Valorisation of Nitrogen-Deficient Wastewater Treatment Systems Using Sludge Enriched with Nitrogen-Fixing Bacteria

PhD

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Declaration

I hereby certify that the work presented in this thesis is my original research work. Due reference is given to literature and any research collaborations where appropriate. No part of this thesis has been submitted previously for a degree at this or any university.

Maria Carolina Ospina Betancourth

Abstract

We live in a world highly dependent on the use of nitrogen fertilisers produced with the Haber-Bosch process (HBP) but their production and deployment are unsustainable. Finding cheaper and greener alternative technologies that can sustain global agricultural production is therefore a research priority. Biological nitrogen fixation (BNF) is a process carried out by some bacteria in which molecular nitrogen (N_2) is converted to ammonia (NH_3) and is an example of sustainable nitrogen fixation. The energy source for biological nitrogen fixation can, in principle, be obtained from industrial or domestic wastewater. In this study a new strategy of nitrogen fixation was developed using bench scale wastewater treatment plants, treating waste with a high carbon-to-nitrogen ratio, to enrich wastewater sludge with N_2-fixing bacteria (NFB). This residual sludge enriched with NFB could contribute to the valorisation of low nitrogen wastewater treatment systems as it can be potentially used as a biofertiliser.

Initially, NFB found in the sludge from industrial and domestic wastewater treatment plants and from an anaerobic digester were used as an inoculum to build replicated nitrogen-fixing sequencing batch reactors (SBR). After 85 days of operation, these reactors were able to fix nitrogen at an average rate of up to 11.8 mg of N L^{-1} day^{-1} and treat the wastewater with a COD removal efficiency of 73%. Additionally, the reactors contained sludge that was enriched with NFB, attaining 13% of the total bacterial population (1:4.2 copies of nifH to 16S rRNA quantified with qPCR).

Though the relationship between nitrogen content and the efficiency of wastewater treatment has been investigated in low nitrogen wastewater, there are no estimates of the inhibition of nitrogen fixation under different ammonia concentrations. Therefore, the nitrogen fixation rates of this sludge enriched with NFB were measured under the effect ammonium in concentrations of up to 78 mg of N L^{-1}. The sludge fed with high C:N wastewater was resistant to inhibition by ammonia with a half maximal inhibitory concentration (IC_{50}) of 54 mg of N L^{-1}. This result suggests that it is reasonable to expect BNF in high C:N wastes that are contaminated with ammonia and that it seems at least plausible that the NFB in the sludge could work as a biofertiliser by fixing nitrogen in soils with modest quantities of nitrogen-based chemical fertilisers.

Finally, the feasibility of using real effluent from pulp and paper mills as a source of carbon to culture NFB in sequencing batch reactors (SBR) was evaluated. Effluent from this industry is produced in large volumes and contains high organic loads with

little to no nitrogen. Four reactors were inoculated with activated sludge enriched with NFB and fed with a high C/N waste (100:0.5) from a paper mill. The reactors were able to reduce the organic load of the wastewater by up to 89% after 114 days of operation but did not have any nitrogen-fixing activity and showed a decrease in the putative number of NFB (quantified with qPCR). Nitrogen fixation was only observed when sucrose in concentration of 3 g L^{-1} was added as a further supplement of carbon source. It is likely that real world biological nitrogen fixation (BNF) will only occur where there is a C/N ratio ≤ 100:0.07. Consequently, operators should actively avoid adding or allowing nitrogen in the waste streams if they wish to valorise their sludge and reduce running costs.

This biotechnology demonstrates the scope and magnitude of nitrogen fixation in wastewaters and provides fundamental insight into a novel green method that can treat pulp and paper mill wastewater using less resources (compared to traditional high C/N wastewater treatment systems) and existing facilities. In addition, this technology has the potential to valorise the sludge from the treatment system by producing NFB which could be used as a sustainable biofertiliser. This investigation sets the basis for future work which should be aimed to demonstrate and quantify the benefits of the nitrogen-fixing sludge as a biofertiliser.

Graphical Abstract

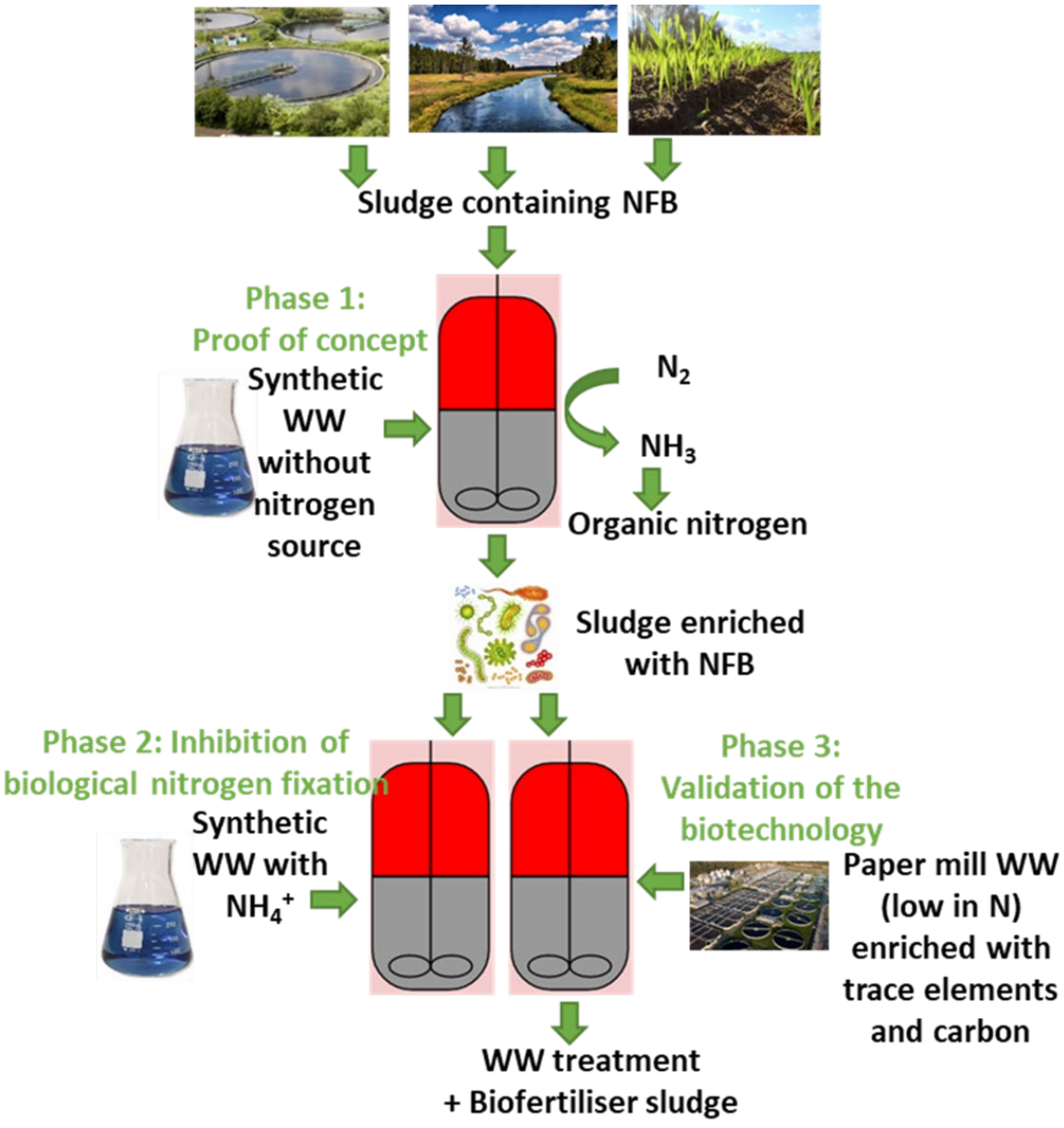

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Table of Contents

List of Figures and Tables

Chapter 1: Introduction

The productivity of many natural ecosystems and the yield of plant biomass in agricultural systems is typically limited by the availability of nitrogen (Elser et al., 2007). This problem was mainly addressed by the industrial “green revolution” at the beginning of the 20th century which was based on using improved plant varieties with high doses of anthropogenic fixed nitrogen added to the soils (Olivares et al., 2013). Anthropogenic nitrogen fixation is made through the Haber-Bosch process (HBP), which has been considered by some as the most important invention of the 20th century (Smil, 1999). The HBP helped to meet global food demands and sustained a 375% increase of the world´s population over the last 110 years (Olivares et al., 2013). However, this process is arguably unsustainable as both the production and use of chemical fertilisers have serious environmental drawbacks. Finding sustainable methods for alternative fertiliser production is correspondingly important.

Nitrogen is an essential chemical element that aids in the formation of amino acids and nucleic acids in living organisms. Bacteria, archaea, and plants use nitrogen compounds such as nitrites, nitrates and ammonia, for cellular growth and reproduction. Pure molecular nitrogen (N_2) is the most common and abundant nitrogen source composing 78.2% of the atmosphere (Lindström et al., 2015). N_2 is very stable because of the high strength of the triple bond, therefore, it is unavailable to most living organisms. The exception being those bacteria and archaea that can carry out biological nitrogen fixation (BNF) by using the enzyme nitrogenase (Bernhard, 2010).

BNF is a remarkably energy-demanding natural process in which N_2 is reduced to ammonium (NH_4^+) or ammonia (NH_3) (Olivares et al., 2013), therefore, N_2-fixing bacteria (NFB) would normally only fix this abundant gas if there were no better, more readily available sources (Burris and Roberts, 1993). NFB are a diverse group of bacterial and archaeal species that include Proteobacteria (Reid et al., 2008), Methanogens (Collins et al., 2016), glycogen- accumulating and polyphosphate-accumulating bacteria (Guo et al., 2017). Free-living and symbiotic NFB have been found in natural terrestrial and aquatic ecosystems and are used as biofertilisers because they can improve nitrogen uptake in plants, enhancing the productivity of agricultural ecosystems (Smercina et al., 2019; Bhattacharjee et al., 2008). Ammonia, which is one of the direct products of BNF,

can inhibit the nitrogenase enzyme (Dixon and Kahn, 2004). The use of nitrogen fertilisers for agriculture has particularly decreased naturally occurring BNF in terrestrial (Fan et al., 2019; Galloway et al., 2004; Smercina et al., 2019) and aquatic (Herbert, 1999; Sohm et al., 2011) ecosystems.

From an industrial perspective, BNF can be very expensive if the source of carbon is costly (Cherkasov et al., 2015). Nevertheless, if the source of carbon is an industrial waste by-product, such as paper mill effluent, then BNF could be considered as low cost and sustainable. Wastewater from the pulp and paper mill industries contains a high load of carbon and is usually low in ammonia and phosphorus (Ashrafi et al., 2015). Therefore this type of wastewater are typically supplemented with nitrogen and phosphorus to ensure adequate biological treatment but nutrient supplementation is expensive and difficult to control. In 2017 the world paper production surpassed the 781 million tons (FAOSTAT, 2017). The paper manufacturing technology uses between 10 and 100 litres of water for each ton of paper produced (Man et al., 2018; Kamali & Khodaparast, 2015). Aerobic processes are the most popular systems used for the treatment of effluents from the pulp and paper mill industry (Ashrafi et al., 2015). However, this technology produces high volumes of sludge that needs to be dewatered and disposed (Hubbe et al., 2016). Sludge treatment comprises more than half of the overall wastewater treatment costs and produces greenhouse gasses (Meyer et al., 2018).

NFB have been used for ammonia production in bioelectrochemical systems (Liu et al., 2017a; Ortiz-Medina et al., 2019) and have been found in activated sludge wastewater treatments systems (Clark et al., 1997; Gapes et al., 1999; Kargi & Ozmihçi, 2004) treating high carbon-to-nitrogen industrial wastewater. BNF occurs naturally in such treatment plants and is favoured by low oxygen and nitrogen conditions (Slade et al., 2003; Dennis et al., 2004; Slade, Gapes, et al., 2004; Slade, Ellis, et al., 2004). Most studies of BNF within paper mill wastewater treatment systems have focused on nitrogen fixation as a treatment of high carbon-to-nitrogen wastewater (Clark et al., 1997; Gauthier et al., 2000; Kargi & Ozmihçi, 2004; Slade, Gapes, et al., 2004; Welz et al., 2018) but have not recognised these systems as a potential source of NFB for biofertiliser production. In theory, sludge enriched with NFB could be cultured in wastewater

treatment systems and used as a biofertiliser, however, very little research has been carried out on nitrogen-fixing bioreactors and the biomass specific fixation rates of NFB from wastewater sludge are not known. Additionally, although the relationship between nitrogen content and the efficiency of wastewater treatment has been investigated previously in synthetic pulp and paper mill wastewater (Kargi and Ozmihçi, 2004; Slade et al., 2011) there are no estimates of the inhibition of nitrogen fixation under different ammonia concentrations.

This thesis focused on researching nitrogen fixation within bench-scale wastewater treatment plant treating wastewater with a high carbon-to-nitrogen ratio. It aims to develop, validate and characterise the principle of N_2-fixing bioreactors that could valorise organic matter reduced in nitrogen by using it to enrich a wastewater sludge with NFB. Such type of reactor, could be operated in a sustainable way and demand lower operation cost compared to a traditionally reduced nitrogen wastewater treatment system.

To this end, firstly the proof of concept for a new strategy for nitrogen fixation was demonstrated by enriching free-living N_2-fixing bacteria in reactors fed with low nitrogen wastewater (Chapter 3). Secondly, the nitrogen fixation rates of this sludge enriched with NFB were measured under the effect of different concentrations of ammonium in batch reactors with the purpose of estimating the scope of biological nitrogen fixation in wastewater and assessing the potential utility of the sludge as a biofertiliser (Chapter 4). Finally, the practical applications of the proposed biotechnology were explored by evaluating the feasibility of using real effluent from pulp and paper mills as a source of carbon to culture NFB in sequencing batch reactors (Chapter 5).

1.1 Main Objective

Develop, validate and characterise a biotechnology process of nitrogen fixing bioreactors, inoculated with mixed microbial cultures, with potential use as a wastewater treatment system and as a source of nitrogen fixing bacteria that could be used as a biofertiliser.

1.2 Specific Objectives

i. To conduct a literature review on BNF in bioreactors (Chapter 2).
ii. To determine and analyse a priori the likely physical makeup of nitrogen-fixing bioreactors from first thermodynamic principles (Chapter 2).
iii. To propose and evaluate a conceptual design for nitrogen-fixing reactors (Chapter 3).
iv. To evaluate the ability of the sludge enriched with NFB to fix nitrogen under the presence of ammonia (Chapter 4).
v. To evaluate the efficiency of the nitrogen-fixing bioreactors as a paper mill wastewater treatment system (Chapter 5).
vi. To characterise the composition of the microbial mixed culture established in the nitrogen-fixing bioreactors (Chapters 3 and 5).

Chapter 2: Literature Review

2.1. Problems Caused by Anthropogenic Nitrogen Fixation

Over the last century, global nitrogen cycling has doubled. Approximately 413 Tg N_2 are fixed per year (Fowler et al., 2013) and anthropogenic activity is responsible for half of it; 40% of the world population now depends on the nitrogen obtained from the Haber-Bosch process (HBP) (Galloway et al., 2008; Smil, 2004). This process was introduced in 1913. It binds nitrogen with hydrogen at high pressures in steel reactors with iron based catalysts and produces nitrogen fertiliser among other products (Cherkasov et al., 2015). Anthropogenic nitrogen fixation has guaranteed the food security of humans but has also caused serious environmental problems associated with greenhouse effect gasses, eutrophication (Olivares et al., 2013), and soil acidification (Galloway et al., 2003).

The HBP consumes 1% of the world's energy production and releases more than 300 million metric tons of CO_2 (Schrock, 2006; Tanabe & Nishibayashi, 2013). It also uses petroleum-based energy, available data estimates that 1 kg of industrially reduced nitrogen requires 1.5 kg of oil (Lindström et al., 2015). In order to improve the yields of nitrogen fixation on an industrial scale, existing methods for nitrogen fixation are being enhanced using metallo-complex and novel catalysts (Cherkasov et al., 2015).

In developing countries, the problems related to nitrogen fertilisation are worse than in developed countries (Austin et al., 2013) because rural areas do not have basic infrastructure and domestic agricultural wastes are released directly into water bodies. This inappropriate wastewater management causes nitrogen and phosphorus enrichment of natural ecosystems. Affecting trophic interactions (Daniel et al., 2002; Ometo et al., 2000), exacerbating public health risks (Hotez, 2008), and leading to the destruction of diverse ecosystems (Nepstad et al., 2009).

2.2. Biological Nitrogen Fixation (BNF)

The reduction of N_2 to ammonium (NH_4^+) or ammonia (NH_3) by living organisms is a process known as BNF. Under optimal conditions it follows this stoichiometric equation:

Equation 2.1:

$$N_2 + 8\,e^- + 16MgATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16\,Mg\,ADP + 16Pi$$

BNF is a self-regulating process that takes place at ambient temperatures, does not cause problems related to environmental contamination by excess fertiliser run-off and consumes renewable substances like carbohydrates and organic wastes from agriculture and forestry (Cherkasov et al., 2015). Only some groups of bacteria and archaea carrying the enzyme nitrogenase are able to run this process. They are known as N_2-fixing bacteria (NFB) or diazotrophs and can be identified by the presence of the marker gene *nifH* (Gaby & Buckley, 2014). NFB fix nitrogen into ammonia (NH_3^+), a simple inorganic compounds that can be used by plants to produce proteins that can be further consumed by animals and humans.

Most fixed nitrogen is rapidly assimilated into biomass (Gapes et al., 1999). The assimilation of ammonia is a fast process, therefore it will be consumed rapidly once it is fixed. Although it was found that in strains of *Azotobacter spp.* between 7 to 13% of the fixed nitrogen (4 to 14 μg N mL^{-1}) was excreted from the cell ~~and~~ but only a small amount was ammonia (Brotonegro, 1974). The excretion of ammonia into the growing media was not affected by the changes in the concentration of amino acids, amides and ammonia (Brotonegro, 1974). Similarly, it was discovered that the NFB *Lysobacter* sp. had the ability to accumulate up 29 mg NH_4^+ L^{-1} in the culture media (Iwata et al., 2010) and that nitrous oxides can be excreted from the cell (Dennis et al., 2004). In general, the excretion of the fixed nitrogen depends on the type of strain and on the growing conditions.

NFB have a very diverse taxonomy with approximately 222 species of identified culturable bacteria an expanding number of uncultured taxa (Gaby & Buckley, 2014). NFB within the Eubacteria domain include the phyla Firmicutes, Cyanobacteria and Proteobacteria. Some examples of free-living NFB include *Clostridium pasteurianum, Azotobacter spp., Beijerinckia sp., Klebsiella spp., Desulfovibrio sp.*, *Azospirillum* spp., some R*hozobium sp.* and Cyanobacteria such as *Anabaena sp..* In the Archaeal domain, there are several genera that fix nitrogen such as *Halobacterium sp., Methanobacterium sp., Methanococcus sp., Methanolobus sp., Methanoplanus sp., Methanosarcina sp. and Methanothermus sp.*(Stacey et al., 1997; Dixon & Kahn, 2004).

2.2.1. The Enzyme Nitrogenase

BNF is catalysed by a family of enzymes called nitrogenases. The enzyme nitrogenase usually comprises up to 15% of the protein content of diazotrophic cells (Dixon & Kahn, 2004) and powered by ATP and electrons (Equation 2.1). The most commonly studied nitrogenase is the molybdenum-iron (MoFe) dependant nitrogenase (Cherkasov et al., 2015). Vanadium and iron-dependant enzymes have also been discovered (Bellenger et al., 2014).

These enzymes are composed by two proteical subunits: the dinitrogenase-reductase and the dinitrogenase. Each subunit has a metal cofactor depending on the microorganisms it comes from (Lindström et al., 2015). The first subunit is the smaller unit and provides the electrons for electrons to the other unit, which at the same time facilitates the binding of N_2 and reduces it to NH_3 (Burris & Roberts, 1993).

Particularly, the dinitrogenase reductase unit of the MoFe nitrogenase (Figure 2.1) is a dimer and is also known as Iron (Fe) protein or component II. Each subunit of the Fe protein contains a nucleotide ATP/ADP binding site and both are bridged by a 4Fe-4S cluster.

The larger complex of the MoFe nitrogenase is a molybdenum-iron (MoFe) protein also named dinitrogenase or component I. It is a tetramer formed by two alpha-beta subunits. Each subunit functions as a catalytic unit and interacts with one Fe protein (Figure 2.1). The MoFe proteins contains two metal centers: a P cluster (8Fe-7S) and a MoFe cofactor (Dixon & Kahn, 2004).

Nitrogenase in Action

In theory the reduction of a dinitrogen molecule requires a 8 electrons but only one electron is transferred at a time (Cherkasov et al., 2015). The temporary interaction between the two complexes of the nitrogenase is the rate limiting step of the enzyme and is dependent on ATP hydrolysis (Thorneley & Lowe, 1985). The mechanism of action of the enzyme is a reduction chain (right part of Figure 2.1) where an electron donor (such as ferredoxiin and flavodoxin) transfers a single electron to the 4Fe-4S cluster. Which in turn transfers it to the P cluster (Seefeldt et al., 2009). Later, internally in one of the subunits of the MoFe component, the electron is transferred from the P cluster to the MoFe cofactor which is the site where the molecular nitrogen binds and gets reduced (Dixon & Kahn, 2004).

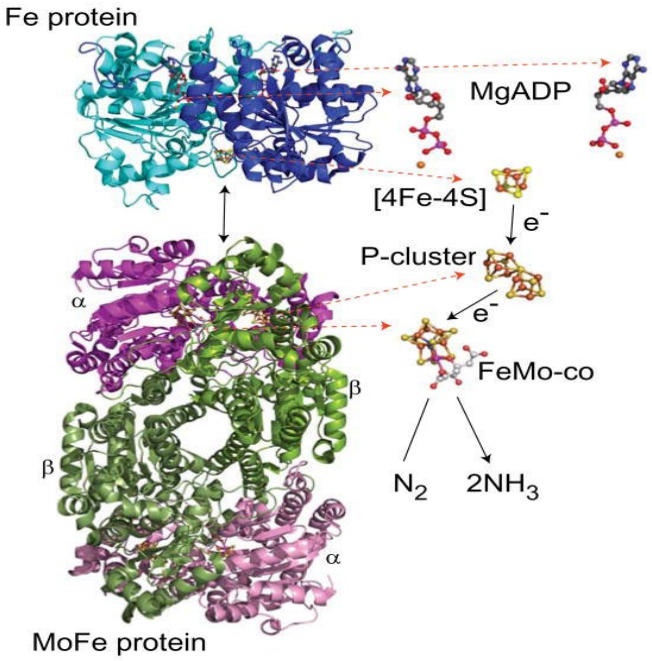

Figure 2.1. Structure of the nitrogenase and electron transfer scheme. Image taken from Seefeldt et al. (2009).

Hydrolysis of MgATP (which is how ATP is found in cells) is also required for BNF and it happens after the electron transfer. The ATP-energy is not used to break directly the strong bond of the dinitrogen molecule but to increase the driving force of the electron transfer between the Fe and MoFe protein complexes (Duval et al., 2013). This is achieved by conformational changes in the interacting complexes (Kurnikov et al., 2001). Although the exact mechanism of action that couples the electron transfer with the ATP hydrolysis has not been completely elucidated yet (Seefeldt et al., 2009).

Environmental Factors Affecting the Nitrogenase Enzyme

The regulation of the enzyme production is diverse, it can be done at both transcription and post-translation levels (Dixon & Kahn, 2004). And is dependent on environmental factors such as temperature, presence of ammonium or oxygen and nutrient availability (Gapes et al., 1999; Shridha, 2012).

Ammonia has a reversible repressing effect on the activity of the nitrogenase enzyme, initially the molecule inhibits the electron transport system to the enzyme and diverts it to the metabolism of ammonia assimilation (Dixon & Kahn, 2004). Furthermore, ammonia can repress the synthesis of the enzyme and trigger nitrogenase degradation when present for more than 4 h (Merrick, 2004). Additionally,

transcriptional or posttranslational regulation of the nitrogen-fixing activity can vary between microorganisms (Smercina et al., 2019).

The presence of molecular oxygen (O_2) destroys nitrogenases and represses its synthesis (Fay, 1992). Therefore, diazotrophs fix nitrogen in anaerobic or micro-aerophilic conditions. NFB have different protecting mechanisms against O_2 including: symbiosis with plants, growth in micro-aerophilic conditions, high respiration rates (causing low biomass growth), conformational protection by protection of the active sites of the enzyme with proteins, exo-polysaccharides production and formation of specialised cells with thick walls (heterocysts) working as vesicles (Bitton, 2003).

2.2.2. The nifH Gene

Diazotrophs are a physiological and phylogenetically diverse group of archaea and bacteria that include anaerobic and aerobic, chemotrophic and phototrophic as well as free-living (Roper & Gupta, 2016) and symbiotic life styles together with other bacteria, plants and algae (Bernhard, 2010). This great diversity is also reflected in the different ecosystem inhabited by NFB, from oceans and rivers to soils. The nitrogenase enzyme complex is encoded by the *nifHDK* genes, but only the *nifH* gene is used as a genetic marker for the identification of NFB. This gene encodes specifically for the nitrogenase reductase component of the enzyme.

The great diversity of NFB is attributed to the lateral gene transfer of the *nifHDK* genes (Raymond et al., 2004); although they are normally transferred vertically. Therefore, the *nifH* gene should be predominantly used to estimate the abundance of nitrogen-fixing bacteria. *nifH* gene sequences should not be used as a unique phylogenetic marker to make taxonomic inferences (Bowers et al., 2008). For example, partial sequences of the *nifH* gene from *Azospirillum brasiliense, Bacillus megaterium and Halomonas mauro* have a 100% homology, but their *16S rRNA* analysis shows that they are phylogenetically distant (Argandoña et al., 2005).

The *nifHDK* genes are highly conserved. And, similarly to the *16S rRNA* gene, the *nifH* gene can be found as a single or multiple copy gene depending on the bacterial species (Hong et al., 2012). All the known diazotrophs have the MoFe nitrogenase,this enzyme havehas a higher specific activity when compared to the alternative nitrogenases (Eady, 2003) and is expressed preferentially when the bacteria contain

more than one isotype of the enzyme. Microorganisms such as Kl*ebsiella pneumoniae*, *Azospirillum brasilense, Gluconacetobacter diazotrophicus* and *Herbasipirillum seropedicae* only contain a single copy of the gene (Hong et al., 2012). Whereas species such as *Paenibacillus durus* (Rosado et al., 1998), *Rhodobacter capsulatus, Desulfobacter curvatus and Clostridium pasteurianum* (Canfield et al., 2005) have multiple gene copies.

It is possible for some bacteria to have the genes for one or more different alternative nitrogenases. These multiple gene copies can be from different phylogenetic origin (Canfield et al., 2005). It is not yet know if all the copies are functional or even if they carry out the same function (Canfield et al., 2005). Wang et al.(1988) suggested that multiple copies could be used as a regulation mechanism of the enzyme's activity under certain ecological conditions.

The diversity of the *nifH* was evaluated by Gaby & Buckley (2014), they constructed a database for the gene with approximately 33,000 sequences. Most of the sequences of the database were obtained from environmental surveys of nifH, although it also contains sequences from bacteria isolates; thus the diversity of diazotrophs is still poorly understood as the majority of the *nifH* sequences do not have representatives isolated through cultivation (Gaby & Buckley, 2011). The aforementioned study also found that most studies of the *nifH* gene are biased towards soil and marine environments, meanwhile micro-aerophilic, anaerobic, fresh water and waste water environments remain poorly characterised.

The variability on the primer specificity together with different PCR reaction conditions can lead to different results of the number copies of the gene *nifH* within the published literature (Gaby & Buckley, 2012, 2017). Gaby & Buckley (2012) analysed different sets of published primers used to amplify the *nifH* gene (Gaby & Buckley, 2011). *nifH* primers are usually designed to be either group-specific (Bürgmann et al., 2004) or universal (Poly et al., 2001). However, they found that most of the universal primers had a unique range of specificities and did not hybridise with the majority of the sequences on the *nifH* database. This type of study is essential for understanding the constraints in data interpreting.

nifH Phylogeny

The gene *nifH* has been clustered into five major homologous groups (I-V). Only, the first three of these groups contain genes encoding for functional enzymes (Figure 2.2) (Raymond et al., 2004; Weber & Legge, 2010). Usually the phylogenies of the other genes that encode for the nitrogenase complex *nifDK*, agree with the *nifH* clusters (Raymond et al., 2004; Young, 2005).

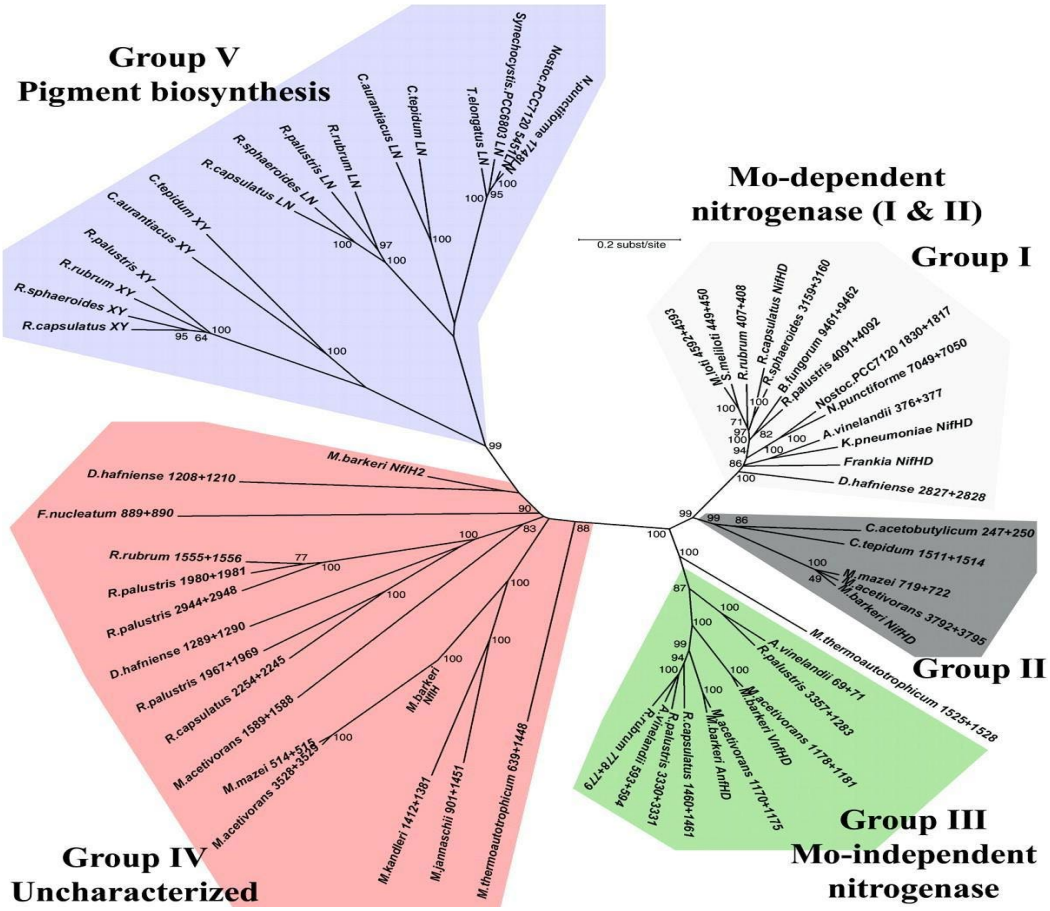

Figure 2.2. *nifH* and *nifD* unrooted phylogenetic tree with the five phylogenetic clusters constructed using the neighbor-joining method. Image taken from Raymond et al. (2004).

Group I contains the most common type of nitrogenase corresponding to the MoFe nitrogenase (Mo dependant cofactor). It includes genes from Proteobacteria, Cyanobacteria, Firmicutes and Actinobacteria (Zehr et al., 2003). Cluster II includes the genes of the alternative nitrogenases FeV (Vanadium-dependent cofactor) and FeFe (Iron-dependent cofactor). This group is relatively small and contains nitrogenases from certain methanogenic archaea. Similarly, Group III mainly contains *nifH* sequences from anaerobic bacteria and archaea such as methanogens,

acetogens and polyphosphate-accumulating and glycogen-accumulating organisms (Zehr et al., 2003; Guo et al., 2017).

On the other hand, Group IV and V contain genes that are not involved in nitrogen fixation. For example, Group V include genes involved in the synthesis of pigments for photosynthesis and electron transport (Young, 2005). Finally, Group VI contain significantly divergent gene sequences from uncharacterised nitrogenases of different origin, belonging to organisms not known to fix nitrogen (Raymond et al., 2004).

The MoFe nitrogenase is the most ancient isozyme. It emerged within the hydrogenotrophic methanogenic archaea when the environment was anoxic, before the great oxidation event (Lindström et al., 2015). The most basal sequences on the phylogenetic tree on Figure 2.3 correspond to hydrogenotrophic methanogens such as *Methanobacteriales* and *Methanococcales* and are classified on Group ~~cluster~~ III. This finding on the phylogenetic tree coincides with the fact that the enzyme is sensitive to oxygen and that the bacterial mechanisms evolved to avoid oxygen near the enzyme are recent innovations (Boyd et al., 2011). Later when oxygen was available in the environment, the MoFe nitrogenases became again prevalent because the molybdenum was once again available (Rayner-Canham & Grandy, 2011).

The alternative nitrogenases FeV and FeFe evolved from a different phylogenetic lineage in response to a Mo limitation in the environment, before the great oxidation event (Lindström et al., 2015).

2.2.3. Nitrogen-fixing Biofertilisers

Biofertiliser is a very broad term, referring to anything from microorganisms and manure, to plant extracts that can help plants to grow (Grageda-Cabrera et al., 2012). More specifically, they are usually defined as products containing living microorganisms like bacteria or fungi which when applied to seed, plant surface or soil, are able to promote plant growth through the conversion of nutritionally important elements (e.g. N_2) or through the decomposition of organic residues (Bhat et al., 2015). This document focuses on NFB functioning as biofertilisers.

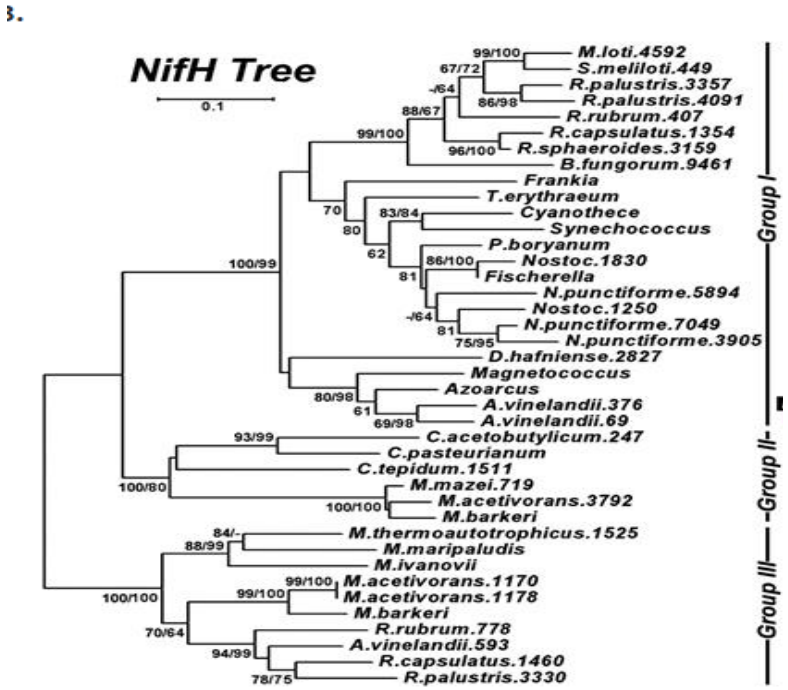

Figure 2.3. *nifH* phylogeny with rooted tree**.** Image taken from Raymond et al. (2004).

The use of biofertilisers will be required in order to sustain food production and at the same time guarantee environmental health for the increasing human population. These alternative fertilisers could play a crucial role in the sustainability and efficiency of agriculture because of their low production costs. The amount of BNF in soils destined for agricultural production is between 33 and 70 million tons N $year^{-1}$ (Smil, 2004; Herridge et al., 2008). Although bacterial biofertilisers currently have a relatively low agricultural significance, they could potentially become an important agricultural advancement (Olivares et al., 2013).

The ability of free-living NFB to improve nitrogen uptake in nonleguminous plants has been demonstrated in cereals (Bageshwar et al., 2017) and sugar cane (Malusà et al., 2016). This type of bacteria can form symbiotic associations promoting plant growth and yield (Bhattacharjee et al., 2008).

Current research work focuses on the isolation and sometimes manipulation in aseptic conditions of diazotrophic bacteria in monocultures with the aim of identifying and culturing the most fitted species. Free-living and symbiotic NFB are commercially available as fertilisers, for example *Rhizobium sp.* (symbiotic) and *Azotobacter sp.* (non-symbiotic) from National Fertilisers Limited (India). Approaches to improving biofertilisers include the development of nitrogen-fixing symbionts, transferring of the

nitrogen-fixing genes to other bacteria and plants, and enhancement of symbiotic nitrogen fixation in legumes (Olivares et al., 2013). However, isolates that are highly efficient in one environment may not be top performers in other conditions due to fitness trade-offs (Kaminsky et al., 2019). Factors such as salinity, temperature, drought, oxygen and nitrogen can limit BNF in soil (Grageda-Cabrera et al., 2012). In some cases, NFB are isolated from natural environments without extreme conditions and are easily eliminated by fitter competitors from the rhizosphere when inoculated elsewhere. Less than 5 % of *Rhizobium* strains are tolerant to stress conditions (above 40°C and 3% NaCl) (Rao, 2014). Therefore, the selection and discovery of NFB tolerant to multiple stress from degraded environments is desirable to guarantee their survival before and after inoculation.

There is evidence suggesting that biofertilisers containing mixed microbial cultures are more productive than pure cultures and their efficiency of nitrogen fixation improves with increasing diversity (Schütz et al., 2018; Hu et al., 2016). Additionally, multispecies inoculant working as biofertilisers may be able to recuperate nitrogen-fixing capabilities of degraded soils and regenerate its natural community structure (Roper & Gupta, 2016; Bashan & Levanony, 1990).

Some of the main challenges when working with natural fertilisers are the low survival and the low nitrogen fixation rates of the bacteria inoculated in agricultural degraded soils (Gutierrez et al., 2020). One reason is because traces of ammonium contained in the soils can inhibit the nitrogenase enzymes (Fan et al., 2019; Smercina et al., 2019). Pérez-Peláez et al. (2011) observed NFB in domestic wastewater treatment plants with high ammonium concentration (>50 mg NH_4^+ L^{-1}) and hinted at the possibility of using these NFB as a biofertiliser in degraded soils by inoculating the sludge. Some disadvantages of the biofertilisers is that the fixed nitrogen is not directly transferred to the plants and the bacteria have low indexes of survival due to the shortage of carbon sources (Olivares et al., 2013). A better understanding of the mechanisms involved in BNF in microbial mixed cultures would help to develop realistic and sustainable short-term biotechnologies that maximise the potential of NFB as biofertilisers (Roper & Gupta, 2016)

Mixed microbial cultures containing NFB can be sourced from natural or disturbed environments and enriched in bioreactors. For example, Reyna et al. (2012) isolated

a nitrogen-fixing consortium from a rice field. When this consortium was cultured in a reactor, it had a higher nitrogen-fixing capacity than pure cultures such as *Anabaena variabilis, Anabaena azollae and Nostoc muscorum*. The biomass from the reactor was employed as a biofertiliser in rice plants grown under laboratory conditions. Plants inoculated with the mixed culture achieved a growth increase of 50% when compared to plants growing with conventional chemical fertilisers. One advantage of this type of biotechnological process using microbial mixed culture is that the bioreactors can be operated using selective culture media in open systems that do not require sterile conditions, thus decreasing operation costs. Therefore the bacteria that remain in the system are the only ones that can survive with the culture media.

Nitrogen fixation and NFB have been observed in treatment plants treating real and synthetic paper mill wastewater(Clark et al., 1997; Gauthier et al., 2000; Kargi & Ozmihçi, 2004; Slade, Gapes, et al., 2004; Welz et al., 2018). However, these studies have viewed nitrogen fixation as a curiosity rather than as an end in itself, even though Clark et al. (1997) estimated that nitrogen was fixed at a rate of 600 kg N day^{-1} in an aerated stabilization basin with a wastewater flow of approximately 200 million liters per day. Aerobic processes are the most popular systems used for the treatment of effluents from the pulp and paper mill industry (Ashrafi et al., 2015). However, this technology produces high volumes of sludge that needs to be dewatered and disposed (Hubbe et al., 2016). Sludge treatment comprises more than half of the overall wastewater treatment costs and produces greenhouse gasses (Meyer et al., 2018). The benefits of sludges enriched with diazothrophs could be twofold; not only can sludges be dewatered and used as a fertiliser but this sludge could also have a benefit because of the presence of large numbers of NFB (Kargi & Ozmihçi, 2004).

2.2.4. BNF in Microbial Fuel Cells

Microbial fuel cells are innovative bioelectrochemical systems used to treat wastewater and produce electricity by direct conversion of the energy contained in organic feed (Logan, 2010). Some of these small-scale biological wastewater treatment systems have used BNF as an input of nitrogen when the wastewater contains it in low quantities. The nitrogen can be fixed by diazotrophic anode respiring bacteria which also consume the organic compounds available in the wastewater (Belleville et al., 2011; Wong et al., 2014). These NFB are inoculated at the anode and use it as the electron acceptor (instead of oxygen). One of the advantages of BNF on

bioelectrochemical systems is that it eradicates the impact of oxygen on the nitrogenase because it happens under anaerobic conditions.

Ortiz-Medina et al. (2019) explored an alternative strategy for ammonium production with NFB using an anaerobic single-chamber microbial electrolysis cells, in the absence and presence of ammonium (104 mg NH_4^+ L^{-1}). Additionally, this biofilm system was able to produce methane gas. Interestingly, the presence of ammonium did not significantly affect nitrogen fixation rates when voltage was applied. The system achieved a nitrogen fixation rate of up to 30 nmol of ethylene min^{-1} mg $protein^{-1}$.

2.3. BNF in Wastewater Treatment Systems

Usually paper mill wastewater contains limited quantities of nitrogen and phosphorus. Therefore, these nutrients are added as a supplement in order to achieve significant and stable performance of organic carbon removal in biological wastewater treatment systems (Slade, Ellis, et al., 2004). The annual costs of adding nitrogen to this type of treatment plants is significant. The operation of some treatment plants without the addition of any supplement nitrogen is explained by the existence of natural occurring NFB (Clark et al., 1997). A wastewater treatment plant located in New Zealand had an increase in the amount of total nitrogen concentration between the raw effluent and the final discharge. BNF supplemented an approximate of 600 kg N day^{-1} to the system (Clark et al., 1997). Research on nitrogen fixation with wastewaters is limited. The occurrence of nitrogen fixation has been studied in systems treating wastewaters from paper mills under different operating conditions, but the production of NFB in mixed culture for use as a fertiliser has been overlooked.

Studies of nitrogen fixation within paper mill wastewater treatment systems have aimed to understand the environmental conditions favouring this process. Nitrogen fixation rates in biological wastewater treatment systems are likely to be influenced by environmental factors including: carbon source and concentration, oxygen levels (Slade et al., 2003; Merrick, 2004), presence of nitrogen in bioavailable forms (Slade et al., 2011), pH (Merrick, 2004), growth stage (Klugkist & Haaker, 1984), metal availability and temperature (Gapes et al., 1999). Table 2.1.1 and Table 2.1.2 summarise the research articles on this topic and show the operational conditions, the type and scale of the treatments as well as the biological oxygen demand (BOD) or

chemical oxygen demand (COD) removal efficiency. Research has been done at different scales from lab bench bioreactors to pilot and full scale treatment plants using real and synthetic wastewaters as a feed (Kargi & Özmihçi, 2002a).

Specifically, nitrogen-fixing wastewater treatment systems such as aerated stabilization basins (ASB) (Clark et al., 1997), activated sludge systems and different type of reactors like sequencing batch (SBR), batch reactors and continuous stirred tank reactors (CSTR) have been investigated. Most current published research on this topic have proved BNF activity in aerobic sludges using the acetylene reduction assay (ARA) and has been done in New Zealand at warm temperatures of 30 °C. A patented technology named N-virotech was developed to treat paper mills wastewater without the addition of nutrients and relying on communities of NFB, which satisfy the nitrogen requirements of the system (Slade et al., 2003). Kargi and Özmihçi (2002a, 2004; 2002b) proved that an aerobic sludge supplemented with the NFB *Azotobacter vinelandii* was more efficient when treating nitrogen deficient wastewater than an Azotobacter-free aerobic sludge.

In the treatment of pulp and paper mill wastewater it is important that there is a very low discharge of ammonia in the effluent (Wiegand et al., 2014). Thus nitrogen-fixing systems have an advantage over the nitrogen-supplemented systems because bacteria can self-regulate the amount of fixed nitrogen according to their requirements. Previous work investigating the stability of high carbon-to-nitrogen aerobic wastewater treatment plants operated under different nitrogen loads, found that the concentration of nitrogen did not have any effect on the organic carbon removal rate of efficient (Slade et al., 2011; Kargi & Ozmihçi, 2004) or inefficient (Penteado et al., 2016) systems but it did influence floc structure and ammonium discharge (Slade et al., 2011). Slade *et al.*(2011) studied a nitrogen-fixing sludge using microscopy and found that the growth of non-nitrogen fixing filamentous bacteria increased together with the nitrogen concentration of the wastewater.

Table 2.1.1. Literature review on studies of nitrogen-fixing waste waters treatment plants.

Reference	Type of wastewater (WW)	Type of treatment	Treatment plant/Pilot/ Bioreactor	Dissolved Oxygen % (mg/L)	BOD/TOC/COD:N:P ratio	Load CODtot kg/m3/d	HRT	total COD removal	Nitrogen Increase (rate kg/d)	T (°C) /pH	Microbiological Characterization	Summary
Dennis, Cotter, Slade and Gapes (2004)	Real WWW from several paper mill sources	SBR and continous stirred tank reactor activated sludge (CSTR-AS)	Lab reactors	0.35	100:0:0.25 (COD)	1-1.43	1.3-1.7	70-78% (CSTR-AS)	0.7-4 mg/L (CSTR-AS)	35/4.5	Evaluted the sludge quality with SBR outperforming CSTR-AS in respect to sludge setteability and dewaterability. The SBR had significant floc size and density with no filament display. The control had smaller size	Compared the efficiency of two systems N fixing and N supplemented (control) under two operating conditions SBR and Continous Stirring Bioreactor treating real wastewater. The two type of systems performed similarly with high rates of COD removal. Nevertheless, concluded that the best regimen is the SBR, which has feast and famine cycles that creates flocs with size appropiate to have a good setteability and density thus lower VSS discharges.
Kargi and Ozmihci (2002)	Synthetic WW supplemented with glucose amd diluted molasses	Batch shake flask experiments	Lab reactors	?	100:0-8:1.5 (COD)	2000-2500mg COD/L	?	80%-95% TOC	?	7.0/25	None, but they use a pure culture	Compared the treatment of synthetic WW using Azotobacter vinelandii single or in combination with activated sludge, they also evaluated different TN/COD ratios. Measured TOC removal efficiency in days.
Kargi and Ozmihci (2004)	Synthetic WW supplemented with glucose	Batch shake flask experiments	Lab reactors	?	100:1-8:1.5(COD)	2000 mg COD/L =900 mgTOC/L	?	90% TOC	?	7.0/25	None	Evaluated the effect of the N:COD/TOC ratio for carbon removal, with treatments containing Azotobacter, sludge or a mix. The efficiency of the treatment evaluated as COD removal for each treatment changed according to the N:COD ratio. In conclusion the sludge together with the pure culture consume the carbon on the media, but the azotobacter works better together with the sludge because they divide roles of fixation and COD removal. Inoculated with a volume of 10%(v/v).
Pratt, Tan, Gapes and Shilton (2007)	Synthetic WW supplemented with acetic acid	SBR shake flask experiments runned for 72 days	Lab reactors	5.0-7.8 mg/L	COD:N:P 100:0.1-3.5:1.5	500mg TOC/L	0.5	>95%	Increased 1mg/L higher than feed solution	30/?	TRFLP profiles show more diversity on the Nfix system, meanwhile a microbial group predominates on the Nsupp system. Communities in both systems did not change abruptly with time. Some groups were present in both treatments.	Compared two systems with and without N in order to evaluate the morphology and settlement characteristics of the flocs. They proved the capability of both systems to substrate carbon. The Nfix system had a decrease in biomass at the beginning of the experiment but after 75 days of operation it had more biomass than the Nsupp. Biomass at an intial concentration of 1.8g/L
Slade, Thorn and Dennis (2011)	Synthetic WW supplemented with Methanol, Glucose and sodium acetate	Continous stirred tank reactor activated sludge (CSTR-AS)	Lab reactors	1.5 (20% saturation)	100:0-4.9:0.5 (BOD)	184 mg BOD/L		1.5		30/?	Microscopic characterisation evaluating the presence of filaments and floc. The microscopic structure changed according to the BOD:N ratio, filaments appeared at higher N concentration as well as protozoa.	Measured the impact of varying concenration of BOD:N ratios whilst maintaining P at a constant level. There was no difference on the organic carbon removal but the floc structure as well as the rates of nitrogen fixation varied when the amount of of N increased. Above the BOD:N ratio of 100:1.9, nitrogen fixation no longer ocurred, nevertheless this cannot apply necesarily to real wastewaters since the organic nutrients contained in the synthetic wastewater might not be available.

Table 2.1.2. Continuation Table 2.1.1

Reference	Type of wastewater (WW)	Type of treatment	Treatment plant/Pilot/ Bioreactor	Dissolved Oxygen % (mg/L)	BOD/TOC/COD:N:P ratio	Load total COD kg/m3/d	HRT (days)	total COD removal	Nitrogen Increase (rate kg/d)	T (°C) /pH	Microbiological Characterization	Summary
Clark, Dare and Bruce (1997)	Combined mill wastewater: refined mechanical pulp, newsprint, groundwood un/bleached kraft pulp	Aerated stabilization basin (treatment system in aerated lagoons)	Plant and laboratory	0.1 (outlet 1.5%saturation)-0.5 (inlet) Plant / 0-50% in bioreactor	100:0.8:0.4 (BOD)	0.629	1.2 (pond) 1.4 (bioreactor)	34% (36 t/d)	157% (0,6 Kg)	35/7.6	Mentions possibility of different bacteria or different resistance to DO.	Proved nitrogen fixation on the different concentration of dissolved oxygen and concluded that high levels do affect the process
Gapes, Frost, Clark, Dare, Hunter and Slade (1999)	Real WWW from bleached kraft mill	Activaed sludge ractor system	Reactors	0.4	100:0.8:0.5 (COD)	1.13	0.5	62	80% biomass attributed to fixation	30/?	Good biomass separation. Higher proportion of filaments in Nfix. than Nsupp. treatment	Compared the efficiency of two systems with and without N in the media taking into acocunt their respective operational conditions. Concluded that the N fixing system is competitive against the N supplemented sustem
Slade, Anderson & Evans (2003)	Real WW from the pulping of Pinus radiata: N and P limited	Activated sludge treatment	Pilot	5-30% (0.3-2.2)	100:0.5-1:0.3-0.5 (BOD)	1.6-2.4	1.4 days	82-87%	55-354 % (4-13)	30/6,4	The bacterial community was dominated by alpha and beta proteobacteria showing a low diversity, despite the changes in wastewater composition and in the dissolved oygen leveles. qPCR showed a ratio of nifH to 16 rDNA of 1.14, therefore the density of the nifH sequences were high.	Patented technology proved in plant with different conditions of dissolved oxygen. Concluded that the best performance is obtained under low DO (5%)
Slade, Gapes, Stuthridge, Anderson, Dare, Pearson and Dennnis (2004)	3 sources of real WW: bleached kraft miLl, thermomechanical pulp and liner board kraft pulp from *Pinus radiata*	Aerated stabilization basin (treatment system in aerated lagoons)	Lab, pilot and full scale plant	0.3-2.2 mg/L(0-2L/min)	100:0.6-1:0.15-0.4 (BOD)	490-3300 mg/L	?	?	0.027-0.035 mg N/mg VSS/ day (400kg/d)	30-39/4.8-8	Reid 2002 a: Microbial community stability	Patented technology for the treatment of nutrient limited WW where they proved the robustness and efficiency of the technology with three treatments with different size. They worked with a range of different wastewater with varying organic charges and P concentrations. The efficiency of the process is increased by operating it at low DO.

Specific C/N/P or BOD/N/P ratios are usually recommended for efficient biological treatment of wastewaters. Conventional C/N/P and BOD/N/P ratios are 100:5:1 (Gray, 2004) and 100:3.5:0.6 (Slade et al., 2003), respectively. Biological wastewater treatment processes require availability of phosphorus (P) in the system as it is essential for bacterial reproduction. The concentration of this nutrient in the literature of nitrogen fixing wastewater treatment systems varied between 0.1 and 8 mg L^{-1} (Table 2.1.1 and Table 2.1.2). Particularly, Slade et al. (2011) used concentrations of P of 1.1 mg L^{-1} while varying the BOD/N ratio in the treatment of synthetic low nitrogen wastewaters between 100:0 and 100:4.9. As a result, it was observed that the BOD/N ratios where nitrogen fixation occurred had a higher P concentration at the discharge than the treatments supplemented with N. Since the organic carbon removal was similar, they concluded that the nitrogen-fixing system had lower phosphorus requirement (Slade et al., 2011). This BOD/N ratio can vary in real wastewaters because the nutrients might not be in an immediately available form.

It is important to provide or make sure that essential minerals such as molybdenum, vanadium and iron are available in the biological wastewater treatment systems because the nitrogenase complex uses them as cofactors, depending on the type of nitrogenase (Gapes et al., 1999). Some experiments evaluating BNF in wastewaters use a positive control consisting of a N supplemented control, which usually operates on the normal conditions in which conventional treatment plants operate (Dennis et al., 2004; Slade, Gapes, et al., 2004).

The enzyme nitrogenase is very sensitive to oxygen even though aerobic NFB require oxygen for cellular respiration, but the optimal dissolved oxygen (DO) concentration differs among diazotrophic species. It is influenced by the supply and demand of oxygen in the cells (Hill, 1988). Brotonegro (1974) found that in *Azotobacter* cultures, the optimum level of oxygen supply for nitrogenase depended on the cell density. Nitrogen fixation occurred in cultures with high cellular concentration (thus high respiration rate) under high oxygen supply and did not happen in cultures with low cellular concentrations. Normally a typical activated sludge system operates at a DO concentration of 2 mg L^{-1} which is considered ~~as~~ a high concentration for NFB (Gapes et al., 1999). Even though diazotrophs have different natural mechanisms to protect the enzyme, research studies on nitrogen-fixing reactors have coincided on the importance of operating them under a low DO concentration in order to achieve high nitrogen-fixing rates.

Slade et al. (2003) evaluated the performance of a nitrogen-fixing bioreactor under different DO conditions and concluded that ~~more~~ greater nitrogen was fixed at the lowest concentration (0.3 mg L^{-1}). Similarly, Clark et al. (1997) observed a variation on the nitrogen fixation rates at different sampling points from the aerated stabilization basin (with differing DO concentrations) using the ARA. When the DO was above 50% of saturation, significantly lower acetylene reduction was observed. But when the DO level was near to zero, the acetylene reduction rate increased up to 10 nmol mL^{-1} $hour^{-1}$. Moreover, the time length in which the culture was exposed to high DO concentration also influenced the rate of nitrogen fixation; the acetylene reduction rate decreased when the culture was exposed to oxygen for more than 190 hours (Clark et al., 1997).

Respiratory protection is a mechanism for protection of the nitrogenase enzyme against oxygen in which the organic carbon sources are consumed in excess in order to get rid of the oxygen and enable BNF (Bitton, 2003). For example, a metabolic model for *Azotobacter vinelandii* revealed that the microorganisms consumed carbohydrates in excess even when the protection was not necessary (when nitrogen fixation was not occurring) (Inomura et al., 2017). Furthermore, the bacteria had a high carbon consumption rate even when the carbon source was low (Inomura et al., 2018). This high respiratory rate of NFB could also be an advantage of nitrogen-fixing systems over nitrogen-supplemented systems, as the efficiency of the COD removal treatment could be benefitted by their presence.

2.3.1. Microbiological Analysis of Nitrogen-Fixing Wastewater Treatment Plants

Studies assessing the microbial composition of bacteria on nitrogen-fixing wastewaters are scarce. Table 2.2.**1** summarises the microbiological research done on these types of systems. Usually molecular biology techniques (such as sequencing, qPCR and dot blot) as well as classic microbiological culture methods have been used.

Overall, the most common types of NFB bacteria in these nitrogen-fixing treatment plants are the Proteobacteria with the classes Alpha, Beta and Gamma proteobacteria prevailing in abundance of both the *nifH* and *16S rRNA* gene sequence libraries. The microbial community from a low nitrogen model plant-based industrial wastewater treatment plant had a profile of *nifH* sequences which was very similar to the profile obtained from samples of forest soil environments (Bowers et al., 2008). Variations in the wastewater composition and dissolved oxygen levels of this same system did not affect the efficiency of the wastewater treatment although changes in the *16S rRNA* composition were observed, suggesting that the evolving microbial community was functionally redundant (Reid et al., 2008).

Likewise, Addison et al. (2011) observed that the wastewater treatment efficiency of a low N system remained optimum even though the microbial community composition varied with the use of different carbon sources (acetate, glucose and methanol) and different levels of phosphorus in the culture media. In general, the bacterial communities in these treatment plants proved to be stable in their function of lowering the BOD level of the wastewaters, despite changes in the wastewater composition and DO concentration.

The detection of NFB with *16S rRNA* gene sequencing can show which NFB are present in a sample, but does not show directly the bacteria that are actively fixing nitrogen in the system. Addison et al. (2010) investigated the active diazotrophs in a microbial community using reverse transcription, cloning and sequencing of *16S rRNA* molecules labelled with $^{15}N_2$. This assay used nitrogen-free paper and pulp wastewater enriched with isotope-labelled nitrogen gas to culture the aerobic sludge from the treatment plant. This study found that the class Gammaproteobacteria were the major active diazotrophs in the treatment plant. This microbial community had three dominant genera~~us~~ identified as *Aeromonas, Pseudomonas and Bacillus,* they all had the potential to fix nitrogen although nitrogen fixation tests were not carried out. Later, when a fluorescent in situ hybridisation (FISH) analysis targeting both the *nifH* and *16S rRNA* genes was carried out on the aerobic sludge, it was revealed that these genera of bacteria were only a small proportion of the total diazotrophic community which was mainly composed of Alpha and Betaproteobacteria. Therefore, by using different methods they could determine that the whole bacterial community was relying on a small subpopulation of NFB for the supply of nitrogen (Addison et al., 2010).

The genus *Klebsiella* was identified as the most abundant NFB within a low nitrogen wastewater treatment plant using classic microbial culture methods (Gauthier et al., 2000). However, *Klebsiella* did not seem to be very abundant when this environment was assessed with culture independent methods (qPCR, FISH, DNA sequencing and ^{15}N-RNA) (Addison et al., 2010; Reid et al., 2008). This genus of bacteria together with some coliforms are easy to cultivate in the lab. Therefore, studies that only use culture methods to describe communities tend to overestimate their importance in the ecosystem (Bowers et al., 2008). This finding points out the importance of also using culture independent techniques when analysing microbial communities.

Table 2.2.1. Literature review on studies of microbiological characterisation of nitrogen-fixing wastewater treatment plants.

Author	Country	Molecular technique	Main groups fixing nitrogen	Summary
Bowers, Reid and Lloyd-Jones (2008)	New Zealand	RFLP and 16rDNA sequencing, qPCR for nifH and 16 rDNA	*Alpha and Beta (Burkolderiales) proteobacteria* dominated the bacterial community, with less ammount of Bacteroidetes.	Proved that the changes on wastewater composition and dissolved oxygen did not affect the the nifH levels and allowed stable water treatment. Connected to Slade (2003). The same microbial diversity profile was seen in forest soils. The nifH to 16sDNA ratio is 1.14, showing low diversity but a high density of the nifH sequences. The primers used are PolF and PolR. Discusses the importance of not doing taxonomic inferences based only on the nifH because this genes can be tranfered horizontally. Also mentions that *Klebsiella* and coliform bacteria are overestimated when using culture based techniques, as it was possible to see that their sequences are low abundant in this type of wastewaters. Studied the temporal stability and change of the dominant phylogenetic groups of bacteria. Changes on the composition and dissolved oxygen did not affect the stability of the community, but did affect the composition thus indicating funcional redundancy.
Addison, Slade and Dennis (2011)	New Zealand	FISH and 16S rDNA sequenciing	*Betaproteobacteria (genus Methilophilus and Methilobacillus) w* as most abundant with methanol, meanwhile for acetate with and without glucose A*lpha and Gammaproteobacteria* competed for growth depending on the amount of N and P.	Community composition and abundance varied along the system, nevertheless despite the changes on the wastewater , temporal stability was achieved. Cell morphology of Alpha and Beta proteobacteria depended on the the concentrations of C:N:P and was independant of the culture duration;playing an important role on culture setteability. Acetate, glucose and methanol were used with different leveles of nitrogen and phosphorus to evaluate the impact on the community composition.
Gauthier, Neufeld, Driscoll and Archibald (2000)	Canada	Bacterial culture, Dot Blot Hybridization of nifH	*Klebsiella (Gammaproteobacteria)* and coliform bacteria	Elucidated the magnitude and microbiology of nitrogen fixation on insitu samples and isolates .Evaluated the presence of nifH genes. The nitrogen-fixing activity was found on the clarifiers where low amount of dissolved oxygen is available
Addison, McDonald and Lloyd-Jones (2010)	New Zealand	stable isotope probing to 16 rRNA, clone library , FISH	*Gammaproteobacteria, firmicutes, alpha and betaproteobacteria;* isolated nitrogen-fixing A*eromonas and Pseudomonas*	The abundance and diversity of active diazothrophs was investigated by enrichment of 15N2. The labelled RNA was analyzed by reverse transcription and sequence analysis of 16rRNA revealed a diversemicrobial community. The active nitrogen fixing commmunity (Gamma proteobacteria) was later compared with the whole community, showing low levels of relative abundance with FISH. Because they found more Klebsiella when culturing than when sequencing, they discuss the importance of using culture independant techniques to accurately analyse the proportions of populations within a community.
Smith, Yu and Mohn (2003)	Canada	Ribosamal intergenic spaces length polymorphism fingerprints, clone libraries	*Proteobacteria* with genus *Acidovorax and Paracraurococcus. Green Nonsulfur bacterium* with the *genus Roseiflexus*	Evaluated the dynamic of a microbial population during the operation of a treatment system, including a period of shutdown. The composition and functional stability during the operation and shutdown of the system was consistent. When comparing the samples from different paper mills , distinct fingerprints and clone libraries were found.

2.4. Energetics of BNF

BNF requires in theory 8 electrons. For every electron transferred, two molecules of ATP are needed. So a minimum of 16 moles of ATP are also required to achieve the BNF process (Equation 2.1) (Bazhenova & Shilov, 1995).

Only 75 % of the electrons needed BNF are used for the actual production of 2 moles of NH_3 as the rest of the electrons (2 out of 6) are utilised for the reduction of hydrogen (Cherkasov et al., 2015). This hydrogen production decreases the efficiency of the BNF process (Broda & Peschek, 1980; Schubert & Evans, 1976), but it is an unavoidable by-product due to the nature of the enzyme which is derived from an ATP-dependent hydrogenase. Approximately 2-3 molecules ATP (Gutschick, 1978) and 2 electrons can be recovered by an uptake hydrogenase from the H_2 produced during BNF (Tamagnini et al., 2002).

The oxidation of a carbon substrate like glucose produces in theory 38 molecules of ATP (Cherkasov et al., 2015). Specifically, the hydrolysis of a mole ATP has a Gibbs free energy of at least 0.046 MJ mol^{-1} (Kammermeier, 1982; Smith et al., 2004). Assuming that the energy required for the production of ATP to ADP is the same amount required for the backward reaction, then the energy required to produce 16 molecules ATP for BNF is approximately 728 KJ mol^{-1} N_2 (or approximately 0.38 MJ mol N^{-1}) (Cherkasov et al., 2015). So, in theory the energy invested in the process of producing ATP to fix nitrogen is lower than the energy used for the Haber-Bosch Process (HBP;0.48 MJ mol^{-1} N) (Cherkasov et al., 2015).

BNF has an approximate Gibbs energy of 0.340 MJ mol N^{-1} (Equation 2.1) meanwhile the hydrolysis of one mole of glucose liberates 3,140 MJ (Gutschick, 1978). Thus in theory, 0.11 moles of glucose can fix one mol of nitrogen. This energy demand makes it a considerably costly process for the bacterial cell and contrasts with the reaction for ammonia produced in standard conditions which liberates 32 kJ (Equation 2.2).

Equation 2.2:

$$N_2 + 3H_2O \rightarrow 2NH_3 \; ; \triangle G_0{}' = -32kJ$$

This difference in the energetic demand is because in BNF the electron transfer

is not concerted and, therefore, some unstable intermediary molecules are formed at the metallo-complex active site of the enzyme. Only biological reductants cannot develop the reaction because the electronic potentials of these intermediary molecules are very negative (Bertini, 1995). These intermediary molecules are diazene (N_2H_2) and hydrazine (N_2H_4). Both molecules have high activation barriers, thus the diazotrophs have to invest energy to overcome these barriers and make the overall reaction favourable (Figure 2.4) (Berg et al., 2002).

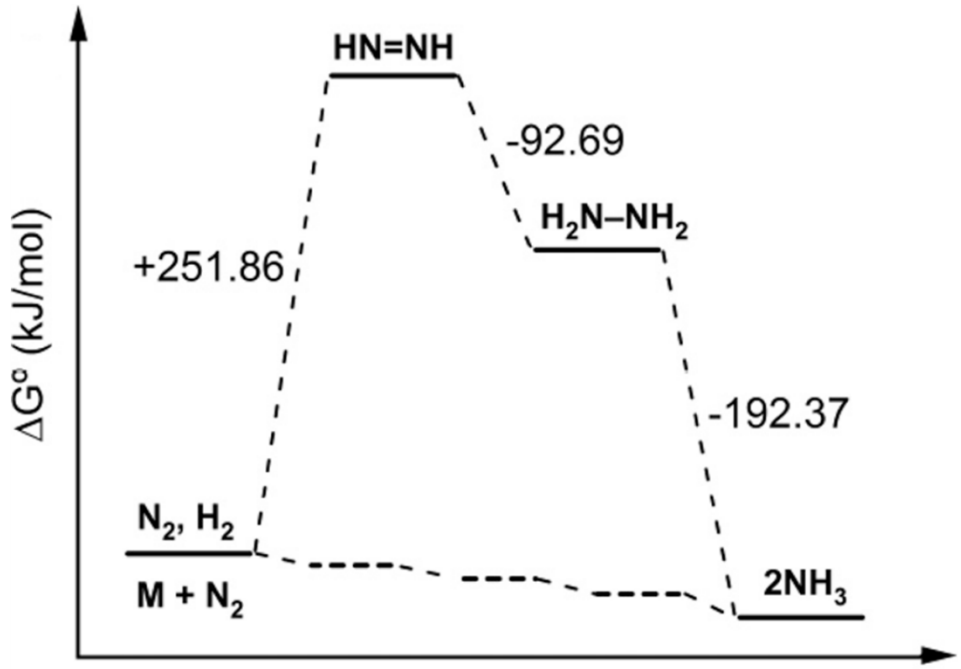

Figure 2.4. Standard Gibb's energy change diagrams for nitrogen fixation pathways in BNF. Possible energy states for metal bound intermediates (M) are shown (lower dashed traces). Image taken from Seefeldt et al. (2013).

The amount of energy used in nitrogen fixation can be seen in the difference between the growth yield of pure bacterial cultures, grown both with and without nitrogen in the culture media. A culture of *Clostridium pasteurianum* produced 1.7 times more biomass when grown with ammonia than without ammonia (Daesch & Mortenson, 1968). *S*imilarly, the growth rates for *Azotobacter chroococcum* fixing N_2 were lower when it had to fix nitrogen (Dalton & Postgate, 1969). The relatively slow turnover time of the nitrogenase enzyme (5 s^{-1}) (Thorneley & Lowe, 1985), forces diazotrophs to synthesise it in large amounts (up to 20% of the protein content of the cell) (Dixon & Kahn, 2004). This additional production of protein implies a change in the cell metabolism, diverting resources that could be used for cell growth.

Practical BNF yields differ from the theoretical balances as the latter do not count for the energy losses and the electron efficiency of the biological process. The oxidation of a carbon substrate like glucose produces in practice approximately 30 molecules of ATP (Cherkasov et al., 2015). Additionally, the productivity of the nitrogenase enzymes and the stoichiometry of the process can vary between different species. For instance the amount of ATP molecules required for BNF can range between 12 and 24 molecules (Gutschick, 1978; Lindström, 2010; Masephl & Kranz, 2009). According to Gutschick (1978), *Azotobacter vinelandii* had a fixation efficiency of 16%. Cherkasov et al. (2015) calculated the fixation efficiency from an industrial perspective at 46.7%. So, the HB process is between 2 to 4.5 times more efficient that BNF when the real energy demands of the two processes are compared (Cherkasov et al., 2015; Gutschick, 1978).

One of the strategies carried out by NFB to avoid the contact of the enzyme with oxygen is respiratory protection (Bitton, 2003). This mechanism increases the substrate consumption in order to achieve high respiratory rates. From an industrial perspective, an increase in substrate consumption involves a decrease in the BNF yields which can be very expensive if the source of carbon is costly. Nevertheless, if the source of carbon is an industrial waste by-produc, such as paper mill effluent, then BNF could be considered as low cost and sustainable.

2.5. Estimation of Nitrogen Production Yields from Wastewater

The energy for BNF can be obtained from different sources like carbohydrates and organic wastes from agriculture and forestry (Cherkasov et al., 2015) and from industrial or domestic wastewater. This section undertakes a theoretical novel exercise to estimate the amount of nitrogen that could be fixed in a sustainable way using the energy derived from domestic wastewater. So to provide some context, the daily dietary nitrogen requirement of a person has been arbitrarily selected as the amount of nitrogen to be produced. For instance, the aforementioned amount of nitrogen could be hypothetically produced by NFB cultured in reactors fed with the energy available in wastewater; or through an alternative HPB powered by biogas produced from from the digestion of wastewater sludge. The following section estimates and compares the volume of wastewater necessary to fix nitrogen through these two methods.

2.5.1. BNF Powered With Wastewater

BNF consumes approximately 0.38 MJ mol fixed N^{-1} (Cherkasov et al., 2015). It has been estimated that domestic waste water from the UK has an energy of 7.6 kJ L^{-1} (Heidrich et al., 2011). This wastewater has sufficient energy to biologically fix the daily nitrogen requirements of a single person (Equation 2.3). An adult with a weight of 70 kg requires 56 g of protein a day (Layman et al., 2015), 16% of which (~9 g) is nitrogen (Ebeling et al., 2006). Therefore, the energy necessary to fix the amount of nitrogen required by a person daily is 243 kJ.

Equation 2.3:

$$56\ g\ of\ prot\ * \frac{0.16\ g\ N}{1\ g\ protein} * \frac{1\ mol\ N}{14\ g\ N} * \frac{380\ kJ}{mol\ of\ N}$$
$$= 243\ kJ\ required\ per\ daily\ person$$

Thus, the energy available in 1000 litres of domestic wastewater could be able to fix the amount of nitrogen required daily by 31 people (Equation 2.4).

Equation 2.4:

$$7600\ kJ\ in\ 1000L\ of\ wastewater\ \div 243\ kJ\ per\ person\ per\ day\ =\ 31x$$

Likewise,, one person can produce around 120 g of COD daily (Kiely, 1997) and each gram of COD contains 14.7 kJ (Shizas & Bagley, 2004). Therefore, the COD produced daily by a single person is equivalent to approximately 1764 kJ (Equation 2.5). This energy could also be used to fix through BNF (Equation 2.3) the nitrogen required daily by 7 people (Equation 2.6).

Equation 2.5:

$$\frac{120\ g\ of\ COD}{per\ person\ per\ day} * \frac{14.7\ kj}{g\ of\ COD}$$
$$= 1764\ kJ\ available\ in\ the\ COD\ produced\ daily\ per\ person$$

Equation 2.6:

$$1764\ kJ\ available\ in\ the\ COD\ produced\ daily\ per\ person$$
$$\div 243\ kJ\ per\ person\ daily\ =\ 7.2$$

2.5.2. Alternative HBP Using Biogas

Alternatively, the nitrogen required daily in the diet of a single person could be obtained through the HBP using the energy available in biogas derived from the anaerobic digestion of sludge (Equation 2.7). Biogas derived from anaerobic digestion can be transformed to electricity and heat. The energy content of the biogas can be boosted by purification of pure methane through a biogas upgrader facility and the purified methane can be supplied to the natural gas grid (Van Haandel, A., & Van Der Lubbe, 2012).

Equation 2.7:

$$56\ g\ of\ prot\ * \frac{0.16\ g\ N}{1\ g\ protein} * \frac{1\ mol\ N}{14\ g\ N} * \frac{480\ kJ}{mol\ of\ N}$$
$$= 307\ kJ\ to\ fix\ with\ HBP\ the\ N\ required\ daily\ per\ person$$

The amount of energy necessary to fix nitrogen for 31 people through the HBP is 9517 kJ (Equation 2.8). This amount of energy is contained in 12 moles of methane (Equation 2.9), considering that the energy content available in methane is 50 MJ kg^{-1} CH_4 % (Van Haandel, A., & Van Der Lubbe, 2012).

Equation 2.8:

$$307kJ * 31\ people = \ 9517\ kJ$$

Equation 2.9:

$$9517\ kJ * \frac{1\ g\ of\ CH_4}{50\ kJ} * \frac{1\ mole\ of\ CH_4}{16\ g\ of\ CH_4} = 11.9\ moles\ of\ CH_4$$

Nevertheless, not all the chemical energy of methane can be used for power generation since the thermal efficiencies vary depending on the type of power produced, for example, for electrical power the conversion efficiency is between 35 and 42% (Van Haandel, A., & Van Der Lubbe, 2012). If we account for an energy conversion efficiency of 35%, then 34 moles of methane would be needed to fix through the HBP the nitrogen required by 31 people. This quantity is equivalent to 0.3 m^3 of methane (Equation 2.10).

Equation 2.10:

$$34\ moles\ of\ CH_4\ *35\ \%\ of\ power\ efficiency\ *\frac{16\ g\ of\ CH_4}{1\ mol\ of\ CH_4}*\frac{1\ kg}{1000\ g}$$

$$*\ \frac{1\ m^3 of\ CH_4}{0.656\ kg}=0.29\ m^3 of\ CH_4$$

Biogas is composed by between 50 and 75% of methane, and the volume of methane produced per kg of digested sludge under normalised conditions is 0.525 m^3 kg^{-1} of volatile suspended solid (VSS) (Van Haandel, A., & Van Der Lubbe, 2012). Therefore, the volume of digested sludge necessary to fix nitrogen for 31 people through the HBP is 0.55 kg of VSS (Equation 2.11).

Equation 2.11:

$$0.29\ m^3\ of\ CH_4\ *\frac{1\ kg\ of\ VSS}{0.525\ nm^3\ of\ CH_4}=0.55\ kg\ of\ VSS$$

Chapter 3: Enrichment of Nitrogen-Fixing Bacteria in a Nitrogen Deficient Wastewater Treatment System

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3.1. Introduction

Nitrogen-based fertilisers are both: essential for global food security in a planet with a 7.7 billion population and a twofold concern. Their deployment causes environmental problems including eutrophication, greenhouse gasses and soil acidification (Galloway et al., 2003; Sutton, 2011). In addition nitrogen fixation by the Haber-Bosch process (HBP) is costly, as it consumes 1% of the world's natural non-renewable energy reserves (oil and natural gas) (Solon et al., 2019; Cherkasov et al., 2015). Fertilisers can, and ideally should, be obtained by nitrogen recovery from wastewater, unfortunately the available technologies at present use more energy than the HBP and currently are only used at small scales (Maurer et al., 2003; Matassa et al., 2015). Consequently, finding alternative methods to sustain and increase current agricultural production whilst maintaining a healthy environment is an important facet of the ongoing search for a more sustainable bioeconomy.

The reduction of molecular N_2 to ammonia (NH_3) by living organisms is a process known as biological nitrogen fixation (BNF). It is a self-regulating process that takes place at ambient pressure and temperature, does not produce greenhouse gasses, is independent from non-renewable energy sources and has an energy efficiency comparable to the energy used in the HBP (Cherkasov et al., 2015). The energy source for BNF can, in principle, be obtained from any organic matter including waste from agriculture and forestry processing (Cherkasov et al., 2015) or industrial or domestic wastewater. It has been estimated that UK domestic wastewater has an energy value of 7.6 kJ L^{-1} (Heidrich et al., 2011), enough to fix 340 mg of ammonia.

N_2-fixing bacteria (NFB) are characterised by the presence of the *nifH* gene which codes for the iron protein subunit of the nitrogenase enzyme complex, responsible for BNF (Gaby & Buckley, 2012). This gene can be found in some organisms from bacterial groups commonly found in wastewater treatment systems, such as Proteobacteria (Reid et al., 2008), methanogens (Collins et al., 2016), polyphosphate-accumulating and glycogen-accumulating organisms (Guo et al., 2017). However, nitrogen fixation is assumed to be of limited importance in conventional domestic wastewater because the presence of abundant amounts of ammonia is thought to inhibit the process (Smercina et al., 2019; Hartmann et al., 1986).

Nonetheless, there are certain industrial processes, such as paper mills, which produce very large amounts of waste with little or no reduced ammonia and with chemical oxygen demand (COD) values of up to 11 grams L^{-1} (Thompson et al., 2001). In fact, the world paper production in 2017 exceeded 781 million tons (FAOSTAT, 2017); each ton of paper produced can use between 5 and 100 litres of water, depending on the type of paper and the manufacturing technology (Doble & Kumar, 2005). These wastes are treated biologically and a specific C/N:P ratio of 100:5:1 is usually recommended for efficient biological treatment of wastewater (Gray, 2004). Therefore, such wastewaters are typically supplemented with nitrogen and phosphorous to ensure adequate treatment.

Nitrogen fixation and NFB have been observed in treatment plants treating real (Clark et al., 1997; Gauthier et al., 2000; Slade, Gapes, et al., 2004) and synthetic paper mill wastewater (Kargi & Ozmihçi, 2004; Welz et al., 2018). However, these studies have viewed nitrogen fixation as a curiosity rather than as an end in itself; even though Clark et al. (Clark et al., 1997) estimated that nitrogen was fixed at a rate of 600 kg N day^{-1} in an aerated stabilisation basin with a wastewater flow of approximately 200 million L^{-1} day^{-1}.

The benefits of nitrogen-fixing sludges could be twofold; not only can sludges be dewatered and used as a fertiliser (Gauthier et al., 2000), this sludge could have a benefit because of the presence of large numbers of NFB (Kargi & Ozmihçi, 2004). The value of adding NFB to a system was hinted by work from Liu et al. (2017a). They developed an approach for the synthesis of ammonia using an inorganic-biological hybrid system that fixes atmospheric N_2 employing hydrogen generated from catalytic water splitting, together with a pure culture of the NFB *Xanthobacter autotrophicus*. This bacterium proved to be an efficient nitrogen-fixing biofertiliser in itself by increasing the root mass storage of radishes by up to 1440%, although the mechanism of interaction of *X. autotrophicus*–plant requires further studies.

This chapter, sought to demonstrate the principle of a nitrogen-fixing biological reactor that would valorise organic matter lacking nitrogen, by using it to enrich a wastewater sludge with NFB. To achieve this objective, nitrogen fixation rates were directly estimated with $^{15}N_2$ gas and the *nifH* gen was quantified with quantitative PCR (qPCR). This chapter demonstrated the scope and magnitude of nitrogen fixation in duplicate bench-scale wastewater treatment plants treating wastewater with a high carbon-to-

nitrogen ratio. An identical pair of reactors with a more conventional C/N ratio were used as a control.

3.2. Methods

3.2.1. Inocula

An environmental survey was done to select sources of NFB in different environments (Table A1, Supplementary Information A1 and Figure **A1**). Reactors were inoculated with 40 mL of the same sludge mix. This sludge was sourced from four different locations and mixed in equal proportions (Figure 3.1a), these sites were selected because they contained NFB and they were accessible for sampling when the experiment was going to start. One sample was obtained from Bran Sands (BS), an industrial and domestic wastewater treatment plant (54°36'32.7"N 1°07'20.0"W, UK). The second and third samples were collected from a paper mill wastewater treatment plant in Prudhoe (54°58'06.2"N 1°51'26.2"W, UK) containing a deep shaft (DS) and a jet-aerated channel (JAC) pond. The fourth source of sludge was a full-scale anaerobic digester (AD) at Cockle Park Farm (55°12'56.6"N 1°40'55.9"W, UK).

3.2.2. Set up and Operation of Reactors

Sequencing batch reactors (SBRs) were assembled and operated for 85 days at 30 °C (to reflect tropical conditions) under two different treatments (Figure 3.1 b and c). Each treatment contained two replicate reactors. One treatment was used as control and contained ammonium ($(NH_4)_2SO_4$) in the culture media with a C/N:P ratio of 100:5:1.5 (low C/N treatment). The second treatment (high C/N) did not contain any source of nitrogen and had C/N:P ratio of 100:0:1.5.

Reactors were fed with synthetic wastewater composed of a minimum salt media (0.5 g L^{-1} K_2HPO_4, 0.4 g L^{-1} $MgSO_4.7H_2O$, 0.2 g L^{-1} NaCl, 0.2 g L^{-1} $CaSO_4.2H_2O$, 5 g L^{-1} $CaCO_3$, 0.02 g L^{-1} $Na_2MoO_4.2H_2O$, 0.02 g L^{-1} $MnSO_4.4H_2O$, 0.05 g L^{-1} $FeSO_4.7H_2O$) supplemented with 1 mL L^{-1} of a trace element solution (Widdel & Pfennig, 1981). Sucrose was used as a carbon source in concentrations of 3 g L^{-1} between day of operations 0 and 59, and then the concentration increased to 6 g L^{-1}. Reactors were operated in 1 L containers with an initial working volume of 0.8 L and a hydraulic retention time (HRT) of 48 hours. Air was pumped into the bottom of the reactors at 0.2 L min^{-1}, a complete mix of the liquor was achieved with mechanical stirrers. At the end of each cycle, the air tab and the stirrers were turned off for 1 hour to settle ~~down~~ the biomass. To start the new cycle, clear supernatant was replaced with 0.76 L of

fresh culture media. Sludge was never removed from the reactors. The pH in each of the four reactors was continuously monitored every 10 min with a pH FermProbe (Broadley James Corp., UK) (Figure A2 a and b). Data was stored using a PicoLog data logger (Pico Technology, UK) and a computer with the associated PicoLog Recorder V5.22.8 software installed. The oxygen concentration in the four reactors was measured every 10 min using a probe-type fibre-optic oxygen meter Fire Sting O_2 (Pyro Science GmbH, Germany) and a computer with Pyro Oxygen Logger software (Figure A2 a and b).

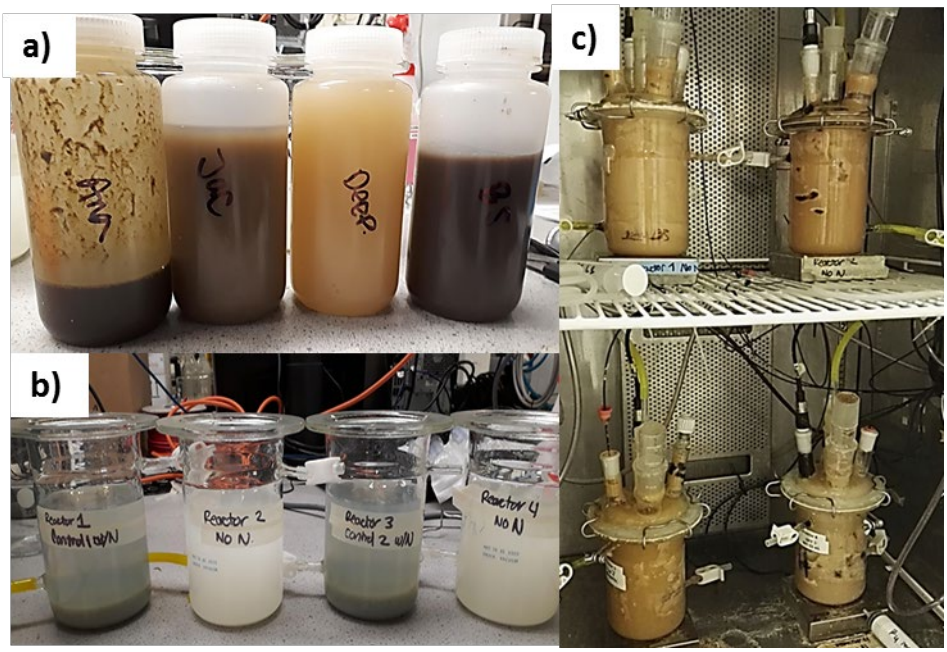

Figure 3.1. Set up of the high and low C/N reactors. Photographs showing **(a)** the bottles containing the inocula of the reactors**,(b)** the reactors just before the start-up of the experiment and **(c)** the reactors directly after mixing on day 85 of operation.

3.2.3. Physicochemical Analyses of Effluent

The soluble fraction of the mixed liquor at the start and end of each HRT is considered as the influent and effluent, respectively. Wastewater treatment efficiency was evaluated as soluble COD removed from the influent at the end of each operation cycle with COD spectroquant test kits (Merck, Germany). Total nitrogen from the influent, mixed liquor and effluent was quantified with spectroquant kits (Merck, Germany). Ammonium, nitrate and nitrite obtained from the influent and effluent were quantified with spectroquant kits (Merck, Germany), ultraviolet *s*pectrophotometry screening (APHA, 2017a) and LCK 341 kits (Hach Lange, Germany), respectively. Total

suspended solids (TSS) and volatile suspended solids (VSS) were quantified according to standard methods (APHA, 2017b) (Table A2).

3.2.4. Acetylene Reduction Assay and $^{15}N_2$ Method

Nitrogen fixation was assessed in parallel during 2 days with the acetylene reduction assay and $^{15}N_2$ methods. The former estimates the rate of acetylene reduction to ethylene by the nitrogenase enzyme and can be used to estimate nitrogen fixation rates indirectly (Hardy et al., 1968). The latter method measures BNF directly by incubating the sample with labelled nitrogen and later monitoring the concentration of this molecule in the biomass over time (Bellenger et al., 2014).

Batch reactors were assembled in crimp-sealed serum 160 mL bottles and were operated under the same two treatments used for the SBRs. The batch reactors were incubated at 30 °C in a shaker at 160 rpm. Biomass was obtained from the mixed liquor of the the SBR and was collected at the start of day 80 of operation (HRT 0). Two technical replicates were obtained from each SBR replicate reactor; therefore, four replicates per treatment. Additionally, a positive control treatment with two replicates was used, these reactors were inoculated with a pure culture of the nitrogen-fixing bacterium *Azotobacter vinelandii* Lipman 1903 (DSMZ 85, DSMZ, Germany) in mid-exponential phase (Figure A3) following Bellenger et al. (2014). The pure strain was cultured as specified by the supplier in nitrogen-free Azotobacter medium 3 (DSMZ, Germany). Negative control reactors containing only culture media from each type of treatment without biomass were included.

For the acetylene reduction assay, 30 mL of mixed liquor from the corresponding SBR was added to each serum bottle. For the positive control treatment, 30 mL of pure culture from *A.vinelandii* was added. Bottles were crimp sealed and the headspace was flushed for 2 min with a gas mix containing 1% acetylene (C_2H_2) in air (Calgaz, UK). A needle attached to the rubber stopper was used as an outlet. Headspace samples of 100 µL were collected at regular intervals using a gas lock syringe (Hamilton, US) and analysed for ethylene (C_2H_4) conversion from acetylene with a GC-FID instrument (Carlo-Erba 5160 GC); employing a HP-PLOT-Q capillary column (30 m x 0.32 mm i.d). The GC-FID machine was operated in split mode (flow rate 100 mL min^{-1} giving a split ratio of 100:1) with the injector at 150 °C and FID at 300 °C. Hydrogen was used as carrier gas at a flow rate through the column of 1 mL min^{-1}. Separation was performed on a HP-PLOT-Q capillary column (30 m x 0.32 mm i.d)

packed with 20 um Q phase. The GC was held isothermally at 75°C for 90 min and finally heated to 250 °C by increasing the temperature 10 °C min^{-1}. Calibration was carried out using gas standards of 1% acetylene in air and 1% ethylene in air (Calgaz, UK). Measurements of ethylene in the headspace were corrected for the proportion of gas dissolved in the liquid phase with Henry´s law (Sander, 2015).

The enriched $^{15}N_2$ tracer experiment was based on Bellenger et al. (2014) with some modifications. Briefly, 27 mL of mixed liquor and 3 mL of corresponding $^{15}N_2$ tracer-enriched assay medium were added into 160 mL crimp sealed serum bottles. For the positive control treatment, 27 mL of pure culture from *A.vinelandii* were used. The $^{15}N_2$ tracer-enriched media were prepared as specified by Klawonn et al. (2015) with some modifications, using minimum salt media with and without ammonium and the Azotobacter medium 3 (DSMZ 85, DSMZ, Germany). Each medium was degassed under vacuum in airtight-closed filtration flask and transferred via siphoning into separate septum glass bottles. Once the bottles were completely filled (headspace-free) and crimp sealed, they were injected with 170 µL of 99% $^{15}N_2$ gas (Sigma-Aldrich, UK) and incubated at 30 °C overnight on a shaker at 160 rpm. This last step was repeated three times. The headspace (previously pre-vacated) of each batch reactor was injected with 20 mL of 99% $^{15}N_2$ gas (Sigma-Aldrich, UK), this volume corresponded to 15% (v/v) of the headspace (Bellenger et al., 2014). Aliquots of 1 mL of the liquid culture were taken over time, cells were collected by filtration on pre-combusted (450 °C overnight) GF/F filters (Montoya et al., 1996). Filters were immediately dried at 50 °C for 6 h, and stored at room temperature until analysis (Mohr et al., 2010). The $^{15}N_2$ content of the biomass was analysed with Elemental Analyser-Isotope Ratio Mass Spectrometry (EA-IRMS) at Iso-Analytical (Cheshire, UK). Samples taken at time 0 of the experiment were used as a control for background $^{15}N_2$.

3.2.5. Microbial Community Analysis

Genomic DNA was extracted from 0.25 mL of mixed liquor from the SBR on day 0 and 85 of operation using the FastDNA® SPIN kit for soil (MP-Biomedicals, US) following the manufacturer's protocol, with a 5 min incubation at 55 °C before final centrifugation at the elution step.

The *nifH* gene was screened in the sludge samples from the environmental survey using a polymerase chain reaction (PCR) method. The primer pair IGK3/DVV (Ando et al., 2005) was used at a final concentration of 1.5 µM. The PCR mixture with a total

volume of 25 µL contained 3 µL of DNA template (or sterile molecular grade water, for the negative control), 13.75 µL of molecular-grade water, 0.5 µL of PCR nucleotide mix, 0.25 µL of FastStart high fidelity enzyme blend and 2.5 µL of FastStart high-fidelity reaction buffer with $MgCl_2$ (Roche, Germany). PCR was conducted on an Alpha Cycler 1 thermocycler (PCRmax, UK) using the following program modified from Gaby et al. (2017): initial hot-start at 95 °C for 15 min, followed by 30 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. The amplification of the 394 bp *nifH* gene was confirmed by a 1.5% agarose gel against use of a size ladder.

The enrichment of the NFB in the reactors was quantified with qPCR from the quantification of the copies of *nifH* and *16S rRNA* genes, assuming that each organism contained one copy of the *nifH* gene and 4.2 copies of the *16S rRNA* gene (Větrovský & Baldrian, 2013). The qPCR composition and the thermocycler program for the *16S rRNA* gene were the same used by Vignola et al. (2018). The temperature profile for the qPCR of the *nifH* gene using the primer pair IGK3/DVV was adapted from Gaby and Buckley (Gaby & Buckley, 2017) and consisted of: 98 °C for 15 min followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s and 72 °C for 30 s. The qPCR mixture used 5 µL of Quantifast SYBR Green mastermix (Qiagen, UK), 2 µL of DNA template (or water for the negative control) and 1.5 µM of each primer. The qPCR assays were performed on a CFX96 real-time PCR detection system (Bio-Rad, UK) with a C1000 thermal cycler iCycler using the software Bio-Rad CFX Manager version 3.0. Each sample was run in triplicate and a calibration curve was generated in every qPCR run. To assess the specificity of the products a melt-curve analysis (between 65 and 95 °C) was performed at the end of each qPCR run. Standards for the calibration curve of both genes were generated independently using genomic DNA of A.*vinelandii* Lipman 1903 (DSMZ 85, DSMZ, Germany) as a template and ranged between 10^3 and 10^8 copies μL^{-1} (Supplementary Information A2). The efficiencies of the qPCR standards for the *nifH* and *16S rRNA* genes were 84.1 and 97.3%, respectively. Previously, the methods were optimised for annealing temperature (Supplementary Information A3 and Table A3) and dilution of inhibitors (Supplementary Information A4 and Figure A4).

The microbial communities within reactors was assessed by next-generation amplicon sequencing of the *16S rRNA* and *nifH* genes in paired end mode. The concentration of the DNA extracts was determined using a Qubit® dsDNA HS assay it (Invitrogen, Life Technologies, Paisley, UK). DNA extracts were sequenced with an Illumina Miseq platform (NU-OMICS, Northumbria University, UK) using ~~a~~ the primer set F515/R806

targeting 294 bp of the V4 region of the bacterial *16S rRNA* gene as described previously in Kozich et al. (2013). The *nifH* gene was sequenced with the same technology, following the protocol developed by Gaby et al. (2018) using a nested PCR with the primers IGK3/DVV.

Sequencing output from the Illumina MiSeq platform was independently processed and quality-filtered for each gene, using the R statistical package DADA2 (version 1.10) (Callahan et al., 2016). Inferred amplicon sequence variants (ASVs) (Callahan et al., 2017) obtained from the *16S rRNA* gene were assigned taxonomic identifiers with the SILVA reference database (version 132) (Quast et al., 2012). *nifH* gene sequences were identified for taxonomy using a curated version of the *nifH* database (annex 1) published in the TaxAdivA pipeline (Gaby et al., 2018); ~~our~~ the version used in this study only contained sequences targeted by the forward primer IGK3. Furthermore, sequences were trimmed to a length of 320 bp following the IKG3 position. To make this database compatible with the DADA2 pipeline, the published fasta file and taxonomy files were unified. Further microbial community analyses of the processed sequencing data were carried out using the packages Phyloseq (1.24.2) (McMurdie & Holmes, 2013) and Vegan (version 2.5-3) (Oksanen et al., 2018).

Group average hierarchical clustering analysis of the Bray-Curtis similarities was performed on a log(X + 1) transformation of the *16S rRNA* sequences using the statistical software Primer 6 (PrimerE, Ivy Bridge, UK). The relative abundance of each taxon or ASV was calculated independently for each gene and corresponds to the proportion of reads obtained from each sample. The total abundance of *nifH* in the reactors was calculated by combining sequencing and qPCR data; *nifH* sequencing reads of each ASV was multiplied by the *nifH* copy number (quantified with qPCR) from the corresponding sample. The phylogenetic neighbour joining tree of the most abundant *nifH* sequences was generated using the software MEGA X (Kumar et al., 2018) with a bootstrap analysis of 1000 replicates. Previously, sequences were aligned with the ClustalW tool of the software.

3.2.6. Statistical Analysis

Variations of the results from the low and high C/N reactors were analysed with nested analysis of variance (ANOVA) using the software Minitab® 17 (Minitab Inc., US).

3.3. Results

3.3.1. Wastewater Treatment Efficiency

The COD removal efficiency in the high and low C/N reactors was statistically indistinguishable (nested ANOVA *p*-value = 0.426) with virtually all of the variance occurring in the replicates (>99%) and not by the type of treatment. On day 85, the high C/N reactors had a COD removal efficiency of 73 ± 4.8% (mean ± standard deviation) corresponding to 3588 ± 238 mg L^{-1} of COD removed from the system (Figure A5). The low C/N reactors had a COD removal efficiency of 83 ± 12.6%. Hence, the availability of nitrogen in the system did not limit the treatment of the organic matter. The average pH values of the low and high C/N treatments were 6.29 ± 0.8 and 6.31 ± 0.78, respectively. Meanwhile, the average dissolved oxygen concentration was 1.6 ± 2.2 mg L^{-1} for the low C/N and 1.18 ± 2 mg L^{-1} for the high C/N treatment (Figure A5).

3.3.2. Fixed Nitrogen in High and Low C/N Reactors

In the high C/N reactors, the amount of total nitrogen, primarily in organic form, increased from less than 10 to 280 ± 57 mg of N L^{-1} after 85 days of operation (Figure 3.2). The amount of ammonium found in these reactors was negligible (less than 0.1 mg of N L^{-1}). Overall, the average rate of organic nitrogen (biomass) produced in the high C/N was 1.6 ± 0.5 mg of N L^{-1} day^{-1}. In the low C/N reactors the total nitrogen after the same period was 175 ± 57 mg of N L^{-1} of which about half (80 ± 14 mg of N^{-1}) was ammoniacal nitrogen (Figure 3.2). The levels of nitrate (less than 6 mg of N^{-1}) and nitrite (less than 0.6 mg of N^{-1}) were low in both treatments.

3.3.3. N_2 fixation in Low and High C/N Reactors

The low C/N reactors had a nitrogen fixation rate of zero by either the $^{15}N_2$ or the acetylene reduction method. The high C/N reactors fixed nitrogen at a rate between 10 and 20 nmoles $^{15}N_2$ hr^{-1} mL^{-1} (on average up to 11.8 mg of N L^{-1} day^{-1}) over the 51-hrs incubation (Figure 3.3). The acetylene reduction test gave analogous results with rates ranging between 13 and 25 nmoles C_2H_4 hr^{-1} mL^{-1} (Figure A6a). The acetylene reduction rate to N_2 fixation rate is known as the *R* ratio, and in the nitrogen-fixing reactors varied between 1.2 and 1.5 (Figure A6b).

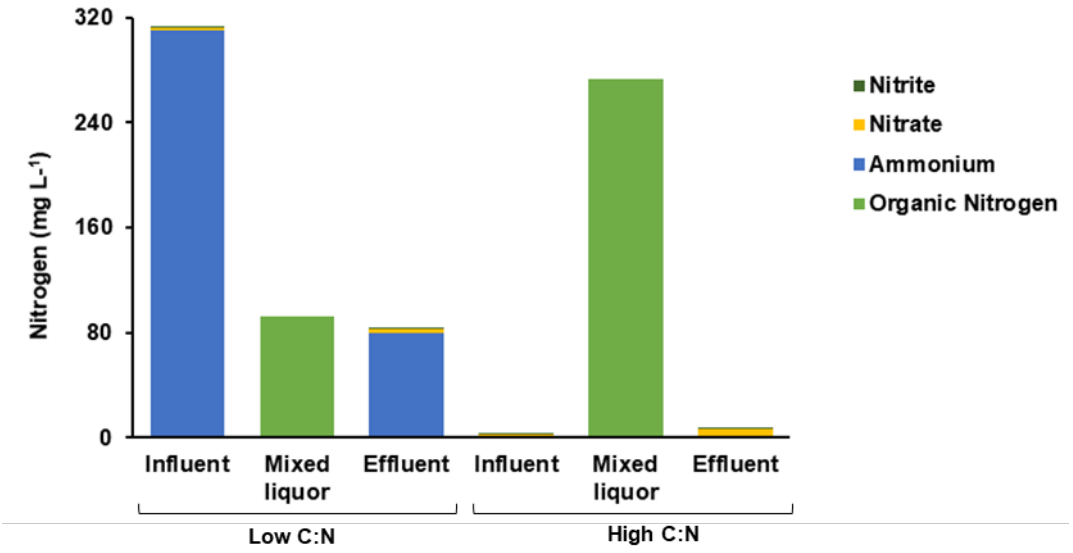

Figure 3.2. Nitrogen speciation profile of the high and low C/N treatments**.** Quantification of nitrogen in the forms of nitrite, nitrate, ammonium and organic nitrogen in the influent, mixed liquor and effluent of the low C/N (control) and high C/N treatments after 85 days of operation.

3.3.4. Abundance of 16S rRNA and nifH Genes in High and Low C/N Reactors

The amount of *16S rRNA* genes in both pairs of reactors increased over time (Figure 3.4a); the number of gene copies was used as a proxy for biomass quantification and increased (relative to day 0) 25 and 19-fold in the high C/N and low C/N treatments, respectively. The low C/N treatment had fewer (29%) *16S rRNA* genes than the high C/N treatment. This difference was modest and not quite significant (two tailed *P*-test = 0.058). There was no difference in the VSS between the test and control reactors (two tailed paired *t*-test, p = 0.32) (Table A2). The seed used to inoculate all the reactors contained 3.9 ± 0.2 x 10^6 *nifH* copies μL^{-1} and after 85 days of operation the high C/N had 8 ± 3.4 x 10^7 *nifH* copies μL^{-1}; meanwhile, the low C/N had 6.8 ± 3.2 x 10^6 *nifH* copies μL^{-1} (Figure 3.4b). The number of NFB in the reactors was calculated by assuming that each bacterial or archaeal cell contained 4.2 copies of *16S rRNA* (Větrovský & Baldrian, 2013) and 1 copy of *nifH;* it comprised about 15% of the biomass in the nitrogen-fixing reactors but decreased from 16% down to 1.5% (Figure 3.4c) in the low C/N. The increase in the influent COD concentration after day 59 of operation did not have a significant impact on the number of copies of either gene in the two treatments.

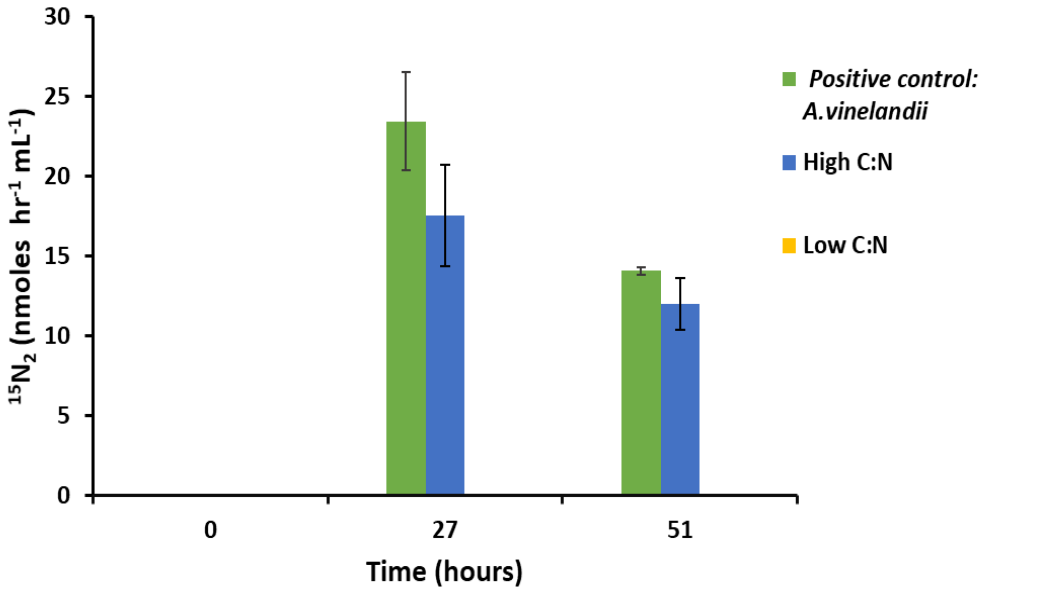

Figure 3.3. Nitrogen fixation rates of the high C/N and positive control treatments**.** $^{15}N_2$ fixation rates in low C/N (control), high C/N and positive control *A.vinelandii* treatments after 0, 24 and 51 hours of incubation in batch reactors. Error bars represent the standard deviation of the sample (*n* = 2 for *A.vinelandii*; *n* = 4 for low and high C/N).

3.3.5. Microbial Community Analysis

16S rRNA Genes

Cluster analysis of the 16S rRNA gene sequence data separated the microbial communities from the reactors into three main clusters (Figure 3.5); the inocula and all samples from day 0 formed a single cluster, and samples from the two treatments (low and high C/N) formed further two distinct clusters. The samples taken on day 0 were biological replicates and the microbial community profiles had high similarity (ca. 80%). The diversity of the microbial communities in all reactors irrespective of treatment, was virtually indistinguishable after 85 days of operation. There were no statistically significant differences in the observed number of species, Simpson or the inverse Simpson indices (nested ANOVA *p*-value >0.05) (Table A4). Nevertheless, the Shannon index was slightly higher in the high C/N reactors and was significantly different from the low C/N reactors (nested ANOVA *p*-value = 0.012) (Table A4).

It is clear that, even when evaluated at the phylum level, the low C/N reactors and nitrogen-fixing reactors developed quite different communities after 85 days of operation (Figure 3.6a). The three most abundant phyla in the high C/N reactors were Chloroflexi (26 ± 7%), Proteobacteria (24 ± 6%) and Firmicutes (22 ± 6%). In the low

C/N reactors, the most abundant phyla were Bacteroidetes (35 ± 0.3%), Actinobacteria (31 ± 4%) and Proteobacteria (21 ± 1%).

The most abundant (19 ± 5.4%) taxon in the high C/N reactors was closely related (85% bootstrap confidence level with Ribosomal Database Project (RDP) classifier (Wang et al., 2007) and 100% percentage of identity) to *Clostridium pasteurianum (*Figure 3.6b): an anaerobic oxygen tolerant NFB from the phylum Firmicutes (Sedlar et al., 2015). Interestingly, this bacterium, has on average, 10 copies of the *16S rRNA* gene (Cole et al., 2014). In the low C/N reactors, the most abundant microbe was *Corynebacterium sp*. representing 27 ± 2% *(*Figure 3.6b); it is a facultative anaerobic bacterium from the phylum Actinobacteria which is commonly found in sewage. This genus contains species that fix nitrogen (Gtari et al., 2012) as well as heterotrophic nitrifiers (Hu & Kung, 2000) and has on average 4.4 copies of the *16S rRNA* gene (Cole et al., 2014).

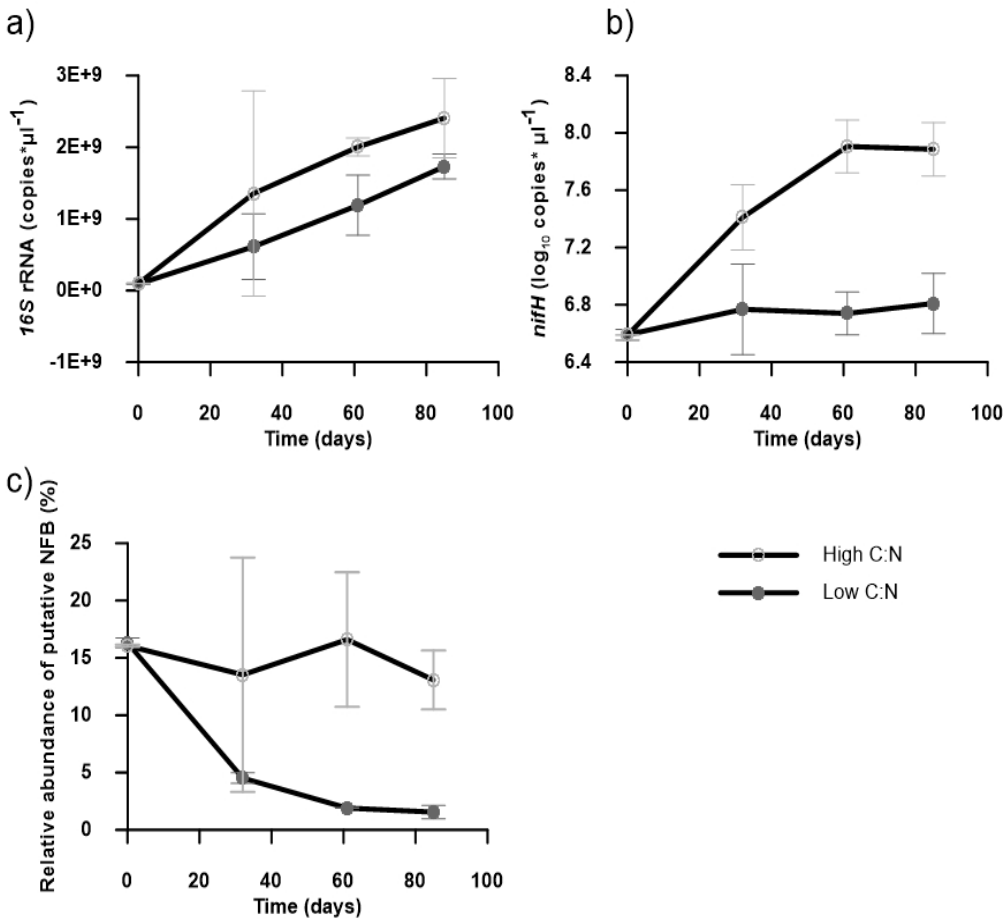

Figure 3.4. Microbial community analysis of the low and high C/N treatments: quantitative PCR (qPCR) results**.** Quantification of **(a)** *16S rRNA* and **(b)** *nifH* gene copies; and **(c)** relative abundance of putative NFB in low C/N (control) and high C/N treatments. Error bars represent the standard deviation of the sample (*n* = 2).

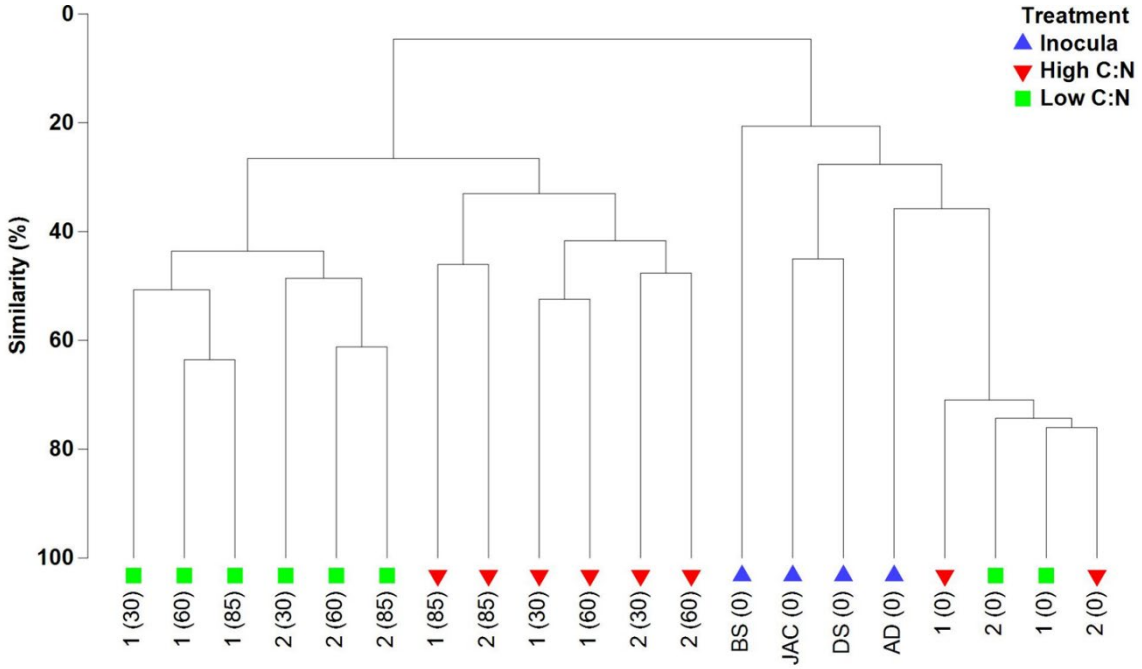

Figure 3.5. Microbial community analysis of the inocula and low and high C/N reactors: *16S rRNA* Dendogram. Comparison of microbial communities in samples from the inocula (BS: Bran Sands, DS: deep shaft, JAC: jet aerated channel, AD: anaerobic digester) low C/N (control) and high C/N reactors using group average hierarchical clustering derived from the Bray-Curtis similarity index of the *16S rRNA* ASVs. Each treatment was run in duplicate reactors (1 and 2) and each reactor was sampled at different time points (indicated in brackets): 0, 30, 60 and 85 days of operation.

nifH Genes

The dominant ASVs of the *nifH* gene on day 85 (ASV 2 and 3) varied between the nitrogen-fixing reactors and differed by just one base in position 339. These two sequences were also more than 99% identical to the nucleic acid sequence of three other ASVs and when all five sequences were translated into amino acids using the same data frame, they formed the same protein sequence composed of 112 amino acids. These results suggest synonymous single nucleotide polymorphisms (SNPs) of the *nifH* gene or sequencing errors rather than competitive exclusion for example two strains of NFB that were highly abundant. Therefore, the numbers of sequencing reads of these ASVs were pooled into ASV 2 and treated as a whole. Specifically, this sequence was present in the high C/N treatment with a total nifH abundance of 3.98 ± 0.73 x 10^7 *nifH* copies μL^{-1}, this number represented 52.13 ± 12.6% of the total *nifH* sequencing reads for both reactors. In the low C/N treatment this sequence was

present with a relative abundance of 25.3 ± 30.7% and a total *nifH* abundance of 2.2 ± 2.9 x 10^6 *nifH* copies μL^{-1}.

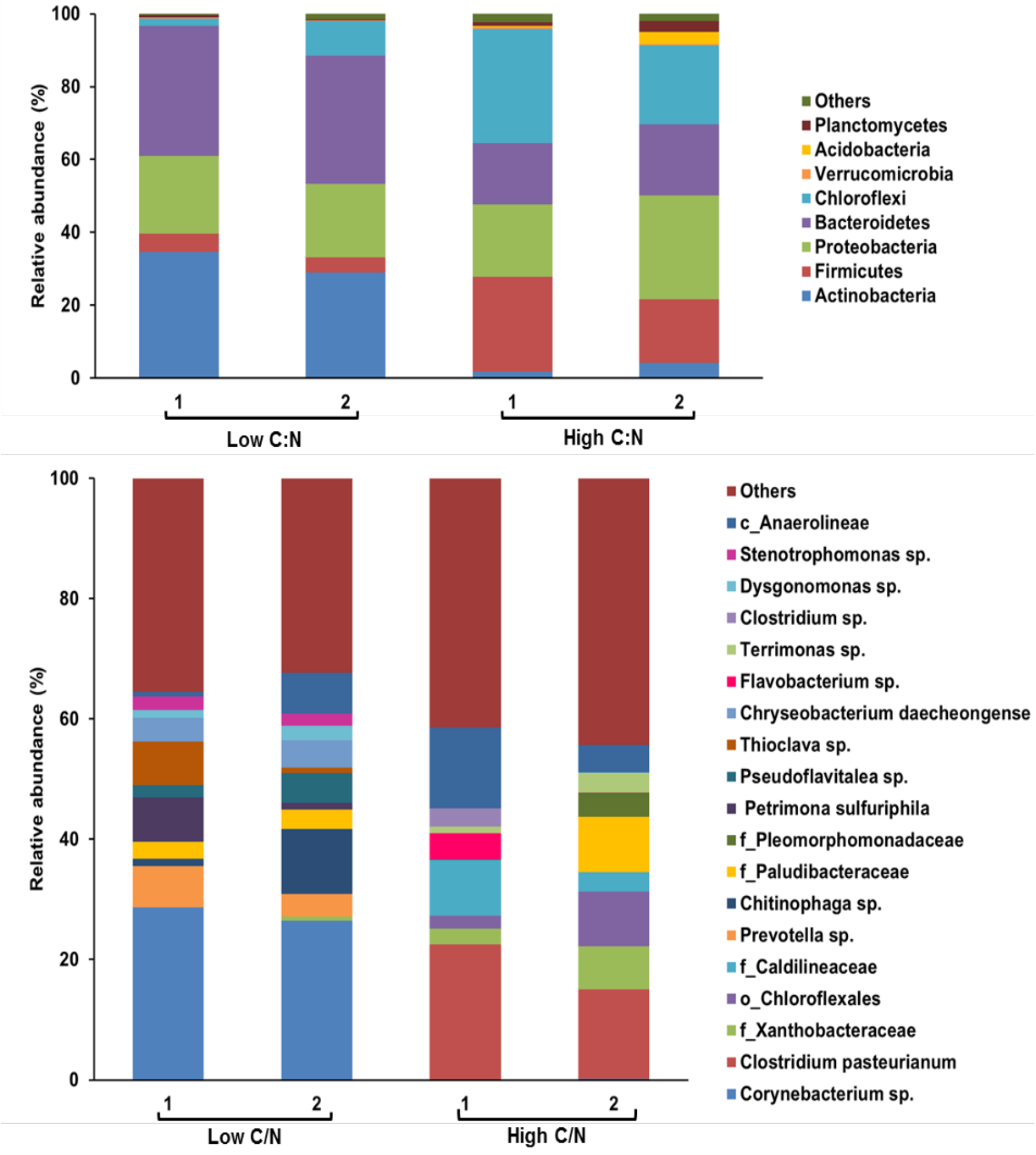

Figure 3.6. Microbial community analysis of the low and high C/N reactors: *16S rRNA* bacterial community composition**.** Bacterial community composition on day 85 of operation at **(a)** phylum and **(b)** genus level or lowest taxonomic rank available for the identified ASVs (c: class; o: order; f: family) of the low C/N (control) and high C/N reactors (*n* = 2).

Six ASVs closely related (<96% with RDP classifier, Wang et al., 2007) to *nifH* from *C. pasteurianum* were also identified; of these particularly ASV 12 had the highest relative

abundance in the nitrogen-fixing reactors representing 10 and 3% of reactors 1 and 2, respectively. ASV 12 was detected in very low abundance in the low C/N reactors (>0.01%).

Though there is apparently no clear agreement between abundant taxa determined by *16S rRNA* and functional *nifH* sequencing data, the results might tentatively suggest the presence a new strain of *Clostridium* sp. in the high C/N reactors. Unclassified *nifH* ASV 2 was aligned with homologous *nifH* sequences and compared in a phylogenetic neighbour-joining tree (Figure 3.7); the sequence grouped within homologous *nifH* sequences from *Clostridium* sp., *C. pasteurianum* and ASV 12 and shared a sequence identity of approximately 66%. A subsequent BLASTn (Johnson et al., 2008) search against the NCBI nucleotide database (Clark et al., 2016) of ASV 2 revealed the highest scoring match was *Paludibacter propionicigenes* WB4 (83% nucleotide sequence identity), which belongs to the phylum Bacteroidetes and was isolated from rice plant residues in an anoxic rice-field soil in Japan (Gronow et al., 2011). *nifH* can be present in multiple copies in a single genome with a high sequence variation, it has been reported that *C. pasteurianum* has six different nonidentical copies with nucleotide sequence identities ranging between 68 and 99% (Wang et al., 1988). Indeed, although ASV 12 clustered with the *C. pasteurianum* X07474 *nifH* reference sequence, it only had a nucleotide sequence identity of 90%.

3.4. Discussion

Most previous work has focused on nitrogen fixation as a treatment of high carbon-to-nitrogen wastewater without focusing on the augmentation of biomass with N fixing bacteria as a consequence of the valorisation method of wastewater sludge (Clark et al., 1997; Gapes et al., 1999; Bowers et al., 2008; Reid et al., 2008; Addison et al., 2011; Slade et al., 2011; Wong et al., 2014). This work shows that wastewater treatment systems operating at a high C/N ratio have the ability to fix appreciable quantities of nitrogen without compromising treatment efficiency and has demonstrated a “proof of concept” for a new strategy for nitrogen fixation. The results from this chapter demonstrated that the reactors, operated without any added nitrogen source, had the ability to produce biomass enriched with NFB and treat the wastewater to the same standard as reactors supplemented with nitrogen.

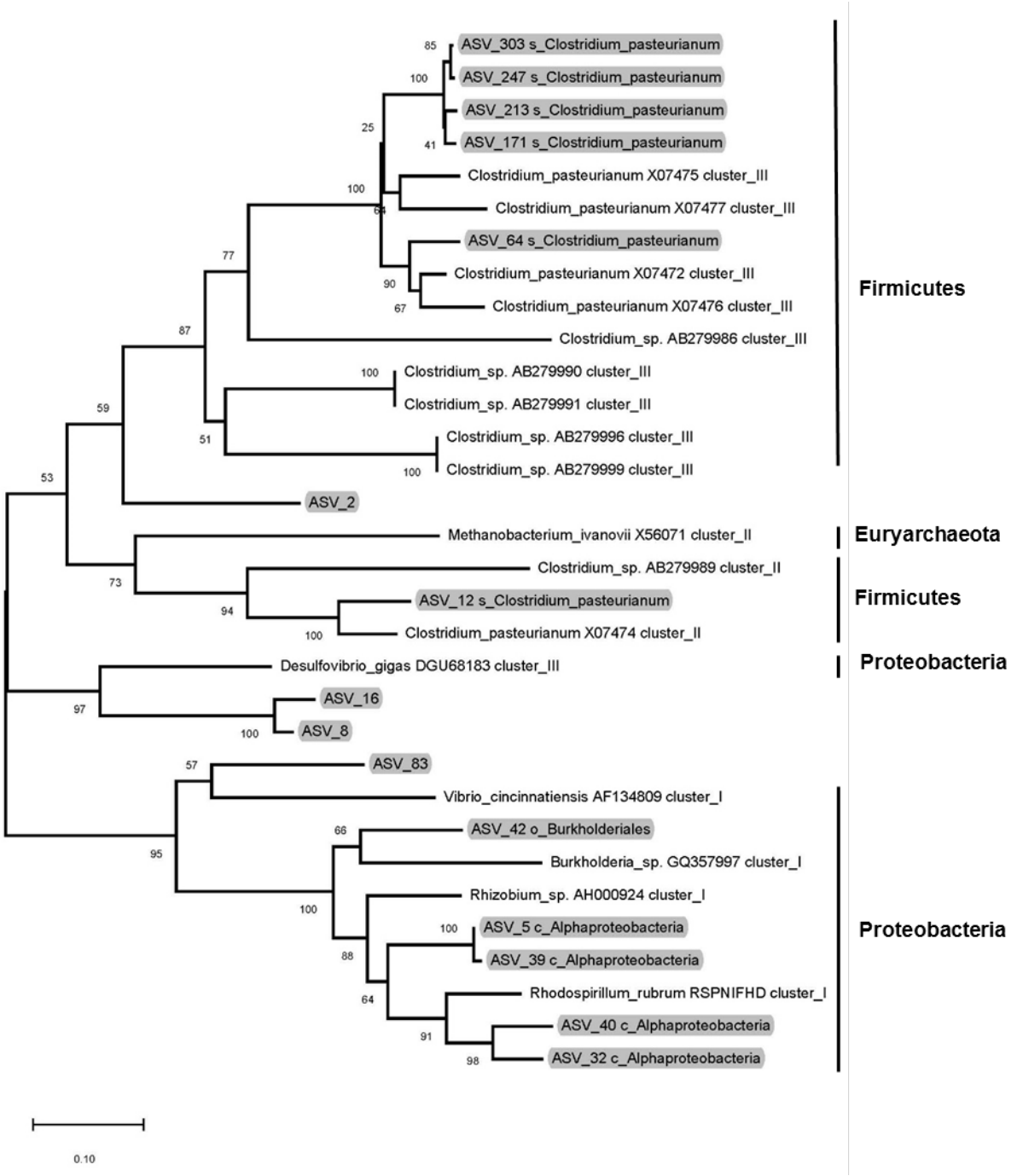

Figure 3.7. Microbial community analysis of the low and high C/N reactors: *nifH* phylogenetic neighbour-joining tree. Phylogenetic neighbour-joining tree of aligned *nifH* ASVs (highlighted in gray) from the high C/N treatment and their closest identified reference taxa from the nifH database (annex 1) published in the TaxAdivA pipeline (Gaby et al., 2018). When applicable, the phylum information and the lowest taxonomic rank available for the identified ASV (c: class; o: order; s: species) are provided. Bootstrap value percentages from 1000 replicate trees are given at each node. Scale bar shows the evolutionary distance of the sequences and corresponds to the fraction of nucleotide substitutions per character.

It is reasonable to infer that the nitrogen was sourced from the air and not from recycled biomass because almost no ammonium or oxidised nitrogen compounds were found in the effluent from the reactors. This type of sludge could, therefore, potentially be used as a biofertiliser; since the ability of free-living NFB to improve nitrogen uptake in plants such as cereals and sugar cane has been demonstrated (Malusà et al., 2016). Moreover some NFB can colonise these non-leguminous plants and form symbiotic associations that promote plant growth and yield (Bhattacharjee et al., 2008). The elemental composition of the dewatered sludge could be a subject of investigation in future studies to assess suitability as a fertiliser.

Our strategy for nitrogen fixation using energy contained in wastewater is sustainable and demands lower operation cost compared to a traditionally reduced nitrogen wastewater treatment system; it does not require the addition of ammonia and operates at low dissolved oxygen concentrations (less than 0.5 mg L^{-1}). Lower dissolved oxygen concentrations are thought to favour NFB (Gapes et al., 1999; Slade et al., 2003) and will obviously reduce running costs (accounts for more than 70% of the electricity consumption in activated sludge plants (Asadi et al., 2017)). The influence of the HRT and sludge retention time on the selection of NFB has not yet been elucidated and should be further investigated with the aim of optimising their reproduction.

The rate of nitrogen fixation observed in the batch test (at an average rate of up to 11.8 mg of N L^{-1} day^{-1}) is comparable to the theoretical amount of nitrogen that could have been fixed in the reactors (9.9 mg L^{-1} day^{-1};Supplementary Information A5) and to that reported in other breakthrough nitrogen fixation technologies. Liu et al. (2017a) obtained a N_2 fixation rate of 12 mg of N L^{-1} day^{-1} in an inorganic-biological nitrogen-fixing hybrid system using a pure culture of *Xanthobacter autotrophicus* and hydrogen obtained from catalytic water splitting (powered with renewable energy). However, the higher rates of this process must be set against the costs and stability of the catalyst and the difficulties of maintaining a pure culture. Milton et al. (2017) produced ammonia by coupling the catalytic protein of the nitrogenase enzyme (MoFe protein) in a cathode together with a bioanode containing a hydrogenase and obtained yields between 0.2 and 0.44 mg of N L^{-1} day^{-1}. Although these yields of nitrogen fixation are lower than the ones obtained in this study, this technology simultaneously produced electrical current with a faradic efficiency of 26.4%.

Previous works evaluating nitrogen fixation in wastewater treatment systems has only estimated nitrogen fixation rates using the acetylene reduction method (Clark et al., 1997), possibly because this method is easy to replicate, rapid and economical. The results obtained in this chapter were similar to the results obtained by Clark et al. (1997) which obtained values of up to 24 nM C_2H_4 mL^{-1} hr^{-1} in samples from an aerobic stabilisation basin treating paper mill wastewater. The *R* ratio corresponds to the acetylene reduction rate divided by the $^{15}N_2$ fixation rate and can be used to estimate the N_2 fixation rate when only the acetylene reduction rate is available. Although the theoretical value of the *R* ratio is 3.2, it varies ranging from 1 to over 30 (Bellenger et al., 2014). Therefore, before extrapolating data to obtain estimates of BNF it is very important to calibrate the acetylene reduction assay with the $^{15}N_2$ method (Chalk et al., 2017). The nitrogen fixation rates of experiments carried in the next result chapters were calculated using R ratio obtained for this experiment (estimated in 1.24) and the acetylene reduction test.

The amount of wastewater produced globally by the paper and pulp industries is estimated to be in at least 3,901 million liters per year, therefore, this high C/N wastewaters could be providing the energy to fix a minimum of 91.38 tons of nitrogen every year. This amount corresponds only to a tiny fraction (4.35×10^{-5}%) of the 210 teragrams of N fixed with the HBP per year (Fowler et al., 2013). Nevertheless, this new strategy of nitrogen fixation could be used with high C/N wastewaters from other industries such as wineries (Vlyssides et al., 2005) and cassava starch (Sreethawong et al., 2010; Liu et al., 2017b) for the production of biofertiliser. The latter could especially benefit farmers from developing countries who cannot afford to buy chemical fertilisers.

The ratio of *nifH* to putative total bacteria in the nitrogen-fixing reactors was 10 times lower than the ratio reported by Bowers et al. (2008). The difference in the level of enrichment in the activated sludge communities, while likely driven by experimental conditions (wastewater composition, HRT, dissolved oxygen level and so on), may also be affected by analysis methodology, such as DNA extraction method, the ratio of *nifH* to putative total bacteria and the primer selection (Albertsen et al., 2015). Particularly, Bowers et al. (2008) used a chemical DNA extraction method with high salt/sodium dodecyl sulphate (Zhou et al., 1996) together with a 1:1 ratio of *nifH* to putative total bacteria and the primer set PolF/PolR (Poly et al., 2001).

The genus *Clostridium* was found in an anaerobic nitrogen-fixing bioelectrochemical system (Wong et al., 2014) but has not been reported in studies held in aerobic wastewater treatment systems; previous studies mainly identified members of the phylum Proteobacteria such as *Azotobacter sp., Geobacter sp. and Klebsiella sp.* (Welz et al., 2018; Bowers et al., 2008). Nonetheless, these studies assessed the diazotrophic bacterial community using the *nifH* gene as a marker together with culture-dependent techniques. Taxonomic classification of bacteria using *nifH* gene is challenging because this gene can be transferred horizontally (Raymond et al., 2004) and consequently its phylogeny has a poor agreement with the *16S rRNA* phylogeny (Zehr et al., 2003), further complicated by the presence of multiple distinct *nifH* copies within individual bacteria. This many-to-one relationship between *nifH* and *16S rRNA* ASVs means that true taxonomic resolution for this system may require the use of whole genome metagenomics, or isolation and culturing to resolve. The most abundant NFB in this reactors do not appear to be classical nitrogen fixers but members, or close relatives, of the genus *Clostridium*. Little is known about the ecology of these bacteria in wastewater. It is possible that when this work is repeated with real wastewater, the community might change. Nevertheless, it seems plausible that this nonclassical NFB will be involved and, therefore, merits further study (Supplementary Information A6, Figure A7 and Table A5).

The HBP (34 MJ kg^{-1} of N) is between 2 and 4.5 times energetically more efficient than BNF (55 MJ kg^{-1} of N)(Gutschick, 1978; Cherkasov et al., 2015) and is also more profitable than the most established nitrogen recovery technologies (29-116 MJ kg^{-1} of N) (Matassa et al., 2015). Nevertheless, its energetic efficiency must be set against the environmental drawbacks associated with the production and use of chemical fertilisers. The challenge of nitrogen comprises an efficient use of fertilisers for food security parallel to the mitigation of its polluting effects (Sanjuán, 2016). Residual sludge from wastewater treatments systems enriched with NFB contributes to the valorisation of high carbon-to-nitrogen wastewater treatment systems as a potential source of nitrogenous fertiliser. In conclusion, the development of a biotechnological solution for the production of diazotrophic-enriched biomass within a waste treatment system provides an innovative sustainable approach to alleviate the environmental problems associated with nitrogen fixed by processes that rely on energy supplied from fossil fuels.

Chapter 4: Low inhibitory effect of ammonia on the nitrogen-fixing activity of a sludge enriched with nitrogen-fixing bacteria

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4.1. Introduction

Biological nitrogen fixation (BNF) is a process carried out by some bacteria in which molecular nitrogen (N_2) is converted to ammonia (NH_3). Globally, every year approximately 198 Tg of nitrogen (N) is fixed by bacteria in natural terrestrial (58 Tg of N) and aquatic ecosystems (148 Tg of N) (Fowler et al., 2013). BNF is being used in traditional agricultural systems, it is estimated that 60 Tg of N is fixed a year in croplands (Herridge et al., 2008). The food security of at least half of the human population depend on nitrogen fertiliser produced by the Haber-Bosch process (HBP) which was developed more than one hundred years ago (Erisman et al., 2008). Currently, approximately 100 Tg of N is fixed anthropogenically each year and used as a fertiliser (Galloway et al., 2013). Although the HBP is essential for the subsistence of a population of 7.7 billion people, it is arguably unsustainable as both the production and deployment of chemical fertilisers cause serious environmental problems. Firstly, because their manufacture consumes 1% of the world´s non-renewable energy supply and produces greenhouse gasses. Secondly, because, excessive use of nitrogen fertiliser causes eutrophication in water bodies, soil acidification and biodiversity loss (Erisman et al., 2008). Consequently, research into alternative and greener methods of nitrogen fixation for sustaining or increasing the present global fertiliser demand for food production is an urgent necessity.

BNF is an example of sustainable nitrogen fixation. This self-regulated process occurs in diverse ecosystems at ambient temperature and pressure; it has energetic efficiencies comparable to the HBP, is independent of primary non-renewable energy resources and does not produce greenhouse gasses (Cherkasov et al., 2015). Free-living and symbiotic N_2-fixing bacteria (NFB) have the ability to fix nitrogen because they possess the enzyme nitrogenase. The most commonly studied such enzyme is the molybdenum-iron (MoFe) dependant nitrogenase (Cherkasov et al., 2015). Vanadium-iron (VFe) and iron only (FeFe) dependant enzymes have also been identified (Bellenger et al., 2014). Some free-living NFB can form symbiotic (commensal or mutualistic) relationships with nonleguminous plants and have proven to increase nitrogen uptake in cereals and sugar cane (Bhattacharjee et al., 2008; Malusà et al., 2016).

Nitrogen is a limiting nutrient in many natural ecosystems but NFB would normally only fix this abundant gas if there were no better, more readily available sources (Burris &

Roberts, 1993). These is thought to be because BNF is an energetically expensive process for the cell as it requires electrons and ATP for the nitrogenase to operate. NFB grown in the absence of reduced nitrogen have a lower growth efficiency than when they are grown in a nitrogen-supplemented culture media (Daesch & Mortenson, 1968; Dalton & Postgate, 1969). Ammonia, which is one of the direct products of BNF, can inhibit nitrogenase activity in the short (post translational level) and long (transcriptional level) term (Dixon & Kahn, 2004). It has been shown that ammonia must be assimilated and the glutamine amino acid formed for the inhibition of BNF to occur (Kennedy et al., 1994). The degree to which ammonia inhibits NFB activity varies considerably with growth conditions such as dissolved oxygen concentration, respiratory rate, pH and growth stage (Klugkist & Haaker, 1984). The use of nitrogen fertilisers for agriculture has particularly decreased naturally occurring BNF in terrestrial (Galloway et al., 2004; Fan et al., 2019; Smercina et al., 2019) and aquatic (Herbert, 1999; Sohm et al., 2011) ecosystems. The concentration of ammonium in fertilized soils can range between from 12.8 to 200 mg kg-1 (Li et al., 2016).

The energy required for BNF can be obtained from organic matter such as industrial (Slade, Gapes, et al., 2004; Welz et al., 2018) or domestic wastewater. Effluent from pulp and paper industries is produced in large volumes, contains little to no ammonia and has high organic loads with chemical oxygen demand (COD) values of up to 11 g L^{-1} (Thompson et al., 2001). These wastes are treated biologically, and a specific C/N/P ratio of 100:5:1 is usually recommended for efficient biological treatment of wastewater (Gray, 2004). Therefore, such wastewater are typically supplemented with nitrogen and phosphorus to ensure adequate treatment. BNF has been detected in several pulp and paper wastewater treatment systems (Clark et al., 1997; Gauthier et al., 2000; Kargi & Ozmihçi, 2004; Slade, Gapes, et al., 2004; Welz et al., 2018). However, it is only recently that these treatment systems have been identified as a source of NFB for biofertiliser production (Liu et al., 2017a; Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020).

Previously in chapter 3 a new strategy for the enrichment of sludge with free-living NFB in sequencing batch reactors (SBR) fed with high carbon to nitrogen (C/N) synthetic wastewater was demonstrated. These reactors contained sludge enriched by 13% with NFB. This NFB-enriched sludge could fix nitrogen at an average rate of up to 11.8 mg L^{-1} day^{-1}. So, in principle, once dewatered, sludge enriched with free-living

NFB could be used as a sustainable and low-cost biofertiliser, alternative to chemical fertilisers in crop production (Gauthier et al., 2000; Kargi and Ozmihçi, 2004).

BNF is insignificant in conventional domestic wastewater treatment systems, presumably, because the feed contains ammonia in concentrations between 14 and 77 mg NH_4^+ L^{-1} (Jafarinejad et al., 2019; Tchobanoglous et al., 1991). However, nitrogen fixation has been observed in the presence of some reduced nitrogen in: pure cultures (45 mg NH_4^+ L^{-1} ;Inomura et al., 2018), the ocean (3.6 mg NH_4^+ L^{-1} (Knapp, 2012), wetlands (90 mg NH_4^+ L^{-1}; Bae et al., 2018), soil (68 kg Urea-N $ha^{-1}day^{-1}$; Smercina et al., 2019) and microbial fuel cells (105 mg NH_4^+ L^{-1}; (Ortiz-Medina et al., 2019).

Though the relationship between nitrogen content and the efficiency of wastewater treatment has been investigated previously in synthetic pulp and paper mill wastewater (Kargi and Ozmihçi, 2004; Slade et al., 2011) there are no estimates of the inhibition of nitrogen fixation under different ammonia concentrations. This type of wastewater is usually supplemented with chemical fertiliser (nitrogen and phosphorus) in order to achieve the recommended C/N/P ratio of 100:5:1 (COD/N/P of 100:3.5:0.8), required for an efficient biological treatment of the organic load. This study aims to find what concentrations of ammonia are inhibitory, this information could also be used to assess the potential utility of a biomass enriched with NFB as a biofertiliser. To this end, the nitrogen fixation rates of this sludge have been estimated under ammonium concentrations of up to 100 mg of N L^{-1} and it has been established that the biomass could fix nitrogen at significant rates whilst treating high C/N wastewater.

4.2. Methods

4.2.1. Setup of Reactors

Batch reactors were assembled and operated simultaneously under different treatments in crimp-sealed 160 mL serum bottles for 48 hours at 30 °C on a shaker at 160 rpm. Each reactor was inoculated with 3 mL of wastewater sludge and fed with 27 mL of synthetic wastewater. The sludge was sourced from nitrogen-fixing reactors operated continuously for 20 months and fed with synthetic wastewater with a C/N/P ratio of 100:0:1.5, specifications are described in Chapter 3 (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020). Sucrose (3 g L^{-1}) was used as a carbon source. Five treatments with ammonium ($(NH_4)_2SO_4$) concentrations of: 0, 30, 55, 80 and 100 mg of NH_4^+ L^{-1} were used. Each treatment contained two pairs of technical replicate

reactors (therefore four replicates per treatment), which were differentiated by the source of the sludge (high C/N reactors 1 and 2 from Chapter 3). Also a negative control reactor containing no biomass (blank) was included. Overall, two identical sets of treatments were assembled, one set of samples were used for chemical analysis and another for the acetylene assay. Therefore, multiple parallel samples were taken from separate replicate reactors for these analyses. This was because the acetylene test could interfere with the other measurements (Brotonegro, 1974).

4.2.2. Determination of Nitrogen Fixation Rates

Nitrogen fixation activity was indirectly measured with the acetylene reduction assay as described in Chapter 3 (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020). The *R* ratio (acetylene reduction rate to $^{15}N_2$ fixation rate) obtained in the aforementioned study (estimated in 1.24) and the acetylene reduction rates obtained in this study, were used to estimate the amount of N_2 fixed during the 48 hours of operation of the reactors. A pure culture of the NFB *Azotobacter vinelandii* Lipman 1903 (DSMZ 85, DSMZ, Germany), grown as specified by the supplier in nitrogen-free Azotobacter medium 3 (DSMZ, Germany), was used as a positive control. Crimp-sealed 160 mL serum bottles were inoculated with 30 mL of the pure culture in mid exponential phase (Bellenger et al., 2014) and different concentrations of ammonium ($(NH_4)_2SO_4$) were added in five treatments: 0, 30, 55, 80 and 100 mg of NH_4^+ L^{-1}; each treatment had two technical replicates and a negative control reactor containing only culture media (blank).

4.2.3. Analytical Methods for Wastewater

Soluble COD and ammonium were quantified in the influent and effluent using Spectroquant kits (Merck, Germany). Total nitrogen was quantified in the influent, effluent and the mixed liquor using Spectroquant kits (Merck, Germany). Nitrate and nitrite in the influent and effluent were measured by ion chromatography (Dionex Integrion High Pressure Ion Chromatography System, Thermo Fisher Scientific, US). The influent and effluent corresponded to the soluble fraction of the mixed liquor at the beginning and end of the batch cycle, respectively.

4.2.4. Statistical Analyses

The experimental data was analysed with nested analyses of variance (ANOVA), one-way repeated measures ANOVA and one-way ANOVA followed by *post-hoc* Tukey test, using Minitab 17 (Minitab Inc., U.S.).

4.3. Results

4.3.1. Nitrogen Fixation Under Different Ammonium Concentrations

The presence of ammonium retarded, but did not necessarily prevent, nitrogen fixation. In the samples of the nitrogen-fixing reactors at 24 hours of operation, where the relationship between presence of ammonium in the culture media and BNF was linear, there was a reduction of 0.36 nmol of C_2H_4 (ethylene) mL^{-1} hr^{-1} in the rate of acetylene reduction for each mg of ammonium. In the 48 hour samples the effect was less pronounced but still linear (Figure 4.1a). For all the treatments, including the positive control, higher rates of ethylene production were observed at 24 hours compared to 48 hours of operation (Figure 4.1a). At 24 hours, the rate of ethylene production in the nitrogen-fixing reactors varied between 45 ± 5.3 (mean ± standard deviation) and 7.6 ± 1.6 nmol of ethylene mL^{-1} hr^{-1} in the absence and presence of 100 mg of NH_4^+ L^{-1} in the culture media, respectively (Figure 4.1a). The rates of acetylene reduction between the treatments of nitrogen-fixing reactors, were significantly different at this timepoint (nested ANOVA *p*-value = 0.001) and the variance was mainly driven by the treatment (92%) followed by the replicate reactors (4%) and the random effect of the error term (4%). Nitrogen fixation activity was reduced by 16 and 83% in the presence of 30 and 100 mg of NH_4^+ L^{-1} in the culture media, respectively (Figure 4.1b). The rates of the nitrogen-fixing reactors were higher than the rates of the positive control reactors. In the positive control treatments, the relationship between ammonium and acetylene reduction was non-linear. After 24 hours of operation, the rates in positive control treatments varied between 28.7 ± 4.5 (mean ± standard deviation) and 3.1 ± 0.04 nmol of ethylene mL^{-1} hr^{-1} in the absence and presence of 100 mg of $NH4^+$ L^{-1} in the culture media, respectively (Figure 4.1a).

The nitrogenase activity in the sludge was resistant to the presence of ammonia with an IC_{50} of 70.2 mg of NH_4^+ L^{-1}, this figure was almost 4 times higher than in *Azotobacter vinelandii* (19.2 mg of NH_4^+ L^{-1}). Specifically, the inhibition process in the sludge was approximately linear with just 0.7% of the nitrogenase activity being lost for each mg of NH_4^+ L^{-1} of wastewater.

4.3.2. Nitrogen Speciation Profiles

In the five treatments the amount of ammonium in the soluble fraction decreased to very low levels (less than 2.8 mg of N L^{-1}; Figure 4.2 a) whilst the amount of organic nitrogen (i.e. biomass produced) in the mixed liquor increased (Figure 4.2 a and b).

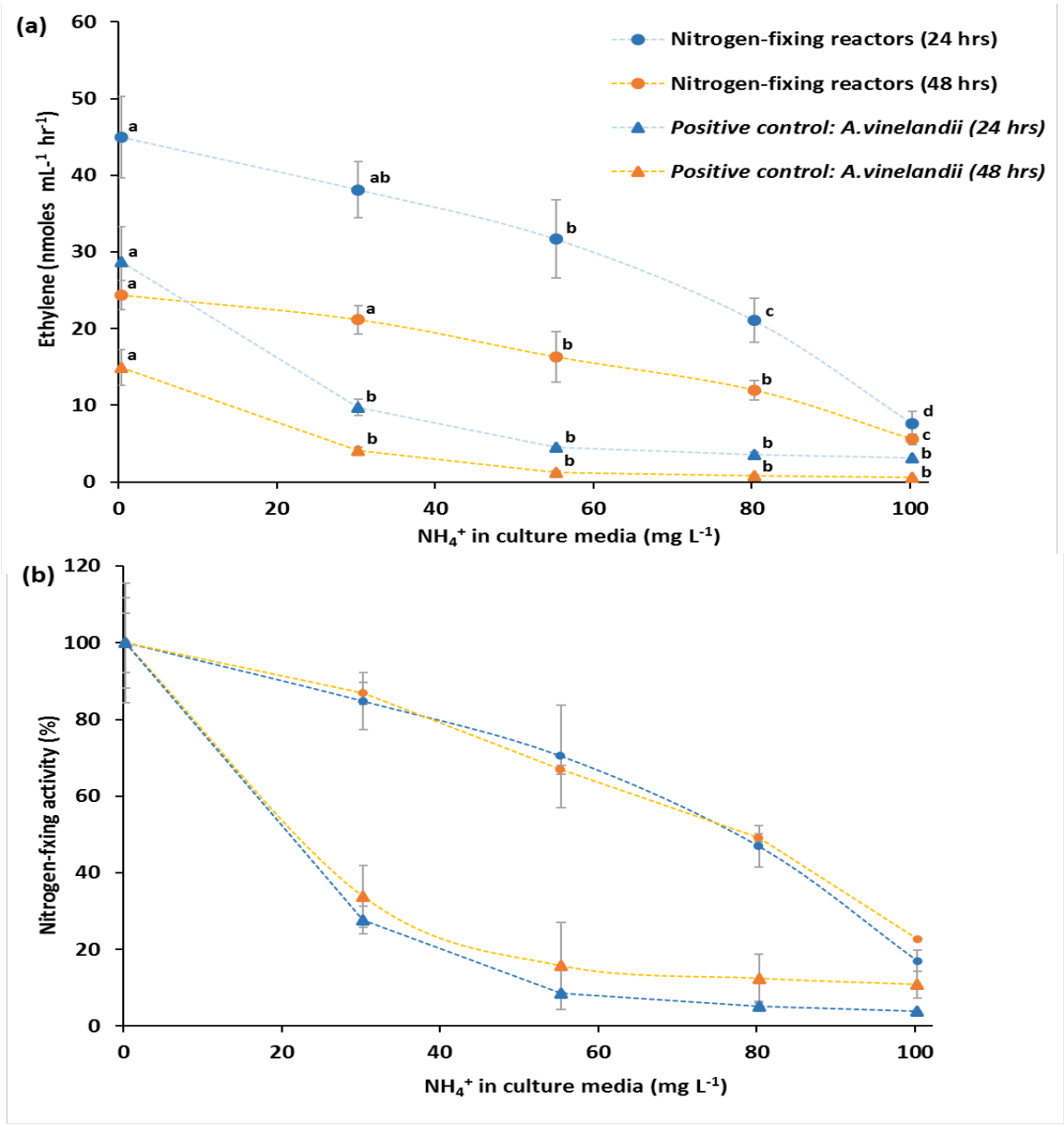

Figure 4.1. Nitrogen fixation rates of the nitrogen-fixing reactors operated under different ammonium concentrations estimated with the acetylene reduction assay. **(a)** Acetylene reduction rates and **(b)** percentage of nitrogen fixation activity (relative to no added ammonium) at 24 and 48 hours of operation in the positive control and nitrogen-fixing reactors operated under different ammonium concentrations. Significant differences between pair of treatments for the positive control and nitrogen-fixing reactors at 24 and 48 hours where identified using the Tukey test and have been indicated with different letters above the markers. Error bars represent the standard deviation of the sample (n = 2 for positive control and n = 4 for nitrogen-fixing reactors). Where the bars are not visible, the standard deviation is obscured by the marker.

Table 4.1. Nitrogen mass balance within treatments of the nitrogen-fixing reactors after 48 hours of operation and corresponding nested analyses of variance (ANOVA) values.

NH_4^+ in culture media (mg L^{-1})	Input (mg N L^{-1}) NH_4^+	N fixed	Sum	Output (mg N L^{-1}) Organic nitrogen produced	Nested ANOVA
0	0 ± 0	26.4 ± 2	26.40	33.22 ±5	0.175
30	19.8 ± 0.5	23 ± 2	42.67	40.5 ± 2.8	0.369
55	41 ± 2	17.7 ± 3.6	58.66	47.8 ± 3.8	0.162
80	62.3 ± 3.4	13 ± 1.4	75.22	64 ± 5.4	0.084
100	75.3 ± 4.3	6 ± 0.7	81.26	61.33 ± 6.8	0.02

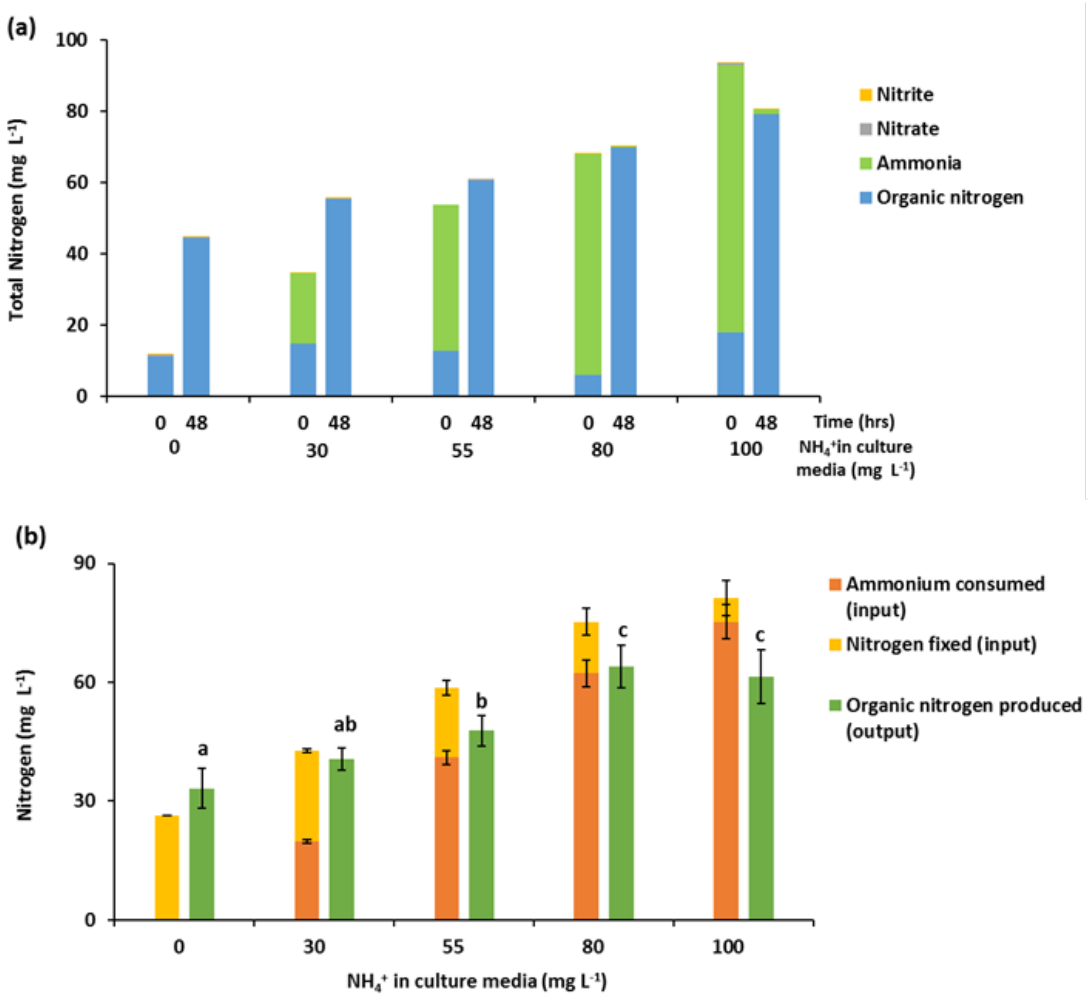

Figure 4.2. Nitrogen speciation profiles and nitrogen mass balance of the nitrogen-fixing reactors operated under different ammonium concentrations**. (a)** Quantification of nitrogen in the forms of nitrite, nitrate, ammonium, and organic nitrogen in the mixed liquor of the nitrogen-fixing reactors operated under different ammonium concentrations at time 0 and after 48 hours of operation. **(b)** Nitrogen mass balance within treatments of the nitrogen-fixing reactors after 48 hours of operation. Fixed nitrogen and ammonium nitrogen consumed correspond to the input sources and organic nitrogen produced correspond to the output. Significant differences in the organic nitrogen produced were identified between pair of treatments of the nitrogen-

fixing reactors using the Tukey test and have been indicated with different letters above the bars. Error bars represent the standard deviation of the sample (n = 4).

The total nitrogen at hour 0 and after 48 hours (Figure 4.2 a) was significantly different within all the treatments (repeated measures ANOVA p-value<0.05), except for the treatment with 80 mg of NH_4^+ L^{-1} in the culture media (repeated measures ANOVA p-value= 0.394). The amount of nitrite and nitrate was negligible (Figure 4.2 a) in the influent (less than 2.4 mg of N L^{-1}) and effluent (less than 1.6 mg of N L^{-1}) for all the treatments. The amount of organic nitrogen produced after 48 hours of operation (Figure 4.2 b), was significantly different between the treatments (nested ANOVA p-value <0.001) and the variance was mainly driven by the treatment (85%) and the random effect of the error term (15%). The average rate of organic nitrogen produced was 16.6 ± 2.5 and 30.7 ± 3.4 mg of N L^{-1} day^{-1} for the treatments with 0 and 100 mg of NH_4^+ L^{-1} in the culture media, respectively. A nitrogen mass balance was carried out for each treatment (Figure 4.2 b) using the average of the ammonium consumed from the culture media and the nitrogen fixed by the sludge as an input and the organic nitrogen produced as an output (assuming that all the incoming nitrogen is converted to biomass). The nitrogen fixed over the 48 hours of operation was estimated from the acetylene reduction rates. The mass balances did not show significant differences between the input and output values within treatments (nested ANOVA p-value >0.05), with the exception of the treatment with 100 mg of NH_4^+ L^{-1} in the culture media (nested ANOVA p-value =0.02) (Table 4.1). Clearly, the production of organic nitrogen was derived from the supply of nitrogen in treatments with up to 80 mg of NH_4^+ L^{-1} in the culture media.

4.3.3. Wastewater Treatment Performance

The COD removal efficiency of the five treatments, operated with an initial load of 3472 ± 126 mg L^{-1} of COD, varied between 95 and 99% (Figure 4.3). The availability of nitrogen did not affect the treatment of organic matter as there were no significant differences between the COD removal efficiencies (nested ANOVA p-value = 0.521) with the variance mainly occurring due to random effect of the error term (73%) and the replicates (27%).

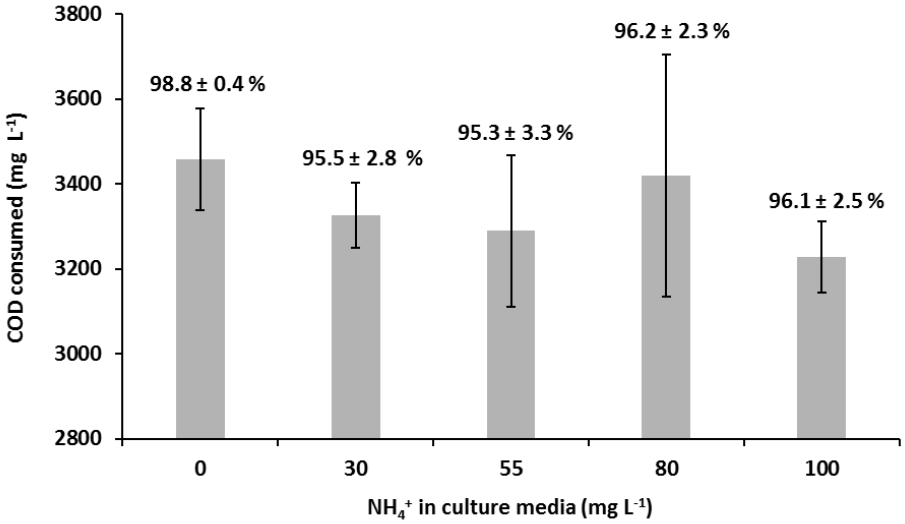

Figure 4.3. Chemical oxygen demand (COD) removal efficiency of the nitrogen-fixing reactors operated under different ammonium concentrations**.** Concentration of soluble COD removed in the treatments with nitrogen-fixing reactors operated under different ammonium concentrations. Error bars represent the standard deviation of the sample (*n* = 4). The numbers (mean ± standard deviation) above each bar correspond to the percentage of soluble COD removed.

4.4. Discussion

BNF occurred in the sludge, even in the presence of relatively substantial amounts of ammonia. The IC_{50} was actually well above the ammonia levels seen in most domestic wastewater, which usually contain ammonium in concentrations between 14 and 77 mg NH_4^+ L^{-1} (Tchobanoglous et al., 1991; Jafarinejad et al., 2019) and where the existence of this process is not well studied. Although the COD removal varied somewhat between treatments (~96–99%), this variation was not associated with the availability of the ammonia in the culture media at the start of the experiment. This suggests that it can be reasonably to BNF in high C:N wastes that are “contaminated” with ammonia. Moreover, it seems at least plausible that the bacteria in the sludge would fix nitrogen in soils with quantities below 100 mg of NH_4^+ L^{-1} of nitrogen-based chemical fertilisers.

BNF under limited presence of ammonium has been observed in marine environments containing concentrations of up to 3.6 mg of NH_4^+ L^{-1} in (Knapp, 2012), in flooded soil systems with 0.7 mg of NH_4^+ g^{-1} of soil (Balandreau et al., 1975) and in pure culture systems of *Lysobacter* sp. and *A. vinelandii* containing concentrations of up to 29 mg of NH_4^+ L^{-1} (Iwata et al., 2010) and 45 mg of NH_4^+ L^{-1} (Bühler et al., 1987), respectively.

The great sensitivity of our pure culture controls to ammonia is consistent with the literature and lends credence to our surprising results. Clearly the microbial ecology of BNF in sludges should not be inferred from studies of pure cultures.

In the present experiment the sludge had been cultured without any nitrogen source in the culture media and was exposed to different concentrations of ammonium for 48 hours. Temporary exposure of the nitrogen-fixing sludge to 100 mg of NH_4^+ L^{-1} showed BNF rates of approximately 4.14 ± 0.8 mg of N L^{-1} day^{-1}. The ammonium inhibition limit for BNF in this system is estimated at 132.4 mg of NH_4^+ L^{-1}, but this tolerance to ammonium could be diminished when there is continuous exposure (Knapp, 2012; Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020). Particularly in wastewater treatment systems, inhibition of nitrogen fixation was observed when reactors were operated for more than 20 days with a HRT of approximately 1.5 days and with nitrogen supplemented in concentrations between 4.7 mg of N L^{-1} (Slade et al., 2011) and 22 mg of N L^{-1} (Dennis et al., 2004). It is energetically cheaper to absorb ammonia (Knapp, 2012; Rittmann & McCarty, 2012) therefore, when carbon and nitrogen are in excess, NFB within a mixed culture will compete evenly with non-nitrogen fixers and could eventually decrease in numbers (Gapes et al., 1999; Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020).

Ammonia has a reversible repressing effect on the activity of the nitrogenase enzyme, initially the molecule inhibits the electron transport system to the enzyme (Laane et al., 1980) and diverts it to the metabolism of ammonia assimilation (Brotonegro, 1974). Furthermore, ammonia can repress the synthesis of the enzyme (Laane et al., 1980; Merrick, 2004; Dixon & Kahn, 2004) and trigger nitrogenase degradation when present for more than 4 hours (Brotonegro, 1974). Transcriptional or posttranslational regulation of the nitrogen-fixing activity can vary between microorganisms (Smercina et al., 2019). The quantification of the nitrogenase messenger RNA would help to elucidate the mechanisms of ammonia inhibition of BNF within a wastewater treatment system but unfortunately the attempts to extract it were unsuccesful.

Nitrogen fixation and ammonia uptake were assessed as concurrent because the highest rate of nitrogen fixation occurred at 24 hours rather than at 48 hours. The rate of nitrogen fixation at 48 h would have been higher than at 24 h if the nitrogen fixation started after the ammonia was depleted in the system. Measurements of ammonia at shorter time intervals and before 48 h would support this hypothesis. All the nitrogen

that entered the system was transformed into new biomass in the treatments with up to 80 mg of NH_4^+ L^{-1} in the culture media. This was not the case for the organic nitrogen produced in the treatment with an initial concentration of 100 mg of NH_4^+ L^{-1} where given the conditions of the experiment (30 °C with mixed aeration), some ammonium could have been lost due to volatilisation. In addition, the results of this study also confirmed that nitrogen concentration does not have any effect on the organic carbon removal rate (Slade et al., 2011; Kargi & Ozmihçi, 2004).

In circumstances where nitrogen is limited and the cost of BNF can be tolerated, being able to simultaneously consume different sources of nitrogen constitutes a fitness advantage for the NFB (Inomura et al., 2018). This could be the case in high C/N wastewater treatment systems from the paper and pulp (Thompson et al., 2001), wineries (Vlyssides et al., 2005) and cassava starch industries (Sreethawong et al., 2010; Liu et al., 2017b), which treat effluent with a high organic load.

This biotechnological solution for production of sludge enriched with NFB, using renewable energy and efficiently treating wastewater, is a viable alternative to chemical fertilisers due to the ability of the sludge to tolerate ammonia. Subsequent research should be focused on practical applications of this sludge as a biofertiliser on a variety of crops in different agricultural settings. In, fact, this technology could especially benefit the food security of low-income farmers from developing countries because it could provide an affordable source of biofertiliser.

Chapter 5: Valorisation of Pulp and Paper Industry Wastewater Using Sludge Enriched with Nitrogen-Fixing Bacteria

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5.1. Introduction

Although artificial nitrogen fertilisers have been said to support up to 42% of the increase in population since 1908, nitrogen fixation by the Haber-Bosch process (HBP) consumes 1% of the global annual non-renewable primary energy supply and emits greenhouse gasses (Erisman et al., 2008). Currently, an estimated 210 Tg of nitrogen per year are fixed through anthropogenic activities such as induced cultivation of legumes, burning of fossil fuels and ammonia production (Galloway et al., 2013). Only 17% of the nitrogen used as fertiliser is consumed by humans, 40% is naturally converted to atmospheric nitrogen; and, because it is applied in excess to crops and fields, the remaining amount runs off into water bodies causing eutrophication and biodiversity loss (Erisman et al., 2008). Nitrogen pollution of the environment (air, soil and water) costs the European Union between €70 and €320 billion per year (Sutton, 2011). At present, alternative technologies to recover ammonia for fertiliser production from domestic and some industrial wastewaters (Solon et al., 2019) require more energy and resources than the HBP and have only been used at small scale (Maurer et al., 2003; Matassa et al., 2015).

Almost as much nitrogen (200 Tg per year) is fixed naturally by bacteria and archaea globally as is by humankind (Fowler et al., 2013). In theory, the energy required for biological nitrogen fixation (BNF; 0.38 MJ mol^{-1} of N) is comparable to that of the HBP (0.48 MJ mol^{-1} of N), but in practice the latter is between 2 and 4.5 times more efficient (Gutschick, 1978; Cherkasov et al., 2015). Nevertheless, BNF is a sustainable natural method for ammonia production which does not emit rely on fossil fuels (Cherkasov et al., 2015) and has been widely studied since its discovery in the nineteenth century (Fowler et al., 2013). Bacterial nitrogen fixation is self-regulated and happens at ambient pressure and temperature (Cherkasov et al., 2015). N_2-fixing bacteria (NFB) can be identified by the presence of the *nifH* gene (which can be transferred horizontally) and is one of the genes encoding for the nitrogenase enzyme, responsible for BNF (Gaby & Buckley, 2012). The energy required for BNF can be sourced from organic matter, for example industrial (Slade, Gapes, et al., 2004) and domestic wastewater or waste from agriculture and forestry processing (Cherkasov et al., 2015).

Effluent from pulp and paper mill industries can have strong chemical oxygen demand (COD) values of up to 11 grams L^{-1} and is usually low in ammonia and phosphorus (Thompson et al., 2001). Consequently, to facilitate biological treatment, this type of wastewater is supplemented with chemical fertiliser (nitrogen and phosphorus) in order to achieve the recommended C/N ratio of 100:5 (COD/N of 100:3.5), required for an efficient biological treatment of the organic load (Pratt et al., 2007; Dennis et al., 2004; Slade, Gapes, et al., 2004; Gray, 2004). But nutrient supplementation is expensive, difficult to control, requires extensive monitoring and sometimes causes an excess of nutrient in the effluent; alternatively, the nitrogen necessary for bacterial growth could be obtained through BNF or nutrient recycling from bental deposits (Slade, Ellis, et al., 2004; Wiegand et al., 2014). Some studies have determined the conditions required to convert and/or operate wastewater treatment plants under nitrogen fixation mode or nutrient recycling from benthal deposits, adding limited or no nutrients to the system (Slade, Ellis, et al., 2004; Wiegand et al., 2014; Pratt et al., 2007; Dennis et al., 2004; Gapes et al., 1999). In 2017 the world paper production surpassed the 781 million tons (FAOSTAT, 2017). The paper manufacturing technology uses between 5 and 100 litres of water for each ton of paper produced (Doble & Kumar, 2005).

NFB have been used for ammonia production in bioelectrochemical systems (Belleville et al., 2011; Wong et al., 2014) and have been found in activated sludge wastewater treatments systems (Clark et al., 1997; Gapes et al., 1999; Kargi & Ozmihçi, 2004) treating high carbon-to-nitrogen industrial wastewater. BNF is generally overlooked in wastewater treatment research as the presence of ammonia in high concentrations can inhibit this process (Hartmann et al., 1986; Smercina et al., 2019; Guo et al., 2017). Bacterial groups containing nitrogen-fixing species usually found in wastewater treatment systems include: Proteobacteria (Reid et al., 2008), Methanogens (Collins et al., 2016), glycogen-accumulating and polyphosphate-accumulating bacteria (Guo et al., 2017). Some free-living NFB can enhance the ability to absorb nitrogen in cereal and sugar cane plants (Bhattacharjee et al., 2008; Malusà et al., 2016), nevertheless, studies of BNF within paper mill wastewater treatment systems (Clark et al., 1997; Gauthier et al., 2000; Kargi & Ozmihçi, 2004; Slade, Gapes, et al., 2004; Welz et al., 2018) have not recognised this systems as a potential source of NFB for

biofertiliser production. Indeed, sludge enriched with NFB could be dewatered (Gauthier et al., 2000) and used as a biofertiliser (Kargi & Ozmihçi, 2004).

Previously in Chapter 3, a new strategy for nitrogen fixation, using sequencing batch reactors (SBR) fed with synthetic high C/N wastewater, to enrich an activated sludge with NFB (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020) was developed. These reactors contained sludge enriched by 13% with NFB and were able to fix nitrogen at an average rate of up to 11.8 mg of N L^{-1} day^{-1}. The present study explores the practical application of a new nitrogen fixation strategy and was developed using two sets of duplicate bench scale wastewater treatment plants treating real wastewater with a high C/N ratio. This chapter evaluates the feasibility of using real wastewater from a pulp and paper mill as a source of carbon to culture NFB contained in a wastewater sludge, valorising therefore the organic matter low in nitrogen and the sludge enriched with NFB. To this end, the conditions necessary for BNF were determined and the nitrogen fixation rates of the sludge were estimated. Additionally, the population of NFB bacteria was assessed by quantifying the *nifH* and *16S rRNA* gene.

5.2. Methods

5.2.1. Setup and Operation of Reactors

SBRs were assembled and operated as previously described in Chapter 3 but with some modifications. The reactors were inoculated with sludge enriched with NFB and operated with high C/N real wastewater for 114 days. Two sets of technical reactors in duplicate (therefore 4 replicate reactors in total) were used. Each set was inoculated with a different source of sludge obtained from the high C/N 1 and 2 reactors operated in Chapter 3 (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020) after 18 months of operation. Sludge from reactor high C/N 1 was used to inoculate the replicate reactors 1.1 and 1.2, meanwhile sludge from reactor high C/N 2 was used to inoculate the replicate reactors 2.1 and 2.2. Each SBR was inoculated with 40 mL of the corresponding sludge. Biomass was never removed from the reactors. The reactors were operated in 1 L containers and had an initial working volume of 0.8 L (Figure 5.1). Raw effluent from a paper mill located in Prudhoe, UK (54°58′06.2″N 1°51′26.2″W) was sampled approximately every 2 months and was stored at 4 °C until needed. Wastewater at room

temperature was fed into the SBRs every 48 hours, which corresponds to the hydraulic retention time (HRT). After 72 days of operation the wastewater fed into the reactors was supplemented with 1 mL L^{-1} of a trace element solution (Widdel & Pfennig, 1981). Dissolved oxygen and pH (Figure B1 a and b) were monitored as described in Chapter 3.

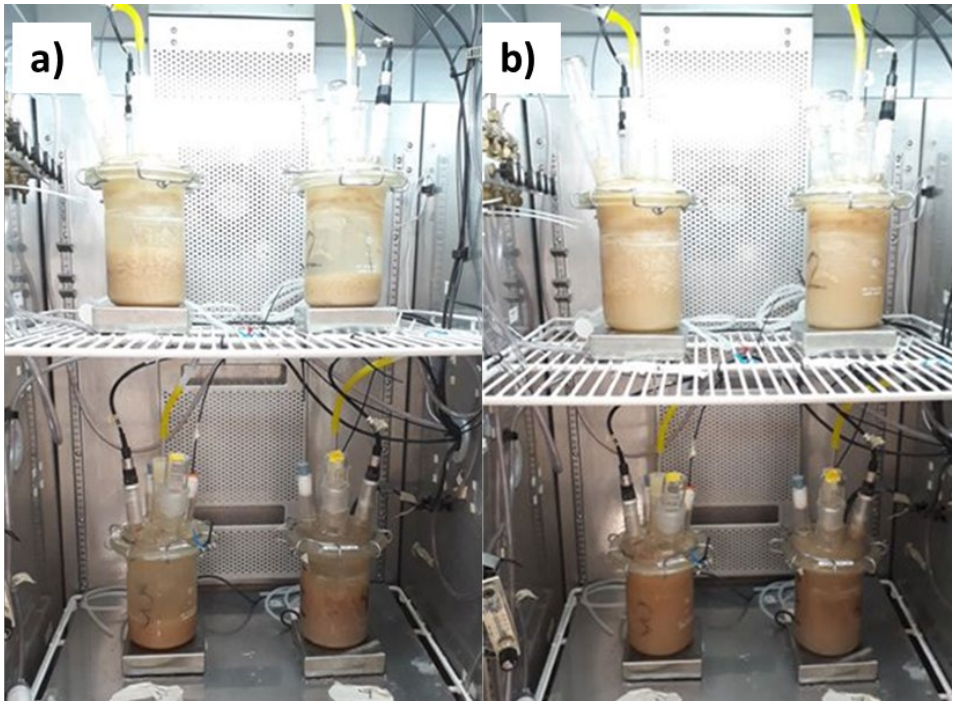

Figure 5.1. Set up of the high C/N reactors treating paper mill wastewater. Photographs showing the high C/N **(a) after a period of** settlement and **(b)** directly after mixing.

5.2.2. Physicochemical Analysis of Wastewater

COD, ammonium and total nitrogen concentration were quantified with spectroquant kits (Merck, Germany). Soluble COD and ammonium were quantified in the influent and effluent wastewater, meanwhile total nitrogen was quantified in the influent, mixed liquor and effluent. Total suspended solids (TSS) and volatile suspended solids (VSS) were quantified following standard methods (APHA, 2017b) (Figure B2). Anion chromatography (Dionex Integrion High Pressure Ion Chromatography System, Thermo Fisher Scientific, US) was used to measure the nitrate and nitrite in the influent and effluent. The total organic carbon (TOC) of the wastewater was analysed using a Shimadzu 5050A total organic carbon analyser, with an ASI-5000A autosampler (Shimadzu, Japan). Metal analysis of the wastewater was conducted by ICP-OES (VISTA-MPX,

Varian, US). The influent and effluent correspond to the soluble fraction of the mixed liquor at the start and end of each batch cycle, respectively.

5.2.3. Nitrogen Fixation Activity

The acetylene reduction assay as specified in Chapter 3 (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020) was used to indirectly measure nitrogen-fixing activity for 48 hours in batch reactors during day 64 and 114 of operation. Briefly, duplicated crimp-sealed 160 mL serum bottles were inoculated with 30 mL of mixed liquor from each SBR reactor collected at the beginning of the HRT. For the experiments made on day 114 of operation, an additional treatment of batch reactors containing paper mill waste water, trace elements and sucrose (as a supplementary source of carbon in concentrations of 3 g L^{-1}) was used. Each trial included negative control reactors containing only the culture media without biomass. Negative results of the acetylene reduction test on day 64 were further confirmed with the $^{15}N_2$ method following the method described in Chapter 3. The amount of N_2 fixed during the 48 hours of operation of the batch reactors was calculated using the acetylene reduction rates obtained in this study and the *R* ratio (acetylene reduction rate to $^{15}N_2$ fixation rate) obtained in Chapter 3, which was estimated in 1.24.

5.2.4. Analysis of Microbial Community

Genomic DNA was extracted from the mixed liquor and copies of the *16S rRNA* and *nifH* genes were quantified with quantitative polymerase chain reaction (qPCR) following the protocols described in Chapter 3. The efficiencies of the qPCR standards were 91% for the *16S rRNA* gene and 82% for the *nifH* gene.

The microbial community of the reactors was elucidated by next-generation amplicon sequencing of the V4 region from bacterial or archaeal *16S rRNA* gene (294 bp) in paired end mode as described elsewhere (Kozich et al., 2013), using the Illumina MiSeq platform (NU-OMICS, Northumbria University, U.K.) with the primer set F515/R806. Sequencing output data was processed using the R statistical package DADA2 (version 1.10) (Callahan et al., 2016), taxonomically assigned with the SILVA reference database (version 132) (Quast et al., 2012), and further processed using the packages Phyloseq (1.24.2) (McMurdie & Holmes, 2013) and Vegan (version 2.5−3) (Oksanen et al., 2018). The statistical software Primer 6 (PrimerE, U.K.) was used to make a group average hierarchical

clustering analysis of the Bray−Curtis similarities on a log(X + 1) transformation of the *16S rRNA* inferred amplicon sequence variants or ASVs (Callahan et al., 2017). The most abundant ASVs (>2.9%) on day 114 of operation were aligned with the ClustalW tool of the software MEGA X (Kumar et al., 2018) and plotted in a phylogenetic neighbour-joining tree with a bootstrap analysis of 1000 replicates.

5.2.5. Statistical Analysis

Repeated measures and nested analysis of variance (ANOVA) tests were used to analyse the variations on the experimental data with the software Minitab 17 (Minitab Inc., U.S.).

1.3 5.3. Results

5.3.1. Wastewater Treatment Performance

The COD removal efficiency in the high C/N reactors, fed with different COD concentrations throughout ~~along~~ the 114 days of operation, ranged between 64 ± 4 to 88.7 ± 2.5% (Figure 5.2). During the first 2 months the wastewater collected from the paper mill had a COD value of 1919 ± 260 mg L^{-1} and for the rest of the experiment it had a COD value of 621 ± 12 mg L^{-1}. Variations in the COD concentration are common and depend on the type of paper produced (Slade et al., 2003). at the time of sampling. On days 46 and 114, the high C/N reactors were able to remove 1262 ± 16.3 and 559 ± 16 mg L^{-1} of COD from the system, respectively. The soluble COD concentration in the effluent obtained from day 72 and 114 of operation was below 72 mg L^{-1} (Figure 5.2). The average pH value and dissolved oxygen concentration in the high C/N reactors was 6.31 ± 0.78 and 1.18 ± 2 mg L^{-1}, respectively (Figure B1 a and b).

5.3.2. Nitrogen Speciation Profiles and Occurrence of BNF

The amount of organic nitrogen in the high C/N reactors increased approximately 3.4-fold, from 18.6 ± 9.2 to 62.8 ± 12.7 mg N L−1 after 62 days of operation (Figure 2). Furthermore, the biomass in the reactors was able to settle immediately after stirrers and aerators were turned off. The total nitrogen in the paper mill wastewater collected during the first 2 months was approximately 3.2 ± 2 mg of N L-1 and contained nitrogen in the form of nitrate (less than 1.75 mg of N L-1), nitrite (less than 0.67 mg of N L-1), ammonium (less than 0.28 mg of N

L-1) and organic nitrogen (less than 2.1 mg of N L-1). A nitrogen mass balance was carried out to examine if the biomass produced (in the form of organic nitrogen) during this time was supported by the incoming nitrogen. The results of the nitrogen fixation assays carried on day 62 on the high C/N reactors were negative, therefore the incoming nitrogen was sourced only from the wastewater. The total incoming nitrogen (106.5 ± 6 mg of N L-1) was enough to support the total biomass produced in the reactors (44 ± 14 mg of N L-1) and the total inorganic nitrogen (nitrate, nitrite and ammonium) in the effluent (49.1 ± 20 mg of N L-1). Although there were no significant differences (repeated measures ANOVA p-value = 0.215) between the amount of total ammonium, nitrate and nitrite in the influent and effluent.

The physicochemical parameters of the paper mill effluent (obtained after the second month of operation) and the synthetic wastewater, used in Ospina-Betancourth et al. (2020) to previously enrich the sludge with NFB, were compared (Table 1). The COD/N/P ratio of the paper mill wastewater ranged between 100:0.2:0.07 and 100:0.8:0.5. This wastewater contained lower concentration of molybdenum, manganese, phosphorus and COD than the synthetic wastewater. Consequently with the aim of promoting biological nitrogen fixation, after 72 days of operation and until the end of the experiment, the wastewater used to feed the reactors was supplemented with the same trace element solution used in the synthetic wastewater (Figure 1). On day 114 of operation, another assay for nitrogen fixation was conducted on the high C/N reactors but the results were negative. The average rate of organic nitrogen produced by the reactors up to this point was 0.6 ± 0.18 mg of N L-1 day-1. There were no significant differences (repeated measures ANOVA p-value = 0.1) between the organic nitrogen concentration in the mixed liquor on days 62 and 114, these time points correspond to the period before and after the addition of the trace elements and to the period in which the incoming COD value decreased. The biomass of the high C/N reactors was only able to fix nitrogen when sucrose in concentrations of 3 g L-1 was supplemented as an additional source of carbon on day 114. This sucrose concentration was used previously in the high C/N reactors fed with synthetic wastewater from Ospina-Betancourth et al. (2020). The modified paper mill wastewater, supplemented with trace elements and sucrose, had a COD/N ratio of approximately 100:0.07. Under this treatment, the

high C/N reactors had an acetylene reduction rate of 20 ± 2.4 nmol C2H4 h^{-1} mL^{-1} after 48 hours of incubation. This acetylene reduction rate is equivalent to a nitrogen fixation rate of 13.4 ± 1.6 mg N L^{-1} day^{-1}.

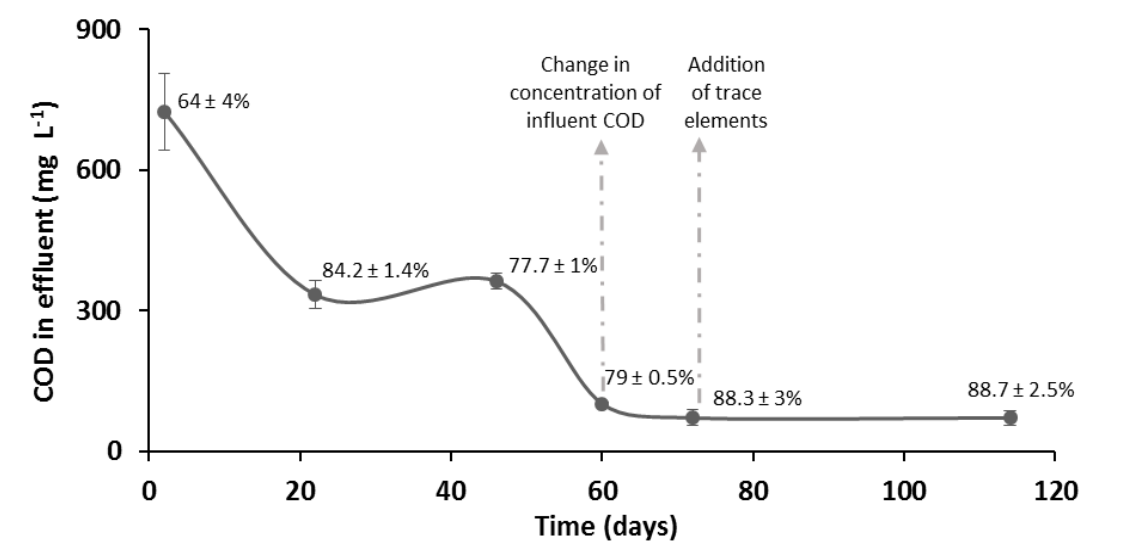

Figure 5.2. COD removal efficiency in the high C/N reactors treating paper mill wastewater. Concentration of soluble COD in the effluent along the days of operation of the high C/N reactors. Error bars represent the standard deviation of the sample (*n* = 4). Where the bars are not visible, the standard deviation is obscured by the marker. The numbers (mean ± standard deviation) above each bar correspond to the percentage of soluble COD removed from the influent.

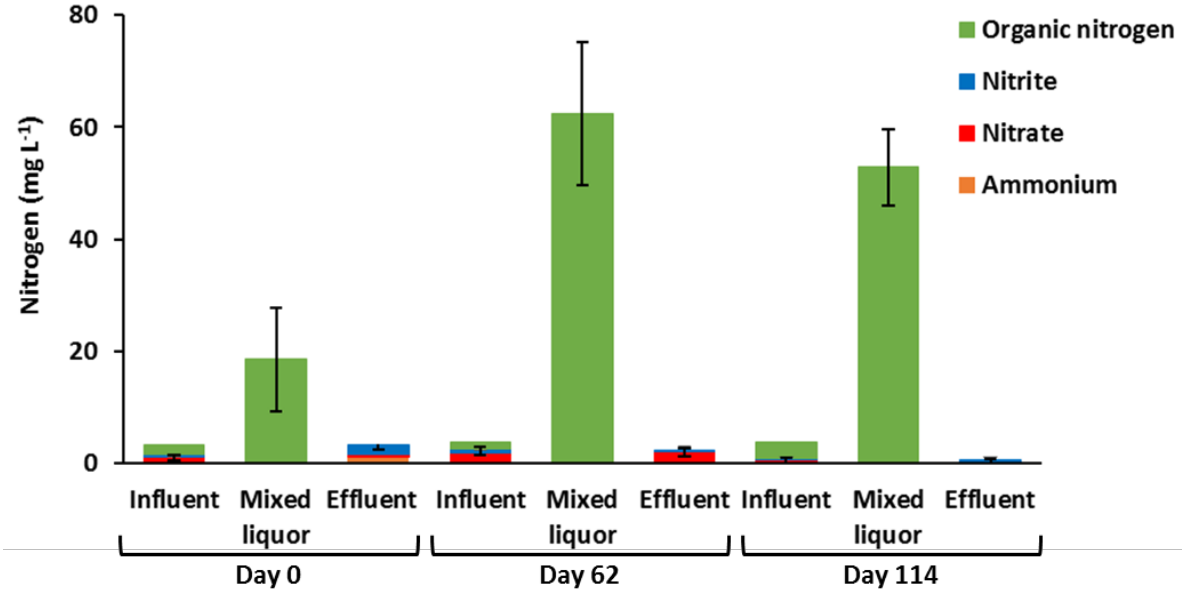

Figure 5.3. Nitrogen speciation profiles in the high C/N reactors treating paper mill wastewater. Quantification of nitrogen in the forms of nitrite, nitrate, ammonium and organic nitrogen in the influent, mixed liquor and effluent of the high C/N reactors on days

of operation 0, 62 and 114 time. Error bars represent the standard deviation of the sample (n = 4).

The biomass of the high C/N reactors was only able to fix nitrogen when sucrose in concentrations of 3 g L^{-1} was supplemented as an additional source of carbon on day 114. This sucrose concentration was used previously in the high C/N reactors fed with synthetic wastewater from Chapter 3 (Ospina-Betancourth, Acharya, B. Allen, et al., 2020)~~e~~. The modified paper mill wastewater, supplemented with trace elements and sucrose, had a COD/N ratio of approximately 100:0.1. Under this treatment, the high C/N reactors had an acetylene reduction rate of 20 ± 2.4 nmol C_2H_4 h^{-1} mL^{-1} after 48 hours of incubation. This acetylene reduction rate is equivalent to a nitrogen fixation rate of 13.4 ± 1.6 mg N L^{-1} day^{-1}.

Table 5.1. Comparison of physicochemical properties of synthetic and paper mill (real) high C/N wastewater (Mo: Molybdenum, Mn: Manganese).

Type of Wastewater	Inorganic-C (mg L^{-1})	Organic-C (mg L^{-1})	COD (mg L^{-1})	Total-N (mg L^{-1})	COD:N ratio	Mo (mg L^{-1})	Mn (mg L^{-1})
Synthetic	25 ± 4.2	964.8 ± 16.6	3790 ± 422	0 ± 0	100:0	1.41 ± 0.3	0.59 ± 0.04
Papermill	35.8 ± 3.8	208.6 ± 4.4	607 ± 12.2	3.2 ± 2	100:0.5	5 ± 1 x 10^{-3}	0.27 ± 0.02

5.3.3. Quantification of 16S rRNA and nifH Genes

Over the 114 days of operation of the high C/N reactors, the amount of *nifH* and *16S rRNA* genes increased by 1.5 ± 0.8 and 4.6 ± 0.8 fold (Figure 5.4a). The amount of *nifH* copies did not change significantly throughout time (repeated measures ANOVA p-value = 0.34). However, the *16S rRNA* copy number changed significantly throughout the operation of the high C/N reactors (repeated measures ANOVA p-value <0.05). In particular, there was a significant decrease (repeated measures ANOVA p-value = 0.03) in the *16S rRNA* copy number between day 72 and 114; this day correspond to the first and last day of the trace metal addition. It was not possible to determine with the data available if there was a change in the *16S rRNA* copy number due to the decrease of the COD in the feed from day 62 onwards. The wastewater contained 1.57 ± .26 x 10^5 *nifH* copies μL^{-1} and 4 ± 0.15 x 10^5 *16S rRNA* copies μL^{-1}. The *16S rRNA* copy

number was used as a proxy of biomass quantification and showed a lower increase when compared to the VSS increment from day 0 to 114 (86-fold; Figure B2). The abundance of NFB (calculated by assuming that each bacterial or archaeal cell contained 4.2 copies of *16S rRNA* (Větrovský & Baldrian, 2013) and 1 copy of *nifH*) was initially estimated as 133 ± 53% and decreased to 37 ± 10% by day 114. The ratio of *nifH* to *16S rRNA* gene copy number varied between 2.8 ± 0.8 x 10^{-2} and 3.3 ± 1.3 x 10^{-1} (Figure 5.4b).

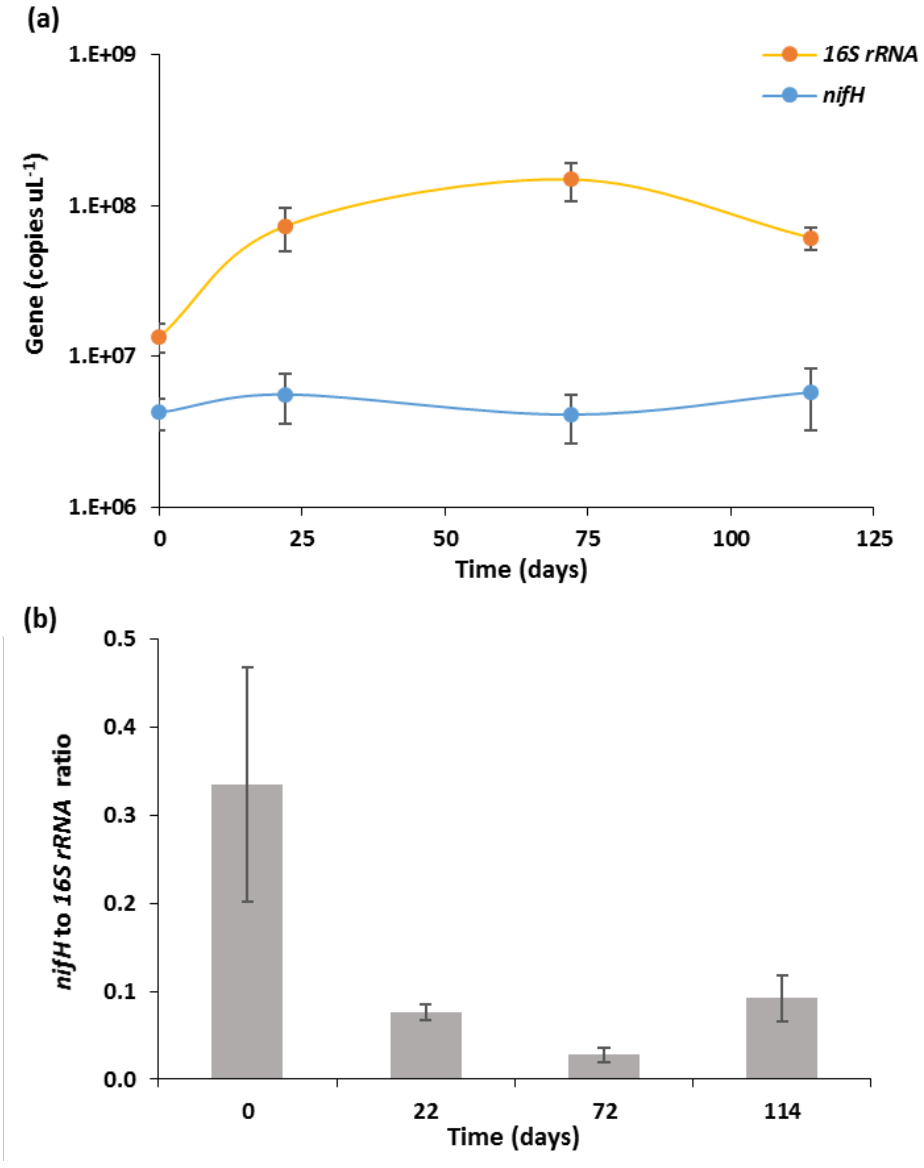

Figure 5.4. Quantification of gene copies and estimation of putative NFB in the high C/N reactors treating paper mill wastewater. **(a)** Quantification of *16S rRNA* and *nifH* gene copies and **(b)** *nifH* to *16S rRNA* copy number ratio in the high C/N reactors. Error bars represent the standard deviation of the sample (*n* = 4).

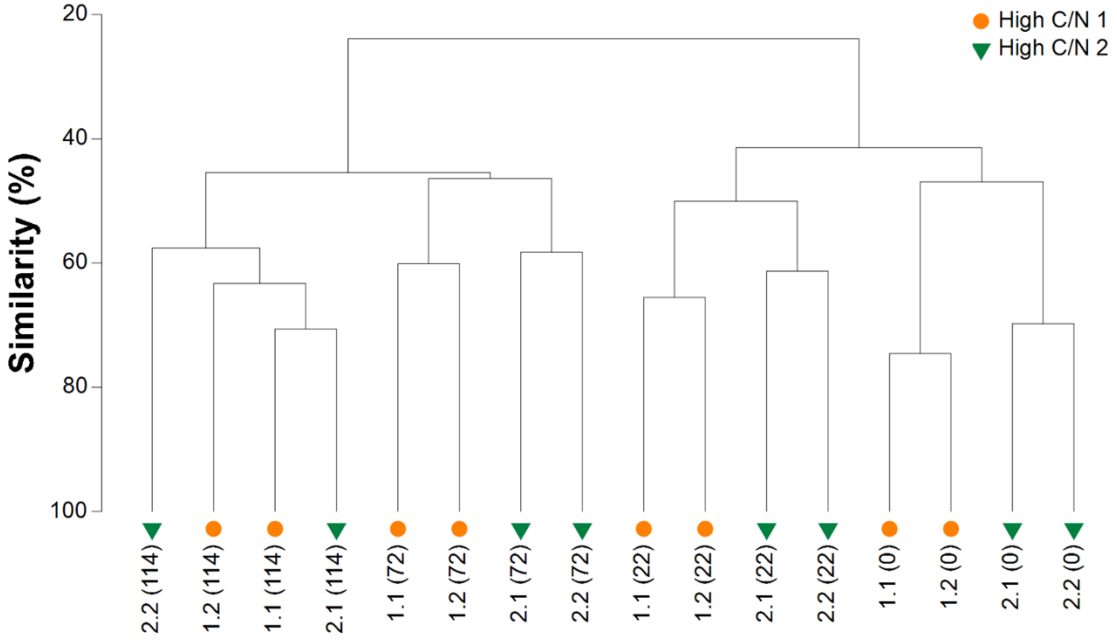

Figure 5.5. Microbial community analysis of the high C/N reactors treating paper mill wastewater: *16S rRNA* dendogram. Comparison of microbial communities in samples from the high C/N treatments using group average hierarchical clustering derived from the Bray−Curtis similarity index of the *16S rRNA* ASVs. All four reactors (1.1, 1.2, 2.1 and 2.2) were sampled at different time points (indicated in brackets): 0, 22, 72, and 114 days of operation.

5.3.4. Microbial Community Analysis

The bacterial communities in the reactors, sampled during different days of operation, were grouped into two main clusters according to a Bray−Curtis similarity index cluster analysis made with the *16S rRNA* gene sequence data. One cluster contained all the samples from day 0 and 22 of operation, meanwhile the other cluster contained all the samples from day 72 and 114 (Figure 5.5). The samples obtained on day 0 from each of the two reactors of the high C/N treatments 1 and 2 corresponded to biological replicates and their microbial community profiles were highly similar (approximately 75 and 70%, respectively). When compared altogether on day 0, the four reactors were at least 43% similar according to the Bray-Curtis similarity index and were statistically different (nested ANOVA *p*-value <0.05) according to the Simpson, Shannon and inverted Simpson diversity indices (Table B1).

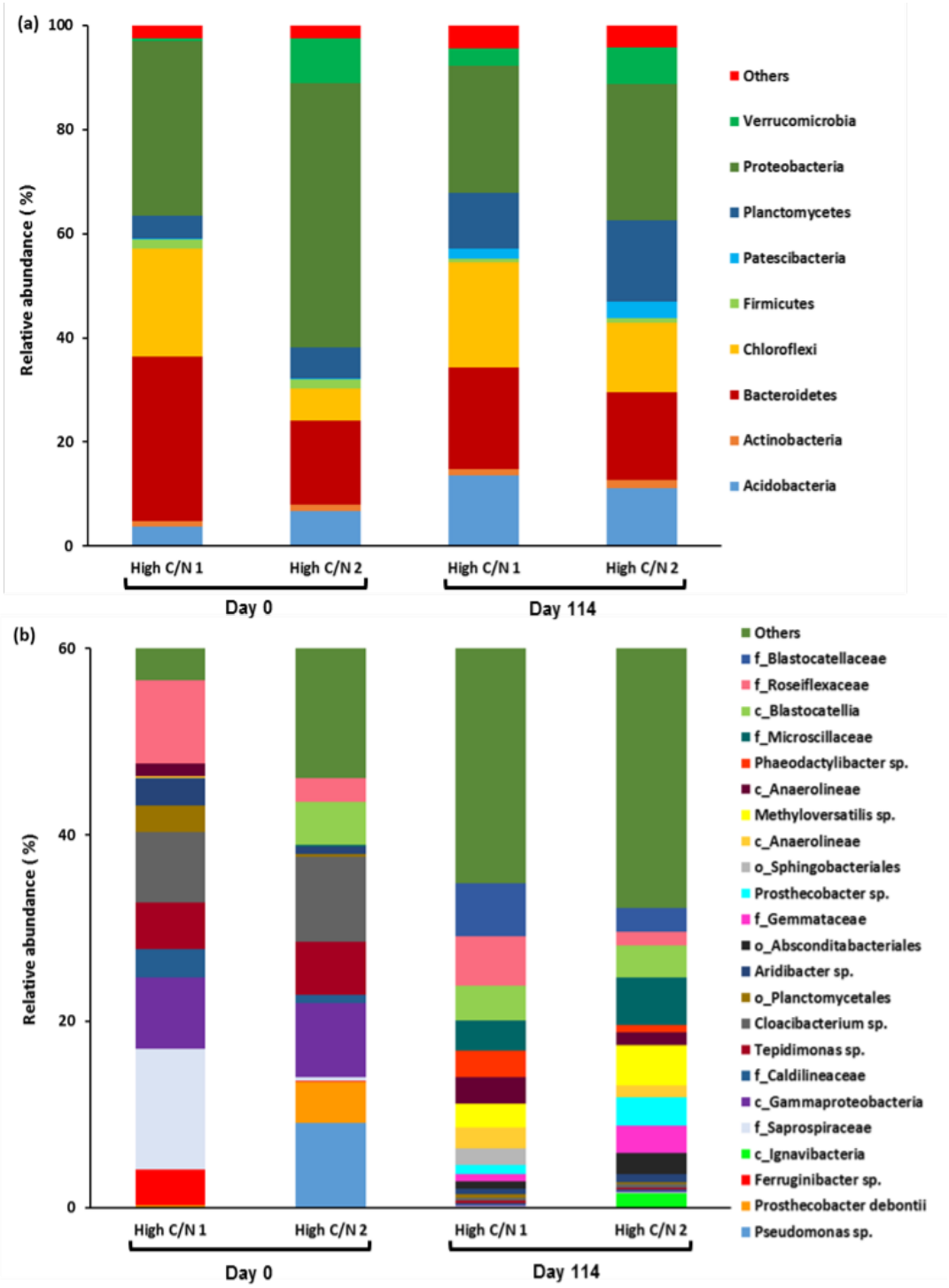

Figure 5.6. Microbial community analysis of the high C/N reactors treating paper mill wastewater: *16S rRNA* bacterial community composition. Bacterial community composition of the high C/N treatments fed with real wastewater on day 0 and day 114 of operation at **(a)** phylum and **(b)** genus levels or lowest taxonomic rank available for the identified ASVs (c: class; o: order; f: family; sp:species or genus). The bar labelled as high C/N 1 represents the average of the replicate reactors 1.1 and 1.2, meanwhile the bar labelled as high C/N 2 represents the average of the replicate reactors 2.1 and 2.2 (n = 2).

The results from the nested ANOVA tests (ran independently for each of the indices), indicated that most of the variance occurred between the treatments (>92%) and not within the replicates (>6%). In contrast, the observed species indices from both treatments on day 0 (Table B1) were statistically indistinguishable (nested ANOVA *p*-value = 0.79) and the variance occurred only within the replicate reactors (100%). On day 114, the microbial community profiles of all the reactors were at least 55% similar (Figure 5.5). and did not have significant differences (nested ANOVA *p*-value >0.4) according to the Simpson, Shannon, inverted Simpson and observed species diversity indices (Table B1). In these nested ANOVA tests (ran independently for each index) most of the variance occurred between the replicates (>92%) and not within the treatments (>6%). The number of ASVs sequenced in the high C/N treatments evidently increased over time (observed species index from Table B1 in supplementary information), initially there were 475 ± 42 and by day 114 approximately 1006 ± 82 sequence variants were identified.

On day 0 the phylum profile of the high C/N treatments showed highly dissimilar communities, but by the end of the experiment the communities looked much more alike (Figure 6.

a). Initially, the most abundant phyla in the high C/N treatments 1 and 2 were Proteobacteria (33.5 ± 3.2 and 50.7 ± 2.1, respectively), Bacteroidetes (31.7 ± 0.3 and 16.1 ± 1.3, respectively), Chloroflexi (20.6 ± 3.1 and 6.1 ± 0.6, respectively) and Verrucomicrobia (0.5 ± 0.01 and 8.6 ± 0.1, respectively). On day 114, the most abundant phyla in the high C/N treatments 1 and 2 were Proteobacteria (24.4 ± 1.4 and 26.3 ± 4.7, respectively), Chloroflexi (20.2 ± 3.5 and 13.3 ± 6.8, respectively), Bacteroidetes (19.5 ± 2.8 and 16.9 ± 3.3, respectively), and Planctomycetes (10.7 ± 2.2 and 15.5 ± 0.6, respectively).

When evaluated at the lowest identified taxon level, the three most abundant taxa of the high C/N reactor setup 1 on day 114 were ASV 11 which belong to the family *Blastocatellaceae* (5.7 ± 1.9%), ASV 2 which belong to the family *Roseiflexaceae* (5.3 ± 2.4) and ASV 5 which belong to the class Blastocatellia (3.7 ±0.1%) (.

In the high C/N reactor setup 2 the most abundant taxa on day 114 were ASV 10 belonging to the family *Microscillaceae* (5 ± 1.2)%, ASV 12 identified as *Methyloversatilis* sp. (4.4 ± 1.2%) and ASV 5 belonging to the class Blastocatellia 3.5 ± 2.1 (Figure 6b). ASV 12 was also present in the high C/N 2 with a relative abundance of 2.5 ±0.01%.The genus *Methyloversatilis* sp. belongs to the phylum Proteobacteria (family *Rhodocyclaceae*), they are versatile methylotrophs able to use a variety of carbon and multicarbon compounds (Oren, 2014). This genus has 2 copies of the *16S rRNA* gene (Cole et al., 2014) and contains the species *Methyloversatilis discipulorum* which is capable of denitrification and nitrogen fixation (Smalley et al., 2015).

Figure 5.7. Microbial community analysis of the high C/N reactors treating paper mill wastewater: *16S rRNA* phylogenetic neighbour-joining tree*. 16S rRNA* phylogenetic neighbor-joining tree of the most abundant ASVs (>2.9%) from the high C/N reactors fed with real wastewater on day 114 of operation (highlighted in grey) and their corresponding closest identified reference taxa. Sequences were aligned prior to the construction of the tree. The phylum information and the lowest taxonomic rank available for the identified ASV or the reference taxa are provided (c: class; o: order; f: family; sp:species or genus). Bootstrap value percentages from 1000 replicate trees are given at each node. Scale bar shows the distance of sequence divergence.

The most abundant taxa (relative abundance in at least one of the reactors >2.9%) sampled in the reactors on day 114 were aligned with homologous *16S rRNA* sequences and compared in a phylogenetic neighbour-joining-tree (Figure 5.7). The majority of these ASV were not identified at genus level using the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) but were all classified at higher taxonomic ranks. The homologous sequences (query cover >96% and percentage of nucleotide sequence identity >84.13%) were obtained from the NCBI database (Clark et al., 2016) with BLASTn (Johnson et al., 2008) and nearly half were closely related to organisn which have not yet been cultured. All of the ASVs and their matching homologous sequences grouped together within their corresponding phyla, except for ASV 84 which belongs to the class Ignavibacteria (Hedlund et al., 1996, 1997).

5.4. Discussion

The present study demonstrated that the sludge had a robust capacity for treating high C/N paper mill wastewater with different COD/N ratios without nitrogen supplementation. Interestingly, although nitrogen fixation was not observed in the reactors, the sludge did not lose its ability to fix nitrogen as it showed acetylene reduction activity when an additional source of carbon was supplemented in the batch reactors. The capability of the sludge to treat wastewater with different COD concentrations is highly beneficial as the COD load of the treatment plant can fluctuate depending on the amount and type of paper been produced.

The changes in the high C/N ratios of the influent paper and pulp mill wastewater used in this study, did not affect the carbon removal efficiency of the treatment system or the settling capacity of the sludge. This favourable treatment performance under limited nitrogen concentration is intriguing since these conditions can cause sludge bulking and treatment failure. Bulking is a common problem when treating pulp and paper mill wastewaters (Thompson & Forster, 2003). Substantial sources of nitrogen were not detected either in the feed or from nitrogen fixation activity. It appears to be the case that the sludge has low nitrogen requirements that could be met by the feed. Low nitrogen requirements have been proposed when there is a low growth rate and/or dual limitation of nitrogen and carbon (Egli, 1991). An observation that is consistent with the low biomass increment in these reactors.

C/N ratios of the treatment system were sufficient to support a 5-fold increase in the total biomass. This increment was much lower than the 25- and 19-fold increases previously observed respectively in the high and low C/N reactors from Chapter 3 (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020). Moreover, the C/N ratios did not favour the NFB (interpreted as the *nifH* to *16S rRNA* gene copy number ratio). By the end of the experiment the nitrogen fixers had decreased by 72%. The *nifH* to *16S rRNA* gene copy ratio on day 114 of operation (0.09) was slightly lower than the one obtained in Chapter 3 (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020) (0.13) and about 10 times lower than in Bowers et al. (Bowers et al., 2008). It appears that even very small amounts of nitrogen (COD/N ratio of 100:0.8 in this study versus 100:0 in earlier work) is sufficient to inhibit the nitrogen fixation genes. Presumably, this modest amount of nitrogen

was the most important factor. However, there were other small methodological variations that could explain the differences in the level of enrichment of *nifH* in the activated sludge communities between the studies. Particularly, Bowers et al. (Bowers et al., 2008) used a chemical DNA extraction method with high salt (Zhou et al., 1996) and the primer set PolF/PolR (Poly et al., 2001).

It is clear that the bacterial community was shaped by the feed as the two different activated sludges used to inoculate the reactors became much more alike and diverse over time. Presumably the presence of nitrogen, albeit at low concentrations, enabled non-NFB to grow. It is likely that these more diverse systems will be more robust and, therefore, resilient to changes in the carbon feed (Kitano, 2004; Curtis & Sloan, 2006; Song et al., 2015).

Interestingly, NFB commonly found in pulp and paper wastewater treatment system such as *Paenibacillus* sp*., Bacillus* sp. *Azotobacter* sp., *Geobacter* sp., *Pseudomonas* sp. and *Klebsiella* sp. (Chiellini et al., 2014; Addison et al., 2010; Welz et al., 2018; Oppong et al., 2003; Ghribi et al., 2016) were not observed in high abundance. At least one representative of the most abundant genus in the reactors, *Methyloversatilis* sp., is capable of nitrogen fixation and denitrification (Smalley et al., 2015) but it is not possible to confirm whether it was fixing nitrogen in the system. Members of this genus have been found in engineered (herbicide (Cai et al., 2011), municipal wastewater (Xu et al., 2020) and drinking water treatment systems (Brumfield et al., 2020)) and natural ecosystems (Doronina et al., 2014; Smalley et al., 2015; Kalyuzhnaya et al., 2006). However, this is the first report of *Methyloversatilis* sp. in paper mill wastewater treatment systems.

The system did not appear to be limited by molybdenum (an essential component of the nitrogenase enzyme (Seefeldt et al., 2009)). Even though molybdenum levels were low, trace element supplementation had no effect in the occurrence of BNF in the reactors. Molybdenum deficiency limited BNF in soils (Ma et al., 2019) and in anaerobic digestors (Lindorfer et al., 2012).

The sludge showed a positive response to biological nitrogen fixation when both sucrose and trace elements was supplemented on the batch reactors assembled on day 114 for the acetylene reduction test. This result suggests that carbon supplementation of the influent wastewater caused the biomass to grow rapidly

and deplete the nitrogen that was inhibiting biological nitrogen fixation in the reactors. The addition of carbon substrates also increased the acetylene reduction rates of different activated sludge treating pulp and paper mill effluent (Gauthier et al., 2000; Knowles et al., 1974). The acetylene reduction rates obtained when sucrose was added in this study were similar to the ones obtained in previous experiments with aerobic sludge (approximately up to 25 nmol C_2H_4 h^{-1} mL^{-1}) fed only with synthetic wastewater in Chapter 3 (Ospina-Betancourth, Acharya, B. D. Allen, et al., 2020) and bleached kraft mill wastewater (Clark et al., 1997). BNF is an energetically expensive process (Cherkasov et al., 2015; Gutschick, 1978), the bacterial cells divert resources, that could be used for bacterial growth for the production (Dixon & Kahn, 2004) and maintenance of the nitrogenase enzyme (Inomura et al., 2018). BNF could be promoted by increasing the COD/N ratio of wastewater by either adding inexpensive sugar refinery wastes (containing sucrose as main constituent) or perhaps scrupulously controlling nitrogen in the production process.

Aerobic processes are the most popular systems used for the treatment of effluents from the pulp and paper mill industry (Ashrafi et al., 2015). However, this technology produces a high volumes of sludge that needs to be dewatered and disposed. Sludge treatment comprises more than half of the overall wastewater treatment costs and produces greenhouse gasses (Meyer et al., 2018).

In principle this excess sludge could be used as a biofertiliser because the free-living NFB therein can improve nitrogen uptake in plants and enhance the productivity of natural and agricultural ecosystems (Smercina et al., 2019; Bhattacharjee et al., 2008). Although this idea was hinted by Liu et al. (2017), more field trials are needed to demonstrate and quantify the benefits of nitrogen-fixing sludge as a biofertiliser. If positive results were obtained, the optimisation of the conditions for reproduction of the NFB within wastewater treatment systems would be desirable.

Moving to a low or no-carbon society requires all of us to think and act differently and to draw on new, or at least non-traditional, solutions for the manufacture of vital products such as paper and fertiliser. Currently, the available methods for obtaining ammonia include recovery from wastewater or ammonia production

using electric fuel cells but unfortunately they are costly and difficult to scale-up (Belleville et al., 2011; Santoro et al., 2019; Logan, 2010; Matassa et al., 2015; Maurer et al., 2003). The application of the technology proposed in this study is timely as the paper industry is expanding whilst environmental policies are becoming more demanding (Toczyłowska-Mamińska, 2017; Reid et al., 2008). This study provides fundamental insight into of a novel green biotechnology that can treat pulp and paper mill wastewater using less resources (compared to traditional high C/N wastewater treatment systems) and existing facilities. In addition, this technology has the potential to valorise the sludge from the treatment system by producing NFB. Hopefully, this could be one of those ideal green technologies that save both money and the environment.

Chapter 6: Final Conclusions and Recommendations

The aim of this project was to develop, validate and characterise N_2 fixation in bioreactors treating wastewaters inoculated with mixed microbial cultures. In summary, this study demonstrates the scope and magnitude of BNF in wastewaters and can contribute to more accurate world-scale predictions of nitrogen fxation. The production of key human resources such as paper and fertiliser must be optimized using more sustainable methods designed with an interdisciplinary approach. This research provides fundamental insight into a novel green biotechnology that can treat pulp and paper mill wastewater using less resources (compared to traditional high C/N wastewater treatment systems) and existing facilities. In addition, it could valorise the use of organic matter low in nitrogen, as selected sludge could potentially be used as a biofertiliser. This green-strategy of nitrogen fixation could both save money and the environment.

6.1 Specific conclusions

- From the literature review it was apparent that BNF occurs naturally in systems treating paper mill wastewaters (Clark et al., 1997) and is favoured by low dissolved oxygen conditions (DO) (Slade et al., 2003) and inhibited by the presence of ammonia (Fan et al., 2019; Merrick, 2004; Sohm et al., 2011).
- From a thermodynamic point of view, it is feasible to fix nitrogen biologically using the energy available in wastewater.
- In principle, pulp and paper wastewater can be used to fixed nitrogen at a rate (11.8 mg of N L^{-1} day $^{-1}$) that is comparable to recent “breakthrough” nitrogen-fixing technologies.
- The nitrogen-fixing bioreactors could treat wastewater efficiently and demand lower operation cost compared to a traditionally reduced nitrogen wastewater treatment system.
- *Clostridium pasteurianum* dominated the microbial community in the reactors high carbon-to-nitrogen wastewater and had a relative abundance of 19%.
- Nitrogen fixation was inhibited by 83% when treating wastewater containing 78 mg of N L^{-1}. The relatively weak effect that significant levels of ammonia has on BNF within wastewater bodes well for the application of this technology.

- Nitrogen-fixing activity and satisfactory organic matter removal is still possible in the presence of modest amounts of ammonia in the reactors.
- In principle, the sludge could function as a biofertiliser in soils by virtue of the high concentrations of NFB in the reactor.

- The organic load of the real pulp and paper mills wastewater was only reduced by 89% without nitrogen supplementation in the treatment system. This treatment system proved to be energetically favourable for the production of biomass despite the low concentration of nitrogen in the influent wastewater.
- The most abundant genus in the reactors treating high C/N paper mill wastewater was identified as *Methyloversatilis* sp. (relative abundance of 4.4 ± 1.2%). This genus is capable of nitrogen fixation and denitrification but one cannot be sure whether it was fixing nitrogen in our system and therefore merits further metagenomic studies.
- Carbon supplementation of the influent wastewater caused the biomass to grow rapidly and deplete the nitrogen that was inhibiting biological nitrogen fixation in the reactors. Consequently operators should actively avoid adding or allowing nitrogen in the waste streams if they wish to valorize their sludge and reduce running costs.
- This technology for nitrogen fixation could be used with high organic loads from other industries such as cassava starch mill (Sreethawong et al., 2010), olive mill (Balis et al., 1996), wineries (Vlyssides et al., 2005) and dairy (Rajesh Banu et al., 2008).

6.2 Future work

Future work should be focused on demonstrating and quantifying the benefits of the nitrogen-fixing sludge as a biofertiliser. This could also include studies on how the NFB in the sludge could integrate and survive within the soil microbial community and its environmental conditions. Furthermore, if a favourable outcome is obtained, subsequent research should aim to optimise the conditions for reproduction of NFB using wastewater as a feed.

The effect of temperature on the nitrogen-fixing reactors should be investigated with the aim of comparing the rates during different seasons and different geographical locations. This type of study could evaluate the viability of the biotechnology under

different climate conditions and can contribute to more accurate world-scale predictions of nitrogen fixation. Future studies should also compare the diversity of NFB in papermills from different locations in order to elucidate the microbial ecology within.

BNF occurred in the sludge, even in the presence of relatively substantial amounts of ammonia. These may have implications on the existence of BNF in typical municipal wastewater treatment systems and their efficiencies as this nitrogen input could affect the mass balances. Therefore, it would also be interesting to investigate the occurrence of BNF in wastewater treatment system containing ammonia.

Annex A: Supporting Information Chapter 3 Enrichment of Nitrogen-Fixing Bacteria in a Nitrogen Deficient Wastewater Treatment System

Table A1. Physicochemical and molecular analysis of samples from the environmental survey of N_2-fixing bacteria (NFB**).** Analyses were carried out following the same chemical analyses methods described for the samples from the reactors (volatile suspended solids: VSS, total suspended solids: TSS). Samples highlighted in grey correspond to the inocula used in the reactors.

Sample	Location	VSS (g/L)	TSS (g/L)	NH_4^+ (mg/L)	COD (mg/L)	pH	NFB (PCR)
Activated sludge (BS)	Bransands WWTP (54°36'32.7"N 1°07'20.0"W)	6.1	14.4	0	112	7.6	+
Jet Aerated Channel (JAC)	Paper Mill WWTP (54°58'06.2"N 1°51'26.2"W)	5.3	5.8	0	81	7.8	+
Deep Shaft Paper Mill (DS)	Paper Mill WWTP (54°58'06.2"N 1°51'26.2"W)	9.0	9.6	0	148	6.4	+
Coledale Beck Sediment	Force Crag metal mine (54°35'00.0"N 3°14'22.4"W)	45.1	4.2	0	0	5.7	+
Compost from Vertical Flow Pond	Force Crag metal mine (54°35'00.0"N 3°14'22.4"W)	48.0	23.3	0	4300	6.1	+
Anaerobic digestor (AD)	Cockle Park (55°12'56.6"N 1°40'55.9"W)	17.7	23.2	800	8125	8.1	+
Soil	Cockle Park (55°12'56.6"N 1°40'55.9"W)	16.6	63.9	0	29400	5.1	-
Soil with Fertilizer	Cockle Park (55°12'56.6"N 1°40'55.9"W)	72.7	61.3	0	27200	4.5	-
Cattle Slurry	Cockle Park (55°12'56.6"N 1°40'55.9"W)	69.4	88.2	380	4300	7.7	-
Mixed liquor	Newton Aycliff WWTP (54°36'11.1"N 1°33'52.9"W)	0.6	2.2	6	108	6.5	-
Recycled and settled activated sludge	Newton Aycliff WWTP (54°36'11.1"N 1°33'52.9"W)	2.7	9.4	5	40	6.4	-

Table A2. Total suspended solids (TSS) and volatile suspended solids (VSS) of the low and high C/N treatments on day of operation 0 and 85.

Sample	Day of operation	TSS (mg L^{-1})	VSS (mg L^{-1})
Initial	0	22.4 ± 3.2	18.6 ± 2.8
Low C/N	85	2.6 ± 0.8	5.2 ± 1
High C/N	85	12.5 ± 4.8	7.3 ± 1.3

Supplementary Information A1: Quantification of NFB in Inocula

The abundance of NFB in the four samples used to inoculate the reactors was quantified with qPCR (Figure A1) following the same methods described for the samples from the reactors. Although the sample from DS showed the highest relative abundance of NFB in the sample (45% of the total population), it can be seen that it contains the second highest amount of *nifH* copies (1.25 x 10^7 copies μL^{-1}) which was an order of magnitude lower than the samples from the AD (1 x 10^8 copies μL^{-1}).

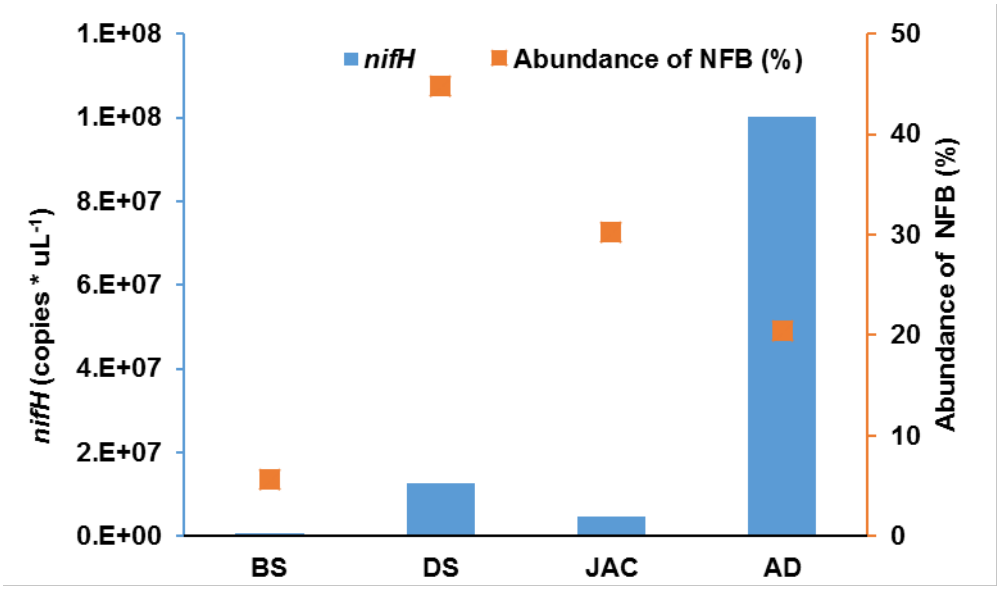

Figure A1. Quantification of *nifH* gene copies and estimation of putative NFB in inocula. Quantification of *nifH* gene copies (blue bars on the left axis) and percentage of abundance of the NFB relative to total bacteria (orange line on the right axis) in the inocula samples (BS: Bran Sands, DS: deep shaft, JAC: jet aerated channel, AD: anaerobic digester).

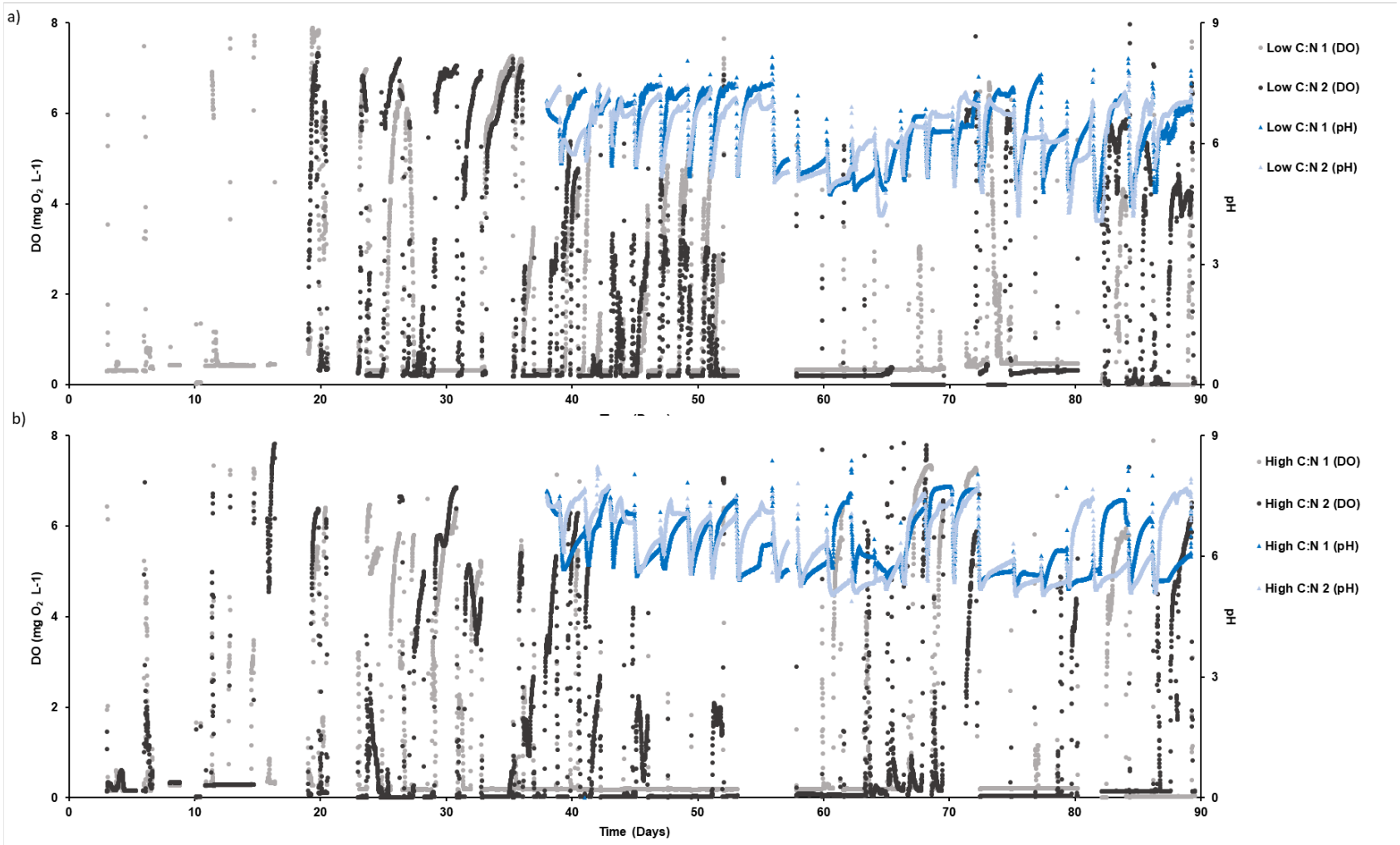

Figure A2. pH and dissolved oxygen in the low and high C/N reactors. pH and dissolved oxygen (DO) concentration along the days of operation of **(a)** low C/N (control) and **(b)** high C/N reactors**.**

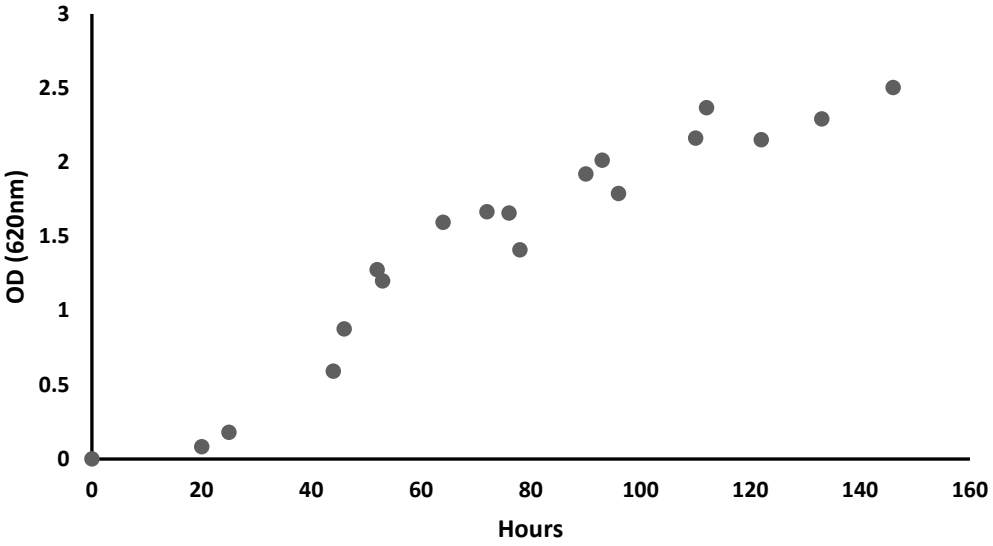

Figure A3. Growth curve of *A. vinelandii* in pure culture. Growth of *A.vinelandii* in pure culture was measured at 620nm (Sekse et al., 2012) in a UV/VIS spectrophotometer (Unicam 8625, UK). Bacterial culture in mid-exponential growth phase (48 hours) was used as a positive control for the ARA and $^{15}N_2$ test.

Supplementary Information A2: Preparation of Standard Solutions for qPCR Calibration Curves

Standards for calibration of qPCR were generated using genomic DNA of *A. vinelandii* Lipman 1903 (DSMZ 85, DSMZ, Germany) as a template. PCR products from the *nifH* and *16S* rRNA gene were obtained independently with two successive PCR using the corresponding primers. The amplicon size of each gene was verified by a 1.5% agarose gel; the *16S* rRNA gene had a length of 708 bp meanwhile, the *nifH* gene was 394 bp long. PCR products were purified using a QIAquick PCR purification kit (Qiagen ,UK) and then quantified using the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, UK). The copy number of standard genes was calculated with equations A1 and A2, based on the PCR product size and assuming a base pair weight of 1.02 x 10^{-21} g molecule^{-1}. Calibration curves for each standard gene were prepared from the stock solution with serial dilutions ranging between 10^{3} and 10^{8} target gene copies µL^{-1}. Stock and calibration curve solutions were stored at -20 °C until used.

Equation A1:

$$\text{Total weight of amplicon } \left(\frac{g}{\text{copy of fragment}}\right) = base\ par\ weight\ \left(\frac{g}{bp}\right) x\ fragment\ length\ \left(\frac{\text{bp}}{\text{copy of fragment}}\right)$$

Equation A2:

$$\frac{\text{Copies}}{\mu\text{L}} = \frac{\text{Concentration of amplicon } \left(\frac{\text{g}}{\mu\text{l}}\right)}{\text{Total weight of amplicon } \left(\frac{\text{g}}{\text{copy of fragment}}\right)}$$

Supplementary Information A3: Optimisation of PCR for *nifH;* PCR Annealing Temperature

A robust assay is necessary to avoid false positive or negatives when screening for genes in environmental samples through PCR (Ishii & Fukui, 2001). Optimisation of PCR conditions will improve the efficiency and sensitivity of the test. When using degenerated primers against mixed templates, it is important to evaluate the best annealing concentration because the kinetics between the primer and the target DNA vary depending on temperature (Zheng et al., 1996). Different annealing temperatures can be tested in the same sample with a temperature gradient thermocycler program.

A temperature gradient *nifH* PCR ranging between 50 and 62 °C was done using the primer pair IGK3/DVV and the environmental samples from the NFB survey as a template (Table A3). This range was selected based on the theoretical annealing temperature (58 °C) of the primer pair. Table A3 shows that all the positive samples for *nifH* amplified at 58 °C. Therefore, this temperature was used for the following analyses.

Table A3. Temperature gradient PCR with samples from environmental survey. Samples highlighted in grey correspond to the inocula used in the reactors.

Location	Temperature (°C)											
	50.8	51	52.1	53.4	54.4	55.6	56.7	58	59	60.2	61.1	61.3
Activated sludge Bransands (BS)	-	+	+	+	+	-	+	+	+	+	-	+
Anaerobic Digestor Cockle Park (AD)	+	+	+	+	+	+	+	+	+	+	+	+
Cattle Slurry Cockle Park	-	-	-	-	-	-	-	-	-	-	-	-
Mixed liquor Newton Aycliff	-	-	-	-	-	-	-	-	-	-	-	-
Activated sludge Newton Aycliff	-	-	-	-	-	-	-	-	-	-	-	-
Jet Aerated Column Paper Mill (JAC)	+	+	+	+	+	+	+	+	+	+	+	+
Deep Shaft Paper Mill (DS)	+	+	+	+	+	+	+	+	+	+	+	+
River Sediment Force Crag	+	+	+	+	+	+	+	+	+	+	+	+
Compost Force Crag	-	+	-	+	+	+	+	+	+	+	+	+
Soil Cockle Park	-	-	-	-	-	-	-	-	-	-	-	-
Soil with Fertilizer Cockle Park	-	-	-	-	-	-	-	-	-	-	-	-
A.vinelandii	+	+	+	+	+	+	+	+	+	+	+	+

Supplementary Information A4: Optimisation of qPCR for *16S* rRNA and *nifH;* Dilution of Inhibitors

The quantification of a target gene with qPCR can be interfered by substances coextracted during the DNA extraction (Mckee et al., 2015). The presence of humic acids and organic matter can decrease the sensitivity of the assay (van Doorn et al., 2009), resulting in underestimation of the DNA concentration or in false negatives. Although PCR inhibitors cannot be avoided, qPCR can be optimised by finding the concentration of the DNA template showing minimum inhibition.

For this assay different dilutions of DNA extracted from high C/N 1 on day 30 were used in independent assays of *nifH* and *16S* rRNA qPCR (Figure A4). Dilution 1 in 100 was used for all the following qPCR analysis.

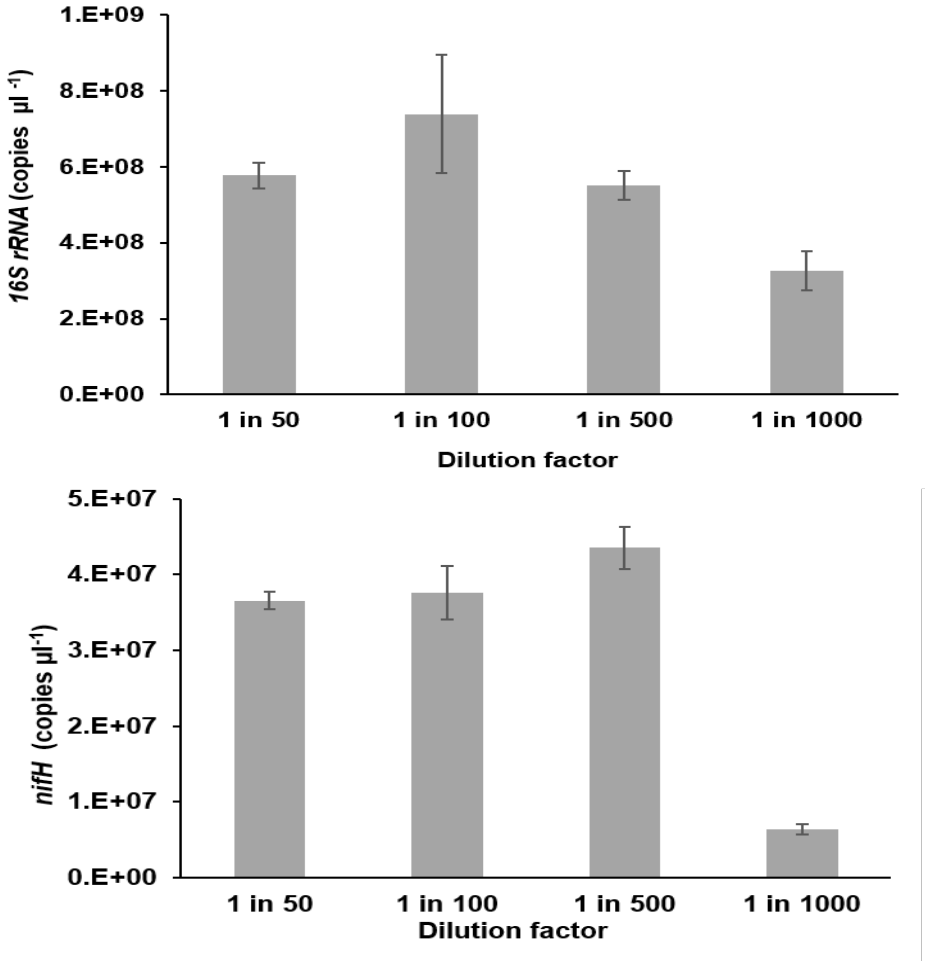

Figure A4. Optimisation of qPCR: Dilution of Inhibitors. **(a)** Dilution assay with *nifH* and **(b)** *16S* rRNA qPCR.

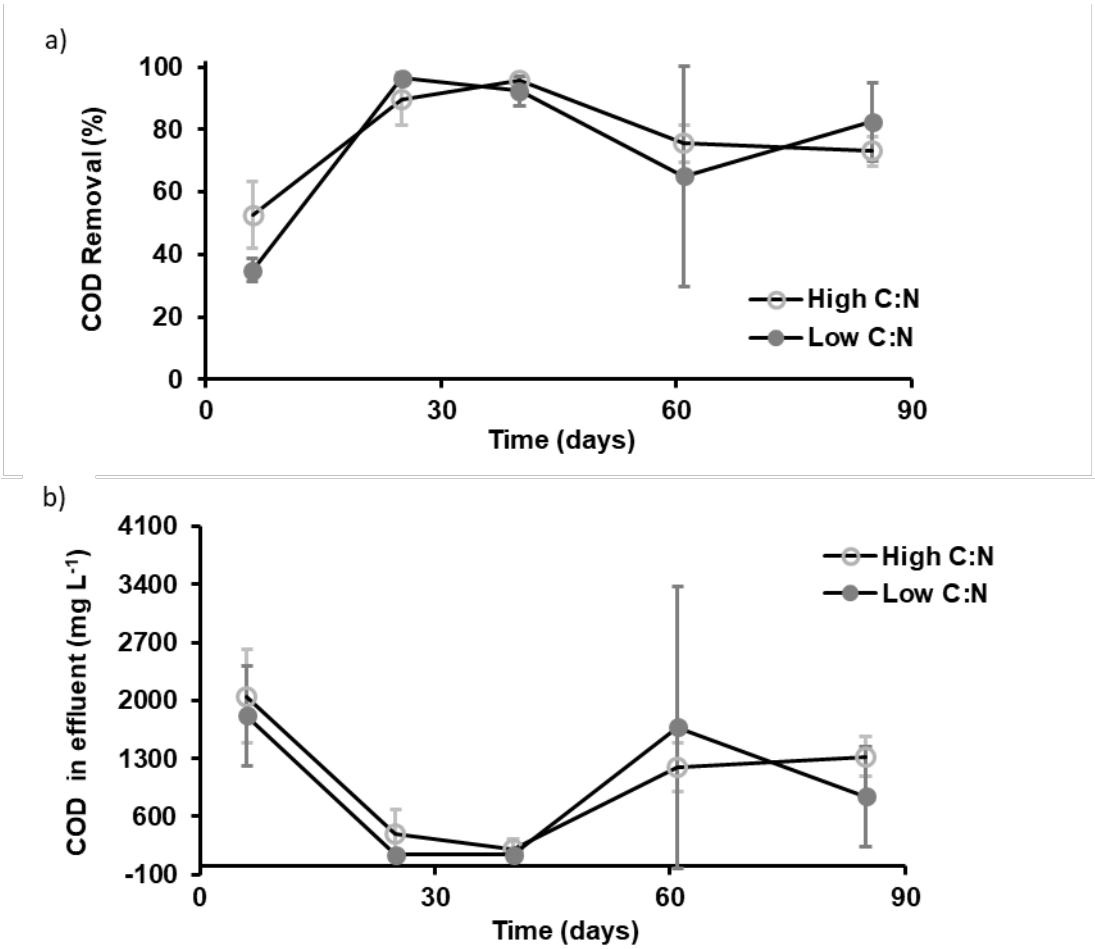

Figure A5. COD removal efficiency of the of high and low C/N treatments. **(a)** Percentage of soluble COD removal and **(b)** concentration of effluent soluble COD along the days of operation of low C/N (control) and high C/N treatments. Error bars represent the standard deviation of the sample (*n* = 2).

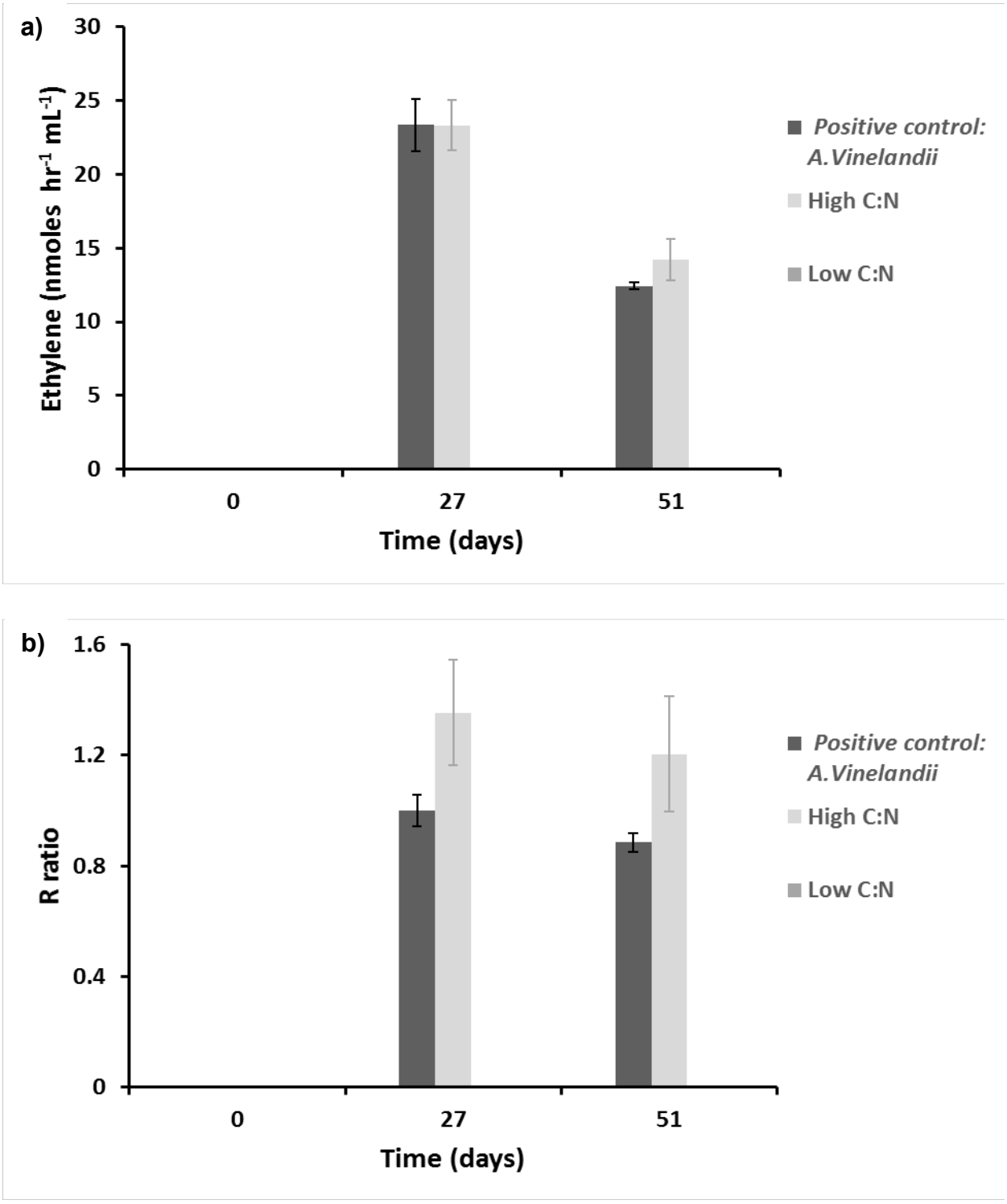

Figure A6. ARA and *R* ratio of *A.vinelandii* and high C/N treatments. **(a)** Acetylene reduction rates and **(b)** *R* ratios in low C/N (control), positive control *A.vinelandii* and high C/N treatments, after 24 and 51 hours of inoculation in batch reactors. Error bars represent the standard deviation of the sample (*n* = 2 for *A.vinelandii* and *n* = 4 for high C/N.

Table A4. Alpha diversity indexes of the reactors on day 85 of operation.

Treatment	Reactor	Observed species	Shannon	Simpson	Inv. Simpson
Low C:N	**1**	244	3.34	0.89	9.51
	2	217	3.38	0.90	10.35
High C:N	**1**	271	3.66	0.93	13.80
	2	259	3.72	0.95	18.53

Supplementary Information A5: Theoretical Rate of Nitrogen Fixation in the high C/N treatment

The theoretical amount of nitrogen that could have been fixed (9.9 mg L^{-1} day^{-1}) was calculated assuming that: the total input of sucrose on the system after 85 days of operation was 165 g of sucrose L^{-1}, the molecules of ATP produced by the oxidation of one mole of sucrose is approximately 57.7 (Rich, 2003), the percentage of NFB in the system was on average 14.8%, the energy percentage used by the nitrogen-fixing system is 39% (Daesch & Mortenson, 1968), the amount of molecules of ATP necessary to fix one mole of ammonia is approximately 20 (Burris & Roberts, 1993) and the efficiency of the nitrogen fixation process is 75% (Cherkasov et al., 2015). See equation below:

$$\left(\frac{3\ g\ of\ sucrose\ *\ 29\ days}{L\ *\ day}+\frac{6\ g\ of\ sucrose\ *\ 13\ days}{L\ *\ day}\right)*\frac{1\ mol\ of\ sucrose}{342\ g\ of\ sucrose}*\frac{57.7\ molecules\ of\ ATP}{1\ mol\ of\ sucrose}$$

$$*\frac{1\ mol\ of\ ammonia}{20\ molecules\ of\ ATP}*\frac{1\ mol\ of\ N}{1\ mol\ of\ ammonia}*\frac{14\ g\ of\ N}{1\ mol\ of\ N}*\frac{14\ g\ of\ N}{1\ mol\ of\ N}*\frac{1000\ mg}{1\ g\ *\ 85\ days}$$

$$*\ 14.8\%\ NFB\ in\ the\ system*39\%\ energy\ used\ for\ N_2\ fixation*75\%\ efficiency\ of\ N_2\ fixation=\frac{9.9\ mg\ of\ N}{L\ *\ day}$$

Supplementary Information A6: Isolation of *Clostridium pasteurianum*

A6.1. Method

Samples from the mixed liquor of the high C/N 1 and 2 reactors (1 mL each) after 14 months of operation were pasteurised for 10 min at 80 °C in a hot block and diluted with 0.85%saline solution (1:10, 1:100 and 1:1000) following Katarzyna et al. (2014). The diluted samples were plated in the same minimum salt media used to operate the reactors but solidified with agarose 1.5%. Plates were incubated in an anaerobic jar using the gas generating kit AnaeroGen (Thermo Scientific, UK) for up to 15 days at 80 °C. To obtain pure culture single colonies were streaked individually into new plates and incubated again for 15 days. This step was repeated several times to make sure that pure cultures were obtained. Pure isolates were confirmed by microscopic observation and Gram staining. Biomass from a single pure culture plate of interest was scraped, collected in a bed tube and sent to Microbes NG (Birmingham, UK) for DNA extraction and whole genome sequencing. Whole genome sequencing was done using Illumina HiSeq technology with 2×250 bp paired-end reads. The genome reads were quality-filtered, assembled and annotated in the software KBase (Allen et al., 2017) using the tools Trimmomatic (Bolger et al., 2014), SPAdes (Bankevich et al., 2012) and RAST (Overbeek et al., 2014), respectively. *16S rRNA* and *nifH* gene sequences were individually searched in the software BLASTn (Johnson et al., 2008) against the NCBI nucleotide database (Clark et al., 2016) and ASV 2.

A6.2. Results

The pure bacterial culture isolated from the high C/N 2 reactor microscopically looked like a sporulated gram positive rod. Colonies of this bacteria were white with a 3 mm diameter and had a regular border (Figure A7). The plates had an acetic acid odour. Results from the whole genome sequencing identified the isolate as a *C. pasteurianum* (Table A5). Five *nifH* gene sequences were found but none of them coincided with ASV 2. Four out of the five sequences had a nucleotide sequence similarity identity with ASV 2 that ranged between 66.57 and 71.47% (Table A5). It is likely that the isolated bacteria does not correspond to the *Clostridium* sp. identified initially in the reactors.

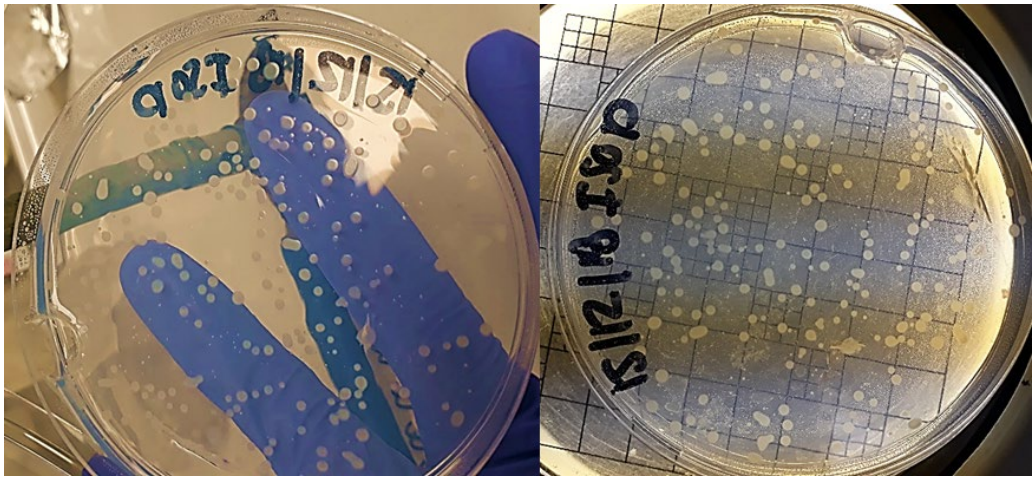

Figure A7. Colonies of *Clostridium pasterianum*.

Table A5. Information from sequences obtained from the isolated *C. pasterianum*.

Sequence ID	Length (bp)	Highest scoring reference sequence	Accession	Region (bp position)	Query cover (%)	Gaps (%)	Percentage of identity (%)	Similarity with ASV 2 (%)
***16S* rRNA**	1504	*Clostridium pasteurianum* strain M150B	CP013019	9694-11197	100	0	99.47	NA
nifH1	822	*Clostridium pasteurianum* strain M150B	CP013019	1514318-1515138	100	0.24	94.28	70.57
nifH2	822	*Clostridium pasteurianum* strain M150B	CP013019	4075168-4075994	99	0	90.81	66.57
nifH3	822	*Clostridium pasteurianum* strain M150B	CP013019	1514318-1515138	100	0	94.28	71.47
nifH4	822	*Clostridium pasteurianum* BC1	CP003261	3451703-3452524	100	0	93.67	67.27
nifH5	186	*Clostridium pasteurianum* strain M150B	CP013019	1514954-1515138	100	1	95.16	none

Annex B: Supporting Information Chapter 5 Valorisation of Pulp and Paper Industry Wastewater Using Sludge Enriched with Nitrogen-Fixing Bacteria

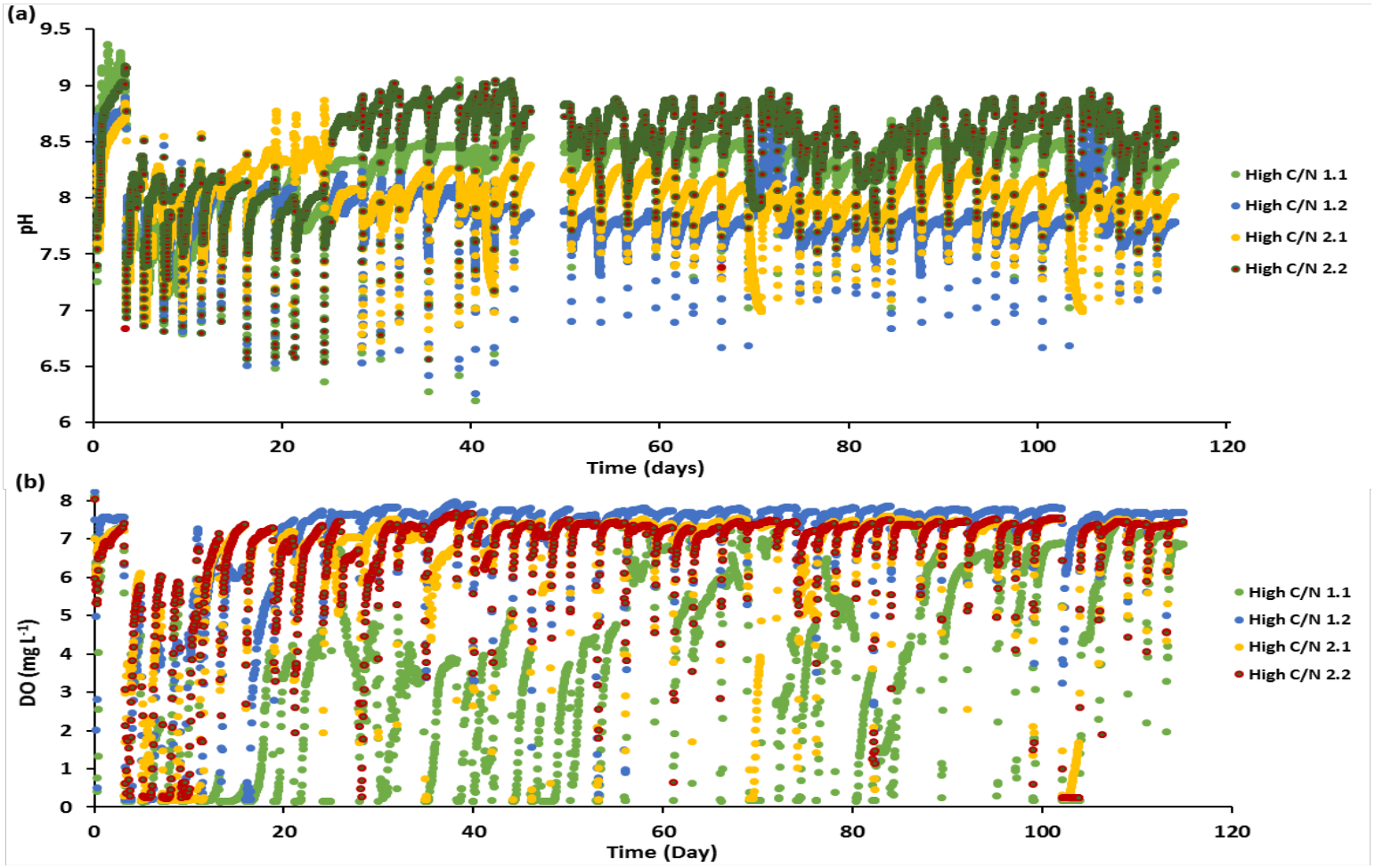

Figure B1. pH and dissolved oxygen in the high C/N reactors treating real wastewater from a pulp and paper mill. **(a)** pH and **(b)** dissolved oxygen (DO) concentration along the days of operation of the four high C/N reactors.

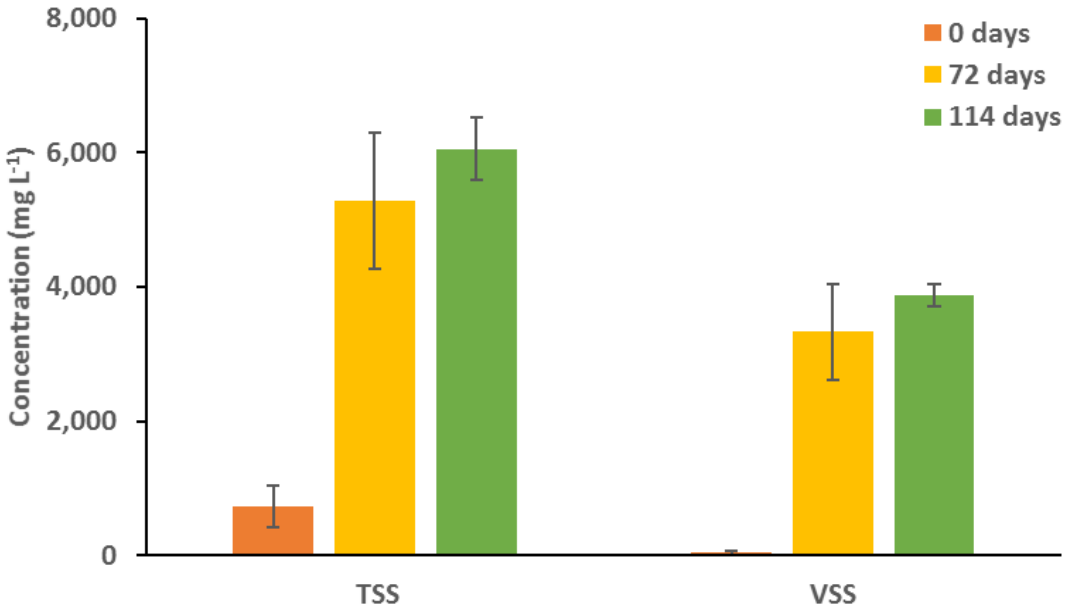

Figure B2. Total suspended solids and volatile suspended solids in the high C/N reactors treating real wastewater from a pulp and paper mill. Total suspended solids (TSS) and volatile suspended solids (VSS) on day of operation 0, 72 and 114 of the high C/N reactors. Error bars represent the standard deviation of the sample (*n* = 4).

Table B1. Alpha diversity indexes of the high C/N reactors on day 0 and 114.

Day	Treatment	Reactor	Observed	Shannon	Simpson	Inv.Simpson
0	**High C/N 1**	**1.1**	472	3.88	0.951	20.4
0	**High C/N 1**	**1.2**	462	3.91	0.954	21.7
0	**High C/N 2**	**2.1**	432	4.10	0.962	26.3
0	**High C/N 2**	**2.2**	533	4.19	0.965	28.2
114	**High C/N 1**	**1.1**	922	5.27	0.985	65.5
114	**High C/N 1**	**1.2**	1121	5.16	0.982	56.2
114	**High C/N 2**	**2.1**	1009	5.20	0.984	62.6
114	**High C/N 2**	**2.2**	972	5.33	0.988	81.4

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