TECHNOLOGICAL ALTERNATIVE TO ENHANCE BIOLOGICAL NITROGEN FIXATION IN THE RHIZOSPHERE OF NON-LEGUMINOUS PLANTS

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Dedicatory

I believe in Spinoza's God who reveals himself in the orderly harmony of what exists, not in a God who concerns himself with the fates and actions of human beings.

Creo en el Dios de Spinoza que se revela en la ordenada armonía de lo existente, no en un Dios que se preocupa por los destinos y las acciones de los seres humanos.

Albert Einstein

I dedicate this work to my family, thanks to their support in every little detail throughout this academic process. In particular, my wife Irene, my son Daniel, my mother Ofelia, and my mother-in-law Doris.

Este trabajo lo dedico a mi familia, gracias a su apoyo en cada pequeño detalle a lo largo de este proceso académico. En particular, mi esposa Irene, mi hijo Daniel, mi madre Ofelia y mi suegra Doris.

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Resumen

Esta tesis doctoral explora la fijación biológica de nitrógeno (FBN) realizada por microorganismos del suelo como alternativa a los fertilizantes químicos nitrogenados en plantas no leguminosas. En primer lugar, se destacan los profundos impactos ambientales provocados por la producción y aplicación de fertilizantes nitrogenados, el último en la agricultura. Se expone la necesidad de alimentar a la humanidad y cómo esto depende de una fertilización adecuada. Luego se propone un enfoque ecológico basado en comunidades microbianas fijadores de nitrógeno para resolver este desafío. En segundo lugar, se revisa el estado del arte para restaurar las funciones microbianas del suelo a través del aislamiento, selección, formulación y aplicación de inoculantes microbianos y consorcios sintéticos. Aquí, se identifican dos deficiencias clave: la dificultad de diseñar biofertilizantes complejos de múltiples especies y el cuello de botella en la ampliación de su producción. Para abordar este desafío se propone fusionar la teoría ecológica con la ingeniería de bioprocesos para producir "comunidades auto ensambladas" enriquecidas, seleccionando gremios funcionales particulares y adaptados a un suelo objetivo y una planta huésped específica, utilizando el problema del nitrógeno como ancla. En tercer lugar, se presenta la construcción de tres reactores de biopelícula de lecho compacto aireado a escala de laboratorio, inoculados con una comunidad microbiana previamente adaptada, derivada de suelo restaurado naturalmente y alimentada con solución modificada de Hoagland. El nitrógeno solo es proporcionado por aire. Los reactores comunitarios fijan nitrógeno y producen más biomasa que el reactor de cultivo puro del fijador de Nitrógeno *Azotobacter vinelandii*. Se obtienen y analizan muestras de ADN, resultando 1616 OTUs, que se asignan a bacterias con un predominio a nivel de filo o familia que varía entre las muestras, pero similares después que los reactores comunitarios alcanzan el estado estacionario. Finalmente, plantas de tomate son fertilizadas con la solución modificada de Hoagland, con N suministrado solo por la biomasa microbiana, a tres concentraciones diferentes. Esto permitió que las plantas crecieran significativamente. Además, los análisis de ADN reflejan una comunidad microbiana compleja fijadora de nitrógeno en las rizosferas en cada caso. Los tratamientos N1 y N2 parecen similares entre sí y con el Inóculo. El suelo original con la diversidad más baja y el N3 con la más alta fueron similares en términos de la ecología funcional. El siguiente paso en futuros estudios será evaluar el nivel de colonización y la interacción entre el microbioma que viene con las semillas y el proporcionado por el tratamiento.

Palabras clave: Fijación de nitrógeno, biotecnología, biorreactores, ecología microbiana, microorganismos diazótrofos, metabolismo microbiano, ecología microbiana, rizosfera del tomate.

General abstract

This doctoral thesis explores the biological nitrogen fixation (BNF) done by soil microorganisms as an alternative to nitrogen-based chemical fertilizers in non-leguminous plants. First, it is highlighted the deep environmental impacts caused by nitrogen-based fertilizers production and application, the last in agriculture. It is exposed the necessity to feed humanity and how this

relies on proper fertilization. Then an ecofriendly approach based on nitrogen-fixing microbial communities to solving this challenge is proposed. Second, the current state-of-the-art technology to restore soil microbial functions through the isolation, screening, formulation, and application of microbial inoculants and synthetic consortia is reviewed. Thus two key deficiencies arose: the difficulty of designing complex multispecies biofertilizers and the bottleneck in scaling up their production are identified. In order to tackle this challenge, herein it is proposed to merge ecological theory with bioprocess engineering to produce 'self-assembled communities' enriched for particular functional guilds and adapted to a target soil and host plant using the nitrogen problem as an anchor. Third, three laboratory-scale aerated packed-bed biofilm reactors were constructed, inoculated with a previously adapted microbial community derived from naturally restored soil, and fed with Hoagland modified solution. Nitrogen was only provided by air. The community reactors could fix nitrogen and produce more biomass than the pure culture reactor of the model N-fixer Azotobacter vinelandii. 1616 OTUs were found and assigned to bacteria with a dominance at phylum or family levels that varied between samples but were similar after the community reactors reached the steady state. Finally, tomato plants were fertilized with Hoagland modified solution with N-supplied only by microbial biomass at three different concentrations. It allowed plants to grow significantly. Also, it reflected a complex nitrogen-fixing microbial community in the rhizospheres in each case. The N1 and N2 treatments were similar to the Inoculum. Original soil with the lowest diversity and N3 with the highest was alike in the functional ecology. The next step will be to evaluate colonization level and interaction between the microbiome that comes with the seeds and the provided by the treatment.

Keywords: Nitrogen fixation, biotechnology, bioreactors, Microbial Ecology, diazotrophs microorganisms, microbial metabolism, microbial ecology, tomato rhizosphere

Introduction

Environmental impacts of nitrogen fertilizer production and application

Ammonium (NH_4^+) is the second world's most manufactured synthetic product, with 80% used by the fertilizer industry (Grinberg et al., 2016; Elishav et al., 2017). This NH_4^+ production is obtained through the Haber-Bosch process, consuming more than 1% of the total energy generated in the world, using about 2% of the natural gas extracted worldwide, and emitting more than 300 million metric tons of carbon dioxide (Cherkasov et al., 2015). The massive demand for N-based chemical fertilizers not only has an impact on global energy use and greenhouse gas emissions but has led to a nitrogen cycle disturbance, illustrated by the increasing accumulation of nitrates in soils and waters and nitrogen oxides in the atmosphere (Vitousek et al., 2013; Lassaletta et al., 2014). Furthermore, N fertilization has affected the corresponding structure of soil microbial communities (Luo et al., 2018). Therefore identifying sustainable sources is needed, and the application of nitrogen-fixing microbes (diazotrophs) has been proposed as a feasible alternative (Gavrilescu & Chisti, 2005).

N-fertilizer is fundamental for crop production that supports the current and projected human population. Global demand for N-fertilizers increased from 105,148 tons in 2016 to 107,424 tons in 2019 (FAO, 2019), and a rise to 111,591 tons is expected by 2022. This N production represents more than 100 Tg N yr^{-1} of reactive N. 50% of this is applied to maize, rice, and wheat crops (Ladha et al., 2016). As a global population expansion of around 10 billion people is projected for 2050 (Ezeh et al., 2012), a higher environmental impact is expected if the current agriculture production model is not switched to ecology-friendly ways.

Biofertilizer potential of diazotrophs

Approximately 49% of total nitrogen fixation on earth is done for diazotrophs (Fowler et al., 2013). With the use of non-culture-dependent methods based on the detection of the nifH gene, a remarkable diversity of these microbes previously uncultivated has been found (Reinhold-Hurek and Hurek, 2011). Furthermore, comparative genomics techniques based on a 'minimal gene set' have identified many additional putative diazotrophs by their genomic signature alone (Dos Santos et al., 2012). The nifH gene is the most widely sequenced marker gene used to identify nitrogen-fixing Bacteria and Archaea (Gaby & Buckley, 2012). A search made on the NCBI website (NCBI, 2021) through the gene option, using the keyword nifH (https://www.ncbi.nlm.nih.gov/gene), yielded 659 species or strains with that gene (May 14, 2021). Importantly, this biological nitrogen fixation (BNF) occurs in nearly every biome, including extreme ecosystems such as hypersaline lakes and hot springs (Abramovich, 2013).

However, despite microbial biofertilizers' world market is growing (Calvo et al., 2014; Timmusk et al., 2017), their application is still marginal compared with chemical fertilizer (Lu and Tian, 2017; Ahmed et al., 2017). Their use in agricultural practice is still hindered by several factors, such as the unpredictability of results, problems to identify and track inoculated strains in the field, the poor understanding of the interrelationships between microorganisms and plants, and the technology of production (Malusà et al., 2016). This is based on the fact that interactions between plants and microbial communities depend on their genotypes, the geochemical characteristics of the soils and the capacity of the microorganisms to compete and colonize their host efficiently. Therefore, the effects of plant species on microbial taxa are often difficult to predict a priori (Ambrosini et al., 2016; Fierer, 2017). Even plants can select a subset of microorganisms at different stages of development, presumably for specific functions (Chaparro

et al., 2014).

Diazotrophic host-plant association

The diazotrophs are associated with plants in a symbiotic, endophytic, or rhizospheric way. Symbiotic and endophytic diazotrophs, whether obligatory or facultative, have an advantage over free-living or rhizospherics, since they have easy access to carbon (energy) sources, plant water and are less vulnerable to competition with other soil microorganisms (Roper and Gupta, 2016). However, their use depends on crop compatibility, microbiome interaction ability, and soil environmental factors (Martínez-Viveros et al., 2010). Symbiotics are able to provide up to 90 % of the N requirements of the legume host plant through atmospheric N_2 fixation (Franche *et al*. 2009). They can also be found in non-legume plants such as maize, cotton, wheat, rice, canola (Hayat *et al.*, 2010), and sugarcane (Yeoh *et al.*, 2016), but not forming root nodules like in the legumes.

Diazotrophs biofertilizer production: Applications and challenges

Most of the bio-inoculants used in 2012 were mainly rhizobium, accounting for 79% of global demand. Nevertheless, of the few products which demonstrated to have a clear (non-substrate induced) plant growth effect, their mechanism of enhancement has not been fully elucidated, or these have a very short shelf-life, whereas for other products, ‘use-by’ dates were completely absent (Owen et al., 2015). Therefore, the stabilisation of the products with time seems to be highly variable.

The PGPR can supply around 20% of the N requirements in sugarcane (Thaweenut et al., 2011) and 24% for maize, rice, and wheat (Ladha et al., 2016). In these cases, the soil diazotrophs resist the high N-chemicals levels applied to those crops. Similarly, one of the problems for the use of non-symbiotic plant biofertilizers is the supply of sufficient microorganisms in a compatible formula (Vilchez and Manzanera, 2011). It is well known that a coordinated molecular communication must be established between them, otherwise there is a rapid decrease of the inoculum due to the pressures that microorganisms must endure, shortly after their application in the rhizosphere. Another aspect to be solved is the convenience of the use of pure strains. Optimized monocultures for high metabolic production in a sterile laboratory environment are usually not economically viable for industrial production. The high cost of substrates, the low resistance to extreme environmental conditions and stability of cultures, directly impact the process of production (Hoffner and Barton, 2014).

Qiao et al. (2017) have shown positive results working with pure strains applied to soils of tomato plants (cultivar 'Moneymaker') in greenhouses. Although this type of inoculation may promote increased crop yields, limitations may also be expected in relation to the compatibility of the strain with the chemicals used for the treatment of seeds, primarily pesticides (Fukami et al., 2016). In fact, some chemicals have been shown to be very damaging to *Rhizobia* (Hungria et al., 2005; Campo et al., 2009) or very toxic (Mohiuddin and Mohammed 2013) for *Azospirillum*. In addition, it is now known that each plant species selects the type of diazotroph with which it establishes relationships (Smith et al., 2015). For that reason, inoculation with consortia has been proposed as an alternative with better results compared to inoculation with individual strains (Leaungvutiviroj et al., 2010, Souza et al., 2015, Bledsoe and Boopathy, 2016).

Searching for appropriate microbes as biofertilizer inoculum

The microbial community of the rhizosphere is influenced by the species, the genotype of the host and even plants can select a subset of microorganisms at different stages of development, presumably for specific functions (Chaparro et al, 2014). Even the colonization of the host's root requires functional abilities, eg, motility, but the selection is more related to specific metabolic activities; therefore, although microbial diversity may be high, the bacterial community may possess a very similar functional profile (Ofek-Lalzar et al, 2014). Additionally, today it is known that plant domestication not only led to a reduction in genetic diversity of cultivars compared to their wild ancestors but also have hampered beneficial interactions between plants and microbes due to loss of rhizosphere microbial diversity (Pérez-Jaramillo et al, 2016). Therefore, led to low self-support production systems with an increased need for external inputs such as chemical pesticides and fertilizers to overcome problems related to pests and diseases, vulnerability to abiotic stress and nutrient depletion. On the other hand, it has been found that the abundance of N-cycling-related prokaryotic groups is drastically affected by long-term mineral fertilization, while organic fertilization clearly increased the abundance of diazotrophs (Wang et al., 2017; Luo et al., 2018).

Research problem and justification

As described above there is no biofertilizer for universal crop application. In addition, most microorganisms in natural environments do not live in isolation but exist as part of complex microbial consortia, where they can exhibit high productivity, resilience and process a wide range

of readily available substrates (Höffner and Barton, 2014). Thus, the biological nitrogen fixation process based on these consortia seems to be an alternative to explore for sustainable production at low cost of biofertilizers. But given all the information gaps, it is necessary to develop a more solid understanding of the ecological relationships between the plant rhizosphere and their nitrogen-fixing microbiome, to provide the basis for exploring the engineering of diazotrophic consortia. Then it is necessary to find the appropriate biofertilizers inocula that can benefit the plant physiology and root exudation patterns, which in turn influence positively the composition of the rhizosphere microbiota (Philippot et al., 2013; de Souza et al., 2015).

Research questions

How does the diversity and activity of the soil microbial community change in response to N-fixation enrichment in bioreactors?

How does diversity and activity of rhizospheric diazotrophs change in response to biofertilizers application?

How do the plant phenotype and its nitrogen content change in response to biofertilizers application?

H_0^1: No significant difference in the diversity, structure, and function is observed in bioreactors when a microbial community inoculant is applied.

H_0^2: No significant difference in diversity, structure, and function is observed in the rhizosphere when the reactor community is applied.

H_0^3: No significant difference is observed in plant phenotypic response when the nitrogen-fixing community is applied.

Objectives

General objective

Engineer the biological nitrogen fixation (BNF) enrichment in the rhizosphere of non-legume plants using N-fixing bioreactors.

Specific objectives

1. Characterize (isolate, identify and validate) microbiologically a nitrogen-fixing community in bioreactors.
2. Study changes in the diversity, structure, and function of the microbial community involved in BNF in the bioreactors.
3. Evaluate the phenotypic response of plants after inoculation of the microbial community coming from bioreactors.
4. Evaluate the diversity, structure, and function of the BNF community in the rhizosphere.

Objectives 1 and 2 are addressed in Chapter 1, while 3 and 4 in Chapter 2.

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REFERENTIAL FRAMEWORK: Restoring degraded microbiome function with self-assembled communities

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Abstract

The natural microbial functions of many soils are severely degraded. Current state-of-the-art technology to restore these functions is through the isolation, screening, formulation and application of microbial inoculants and synthetic consortia. These approaches have inconsistent success, in part due to the incompatibility between the biofertilizer, crop, climate, existing soil microbiome and physicochemical characteristics of the soils. Here, we review the current state of the art in biofertilization and identify two key deficiencies in current strategies: the difficulty in

designing complex multispecies biofertilizers and the bottleneck in scaling the production of complex multispecies biofertilizers. To address the challenge of producing scalable, multispecies biofertilizers, we propose to merge ecological theory with bioprocess engineering to produce 'self-assembled communities' enriched for particular functional guilds and adapted to a target soil and host plant. Using the nitrogen problem as an anchor, we review relevant ecology (microbial, plant and environmental), as well as reactor design strategies and operational parameters for the production of functionally enriched self-assembled communities. The use of self-assembled communities for biofertilization addresses two major hurdles in microbiome engineering: the importance of enriching microbes indigenous to (and targeted for) a specific environment and the recognized potential benefits of microbial consortia over isolates (e.g. functional redundancy). The proposed community enrichment model could also be instrumental for other microbial functions such as phosphorus solubilization, plant growth promotion or disease suppression.

Keywords: biological nitrogen fixation; biotechnology; biofertilization; microbial ecology; diazotrophs; soil fertilization; restoration ecology; community assembly

A century of degradation: the impact of synthetic fertilizer and alternatives

The use of synthetic nitrogen (N) for crop fertilization contributes tremendously to the world's food supply, but the production and application of these fertilizers also contribute to a global environmental imbalance. It is estimated that synthetic N is the second most manufactured synthetic product in the world (Elishav et al. 2017), and the Haber–Bosch process used to drive this production is estimated to consume 1% of the global primary energy produced (Cherkasov, Ibhadon and Fitzpatrick 2015). In addition to the high energy cost, N fertilizers alter microbiome function, increasing soil respiration and nitrous oxide emissions (Bicer et al. 2017) equivalent to 1–2.5% of global greenhouse gas emissions (Pfromm 2017), all the while reducing soil diversity (Dai et al. 2018) and degrading the natural community structure and nitrogen-fixing capabilities of soils (Wang et al. 2017; Luo et al. 2018). Paradoxically, the increased use of N fertilizers has coincided with a significant global decrease in the efficiency of N use over the last 50 years (Lassaletta et al. 2014). Only 4–14% of synthetic N applied to croplands enters the economy as a downstream final product (Pikaar et al. 2017). Moving forward, increasing N-use efficiency is integral to increased agricultural sustainability (Schils et al. 2018).

One approach to reduce the impact of synthetic N is to reduce the energy footprint of its production by increasing the use of renewable resources to drive the Haber–Bosch process (Wang et al. 2018), or by identifying alternative low-energy technologies for synthetic N production (Qiu et al. 2018). However, these approaches do not address the downstream ecological

consequences of synthetic N use listed above. Another approach is to increase the use of organic fertilizers (Celestina et al. 2019), and there is considerable demand for the transformation of waste streams into organic fertilizers through composting, fermentation or thermochemical processing (Sudharmaidevi, Thampatti and Saifudeen 2017). However, the use of organic fertilizers has been controversial and restricted because organic fertilizers have also been associated with increased generation of greenhouse gas emissions, heavy metal accumulation/leaching and as a source of emerging contaminants (Moran-Salazar et al. 2016; Lekfeldt et al. 2017; Pan and Chu 2017; Ibekwe, Gonzalez-Rubio and Suarez 2018; Weithmann et al. 2018).

The advent of synthetic biology has also offered some possible 'silver bullets' that are being investigated intensely. The most drastic approach would be through 'extreme bioengineering' of N fixation directly into the plants (Buren, Lopez-Torrejon and Rubio 2018). Although efforts to engineer plants for N fixation have made strides through eukaryotic nitrogenase expression, achieving significant N fixation under field conditions is likely decades away and would require a concerted effort and investment (Good 2018). Furthermore, the ecological consequences of cultivating such plants are completely unknown but would undoubtedly alter global N cycles. Another such 'silver bullet' is to engineer symbiont nodules into cereal roots (Rosenblueth et al. 2018). Strategies for engineering rhizobial infection and nodule organogenesis so far remain elusive (Bloch et al. 2020), and again, the ecological consequences of engineering such interactions are unknown but would likely have global impacts.

Restoring natural populations of free-living diazotrophs

Biological nitrogen fixation (BNF), through either symbiotic or free-living organisms, provides another route for N into the agroecosystem. For example, synthetic N demand may be decreased by intercropping cereals with nodule-forming legumes (Brooker et al. 2015; Iannetta et al. 2016). A strength of intercropping is the relatively low cost of entry and scalability (Reckling et al. 2020). Another approach to leverage BNF is through biofertilization with either symbiotic or free-living inoculants (Schütz et al. 2018). Research efforts in biofertilization are generally small scale and are primarily focused on screening, characterizing and formulating isolates, and more recently synthetic communities (Kong, Hart and Liu 2018; Schütz et al. 2018; Toju et al. 2018). The first commercial biofertilizer was already patented in the 19th century, and there are now many products on the market (Garc´ıa-Fraile, Menendez and ´ Rivas 2015).

Generally speaking, the goal of biofertilization efforts is to identify isolates that exhibit the highest ability for a particular function. Once these 'top performers' have been identified, they may be mass produced for field application. A meta-analysis of nodule-forming rhizobia highlights a major challenge with this approach: the effectiveness of individual rhizobial isolates is only observed where the indigenous population of rhizobia is low (Thilakarathna and Raizada 2017). One cause of this may stem from fitness trade-offs: isolates that are 'top performers' in one environment are maladapted to others (Kaminsky et al. 2019). Indeed, the relationship between the competitiveness of rhizobial isolates that form nodules is often not correlated with their nitrogen-fixing capacity (Bourion et al. 2018). A different meta-analysis highlights another challenge: biofertilizers seem to be most effective when multiple inoculant species are applied (e.g. as co-inoculants) (Schütz et al. 2018), and the efficacy of multispecies inoculants has been shown to improve with increasing diversity (Hu et al. 2016).

An alternative approach to biofertilization is soil transplantation, which aims to leverage the function and diversity of one ecosystem (with a desired function) to restore (a subset) these functions in a target ecosystem (Wubs et al. 2016). A classic example of a soil transplantation experiment is the case of disease suppressive soils. This function has been shown to be transferable even at dilutions of 1/1000 from the source soil (Cook and Rovira 1976). However, soil transplantation experiments are not always successful, and may require much higher proportions for crop application (Ma et al. 2020). One of the main drawbacks of soil transplantation is the logistical problems associated with scaling and implementing such large-scale projects. Another important consideration is that scaling soil transplants would put further strain on the natural ecosystems, especially as long-term agricultural practices will require repeated biofertilization. Furthermore, as there are few soil transplantation field studies, the success rate and the mechanisms are relatively unknown.

The practical trade-offs between biofertilization approaches may be summarized based on the following characteristics: the complexity of the inoculant and the scalability of the approach (Fig. 1). On one end of the spectrum, 'top-performing' isolates are identified and produced at industrial scale. By producing synthetic microbial communities (SynComs), it is possible to increase the complexity of microbial inoculants, and there have been significant inroads in the design and mechanisms of complex SynComs (Finkel et al. 2020). However, there is a trade-off

between the complexity and scalability of SynCom production, as each isolate must be produced individually before formulation. On the other end of the spectrum, soil transplantations provide an inoculant with a much higher number of species, but with a trade-off in scalability, as any particular soil is finite. Thus, the current methods for biofertilization represent two extremes of the spectrum in the complexity and scalability of the product. While diverse microbial inoculants are not always better than 'top performers', approaches that leverage multiple inoculants often do outperform single isolates (Hu et al. 2016; Schütz et al. 2018), highlighting the need for an intermediate approach that can produce biofertilizers that are complex and scalable (Fig. 1). To address this gap in the production of scalable and complex microbial biofertilizers, we propose a novel approach that leverages ecological theory and process engineering to produce a 'self-assembled' community enriched in particular functions related to plant growth promotion, nutrient acquisition or disease suppression, and adapted to a target environment.

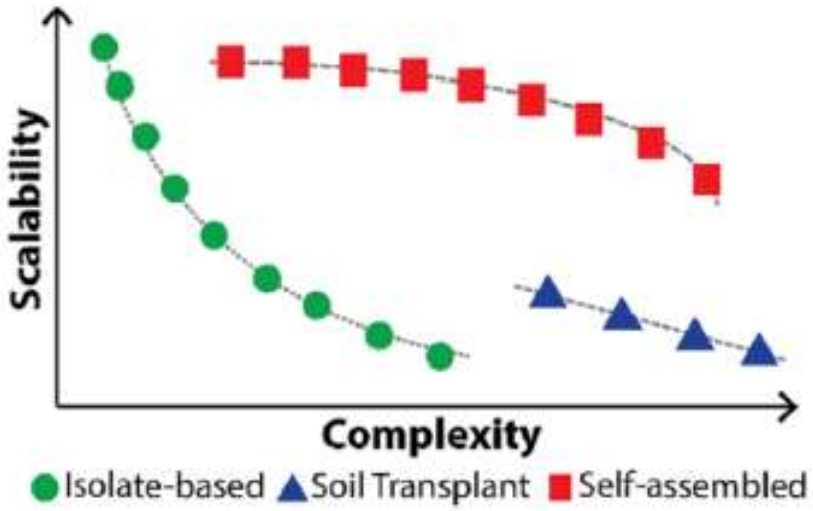


Figure 1. The trade-off between the complexity and scalability of production for isolate-based (•), soil transplant (), and self-assembled () biofertilizers. Irrespective of the efficacy of a biofertilizer, a key challenge is the scalability of production. For a single isolate, mass production is relatively straightforward. However, as the number of species in an isolate-based inoculum increases, the ability to scale production decreases, as each isolate must be produced separately and subsequently formulated together. In the case of soil transplantation, an immensely complex and diverse inoculum is available; however, the trade-off is that any given source soil is finite, and thus, scaling must be achieved by diluting the soil. Eventually, a dilution is reached in which the complexity of the soil is so diluted that the beneficial aspects of the soil are lost. In contrast, to isolate-based approaches, self-assembled communities may be mass-produced in a single reactor, thus allowing the scalable production. As detailed in this review, community assembly may be guided toward a desired output through careful bioprocess engineering and ecological parameterization. Thus, self-assembled communities represent a highly scalable approach to produce complex biofertilizers.

The use of bioreactors provides many additional levels of real-time control on environmental and biological parameters such as pH, gas flux, temperature, growth rate, and form, and may thus be used to recapitulate the ecological parameters required to drive the assembly of communities with even highly specialized metabolisms (Imachi et al. 2020). The improvements that bioreactors provide over traditional plate-based techniques have long been recognized as a powerful tool to investigate isolated rhizosphere diazotrophs (Fritzsche, Ueckert and Niemann 1991; Ueckert, Huckfeldt and Fendrik 1995; Liu et al. 2017). However, there are only few examples of bioreactors used to enrich communities under simulated rhizospheric conditions (Peacock et al. 2014; Xiao et al. 2018), and none investigating rhizospheric diazotroph communities. Despite this, free-living diazotrophs are commonly enriched in other community-based biotechnologies such as wastewater treatment (Welz et al. 2018; Ospina-Betancourth et al. 2020) or for the production of the bioplastic polyhydroxyalkanoate (Patel et al. 2009). Thus, there is ample evidence that not only bioprocess engineering may enrich functionally diverse diazotrophs (Bowers, Reid and Lloyd-Jones 2008; Reid, Bowers and Lloyd-Jones 2008) but also such an approach is generalizable and may be used to produce self-assembled communities with other functions of agro-ecological interest such as phosphorus (P) solubilization and chitin degradation (Cretoiu et al. 2013), or the simultaneous selection of multiple functions (Oyserman et al. 2017). To root our perspective, we use nitrogen fixation as a target function for restoration. We first discuss the key ecological parameters that must be recapitulated in order to select for diazotrophs based on not only what is known about the microbial physiology of diazotrophs but also the target environment (soil/rhizosphere) and host (crop). We then consider how these ecological parameters may be integrated with bioprocess engineering and reactor design strategies for the production of self-assembled communities (Fig. 2).

Ecological parameters for community self-assembly

To engineer a community-based bioprocess, it is important to recapitulate the ecological conditions that select for the natural assembly of a community with a desired function. In the case of biofertilizers, this must be taken one step further, as selection for traits related to survivability in the target soil and rhizosphere must also be accounted for. With regard to nitrogen fixation, the most obvious selective parameter is nitrogen limitation. However, what constitutes ‘N-limiting’ is based on the stoichiometry of various elements and are species specific

(Dynarski and Houlton 2018; Inomura et al. 2018; Zheng et al. 2018, 2020). For example, recent work developing a quantitative model of nitrogen fixation in the heterotrophic bacteria *Azotobacter vinelandii* shows that nitrogen fixation may occur in the presence of ammonium as long as the C:N ratios are sufficiently high at a given O2 availability (Inomura et al. 2018).

As a starting guide, C:N ratios in soil typically fall between 8:1 and 16:1 (Tian et al. 2010). Interestingly, despite the broad range of soil C:N ratios, a meta-analysis of soil microbial stoichiometry suggests that soil microbial stoichiometry is uncoupled from these ratios and a stoichiometry of 60:7:1 C:N:P has been proposed for soil microbes (Cleveland and Liptzin 2007). Furthermore, the C:N ratios of leaf litter are much higher and can reach between 50:1 and 90:1 (McGroddy, Daufresne and Hedin 2004). These ratios may be used as a starting point, but currently little is known about how C:N ratios impact the selection of different diazotroph lineages, nor the outcome of selection when different ratios are interplayed between other parameters such as oxygen.

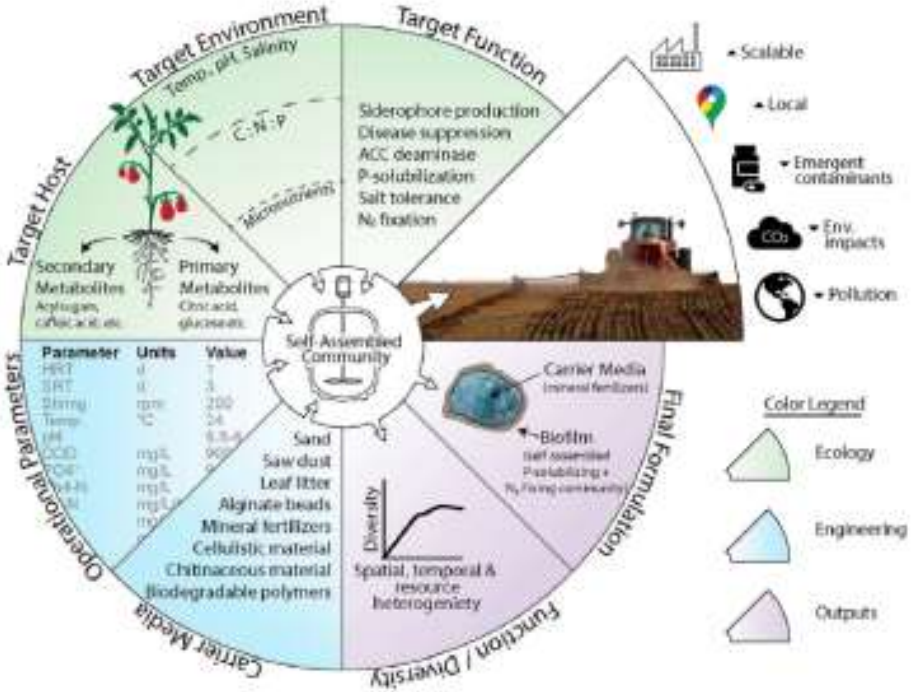


Figure 2. A conceptual overview of the design, production and application of self-assembled communities. First, the ecology (in green) of a system must be investigated. A target host and environment are identified, as well as the desired function(s) to be enriched. Based on the target environment, host and function, and the desired end formulation, operational parameters are determined and other bioprocess engineering considerations are made, such as the choice of the carrier media (in blue). The final formulation, level of diversity and function produced are dependent on the operational parameters. For example, a P-solubilizing and nitrogen-fixing community may be enriched using N- and P-limited media on a P-rich mineral fertilizer as a carrier medium (in purple). With increased understanding of the ecology of both target system and bioprocess design, increasingly tailored and effective

self-assembled communities may be produced.

Another major selective parameter for diazotrophs is oxygen. Broadly speaking, diazotrophs may be classified as anaerobic, microaerophilic and aerobic. This broad range of oxygen tolerance is surprising given that nitrogenase, the enzyme responsible for nitrogen fixation, is irreversibly inhibited by oxygen. Therefore, all diazotrophs regardless of their life history must employ mechanisms to maintain anaerobic conditions for a functional nitrogenase (Gallon 1981). However, even within a species, the level of sensitivity and responses to oxygen are rather plastic and adaptive (Dingler and Oelze 1985). Indeed, the scavenging mechanisms employed are quite costly, and because of this, nitrogen fixation is believed to be most efficient under microaerophilic conditions as shown in *A. vinelandii* (Inomura et al. 2018) and *Crocosphaera watsonii* (Großkopf 2012). Thus, on the one hand, by finely tuning bioreactor oxygen concentrations, a highly specialized subset of diazotrophs may be selected for. Conversely, by engineering conditions with varied concentrations of oxygen through time, space or both, diazotroph communities that may invade various oxygen niches in the soil may be enriched. Spatially, such niches may range from microaerophilic in the endosphere or rhizoplane where respiration is likely highest, to much higher concentrations of oxygen further away from the roots. Temporally, oxygen concentrations likely follow cyclic diurnal rhythms based on the photosynthetic status of the plant. Such patterns may be mimicked by careful selection of reactor type and operational parameters as described in subsequent sections.

Many other factors exist that are related to the efficiency of nitrogen fixation at a molecular level. For example, while the primary nitrogenases are molybdenum dependent (Mo-nitrogenase), alternative vanadium- or iron-dependent nitrogenases may also be used to fix nitrogen (V-nitrogenase and Fe-nitrogenase, respectively) when molybdenum is limiting (Smercina et al. 2019). While these alternative nitrogenases are less efficient under most conditions, V-nitrogenase appears to be more efficient under low temperatures (Bellenger et al. 2014). As with the case above with determining nitrogen limitation, what constitutes molybdenum deficiency is also dependent on the environmental context, as P availability may play a large interacting role, especially when both nutrients are limiting (Reed, Cleveland and Townsend 2013). Indeed, P availability in itself can also be a limiting factor of nitrogen fixation (Reed et al. 2007), and therefore it is not surprising that many diazotrophs tested for biofertilization are also phosphorus solubilizers (Schütz et al. 2018). Thus, by applying selective pressures matching

target soil characteristics such as trace element and nutrient stoichiometry, scalable production of pre-adapted biofertilizers may be achieved decoupled from limitations of enriching these communities directly in the soil (e.g. seasonality) (Fig. 2).

The availability of soil carbon was once thought to be a limitation for free-living diazotrophs; however, it is now known that plants transfer around 20–50% of the total assimilated carbon into the soil to recruit microbes using root exudates (Haichar et al. 2014). Indeed, the quantity and quality of root exudation is now considered an important functional trait associated with high growth rates and N acquisition (Guyonnet et al. 2018). Up to 90% of root exudate carbon may be taken up by microbial biomass (Smercina et al. 2019). The current state of the art in the quantity and quality of root exudates is by studies implementing axenic systems (Kuijken et al. 2015). From such systems, it is clear that organic acids and sugars are the primary components of root exudates, but the concentrations and make-up are both species-specific and context-dependent (Johnson et al. 1996; Kravchenko et al. 2003). Advances in methodology continue to put this exciting field into focus, such as the role of primary and secondary metabolites in shaping the microbiome (Szoboszlay, White-Monsant and Moe 2016; Girkin et al. 2018; Stringlis et al. 2018) and the use of split root systems to investigate how microbial inoculation alters root exudate profiles (Korenblum et al. 2020). Therefore, the selection of dominant carbon sources and dosing of secondary metabolites matching the host plant are important controls that will impact the diversity and function of self-assembled communities (Fig. 2). In particular, a key focus here should be investigating how biotic/abiotic stresses alter root exudates, and how such a "cry for help" may be leveraged to select communities adapted to them (Berendsen et al. 2018). For example, addition of caffeic acid, a phenolic root exudate and important intermediate in the biosynthesis of lignin, has been used to target the isolation of endophytic diazotrophs (Adachi, Nakatani and Mochida 2002). A recent review has summarized the numerous formulations of N-free media that have been used to isolate diazotrophs (Baldani et al. 2014), and experiments using artificial root exudates may also serve as an initial guide (Baudoin, Benizri and Guckert 2003). Altering these formulations to match soil chemistry and crop exudate specifics may serve as the ecological basis for selection of self-assembled diazotrophic communities (Fig. 2).

Bioprocess engineering for community self-assembly

In the previous section, we detailed how environmental parameters such as C:N:P ratios, oxygen

concentrations, trace elements, primary carbon sources and secondary metabolites may be used to steer diazotrophic community assembly. In this section, we explore how, given these parameters, different reactor types (attached versus suspended growth), mode of operation (batch, fed batch or continuous) and process parameters (hydraulic retention time, solid retention time and particle size distribution) may be used to further control community assembly. We highlight how these parameters may be tuned to obtain a desired functionality and level of diversity, and contextualize these technical aspects with practical decisions related to the production of diverse self-assembled biofertilizers (Fig. 2). While product consistency will be a major challenge for self-assembled communities, it is important to note that complex bioprocesses often have high spatial and temporal habitat heterogeneity but still can lead to predictable community structures (Nielsen et al. 2012). Furthermore, open reactor systems operating with high degrees of spatial and temporal heterogeneity may still be consistently enriched with the same target taxa (~90% relative abundance), and their growth state highly characterized (Oyserman et al. 2016a,b). Thus, while there are certainly additional challenges to using mixed communities rather than single isolates, there is also a long history of successful innovation in novel reproducible bioprocesses, as exemplified by the discovery and now widespread implementation of Anammox (Kuenen 2008).

Broadly speaking, reactors may be categorized into attached growth or suspended growth systems. An attached growth design employs a carrier medium, or packing material, to support biofilm growth. This carrier medium may be chosen based on a combination of practical considerations related to biological properties, costs, and downstream application. Essentially, any material may be used as a carrier and can either be fixed or suspended in the reactor (Loupasaki and Diamadopoulos 2013). Similarly, packed bed columns or soil columns may be used (Wery et al. 2003; Wei et al. 2016). The choice of carrier medium is important, especially when it is desired to apply the carrier medium containing the biofertilizer applied directly to the soil (Akay and Fleming 2012). One advantage of using an attached growth system is that existing and emerging agricultural inputs such as phosphate rocks, struvite, sand, wood chips, sawdust and chitinaceous material may be used as a carrier medium and hence ‘activated’ with key agro-ecologically relevant functions such as phosphorus solubilization, or chitin degradation before their application into the agroecosystem. Another type of attached growth system that has been used for the production of biofertilizer isolates is solid-state fermentation (Jin et al. 2019).

Solid-state fermentation is an especially alluring process, as it uses minimal water and may therefore be more suitable for mimicking soil conditions or even cycling drought conditions. In contrast, suspended growth reactors do not contain carrier mediums. Growth is suspended within the reactor as either free-living cells or as floccular and granular structures. Suspended growth systems may subsequently be applied directly to the soil or formulated with a natural or synthetic carrier and then applied to the soil (Malusá, Sas-Paszt, and Ciesielska 2012).

The level of diversity that develops within a community may be managed by adjusting the level of spatial and temporal heterogeneity. Spatial heterogeneity may be impacted on both macro and microscale. On the macroscale, heterogeneity may be achieved when the packing material is fixed in relation to the flow of the media, such as in a soil column reactor or trickle bed reactor. In such systems, influent concentrations are spatially altered through the flow of the reactor and thus organisms at the beginning generally experience high concentrations of influent substrates, whereas organisms at the end of the process are exposed to low concentrations of influent substrates, but high concentrations of waste products. In these systems, it is common for diverse organisms carrying out differential biogeochemical processes to establish through the column (Wery et al. 2003). Thus, packed bed and soil column reactors promote spatial segregation of microorganisms optimized for specific environments.

Microscale spatial heterogeneity may be promoted through the development of biofilm (Stewart and Franklin 2008). In suspended growth systems, spatial heterogeneity is dependent on the particle size distribution (i.e. the thickness of the biofilm). As particle size increases in floccular or granular communities, diffusion through these biofilms contributes to increasing environmental heterogeneity. Biofilm formation is an important rhizosphere process and associated with disease suppression (Bais, Fall, and Vivanco 2004), and therefore, particle size distribution and biofilm thickness is likely a key operational parameter in selecting for self-assembled biofertilizers. Indeed, the choice of carrier media may also be done to promote such heterogeneity. For example, using mineral fertilizers containing insoluble P as a carrier medium in a P-depleted system would favor the formation of a biofilm on this mineral fertilizer and the establishment of two countering gradients of oxygen (into the biofilm) and P (out of the biofilm).

In addition to the spatial heterogeneity discussed above, the temporal heterogeneity of a process

may also be established. The simplest design is a batch system, in which influent addition occurs at a single time point, operation occurs until a reaction has gone to completion and concentrations of resources vary through the time of production. Many industrial bioprocesses such as beer production and other microbial fermentation products are operated as batch systems. In this manner, community members adapted to different concentrations may be differentially active at the beginning and end of a bioprocess. Such temporal heterogeneity may be important for selecting diverse communities and must be accounted for when considering product consistency. However, this heterogeneity may also be exploited to select the appropriate growth state, such as the production of storage polymers, which has been shown to improve inoculant survivability. Furthermore, as community structure increasingly diverges from the original community structure after inoculation before reaching stability (Trebuch et al. 2020), by operating over a single reaction cycle, the community that develops is not so far removed from the original inoculant. Determining the length of batch operation is also necessary. This may be determined by monitoring growth or substrate use.

A sequencing batch reactor is a simple extension of the batch reactor. To operate a sequencing batch reactor, the reactor must simply undergo consecutive fill and draw cycles in which the biomass is retained but the bulk fluid replaced with new influent. In this way, biomass may be accumulated to a desired concentration. The repeated fill and draw cycles may be advantageous when enriching communities for dynamic physiologies such as polymer cycling and storage (Oyserman et al. 2016b), which has been shown to be an important factor in the successful application of biofertilizers (Okon and Itzigsohn 1995). After consecutive cycles, the community structure may take on emergent properties such as the formation of floccular and granular biomass (Jarvis et al. 2005), which may be leveraged in downstream processes such as harvesting and application. For example, one of the key features of floccular and granular structures is their high content of extracellular polysaccharides and alginate-like substances (Trebuch et al. 2020), which, again, has been shown to improve biofertilizer viability after application (Okon and Itzigsohn 1995). Finally, dual approaches that combine isolates with community based-approaches may also be successful. For example, starting inoculants may include ‘top-performing isolate(s), or may be inoculated at high densities after community assembly has already been initiated. Indeed, understanding how/when isolates can invade established communities remains a key challenge in microbiome engineering. Such challenges will also have

to be addressed with the application of self-assembled communities.

Conclusions and future perspectives

Intensive agricultural practices have resulted in the global degradation of microbial functions in soils. Restoring these microbial communities is important for the future sustainability of agriculture. Furthermore, restoring microbial function is important when these agricultural systems are converted back into natural systems. Current methods for biofertilization are limited in their diversity and scalability. On the one hand, the production of isolate-based biofertilizers may take advantage of the scales of industry. However, isolate-based biofertilizers are inherently limited in their complexity, even in the context of synthetic communities. On the other hand, soil transplantation experiments are at the other end of the spectrum. While they are diverse, scaling soil transplantations is logistically not feasible because natural soils are a limited natural resource that may also be depleted. To address these deficiencies, we present a novel approach to produce functional, diverse and scalable biofertilizers by merging bioprocess engineering and ecological theory.

There are many microbial functions that could be targeted by such an approach, and ecological selection is often already applied to screen isolates with these characteristics. For example, to identify microbes with ability to degrade 1-aminocyclopropane-1-carboxylic acid (ACC), isolates are often grown with ACC as a sole nitrogen source. Such selective strategies can be directly applied to select self-assembled communities enriched in such functions. In this manner, communities with enhanced abilities for phosphorus solubilization, siderophore production, chitin degradation and many other agro-ecologically relevant traits may be targeted. Interestingly, BNF organisms often have many of these functions, and therefore, it can be hypothesized that selecting for key functions such as BNF may act as a ‘Trojan Horse’, whereby many desired traits accompany the selection of a key function.

Selection may also take a deliberately modular approach and select for compatibility between community members with different functions (Oyserman, Medema and Raaijmakers 2018). For example, a carrier medium composed of recalcitrant insoluble phosphorus in nitrogen-free media would select for phosphorus-solubilizing and nitrogen-fixing organisms. Thus, by moving beyond classic isolate-based approaches and leveraging ecological theory merged with

bioprocess engineering, it is likely that current challenges related to soil health may be addressed. Furthermore, bioprocesses such as anaerobic digestion are increasingly operated by small farms (Wang 2014; Imeni et al. 2020). Thus, advances in the production of self-assembled communities for biofertilization not only provide a means to produce highly complex and scalable production of biofertilizers but also may provide farmers and land managers a new tool in managing soil health at the local level. Currently, the potential for self-assembled communities as biofertilizers is completely unexplored and we hope that outlining the ecological and engineering considerations here will spur further research on this topic.

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authors' contributions
BOO and CFGL conceived and drafted the manuscript. JSG and JMR provided critical feedback and discussion. All authors contributed critically to the drafts and gave final approval for publication.

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CHAPTER 1. EXPLORING NITROGEN FIXATION BY A SOIL MICROBIAL COMMUNITY ENRICHED IN AERATED PACKED-BED BIOFILM REACTORS IN A NITROGEN-FREE SOLUTION

Abstract

It is necessary to find solutions to the environmental impacts of the production and application of chemical fertilizers. One way to do this is to explore natural solutions to produce nitrogenous fertilizers. For this reason, three laboratory-scale aerated packed-bed biofilm reactors were constructed and inoculated with a previously adapted microbial community derived from naturally restored soil. The community reactors (with the influent COD of 1200 mg/L reduced by 90–94%) performed comparable to the pure culture reactor of the model N-fixer *Azotobacter vinelandii*. We use 16S gene sequencing to estimate the microbial composition and diversity of samples collected from the soil and at different run times from community reactors. We found 1616 operational taxonomic units (OTUs) assigned to bacteria with a dominance at phylum or family levels that varied between samples but were similar after the community reactors reached the steady-state. We conclude that the soil microbial community adapted to fix nitrogen in nitrogen-free reactors, and it could become an ecological alternative to chemical nitrogen fertilizers. Furthermore, a soil microbial community can use this solution to produce more biomass than a microbial pure-culture. The next step of research should be to test this approach in non-legume plant rhizospheres to evaluate the plant and microbiome response.

1.1. Introduction

The intensification of agriculture has highly increased food availability over recent decades. However, it has led to considerable adverse environmental impacts, such as increases in eutrophication of land and water bodies, greenhouse gas (GHG) emissions, and biodiversity losses (Fowler et al., 2013; Bender et al., 2016; Muller et al., 2017; Pfromm, 2017; Bowles et al.,

2018). Given that by 2050 world population could increase by 9 to 10 billion (Foley et al., 2011; Alexandratos & Bruinsma, 2012; Rockström et al., 2017), some ecological alternatives to the intensive use of crops are open, such as leguminous-based fertilized systems, and organic farming. However, they have some restrictions. There are concerns because not all non-leguminous plants are liable to intercrop with legumes, rotation can take longer than expected, or it is a challenge to a large-scale application (Duchene et al., 2017; Stagnari et al., 2017; Bordonal et al., 2018). In relation to organic agriculture, it uses manure, composts, and plant residues in crop production systems to restore degraded soils and ameliorate physicochemical constraints (Celestina et al., 2019). In addition, neither synthetic fertilizers nor synthetic pesticides are applicable and heavily rely on an active microbial community to break down organic matter into plant-available nutrients (Lori et al., 2017; Martínez-García et al., 2018). Conversely, the use of organic fertilizers has been controversial and restricted because they have also been associated with increased accumulation of pharmaceuticals and antibiotics in the agricultural fields (Pan and Chu 2017). Moreover, there is not enough animal manure to supply nutrients to a much larger area under organic production (Meemken & Qaim, 2018). As a result, abandoning synthetic N-fertilizers could lead to nutrient undersupply, even with increased legume cropping. Therefore, the ability of organic agriculture to sustainably feed the world is not reliable for the time being (Muller et al., 2017).

A feasible solution to this challenge is microbiome engineering since organic agriculture depends on an active microbial community. This method allows the manipulation and study of microbial communities (Lawson et al., 2019; Roell et al., 2019; Kerfeld et al., 2020), even in their native context without the need for the domestication of individuals in the laboratory (Sheth et al., 2016), and can promote plant fitness and health (Pascale et al., 2020). Focused on the top-down design process and self-assembled construction methods approached by Lawson et al. (2019), Gutierrez et al. (2020) merged microbiome engineering theory with bioprocess engineering to propose the self-assembled microbial communities, enriched for Nitrogen-fixation (diazotrophs) functional guild and adapted to a target soil and host plant. That proposal uses environmental parameters such as C: N: P ratios, oxygen concentrations, trace elements, primary carbon sources, and secondary metabolites that could be used to steer diazotrophic communities assembly in bioreactors. In the present study, we aimed at testing these self-assembled diazotroph communities approach, explore its production at a laboratory scale, and obtain operational data

from this system. Therefore, using the nitrogen fixation guild as an anchor, move forward in the microbiome engineering and production knowledge to provide alternatives that support organic agriculture.

1.2. Materials and methods

1.2.1. *Bioreactors configuration*. Five laboratory-scale Biologically Aerated Filter (BAF) reactors were used. Each reactor consisted of a sterilized acrylic tube (0.42 m high, 0.102 m in diameter) containing sterilized clinoptilolite zeolite as carrier media (4 mm in diameter). The total volume of reactors was 1.8 L with a load volume of 1.2 L. The details of the reactors are presented in Figure 1. Three reactors were designed as experimental replicates (R2, R3, and R5). The other two reactors were operated as negative (C-) and positive (C+) controls (R1 and R4), respectively.

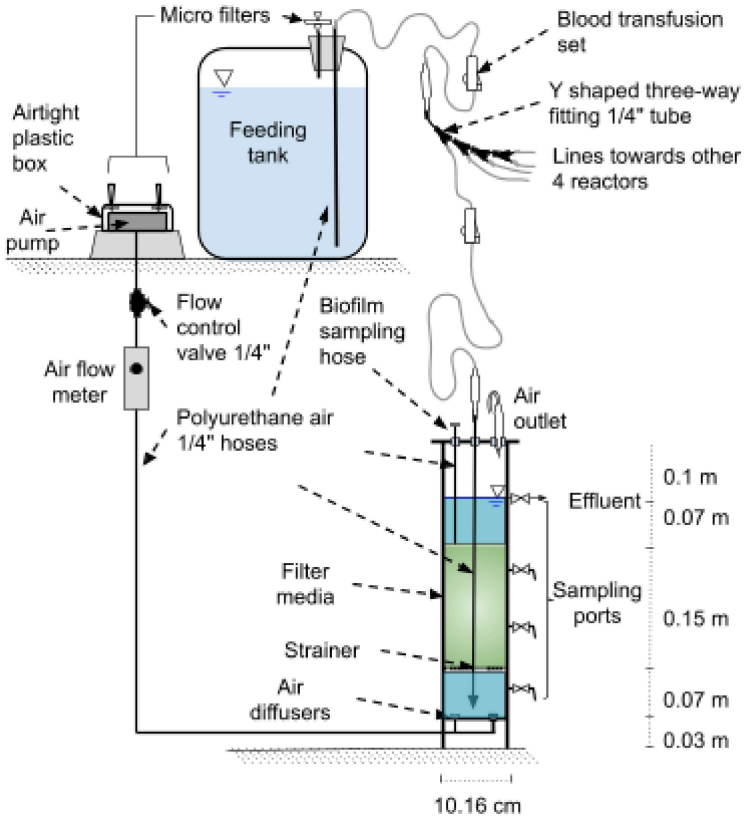


Figure 1. BAF reactors configuration used during the experiments

1.2.2. *Soil selection and conditions*. For the experimental systems, 5 kg of soil was collected (0.2 m depth) from a tropical dry forest with more than 30 years in a naturally successional process, located next to the Experimental Station at the campus of Universidad del Valle (3°22’23’’N,

76°31’51”W; Cali, Colombia). The soil type on the site is hydrogenous clastic surface deposits with the texture of clay loam. The soil was dried by air and sieved (<2 mm) to remove root fragments and debris. The soil samples were analyzed for total phosphorus (TP) and total organic carbon (TOC), using colorimetric methods. Total Kjeldahl Nitrogen (TKN) concentration was determined by Kjeldahl digestion of an unfiltered sample according to the APHA method (APHA 1998). The pH was measured in distilled water at a 1:4 soil-to-water volume ratio with a glass electrode. All the analyses were carried out in triplicates. The soil had a pH of 6.09, total organic carbon of 29.71 g/kg soil, TKN of 3.045 g/kg soil, and TP of 55.73 mg/kg soil.

1.2.3. *Inoculum acclimation*. 1 gr of pulverized soil was diluted in 2 L of sterilized RBA solution (DSMZ - Medium 441). It is an ammonium-free solution that has successfully been used for the isolation, growth, and purity check of a broad spectrum of nitrogen-fixing bacteria. In the darkness, it was placed on a stirring plate at 100 rpm. The bottle cap was connected to a 0.2 mm microfilter to avoid airborne microbial contamination. Glucose (8 g) was used as the only Carbon source. After two days in the complete stirring process, 0.5 L nutrient solution was switched to sterilized Hoagland N-free solution, 80% glucose - 20% citrate (w/w = 6.4 gr glucose/1.7 gr citrate), according to the molecular weight of each compound. Every two days, 0.5 L of Hoagland solution was switched (20%) until reaching 100% (8.5 gr) citrate as the only Carbon source. In this solution, nitrates in the form of potassium nitrate (KNO_3) and calcium nitrate $Ca(NO_3)_2$ were removed and replaced by potassium chloride (KCl) and calcium chloride (CaCl2), respectively. The concentration of nutrients (g/L) were 1.6 $C_6H_8O_7$ (citrate as the carbon source), 7.5 KCl, 5.55 $CaCl_2$, 2.0 KH_2PO_4, 2.0 $MgSO_4$, 1.5 $FeSO_4$, and 1 mL trace element solution SL-6 (DSMZ medium 27). It was expected to have a Chemical Oxygen Demand (COD) concentration around 1200 mg/L.

Citrate was applied (pH 5,5) since it is one of the principal root exudates of some non-legume plants (Kravchenko et al., 2003, Kamilova et al., 2006). For the positive control (C+) reactor, 2 mL of a pure culture of *Azotobacter vinelandii* (DSM 2290, a well-known N-fixer bacteria), obtained from the DSMZ (Braunschweig, Germany) was reactivated in a bottle with 2.0 L of sterilized RBA solution (DSMZ medium 441). During ten days, the solution was switched as was done for the experimental reactors. Subsequently, 120 mL soil inoculum was applied to the

experimental reactors; same volume of pure culture of *A vinelandii* for the C+ reactor, respectively. The reactors were fed by drip gravity with the N-free sterilized Hoagland solution, modified to favor N-fixation, and operated in sequencing-batch mode with a Hydraulic Retention Time of 7 days. No inoculum was applied to the C- reactor. Finally, in each reactor, the air flow was microfiltered to avoid microbial contamination, an air pump maintained at a rate of 4 mL/min was used, at room temperature (22 ± 2 °C). Finally, in each reactor, the airflow was microfiltered to avoid microbial contamination, and an air pump maintained at a rate of 4 mL/min was used at room temperature (22 ± 2° C).

1.2.4. *Samples Collection and Processing*. 850 mL samples were taken from each reactors' effluent every two weeks, centrifuged for 60 min at 9000x g, and the pellets were stored in ethanol (70%) to further processing for DNA. Other unfiltered 50 mL were used for TKN, ammonium (NH_4^+), nitrites (NO_2^-), nitrates (NO_3^-), and Chemical Oxygen Demand (COD) analysis, respectively. Physicochemical analyses were done according to Standard Methods (APHA et al. 2005). Wet microbial biomass was obtained by centrifuging 100 mL sample at 13000g for 20 min (Thermo Scientific Heraeus Multifude X1, Germany). The empty centrifugation tubes and tubes with pellets after centrifugation were weighted. The microbial wet weight was calculated as the weight difference between the tube with pellet and the empty one. Since the samples obtained for wet microbial biomass were not enough to measure the total biomass through time, the biomass estimation was also complemented by Optical Density (OD) at 600 nm using a Vision Spectrophotometer SP-2001SD manufactured by Hoefer, Inc.

1.2.5. *DNA extraction*. 250 mg of inoculating soil samples and 0.8 mL aliquots from reactors were processed using a NucleoSpin® soil genomic DNA extraction kit (Machery-Nagel, Germany) following its standard protocols. DNA yield and purity were measured using a micro-volume fluorospectrometer (Thermo Scientific, NanoDrop Technologies, USA). DNA samples were frozen at −20 °C until shipment to MrDNA laboratories (MrDNA, Shallowater, TX, USA). The raw sequencing data of the 16S rRNA gene amplicon (hypervariable region V4) Illumina sequencing were processed using Qiime2, version 2019.1 (Hall and Beiko, 2018). Denoising, quality filtering, and chimera checking ("consensus") were performed by the 'dada2' plugin (Callahan et al., 2016), and taxonomy was assigned against the SILVA database (132 released; 2018).

1.2.6. *Statistical analyses*. All statistical tests were performed using the RStudio v.4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). All tests were two-sided with a significance level set at $p < 0.05$ and included Kolmogorov – Smirnov & Lilliefors test for homogeneity of variance and normality. Also, Kruskal–Wallis for analysis of variance (ANOVA).

2.7. *Diversity Analysis*. We calculated three aspects of the alpha diversity (richness, evenness, and dominance) for each sample at the experimental and C+ reactors. Specifically, we analyzed observed species, Shannon's index, Simpson's index, and Chao1's index using the phyloseq R package. Principal component analysis (PCA) was conducted using Rstudio (version 4.0.2) to examine the effect of treatments on nitrogen fixation and microbial community composition.

1.3. Results

1.3.1. *Reactors startup and performance*. The start process for the three types of reactors was notably different. The negative control reactor (C-) did not show any OD or biomass increase during the entire operation, while in the C+ reactor, the increment rates in OD and biomass had many ups and downs (Fig. 2). A similar trend to C+ were shown by the soil community (SC) reactors (R2, R3 and R5, respectively). However, the time to reach steady-state (Fig. 2) took different periods, at least 80 (SC) and 90 (C +), respectively; SC reactors achieved the highest OD and biomass (Tables 1 – 4 in Support material). The final OD results were 0.84 (± 0.085) and 0.63 (t = 197 d). Meanwhile, the biomass increased from the undetectable at the beginning to 1609,7 (± 478.4) and 865 mg/L for SC and C+ reactors, respectively. The results indicate both *Azotobacter* pure culture and soil community consumed the Hogland (N-free) solution, enriched with citric acid as the sole source of carbon for growth. Therefore, in micro-aerated bioreactors, a soil microbial community can use this solution to produce more biomass than a pure culture.

The COD results were in agreement with the OD results. During the first few weeks of operation, the COD removal efficiencies were very low, and its increase was with ups and downs. It is assumed that the C- reactor did not have citrate consumption since the respective C- points (Figure 3) over the zero line are related to the equipment error range, very close to 10%. The SC reactors reached the highest COD removal efficiency (Table 1) compared to the Control (+ and -) reactors (Statistical analysis in Tables 5 – 6, Support material). The nitrogen fixation process, represented by the sum of TKN, NH_4, NO_2, and NO_3, showed higher values in replicates reactors

(Fig 4; Tables 7 – 8, in Support material). In our results, NH_4, NO_2, and NO_3 concentrations were negligible. The nitrogen fixed corresponded almost totally to TKN, uptaken into the microbial biomass.

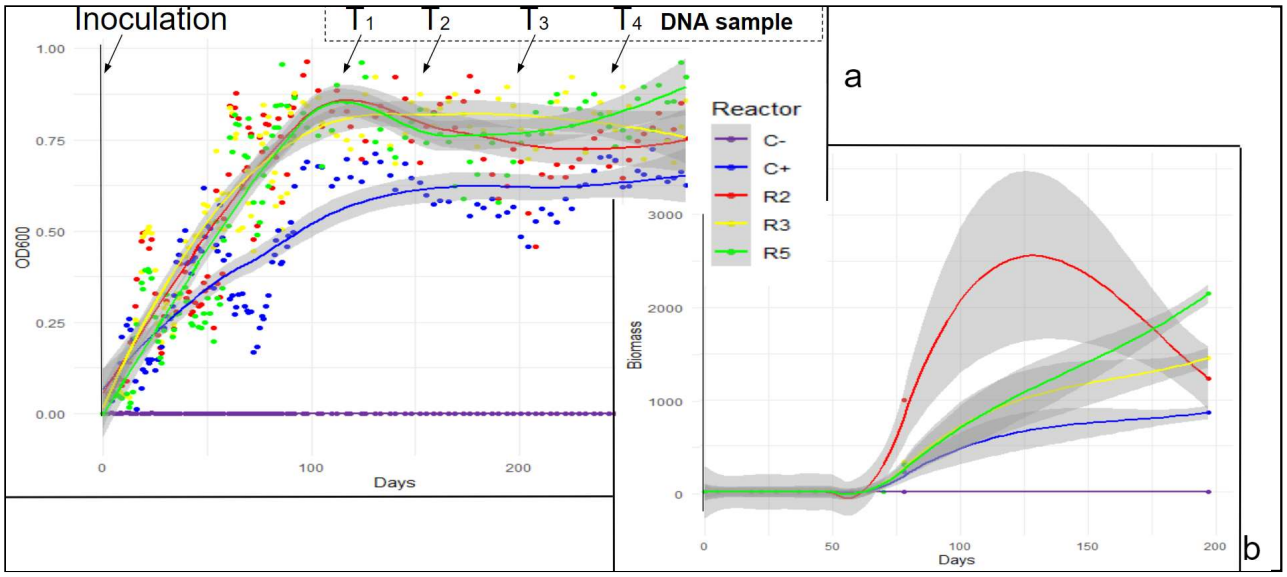


Figure 2. Comparing bioreactors operation: Optical Density (OD_{600}) Biomass (mg/L). R2, R3 and R5 reactors for replicates of soil diazotrophic community. Negative and positive control (C-, C+) reactors, respectively.

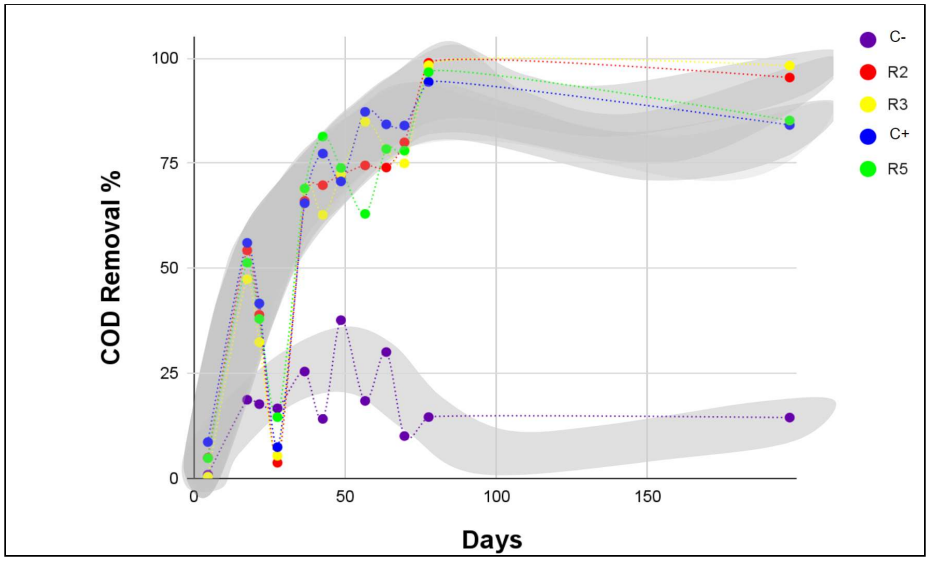


Figure 3. Comparing bioreactors operation. Chemical Oxygen Demand removal efficiency (COD removal %). R2, R3 and R5 reactors for replicates of soil diazotrophic community. Negative and positive control (C-, C+) reactors, respectively.

1.3.2. *General structure and composition of the bacterial community*. We obtained 1510 OTUs assigned to 12 bacterial phyla, 25 classes, 43 orders 88 families, 210 genera and 373 species. The prevalent phyla (99.9% of all sequences) were Proteobacteria (50.9%), Firmicutes (26.02%), Cyanobacteria (12.52%), Bacteroidetes (5.82%), Verrucomicrobia (3.84%) and others:

Actinobacteria, Acidobacteria, Chlamydiae, Chloroflexi, Fusobacteria, Planctomycetes and Spirochaetes (1.48%). Phyla abundance in samples changed from the soil to the acclimation until definitive settlement in the reactor (samples T1 to T4).

Table 1. Summary of average physical and chemical parameters.

Average values +/- Standard Deviation

		Start [a]	Steady-state [b]
OD600 (nm)	C-	0.00	0.00
	C+	0.28 +/- 0.15	0.59 +/- 0.09
	R2	0.39 +/- 0.24	0.76 +/- 0.1
	R3	0.41 +/- 0.23	0.78 +/- 0.09
	R5	0.28 +/- 0.15	0.59 +/- 0.09
Biomass (mg/L)	C-	0.00	0.00
	C+	0.09 +/- 0.04	541 +/- 457
	R2	0.1 +/- 0.04	1114 +/- 164
	R3	0.1 +/- 0.05	888 +/- 797
	R5	0.8 +/- 0.04	1223 +/- 1305
COD removal (%)	C-	18.57 +/- 9.84	13.01 +/- 2.06
	C+	57.92 +/- 30.97	95.87 +/- 0.64
	R2	56.92 +/- 32.01	97.98 +/- 0.36
	R3	61.56 +/- 30.43	84.01 +/- 0.16
	R5	59 +/- 29	88.28 +/- 4.38
TKN (mg/L)	C-	0.95 +/- 1.94	0.83
	C+	0.14 +/- 0.13	13.75
	R2	0.16 +/- 0.16	15.00
	R3	0.07 +/- 0.10	6.87
	R5	0.88 +/- 1.67	23.96
N-NH4 (mg/L)	C-	1.14 +/- 1.87	0.2
	C+	0.53 +/- 2.16	0.08
	R2	1.55 +/- 2.35	0.1
	R3	1.67 +/- 2.48	0.07
	R5	1.97 +/- 2.56	0.08
N-NO3 (mg/L)	C-	0.06 +/- 0.11	0.01
	C+	0.06 +/- 0.1	0.43
	R2	0.07 +/- 0.1	0.14
	R3	0.07 +/- 0.1	0.09
	R5	0.08 +/- 0.11	0.84
N-NO2 (mg/L)	C-	0.02 +/- 0.01	0.09
	C+	0.02 +/- 0.01	0.11
	R2	0.02 +/- 0.01	0.12
	R3	0.02 +/- 0.02	0.14
	R5	0.02 +/- 0.01	0.14
N Fixed = TKN + NH_4 + NO_2 + NO_3	C-	0.02 +/- 0.01	1.13
	C+	0.02 +/- 0.01	14.37
	R2	0.02 +/- 0.01	15.34
	R3	0.02 +/- 0.02	7.16
	R5	0.02 +/- 0.01	25.02

[a] Start is referring to the process of reactors inoculation until they reach the steady-state after 80 days of operation.

[b] Steady-state is referring to the process in which there are no significant changes in the values (less than 5%).

In the soil sample, Firmicutes was the most abundant phyla (90.87%), followed by Proteobacteria (9,08), the remaining accounted for less than 1% of the community (Fig. 6a). The soil community changed its composition, during the acclimation process, to Cyanobacteria (50.70%), Proteobacteria (45.98%), and other phyla for 3.39%.

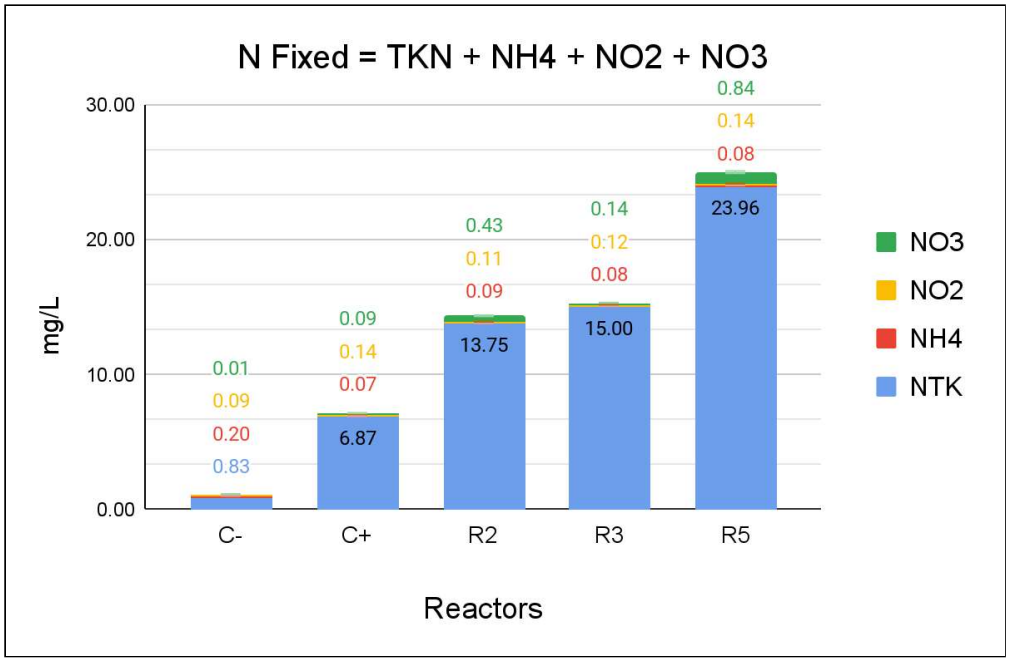


Figure 4. Comparison of nitrogen forms in the last sampling in the bioreactors. Average diazotrophic reactors (R2, R3 and R5), negative control (C-), and positive control (C +), respectively. C- values over the zero line are related to the equipment error range.

Finally, the samples obtained during the rest of the operation (steady-state from T1 to T4) showed that the most abundant phylum was the Proteobacteria, followed by Bacteroidetes and Verrucomicrobia (Fig. 5a). Furthermore, only Proteobacteria was significantly present in all the samples. In the soil, the most abundant families (Fig. 5b) were Clostridiaceae, Sporolactobacillaceae (Firmicutes: 70.57 and 17.02%, respectively), Enterobacteriaceae, Pseudomonadaceae (Proteobacteria: 5.73 and 2.52%, respectively). In the inoculum applied to the reactors, the most abundant families were Cyanothecaceae (Cyanobacteria, 50.5%), Rhodocyclaceae, Rhodospirillaceae, Xanthomonadaceae (Proteobacteria: 22.17, 5.87 and 4.1%, respectively). Some Cyanothecaceae have received attention for their distinctive advantages of hydrogen fuel production (Zhang et al., 2015a; Zhang et al., 2015b). During steady-state (T1 to T4), the most abundant families were the Proteobacteria Sphingomonadaceae (33.9, 36.7, 21.9 and 19%, respectively), Xanthobacteraceae (4.80, 5.76, 18.42 and 7,69%, respectively),

Rhodospirillales *incertae sedis* (4.18, 4.30, 14.82 and 2.59%, respectively). Belonging to Bacteroidetes, the most abundant was Sphingobacteriaceae (7.99, 5.85, 3.92, and 4.12%, respectively). The most abundant Verrucomicrobia was Verrucomicrobiaceae (1.73, 10.74, 9.01, and 15.04, respectively).

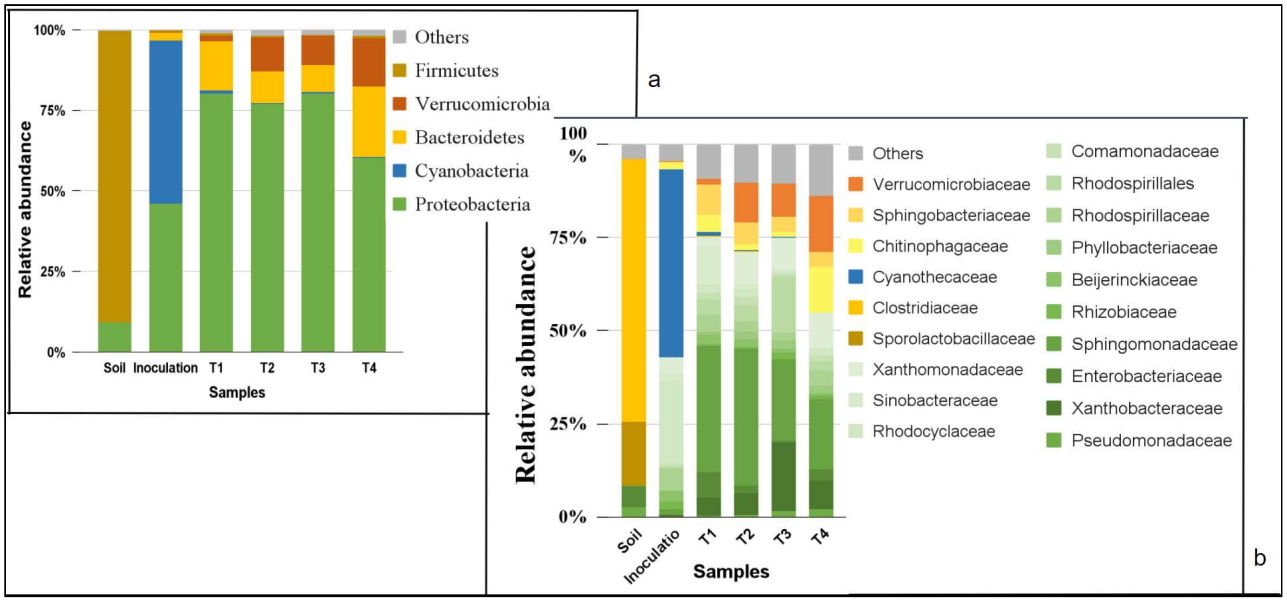


Figure 5. Relative abundance of bacteria in the samples: a) Phylum, b) Family.

Using 20 families with the highest relative abundance, which sum more than 86% in each sample, we plotted Principal Component Analysis (PCA). This analysis confirms that the microbial community composition significantly changes (Figure 6) from the soil to the acclimatization reactor and finally to a cluster formed by the samples (T1 - T4) in the experimental reactors. The acclimatization sample corresponds to the community inoculated to the experimental reactors. These changes were related to the substrate type and growth pattern, that is, attached to the media or suspended in the water column: solid for soil community and exclusively free in liquid in acclimatization, however from T1 to T4, the community corresponds to biofilm attached to the filter media. On the other hand, each substrate had a significant dominant family: Clostridiaceae, Cyanothecaceae, and Sphingomonadaceae (soil, acclimatization, and experimental reactors, respectively). The remaining families had a low relative abundance and, according to the PCA quadrants (Figure 6), some of them did not have records on some substrate.

1.3.3. *Bacterial Diversity*. The number of microbial species grew from the Soil (83) to the T2 sample (276), reduced in T3 (199), finally increasing to 246 in T4 (Figure 7). In the same way,

the diversity of Shannon showed a low level of diversity in the Soil sample (0.63) but increased during the acclimatization process (0.88). Through the steady-state, it varied from 1.48 (T1), 1.43 (T2), 1.41 (T3), and 1.52 (T4), respectively (Figure 8). For its part, the Simpson index showed a high dominance in the Soil sample (0.44), which decreased during the acclimation process (0.30) and reached even lower levels during the stationary state from T1 to T4 (0.07, 0.1, 0.09, and 0.06, respectively). Finally, the Chao1 index showed few species but dominant in the soil sample (low index: 123.33) and many species with equal or low abundance in the Acclimation and T1 to T4 (218.78, 266.02, 310.72, 224.92, and 300.86, respectively).

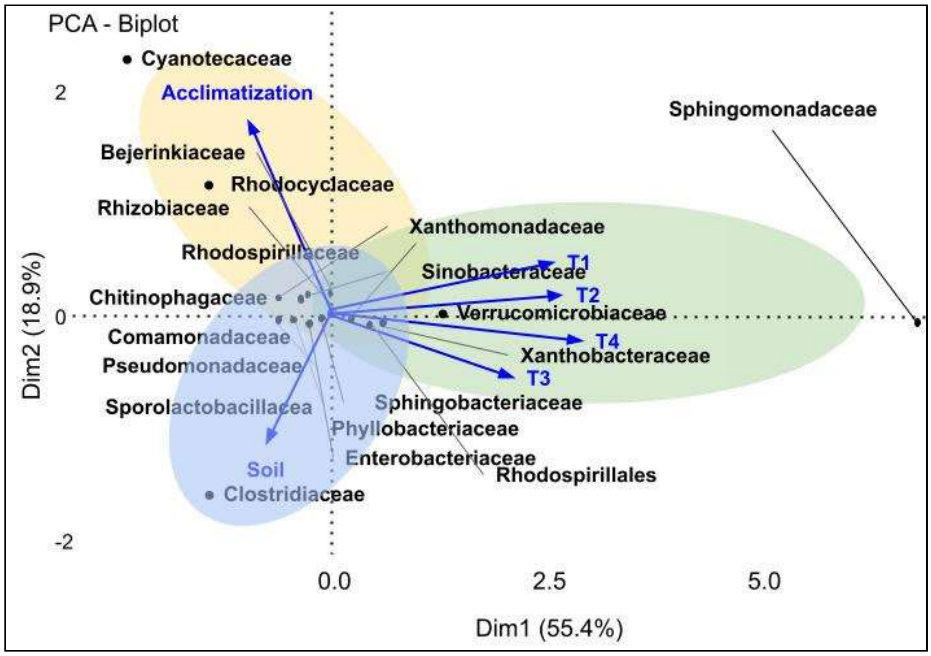


Figure 6. Principal component analysis (PCA) of the samples

1.3.3. *Bacterial Diversity*. The number of microbial species grew from the Soil (83) to the T2 sample (276), reduced in T3 (199), finally increasing to 246 in T4 (Figure 7). In the same way, the diversity of Shannon showed a low level of diversity in the Soil sample (0.63) but increased during the acclimatization process (0.88). Through the steady-state, it varied from 1.48 (T1), 1.43 (T2), 1.41 (T3), and 1.52 (T4), respectively (Figure 8). For its part, the Simpson index showed a high dominance in the Soil sample (0.44), which decreased during the acclimation process (0.30) and reached even lower levels during the stationary state from T1 to T4 (0.07, 0.1, 0.09, and 0.06, respectively). Finally, the Chao1 index showed few species but dominant in the soil sample (low index: 123.33) and many species with equal or low abundance in the Acclimation and T1 to T4 (218.78, 266.02, 310.72, 224.92, and 300.86, respectively).

1.4. Discussion

1.4.1. *Reactors startup and performance*. In biological nutrient removal for treatment of low strength organic wastewaters, Mielcarek et al. (2015) achieved a significant use of citrate as the sole carbon source after 2.5 months of operations when they obtained the steady-state condition. Similar to the time used by our study, but with a media exclusively nitrogen-free. Therefore, nitrogen-fixers must have acquired it from the air. Regarding the nitrogen fixation process with higher values in SC reactors, this achievement is similar to recent results (Welz et al., 2018; Holtman et al., 2018; Tikariha & Purohit, 2019; Ospina-Betancourth et al. al., 2020). They obtained, by systems under nitrogen limiting conditions and a high C: N ratio, appreciable amounts of nitrogen in reactors showing that microbes in mixed cultures or communities can fix it from the air. According to the composition of nitrogen forms in the reactors, Welz et al. (2018) reported nitrate was the principal contributor to total nitrogen in sand bioreactors. However, diazotrophs also convert almost all N-fixed to biomass (Liu et al., 2017). Finally, biofilms grown with packed inorganic nitrogen (N-repleted) or without exogenous supply (N-deficient) showed that N-rep consisted of more denitrifying and nitrogen-consuming microbes, while N-def of diazotrophs that increased nitrogen in the substrate (Li et al., 2017).

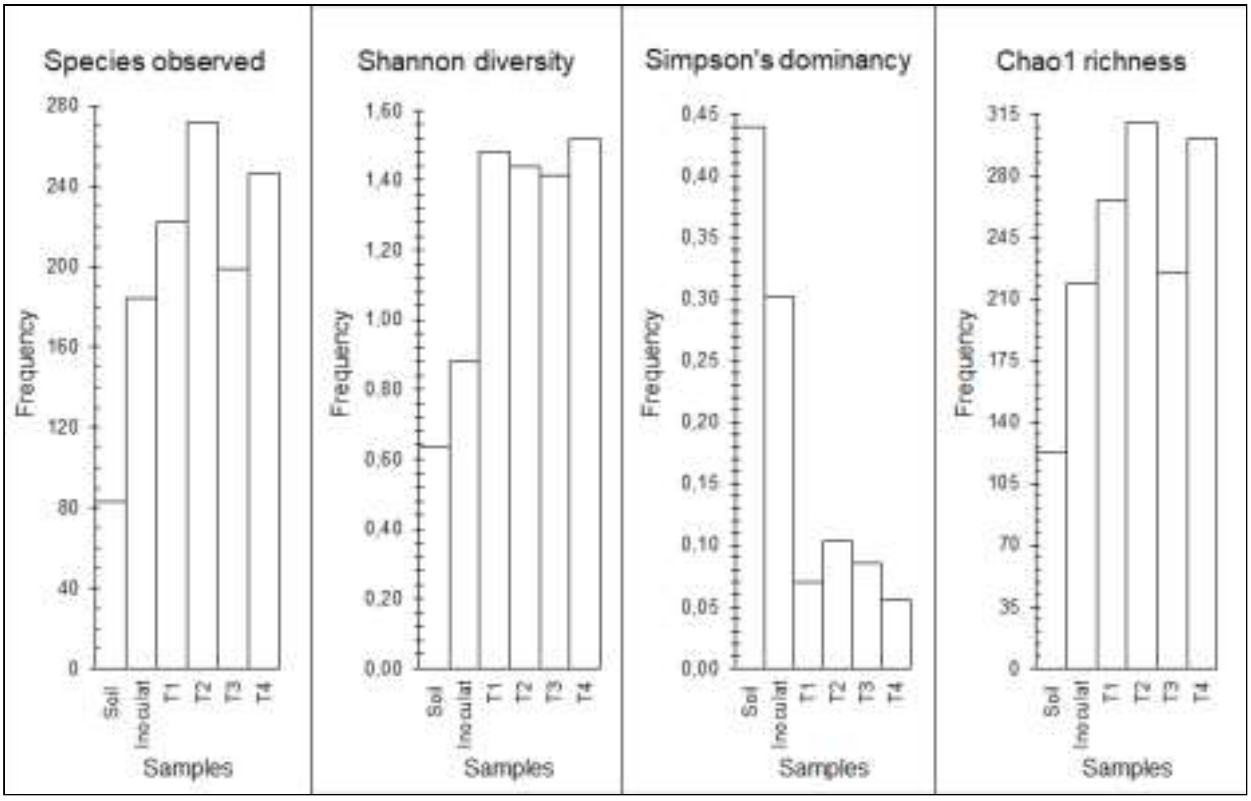


Figure 7. Patterns of species richness (observed), diversity (Shannon's index), dominancia (Simpson's index), and dominance (relative) of bacterial communities.

Since in our experiment the difference between the two types of reactors were the inocula source (pure culture of *Azotobacter* in C+, soil microbial community in SC, respectively), the higher biomass/OD600 and N-fixed/COD removal efficiencies values in SC reactors was likely due to the community complex. In biofilm communities, growth rates exceed those predicted by the Monod model for a single population (Sinsabaugh et al., 2012). Hassan et al. (2012), Kallistova et al. (2014), and Hoelzle et al. (2014) found bacteria in mixed cultures are more efficient than in pure culture. Systems with highly diversified microorganisms in organic materials treatment provide a more stable process, even under fluctuating environmental conditions.

1.4.2. *General structure and composition of the bacterial community*. Firmicutes and Proteobacteria belong to bacteria preferring nutrient-rich (copiotrophic) environments. However, Firmicutes is known to be involved in the degradation in soils of complex organic compounds like manure (Francioli et al., 2016; Hartmann et al., 2015). Firmicutes also have a high predominance in soils after biochar amendment (Liao et al., 2019). For their part, Proteobacteria prefer environments rich in labile - easily transformed - carbon (Bu et al., 2018). These bacterial proportions result from previous land use and climate influence differences in soil organic carbon following reforestation of former agricultural land (England et al., 2016). Plant litter composition selects different soil microbial structures and, in turn, drives different litter decomposition patterns and soil carbon sequestration capability (Yan et al., 2018).

Relevant to the microbial community composition change after placing the soil sample in the acclimatization reactor, regardless of ecosystem type of soil, a large bacteria fraction (69–74%) responding to rewetting are below detection limits in the dry soils (Aanderud et al., 2015). Some of them become dominant members of the rewetted communities, and it explains why, in our experiment, Cyanobacteria became the taxon prevalent at the end of the microbial acclimatization process. Cyanobacteria are a significant component of terrestrial and aquatic ecosystem structure and function. These CO_2 and N_2 fixing bacteria can survive in extreme environmental conditions, tolerate high temperatures, desiccation, extreme pH, and salinity (Singh et al., 2016; Williams et al., 2014).

In relation to the last community composition change occurred when samples were placed into

the experimental reactors coming from the acclimatization reactor, the Phylum Proteobacteria have been found fixing nitrogen in bioreactors treating wastewater with a high carbon to nitrogen ratio (Reid et al., 2008; Bowers et al., 2008; Conthe et al., 2018; Welz et al., 2018). In these reactors, Bacteroidetes are less abundant but significant accompanying bacterial microbioma. Meng et al. (2018) evaluated variations in the diazotrophic community of rhizospheric soil. They demonstrated, under long-term monoculture rotations, the phylum Proteobacteria increased while Verrucomicrobia decreased, which is related to minerals (N, P, K) availability in the soil. This evidence suggests that, in our experimental reactors, the phyla abundances depended on the mineral nutrients ratio and type of C compound.

Concerning the abundance of families during the different experimental stages until the reactors reached the steady-state, we found that community composition and prevalent families changed significantly in each one, reflecting the differences in the substrate state (dry-soil, liquid, biofilm), and mineral and carbon compounds. In the soil context, Pepe-Ranney et al. (2016) reported Clostridiaceae (Firmicutes) is an abundant diazotroph in early successional soil crusts. Ding et al. (2017) have reported a large proportion of Clostridiaceae are capable of N fixation in non-fertilized soil. On the other hand, (Macias-Benitez et al., 2020) found that lactic acid present in some rhizospheres supports the proliferation and the Clostridiaceae dominance. In turn, some family Sporolactobacillidae (Firmicutes) members are part of the endophytic diazotrophs strains from some forest soils (Detmont et al., 2015; Puri et al., 2018).

It is common to find the Enterobacteriaceae (Proteobacteria) members in forest soils, related to plant litter decomposition worldwide (Orellana et al., 2017), and playing a role in the catabolism of low-molecular-weight, partially-degraded forms of lignin (DeAngelis et al., 2011; Díaz-García et al., 2020). Enterobacteriaceae are considered as promising nitrogen-fixing organisms to enhance the fertility of the soil, however, checking first their pathogenicity is required before to be used as a potential biofertilizer for sustainable agricultural practice (Kumar et al., 2017; Chakraborty et al., 2019). Finally, Pseudomonadaceae (Proteobacteria) often have similar ecological functions as Enterobacteriaceae in forest soils, but the dominant driving factors for these two families are C/N and N/P ratios, where the soil N/P ratio affects Enterobacteriaceae abundance, while the C/N for Pseudomonadaceae (Xun et al., 2018).

During the acclimatization stage, reactor conditions favored the formation of a bacterioplankton

community instead of biofilm (Vrede et al., 2002; Sinsabaugh et al., 2010). This was mainly due to the absence of attachment material on which the community could grow. Regarding changes in the prevalence of families presented after soil sample application to the acclimatization reactor, Cyanothecaceae (Cyanobacteria) have received attention for their distinctive advantages in hydrogen fuel production in reactors (Zhang et al., 2015a; Zhang et al., 2015b). In fact, some Cyanothecaceae strains use the nitrogen-fixing metabolic pathway to produce hydrogen and fix nitrogen in darkness or at night when both the oxygen and nitrate levels are low (Toepel et al., 2009; Aryal et al., 2011). Rhodospirillaceae (Proteobacteria) grow photoheterotrophically, chemotrophically. The above depending on anoxic and light conditions or chemoheterotrophically under air effect (Baldani et al., 2014). This metabolic versatility has allowed them to be also used to produce hydrogen fuel (Kim et al., 2008a; Kim et al., 2008b; Wang et al., 2017). The Rhodocyclaceae (Proteobacteria) family can fix nitrogen or denitrify (Smalley et al., 2015), depending on the substrate nitrogen concentration in reactors (Conthe et al., 2018). Its relative abundance over other groups relies on aerobic conditions (Liu et al., 2017), phosphorus concentration, a shorter retention time of sludge (Yang et al., 2018), and light concentration (Gebicki et al., 2010). The Xanthomonadaceae (Proteobacteria) family is also predominant in aerated nitrate-reducing environments (Sarkar et al., 2016; 2020). Nitrogen fixer wastes were the only possible source of nitrates in the acclimatization reactor, and it was significantly low, hence the possible low Xanthomonadaceae concentration level in that reactor.

Finally, the last community composition change occurred when samples were placed into the experimental reactors coming from the acclimatization reactor and favored the biofilm formation. Many representatives of the Sphingomonadaceae are aerobic heterotrophs that exhibit an extreme ability to use organic substrates ranging from acetate to aromatic hydrocarbons (Ebrahimi et al., 2010; Szabó et al., 2017; Oh & Choi, 2019) in air granular sludge reactors. In biofilms under an N-deficient substrate, significant amounts of Sphingomonadaceae fix nitrogen (Li et al., 2017). Some Xanthobacteraceae are aerobic, chemolithoautotrophic, and nitrogen-fixers able to use aromatic hydrocarbons as substrate (Wu et al., 2018). Xanthobacteraceae are significant in the NH_3 synthesis from N_2 and H_2O at ambient conditions in a single reactor by coupling hydrogen generation from catalytic water splitting to an H_2-oxidizing bacterium, which performs N_2 and CO_2 reduction to solid biomass (Liu et al., 2017; Hu et al., 2020). Yuan et al. (2021) found that, in some reactor conditions, *Xanthobacter* is intolerant to NH_4^+ and is more related to fixing nitrogen

in micro-oxygenic environments than in the N removal function.

Reyranella, a genus belonging to Rhodospirillales *incertae sedis* and significantly presented in most of our samples, has been collected from fresh (Pagnier et al., 2011; Cui et al., 2017) and ground eutrophic waters (Kausch et al., 2020) and soils (Kim et al., 2013; Lee et al., 2017). Furthermore, *Reyranella* is a keystone taxon observed under manure-mowing, with ecological functions mainly involved in organic carbon degradation and nitrogen fixation in soils (Xue et al., 2020). Sphingobacteriaceae (genus *Sphingobacterium*) is a dominant member in the nitrate-reducing biocathode of a microbial fuel cell fed with mixed liquor from a domestic activated sludge plant (Rabaey et al., 2008). Similarly, the microbial community structure of a freshwater system receiving tertiary wastewater treatment effluent showed that both planktonic and benthic communities near the effluent had an increased abundance of Sphingobacteriaceae (Hladilek et al., 2016). However, *Sphingobacterium* is also abundant in some higher C/N ratio environments promoting nitrogen fixation, such as chicken manure and corn stover mix composting (Zhang et al., 2020). Also in the rhizosphere of some legume plants where they have an operative nifH (N-fixing check) gene (Ahmed et al., 2014). Some freshwater representatives of phylum Verrucomicrobia (Spartobacteria and Opitutae classes with aerobic and heterotrophic metabolism) show at the genomic, taxonomic, and ecological levels are capable of nitrogen fixation (Cabello-Yeves et al., 2017) and carbohydrates degradation (Martinez-Garcia et al., 2012; Herlemann et al., 2013). In fact, the Verrucomicrobia, in contrast to the Proteobacteria, is one of the more abundant taxa in some natural wetlands compared to those constructed to treat municipal wastewater, where the organic matter and nutrient contents are significantly higher (Ansola et al., 2014; Li et al., 2021).

As expected, in the absence of significant amounts of NH_4, NO_2, and NO_3in samples, there were no detected ammonium-oxidizing bacteria (AOB) or nitrite-oxidizing bacteria (NOB) as they were in other studies (Ma et al., 2016; Sepehri et al., 2018). Consequently, although the composition of the community, therefore the prevalent families in the acclimatization and experimentation stages of the reactors were significantly different, as is confirmed by the PCA plot, all of them had both nitrogen-consuming and fixing gen capacities. Given the nutrient condition of the reactors with the presence of nitrogen coming exclusively from the atmosphere, then favored fixation instead of consumption. In biofilm communities, the assimilation of a limiting nutrient is mediated by populations having the highest affinity for the substrate. But once

acquired, it can be internally recycled, and nutrient concentrations can be orders of magnitude greater than the environmental concentration (Sinsabaugh et al., 2012).

1.4.3. Bacterial Diversity. In all cases, the support substrate type significantly affected the alpha-diversity (Shannon and Simpson indices) of the bacterial community, revealing a complexity gradient. The soil at the lowest, and the biofilm at the highest. Biofilm communities have a more complex physical structure and trophic organization than others, despite having similar ecoenzymatic stoichiometry about productivity and biomass composition (Sinsabaugh et al., 2010). The biofilm communities are more biodiverse since they maintain selected microenvironments (Brenner et al., 2008). So they resist challenges such as nutrient limitation, desiccation, antimicrobial agents. Additionally, they offer better chances for interactions such as horizontal gene transfer and cometabolism (Burmølle et al., 2006). However, the review done by Nielsen et al. (2011) found that, at low levels of biodiversity, additional species often improved ecosystem functioning, while at higher-diversity levels, effects of species richness on functioning were less frequent. It means that community composition often has stronger effects compared with species richness.

1.5. Conclusions

The reactors fed with a Hoagland modified solution with N-supplied only by air and citrate as the carbon source allowed the growth of a complex nitrogen-fixing microbial community.

The process to reach the steady-state lasted around 80 days.

NH_4, NO_2, and NO_3 concentrations were negligible, and TKN corresponded to that uptaken by microbial biomass.

The different communities' compositions revealed the effect of nutrient and substrate state (soil, liquid, biofilm).

As expected, the soil community composition reflected its forest origins.

The acclimatization process selected a free-swimmer community dominated by cyanobacteria.

The community in nitrogen-fixing reactors was different from those related to nitrogen-deficient wastewater treatment processes, although the nutrient conditions were similar.

The microbial community in the biofilm from the nitrogen-fixing reactors was more diverse than

those coming from soil or the Acclimatization process.

There were no detected ammonium-oxidizing or nitrite-oxidizing bacteria, and we related this to the absence of significant amounts of ammonium, nitrites, or nitrates dissolved in nutrient media samples.

The next step of research must be to test this approach in non-legume plant rhizospheres to evaluate the plant and microbiome response.

The community enrichment model tested in this experiment involved a bacterial metabolic diversity-specific selection and can be applied for other microbial functions such as phosphorus solubilization, plant growth promotion, or disease suppression. In this way, to foster soil microbial community function restoration for agricultural or ecological proposes

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Support material

Coefficients for transformed OD_{600} response

Term	Coef	SE coef.	T	p	VIF
Constant	-2,332	0,117	-19,99	0,000	
Days	0,01683	0,00197	8,54	0,000	2,00
Treatment R	-0,099	0,165	-0,60	0,548	3,42
Days * Treatment SC reactor	0,00560	0,00279	2,01	0,047	4,42

Table 1. Anova for OD600 response

Equation of regression Treatments	
OD C+	ln(Response) = -2,332 + 0,01683 Days
OD SC reactor	ln(Response) = -2,431 + 0,02243 Days

Table 2. Equation of regression for OD_{600} response

Coefficients for transformed biomass (mg/L) response

Term	Coef	SE coef.	T	p	VIF
Constant	-1,716	0,112	-15,30	0,000	
Days_1	0,00767	0,00159	4,81	0,000	2,00
Treatment					
SC reactor	0,008	0,159	0,05	0,960	2,14
Days_1*Treatment					
SC reactor	0,00012	0,00225	0,05	0,958	3,14

Table 3. Anova for biomass response

Equation of regression Treatments	
C+	-Response^-0,145643 = -1,716 + 0,00767 Days_1
SC reactor	-Response^-0,145643 = -1,708 + 0,00779 Days_1

Table 4. Equation of regression for biomass response

Coefficients for COD % removal efficiency response

Term	Coef	SE coef.	T	p	VIF
Constant	10,87	3,95	2,75	0,010	
Days_1	0,1665	0,0863	1,93	0,063	3,00
Treatments					
C+	6,46	5,59	1,16	0,257	4,76
SC reactor	2,02	5,59	0,36	0,720	4,76
Days_1*Treatments					
C+	0,868	0,122	7,11	0,000	5,43
SC reactor	0,889	0,122	7,29	0,000	5,43

Table 5. Anova for COD % removal efficiency response

Equation of regression Treatments	
C+	Response = 17,32 + 1,0343 Days
SC reactor	Response = 12,89 + 1,0556 Days

Table 6. Equation of regression for COD % removal efficiency response

Coefficients for transformed NT response

Term	Coef	SE coef.	T	p	VIF
Constante	-1,53	1,01	-1,51	0,165	
Days_1	0,0014	0,0117	0,12	0,905	3,00
Treatment					
C+	-2,43	1,43	-1,70	0,123	2,67
SC reactor	-0,33	1,43	-0,23	0,823	2,67
Days_1*Treatment					
C+	0,0234	0,0166	1,41	0,193	3,33
SC reactor	0,0194	0,0166	1,17	0,272	3,33

Table 7. Anova for COD % removal efficiency response

Equation of regression

Treatment	
C+	ln(Response) = -3,96 + 0,0248 Days_1
SC reactor	ln(Response) = -1,85 + 0,0209 Days_1

Table 8. Equation of regression for COD % removal efficiency response

CHAPTER 2. TOMATOES PLANTS PHENOTYPE RESPONSE TO DIAZOTROPHIC SOIL ENRICHMENT AND THEIR RHIZOSPHERE MICROBIAL COMMUNITY STRUCTURE

Abstract

The intensification of agriculture has highly increased food availability over recent decades. However, it has led to considerable adverse environmental impacts and biodiversity losses mainly related to Nitrogen-fertilizers production and consumption in agriculture. The challenge is to feed an increasing world population in an ecology-friendly way. Merging ecological theory with bioprocess engineering has been demonstrated for production in reactors of 'self-assembled communities' enriched for N-fixer functional guild. Here, tomato plants were fertilized with Hoagland modified solution with N-supplied only by reactor microbial biomass at three different concentrations. It allowed plants to grow significantly. Also, it reflected a complex nitrogen-fixing microbial community in the rhizospheres in each case. The N1 and N2 treatments' communities seemed similar to each other. Original soil with the lowest diversity and N3 with the highest was alike in the functional ecology. The next step will be to evaluate colonization level and interaction between the microbiome that comes with the seeds and the one provided by the treatment.

2.1 Introduction

The intensification of agriculture has highly increased food availability over recent decades. However, it has led to considerable adverse environmental impacts, such as increases in eutrophication of land and water bodies, greenhouse gas (GHG) emissions, and biodiversity losses (Fowler et al., 2013; Bender et al., 2016; Muller et al., 2017; Pfromm, 2017; Bowles et al., 2018). Given that by 2050 world population could increase by 9 to 10 billion (Foley et al., 2011; Alexandratos & Bruinsma, 2012; Rockström et al., 2017), some ecological alternatives to the intensive use of crops are open, such as leguminous-based fertilized systems, and organic farming. However, they have some restrictions. There are concerns because not all non-leguminous plants are liable to intercrop with legumes, rotation can take longer than expected, or it is a challenge to a large-scale application (Duchene et al., 2017; Stagnari et al., 2017; Bordonal et al., 2018). In relation to organic agriculture, it uses manure, composts, and plant residues in crop production systems to restore degraded soils and ameliorate physicochemical constraints (Celestina et al., 2019). In addition, neither synthetic fertilizers nor synthetic pesticides are applicable and heavily rely on an active microbial community to break down organic matter into plant-available nutrients (Lori et al., 2017; Martínez-García et al., 2018). However, the use of organic fertilizers has been controversial and restricted because they

have also been associated with increased accumulation of pharmaceuticals and antibiotics in the agricultural fields (Pan and Chu 2017). Likewise, there is not enough animal manure to supply nutrients to a much larger area under organic production (Meemken & Qaim, 2018). As a result, abandoning synthetic N-fertilizers could lead to nutrient undersupply, even with increased legume cropping. Therefore, the ability of organic agriculture to sustainably feed the world is not secured (Muller et al., 2017).

A feasible solution to this challenge is microbiome engineering since organic agriculture depends on an active microbial community. This method allows the manipulation and study of microbial communities (Lawson et al., 2019; Roell et al., 2019; Kerfeld et al., 2020), even in their native context without the need for the domestication of individuals in the laboratory (Sheth et al., 2016), and can promote plant fitness and health (Pascale et al., 2020). Focused on the top-down design process and self-assembled construction methods approached by Lawson et al. (2019), Gutierrez et al. (2020) merged microbiome engineering theory with bioprocess engineering to propose the self-assembled microbial communities, enriched for Nitrogen-fixation (diazotrophs) functional guild and adapted to a target soil and host plant. That proposal uses environmental parameters such as C: N: P ratios, oxygen concentrations, trace elements, primary carbon sources, and secondary metabolites that could be used to steer diazotrophic communities assembly in bioreactors. In the present study, we aimed at testing these self-assembled diazotroph communities approach, explore its production at a laboratory scale, and obtain operational data from this system. Therefore, using the nitrogen fixation guild as an anchor, move forward in the microbiome engineering and production knowledge to provide alternatives that support organic agriculture.

2.2. Materials and methods

2.2.1. *Substrate selection and seed sow*. Soil samples were collected from a naturally restored forest, as it was described previously (Chapter 1). 50 kg of mixed sample was gently air-dried, sieved (2 mm), placed on ten tin trays (5 x 25 x 50 cm), wrapped with kraft foil, autoclaved (120 °C for 2 h), and dried in an oven (50 °C for 4 h). Wild tomato (*Solanum pimpinellifolium*) seeds were purchased from a local market. The seeds were surface sterilized by gently shaking with 70% ethanol (2 min), 2.5% sodium hypochlorite solution (5 min) followed by five rinses in sterile distilled water (SDW). Thereafter seeds were soaked overnight in SDW and germinated in the dark (22 °C) on sterile Petri dishes. After three days, 72 germinated seeds were planted in an

alcohol sterilized tray (6 x 12 cells) previously filled with sterile soil. Once planted, the germination tray was translated to a net house located in the Experimental Station at Universidad del Valle's campus (Chapter 1). Treatments consisted of four fertilizer applications: positive control (C+) for Hoagland modified solution; N1 for N-free Hoagland modified solution with a microbial optical density (OD600) of 0.1; N2, similar to N1 but with an OD of 0.2; and N3 with an OD of 0.3. The negative control (C-) consisted just of sterilized deionized water (SDW). The treatments were randomly applied to the surface on each tray cell. Each plant was then inoculated with a volume of 200 µL, applied three times per week, and the dose was increased another 200 µL every week as plants grew. Given the reactors' volume limitation to prepare biofertilizer, a total of eleven plant replicates were used for each treatment.

2.2.2. *Fertilizer preparation*. The microbial community for OD treatments was obtained from a steady-state nitrogen-fixer reactor effluent having 208 days in operation. A detailed description of the reactor's operating conditions is presented in Chapter 1. Briefly, the reactors received Hoagland (N-free) modified solution (Chapter 1) to favor Nitrogen fixation, in which nitrates in the form of potassium nitrate (KNO_3) and calcium nitrate $Ca(NO_3)_2$ were removed and replaced by potassium chloride (KCl) and calcium chloride ($CaCl_2$), respectively. Nutrient concentrations (g/L) were 7.5 KCl, 5.55 $CaCl_2$, 2.0 KH_2PO_4, 2.0 $MgSO_4$, 1.5 $FeSO_4$, and 1 ml trace element solution SL-6 (DSMZ medium 27). The effluent biomass was collected, centrifuged, and resuspended in the Hoagland modified N-fixer solution to normalized OD_{600} treatments. Throughout the experiment, the pH of each treatment was adjusted to 7.0 ± 0.05 using a pH meter (Orion Star A211) with 0.1 M NaOH or 0.1 M HCl. Soil moisture was maintained by watering with SDW when necessary.

2.2.3. *Tomato plant assessments*. After sowing, once a week, the stem length of each plant was measured. Other phenotypic responses were measured in two different harvests. The first one was in the seventh week. Seven random plants per treatment were carefully uprooted, root length, and width were measured. The remaining randomly selected plants were carefully transferred to a garden pot (15 x 30 cm, diameter x height), filled with 1,5 kg of sterilized soil. The fertilizer dosing scheme continued with 1.2 ml three times per week and increasing 400 µL every week as the plants continued to grow. Stem length continued to be measured weekly. The final harvest was made at week 24, repeating the same process carried out in week 7. DNA extraction, statistical analysis (included a Tukey multiple pairwise-comparisons), and diversity analyzes

were processed as described in Chapter 1.

2.3. Results

2.3.1. *Biofertilizer characteristic*. As described in detail in Chapter 1, at the starting time of the biofertilizer application to tomato plants, the reactors had reached an average Nitrogen-fixed (NTK + NH_4 + NO_2 + NO_3) concentration of 18.24 (± 5.89) mg/l, where only the NTK contained by the microbial biomass had a significant contribution while the others N-forms were negligible. The effluent biomass was 1609.7 (± 478.4) mg/l and had an OD_{600} of 0.67 (± 0.19). During the tomato fertilization process, the reactors kept an average OD of 0.78 (± 0.09) without significant concentration changes until the tomatoes were harvested at 280 days of reactor's operation (Figure 1). Based on the above, we assumed that we applied to rhizospheres N concentrations around 2.39, 4.67, and 7.01 mg/l (treatments N1, N2, and N3, respectively).

2.3.2. *Tomatoes phenotypic response*. The time series analysis of stem length measurement showed that, during the first week of growth, unfertilized plants (Treatment C-) sprouted in the shortest average stem length (0.77 ± 0.21 cm). Likewise, the growth curve equation had the lowest value (Table 1 in Support material). Finally, this treatment had the lowest average length (7.41 ± 1.28 cm) in the seventh week (Figure 2). Concerning plants fertilized with the Hoagland modified and N-supplied solution (C+), at the first week, they showed the second-lowest average stem length (0.79 ± 0.24 cm). Presented the second-highest growth curve equation value and reached the second-highest average stem length with 10.2 ± 2.46 cm (Table 1 in Support material). For their part, the plants fertilized with the N-fixing community at OD 0.1 (N1) treatment showed an average stem length, at the first week, of 0.89 ± 0.30 cm (Figure 2), and these ones reached an average stem length of 9.51 ± 1.49 cm (Table 1 in Support material).

On the other hand, the plants fertilized with the N2 treatment (OD = 0.2) presented the highest average stem length, at the first week, of 1.43 ± 0.57 cm (Figure 2), showed the highest growth curve equation value, and reached the third-highest average stem length with 10.93 ± 2.77 cm (Table 1 in Support material). Finally, the plants fertilized with the N3 treatment (OD = 0.3) presented an average stem length, at the first week, of 1.17 ± 0.6 cm (Figure 2), showed the second-lowest growth curve equation value, and reached the second-lowest average stem length with 9.14 ± 1.67 cm (Table 1 in Support material).

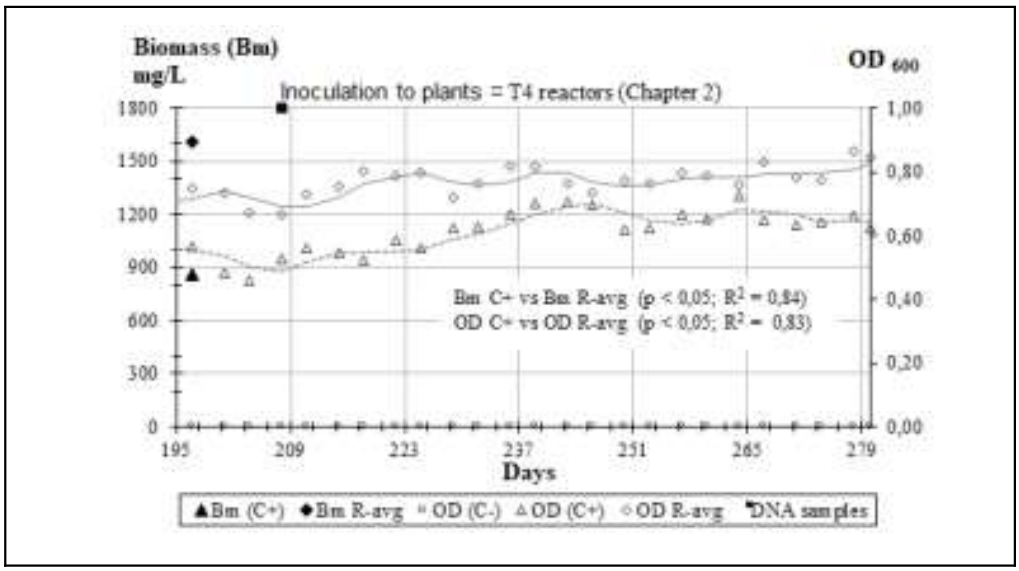


Figure 1. Display of bioreactors operation: Biomass (Bm), Optical Density (OD), Average of soil community (SC), and positive control (C+) reactors, respectively.

At the first harvest, ANOVAs were used to compare significant differences in root length and width, and total length (Figure 3). In all cases, there were differences, summary results in Table 2 (Supporting material). By comparing between stem lengths a difference between treatments N2 and C- was found (Table 3, in Support material). For root length, there were differences between N1:C-, N2:C-, and N2:N3 treatments, respectively (Table 4, in Support material). Regarding total length, there was a difference between N2:C- (Table 5, in Support material). Finally, the root width and volume results (Figure 4a and 4b) showed no differences between N1 and N2 in both cases.

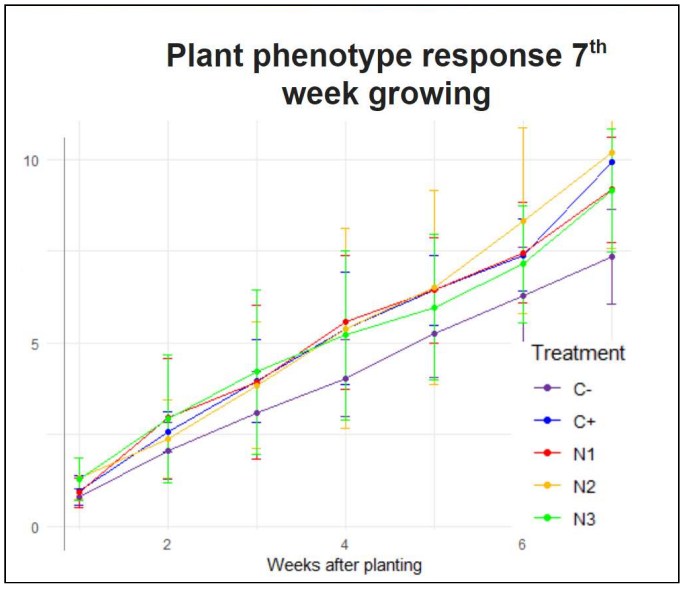


Figure 2. Comparing average plant stem growth before the first harvest: Negative and positive control (C- and C+, respectively); Treatment 1 (N1), Treatment 2 (N2), and Treatment 3 (N3), respectively.

In the second harvest, 24 weeks after planting, we must mention first that C- plants died in the 9th week after planting, then we excluded them from the analysis. C+ plants showed the highest growth curve equation value (Table 8 in Support material) but reached the second-highest average stem length with 167.73 ± 7.71 cm (Figure 5). The N1 plants showed the lowest growth rate (Table 8 in Support material) and reached the stem length with 158.55 ± 16.58 cm (Figure 5). Regarding the N2 plants, they showed the second-highest growth curve equation value (Table 8 in Support material) and reached the highest stem length with 181.55 ± 8.1 cm (Figure 5). Regarding N3 plants, they showed the second-lowest growth curve equation value (Table 8 in Support material) and reached the lowest stem length with 158.55 ± 16.58 cm (Figure 5). At the second harvest, ANOVAs (Figures 6 and 7) show in all cases there were not significant differences (summary results in Table 9, Supporting material).

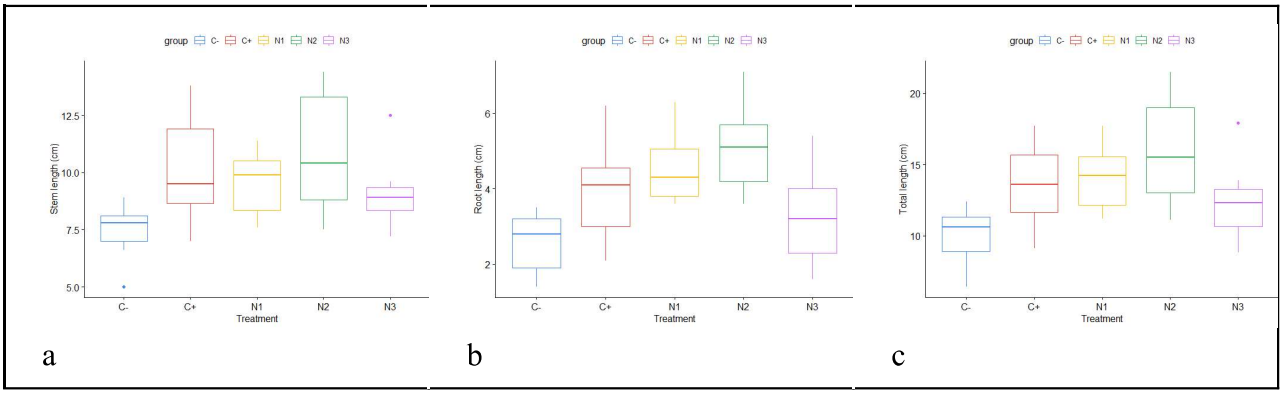


Figure 3. Comparing average plant stem length (a), root length (b), and total plant length (c) at the first harvest: Negative and positive control (C- and C+, respectively); Treatment 1 (N1), Treatment 2 (N2), and Treatment 3 (N3), respectively.

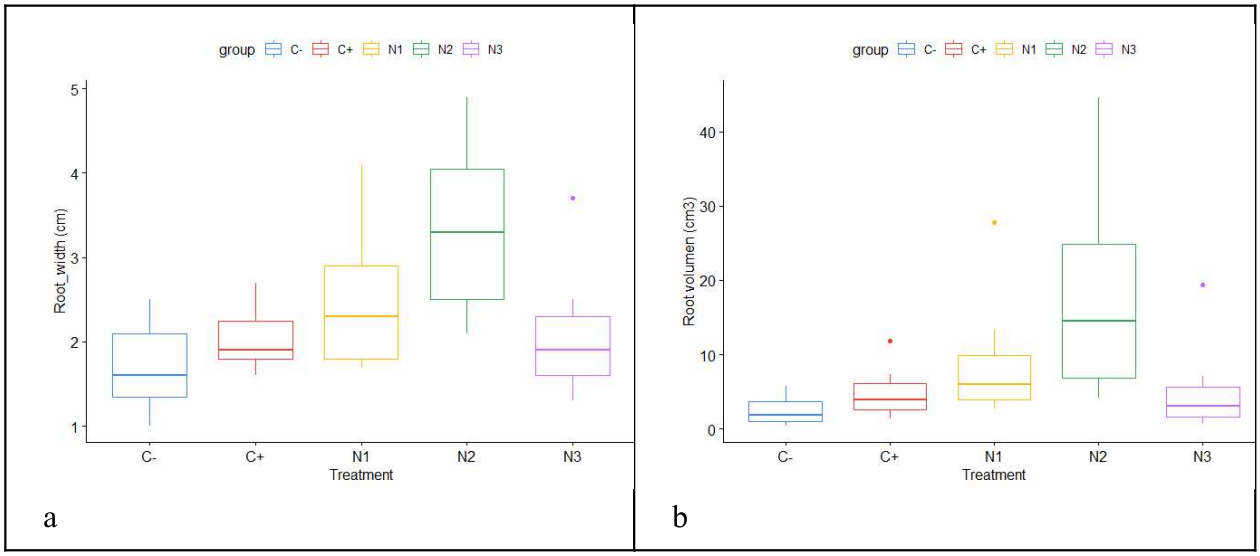


Figure 4. Comparing average plant root width (a), and root volume (b) at the first harvest: Negative and positive control (C- and C+, respectively); Treatment 1 (N1), Treatment 2 (N2), and Treatment 3 (N3), respectively.

2.3.3. *General structure and composition of the bacterial community*. We obtained 4094 OTUs assigned to 19 bacterial phyla, 53 classes, 92 orders, 200 families, 543 genera and 1108 species. The prevalent phyla (99.9% of all sequences) were Firmicutes (31.89%), Proteobacteria (28.77%), Verrucomicrobia (12.56%), Bacteroidetes (8.41%), Actinobacteria (7.95%), Acidobacteria (3.47%), Cyanobacteria (2.25%), and others (Table 10 in support material). The bacterial phyla abundance switched from the soil sample through the acclimation and the process of reaching the steady state in the reactors (Chapter 2). The microbial community inoculated to tomato plants corresponded to the Time 5 (T5) community from N-fixing reactors.

At 24 weeks after planting and continuous fertilization with the N-fixer community at three concentrations: N1, N2, and N3, the proportion of Proteobacteria members is quite similar (35.57 and 35.77%, respectively) in tomatoes rhizospheres fertilized with N1 and N2 treatments (Figure 8a). The Proteobacteria proportion between the original soil and N3 treatment is also very similar (9.06 and 9.71%, respectively). Bacteroidetes and Verrucomicrobia phyla showed similar proportions (Figure 8a) between rhizospheres fertilized with N1 and N2 treatments (13.81 and 8.84%; 20.76 and 26.93%, respectively). The relative abundance of the phylum Firmicutes significantly switched from 90.7% in original soil to 0.6%, 1.0%, and 0.8% (Inoculum, N1, and N2, respectively) until 57.4% in N3.

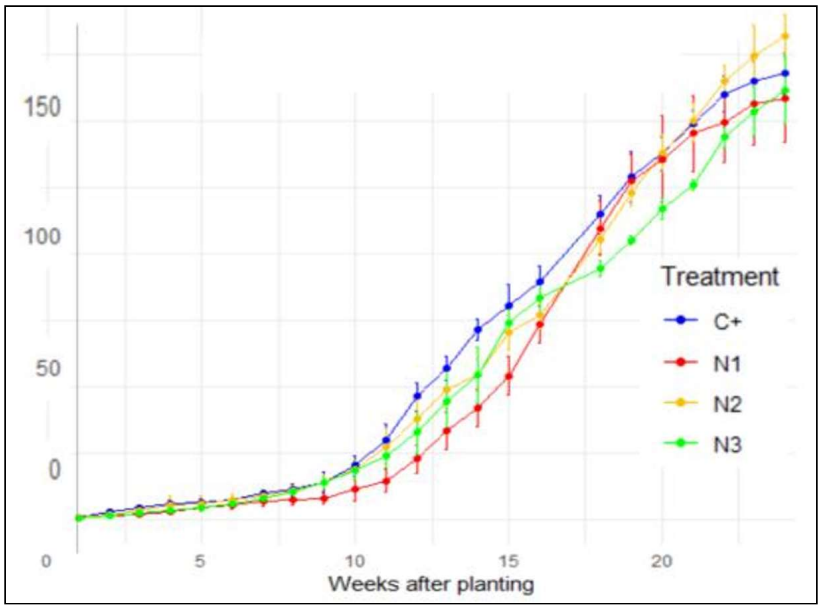


Figure 5. Comparing average plant stem length at the second harvest: Negative and positive control (C- and C+, respectively); Treatment 1 (N1), Treatment 2 (N2) and Treatment 3 (N3), respectively.

At the family level (Figure 8b, Table 11 in Support material), Clostridiaceae and Sporolactobacillaceae (Firmicutes: 70.57 and 17.02%, respectively) were the most abundant in the Soil sample. In the Inoculum sample, Sphingomonadaceae, Xanthomonadaceae, and Xanthobacteraceae (Proteobacteria: 19.51, 9.73, and 7.89%, respectively); Sphingobacteriaceae and Verrucomicrobiaceae (Bacteroidetes and Verrucomicrobia: 13.73 and 15.44%, respectively) were the most abundant.

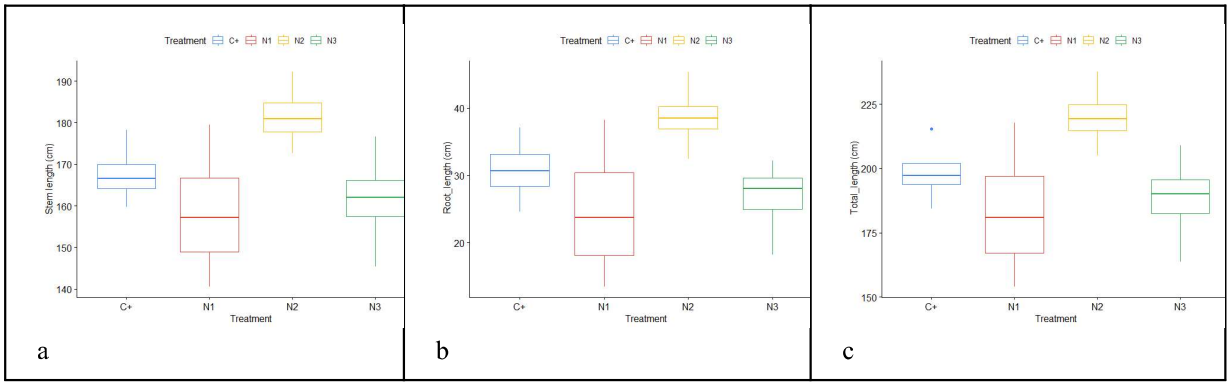


Figure 6. Comparing average plant stem length (a), root length (b)and total length (c). Positive control (C+), Treatment 1 (N1), Treatment 2 (N2) and Treatment 3 (N3), respectively.

In the N1 sample, the families Opitutaceae (Verrucomicrobia: 20.24%), Fulvivirgaceae, and Chitinophagaceae (Bacteroidetes: 8.27 and 4.55%, respectively) were the most abundant families. In the N2 sample, the families Opitutaceae, Verrucomicrobiaceae (Verrucomicrobia: 19.36 and 15.436%, respectively), and Rhodospirillaceae (Proteobacteria: 5.691%) were the most abundant. In the N1 and N2 samples, the Proteobacteria families: Sphingomonadaceae and Xanthomonadaceae showed similar proportions. On another hand, Paenibacillaceae and Clostridiaceae families (Firmicutes: 25.07 and 8.09%, respectively) were the most abundant in the N3 sample. Finally, we underline that Pseudomonadaceae was in the Soil, Inoculation, N1, and N2 samples in similar proportions, around 2%.

Principal Component Analysis (PCA) confirms that the microbial community composition significantly changed (Figure 9) from the Soil sample to the adaptation process, and finally to rhizosphere treatments. Three clusters were formed from. Inoculum (T4 sample in Chapter 1) for one side, other by N1 and N2 treatments. Finally, it is highlighted that N3, despite difference, in terms of families' relative abundance, seems similar in composition to the Soil since they share some of the most abundant families in both samples.

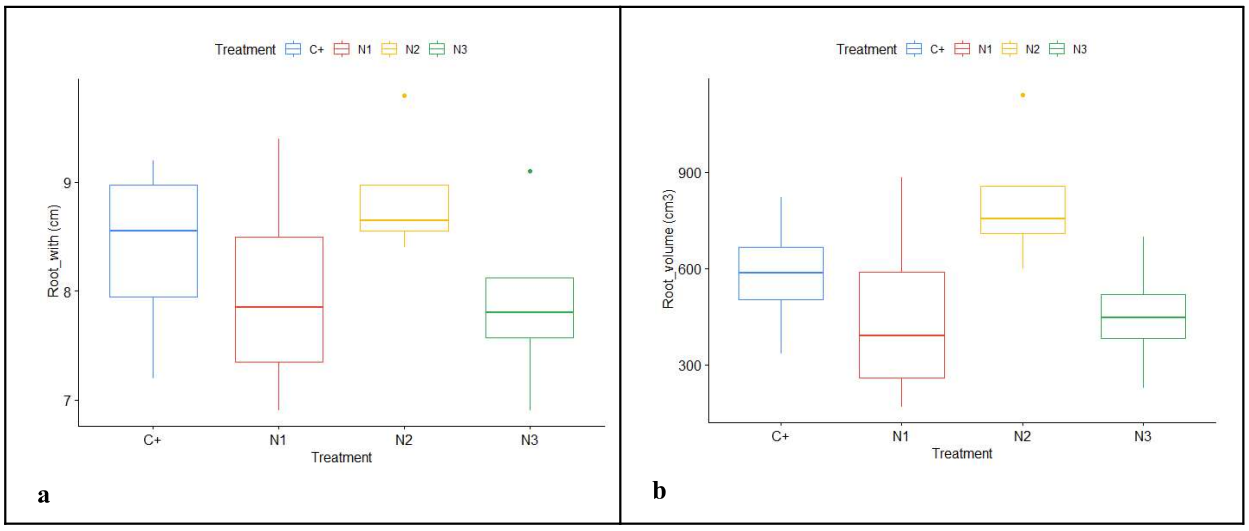


Figure 7. Comparing average plant root with (a) and Root volume (b). Positive control (C+), Treatment 1 (N1), Treatment 2 (N2) and Treatment 3 (N3), respectively.

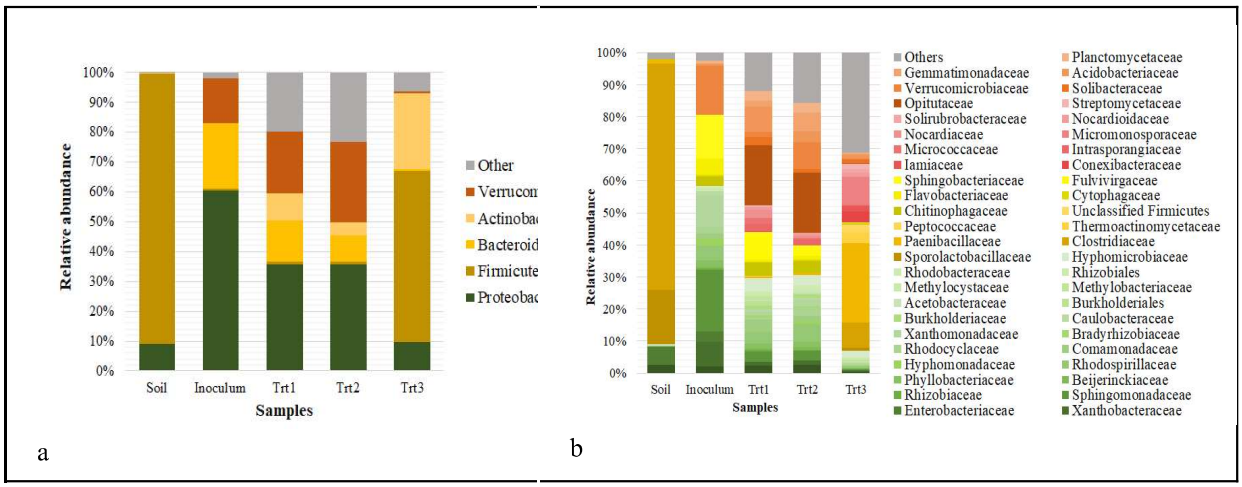


Figure 8. Relative abundance of bacteria in the samples: a) Phylum, b) Family.

Same as exposed in Chapter 1, different clusters are related to substrate type and lifestyles, but they also reflect some ecological traits. Despite Soil and the N-treatment samples were obtained from the same solid substrate, the different community assemblages show treatments effect over that substrate. N1 and N2 showed the biofilm community inoculation and the plants' microbial selection in the rhizosphere. The N3 community assemblage showed first a selection by inoculum decomposition and later that made by the plants.

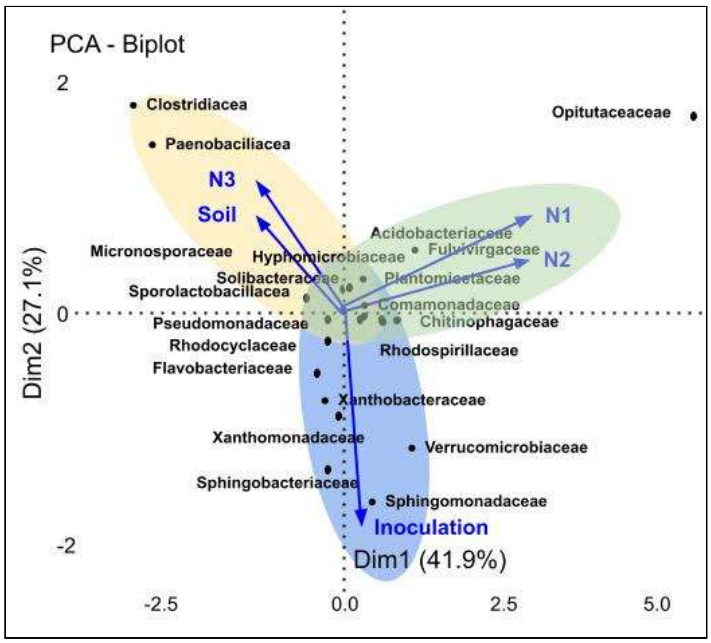


Figure 9. Principal component analysis (PCA) of the five samples

2.3.4. *Bacterial Diversity*. Chapter 1 presents, in detail, the variation in the number of microbial species from the Soil (83) to the Inoculum (T4: 246) sample. Similarly, it presents the variation in the indexes of diversity: Shannon, Simpson's dominance and Chao1 richness. In the rhizosphere, the number of species was 511 in N1, 499 in N2, and 610 in N3, respectively, and much higher than in Soil and Inoculum samples (Figure 10). Regarding the other indexes, the ratio between treatments was similar, with N3 having the highest diversity and lowest dominance.

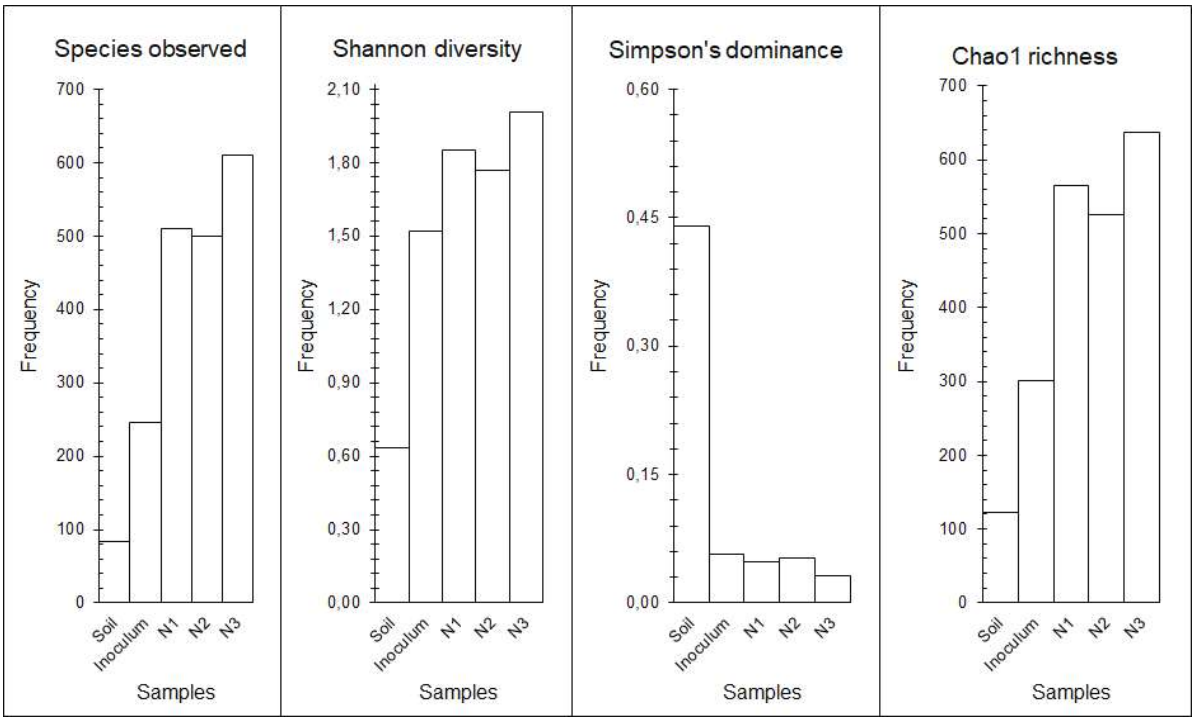


Figure 10. Patterns of species richness (observed), diversity (Shannon's index), evenness (Pielou's index), and

dominance (relative) of bacterial communities in the rhizospheres.

2.4. Discussion

The N concentrations used in our treatments were in the middle range because we wanted to test if the diazotrophic (N-fixer) community would make that substitution more than replacing the N-synthetic for N-organic. The microbial N content of planktonic communities in oligotrophic waters varies substantially due to taxa involved, growth conditions, or ecological restrictions (Vrede et al., 2002; Fukuda et al., 1998), but with a maximum of 0.035 mg/l. In wastewater treatment plants, where N-fixation occurs, effluents have N concentrations around 12 mg/l (Welz et al., 2018; Ospina-Betancourth et a., 2020). The Hoagland and Arnon nutrient solution in tomato cultures often uses a total N concentration of 5 mM (Lu et al., 2009; Dong et al., 2002), meaning 70 mg/l, and providing NO_3 as KNO_3 and $Ca(NO_3)_2$, and NH_4^+ as $(NH_4)_2SO_4$ in a 75:25 ratio (Nitrate/Ammonium). Cordovez et al. (2021) used 1/2th or 1/4th Hoagland's solution in different growth cycles, meaning an N concentration around 2.5 or 1.25 mM (35 and 17.5 mg/l), respectively.

Here, our idea was to obtain a self-assembled microbial community (Yu et al., 2019; Gutierrez et al., 2020), the closest to a rhizobacteria biofilm helping non-legume plants to increase the N-availability. We were looking for an appropriate threshold since the perfect formulation does not exist, and each type has its advantages and limits (Herrmann and Lesueur, 2013). Besides, excessive concentrations may negatively affect the inoculation effect (Rossi 2020; Bashan et al. 2014). There is even growth inhibition when using seaweed extracts to fertilize tomatoes with increasing extract concentrations (Hernández-Herrera et al., 2013; Garcia-Gonzalez and Sommerfeld, 2016) or conventional fertigation rates (Moncada et al., 2020).

The growth rate presented in figure 2 shows there was no significant difference between treatments, at least until the 4th week when C- and N3 treatments began to get left behind at the lowest average heights levels. In the C- case, the growth rate despite lack of fertilization could be because the soil still held enough nutrients to support those unfertilized plants. For the N3, the reduced growth rate suggests an excessive biofertilizer concentration having adverse effects on plants (Rossi 2020; Moncada et al., 2020; Bashan et al. 2014).

In our study, the highest root and stem length obtained, after seven weeks of growing, were 5.09 ± 1.2 and 10.93 ± 2.77 cm, respectively. Comparing our results to some obtained by several

authors that worked with different tomato plant strains, cultivars, or varieties, and fertilization regimes, we observed the N2 treatment performing well with *Solanum pimpinellifolium*. In tomato farming on sterile soil under net house conditions, Mehta et al. (2015), after two months, obtained root length of 9.07 ± 0.06, and shoot length of 21.93 ± 0.06 cm, respectively. They worked with the Pikovskaya (PVK) medium inoculated with 0.1 ml of *Bacillus circulans* culture (106 cells/ml) and a no-mentioned tomato strain, in a mixed ratio of 1:1:1 that included sand and farm-yard manure.

In greenhouse conditions, Souri and Bakhtiarizade (2019) using an essential oil spray of 500 or 1000 ppm oil, a soil application of 500 µL oil/kg soil, and a no application control on tomato (*Lycopersicon esculentum* var Green Supper), obtained a plant height around 18 cm after eight weeks sowing. Moncada et al. (2020) worked with *Solanum lycopersicum* 'Marmande'. They obtained a maximal plant height of 21.7 cm with seeds sprayed with gibberellic acid and 28 days of fertigation (2g/l) of NPK 20-20-20. Finally, Ramírez-Guerrero and Meza-Figueroa (2019) used wye nutrient solution 50% diluted and 30 days after seeding, obtained a maximal plant height of 16.6 cm. The differences between our study with those cited here can be more due to the phenotypic diversity in between the species *pimpinellifolium* and *lycopersicum* (Knapp and Peralta 2016) and varieties of tomato (D'Angelo et al., 2019; Figàs et al., 2015) since this plant has undergone intensive selection through domestication and breeding (Sim et al., 2011).

Microbial community composition shifts in the plant rhizospheres driven by soil type at early stages, whereas at late stages, community composition is also affected by the plant genotype (Schlemper et al., 2017; Marques et al., 2014). That could explain the variations in tomato growth rate under different treatments. During the first four weeks (Figure 2), there were no significant differences in the growth rates, but since there began differentiation in rates according to the treatments (Figure 5). Effects of different fertilizer formulas on the growth of loquat showed that under some fertilization schemes, the growth rates could increase or decrease according to the plant growth stage, resulting in a moment one formula is more effective than others, but the trend changed in the next grade (Xu et al., 2020). The highest root and stem length obtained in our study, after 24 weeks of growing, was 0.39 ± 0.05 and 1.81 ± 00.8 m, respectively. Similarly, in the previous seven-week comparison, these results show that the N2 treatment performs again well with *Solanum pimpinellifolium*. Working with tomato (*S. Lycopersicon* variety Arthaloka), under a non-reported fertilization regime, and after 14 weeks of

planting, Esyanti et al. (2016) obtained plants' height of around 1.2 m.

Dasgan et al. (2016) worked with the cultivar Jaledo under a fertilization regime of Mycorrhiza, vermicompost, and fermentation bacteria. They obtained at 21 weeks the longest plant height of 1.07 m. With a combination of vermicompost and chicken manure compost applied on a non-reported tomato variety, Wang et al. (2017) obtained a plant height of around 0.9 m after 15 weeks of transplanting. With an application of vermicompost from tannery waste-animal fleshing on the tomato variety PKM I, Ravindran et al. (2019) observed a maximal height of 1.25 ± 0.09 cm after 13 weeks of experimental studies. Using the Hoagland nutrient solution on the variety Green Supper, after 22 weeks, Dehnavard et al. (2017) obtained a maximal plant height of around 1.8 m. With this same formula on variety Money Maker but adding a foliar spraying treatment with calcium nitrate, Souri and Dehnavard (2018) obtained a plant height of 2.25 ± 0.22 m after four months of planting.

Our findings agree with several other studies showing that the phyla Proteobacteria and Bacteroidetes are the predominant taxa in the tomato plant rhizospheres. It can range around 40% and 20%, of relative abundance, respectively (Qiao et al., 2017) regardless of the variety, plant developmental stage, and susceptibility to the soil-borne pathogens that may switch some rhizosphere microbiome structure aspects between distinct genotypes (Li et al., 2014; Kwak et al., 2018; Cheng et al., 2020; French et al., 2020). However, although Proteobacteria is the most ubiquitous and usual group, they can be more abundant in tomato soils characterized by a much higher content of both soil organic C and total N (Pii et al., 2016). Even when Jaiswal et al. (2017) used biochar to amend the tomato rhizospheres, the Proteobacteria and Bacteroidetes relative abundance ranged around 60% and 17%, respectively.

In addition, the phyla Verrucomicrobia, Firmicutes, and Actinobacteria, although common in tomato plant rhizospheres, are usual in low relative abundances (Lee et al., 2019; Cheng et al., 2020). Jaiswal et al. (2017) reported for the phyla Verrucomicrobia and Firmicutes relative abundance that ranged between 2.9% and 0.7%, respectively. Similarly, Jaiswal et al. (2017) found no differences in the relative abundance of Actinobacteria (around 7.1%) in the non-amended and biochar-amended rhizosphere. Lee et al. (2016) found an Actinobacteria relative abundance of 17.7%, and after a culture-dependent approach, that abundance was 8.5%. Qiao et al. (2017) reported Actinobacteria with a significant 9% of relative abundance. The

Firmicutes frequency in the initial soil (chapter 1) is related to their predominance in degrading complex organic compounds with high organic matter content and concentrations of nutrients, such as manure-amended soils (Francioli et al., 2016; Hartmann et al., 2015). Based on this, we suppose that the higher microbial community concentration applied in the N3 treatment significantly transformed into decomposing biomass and served as an organic matter source. Despite this, that treatment helped the plants to grow. On the other hand, since Actinobacteria is significantly higher in soils with conventional fertilization (NPK) than in organically fertilized soils (Francioli et al., 2016; Li et al., 2012), allows presuming that in the tomato rhizospheres environment, the minerals nutrients from the Hoagland solution had a higher effect in N3 treatment than in N1 or N2.

See Chapter 1 for a more comprehensive discussion about the structure of the microbial communities in the soil and their evolution to reach the inoculum. Regarding the abundance of families after the treatments with nitrogen communities, Hernández et al. (2015), Breidenbach et al. (2016), and Hester et al. (2018) found the Opitutaceae in different water-saturated rhizospheres, growing by fermentation and nitrate reduction, taking advantage of the abundant plant-derived organic carbon under anoxic conditions. On the contrary, in a sugarcane rhizosphere, a higher abundance of Opitutaceae was related to a low N availability (Navarrete et al., 2015), as in other soils with low water saturation, such as grass, potato, and leek (Da Rocha et al., 2010; 2013). Moreover, the genus Fulvivirga, a strictly aerobic, heterotrophic bacterium (Nedashkovskaya et al., 2007), has been identified as a keystone genus in low-Phosphorus fertilization treatments (Liu et al., 2020). A similar P-function has been stated for Chitinophagaceae in N1 (Gautam et al., 2020) and Verrucomicrobiaceae in N2 (Grafe et al., 2018; 2021; O'Brien et al., 2018), but under high nitrogen fertilization, primarily organic-N (Li et al., 2017; 2018). For the family Rhodospirillaceae in N2, Ding et al. (2018) associated its relative abundance with the N-deficiency fertilization suggesting N-fixation, while Wang et al. (2017) with the N-organic enriched soil, meaning nitrate oxidation. Based on the above, in our case, the N-depleted fertilizer conditions suggest the Opitutaceae and Rhodospirillaceae families were involved in the nitrogen fixation process, while Fulvivirgaceae, Chitinophagaceae, and Verrucomicrobiaceae at P-depleted environment then favoring the phosphate-solubilization. It would mean a P-deficient medium was unintentionally applied. We sought an intermediate P-concentration between the Hoagland solution formula and the RBA, a nutrient medium known

to favor the enrichment of nitrogen fixers in a substrate, so we decided to increase the P concentration in our experiments. However, the community self-assemblaged results suggest that although it was not apparently enough, in the end, the community managed to supply both nitrogen and phosphorus sufficiently.

Regarding the relative abundance of Sphingomonadaceae and Xanthomonadaceae families in N1 and N2 treatments, they are prevalent in some rhizospheres significantly enriched by N fertilization (Wu et al., 2021), mainly HNPK (high rate of N fertilizer, regular PK fertilizers) for Sphingomonadaceae and MNPK (organic manure plus NPK fertilizers) for Xanthomonadaceae (Li et al., 2017). In fact, Ding et al. (2016) reported Sphingomonadaceae relative abundance is markedly lower in MNPK than in potassium-inorganic fertilizer (NPK). The aforementioned suggests that a small portion of the microbial community added to the nutrient substrate in soils during treatments N1 and N2 preferred inorganic nutrients despite the high organic load evidenced by the remaining community composition. Because all of the N and a significant amount of P were contributed by community members, those exclusively inorganic compound consumers would be using by-products mineralized by N-fixers and P-solubilizers microbes. Regarding the most abundant families in the N3 sample, the abundance of Paenibacillaceae was associated with nitrogen fixation processes in some legume crop rhizospheres (Coelho et al., 2007; 2009; Rodrigues Coelho et al., 2008). In relation to the Clostridiaceae prevalence, Rui et al. (2009) reported that this family is most dominant in the early stages during decomposition processes of plant residues in rice field soil, which suggests it is responsible for the breakdown of the easily degradable fraction of residues. Other authors (Wakase et al., 2008; and Schlatter et al., 2018) have reported that the extensive distribution of this family in agricultural soils is due to the wide application of manure fertilizer. Applying the reductive soil disinfestation (RSD) method during tomato planting, Tan et al. (2019) found the relative abundance of *Clostridium* was notably higher for all RSD treatments than those without that substrate. This method refers to creating anaerobic reducing conditions by adding easily decomposable organic matter with water to saturate the soil, then covering it with a plastic film and incubating at a high temperature for 3–4 weeks to kill plant pathogens (Tan et al., 2019). After that process, the substrate can be used for sowing. The aforementioned factor supports our presumption that higher microbial community concentration applied in the N3 treatment significantly transformed into anaerobic decomposing biomass and served as an organic matter source. However, since the nitrogen was

almost exclusively in the microbial biomass and the high abundance of N-fixers suggest microbial biomass decomposition simultaneously occurred with N-fixation. Thus, during the N1 and N2 treatments, a small portion of the microbial community preferred inorganic nutrients despite the high organic load. About the presence of the Pseudomonadaceae family throughout all the samples, authors such as McManus et al. (2002), Innerebner et al. (2011), Ardanov et al. (2012), and Stockwell and Duffy (2012) report some family members are related to plant diseases, infecting them as soil-borne pathogenic microbes in some cases. However, they are also involved in protecting host plants as natural control to various fungal pathogens (Mendes et al., 2011; Mavrodi et al., 2012; Blaustein et al., 2017). Furthermore, this family is associated with the nitrogen fixation process in several crops and grasses (Dalton et al., 2004; Montañez et al., 2009). Given rhizosphere conditions and tomatoes did not show any disease signs, we assumed this family was supporting plants' health condition and nitrogen fixation.

Finally, the Principal Component Analysis (PCA) and bacterial diversity first show the differential effect caused by substrate type, biofilm for Inoculum, and solid for the remaining. However, the solid-substrate communities also show the N-microbial concentration effects. While soil community origin had the lowest diversity of all samples, N-treatments obtained a higher one, even over the biofilm inoculum community. However, the N3 treatment achieved the highest microbe diversity of all, although, from functional ecology, this community appeared very similar to the original soil sample, except for its high abundance of Actinobacteria.

2.5. Conclusions

The plants fertilized with Hoagland modified solution with N-supplied only by microbial biomass coming from N-fixer reactors at different concentrations allowed the tomato plants to grow significantly. Likewise, it reflected a complex nitrogen-fixing microbial community in the rhizospheres in each case. The N1 and N2 treatments seemed similar to each other and with the Inoculum, whilst original Soil had the lowest diversity and N3 had the highest but were (in the functional ecology) similar amongst them.

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Support material

Trend line equations and R square values	
C- = 1,1117x - 0,3918	R^2 = 0,9982
C+ = 1,4638x - 0,8408	R^2 = 0,9802
N1 = 1,3189x + 0,3694	R^2 = 0,9709
N2 = 1,5791x - 0,398	R^2 = 0,9962
N3 = 1,2194x + 0,2286	R^2 = 0,9841

Table 1. Comparing fertilizer treatment effects of the tomato growth rates in the first gathering.

	diff	lwr	upr	p adj
C+-C-	2.7857143	-0.3451074	5.916536	0.0997065
N1-C-	2.1000000	-1.0308216	5.230822	0.3163822
N2-C-	3.5142857	0.3834641	6.645107	**0.0217685**
N3-C-	1.7285714	-1.4022502	4.859393	0.5080016
N1-C+	-0.6857143	-3.8165359	2.445107	0.9680413
N2-C+	0.7285714	-2.4022502	3.859393	0.9603222
N3-C+	-1.0571429	-4.1879645	2.073679	0.8623589
N2-N1	1.4142857	-1.7165359	4.545107	0.6870557
N3-N1	-0.3714286	-3.5022502	2.759393	0.9968156
N3-N2	-1.7857143	-4.9165359	1.345107	0.4760769

Table 2. Tukey multiple pairwise-comparisons of Stem length in the first gathering.

	diff	lwr	upr	p adj
C+-C-	1.3714286	-0.4462773	3.18913445	0.2115377

N1-C-	2.0000000	0.1822941	3.81770588	**0.0254190**
N2-C-	2.5285714	0.7108655	4.34627731	**0.0029723**
N3-C-	0.7000000	-1.1177059	2.51770588	0.7963371
N1-C+	0.6285714	-1.1891345	2.44627731	0.8518911
N2-C+	1.1571429	-0.6605630	2.97484874	0.3670490
N3-C+	-0.6714286	-2.4891345	1.14627731	0.8195156
N2-N1	0.5285714	-1.2891345	2.34627731	0.9147368
N3-N1	-1.3000000	-3.1177059	0.51770588	0.2572031
N3-N2	-1.8285714	-3.6462773	-0.01086555	**0.0480815**

Table 3. Tukey multiple pairwise-comparisons of Root length in the first gathering.

	diff	lwr	upr	p adj
C+-C-	3.600000	-0.9800697	8.180070	0.1794070
N1-C-	4.100000	-0.4800697	8.680070	0.0965278
N2-C-	6.042857	1.4627875	10.622927	**0.0051454**
N3-C-	2.428571	-2.1514983	7.008641	0.5468984
N1-C+	0.500000	-4.0800697	5.080070	0.9976950
N2-C+	2.442857	-2.1372125	7.022927	0.5413182
N3-C+	-1.171429	-5.7514983	3.408641	0.9447722
N2-N1	1.942857	-2.6372125	6.522927	0.7340233
N3-N1	-1.671429	-6.2514983	2.908641	0.8258500
N3-N2	-3.614286	-8.1943554	0.965784	0.1764419

Table 4. Tukey multiple pairwise-comparisons of Total length in the first gathering.

	diff	lwr	upr	p adj
C+-C-	0.32857143	-0.87648590	1.53362876	0.9312654
N1-C-	0.78571429	-0.41934304	1.99077161	0.3436463
N2-C-	1.62857143	0.42351410	2.83362876	**0.0040306**
N3-C-	0.38571429	-0.81934304	1.59077161	0.8835997
N1-C+	0.45714286	-0.74791447	1.66220019	0.8049507
N2-C+	1.30000000	0.09494267	2.50505733	**0.0294874**
N3-C+	0.05714286	-1.14791447	1.26220019	0.9999144
N2-N1	0.84285714	-0.36220019	2.04791447	0.2772783
N3-N1	-0.40000000	-1.60505733	0.80505733	0.8694778
N3-N2	-1.24285714	-2.44791447	-0.03779981	**0.0406474**

Table 5. Tukey multiple pairwise-comparisons of Root width in the first gathering.

	diff	lwr	upr	p adj
C+-C-	2.4549360	-10.64491224	15.554784	0.9819044
N1-C-	6.6637450	-6.43610324	19.763593	0.5856284
N2-C-	15.6326016	2.53275336	28.732450	**0.0131211**
N3-C-	2.9239694	-10.17587884	16.023818	0.9657966
N1-C+	4.2088090	-8.89103924	17.308657	0.8822008
N2-C+	13.1776656	0.07781736	26.277514	**0.0480932**
N3-C+	0.4690334	-12.63081484	13.568882	0.9999720
N2-N1	8.9688566	-4.13099164	22.068705	0.2970215
N3-N1	-3.7397756	-16.83962384	9.360073	0.9197947
N3-N2	-12.7086322	-25.80848044	0.391216	**0.0606439**

Table 6. Tukey multiple pairwise-comparisons of Root volume in the first gathering.

Trend line equations and R square values

C+ = $-0{,}002x^4 + 0{,}0718x^3 - 0{,}3251x^2 + 0{,}2514x + 3{,}6092$	$R^2 = 0{,}9978$
N1 = $-0{,}0021x^4 + 0{,}093x^3 - 0{,}8747x^2 + 3{,}8588x - 2{,}0546$	$R^2 = 0{,}9992$
N2 = $-0{,}0049x^4 + 0{,}2217x^3 - 2{,}7156x^2 + 12{,}122x - 12{,}06$	$R^2 = 0{,}9976$
N3 = $-0{,}0007x^4 + 0{,}0227x^3 + 0{,}2141x^2 - 1{,}9876x + 4{,}8939$	$R^2 = 0{,}9955$

Table 7. Comparing fertilizer treatment effects of the tomato growth rates in the second gathering.

Stem length	Df	Sum Sq	Mean Sq	F value	Pr(>F)
group	3	499	166.5	0.116	0.949
Residuals	12	17192	1432.7		
Root length	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
group	3	77.8	25.94	0.532	0.669
Residuals	12	585.6	48.80		
Total length	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
	3	788	262.8	0.133	0.939
	12	23754	1979.5		
Root width	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**

	group	3	30.55	10.184	3.262	0.0594
	Residuals	12	37.46	3.122		
Root volume		Df	Sum Sq	Mean Sq	F value	Pr(>F)
	group	3	575361	191787	1.836	0.194
	Residuals	12	1253487	104457		

Table 8. Summary of the analysis of variance models for the second gathering.

Phylum	Soil	Inoculum	N1	N2	N3
Firmicutes	43190	232	421	410	26279
Proteobacteria	4316	22736	14418	17717	4445
Verrucomicrobia	1	5668	8413	13340	350
Bacteroidetes	7	8280	5637	4378	308
Actinobacteria	123	52	3660	2242	11502
Acidobacteria	1	251	4226	2413	791
Cyanobacteria	0	77	832	4037	28
Gemmatimonadetes	0	0	684	2793	281
Planctomycetes	0	302	1332	1772	200
Chloroflexi	0	0	440	313	1262
Nitrospirae	0	0	142	2	157
Ignavibacteriae	0	0	153	30	0
Chlamydiae	0	88	8	40	3
Candidatus Dependentiae	0	0	0	0	117

Candidatus Saccharibacteria	0	0	11	9	45
Deinococcus-Thermus	0	0	63	2	0
Fusobacteria	0	0	49	10	0
Candidatus Melainabacteria	0	0	24	0	0
Fibrobacteres	0	0	4	8	1
Spirochaetes	0	0	5	7	0
Thaumarchaeota	0	0	9	2	0
Thermotogae	0	0	0	5	0
Euryarchaeota	0	0	0	0	4

Table 9. Number of individuals of each Phylum present in samples.

Phylum	Family	Soil	Inoculum	N1	N2	N3
Proteobacteria	Sphingomonadaceae	0,13225	19,5095	3,52995	2,94872	0,403
Proteobacteria	Xanthomonadaceae	0,08817	9,73025	1,3063	2,04708	0,30779
Proteobacteria	Enterobacteriaceae	5,72664	3,07357	0,03487	0,02522	0,22807
Proteobacteria	Xanthobacteraceae		7,89101	1,2446	1,51114	0,23472
Proteobacteria	Rhodospirillaceae	0,02729	4,61035	3,9323	5,69147	0,51372
Proteobacteria	Pseudomonadaceae	2,51905	2,02452	2,43019	2,6881	0,40743
Proteobacteria	Phyllobacteriaceae	0,0063	2,11717	1,6094	1,65406	0,19486
Proteobacteria	Comamonadaceae		1,6376	3,85719	1,62673	0,05979
Proteobacteria	Caulobacteraceae	0,0105	1,38965	0,67595	0,47499	0,14614
Firmicutes	Clostridiaceae	70,567	0,01635	0,04828	0,02102	8,09769
Firmicutes	Sporolactobacillaceae	17,0162	0,00272		0,0021	0,75729
Firmicutes	Paenibacillaceae	1,19235	0,00272	0,50696	0,67255	25,0747
Bacteroidetes	Sphingobacteriaceae	0,0063	13,733	1,02465	1,24422	0,03764
Bacteroidetes	Flavobacteriaceae		5,11172	0,57938	1,2148	0,1085
Bacteroidetes	Chitinophagaceae	0,0084	3,00817	4,5546	3,86297	0,20814
Bacteroidetes	Fulvivirgaceae			8,26963	2,05969	0,03764

Actinobacteria	Nocardiaceae		0,0327	2,80572	0,70828	0,19707
Actinobacteria	Intrasporangiaceae	0,0021		2,41946	1,96721	0,14836
Actinobacteria	Micrococcaceae	0,19942		2,0654	0,38041	0,19707
Verrucomicrobia	Opitutaceae			20,2409	19,3611	0,248
Verrucomicrobia	Verrucomicrobiaceae	0,0021	15,436	1,94469	8,52249	0,28122
	Others	2,49596	10,673	36,9196	41,3157	62,1111

Table 10. Proportion of most abundant families present in samples.