterium] tenue)	**0.9%**	2.0%
s	**Acidobacterium sp. WY65** (1 OTU with 95% identity in 404bp to: Acidobacterium sp. WY65)	**0.9%**	0.5%
s	**Steroidobacter sp. JC2953** (1 OTU with 96% identity in 429bp to: Steroidobacter sp. JC2953)	**0.8%**	0.9%
s	**Pseudobutyrivibrio sp.** (1 OTU with 100% identity in 404bp to: Pseudobutyrivibrio sp.)	**0.8%**	1.1%
f	**Lachnospiraceae** (1 OTU with 100% identity in 404bp to: Enterocloster clostridioformis, Fusicatenibacter saccharivorans)	**0.7%**	1.0%
s	**Bifidobacterium longum** (1 OTU with 100% identity in 409bp to: Bifidobacterium longum)	**0.7%**	1.2%
s	**Bacteroides stercorirosoris** (1 OTU with 97% identity in 424bp to: Bacteroides stercorirosoris)	**0.6%**	1.0%
f	**Thermomonosporaceae** (1 OTU with 94% identity in 405bp to: 11 unclassified Actinocorallia strains, 3 unclassified Actinomadura strains, Actinocorallia aurantiaca, Actinocorallia aurea, Actinocorallia herbida, Actinocorallia libanotica, Actinocorallia spatholoba)	**0.6%**	0.7%
s	**Actinomadura sp. 2602GPT1-42** (1 OTU with 93% identity in 409bp to: Actinomadura sp. 2602GPT1-42)	**0.6%**	0.7%
s	**Actinomadura meyerae** (1 OTU with 94% identity in 409bp to: Actinomadura meyerae)	**0.5%**	0.6%

s	Lachnoclostridium [Clostridium] symbiosum (1 OTU with 100% identity in 404bp to: [Clostridium] symbiosum)	0.5%	0.7%
s	Acidobacterium sp. BK18 (1 OTU with 94% identity in 410bp to: Acidobacterium sp. BK18)	0.5%	0.3%
s	Iamia majanohamensis (1 OTU with 91% identity in 397bp to: Iamia majanohamensis)	0.4%	0.5%
s	Mediterraneibacter massiliensis (1 OTU with 100% identity in 404bp to: Mediterraneibacter massiliensis)	0.4%	0.6%
g	Slackia (1 OTU with 100% identity in 405bp to: Slackia isoflavoniconvertens, Slackia sp. NATTS)	0.4%	0.3%
g	Dorea (1 OTU with 100% identity in 404bp to: Dorea formicigenerans, Dorea phocaeensis)	0.4%	0.2%
g	Pseudomonas (2 OTUs with 99-100% identity in 429bp to: 2 unclassified Pseudomonas strains, Pseudomonas caeni, sulfide-oxidizing bacterium ISW_10)	0.4%	0.9%
f	Xanthomonadaceae (1 OTU with 100% identity in 429bp to: 22 unclassified Stenotrophomonas strains, Stenotrophomonas acidaminiphila, Stenotrophomonas maltophilia, Stenotrophomonas nitritireducens, Xanthomonas sp. HH69)	0.4%	0.6%
s	Bdellovibrio sp. (1 OTU with 96% identity in 406bp to: Bdellovibrio sp.)	0.4%	0.5%
s	Aciditerrimonas ferrireducens (1 OTU with 94% identity in 406bp to: Aciditerrimonas ferrireducens)	0.4%	0.4%
c	Alphaproteobacteria (1 OTU with 100% identity in 404bp to: 12 unclassified Brevundimonas strains, 5 unclassified Mycoplana strains, Brevundimonas subvibrioides)	0.4%	0.4%
s	Paenibacillus sp. 27-9 (3 OTUs with 97-98% identity in 404bp to: Paenibacillus sp. 27-9)	0.3%	1.7%
s	Povalibacter uvarum (1 OTU with 91% identity in 429bp to: Povalibacter uvarum)	0.3%	0.3%
s	Lachnospira eligens (1 OTU with 100% identity in 404bp to: Lachnospira eligens)	0.2%	0.6%
s	Pseudonocardia alaniniphila (1 OTU with 83% identity in 419bp to: Pseudonocardia alaniniphila)	0.2%	0.2%
s	Actinomadura sp. Y218 (1 OTU with 95% identity in 409bp to: Actinomadura sp. Y218)	0.2%	0.2%
s	Haliangium tepidum (1 OTU with 90% identity in 429bp to: Haliangium tepidum)	0.2%	0.2%
s	Turicibacter sanguinis (1 OTU with 100% identity in 421bp to: Turicibacter sanguinis)	0.2%	0.6%
s	Phocaeicola vulgatus (1 OTU with 100% identity in 424bp to: Phocaeicola vulgatus)	0.2%	0.3%
s	Rhodovastum atsumiense (1 OTU with 95% identity in 404bp to: Rhodovastum atsumiense)	0.2%	0.3%
c	Actinobacteria (1 OTU with 94% identity in 409bp to: Actinomadura sp. Y218, Kineosporia sp. OTSz_A_216)	0.1%	0.2%
s	Eubacterium coprostanoligenes (1 OTU with 92% identity in 406bp to: Eubacterium coprostanoligenes)	0.1%	0.4%
s	Dialister succinatiphilus (1 OTU with 99% identity in 429bp to: Dialister succinatiphilus)	0.1%	0.4%
g	Burkholderia (1 OTU with 100% identity in 429bp to: 15 unclassified Burkholderia strains)	0.1%	0.3%
s	Oscillibacter valericigenes (1 OTU with 96% identity in 406bp to: Oscillibacter valericigenes)	0.1%	0.2%
g	Dialister (1 OTU with 100% identity in 429bp to: Dialister hominis, Dialister massiliensis, Dialister sp. S7D)	0.1%	0.4%
	Other	0.4%	0.8%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.26: EBBR-Anammox microbial communities identified by NGS (**14.A3V4a)**

Filtered (0 reads)

15.AV3V4a (44 277 reads)

s	**Tepidiforma bonchosmolovskayae** (28 OTUs with 85-95% identity in 402-407bp to: Tepidiforma bonchosmolovskayae)	**21.6%**	23.2%
s	**Nitrosomonas sp.** (7 OTUs with 97% identity in 429bp to: Nitrosomonas sp.)	**11.9%**	6.1%
s	**Stenotrophobacter terrae** (5 OTUs with 93-96% identity in 404bp to: Stenotrophobacter terrae)	**10.8%**	6.5%
f	**Chitinophagaceae** (6 OTUs with 79% identity in 424-426bp to: Arachidicoccus sp., Haoranjiania flava)	**8.9%**	11.9%
s	**Litorilinea aerophila** (6 OTUs with 83-93% identity in 398-405bp to: Litorilinea aerophila)	**4.7%**	5.1%
s	**Terrimonas sp.** (3 OTUs with 96-97% identity in 424bp to: Terrimonas sp.)	**3.5%**	4.7%
s	**Denitratisoma oestradiolicum** (3 OTUs with 97-98% identity in 429bp to: Denitratisoma oestradiolicum)	**3.4%**	1.7%
g	**Comamonas** (4 OTUs with 96-99% identity in 429bp to: 32 unclassified Comamonas strains, Comamonas aquatica, Comamonas denitrificans, Comamonas jiangduensis, Comamonas phosphati, Comomonas sp. AT4)	**2.6%**	2.6%
s	**Caldilinea aerophila** (3 OTUs with 88-92% identity in 405bp to: Caldilinea aerophila)	**2.0%**	2.2%
s	**Nitrospira sp.** (1 OTU with 98% identity in 419bp to: Nitrospira sp.)	**1.9%**	1.0%
s	**Thermomarinilinea lacunifontana** (4 OTUs with 82-84% identity in 402bp to: Thermomarinilinea lacunifontana)	**1.9%**	1.9%
f	**Comamonadaceae** (2 OTUs with 79-100% identity in 429-430bp to: 2 unclassified Alicycliphilus strains, 5 unclassified Acidovorax strains, Acidovorax aerodenitrificans, Alicycliphilus denitrificans, Comamonas granuli, Melaminivora alkalimesophila, Variovorax paradoxus)	**1.6%**	1.6%
s	**Sphaerobacter thermophilus** (5 OTUs with 87-91% identity in 406-407bp to: Sphaerobacter thermophilus)	**1.5%**	1.5%
s	**Dehalococcoides sp. BHI80-52** (2 OTUs with 82-84% identity in 406-409bp to: Dehalococcoides sp. BHI80-52)	**1.4%**	1.5%
s	**Bellilinea caldifistulae** (1 OTU with 90% identity in 399bp to: Bellilinea caldifistulae)	**1.2%**	1.2%
s	**Desulfotalea psychrophila** (4 OTUs with 79-80% identity in 418bp to: Desulfotalea psychrophila)	**1.1%**	4.0%
s	**Terrimonas sp. YJ03** (2 OTUs with 96-98% identity in 424bp to: Terrimonas sp. YJ03)	**1.1%**	1.5%
s	**Brevefilum fermentans** (1 OTU with 83% identity in 402bp to: Brevefilum fermentans)	**1.0%**	1.0%
s	**Thermomicrobium roseum** (1 OTU with 94% identity in 388bp to: Thermomicrobium roseum)	**0.9%**	1.0%
s	**Chryseotalea sanaruensis** (1 OTU with 91% identity in 415bp to: Chryseotalea sanaruensis)	**0.9%**	1.1%
s	**Ideonella sp.** (1 OTU with 97% identity in 429bp to: Ideonella sp.)	**0.8%**	1.4%
g	**Nitrosomonas** (1 OTU with 100% identity in 429bp to: 4 unclassified Nitrosomonas strains, Nitrosomonas europaea)	**0.8%**	0.4%
s	**Acidobacterium sp. WY65** (2 OTUs with 92-96% identity in 404bp to: Acidobacterium sp. WY65)	**0.8%**	0.5%
s	**Sterolibacterium sp. TKU1** (1 OTU with 92% identity in 431bp to: Sterolibacterium sp. TKU1)	**0.7%**	0.4%
s	**Candidatus Solibacter usitatus** (1 OTU with 92% identity in 404bp to: Candidatus Solibacter usitatus)	**0.7%**	0.4%
s	**Limisphaera ngatamarikiensis** (1 OTU with 88% identity in 428bp to: Limisphaera ngatamarikiensis)	**0.6%**	1.2%
s	**Haematococcus lacustris** (1 OTU with 90% identity in 424bp to: Haematococcus lacustris)	**0.6%**	0.3%
s	**Fimbriimonas ginsengisoli** (2 OTUs with 87% identity in 406-407bp to: Fimbriimonas ginsengisoli)	**0.6%**	0.7%
s	**Thermomarinilinea sp.** (2 OTUs with 92-93% identity in 397-398bp to: Thermomarinilinea sp.)	**0.6%**	0.6%
s	**Ferruginibacter sp.** (1 OTU with 92% identity in 424bp to: Ferruginibacter sp.)	**0.6%**	0.7%
s	**Thermoflexus hugenholtzii** (1 OTU with 82% identity in 405bp to: Thermoflexus hugenholtzii)	**0.5%**	0.6%

	405bp to: Thermoflexus hugenholtzii)		
p	**Chloroflexi** (1 OTU with 83% identity in 405bp to: Dehalococcoides sp. BHI80-52, Longilinea sp.)	**0.5%**	0.6%
s	**Steroidobacter sp.** (1 OTU with 93% identity in 429bp to: Steroidobacter sp.)	**0.5%**	0.6%
s	**Longilinea sp.** (1 OTU with 83% identity in 407bp to: Longilinea sp.)	**0.5%**	0.5%
s	**Chryseolinea soli** (1 OTU with 91% identity in 416bp to: Chryseolinea soli)	**0.5%**	0.7%
s	**Hyphomicrobium sp. WG6** (1 OTU with 98% identity in 405bp to: Hyphomicrobium sp. WG6)	**0.4%**	0.3%
s	**Ralstonia sp. P-4CB2** (2 OTUs with 97-98% identity in 430bp to: Ralstonia sp. P-4CB2)	**0.4%**	1.0%
s	**Aquincola sp. HME6814** (1 OTU with 97% identity in 429bp to: Aquincola sp. HME6814)	**0.4%**	0.6%
s	**Thermanaerothrix daxensis** (2 OTUs with 82-86% identity in 406bp to: Thermanaerothrix daxensis)	**0.4%**	0.4%
s	**Candidatus Kuenenia sp.** (1 OTU with 97% identity in 429bp to: Candidatus Kuenenia sp.)	**0.3%**	0.5%
s	**Roseisolibacter agri** (1 OTU with 91% identity in 421bp to: Roseisolibacter agri)	**0.3%**	0.2%
s	**Pseudorhodoplanes sinuspersici** (1 OTU with 97% identity in 404bp to: Pseudorhodoplanes sinuspersici)	**0.3%**	0.3%
s	**Thioprofundum hispidum** (1 OTU with 91% identity in 429bp to: Thioprofundum hispidum)	**0.3%**	0.3%
f	**Caldilineaceae** (1 OTU with 88% identity in 405bp to: Caldilinea aerophila, Litorilinea aerophila)	**0.3%**	0.3%
s	**Labilithrix luteola** (1 OTU with 88% identity in 431bp to: Labilithrix luteola)	**0.3%**	0.3%
s	**Ferruginibacter alkalilentus** (1 OTU with 95% identity in 424bp to: Ferruginibacter alkalilentus)	**0.2%**	0.3%
s	**Opitutus sp. VeSm13** (1 OTU with 96% identity in 428bp to: Opitutus sp. VeSm13)	**0.2%**	0.1%
s	**Vibrio sp. Gp-3-5.1** (2 OTUs with 87% identity in 430bp to: Vibrio sp. Gp-3-5.1)	**0.2%**	0.7%
g	**Haliscomenobacter** (1 OTU with 92% identity in 424bp to: 2 unclassified Haliscomenobacter strains)	**0.2%**	0.3%
f	**Bradyrhizobiaceae** (1 OTU with 100% identity in 404bp to: 2 unclassified Rhodopseudomonas strains, Nitrobacter sp. NS5-5, Rhodopseudomonas faecalis)	**0.2%**	0.2%
f	**Sphingomonadaceae** (1 OTU with 100% identity in 404bp to: 9 unclassified Sphingopyxis strains, Sphingomonas sp. HPC808, Sphingopyxis granuli)	**0.2%**	0.2%
p	**Bacteroidetes** (1 OTU with 79% identity in 426bp to: Arachidiccoccus sp., Eisenibacter elegans, Flexibacter sp. JB 251, Haoranjiania flava)	**0.2%**	0.3%
s	**Owenweeksia hongkongensis** (1 OTU with 90% identity in 424bp to: Owenweeksia hongkongensis)	**0.2%**	0.2%
s	**Thioalkalivibrio paradoxus** (1 OTU with 91% identity in 429bp to: Thioalkalivibrio paradoxus)	**0.2%**	0.1%
s	**Pandoraea sp.** (1 OTU with 94% identity in 429bp to: Pandoraea sp.)	**0.2%**	0.5%
g	**Mesorhizobium** (1 OTU with 100% identity in 404bp to: 2 unclassified Mesorhizobium strains)	**0.2%**	0.1%
g	**Thermomonas** (1 OTU with 98% identity in 421bp to: 2 unclassified Thermomonas strains)	**0.1%**	0.2%
s	**Racemicystis persica** (1 OTU with 92% identity in 428bp to: Racemicystis persica)	**0.1%**	0.3%
g	**Azoarcus** (1 OTU with 93% identity in 429bp to: 3 unclassified Azoarcus strains, Azoarcus olearius)	**0.1%**	0.2%
o	**Rhodocyclales** (1 OTU with 92% identity in 429bp to: 3 unclassified Thauera strains, Azonexus caeni)	**0.1%**	0.2%
s	**Steroidobacter denitrificans** (1 OTU with 97% identity in 429bp to: Steroidobacter denitrificans)	**0.1%**	0.1%
	Other	**0.5%**	1.0%
	Unclassified (0 reads)		
	Filtered (0 reads)		

SI 5.27: EBBR-Anammox microbial communities identified by NGS (**15.A3V4a)**

List of conferences attended and presentations

1) The School of Science and Environment research symposium Manchester metropolitan University June 2016

2) Research conference at Manchester metropolitan University September 2016

3) IBBS Conference Manchester metropolitan University September 2017

4) ASM Early Career Conference at Trinity college Ireland June,2019

Research output 1 -posters presented at conferences

Anaerobic ammonia oxidation (anammox) in an expanded bed biofilm reactor (EBBR).

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Manchester Metropolitan University

Abstract

Anaerobic ammonium oxidation (anammox) is a denitrification process in which nitrite (NO_2^-) and ammonia (NH_3) are directly converted to nitrogen gas (N_2) by Planctomycetes bacteria. Anammox, important for nitrogen removal from wastewater containing a high concentration (*e.g.*500 mgL^{-1}) of NH_3 has not yet been investigated using the expanded bed biofilm reactor (EBBR) technology despite its simplicity and energy-efficiency.

This study is focused on investigating the conditions for anammox to treat synthetic wastewater containing 500 mgL^{-1} (ammonia) using the EBBR. A lab scale EBBR was inoculated with anammox granules operated as continuous culture with process conditions: 30°C, pH 7.7- 8.3, influent NH_4^+-N (500 mg L^{-1}) and NO_2^--N (660 mg L^{-1}). Observations of a simultaneous decrease in NH_4^+-N and NO_2^--N outlet concentrations with consistent low nitrate-nitrogen (NO_3^-N) production was taken as evidence of anammox activity, alongside early stage biofilm formation. Max N removal (44%) was observed with average nitrogen removal rate of 0.7 $KgNm^{-3}d^{-1}$.

Introduction

Nitrogen pollution of aquatic environments can lead to eutrophication and oxygen depletion with detrimental effects on aquatic lives e.g. fishes (Rodriguez-Sanchez *et al.*, 2014). The removal of nitrogen from wastewater before discharge is important to protect the aquatic environment. Anaerobic ammonia oxidation (anammox) is a denitrification wastewater treatment (WWT) process discovered by Mulder *et al.*, (1995) during which nitrite and ammonia are converted directly into nitrogen (N_2) under anoxic conditions (Strous *et al.*, 1999; Lagone *et al.*, 2014). Anammox bacteria (AB) depend on ammonia oxidizing bacteria (AOB) to remove up to 90% of wastewater ammonia and nitrite, with 10% converted to nitrate (Kartal *et.al*, 2007; van Hull *et al.*, 2010). Anammox process has been successfully established using WWT technologies, *e.g.* sequencing batch reactor (SBR), but is yet to be investigated using the expanded bed biofilm reactor (EBBR), despite successful pilot-plant scale implementation for high-rate nitrification (1.7 ± 0.6 kg NH_3-N $m^{-3}{}_{EBBR}$ d^{-1}). The process intensification resulting in a compact reactor that occupies 10 times less space compared to other WWT technologies (Akhidime and Dempsey, 2009).

Aim

To establish for the first time the conditions for the anaerobic ammonia oxidation (anammox) process in the EBBR.

Methods

The 700 cm^3 EBBR (Fig.1) was filled up to one third with 0.7-1mm biomass support particles and seeded with anammox granules. The reactor was supplied with synthetic wastewater containing NH_4^+-N: 500 mgL^{-1}; NO_2^- -N: 660 mgL^{-1}; maintained at 30° C; pH: 7.7 - 8.0 and nitrogen loading rate: 1.1 Kg N m^{-3} d^{-1}. Nitrogen gas was injected to minimise dissolved oxygen and EBBR covered with black sacks to prevent light penetration. Samples were removed from bed to investigate biofilms formation. Ammonia concentrations measurement was by ISO 7150-1:1984, nitrite (ISO 6777-1984) and nitrate (APHA 4500-NO_3^-) spectrophotometric methods.

Fig.1 Picture of the lab-scale expanded bed bioreactor (**1**- Expanded bed column, **2** - Recycle, **3** - Outlet, **4** -recirculation column, **5** - N_2 gas inlet, **6** - Feed pump, **7** - Fluidizing pump, **P** – pH sensor, **R**- refuse bag , **T** – Temperature sensor, **W** – Water jacket).

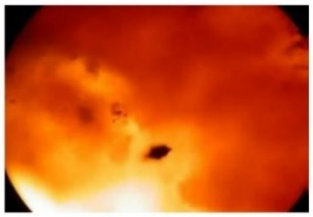

Fig.2 Fully developed bioparticles taken from the EBC observed under a phase contrast microscope (x400)

Fig.3 Initial stage of biofilm formation on carbon support particles taking from the EBBR with (x400) .

Results

The indicators of anammox activity are simultaneous consumption of NH_4^+-N and NO_2^--N coupled with minimal production of NO_3^--N (Suneethi *et al.* 2014). Results obtained from this study revealed a 74 % (NH_4^+-N) and 92 % (NO_2^--N) removal; 73 mgL^{-1} (NO_3^--N) production with 0.7 $kg\text{-}Nm^{-3}d^{-1}$ average nitrogen removal rate (Fig.4). Repeated consumptions of ammonia and nitrite simultaneously and production of minimal nitrate in phases A and B were consistent with anammox activity in the EBBR. Observation of some fully formed bioparticles (Fig 2) and initial biomass attachment on support media (Fig 3), suggest that process conditions in the reactor were suitable for anammox activity. This was in contrast to phase C, in which a decrease in ammonia concentration was coincident with an increase in NO_2^--N (> 601 mgL^{-1}), presuming NO_2^--N inhibition of anammox within the EBBR.

Discussion

The formation of fully developed bioparticles and observation of initial biofilm attachment on carbon support indicates that process conditions might be ideal for growth of anammox microbial consortium in the EBBR. The simultaneous NO_2^--N and NH_4^+-N removal with little NO_3^--N production is consistent with anammox activity in the reactor. Slow growth rate of AB and possible inhibition by NO_2^--N - a major suppressor of anammox bacteria (van der Star *et al.*, 2007), highlight the challenges of creating optimum conditions for anammox process using the EBBR technology for the first time.

Conclusion

The consistent evidence for anammox activity in the EBBR was the simultaneous decrease in NH_4^+-N and NO_2^-N and minimal NO_3^--N production. Findings from the initial stage of this work have demonstrated the potential of using EBBR technology for denitrification through the anammox process. The expanded bed comprising anammox bioparticles has not been developed possibly due to inhibition caused by nitrite accumulation. Results from this study have revealed some operating conditions (i.e. 30°C, pH 8) important to maintain anammox in the reactor, hence the possibility of a viable industrial anammox process using the EBBR technology. Further work will be undertaken to identify the threshold concentration of nitrite that causes anammox inhibition and conditions that allow full development of bioparticles.

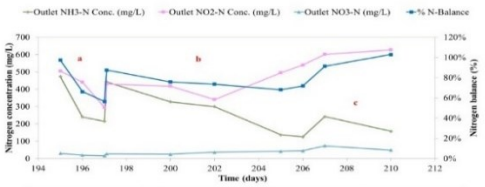

Fig.4: Outlet N- concentration and N-balance in continuous culture

Key	Days	NH4+-N	NO2- -N	NO3- -N	N-balance	Average, NRR ($kg\text{-}Nm^{-3}d^{-1}$)
a:	95-197	484 -214	505- 295	30 - 16	97- 56	0.5
b:	197.1- 206	442 -125	430 -520	27 - 36	87 - 72	0.6
c:	207- 210	242 -157	601 -627	73-49	91 – 103	0.4

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P1: Poster presentation at MMU research conference June 2016

Anaerobic ammonia oxidation (anammox) in an expanded bed biofilm reactor (EBBR).

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Introduction

The removal of nitrogen from wastewater before discharge is important as nitrogen pollution of aquatic environments can lead to eutrophication and oxygen depletion with detrimental effects on aquatic lives e.g. fish (Rodriguez-Sanchez *et al.* 2014). Anaerobic ammonia oxidation (anammox) is a denitrification wastewater treatment (WWT) process which converts nitrite and ammonia directly into nitrogen (N_2) gas under anoxic conditions. Anammox bacteria (AB) depend on ammonia oxidizing bacteria (AOB) to oxidize up to 90% of wastewater ammonium, with ultimately 10% converted to nitrate (Langone *et al* 2014). Anammox process has been successfully established using other WWT technologies, but is yet to be investigated using the expanded bed biofilm reactor (EBBR). The process intensification in EBBR allows for compact reactor that occupies 10 times less space compared to other WWT technologies (Akhidime and Dempsey, 2009).

Aim

To establish for the first time anaerobic ammonia oxidation (anammox) process in the EBBR.

Methods

A 700 cm^3 lab scale EBBR was filled with 0.7-1.0 mm biomass support particles and seeded with anammox granules (Fig1 (1)) The reactor was supplied with synthetic wastewater containing NH_4^+-N: 500 mg L^{-1}; NO_2^--N: 500 mg L^{-1} and maintained alternatively at 30° C; pH: 7.7 - 8.0 in batch (B) or continuous (C) culture conditions.

The EBBR was flushed with nitrogen gas during the recycle phase (Fig 1, (5)) to minimise dissolved oxygen. Samples were removed from the bed to investigate biofilms formation. Ammonium, nitrite and nitrate nitrogen concentrations were measured using ISO 7150-1:1984, ISO 6777-1984 and APHA 4500-NO_3^--N, which are standard spectrophotometric methods, respectively.

Results

The indicators of anammox activity are simultaneous consumption of NH_4^+-N and NO_2^--N coupled with minimal production of NO_3^--N (Suneethi *et al.* 2014).

Results obtained from this study revealed > 98% (NH_4^+-N), > 94% (NO_2^--N) conversion with a maximum of 0.18 kg-N $m^{-3}d^{-1}$ nitrogen removal rate (NRR) in batch culture (Fig.3). The continuous culture (C) had a maximum of 74% (NH_4^+-N), > 92% (NO_2^--N) conversion with 0.62 kg-N $m^{-3}d^{-1}$ NRR (Fig.4). Repeated consumptions of ammonia and nitrite with negligible nitrate production in both cultures were consistent with anammox activity, demonstrating the potential of the EBBR technology for the anammox process. Even though NRR was higher in C, the three indictors of anammox activity were evident in both the batch and continuous operations.

Although, fully developed anammox bioparticles to the naked eye are yet to be identified in the EBBR, observation of the initial stage of biomass attachment on support media (Fig 2), suggest that process conditions in the reactor support anammox activity within the EBBR, leading to simultaneous consumption of NH_4^+-N and NO_2^--N with a small amount of NO_3^--N produced.

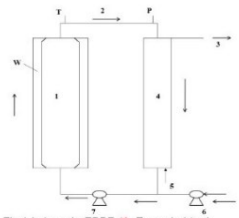

Fig.1 Lab-scale EBBR (1- Expanded bed column, 2 - Recycle, 3 - Outlet, 4 - recirculation column, 5 - N_2 gas inlet, 6 - Feed pump, 7 - Fluidizing pump, P – pH sensor, R- refuse bag T – Temperature sensor, W – Water jacket).

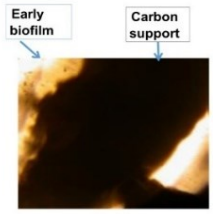

Fig.2 Initial stage of biofilm formation on carbon support particles taking from the EBBR with (x400) .

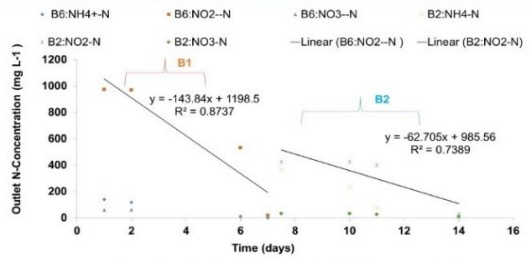

Figure 3: Outlet N - concentration in batch cultures (B1 and B2)

Key	Days	NH_4^+-N	NO_2^--N	NO_3^--N	NRR (kg-N $m^{-3}d^{-1}$)
B1:	1- 7	119 – 40	969-19	61-11	0.18
B2:	8-14	367- 40	530-25	11-10	0.11

Discussion

The observation of initial biofilm attachment on carbon support indicates that conditions might be ideal for growth of anammox microbial consortium in the EBBR. The simultaneous NO_2^--N and NH_4^+-N removal with little NO_3^--N production evident in this study is consistent with anammox activity (Lagone *et al* 2014). The production of little nitrate indicates that the ammonium and nitrite removed, were converted to nitrogen gas, seen as bubbles in the EBBR. For example, in B2 (Fig.3) out of the 367 (NH_4^+-N), only 11 mg L^{-1} of nitrate was produced. These results are comparable to those obtained by Speth *et al.* (2016) in which an average of 41 mg L^{-1} (nitrate) was produced from 266 mg L^{-1} of ammonium. This study (Speth *et al.* 2016), seems to support that the results obtained using the EBBR were due to anammox bacterial activity; demonstrating the possibility of using the EBBR for anammox process in future wastewater treatment, that is both cost effective and environment friendly (Rodriguez-Sanchez *et al.* 2014).

Although anammox bacteria have slow growth rate, there was a possible inhibition of anammox activity by NO_2^--N accumulation in this study, as has previously been reported by van Hulle *et al.* (2010), which will be addressed in the next phase of this work.

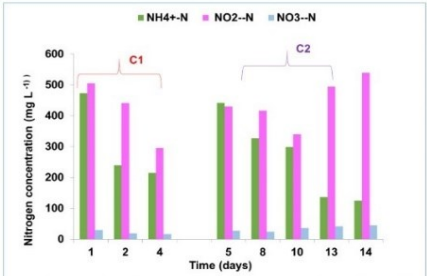

Figure 4: Outlet N- concentration in continuous culture s (C1 & C2).

Keys	Days	NH_4^+-N	NO_2^--N	NO_3^--N	N-balance	Av. NRR (kg-N $m^{-3}d^{-1}$)
C1:	1- 4	484-214	505-295	30-16	97-56	0.54
C2:	4 -14	442 -125	430-520	27-36	87-72	0.62

Conclusion

There is evidence of anammox activity in the EBBR displayed by the simultaneous consumption of NH_4^+-N and NO_2^--N and minimal NO_3^--N production. Findings from this work have:

- demonstrated the potential of using EBBR technology for denitrification through the anammox process;
- revealed the operating conditions of 30°C, pH 8 is important to maintain anammox in the EBBR and
- Identified inhibition of anammox activity by NO_2^--N accumulation.

Further work will be undertaken to identify:

- the threshold concentration of nitrite inhibition;
- conditions that allow full development of bioparticles and
- key microbial communities within the EBBR by molecular techniques.

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P2: Poster presentation at research conference, MMU September 2016

Application of ABDITE® support for nitrification for Anammox activity in Expanded Bed Biofilm Reactor (EBBR)

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Introduction

ABDite® is a porous carbon, biomass support material ideal for use in expanded bed biofilm reactors (EBBRs) **[1].** ABDite® has surface pores protected from hydrodynamic shear and, are more easily colonized by bacteria than the exposed surface of solid materials, such as sand. Growth beyond the pores leads to the formation of an enveloping biofilm and, then, "bioparticles" **[2].**

The Anammox denitrification process involves several bacteria including Ammonia oxidizing bacteria (AOB) Anammox bacteria and Comammox bacteria **[3].** The hybrid Anammox process is inhibited by many factors, which hinders the process improvement in continuous culture bioreactors **[4].**

Anammox process has been established in several types of fixed film bioreactors, but not fully established in EBBRs.

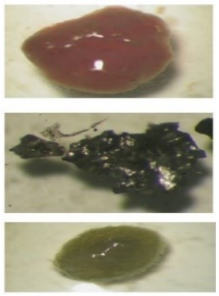


Figure 1 Phase contrast image of (a) anammox bacteria, (b) ABDite® granules and (c) ammonia oxidizing bacteria bioparticles for nitrification

Aim

To establish Anammox activity in lab scale EBBR using high strength Nitrogen synthetic waster

Methods

A lab-scale EBBR (700 cm^3) established with ABDite® particles (0.7 – 1.0 mm diameter) and seeded with Anammox granules from an active wastewater treatment plant.

To simulate high-strength N wastewater, the EBBR was feed with NH_4^+-N and NO_2^--N in a ratio of 1:1.3 (NH_4^+-N : NO_2^--N); maintained in continuous culture.

Scanning Electron Microscopy (Supra 40VP) images were obtained from particles harvested from the EBBR

Bacterial DNA were extracted from the ABDite® particles using species-specific PCR, Sanger sequencing of products and BLAST analysis.

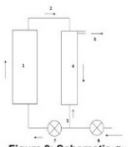


Figure 2: Schematic diagram of Lab-scale EBBR

Figure 3: ABDite particles from EBBR-Anammox process coated with a thin layer of Au/Pd metal using a sputter coater (Manufacturer: Polaron; Model: SEM Coating System; Target: Au)

Results and Discussion

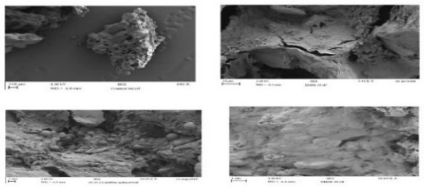


Figure 4: SEM image of anammox bacteria biofilm on ABDite particle a:uncolonised ABDite; b: thin biofilm; c: partially formed biofilm; d: fully formed biofilm

Results and Discussion

Following 60 weeks of continuous culture, layers of brick-red bioparticles consistent with Anammox and nitrifying bacteria bioparticles developed in the EBBR (Fig.5). The EBBR-Anammox process achieved 44 to 96 % maximum nitrogen-removal, at a rate of 1.9 to 6.8 Kg N/$m^{-3}$$d^{-1}$ (Fig.6); NH_4-N and NO_2-N consumption with minimal NO_3-N production (Fig7), which indicate the presence of Anammox activity.

SEM analysis revealed bacterial attachment in the pores of the particles (Fig.4); however, few particles were completely enveloped by biofilms to form bioparticles.

Bacterial DNA was successfully extracted from the ABDite® particles; using species-specific PCR, Sanger sequencing of products and BLAST analysis, (*Nitrosomonas sp.* clone A28 and clone M22 (99 – 100 %), *Nitrobacter* sp. clone BR_02, *Nitrobacter* sp. FGPS_EFE_3b (75 -99 %), Candidatus Kuenenia, Candidatus Brocadia clone ANA19042 (97 -99 %) and *Nitrospira inopinata)* (Fig.8) were identified.

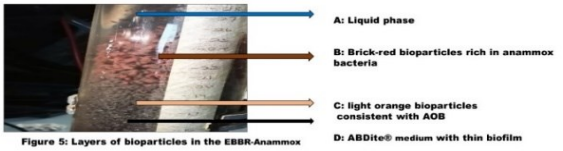


Figure 5: Layers of bioparticles in the EBBR-Anammox

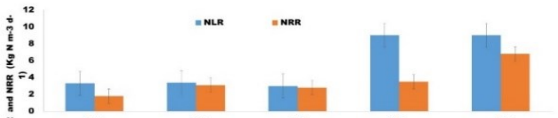
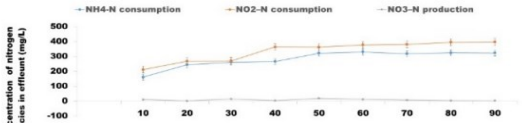


Figure 6: Nitrogen loading (NLR) and removal rate (NRR) (Kg N m-3 d-1). Blue (NLR) and orange (NRR)



Concentration (mg L-1) of nitrogen species in EBBR-Anammox process effluent. Blue (NH4+-N); orange (NO2- -N); grey (NO3- -N).

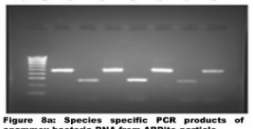


Figure 8a: Species specific PCR products of anammox bacteria DNA from ABDite particle. *Lane* 1:100 bp hyper ladder; 2, 4, 6 & 8: Amx368F/ Amx820R (*Brocadia & Kuenenia*); 3,5 & 7: Brod541F/ Amx820R (*Brocadia & Scalindua*)

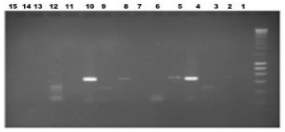


Figure 8b: Species specific PCR products of anammox bacteria DNA from ABDite particle *Lane* 1: 100 bp hyper ladder; 2, 8 & 15: empty; 3 & 9:Amx368F/ Amx820R (*Brocadia & Kuenenia*); 4 &10: Brod541F/ Amx820R (*Brocadia & Scalindua*); 5 & 11: amoA-1F and amoA-2R (*Nitrosomonas*); 6 & 12: FGPS 872 and FGPS 1269 (Nitrobacter); 7 & 13: Nino_amoA_1/ Nino_amoA_252R 9F *Nitrospira inopinata*); 14: Negative control

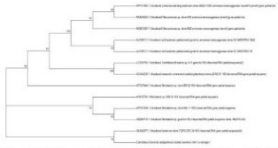


Figure 9: Phylogenetic tree of EBBR-Anammox bacteria The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The bootstrap consensus tree inferred from 500 replicates [3] is taken to represent the evolutionary history of the taxa analysed.

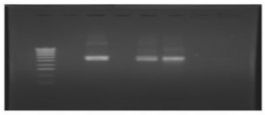


Figure 8c:Repeated *Nitrospira inopinata* PCR products. *Lane* 1:100 bp hyper ladder

Conclusion

- There is evidence of active Anammox bacteria in the lab scale EBBR
- Conditions in the EBBR have not been optimized to ensure complete biofilm formation.
- Further work is required to optimize the Anammox activity in the EBBR.

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[2]. Akhidime, I.D. and Dempsey, M.J. (2009) Influence of biofilm thickness on ammonia oxidation rate of bioparticles from expanded bed process for tertiary nitrification. IWA Nutrient Removal conference, Krakow, Poland, Sept. 2009.
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P3: Poster presentation at ASM Early Career Conference at Trinity college Ireland June,2019

Research output 2- Oral presentation at conference

Establishing anaerobic ammonia oxidation (anammox) process in an expanded bed biofilm reactor (EBBR).

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ABSTRACT

Establishing anaerobic ammonia oxidation (anammox) process in an expanded bed biofilm reactor (EBBR).

Anyanwu, C. C., Akhidime I.D. and Dempsey M.J

Anaerobic ammonium oxidation (anammox) is a denitrification process in which nitrite and ammonia are directly converted to nitrogen gas by bacteria genera (*Brocadia, Kuenenia, Jettenia, Anammoxoglobus and Scalindua).* Application of anammox process in wastewater treatments reduces energy consumption (60%) and carbon dioxide emission (90%).

Bioreactors designed for wastewater nitrification include sequencing batch (SBR), membrane bioreactor (MBR) and expanded bed biofilm reactor (EBBR). Despite the slow growth rate (0.33 d^{-1}) of anammox bacteria, this process has successfully been established in MBR and SBR (Lotti et al., 2015) but not in EBBR systems.

The aim of this research is to establish the anammox process in EBBR using ABDite® support medium. Anammox is dependent on an initial aerobic ammonia oxidation to nitrite; resulting in a complex interplay of conditions (temperature, pH) required for a successful anammox process.

In this work, anammox granules from a municipal plant were inoculated into a lab scale EBBR, fed continuously (9 months) with synthetic wastewater (NH_4^+-N (300 ± 20 mg L^{-1}) and (NO_2^{-1}-N (400 ± 20 mg); controlled at pH 8.1- 8.3, 30 °C and loading rate (3.0 – 9.8 kg N m^{-3} d^{-1}). Evidence for anammox activity were development of brick-red anammox-rich bioparticles, gas evolution, simultaneous decrease in [NH_4^+-N] and [NO_2^{-1}N], negligible [NO_3^{-1}-N] production and 4.9 kg N m^{-3} d^{-1} nitrogen removal.

Although ABDite® support medium biofilm colonization is slow; these findings give evidence for the establishment of the anammox process in EBBR.

Future studies include identification of the presence of species DNA (PCR), and optimum conditions for full-scale EBBR- anammox process.

Content

- Anammox
- Anammox reactors and n
- Microorganisms involved
- Advantages of anammox process and EBBR
- Experimental set up
- Results
- Discussion and conclusion
- Further work

Anammox

- Anammox stands for anaerobic ammonium oxidation
- Anammox is a wastewater treatment (WWT) process that converts ammonia to nitrogen gas in 2 steps
- Nitritation: ammonia oxidizing bacteria (AOB) partially convert ammonia (50 %) into nitrite
- Denitrification: anammox bacteria (AnAOB) convert remaining ammonia and nitrite into nitrogen gas.

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Anammox

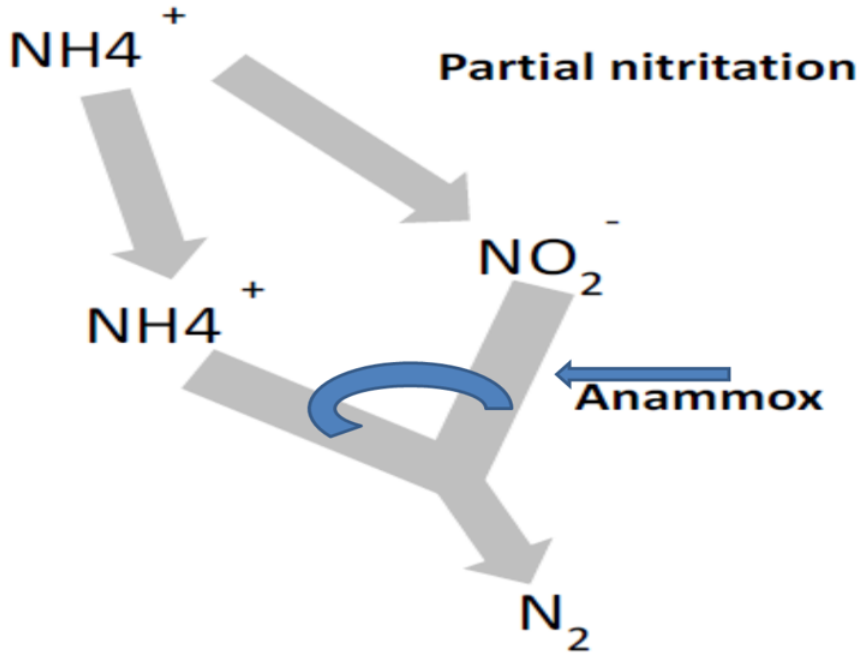

Fig 1.1 Simultaneous partial-nitrification and anammox process modified (Rodriguez-Sanchez et al., 2014)

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Anammox organisms

- Microorganisms involved in the anammox belong to two groups
- Ammonia oxidizing bacteria (AOB) such as *Nitrosomonas* and *Nitrosospira*
- Anaerobic ammonia oxidizing bacteria (AnAOB) of the genera
  - *Brocadia*
  - *Kuenenia*
  - *Jettenia*
  - *Anammoxoglobus and*
  - *Scalindua*

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Co-existence of anammox microorganisms

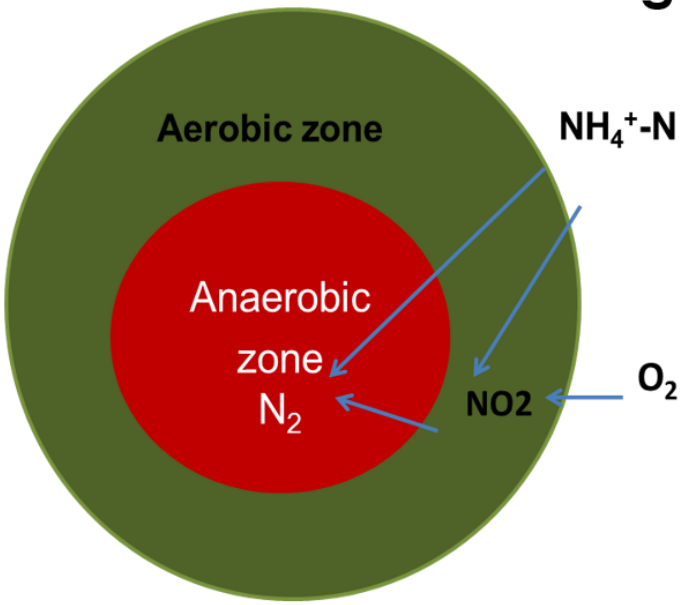

Figure 1.2 Schematic diagram of anammox granule showing co-existence of ammonia oxidizing bacteria (AOB) in the aerobic zone and anammox bacteria (AnABO) in the inner anaerobic zone (adapted from Sanitas, 2013).

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Advantages of anammox

- **Anammox is an essential wastewater treatment process because**

  high NH_3 concentration in discharged effluents is

  - **toxic to aquatic life**
  - **places a high oxygen demand on receiving waters**
  - **leads to eutrophication in water bodies.**

- **Application of anammox in wastewater treatments:**

  - **completely removes ammonia**
  - **eliminates carbon source requirement**
  - **reduces**
    - *** energy consumption by 60%**
    - *** carbon dioxide emission by 90%**
    - *** oxygen requirement by 50 %**

Advantages of (EBBR)

- High biomass concentrations are achieved within the reactors (over 40 Kg m^{-3})
- The biomass is retained in the reactor, as the bulk fluid flows through the bed
- Eliminates recovery of biomass before effluent discharge
- Large surface area for reaction (up to 3 000 m^2 m^{-3}).

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Advantages of EBBR-Anammox process

- Expanded bed biofilm reactor (EBBR) technology is a process intensification method that can result in 10-fold increase in volumetric rate of reaction.
-
- This improved rate leads to more compact bioreactors with a smaller footprint and lower capital cost and EBBR technology has been applied successfully to wastewater nitrification systems.
- The nitritation–anammox process is reported to be a good low energy consumption N-removal process, achieving up to 50% less oxygen consumption saving on nitrification without requiring a carbon source for denitrification.
- The anammox process is 60% more energy efficient than the conventional activated sludge system for N-removal.

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Aim of study

- The aim of this research is to establish the anammox process in EBBR using ABDite® support medium.
- Anammox is dependent on an initial aerobic ammonia oxidation to nitrite; resulting in a complex interplay of conditions (temperature, pH) required for a successful anammox process.

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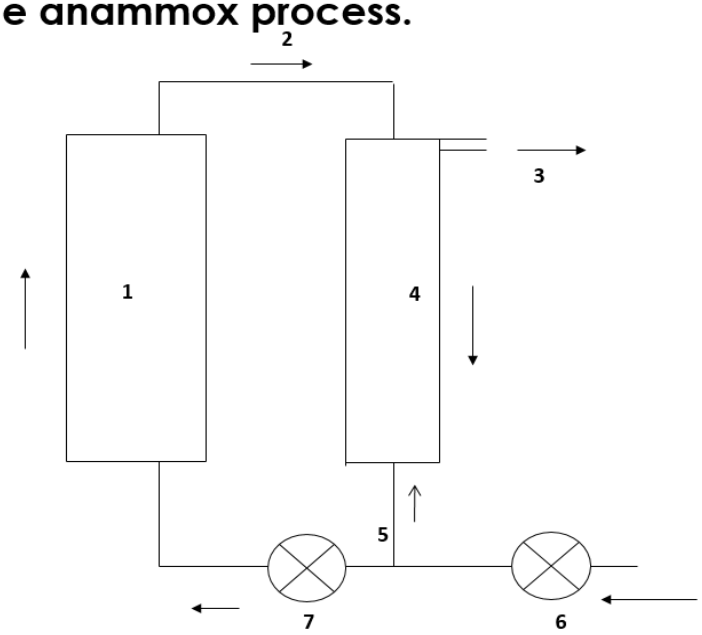
Experimental set up

- **Anammox granules from a municipal wastewater treatment plant were inoculated into a lab scale EBBR**
- **Fed continuously for 9 months with synthetic wastewater (NH_4^+-N (300 ± 20 mg L^{-1}) and (NO_2^{-1}-N (400 ± 20 mg)**
- **Controlled at pH 8.1- 8.3, 30 °C and loading rate (3.0 – 9.8 kg N m^{-3} d^{-1}).**
- **Dissolved oxygen controlled by nitrogen gas injection through the air-inlet into the recirculation column.**

Experimental set up

- We designed a lab –scale expanded bed bioreactor for the anammox process.



1 – Expanded bed
2 – Recycle
3 - Outlet
4-Recirculation column
5 - Air inlet
6 - Feed pump
7 - Fluidizing pump

Figure 2.1 Schematic diagram of Lab-scale expanded bed bioreactor

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Sampling and analysis

Sampling

-a 5 mL syringe was used to collect 2 cm^3 inlet and outlet samples at sampling points

- **The specific denitrification rates**
  - concentrations of NH_3, NO_2 and NO_3 measurements was by spectrophotometric methods (ISO-7150-1:(1984),ISO-6777 (1984) and ISO-7890-3 (1988))

- **The image of bioparticles**
  - using a phase contrast light microscope

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Results and discussion

- Evidence for anammox activity were

- development of brick-red anammox-rich bioparticles

- gas evolution

- simultaneous consumption of [NH_4^+-N] and [NO_2^{-1}N] and negligible [NO_3^{-1}-N] production

- 1.8 – 5.0 kg N m^{-3} d^{-1} nitrogen removal rate (NRR)

Table 1: Nitrogen loading (NLR) and nitrogen removal rate (NRR)

Time (days)	NLR Kg N m^{-3} d^{-1}	NRR Kg N m^{-3} d^{-1}
157	3.3	1.8
158	3.3	2.0
167	3.4	3.1
168	3.4	3.0
187	3.0	2.8
188	3.0	2.5
194	3.2	2.7
196	3.2	3.2
198	9.0	3.5
199	9.0	5.3
200	9.0	6.8

Nitrogen loading (NLR) and nitrogen removal rate (NRR)

• The nitrogen removal rate (NRR) of the anammox process is directly influenced by the loading rate (NLR) nitrite ($NO_{2)}$ concentration and biomass (microbial concentration.

• In this work, increase in NLR led to increase in NRR

• High nitrogen loading rate causes
  - Nitrite accumulation in the EBBR
• NO_2 concentration > 200 mg L^{-1}
  - inhibits anammox bacterial activity
  - decrease in NNR

•Nitrite concentration has to be controlled in EBBR-Anammc process.

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Nitrogen loading (NLR) and nitrogen removal rate (NRR)

- Low nitrogen loading rate (NLR) causes

- nutrient limitation

- reduces

*growth rate of anammox bacteria (have very slow growth rate)

*microbial (biomass) concentration

*nitrogen removal rate (NRR)

Development of bioparticles in EBBR – Anammox process

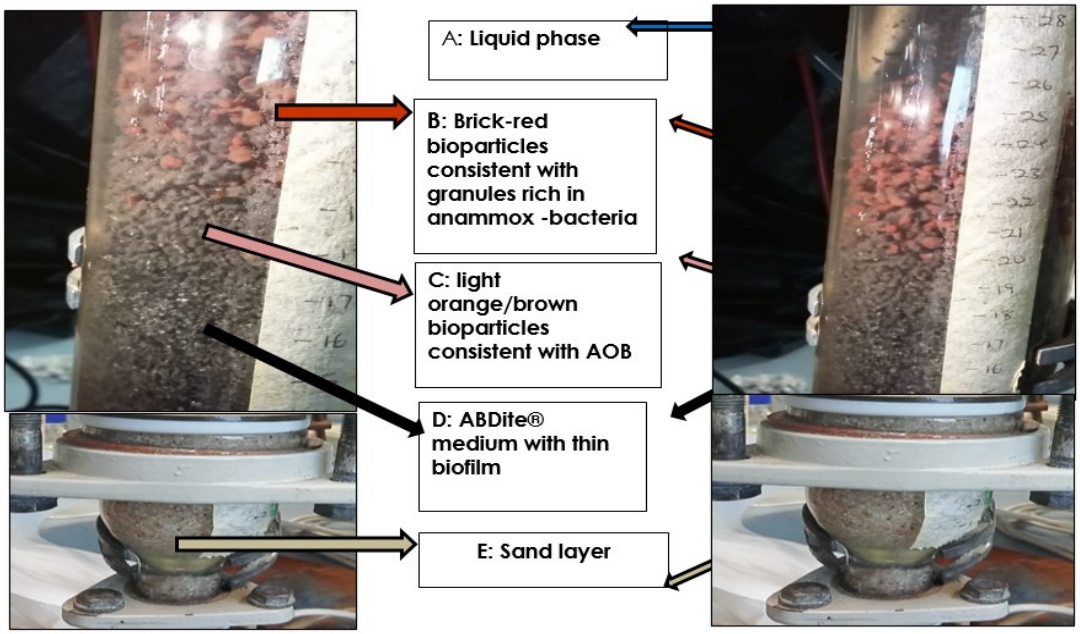

Figure 3:1 Layers of bioparticles in the EBBR-Anammox

Gas evolution in EBBR-Anammox process

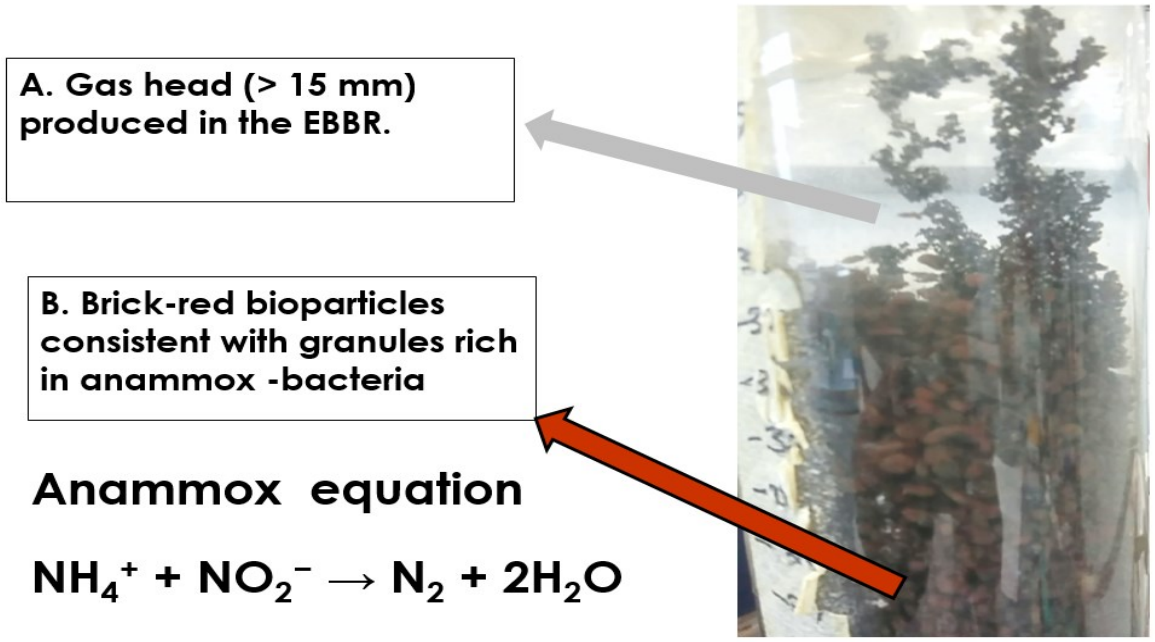

Figure 3:2 Gas production in the EBBR-Anammox process

% NH_4-N & NO_2-N consumption and NO_3-N production

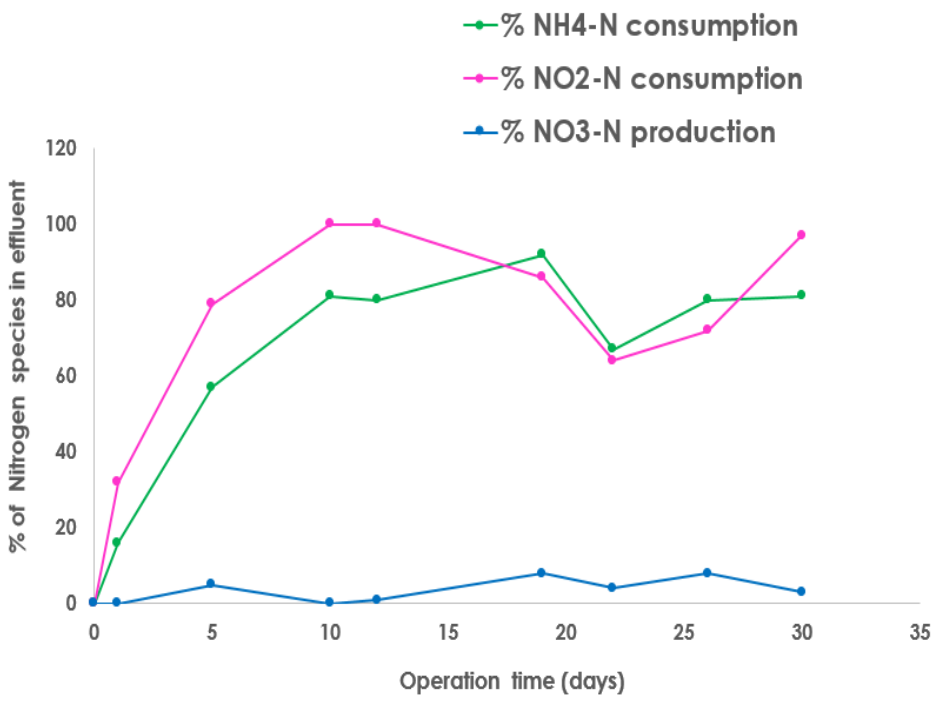

Figure 3.3: Percentage (%) of nitrogen species in the EBBR-Anammox process. . Green (NH_4^+-N), pink (NO_2^--N) blue (NO_3^--N).

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Table 2: % NH_4-N & NO_2-N consumption and NO_3-N production

Time (days)	% NH_4-N consumption	% NO_2-N consumption	% NO_3-N production
0	0	0	0
1	16	32	0
5	57	79	5
10	81	100	0
12	80	100	1
19	92	86	8
22	67	64	4
26	80	72	8
30	81	97	3

- Simultaneous NH_4-N & NO_2-N, and minimal NO_3-N production
- NO_3-N production 0 - 8 %
- Denitrification rate was > 90 %

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% NH_4-N & NO_2-N consumption and NO_3-N production

- Anammox process removes 90% of wastewater ammonia, with 10% converted to nitrate (Langone *et al* 2014).
- In EBBR-Anmmox process there were
  - simultaneous consumption of NH_4-N (& NO_2-N (sometimes, 100 %)
  - minimal nitrate (NO_3-N) production (1 - 8 %)
- Denitrification rate was > 90 %

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Conclusion

- The results showed ABDite® support medium biofilm colonization is slow.
- Findings give evidence for complete establishment of the anammox process in EBBR.

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Further work

- These findings highlight the potential of the lab scale EBBR-anammox process for wastewater treatment
- PCR amplification and sequencing of DNA (next gen) of species in the EBBR-anammox.
- Investigation of optimum conditions for pilot and full-scale EBBR- anammox process.

Manchester Metropolitan University

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Establishing the anammox process in EBBRs.

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Keywords

Anaerobic bioreactors; Biofilms; Denitrification; EBBR bioreactors, Nutrient removal; Wastewater treatment

Abstract

Anaerobic ammonium oxidation (anammox) is a denitrification process in which nitrite and ammonia are directly converted to nitrogen gas by bacteria genera, including Brocadia, Kuenenia, Anammoxoglobus and Scalindua). The application of the anammox process in wastewater treatments reduces energy consumption (60%) and carbon dioxide emission (90%). Bioreactors designed for conventional wastewater nitrification include sequencing batch (SBR), membrane bioreactor (MBR). This work investigates the potential for establishing the anammox process in expanded bed biofilm reactors (EBBR's). Anammox granules from an active wastewater treatment plant were seeded into a lab scale EBBR and maintained using synthetic wastewater (300 mg L^{-1} [NH_4^+-N] and 400 mg L^{-1} [NO_2^{-1}-N]) to support the growth of anammox bacteria at pH 7.7 - 8.3 and 30 ° C. Despite the slow growth rate of anammox bacteria, there was evidence of anammox activity and the establishment of early-stage biofilm formation on the pores of the ABDite ® support medium used in this investigation.

1.0 Introduction

Anaerobic ammonium oxidation (anammox) is a denitrification process in which nitrite and ammonia are directly converted to nitrogen gas by bacteria genera, including Brocadia, Kuenenia, Anammoxoglobus and Scalindua). The application of the anammox process in wastewater treatments (WWT) is reported to achieve a 60 % and 90 % reduction in energy consumption and carbon dioxide emission respectively (Du *et al.*, 2015). Anammox reactors also eliminate the requirement of a carbon source in Nitrogen removal (N-removal systems (Persson *et al.*, 2014) and reduces the oxygen requirement of bioreactors by 50 % (Carvajal-Arroyo *et al.*, 2016). This is therefore an efficient means of achieving nitrogen removal from nitrogen rich wastewaters (van Hulle *et al.*2010).

Expanded bed biofilm reactors (EBBRs) have been successfully applied to wastewater nitrification systems (Dempsey *et al.*, 2005; Dempsey *et al.*, 2006). The novel EBBR technology are able to achieve large surface area for reaction (up to 3 000 $m^2 m^{-3}$) in EBBR's in compact bioreactor systems achieving high biomass concentrations within EBBR reactors (over 40 Kg m^{-3}). EBBR's also effectively retain active biomass in the reactor leading to a process intensification and a low footprint (Dempsey *et al.*, 2005; Dempsey *et al.*, 2006)

Anammox bacteria are reported to have slow growth rates (0.33 d−1) (Suneethi *et al.*, 2014). Despite the slow growth rate anammox process has successfully been established using several WWT technologies including Sequencing batch reactor (SBR), Membrane biofilm reactor (MBR) and Internal-loop-airlift bioparticle reactor (ILAB), (Abbas *et al.*, (2015); Langone *et al.*, (2014); Van Hull *et al.*, (2010)). However, the anammox process has not yet been investigated using the expanded bed biofilm reactor (EBBR) despite reported the simplicity and energy-efficiency advantages of this technology. This work investigates the potential establishment of anammox activities in the novel EBBR system.

2.0 Methods

2.1 EBBR design and operation

A scale expanded bed biofilm reactor (EBBR) was adapted from EBBR for nitrification comprised of two connected vertical columns; a 0.7L expanded bed column and a recycle column connected by tubing and sealed to maintain anaerobic conditions and continuous culture (Fig. 1).

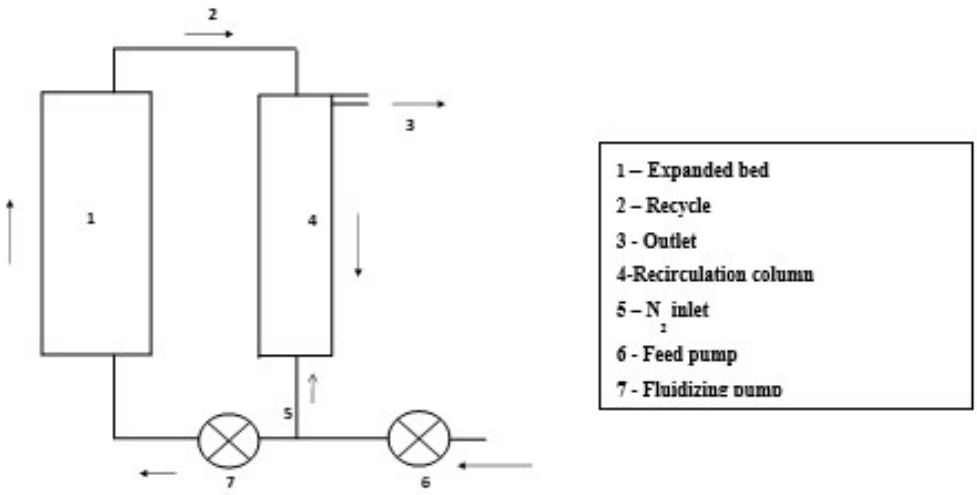
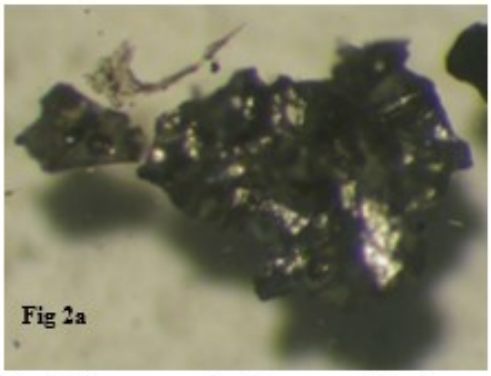


Fig 1 Schematic diagram of Anammox EBBR (adapted from EBBR for established for nitrification of wastewaters)

2.2 Seeding the anammox EBBR

ABDite® biomass support particles (ABD Ltd., Manchester, UK), (0.7 - 1.0 mm) were rinsed with water and 100 cm^3 particles placed into the expanded bed column of the EBBR. The expanded bed column was then seeded with anammox granules (5 cm^3) from an active wastewater treatment plant in the UK (Fig 2)



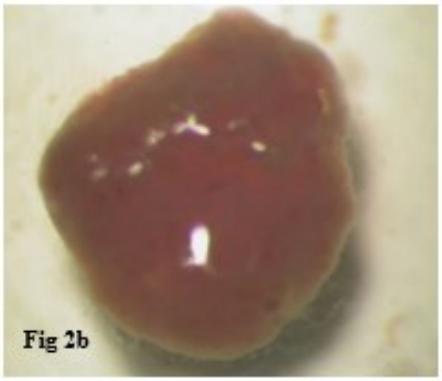


Fig 2 Phase contrast image of (a) uncolonized ABDite® granule (b) Anammox granules form wastewater treatment plant seeded into the lab scale EBBR

The bioreactor was maintained at 50 % bed expansion, 1 cm s^{-1}upward velocity with synthetic wastewater (SWW) (300 mg L^{-1} [NH_4^+-N] and 400 mg L^{-1} [NO_2^{-1}-N]) to support the growth of anammox bacteria. The anammox operation conditions were maintained at 30 °C using a thermocirculator (Thermo Haake K10, USA) and pH 7.7 – 8.3 using a pH controller (Electrolab 260, UK) and the EBBR was maintained in continuous culture for nine months with synthetic wastewater

Anaerobic growth conditions was encouraged in the lab scale EBBR by bubbling nitrogen through the recycle column to strip away dissolved oxygen from the recycling wastewaters before it arrives at the fluidizing point at the expanded bed (Fig 1).

2.4 Chemical analysis

The ammonia [NH_4^+-N], nitrite [NO_2^--N] and nitrate [NO_3^--N] concentrations were determined using the standard method ISO 7150-1:1984; ISO 6777-1984 and (APHA 4500-NO_3^-) respectively and based on spectrophotometric analysis.

The Nitrogen removal rate (NRR) was calculated to determine the anammox activity and involved determining the [NH_4^+-N], [NO_2^--N] and [NO_3^--N] in both the inlet and outlet points of the bioreactor. The simultaneous consumption of NH_4^+-N, NO_2^--N with low of NO_3^--N production is an indication of anammox activities in the bioreactors ((Trigo *et al.,* 2006; Van der star *et al.,* 2008)).

2.5 Snapshot anammox activity investigation

A 30-day snapshot investigation to highlight anammox activity (consumption of NH_3-N, NO_2-N and production of NO_3-N) was set up. At day 0, the anammox EBBR was flushed with SWW simulate a freshly started EBBR system conditions with 321 mg L^{-1} [NH3-N], and 410 mg L^{-1} [NO_2-N and 0 mg L^{-1} [NO_3-N] influent SWW to the EBBR system. The EBBR was maintained at pH 8.1 – 8.3, 30 °C.

3.0 Results and Discussion

The NRR of the anammox process is directly influenced by the NLR, [NO_2^--N] and microbial biomass available for reaction (Jin *et al.,* 2012; Suneethi *et al.,* 2014). The anammox EBBR achieved NRR of (0.5 - 6.1 kg N m^{-3} d^{-1}) at operation conditions of 1.2 - 3.6 d^{-1} dilution rate (D), 0.3 - 0.9 d^{-1} hydraulic retention time (HRT) and 1.1- 3.9 Kg N m^{-3} d^{-1}nitrogen loading rate (NLR).

Clear evidence of anammox activity in the bioreactor was shown as there was simultaneous consumption of NH_4-N and NO_2^--N with minimal production of NO_3^--N. The bioreactor attained maximum anammox activity with 81 % NH_3-N and 100 % NO_2^--N consumption on day 10 and minimum anammox activity with 67 % NH_3-N and 64 % NO_2^--N on day 22 (Fig 3). However, the reduction in activity was due to some maintenance work carried out on the reactor equipment.

Another outcome revealed by this investigation is a strong indication that for anammox EBBR reactors, anammox activities are affected than ammonia oxidizing activities when reactor dynamics are disturbed. The anammox activity steadily increased to 80% and 72 % for NH_3-N and 64 % NO_2^--N consumption respectively by day 26 an indication of a more robust ammonia oxidation activity than anammox activity in the developing biofilms. Therefore, [NO_2-N] production has to be carefully monitored in anammox EBBR process. During the 30-day investigation, [NO_3^--N] production was maintained at 1- 8 % (Fig 3). Langone *et al.,* (2014) reported anammox activity can achieve 90% of ammonia removal in wastewater with only a 10 % conversion of nitrite to nitrate.

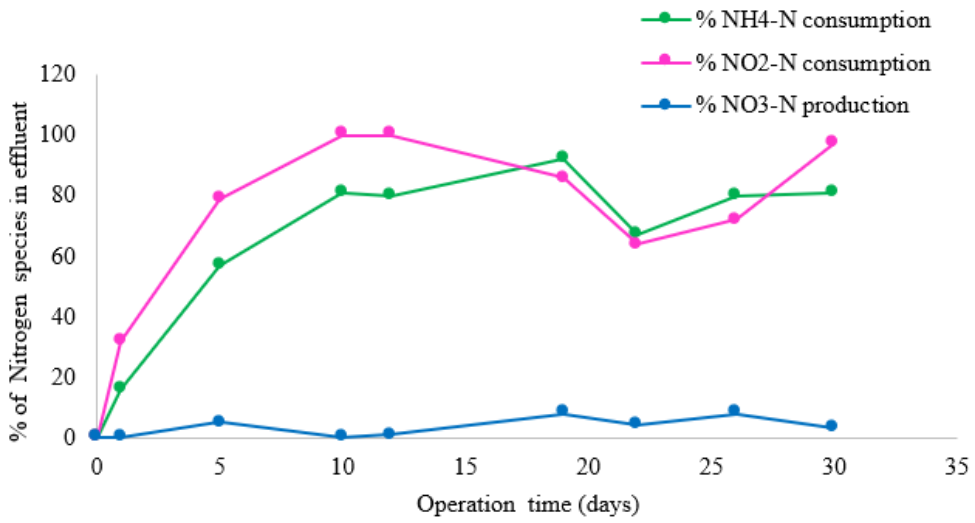


Fig 3 Anammox activity from 30- day snapshot investigation to highlight anammox activity showing of [NH_3-N], [NO_2^--N] consumption and production of NO_3^--N in lab scale anammox EBBR.

Other evidence of anammox activity in the lab scale EBBR include the development of brick-red anammox-rich bioparticles (Fig 4) and the production of gas on the surface of the ABDite® particles leading to the suspension of ABDite® above the surface of the expanded bed biomass (Fig 4). The gas produced within the ABDite® particles in the anammox bioreactor is a strong indication of the establishment of biomass within the pores of the particles. The biomass eventually grow out of the pores of the support and envelope the entire particles in a complete biofilm forming a bioparticle which is different from the anammox granules used to seed the anammox reactor. Due to the slow growth rate of anammox bacteria, complete colonization of the ABDite® particles was not still evident after nine months of continuous culture of the Anammox EBBR reactor. However, evidence of anammox activity in EBBR's was evident

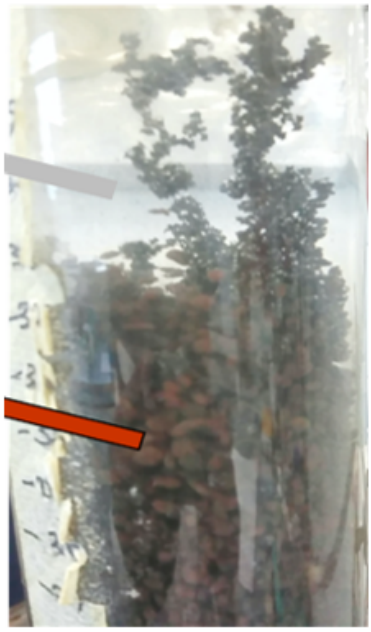


Fig 4 Lab scale anammox EBBR showing the suspension of ABDite® particles above the expanded bed due to the attachment of gas bubbles produced by anammox activity in the bioreactor.

4.0 Conclusion

Overall, the findings from the initial stage of this work have demonstrated the potential of using EBBR technology for denitrification using the anammox process. Although biofilm colonization of the ABDite® support was minimal, these findings give evidence for potential establishment of the anammox process in EBBR. Further studies include identification of anammox species using molecular technique (e.g., PCR) and optimization of process conditions for pilot-scale EBBR- anammox.