Valorization of pulp and paper industry wastewater using sludge enriched with nitrogen-fixing bacteria

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Abstract
Nitrogen-fixing bacteria (NFB) can reduce nitrogen at ambient pressure and temperature. In this study, we treated effluent from a paper mill in sequencing batch reactors (SBRs) and monitored the abundance and activity of NFB with a view to producing a sludge that could work as a biofertilizer. 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. Though the reactors were able to reduce the organic load of the wastewater by up to 89%, they did not have any nitrogen-fixing activity and showed a decrease in the putative number of NFB (quantified with qPCR). The most abundant species in the reactors treating high C/N paper mill wastewater was identified by Illumina MiSeq 16S rRNA gene amplicon sequencing as Methyloversatilis sp. (relative abundance of 4.4%). Nitrogen fixation was observed when the C/N ratio was increased by adding sucrose. We suspect 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 valorize their sludge and reduce running costs.

Practitioner points
• Efficient biological wastewater treatment of low nitrogen paper mill effluent was achieved without nutrient supplementation.
• The sludge was still capable of fixing nitrogen although this process was not observed in the wastewater treatment system.
• This high C/N wastewater treatment technology could be used with effluents from cassava flour, olive oil, wine and dairy industries.

KEYWORDS
biofertilizer, biological nitrogen fixation, biological treatment, high C:N wastewater treatment, nitrogen-fixing bacteria, pulp and paper mill effluent

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INTRODUCTION

Although artificial nitrogen fertilizers 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., 2015). Currently, an estimated 210 Tg of nitrogen per year is 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 fertilizer 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., 2015). 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 fertilizer 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 (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 (Cherkasov et al., 2015). Nevertheless, BNF is a sustainable natural method for ammonia production which does not rely on fossil fuels 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). Nitrogen-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 et al., 2004) and domestic wastewater or waste from agriculture and forestry processing (Cherkasov et al., 2015).

Effluent from pulp and paper mill industries is strong with chemical oxygen demand (COD) values of up to 20 g L$^{-1}$ and usually low in ammonia and phosphorus (Ashrafi et al., 2015). Consequently, to facilitate biological treatment, this type of wastewater is supplemented with chemical fertilizer (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 (Dennis et al., 2004; Gray, 2004; Pratt et al., 2007; Slade et al., 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 benthal deposits (Hubbe et al., 2016; Slade et al., 2004; Wiegand et al., 2014). Some studies have determined the conditions required to convert and/or operate wastewater treatment facilities under nitrogen fixation mode or nutrient recycling from benthal deposits, adding limited or no nutrients to the system (Pratt et al., 2007; Slade et al., 2004; Wiegand et al., 2014).

In 2017, the world paper production surpassed the 781 million tons (FAOSTAT, 2017). The paper manufacturing technology uses between 10 and 100 L of water for each ton of paper produced (Kamali & Khodaparast, 2015; Man et al., 2018). 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., 2017; 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 is generally overlooked in wastewater treatment research as the presence of ammonia in high concentrations can inhibit this process (Guo et al., 2017; Smercina et al., 2019). Bacterial and archaeal groups containing some representative nitrogen-fixing species usually found in wastewater treatment systems include the following: Proteobacteria (Welz et al., 2018), Methanogens (Collins et al., 2016), glycogen-accumulating bacteria, 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 (Mahanty et al., 2017; 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 et al., 2004; Welz et al., 2018) have not recognized these systems as a potential source of NFB for biofertilizer production. Indeed, sludge enriched with NFB could be dewatered (Gauthier et al., 2000) and used as a biofertilizer (Kargi & Ozmihçi, 2004).

In previous work, we developed 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 et al., 2020). 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$^{-1}$. This study explores the practical application of our new nitrogen fixation strategy. It 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, valorizing therefore the organic matter low in nitrogen and the sludge enriched with NFB. For this study, we have quantified the \textit{nifH} gene, determined the conditions necessary for BNF, and estimated the nitrogen fixation rates in two sets of duplicate bench scale water resource recovery facility treating wastewater with a high C/N ratio.

\section*{METHODS}

\subsection*{Setup and operation of reactors}

SBRs were inoculated with activated sludge enriched with NFB and operated with high C/N paper mill wastewater for 114 days. We used two sets of biological reactors in duplicate (therefore 4 reactors in total). Each set was inoculated with a different source of sludge obtained from reactors high C/N 1 and 2 from Ospina-Betancourth 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. The reactors were assembled in 1 L containers with an initial working volume of 0.8 L and a hydraulic retention time (HRT) of 48 h. Each SBR was inoculated with 40 ml of the corresponding sludge. Complete mix of the liquor in the reactors was achieved with mechanical stirrers, and air was pumped into the bottom of the reactors at 0.2 L min$^{-1}$. At the end of each cycle, the stirrers and the air tab were turned off for 1 h to settle down the biomass. Before starting a new cycle, clear supernatant was replaced with 0.76 L of wastewater at room temperature. Biomass was never removed from the reactors. 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.

\subsection*{Physicochemical analysis of wastewater}

COD, ammonium, and total nitrogen concentration were quantified with spectroquant kits (Merck, Germany) every two or three weeks. 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, 2017) (Figure S2). Anion chromatography ( Dionex Integron High Pressure Ion Chromatography System, Thermo Fisher Scientific, US) was used to measure the nitrate and nitrite in the influent and effluent. The concentration of phosphorus in the influent was estimated from phosphate measured with anion chromatography. The total organic carbon (TOC) of the wastewater was analyzed using a Shimadzu 5050A total organic carbon analyzer, 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.

\subsection*{Nitrogen fixation activity}

The acetylene reduction assay as specified elsewhere (Ospina-Betancourth et al., 2020) was used to indirectly measure nitrogen-fixing activity for $48$ h in batch reactors during day 62 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 wastewater, 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 62 were further confirmed with the $^{15}$N$_2$ method following Bellenger et al., (2014) The amount of N$_2$ fixed during the 48 h of operation of the batch reactors was estimated in 1.24 and 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 previously in Ospina-Betancourth et al., (2020).

\subsection*{Analysis of microbial community}

Genomic DNA was extracted from the mixed liquor, and copies of the \textit{16S rRNA} and \textit{nifH} genes were quantified with quantitative polymerase chain reaction (qPCR) following the protocols described by Vignola et al., (2018) and Gaby and Buckley (2017), respectively. The efficiencies of the qPCR standards were 91% for the \textit{16S rRNA} gene and 82% for the \textit{nifH} gene.

The microbial community of the reactors was elucidated by next-generation amplicon sequencing of the V4
region from the bacterial 16S rRNA gene (294 bp) in paired-end mode as described elsewhere (Kozich et al., 2013), using 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 identified 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 and a non-metric multidimensional scaling (nMDS) plot 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 neighbor-joining tree with a bootstrap analysis of 1000 replicates.

Statistical analysis

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

RESULTS

Wastewater treatment performance

The COD removal efficiency in the high C/N reactors, fed with different COD concentrations along the 114 days of operation, ranged between 64 ± 4 and 88.7 ± 2.5% (Figure 1). 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. This variable was not considered when planning the experiment. 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 1). The average pH value and dissolved oxygen concentration in the high C/N reactors were 6.31 ± 0.78 and 1.18 ± 2 mg L$^{-1}$, respectively (Figure S1a,b).

Nitrogen speciation profiles and occurrence of BNF

The amount of organic nitrogen in the high C/N reactors increased approximately 3.4 folds, 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 h of incubation. This acetylene reduction rate is equivalent to a nitrogen fixation rate of 13.4 ± 1.6 mg N L⁻¹ day⁻¹.

### 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 folds (Figure 3a). 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 ± 0.26 × 10^5 nifH copies μl^{-1} and 4 ± 0.15 × 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 folds; Figure S2). The number of NFB (calculated by assuming that each bacterial cell contained 4.2 copies of 16S rRNA (Větrovský & Baldrian, 2013) and 1 copy of nifH) was initially estimated in 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 × 10^{-2} and 3.3 ± 1.3 × 10^{-1} (Figure 3b).

**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 (Figure 4a) and a nMDS plot (Figure 4b) 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. The samples obtained on day 0 from each of the two reactors of the high C/N treatments 1 and 2 were replicates and their microbial community profiles were highly similar: 75% of similarity for reactors 1.1 and 1.2, and 70% of similarity for reactors for 1.1 and 1.2 (Figure 4b). 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 S1). The results from the nested ANOVA tests (ran independently for each of the indices) indicated that most of the variance occurred between the treatments (biological replicates; >92%) and not within the replicates (replicate reactors; >6%). In contrast, the observed species indices from both treatments on day 0 (Table S1) 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 the reactors were at least 55% similar (Figure 4a) 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 S1). 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 S1); initially, there were 475 ± 42 and by day 114 approximately 1006 ± 82 sequence variants were identified.

Likewise, 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 5a). 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...

![Figure 4](image-url)
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 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%) (Figure 5b). In the high C/N 2, the most abundant taxa on day 114 were ASV 10 belonging to the family Microscillaceae (5 ± 2.4), ASV 12 identified as Methyloversatilis sp. (4.4 ± 1.2%), and ASV 5 belonging to the class Blastocatellia 3.5 ± 2.1 (Figure 5b). 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).

The most abundant taxa (relative abundance in at least one of the reactors >2.9%) on 114 were aligned with homologous 16S rRNA sequences and compared in a phylogenetic neighbor-joining tree (Figure 6). 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 uncultured bacteria. 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.
**DISCUSSION**

In the present study, we demonstrated that the sludge had a robust capacity for treating high C/N paper mill wastewater with different COD/N ratios without nitrogen supplementation. Although nitrogen fixation was not observed in the reactors, we confirmed that the sludge did not lose its ability to fix nitrogen. The capability of the sludge to treat wastewater with different COD concentrations is highly beneficial as the COD load of the treatment facility 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 favorable treatment performance under low 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). Furthermore, it is likely that some of the total nitrogen entering the system was lost due to volatilization or denitrification as the incoming nitrogen was higher than the organic nitrogen produced and the inorganic nitrogen leaving the system. 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, 2015), an observation that is consistent with the low biomass increment in these reactors.

According to the 16S rRNA qPCR results, the 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 high and low C/N reactors from Ospina-Betancourth et al., (2020). Moreover, the C/N ratios did not favor 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 by Ospina-Betancourth et al., (2020) (0.13) and about 10 times lower than in Bowers et al., (2008). It appears that even very small amounts of nitrogen (COD/N ratio of 100:0.5 in this study versus 100:0 in our earlier work) are sufficient to inhibit the nitrogen fixation genes. We believe that 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., (2008) used a chemical DNA extraction method with high salt (Zhou et al., 1996) and the primer set PolF/PoR (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. We suspect that these more diverse systems will be more robust (Curtis & Sloan, 2006; Kitano, 2004; Song et al., 2015).

Interestingly, we did not observe an abundance of NFB commonly found in pulp and paper wastewater treatment systems such as *Paenibacillus* sp., *Bacillus* sp. *Azotobacter* sp., *Geobacter* sp., *Pseudomonas* sp., and *Klebsiella* sp. (Addison et al., 2010; Chiellini et al., 2014; Ghribi et al., 2016; Oppong et al., 2003; Welz et al., 2018). 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 we cannot be sure whether it was fixing nitrogen in our 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; Kalyuzhnaya et al., 2006; Smalley et al., 2015). However, this is the first report of *Methyloversatilis* sp. in a paper mill wastewater treatment system.

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).

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 activated sludges 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 sludges (approximately up to 25 nmol C$_2$H$_4$ h$^{-1}$ ml$^{-1}$) fed only with synthetic wastewater (Ospina-Betancourth et al., 2020) and bleached kraft mill wastewater (Clark et al., 1997). BNF is an energetically expensive process (Cherkasov et al., 2015); 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., 2014; Kalyuzhnaya et al., 2006; Smalley et al., 2015). Therefore, it is likely that NFB will only fix nitrogen when they do not have it immediately available in their environment. 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.

In principle, the sludge produced in our reactors could be used as a biofertilizer because the free-living NFB therein can
improve nitrogen uptake in plants and enhance the productivity of natural and agricultural ecosystems (Bhattacharjee et al., 2008; Smercina et al., 2019). Nevertheless, field trials are needed to demonstrate and quantify the benefits of nitrogen-fixing sludge as a biofertilizer. If positive results were obtained, the optimization of the conditions for reproduction of the NFB within wastewater treatment systems would be desirable (e.g., the effect of phosphorus).

Currently, the available greener methods for obtaining ammonia for fertilizers 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; Logan, 2010; Matassa et al., 2015; Maurer et al., 2003; Santoro et al., 2019). Thus, the application of our technology is timely as the paper industry is expanding while environmental policies are becoming more demanding (Reid et al., 2008; Toczyłowska-Mamińska, 2017).

CONCLUSIONS

The production of key human resources such as paper and fertilizer must be optimized using more sustainable methods designed with an interdisciplinary approach. This study 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, this technology has the potential to valorize the sludge from the treatment system by producing NFB which could be used as a sustainable biofertilizer. We are hopeful that this could be a green technology that saves both money and the environment.

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AUTHOR CONTRIBUTIONS

Carolina Ospina-Betancourth: Conceptualization (lead); data curation (lead); formal analysis (equal); supervision (lead); writing-original draft (equal); writing-review & editing (equal). P. Curtis: Conceptualization (equal); methodology (equal); supervision (lead); writing-original draft (equal); writing-review & editing (equal).

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.