Kinetic analysis of the evolution of the microbiota of the PAFF Box

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Diplôme : Master en bioingénieur : sciences agronomiques, à finalité spécialisée
Année académique : 2018-2019
URI/URL : http://hdl.handle.net/2268.2/8190

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IRIS SZEKELY

TRAVAIL DE FIN D'ÉTUDES PRÉSENTÉ EN VUE DE L'OBTENTION DU DIPLÔME DE

MASTER BIOINGÉNIEUR EN SCIENCES AGRONOMIQUES

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Acknowledgments

Firstly, I would like to thank my parents for allowing me to carry out those studies and for always supporting me.

Thank you to my supervisor, Mathilde Eck, for helping me throughout this entire thesis and for giving me so much support and positiveness.

I would like to sincerely thank my two co-promotors, H. Jijakli and S. Massart, for having allowed the development of this experiment, for guiding me throughout this thesis and for all the good advices that they gave me in order to improve this work.

Thank you to Gilles Stouvenakers for helping me with all the technical aspects of the PAFF Box and the general advices.

I would also like to thank Jimmy Bin and François Maclot for helping me with their laboratory skills, and Abdoul Razak for his enthusiasm and help during sequencing data analysis. Thank you to the all team of the Integrated and Urban Plant Pathology Laboratory in general, for their welcome.

Finally, I would like to thank my family and friends, Corentin, Clément, Pauline, Marie and Félix for helping me and supporting me during those past 5 years.

Abstract

Aquaponics is an innovative farming concept that integrates both fish and hydroponics crop productions in a recirculating water system. Functioning as a closed nutrient loop, this technic could play an important role in the environmental and socio-economical sustainable challenges of urban areas. This method is based on a tripartite symbiosis made of fish, plants and microorganisms. Mainly by transforming the fish wastes and making them bioavailable for the plants, microorganisms in aquaponics are crucial and an increasing number of studies aim to characterise their diversity and functionalities. The present survey analyses bacterial and fungal communities over time in a small scale aquaponic system, i.e. the Plant and Fish Farming box (PAFF Box), in order to assess the stability of the microbiota observed in aquaponics. To this end, samples of four different "compartments" were taken twice and then once a week for 9 weeks after the introduction of lettuces (*Lactuca sativa*) in the system, i.e. the circulating water, the biofilter, the lettuce endosphere and rhizoplane. The bacterial communities were analysed by 16s rRNA gene sequencing and the fungal communities by ITS gene sequencing, using Illumina MiSeq technology and the QIIME bioinformatic software. As a result, the biofilter and lettuce roots (both endosphere and rhizoplane) to a larger extent, underwent a bacterial shift respectively 11 to 15 days and 18 to 25 days after the introduction of the lettuces in the system. This shift was mainly characterised by the disappearance of *Lactobacilli* and *Streptococci* genera from the relative abundances, in both the biofilter and roots. Afterwards, the bacterial communities remained fairly stable in those compartments. In the circulating water, bacterial communities fluctuated a lot throughout the 9 weeks of experiment, but with no drastic shifts. The fungal communities were difficult to study due to a lack of fungal assignment. However, the biofilter appeared as a much more fluctuating compartment than the water and roots compartments, in term of fungal communities. Other studies should be made in order to further characterise and understand the microbial shifts that can occur in such system, as well as better characterise the fungal communities in AP.

Key words: aquaponics, bacterial communities, fungal communities, high throughput sequencing, 16s rDNA, ITS, kinetic analysis

Résumé

L'aquaponie est une technique d'agriculture innovante qui combine la production de plantes hydroponiques et l'élevage de poissons, dans un système recirculé. Fonctionnant en boucle fermée, cette méthode s'inscrit dans les défis environnementaux et socio-économiques actuels des zones urbaines. Ce système est basé sur une symbiose tripartite entre les plantes, les poissons et les microorganismes. Principalement en transformant les déchets d'aquaculture et en les rendant biodisponibles pour les plantes, les microorganismes ont un rôle crucial en aquaponie et de plus en plus d'études tentent de caractériser leur diversité et fonctionnalités. Cette présente étude analyse les communautés bactériennes et fongiques dans le temps d'un système aquaponique de petite échelle, i.e. la Plant and Fish Farming Box (PAFF Box), dans le but d'évaluer la stabilité du microbiote observé en aquaponie. Pour cela, des échantillons de quatre « compartiments » différent ont été relevés deux puis une fois par semaine durant 9 semaines après l'introduction de laitues (*Lactuca sativa*) dans le système, i.e. l'eau circulante, le biofiltre, l'endosphère et la rhizoplane des laitues. Les communautés bactériennes ont été analysées par séquençage du gène 16s rRNA et les communautés fongiques par séquençage du gène ITS, en utilisant la technologie Illumina MiSeq et le logiciel bioinformatique QIIME. Ainsi, le biofiltre et les racines de laitues (endosphere et rhizoplane) dans une plus large mesure, ont subi un important changement bactérien respectivement 11 à 15 jours et 18 à 25 jours après l'introduction des laitues dans le système. Ce changement était principalement caractérisé par une disparition des genres *Lactobacillus* et *Streptococcus* des abondances relatives, aussi bien dans le biofiltre que dans les racines. Après ce changement, les communautés bactériennes sont restées relativement stables dans ces compartiments. Dans l'eau circulante, les communautés bactériennes ont beaucoup fluctué au cours des 9 semaines d'expérience, mais sans changement drastiques. De leurs côtés, les communautés fongiques ont été difficiles à étudiées à cause d'un manque

d'assignation des séquences. Cependant, le biofiltre semblait être un compartiment bien plus fluctuant que ceux de l'eau ou des racines, en termes de communautés fongiques. D'autres études devraient être menées pour caractériser et comprendre les grands changements microbiens qui peuvent survenir dans un système aquaponique, et mieux caractériser également les champignons en aquaponie.

Mots clés : aquaponie, communautés bactériennes, communautés fongiques, séquençage haut débit, 16s rDNA, ITS, analyse kinétique

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List of abbreviations

µL: microlitre
µm: micrometre
ADEME: agence de l'environnement et de la maîtrise de l'énergie
ADN: acide désoxyribonucléique
AMF: arbuscular mycorrhizal fungi
ANAMMOX: anaerobic ammonium-oxidizing bacteria
AOB: ammonia oxidizing bacteria
AP: aquaponics
ATP: Adenosine triphosphate
B: boron
BC: before Christ
BLAST: basic local alignment search tool
Bp: base pair
C: Celsius
Ca: calcium
Cm: centimetre
COMAMMOX: complete ammonia oxidizer
Cu: copper
ddNTP: dideoxynucleotide
DNA: deoxyribonucleic acid
dNTP: deoxyribonucleotides
DO: dissolved oxygen
DWC: deep water culture
EC: electro conductivity
FAO: Food and Agriculture Organisation
FCR: feed conversion ratio
Fe: iron
G: gram
H: hour
HTS: high throughput sequencing
ID: identifier
ITS: internal transcribed spacer
IUPPL: Integrated and Urban Plant Pathology Laboratory
K: potassium
KPBT: Potassium Phosphate Buffer
L: litre
LAB: lactic acid bacteria
LBVD: lettuce big-vein disease
Mg: magnesium
mg: milligram
ml: millilitre
mM:millimolar
Mn: manganese
Mo: molybdenum

N: nitrogen
NFT: nutrient film technique
ng: nanogram
NOB: nitrite oxidizing bacteria
NOB: nitrite oxidizing bacteria
OTU: operational taxonomic unit
P: phosphorus
PAFF: Plant and Fish Farming
PC: physicochemical
PCA: principal component analysis
PCoA: principal coordinates analysis
PCR: polymerase chain reaction
PD: phylogenetic diversity
PGPF: plant growth promoting fungi
PGPM: plant growth promoting microorganisms
PGPR: plant growth promoting rhizobacteria
pH: potential of hydrogen
QIIME: quantitative insight into microbial ecology
RAS: recirculating aquaculture system
rDNA: ribosomal deoxyribonucleic acid
RNA: ribonucleic acid
rRNA: ribosomal ribonucleic acid
S: sulphur
Sec: second
T: temperature
TAN: total ammonium nitrogen
TSB: Tryptic Soy Broth
Tw: tween
UA: urban agriculture
Zn: zinc

1. Introduction

1.1 General introduction

Mankind is facing a major demographic challenge in this 21st century. The world population, estimated at 7.3 billion in 2015, is expected to reach 9.7 billion by 2050 (United Nation, 2017), which represents a growth of 32%. This will lead to a significant rise in food demand, particularly in South Asia and Africa where the population growth is predicted to be constant and more important (FAO, 2018). The changes in demographic dynamics will also play a major role in this demand. For instance, the higher incomes, the technological progress, the globalization and the urbanization have led, among other aspects, to an increase in the calorie intake per capita as well as a greater consumption of meat, fish and dairy products (FAO, 2018). However, 821 million people are still suffering from chronic hunger and two billion people are having diverse micronutrient deficiencies (FAO, 2018). In addition, this contrasting situation must also address the challenges associated with climate change. While agriculture has significantly contributed to the production of greenhouse gases, representing 10% of the total emissions in 2016 in Europe for instance (European Commission, 2019), it is now facing its backlash, being extremely vulnerable to environmental modifications (Nelson, 2009; FAO, 2018; Howden *et al*, 2007). More specifically, enteric fermentation, manure left on pasture as well as fertilizers appear to be the main contributors to those emissions and the prospects do not bode well (FAO, 2014; Lenka *et al*, 2016). Eutrophication, important emissions of N_2O and nitrate pollution in groundwater are some of the negative aspects that can be intensified with inappropriate use of fertilizers (Lenka *et al*, 2016).

Hence, it seems crucial to find alternatives and encourage agricultural systems that minimize their environmental impacts while being productive enough to address the increasing food demand.

1.2 Urban agriculture

The percentage of population living in urban areas has evolved from 29.6% in 1950, to 53.9% in 2015 and will likely reach 68.4% by 2050 (United Nation, 2018). Greater opportunities and less poverty in urban area or globalization that tends to connect cities to each other; the causes are many and diverse (Montgomery, 2008). As the cities expand with more and more people having better incomes, their food demand keeps growing while they are strongly relying on rural areas, providing them most of their needs (Miccoli *et al.*, 2016). However, urbanization itself is indirectly destroying the hand that feeds, causing a reduction in fertile soils, water pollution and deforestation (Orsini *et al.,* 2013). Thus, it seems necessary to develop agriculture within the cities to increase self-sufficiency and limit environmental impacts. Urban agriculture (UA) has multiple definitions depending on its localisation, functions, local dynamics and activities (Daniel, 2013). A general description was given by Mougeot (2000, p. 10): "Urban agriculture is an activity located within or on the fringe of an urban area, which cultivates, processes, and distributes a diversity of products, re-using resources, and services found in and around that urban area, and supplies resources, products, and services largely to that urban area". However, a distinction can be made between UA located in developed or developing countries.

In developing countries, UA provides an important part of the food supply and is crucial for subsistence and food security (ADEME, 2017). For instance, in Hanoi (Vietnam), "80% of fresh vegetables, 50% of pork, poultry and freshwater fish, as well as 40% of eggs, originate from urban and peri-urban areas" (ETC, 2003). A significant part of the production is thus dedicated to domestic consumption, the surpluses being placed on the market afterwards. Developing countries have specific challenges towards this agriculture since they are the ones that undergo a massive urban population growth as well as an increasing urban poverty and social disparity (ETC, 2003). UA thus aims to reduce this poverty as well as improve the city dwellers health conditions with easier access to fresh and safe food (Orsini *et al.*, 2013). By including women, unemployed or immigrants, it can also contribute to better gender relationships and social inclusion (ETC, 2003). In this case, as it plays an important socio-economical role, many of urban inhabitants are being involved since it offers many job opportunities. In Brazzaville (Congo), 80% of the urban population is implicated in urban farming

(Daniel, 2013). In these countries, UA is usually in the form of horticultural systems through mini-gardens, community garden and simplified soilless systems within the urban area. Intensive horticulture systems occur too, mostly in peri-urban areas, with landowners (Orsini *et al.,* 2013).

In developed countries, the expansion of UA is in fact recent, starting 20 years ago in the United States and 10 years ago in Europe, showing the reconsideration of these countries towards their urban organization, ways of production and consumption (Daniel, 2013). In a social-oriented purpose, urban farming is usually in the form of non-commercial micro-farms with reemployment and rehabilitation processes, rooftop productions, community gardens and collective henhouses (ADEME, 2017; Daniel, 2013). The benefits are social but also economic, environmental and educational, reinforcing social bonds, reconnecting the children with food production, promoting local and sustainable agriculture. In a more productive perspective, UA can be in the form of farm businesses in peri-urban areas, using conventional or unconventional technics, being more or less linked to the city (Daniel, 2013). More technological systems are also being developed, such as hydroponics. This method consists of "growing plants using mineral nutrient solutions, without soil. Terrestrial plants may be grown with their roots in the mineral nutrient solution only or in an inert medium, such as perlite, gravel or mineral wool." (Sengupta & Banerjee, 2012, p. 103). In comparison to soil-based culture, this method saves water and space, since plants can be grown vertically, while obtaining higher yields thanks to controlled conditions that can meet the optimal plant requirements (Somerville *et al.,* 2014). Pests and soil-borne diseases can also be reduced as the contact between soil and plants is avoided and the soilless-media can be sterilised (Somerville, 2014). It also offers the possibility to cultivate crops in areas with non-arable soils (Putra & Yuliando, 2015; Sengupta & Banerjee, 2012).

Concerning UA in general and focusing on ecological aspects, the presence of green areas within the cities can help preserving biodiversity as well as reducing the temperature, air pollution, suspended dust and noise pollution (Harris, 2010; Daniel, 2013). It can also address the waste management issue, by producing compost and recycling inorganic waste, using plastic tanks and plastic bottles for soilless cultivation for example. Since the production is close to the consumers, it leads to a significant reduction of goods transportations that are not only polluting but are also a major source of food waste (Orsini *et al.*, 2013). However, UA can also have weaknesses. Food contaminations could occur, caused by polluted soil and water or inappropriate use of urban compost. The proximity between animal production and humans also facilitates disease transmission with an additional higher amount of insect's vectors, mostly in the case of developing countries (Orsini *et al.*, 2013).

In conclusion, UA has multiple challenges. With the considerable urban population growth that the world will have to face, this activity needs to take on ever greater prevalence while being sustainable and respectful towards the environment.

1.3 Fish production

The food fish consumption went from an average of 9 kg per capita in 1961 to 20.2 kg in 2015, peaking a total production of 171 tonnes in 2016 (FAO, 2018; Giuliani G. *et al*, 2004). Aquaculture, which is the farming of aquatic organisms, represents nearly half of the production and is the reason why fish production continues to impressively grow, the capture fishery being stagnant since the late 1980s (FAO, 2018). Fish is an important part of the general diet, representing 17% of animal proteins that were consumed by the world population in 2015 (FAO, 2018). They are also a source of fundamental nutrients, giving many health benefits in the prevention of cardiovascular diseases or mental health for instance (FAO, 2018). With the worrying situation of overfishing and its massive impacts on aquatic ecosystems, aquaculture can appear as an alternative to release the pressure put on ocean fisheries. However, the traditional flow through system is widely criticized due to several environmental impacts. Indeed, it releases a high amount of wastes that can be a source of eutrophication in watersheds if the effluents are not well treated (Somerville *et al.*, 2014; Martinez-Porchas & Martinez-Cordova, 2012). This water enrichment could lead to an excessive growth of macroalgae in coral reefs and other environmental perturbations (Somerville *et al.*, 2014). This can be due to overfeeding which results in a high amount of unconsumed feed and the decomposition of organic matter. From the total nitrogen that is given to the farmed organisms, only a fraction of it is trapped as biomass by those organisms, ranging from 20 to 50% (Martinez-Porchas & Martinez-

Cordova, 2012). The rest is thus incorporated into the sediments or into the water column while a part of it can also be discharged into the environment and cause adverse effects. Amongst the release of nitrogenous metabolites, ammonia is highly toxic to most of aquatic organisms (Martinez-Porchas & Martinez-Cordova, 2012).

Considering those negative aspects, closed loop systems have been developed, called recirculating aquaculture system (RAS), where the water is being re-used in the production. These systems can "effectively manage, collect and treat wastes that accumulate during fish growth and, under optimal conditions, do not require water replacement except to account for losses because of evaporation" (Schreier *et al.*, 2010, p.1). It can thus significantly reduce the water requirements while drastically increase the productivity per unit of land (Somerville *et al.*, 2014). The toxic products are being treated with the use of mechanical and biological filters, which limit the environmental impacts (Schreier *et al.*, 2010; Rakocy, 2012). Hence, RAS manage to combine high fish production with more sustainable methods and can thus be seen as an interesting eco-intensive agricultural practice (Delaide, 2017).

1.4 Aquaponics

1.4.1 What is aquaponics?

According to Somerville *et al.* (2014, p. 4): "Aquaponic is the integration of recirculating aquaculture and hydroponics in one production system. In an aquaponic unit, water from the fish tank cycles through filters, plant grow beds and then back to the fish". This method combines those two systems while limiting their respective disadvantages. Indeed, one of the major problems of all aquaculture systems, including RAS, is the waste management. Although RASs reduce the total volume of waste discharged and toxic products into the environment, the organic matter and dissolved nutrients per unit are actually increased (Rakocy, 2012). This is due to the farming of large quantities of fish in small volumes. While the water is being treated by removing toxic wastes, nontoxic nutrients and organic matter are accumulating since the water is being reused many times (Rakocy, 2012). Those highly concentrated effluents can threaten the environment if they are not well treated or represent an additional cost if they need further treatments (Rakocy, 2012). Concerning hydroponics, even though the water and nutrient managements are much more efficient than soil-based agriculture, this method still relies on fertilizers to operate. Those nutrients can come from manufactured and chemical sources which can be very expensive and often come from unsustainable practices (Somerville *et al.*, 2014).

Hence, using the aquaculture effluents as fertilizers for the plants not only represents a sustainable, non-chemical and low-price source of nutrients but it also allows not to discharge the aquaculture wastes into the environment (Somerville *et al.*, 2014). Thus, the wastes of one represent the needs of the other. Firstly, the solid fish wastes are being removed from the water by going through a mechanical filter. Then, the dissolved wastes, usually in the form of ammonia coming from the fish, are being processed by a biofilter which provides a medium for bacteria to convert ammonia to nitrite and then to nitrate (Somerville *et al.*, 2014). The ammonia, which is toxic for the fish, is thus converted into nitrate, which is the preferred form of nitrogen for the plants and is rather harmless (Rakocy, 2012). As the water flows through the plant grow beds, the crops uptake the nitrate and other nutrients which purifies the water, that can than go back to the fish tank. Compared to aquaculture systems, the nitrate accumulation in an aquaponic water can be reduced by up to 97 % (Rakocy, 2012). In this way, fish, plants and microorganisms live in symbiosis, providing a balanced system (Somerville *et al.*, 2014) (Fig.1).

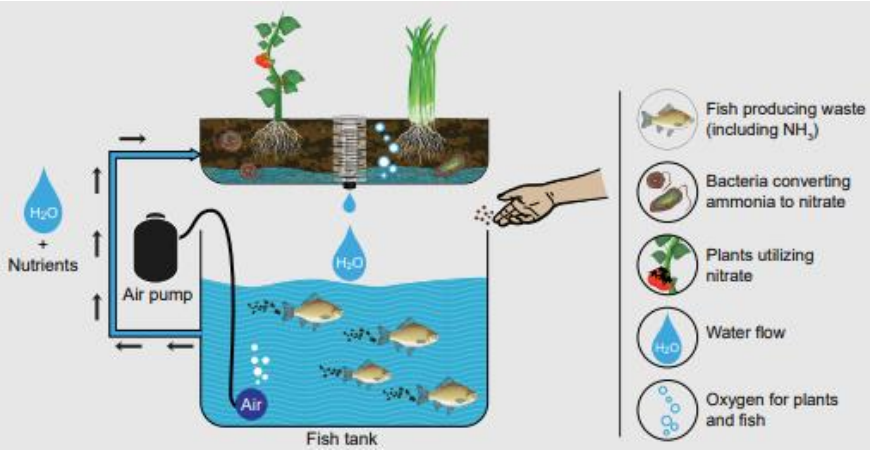


Figure 1 *The biological components in the aquaponic process: fish, plants and bacteria (Somerville et al., (2014))*

1.4.2 A brief history

The idea of using faeces and more particularly fish faeces as fertilizers for plants, is actually very old, existing in early civilizations of Asia and South America thousands of years ago. In Mexico, 1150-1350 BC, the Aztecs cultivated plants on islands in shallow lakes using wastes coming from surroundings cities and canals to irrigate and fertilize the plants (Turcios *et al.,* 2014). Indeed, the canals were used to produce fish which resulted in the accumulation of faeces at the bottom of the canals. Those discharges could then be collected and spread over the crops (Turcios *et al.,* 2014). Likewise, in South-East Asia 1500 years ago, fish effluents were channelled out to paddy rice fields (Goddek *et al.,* 2015). In the late 1970s and early 1980s, the New Alchemy Institute at the North Carolina State University developed the principles of modern aquaponics (Goddek *et al.,* 2015). In the following decades, the systems became productive and efficient thanks to technological improvements concerning the general design and biofiltration as well as better knowledge of the optimal conditions (Turcios *et al.,* 2014; Somerville *et al.,* 2014)

Today, aquaponics is mostly represented by small-scale units, providing food for domestic consumption but also serving as educational material, teaching sustainable agricultural techniques for schools and universities (Somerville *et al.,* 2014). Commercial aquaponics frequently use monocultural practices with large productions of lettuce and basil, the two main productions of commercial-scale aquaponics (Love *et al.,* 2015). However, those systems remain a minority since the profits are often too low comparing to the initial investment that can be extremely high (Somerville *et al.,* 2014). Indeed, besides all the advantages of aquaponics, this agriculture also has weak points.

1.4.3 Benefits and weaknesses

Aquaponics is a combination of two of the most productive systems in their fields. The production is thus intensive while using sustainable practices, meeting the increasing food demand as well as the environmental challenges (Goddek *et al.*, 2015). As it has already been discussed, the effluents of the fish are being used as a value-added product for the plants, only requiring fish-feed inputs and resulting in two very different agricultural products (Rakocy, 2012). If there is a good market for vegetables, transforming a RAS into an aquaponic system can thus significantly improve the profitability (Rakocy, 2012). Compared to conventional agriculture, aquaponics is extremely water-efficient, using less than 10% of water (Goddek *et al.*, 2015). Those systems can be developed within cities since it doesn't require soil or a lot of space, which adds the benefits of UA. It can also be extended to general locations where the soil-based farming is difficult or impossible such as deserts or degraded and salty soils (Somerville *et al.,* 2014). The control over the production is also higher, leading to fewer losses as well as lower risk of contaminations (Somerville *et al.,* 2014).

However, as it is a symbiotic system, a strong knowledge of fish, plants and microorganisms is needed as well as a daily management since the conditions have to be suitable for all the organisms. The

system can thus rapidly collapse if mistakes are being made or if the producer is not cautious enough (Goddek *et al.*, 2015; Somerville et al., 2014). Still in the aim of maintaining the symbiotic balance, pest and disease managements can also be more complicated since no pesticides and no antibiotics can be used, although this can also be seen as an advantage (Rakocy, 2012). The initial costs are also very expensive compared to soil productions or hydroponics and the energy needed to operate can be high in order to maintain stable conditions (Delaide *et al.*, 2017).

1.4.4 General components of aquaponic systems

Several common components are being shared within all aquaponic systems, that is, fish tank(s), a mechanical filter, a biofilter, some hydroponic containers and a pump that will allow the water circulation through all the compartments (Somerville *et al.*, 2014).

The **mechanical filter** removes the solid and suspended wastes from the fish tank, such as faeces and uneaten feed. This organic matter needs to be removed since its decomposition produces CO_2 and ammonia while decreasing the dissolved oxygen (DO) levels (Rakocy, 2012). If this matter accumulates in the bottom of the tank, it can also lead to anaerobic decay, producing harmful gases such as methane and hydrogen sulphide (Rakocy, 2012; Somerville *et al.*, 2014). If suspended solids enter the hydroponic compartment, they could also accumulate on the plant roots and create anaerobic area, killing the roots and blocking the nutrient absorption (Rakocy, 2012). However, their mineralisation also produces essential nutrients for the plants and the involved decomposing microorganisms are usually antagonistic to root pathogens (Rakocy, 2012). A challenging balance should thus be reached between insufficient and excessive suspended solids. Multiple mechanical filter methods exist such as sedimentation devices which are usually in the form of settling basins or tube separator. Filtration devices are also used, such as microscreen drum filters and bead filters (Somerville *et al.,* 2014; Racoky, 2012).

The **biofilter**, located after the mechanical filter, is a major component as it is the centre of the nitrification process. Indeed, thanks to nitrifying bacteria, the toxic ammonia excreted by the fish is being oxidized into nitrite, which is also toxic. This nitrite can then be oxidized into nitrate, a rather harmless form of nitrogen that is being uptaken by the plants (Rakocy, 2012). The aim of the biofilter is thus to have a media with a large surface area in order to promote bacteria growth via the formation of biofilms (Rakocy, 2012). In the form of biochips or beads for instance, assembled in a separate compartment, the biofilter can also be combined with the hydroponic compartment, using granular plant support media such as gravel (Somerville *et al.*, 2014; Rakocy, 2012). As some chemical processes need high levels of DO, a good oxygenation is required which can be achieve with the use of air pumps for instance (Rakocy, 2012).

Concerning the **hydroponic compartment**, three main types are commonly used in aquaponics: the media bed units where the crops grow within a substrate; the nutrient film technique (NFT) where the roots are in contact with both air and culture water, the water flowing inside wide pipes; and the deep water culture (DWC) units, also called floating-bed systems or raft aquaponics, where the plants are placed in floating platforms with holes, allowing the roots to be submerged in the culture water (Pattillo, 2017; Maucieri *et al.,* 2018; Somerville *et al.,* 2014) (Fig. 2). Other methods are used such as vertical growing systems or drip irrigation, where a drop by drop water culture is provided for each plant being placed in a substrate (Pattillo, 2017).

In media bed units, the media usually provides enough surface area for the filtration and the bacteria growth, making this system suitable for developing countries as it the simplest method, not requiring any separate biofilter or mechanical filter (Wongkiew *et al.*, 2017). The NFT usually produces higher yields, as the roots are well oxygenated, and induce less evapotranspiration loses. However, an efficient solid removal is crucial for this method, as the pipes must not get clogged and the nutrient-rich water flow needs to be constant (Wongkiew *et al.*, 2017). Similarly, this technique is not suitable for big vegetables since their roots could block the water flow. In this way, DWC are often used in more commercial scale, growing only one type of crop, as the roots can freely have access to a large volume of culture water, without blocking the flow (Wongkiew *et al.*, 2017; Love *et al.,* 2015; Somerville *et al.*, 2014).

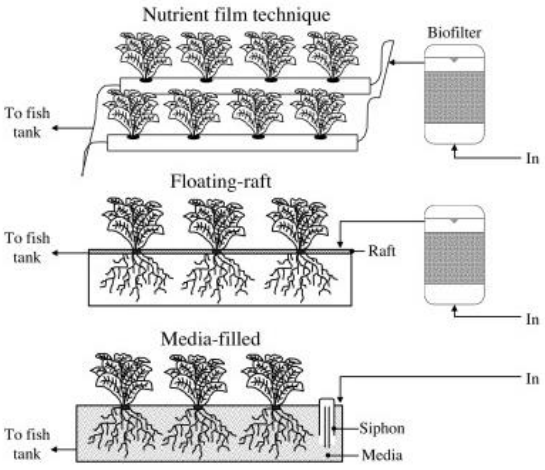


***Figure 2**: The three main types of hydroponic systems in aquaponics (Wongkiew et al., 2017)*

Hence, the type of hydroponic compartment appears as a way of characterizing and classifying an aquaponic system.

1.4.5 The main actors

Aquaponic systems are based on a balance between three major actors: the fish, the plants and the microorganisms. What are these living groups made of and what are their characteristics?

1.4.5.1 Fish

Given that plant growth is usually inhibited by high salinity, most cultivated fish are freshwater species that also fit well with aquaponics because they can easily tolerate crowding (Wongkiew *et al.*, 2017; Rakocy, 2012). However, some of them are most commonly used, as they show great growth rate in aquaponic systems, such as tilapia, carp, catfish, trout or largemouth bass (Somerville *et al.*, 2014). Well adapted to this kind of agriculture, tilapia is by far the most used species. Indeed, their growing cycle is short (6-9 months), they resist to many pathogens and they have good tolerance towards big fluctuations in water quality and low DO (Jones, 2002; Somerville *et al.*, 2014). Mostly produced and consumed in Asia, South America and Africa, the European market is slowly growing due to its ease of cultivation and attractive price, particularly for the Nile tilapia (*Oreochromis niloticus)* (FAO, 2018). Given that their ideal temperature is between 27 and 30°C and that the market in Europe is still small, mixing several species throughout the year could be more profitable and energy-efficient, using tilapia only in the warm season for instance (Somerville *et al.*, 2014; Jones, 2002).

In an ideal aquaponic system, the feeds should be the only nitrogen inputs. Having an important role, the feeds composition typically contains carbohydrates and lipids for the source of energy, proteins to bring essential amino acids and to build the fish biomass as well as minerals and vitamins that are fundamental for the fish health (Somerville et al., 2014). However, the exact components and their proportions depend on the nature of the fish, whether they are omnivorous, carnivorous or herbivorous (Bittsanszky *et al.*, 2016). The feed conversion ratio (FCR) can be a useful parameter as it represents the efficiency of the fish to convert feed into body weight (Somerville *et al.,* 2014). Once again, tilapia culture is advantageous as its FCR is quite low (1.4-1.8) meaning only 1.4-1.8 kg are required to grow 1 kg of tilapia (Somerville *et al.,* 2014). In term of outputs, fish discharge wastes in several pathways. Firstly, some ammonia is being passively diffused in the water from their gills (Wongkiew *et al.*, 2017; Somerville *et al.,* 2014). Secondly, the vents of freshwater fish excrete big volumes of diluted urine as well as faeces, containing 10 to 40% of nitrogen content in the form of ammonia, amino acids and proteins (Somerville *et al.,* 2014; Wongkiew *et al.*, 2017).

1.4.5.2 Plants

Many types of plants have been successfully grown in aquaponics. The main condition is for the culture to induce the highest income per unit area per unit of time (Rakocy, 2012). In this way, the best option is culinary herbs, such as basil or cilantro, growing rapidly and having a very high market price (Rakocy, 2012). On the other hand, leafy greens usually fit very well to this type of agriculture, lettuce being one of the main crops cultivated in aquaponics (Rakocy, 2012). Indeed, their growing cycle is very short (usually 4 weeks in the system) which lowers the pest pressure and their demand in nutrients is low (Rakocy, 2012; Somerville *et al.*, 2014). Fruiting crops such as tomatoes or cucumbers are also used in aquaponics but they require a longer growing period (more than 90 days) subjecting them to higher pest pressure, their edible proportion of harvested biomass is lower and they are high-nutrient demanding (Rakocy, 2012; Somerville *et al.*, 2014).

1.4.5.3 Microorganisms

Last but not least: the microorganisms. Establishing the bridge between fish and plants, this third group is a key actor and multiple aspects can be discussed. It will thus be explored in a separate section.

1.5 Focus on microorganisms

Mainly composed of *Eubacteria*, *Fungi* and *Archae*, this group is extremely diversified and has multiple functions (Somerville *et al.,* 2014). In our case, we will focus on *Eubacteria* and *Fungi*. Furthermore, studies that not only focus on the taxonomic diversity of those microorganisms in the general system but also on the diversity between different compartments within an AP system and the diversity between types of AP systems are also very interesting, as they expand the general knowledge and possible improvements of aquaponics.

1.5.1 Characterisation of the microbiota

In order to characterise this large group, a subdivision into several functional groups can be made.

1.5.1.1 Nitrifying bacteria

The nitrifying bacteria have been the most studied group in AP since they are responsible for the crucial process of nitrification (Schmautz *et al.,* 2017). During that 2-step aerobic process, the total ammonium nitrogen (TAN) is firstly oxidized into nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB) such as *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* sp., all of them coming from the *Proteobacteria* phylum (Wongkiew *et al.*, 2017; Schmautz *et al.*, 2017). Some *Archaebacteria* also carry out this transformation but they do not seem to have a role in AP systems as they mainly occur in extremely nitrogen-poor environment (Wongkiew *et al.*, 2017). TAN is the summation of unionized ammonia (NH_3) and ionized ammonia (NH_4^+) which are both in equilibrium in the water, their respective proportion depending on the ambient pH and temperature (Wongkiew *et al.*, 2017). As previously mentioned, this TAN comes either directly from the fish gills, or from the hydrolysis of organic nitrogen, e.g. fish urine and faeces (Wongkiew *et al.*, 2017). Once the first nitrification step is completed, the resulting nitrite (NO_2^-) is oxidized into nitrate (NO_3^-) by nitrite oxidizing bacteria (NOB) such as *Nitrobacter*, *Nitrococcus*, *Nitrospira* and *Nitrospina* spp. (Wongkiew *et al.*, 2017). Besides, some *Nitrospira* population can actually achieve the complete two-steps nitrate transformation, thus belonging to the complete ammonia oxidizers (COMAMMOX) (Pinto *et al.*, 2016).

In general, AOB and NOB can be found in the biofilter as it is primarily designed for nitrification by promoting the development of biofilm (Wongkiew *et al.*, 2017). However, they can also be found on the surface of plant roots in AP systems (Schmautz *et al.,* 2017; Wongkiew *et al.*, 2017).

The nitrifying bacteria also named autotrophic bacteria in AP, i.e. using inorganic forms of carbon (CO_2 and bicarbonate), can be distinguished from the heterotrophic bacteria (Wongkiew *et al.*, 2017).

1.5.1.2 Heterotrophic bacteria

Fish wastes that are not released in the form of ammonia are a mixture of proteins, lipids, carbohydrates, minerals and vitamins. A large amount of solid wastes thus remains a certain amount of time in the AP system, composed of those faeces as well as uneaten food, plant residues and dead microorganisms (Somerville *et al.*, 2014). Thanks to heterotrophic bacteria, this organic matter undergoes an important decomposition process known as mineralisation, also named solubilisation in more recent reports. It releases the nutrients that were being detained in solid wastes, which is vital for the plants as they can only uptake dissolved nutrients (Somerville *et al.*, 2014). "In particular, phosphorus, calcium, magnesium, and most of the micronutrients are not bioavailable and must be mineralized prior to delivery in hydroponics systems" (Delaide, 2017, p. 61). In that way, systems in which this process is enhanced could be very interesting for plant growth as more essential nutrients would be made available. Goddek *et al.* (2016, p. 2) have studied decoupled AP systems, "where fish, plants and, if applicable, remineralization are integrated as separate functional units comprising individual water cycles that can be controlled independently". Improving mineralisation from fish sludge via heterotrophic bacteria, can thus ameliorate the sustainability of AP systems as the resource utilization is being optimized (Goddek *et al.*, 2016).

Those bacteria can be found in all compartments but evidently accumulate in area highly concentrated in solid wastes, such as mechanical filters or canals (Somerville *et al.*, 2014). In that way, they coexist with autotrophic bacteria, becoming predominant when concentration in organic matter or C:N ratio is high (Wongkiew *et al.*, 2017). However, their development can be at the expense of nitrifying bacteria. Indeed, heterotrophic bacteria's growth rate is higher, and their development might block the oxygen access to the nitrifying bacteria, as they can both accumulate in the form of biofilm and heterotrophic bacteria rapidly develop on outer layers (Rurangwa & Verdegem, 2013). Thus, the organic matter content in biofilter is usually limited in order to maintain a good nitrification process (Rurangwa & Verdegem, 2013). Heterotrophic bacteria can also be a source of nitrogen loss when gaining in importance as they use NH_4^+ and NO_3^- for their cell growth (Wongkiew *et al.*, 2017). A balance should thus be maintained between those two types of bacteria.

Although their most well-known role is the mineralisation and/or solubilisation process, some of them could also have positive biocontrol effects on plants, by occupying niches and competing with pathogens or by being identified as potential plant growth promoting microorganisms (PGPM) (Schmautz *et al.*, 2017). "In soil-based environments, PGPMs are known to enhance plant growth via a number of mechanisms, including: nitrogen fixation, organic matter mineralization, root growth promotion, protection against pathogens, and increasing the bioavailability of nutrients, including micronutrients such as iron. In soilless environments, PGPM research is limited, but existing studies suggest PGPMs also play a significant role in plant growth and health" (Bartelme *et al.,* 2018, p. 2). In hydroponics, genera such as *Bacillus*, *Enterobacter* and *Streptomyces* have been identified as potential PGPM (Bartelme *et al.,* 2018). In Schmautz *et al.* (2017) experiment, they reported a high proportion of *Pseudomonas spp* on the surface of plant roots, a large group able to compete for nutrients as well as produce antimicrobial and antifungal compounds (Stouvenakers *et al.,* 2019). These modes of action are also found in the *Bacillus* genus. Moreover, *Pseudomonas* and *Bacillus* can indirectly bio stimulate the plants or elicit plants defences (Stouvenakers *et al.,* 2019). Besides, those two genera were also be found in RAS and AP biofilters (Rurangwa & Verdegem, 2013; Munguia-Fragozo *et al.*, 2015). However, members of those same groups can also be identified as plant pathogens, e.g. *Pseudomonas syringae* pathovars.

Thus, studying the microorganisms located on the surface and in the internal part of plants roots, two separate ecological niches respectively named rhizoplane and endosphere, seem very interesting. Indeed, some plant-associated microbes, called endophytes, are able to penetrate into the tissues and live within the plant (Goel *et al.*, 2017). Bacterial endophytes can actually be used for biocontrol since phytopathogens reside in similar niches, but they are also known as "possible useful sources of

bioactive secondary metabolites and as medicinally important agents in agriculture and industries as well" (Goel *et al.*, 2017, p. 169).

The potential as well as challenges surrounding heterotrophic bacteria are large, making them an important part of the microbial communities.

1.5.1.3 Unwanted bacteria

Not all bacteria have beneficial roles in AP systems and their diversity can also be explored.

Firstly, denitrifying bacteria can be a source of nitrogen loss for the plants as they transform the nitrate into atmospheric nitrogen, i.e. N_2 (Somerville *et al.,* 2014). Growing in anaerobic conditions, these bacteria can mostly be a problem in the case of deep water grow beds, where the large surfaces are not well oxygenated (Somerville *et al.,* 2014). This process can be achieved by several *Archae* members and facultative heterotrophic bacteria such as members of *Flavobacterium, Pseudomonas, Protus, Micrococcus, Acinetobacter, Bacillus, Brevibacterium, Achromobacter, Aerobacter* genera (Wongkiew *et al.*, 2017). Schmautz *et al.* (2017) also found the presumed denitrifiers *Dokdonella* and *Thermomonas* in AP.

A second pathway of nitrogen loss can occur via anaerobic ammonium-oxidizing bacteria (ANAMMOX), transforming ammonia and nitrite into N_2O and N_2 gases (Eck *et al.*, 2019). They can coexist with AOB in oxygen-depleted zones of AP biofilters (Wongkiew *et al.*, 2017). In a marine RAS biofilter, "three genera of anammox planctomycetales (*Brocadia, Kuenenia and Scalindua*) were identified" (Rurangwa & Verdegem, 2013, p. 119). Likewise, three species of anammox *Nitrosomonas* (*N. aestuarii, N. marina, N. oligotropha*) were found in RAS (Munguia-Fragozo *et al.*, 2015).

Pathogenic bacteria can also be present in the system. Some bacteria associated to fish pathogenicity can be found in the biofilter, e.g. strains of *Bacillus* (e.g. *B. cereus, B. mycoides, B. subtilis*), *Pseudomonas* (e.g. *P. anguilliseptica, P. aeruginosa, P. fluorescens*), *Aeromonas* (e.g. *A. hydrophyla, A. salmonicida*), *Flavobacterium* (e.g. *F. psychrophilum, F. columnare*) and *Vibrio* (e.g. *V. alginolyticus, V. anguillarum*) (Munguia-Fragozo *et al.,* 2015; Schneider et al., 2007; Austin B. & Austin DA., 2007). Some of the fish or human pathogens are also used as indicators for safe products and water quality, i.e. *Escherichia coli* and *Salmonella spp.,* as they typically occur during faecal contamination of warm-blooded animals such as birds and mice (Munguia-Fragozo *et al.,* 2015). Focusing on plants, some root pathogens are well adapted to the continuous water environment found in AP systems such as members of *Ralstonia*, *Pseudomonas*, as already mentioned, *Erwinia*, *Xanthomonas* and *Clavibacter* (Stouvenakers *et al.,* 2019)

Another group of unwanted bacteria is made of the sulphate reducing bacteria. Using sulphur throughout an oxidoreduction process in order to obtain energy, they produce H_2S in anoxic conditions, which is very toxic to fish (Somerville *et al*, 2014). For instance, members of *Flavobacteriales* and *Bacteroides plebeius* have been found in RAS (Munguia-Fragozo *et al.*, 2015). The sulphate reducing bacteria occur when excessive solid wastes are present in the system compared to mineralizing bacteria, thus creating anaerobic festering conditions which increase their development (Somerville *et al*, 2014). This phenomenon is responsible for the particular smell of rotten egg, thus becoming a sign of their expansion (Somerville *et al*, 2014).

Likewise, some bacteria can be responsible for off flavours in the fish. They are caused by the accumulation of two secondary metabolites: geosmin and 2-methylisoborneol (Rurangwa & Verdegem, 2013). These compounds are mainly produced by members of *Cyanobacteria*, *Myxococcales*, *Actinomycetes* and some fungi. More particularly, *Streptomyces* (*Actinomycetes*), *Sorangium* and *Nannocystis* (*Myxococcales*) as well as *Micromonospora* species have been found in RAS and are suspected to induce those off-flavour (Rurangwa & Verdegem, 2013).

1.5.1.4 Fungi

It is known that fungi are a part of the microbial communities of AP, having certain functions and performing some processes. However, very few studies mention their presence and/or roles which makes it very difficult to characterise this group. According to Somerville *et al.* (2014, p. 77), "naturally occurring fungi help decompose the solid portion of the fish waste", thus participating to the mineralisation and/or solubilisation process. In RAS, fungi have also been associated with assimilatory nitrate reduction with the example of *Aspergillus niger* NBG5, removing "ammonium, nitrite and protein at low temperature but shifting to metabolize carbon at high temperature" (Rurangwa & Verdegem, 2013, p. 119), this process being confirmed in Munguia-Fragozo *et al.* (2015) review. As mentioned previously, fungi could also be responsible for off flavours in fish products (Rurangwa & Verdegem, 2013).

They are also associated to plant pathogenicity, some of them being very well adapted to the water environment found in AP. This is particularly the case for the pseudo-fungi *Oomyctes,* such as members of *Pythium (*e.g. *P. dissocotum* and *P. myriotylum* in lettuces) and *Phytophtora* genera, producing motile zoospores and causing root rot diseases (Stouvenakers *et al.,* 2019). Concerning "real" fungi, some members of the *Fusarium*, *Colletotrichum*, *Rhizoctonia* and *Verticillium* genera can induce several plant damages on the leaves, fruits and stem for instance (Stouvenakers *et al.,* 2019).

Some fungi are also identified as plant growth promoting fungi (PGPF). Indeed, arbuscular mycorrhizal fungi, who are well-documented in soil-based cultures, appeared to have positive impacts on the health of hydroponic plants, preventing the development of tomato root rot for instance, caused by *Fusarium oxysporum* (Bartelme *et al.,* 2018). Likewise, members of *Gliocladium* and *Trichoderma* genera have also been identified as PGPF (Bartelme *et al.,* 2018)

In conclusion, fungi do seem to play significant roles in AP although they are very little studied to date.

1.5.2 Microbial diversity within AP systems

The microbiota characterisation showed how diverse the microorganisms of AP could be, in term of taxonomy and functionalities but it also pointed the link between certain types of microorganisms and the place in which they were mostly found. Indeed, the various components of AP systems offer different kind of micro-environments for the microbial communities, depending on nutrients concentrations and oxygen levels for instance (Munguia-Fragozo *et al.*, 2015). This could result in the development of different microbiota according to the system area. From this perspective, Schmautz *et al.* (2017) studied the microbial diversity in four different compartments of an AP system: the biofilter, the plant roots, the fish faeces and the periphyton. The Graph at Figure 3 shows this diversity.

It resulted that the biofilter, plant roots and periphyton essentially shared the same phyla, but in different proportion. The fish faeces on the other hand, appeared as a separate ecosystem, with much less diversity and a dominance of *Fusobacteria* as well as other gut-type bacteria. The plant roots seemed to have specific root-associated bacteria, some of them being exclusively found on this sample and others being identified as potential PGPM. Thus, it seems like each of the compartment has its specific microbiota which could be linked to its physiological role in that specific niche sample. However, Schmautz *et al.* (2017) also suggest that, in order to establish this link, further experiments should be made, linking the microbial communities to system and biochemical parameters.

Finally, Schmautz *et al.* (2017, p. 618) pointed that the dominance of some bacteria discovered in specific compartments "might be correlated with a difference in the basic setup of the AP system", which leads us to our next point.

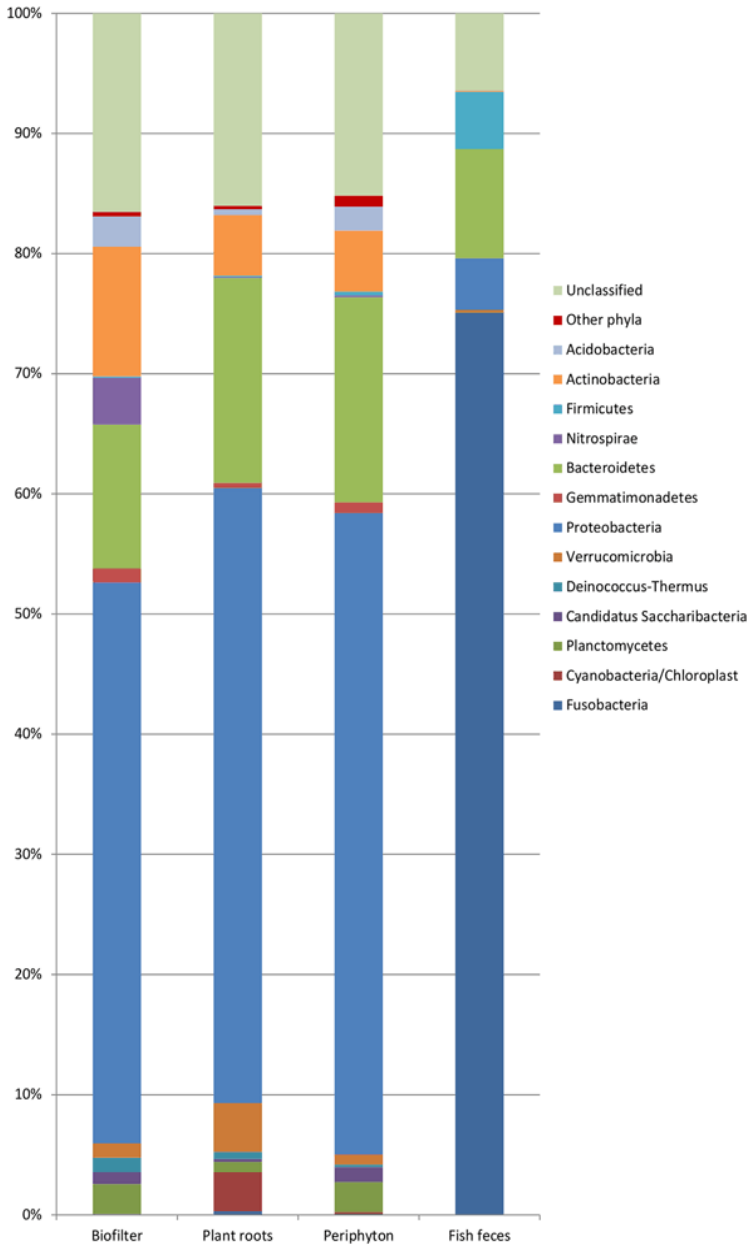


Figure 3 *Classification of reads from biofilter, plant roots, periphyton and fish faeces to level phylum indicated as percentage of the population (Schmautz et al., 2017)*

1.5.3 Microbial diversity between AP systems

Multiple system designs exist with a certain combination of plant and fish species. Studying and comparing the microbial communities between distinct systems thus seems interesting, as a potential link could be done between them as well as a better understanding of the functionalities of each microbial group. Furthermore, RAS are often used as references when describing AP microbial communities, as modern AP is a very recent method and they share many characteristics (Rurangwa & Verdegem, 2013; Munguia-Fragozo *et al.*, 2015). However, a direct comparison between similar RAS and AP system seems useful as it could help understand the potential influence of plants in this kind of systems. In this regard, Eck *et al.* (2019, p. 1) "compared different aquaculture and AP systems, which differ in terms of plant and fish species and/or feed type. AP designs included both "coupled" or closed loop AP (one loop containing fish and plants) and "decoupled" or open loop AP systems (two separate loops for fish and plants)". To this purpose, the bacteria of biofilter and water samples were analysed and compared.

As a result, it appeared that the bacterial communities were specific to each system, even when the systems where similar in terms of general design and setup. However, the five aquaponic systems had more bacteria in common than with the three aquaculture systems, showing that AP plants have an influence on the bacterial communities and that "not yet identified conditions are specific to AP systems, independently of the setup" (Eck *et al.*, 2019, p. 9). That was confirmed with the detailed comparison of a RAS and AP system, highly comparable as they were similar in "size, fish, feed type and incoming water quality" (Eck *et al.*, 2019, p. 10), both developed at the University of Gembloux Agro-Bio Tech (Gembloux, Belgium). The graph at Figure 4 shows this comparison in term of identified families and genera.

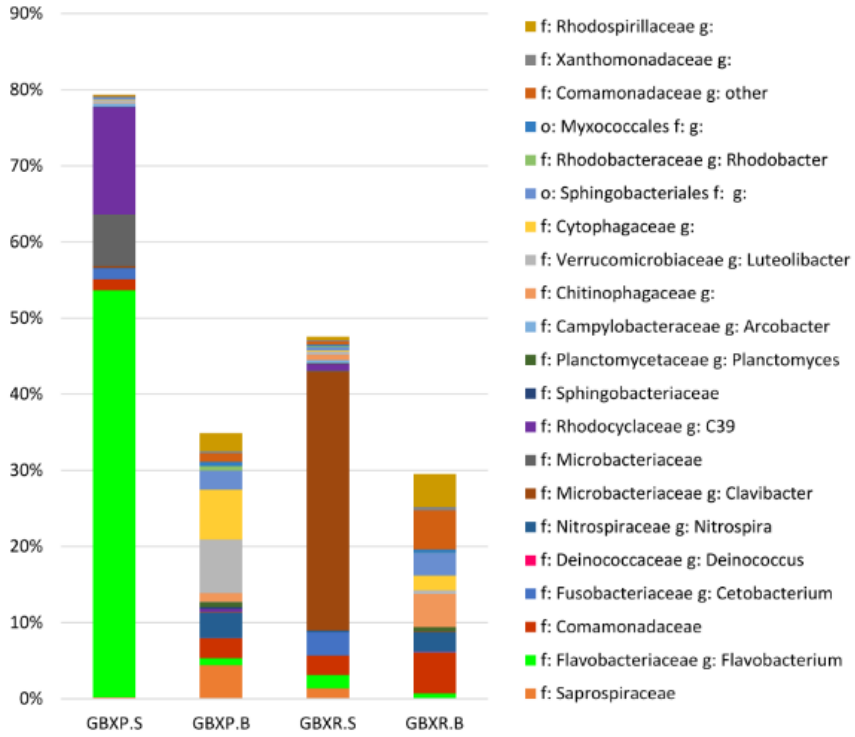


Figure 4 *Bar chart showing the relative abundances of the families and genera representing more than 1% of the total reads within each sample, for the biofilter and sump samples of the RAS (GBXR) and plant and fish farming (PAFF) Box systems (GBXP) of Gembloux Agro-Bio Tech. The groups representing less than 1% are not shown on this graph.*

This graph shows that the two types of samples, i.e. biofilter and water, both had specific microbial communities at the genus and family levels even the two systems were highly comparable.

Finally, Eck *et al.* (2019) suggested that further research could be conducted in order to better understand the role of each bacterial community and the biological processes within a single system, by following the microbiota over time and in more compartments.

1.5.4 Studying the microbiota

1.5.4.1 High throughput sequencing

The study of microbial communities has evolved tremendously, from the isolation of single specimen and laboratory cultivation to the actual genomic analyses. The first major improvement occurred in the late 1970s, with the development of the "Sanger sequencing method" (Heather & Chain, 2016). It consisted in mixing deoxyribonucleotides (dNTPs) with a small fraction of fluorescently or radio labelled dideoxynucleotides (ddNTPs), i.e. dNTPs lacking the 3'hydroxyl group required for the

extension of the DNA molecule, into a DNA polymerisation reaction (Heather & Chain, 2016). Performed in parallel for the four types of ddNTPs, it resulted in the formation of DNA strands of all possible length with an ultimate labelled nucleotide, as the ddNTPs were introduced randomly in the DNA chain (Heather & Chain, 2016). The four resulting reactions could then be run on a polyacrylamide gel in order to separate the fragments by size, followed by a visualization of the DNA bands using autoradiography or UV light. This could thus show "what the nucleotide sequence in the original template was, as there was a radioactive band in the corresponding lane at that position of the gel" (Heather & Chain, 2016, p. 2-3).

Since then, massive advances have been made in term of parallel sequencing, leading to the term of "high throughput sequencing" (HTS). Although several HTS techniques exist, the most important one is by far the Illumina technology, due to its high accuracy (Shokralla *et al.*, 2012). Among the advances, the polymerase chain reaction (PCR) developed in the 1980s allowed the development of HTS by providing high concentrations of DNA fragments (Heather & Chain, 2016). In practice, the HTS principles are similar to the "first generation sequencing". Indeed, "DNA polymerase catalyses the incorporation of fluorescently labelled dNTPS into DNA template strand during sequential cycles of DNA synthesis. During each cycle, at the point of incorporation, the nucleotides are identified by fluorophore excitation" (Illumina, 2016, p. 4). The major difference is that this process is extended to millions of fragments at the same time and not only for one fragment (Illumina, 2016). Thus, in addition to a drastic reduction in duration and cost, HTS allowed to sequence samples having a mixture of DNA coming from a variety of specimens, and not only from one microorganism (Shokralla *et al.,* 2012). In this way, "by comparing obtained sequences to a growing standard reference library of known organisms, taxa present in an environmental sample can be identified with high confidence" (Shokralla *et al.*, 2012, p. 1795). This data analysis can be achieved with the use of bioinformatic softwares such as QIIME (quantitative insight into microbial ecology).

1.5.4.2 Species specific regions

In order to identify microorganisms without having to sequence their entire genome, genomic regions that were species-specific have been identified (Shokralla *et al.*, 2012). More specifically, the 16S ribosomal RNA gene is typically used for the identification of bacteria as it contains 9 hypervariable and 9 conserved regions (Nikolaki & Tsiamis, 2013). Indeed, the hypervariable regions differ between the taxa while being surrounded by very conserved regions which allows the design of PCR primers (Nikolaki & Tsiamis, 2013). Those broad-range 16S rDNA primers can then bind to the conserved areas and amplify the variable regions, distinguishing the different bacteria taxa (Nikolaki & Tsiamis, 2013). They are specifically used in microbiome research and more than a hundred different primers exist (Klindworth *et al.*, 2013; Patel *et al.*, 2017). Likewise, the noncoding internal transcribed spacer (ITS) region of the ribosomal DNA is the most used locus for the identification of fungi (Guo, 2010; Nilsson *et al.*, 2008). Indeed, this region shows variability at the genus or species level, going up to intraspecific differentiation in some cases (Guo, 2010; Nilsson *et al.*, 2008).

1.5.4.3 Diversity indices

Once the sequencing completed and the microorganisms or operational taxonomic unit (OTU) identified, diversity indices can be used to describe the microbial communities.

In order to characterise the diversity observed within a certain community or sample, alpha-diversity indices are used. Firstly, the species richness of a sample can be estimated via the number of observed OTUs, i.e. count of unique OTU in each sample, or via Chao 1 richness estimator for instances. This non-parametric estimator calculates the minimal number of OTUs in each sample, which estimates the diversity from abundance data (Lemos *et al.*, 2011). Only considering the species richness of a sample would give a strong importance to rare OTUs, which discriminates the more abundant species. Thus, the evenness can be taken into account, referring to how evenly the OTUs present in the sample are distributed (Kvålseth, 2015). Several indices incorporate both the richness and the evenness of a sample, such as Shannon and Simpson indices. In this case, the calculated diversity value increases both when the number of OTUs and the evenness increases. More particularly, the Shannon index

"measures the average degree of uncertainty in predicting as to what species an individual chosen at random from a collection of S species and N individuals will belong" (Lemos *et al.*, 2011, p. 2). For its part, the Simpson index indicates the "species dominance and reflects the probability of two individuals that belong to the same species being randomly chosen" (Lemos *et al.*, 2011, p. 2). Hence, the Shannon index is more influenced by the rare OTUs while the Simpson index is more influenced by the abundance or dominance of the OTUs, which is the reason why we often refer as the "Shannon richness estimation" and the "Simpson dominance estimation" (Lemos *et al.,* 2011). Another alpha-diversity estimator that can be used is the phylogenetic diversity (PD), considering the phylogeny of the OTUs in order to value the diversity across the phylogenetic tree (Faith & Baker, 2007). For instance, if all OTUs present in the samples are closely related, the PD will be low.

On another hand, the beta-diversity is used to study the diversity observed among samples. To this end, the phylogenetic tree can also be considered, using the UniFrac distances. UniFrac measures the difference between two samples "as the amount of evolutionary history that is unique to either of the two, which is measured as the fraction of branch length in a phylogenetic tree that leads to descendants of one sample or the other, but not both" (Lozupone *et al.*, 2011). This measure is actually called "unweighted UniFrac", only considering the presence or absence of lineage. However, another version exists, named "weighed UniFrac" that directly consider the differences in relative abundances of observed OTUs (Lozupone *et al.*, 2011). Hence, the 2nd version is quantitative while the 1st version is qualitative. Afterwards, these distances can be used in order to cluster several samples, using multivariate statistical analyses.

In conclusion, both beta and alpha-diversity indices can be used in order to characterise microbial communities among samples or within samples.

1.6 Crucial parameters in AP systems

We talked about the diversity and characteristics of the three main actors that compose the symbiosis of AP systems. However, in order to further understand them and optimize the AP system, we need to focus on what they need and/or tolerate.

1.6.1 Water quality

Water is one of the most important points in AP. It is the medium in which all the nutrients are being transported to the plants, establishing a pathway between the fish and hydroponic compartments, as well as hosting all the microbial communities (Somerville *et al.*, 2014). Understanding the main water quality parameters is thus essential as all of them have an important impact on each of the three main groups of organisms. Dealing with a tripartite symbiosis implies to work in the tolerance range of each organism. Table 1 is a literature review, showing all the optimal parameters for each of the organisms and possibly, the resulting compromise. It focuses on two commonly used species in AP: lettuces (*Lactuca sativa*) and Nile tilapia (*Oreochromis Niloticus*).

1.6.1.1 Oxygen

All three organisms need oxygen in order to live. In water, this parameter can be expressed as dissolved oxygen (DO) levels, describing "the amount of molecular oxygen within the water, measured in mg/L" (Somerville *et al.,* 2014, p. 22). It has immediate effects on AP, as the fish may die within a few hours if the DO is insufficient. The nitrification process also needs high DO levels at all times since it is an aerobic reaction (Rakocy, 2012). Likewise, the plants roots need oxygen as a part of the respiration process and to avoid root-rot (Somerville *et al.*, 2014). The simple process in which the oxygen from the air dissolves into the water surface is sufficient in natural environments. However, in intensive farming system, fish densities are higher which requires water pumps, aerators and other methods to produce water movements and aerate (Somerville *et al.,* 2014). Additionally, the temperature needs to be monitored as it reduces the oxygen solubility when rising (Somerville *et al.,* 2014). The optimum DO for the three types of organisms are shown in Table 1.

1.6.1.2 pH

pH has major impacts on plants and bacteria of AP. This parameter controls the accessibility of all nutrients to the plants. For instance, above 7.5, plants can go through some "nutrient lockout", meaning that the nutrient is present in the system, but the crops are not able to utilize it (Fig. 5) (Rakocy, 2012; Somerville *et al.,* 2014). As well, nitrifying bacteria activity is reduced in acid conditions, below a pH of 6, which might induce an increase in ammonia in the system (Wongkiew et al., 2018; Somerville *et al.*, 2014). Two main processes can slowly lower the pH of the water: the nitrification that releases H^+ ions through the process; the fish respiration that releases CO_2 which is naturally converted into carbonic acid (H_2CO_3) in the water (Somerville *et al.,* 2014).

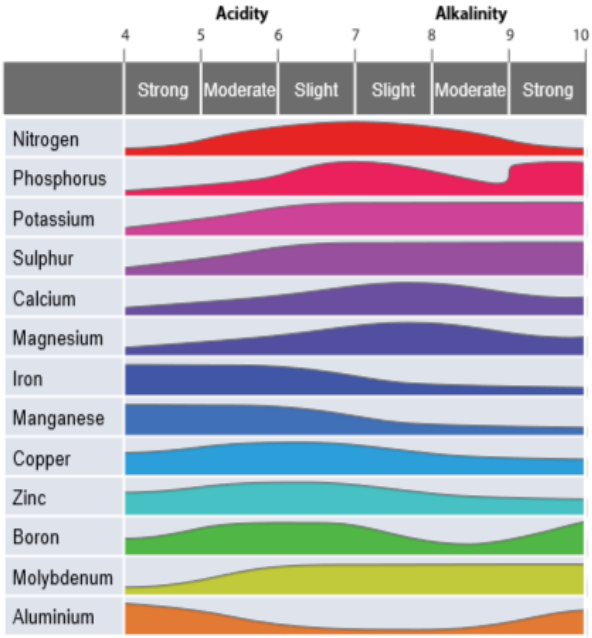


***Figure 5**: The impact of pH on nutrient availability for plants (Somerville et al., 2014)*

1.6.1.3 Temperature

The optimal temperature for a system highly depends on the chosen fish and plant species, minimal fluctuations being preferable for an efficient system. In addition to the effects on DO, temperature also influences the ionization of ammonia. Indeed, if it rises, toxic unionized ammonia increases too (Somerville *et al.,* 2014).

1.6.1.4 Total nitrogen

Nitrogen enters the AP system via fish feed, usually through proteins. It is then excreted in the form of ammonia (NH_3) which can then be oxidized into nitrite (NO_2^-) and nitrate (NO_3^-), as already explained. While remaining in the water, NH_3 can also binds with ambient H^+, depending on the pH, and create ammonium (NH_4^+), the less toxic form of ammonia (Wongkiew *et al.*, 2017). But first of all, why is NH_3 toxic to fish? Fish can excrete this component via passive diffusion from their gills into the water. Thus, if surrounding levels of NH_3 are high, this diffusion won't happen as easily, leading to a potential accumulation in the blood flow and negative impacts on the fish organs (Rakocy, 2012; Somerville *et al.,* 2014). Nitrite on another issue, is also toxic to fish as it can inhibit the oxygen transport in the blood. In that way, "ammonia and nitrite are approximatively 100 times more poisonous than nitrate" (Somerville *et al.,* 2014, p. 25) and their concentrations should thus be kept close to zero. However, too much nitrate could also lead to unreasonable vegetative growth and excessive concentrations of nitrate in the leaves (Somerville *et al.,* 2014).

1.6.2 Plants' nutrients requirements

Plants need inorganic salts in order to grow, reproduce and complete photosynthesis. Those minerals can be divided into two main categories: the macronutrients and the micronutrients. The macronutrients are needed in much greater quantities than the micronutrients which are required only in trace amounts, but both categories are essential (Somerville *et al.,* 2014). The total amount

of dissolved nutrients can be measured "as the capacity of nutrient solution to conduct an electrical current (EC)" (Rakocy, 2012, p. 368), usually expressed in microSiemens per centimetre (µS/cm).

1.6.2.1 Macronutrients

There are 6 required macronutrients: nitrogen (N), phosphorus (P) and potassium (K), which are considered as primary nutrients; and sulphur (S), magnesium (Mg) and calcium (Ca), that are considered as secondary nutrients (Bittsanszky *et al.,* 2016). Among them, nitrogen is the most important one for plant growth, used for the synthesis of amino acids and proteins (Licamele, 2009). Phosphorus is crucial for the synthesis of nucleic acids, ATP and phospholipid membranes for instance (Licamele, 2009). Potassium is involved in many processes such as cell signalling, stomatic opening, water use and disease resistance. On the other hand, magnesium plays major role in photosynthesis, calcium in cell structures and sulphur in the production of proteins such as chlorophyll (Somerville *et al.*, 2014). AP systems can often face K, P or Ca deficiencies, requiring some supplemental fertilizers. However, crops with short growing cycle that do not produce fruits such as lettuce, usually do not need those additions (Bittsanszky *et al.,* 2016). The optimal values of macronutrients are shown in Table 1 for *Lactuca sativa*.

1.6.2.2 Micronutrients

Micronutrients are composed of iron (Fe), manganese (Mn), boron (B), zinc (Zn), copper (Cu) and molybdenum (Mo) (Bittsanszky *et al.,* 2016). Iron is by far the most critical micronutrients as it is the most frequent deficiency in AP due to its limited presence in fish feed and his reduced availability at a pH superior to 7 (Bartelme *et al.,* 2018; Somerville *et al.*, 2014). In this case, intervenous chlorosis on young leaves can appear since this mineral is crucial for proper photosynthesis (Licamele, 2009).

Tableau 1: Optimal parameters for the three types of organisms in AP

Parameter	Optimal value				Source	Type of system
	Plant *Lactuca sativa*	Fish *Tilapia nila*	Bacteria	Compromise		
pH	6-7	6,5 - 8,5	6-8,5	6-7	Somerville *et al.*, 2014	Aquaponics
	5,5-6,5				Resh, 2013	Hydroponics
			7-7,8		Licamele, 2009	Aquaponics
		7-8,0			Graber & Junge, 2009	Aquaculture
Electro-conductivity (µS/cm)				< 1500	Somerville *et al.*, 2014	Aquaponics
	1500-2000				Resh, 2013	Hydroponics
		< 1200			Graber & Junge, 2009	RAS
	1000-2000				Licamele, 2009	Aquaponics
Dissolved oxygen (mg/L)	> 3	4-6	4-8	> 5	Somerville *et al.*, 2014	Aquaponics
		4-6	> 1		Goddek *et al.*, 2016	RAS
		> 2	> 2		Licamele, 2009	Aquaponics
Temperature (°C)				25	Delaide *et al.*, 2017	Aquaponics
	16-30	27-30	14-34	27- 30	Somerville *et al.*, 2014	Aquaponics
		27-30			El-Sayed, 2006	RAS
	21-25	28-35	20-30		Licamele, 2009	Aquaponics
Ammonia nitrogen (mg/L)	< 30 (TAN)	< 3 (TAN)	< 3 (TAN)	< 1 (TAN)	Somerville *et al.*, 2014	Aquaponics
		< 1 (TAN)			Al-Hafedh *et al.*, 2003	RAS
		< 0,1 (NH_3-N)			El-Sayed, 2006	RAS
	25 (NH_4^+)				Resh, 2013	Hydroponics
Nitrite (mg/L)	< 1	< 1	< 1	< 1	Somerville *et al.*, 2014	Aquaponics
		< 0,5 (NO_2^--N)			Al-Hafedh *et al.*, 2003	RAS
		< 0,2 (NO_2^--N)			Graber & Junge, 2009	RAS
Nitrate (mg/L)				120 (NO_3 - N)	Schmautz *et al.*, 2017	Aquaponics
		< 400	< 400	5-150	Somerville *et al.*, 2014	Aquaponics
	165 (NO_3^-)				Resh, 2013	Hydroponics
		< 150 (NO_3-N)			Graber & Junge, 2009	RAS
Phosphate (mg/L)				35 (PO_4- P)	Schmautz *et al.*, 2017	Aquaponics
	50				Resh, 2013	Hydroponics
	35-80				Licamele, 2009	Hydroponics
Potassium (mg/L)				150	Schmautz *et al.*, 2017	Aquaponics
	210				Resh, 2013	Hydroponics
Magnesium (mg/L)				40	Schmautz *et al.*, 2017	Aquaponics
				60-140	Somerville *et al.*, 2014	Aquaponics
	40				Resh, 2013	Hydroponics
Sulfate (mg/L)	113 (SO_4^{2-})				Resh, 2013	Hydroponics
Calcium (mg/L)				200	Schmautz *et al.*, 2017	Aquaponics
				60-140	Somerville *et al.*, 2014	Aquaponics
	200				Resh, 2013	Hydroponics
Iron (mg/L)				3	Schmautz *et al.*, 2017	Aquaponics
	5				Resh, 2013	Hydroponics

As a conclusion, nutrients are important parameters to monitor in AP as they have direct impacts on the crops and consequently, on the tripartite symbiosis made of fish, plants and microbial communities. Hence, nutrients and water quality parameters appear as key factors to the characterisation and evolution of the microbiota of an AP system. However, as Bittsanszky *et al.* (2016, p. 19) mentioned, "very little is known about the nutrient requirements and potential sensitivities of the microbial communities to variations in the availability of nutrients". Similarly, the potential impacts of variations in water quality parameters on the microbiota of an AP system are rather unknown.

2. Problematic and aims of the experiment

We saw the crucial role of microorganisms in aquaponics, establishing the bridge between fish and plants.

The aim of this experiment is to analyse the bacterial and fungal communities of an aquaponic system over time, starting at the introduction of plants into the system. The main goal is to study the stability of the microbiota of such systems over a period of time, while following important parameters, e.g. water quality, nutrients and plants introduction, in order to possibly draw tendencies and potentially correlate parameters variations to microbiota variations.

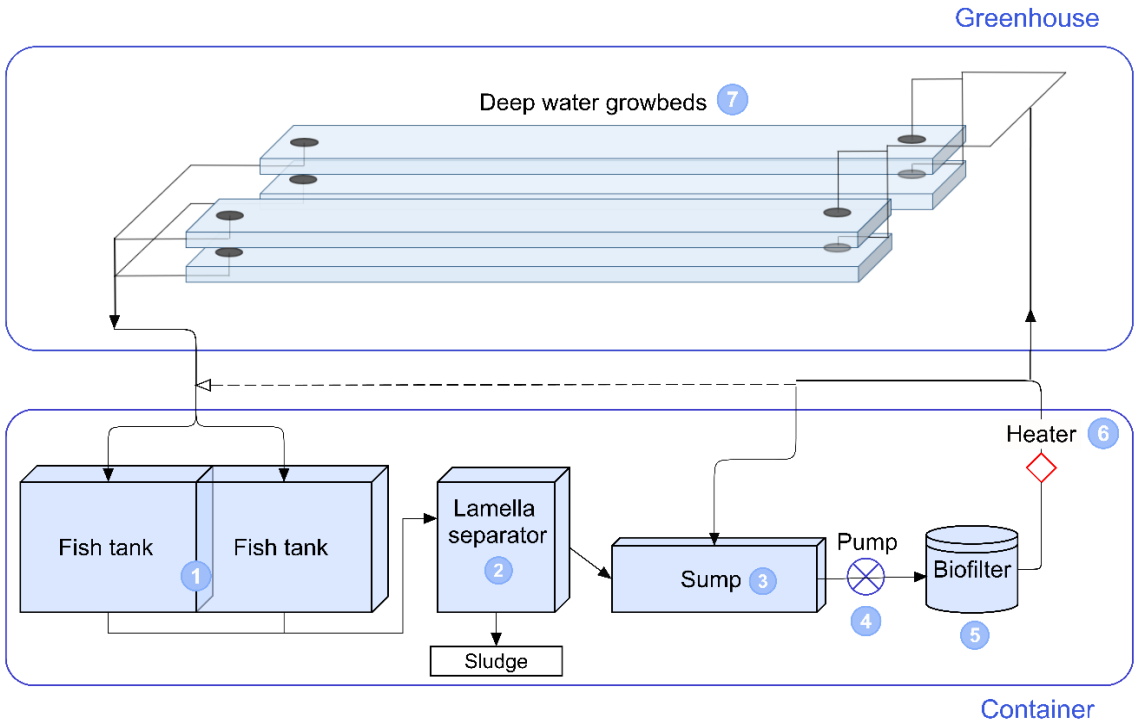
Different compartments will be sampled in order to have a more complete view of the microbiota which adds the possibility to compare the communities between separate microniches and expand the already existing knowledge. Among the microniches, plant roots will be studied at the rhizoplane and endopshere levels which can be interesting in several ways. Being the main centre of potential PGPM, comparisons between the microbiota of plants from different growth stages can also be made in order to further understand the relationship between microbial communities and plants.

In addition, as knowledge in fungi present in AP are very limited, this study also aims to discover, characterize and further understand the possible roles of these organisms in AP. Concerning bacteria, it aims to further research and characterise the bacterial communities present in an aquaponic system.

3. Materials and Methods

3.1 Description of the aquaponic system

The experiment was conducted in the small scale aquaponic system of the Integrated and Urban Pathology Laboratory (IUPPL) of Gembloux Agro-Bio Tech (Gembloux, Belgium), named the Plant and Fish Farming box (PAFF Box). As explained in detail in Delaide *et al.* (2017) experiment, the system consisted in a steel container carrying two fish tanks, a mechanical filter (lamella separator), a sump and a biofilter, topped with a greenhouse containing the hydroponic units, i.e. deep water grow beds. Thanks to a pump with permanent flow (1.2 m^3/h), the water could move continuously from the fish tanks to the hydroponic compartment, going through the sump and the two filters. After the biofilter, the water could also return to the sump, the lowest point of the system that was maintaining the water lever constant. It was also the compartment in which water and nutrients were added. A heater ensured constant water temperature. The system also had the possibility to be converted into a RAS by making the water flow back to the fish tanks after going through the biofilter. Figure 6 summarizes the functioning of the PAFF Box and provides additional technical information, based on Delaide *et al.* (2017) and Delhaye (2015).



Dimension and details

Container: 5.72 x 2.17 x 2.21 m, volume of 27.43 m^3

Greenhouse (Euro-Maxi, Euroserre, Genk, Belgium): 6 x 2.40 x 2.40 m, north-south oriented with 4 windows of 0.70 x 0.90 m

1. Fish tank: 1.06 x 1.06 x 0.42 m, water volume of 380L
2. Lamella separator (Landing Aquaculture, LZ Oirschot, Netherlands): total volume of 320L
3. Sump: 1.14 x 0.94 x 0.72 m
4. Pump (Pro-jet SE 20/8 Tri, Aquatic science, B-4040 Herstal, Belgium): power of 0.55 Kw, regulated by an electric flow variator (VARFLW001B, Aquatic science, B-4040 Herstal, Belgium)
5. Biofilter : microbead filter: 0.115 m^3 water volume with 0.07 m^3 of microbeads (SHARK BEAD 45/25, Aquatic science, Herstal, Belgium)
6. Heater (Elecro Engineering Ltd, Hertfordshire, England): 230 V, 3kW
7. Deep water grow beds: 2 x 0.275 m^3 of water volume, 4.23 x 0.50 x 0.20 m, 65 plant holes and 2 x 0.345 m^3 , 5.31 x 0.50 x 0.20 m, 83 plant holes; 20 cm between holes; 300L/h of water flow per bed
   Total system water volume: 2.673 m^3
   Total land area: 12.41 m^2

***Figure 6**: Functioning of the PAFF Box and technical information*

3.2 Experimental setup and collection of the samples

The experiment started on March 11, 2019 and ended 9 weeks later, on May 13. The main objective was to follow the evolution over time of the microbiota from the PAFF Box after the introduction of lettuces in the aquaponic system. The samples collected for the isolation of bacterial and fungal communities were taken from 4 different compartments of the system: 1) the water entering the sump, after it went through the biofilter; 2) the biofilter itself; 3) the lettuce rhizoplane, i.e. the root surface; 4) the lettuce endosphere. The sampled plants were followed over their 6-week lifecycle.

Prior to the experiment, the PAFF Box had been running for three months without plants, from mid-December 2018 to the end of February 2019. The box was operating as a RAS, using only the container compartment. The biofilter was maintained thanks to the presence of 34 Nile Tilapia (*Oreochromis niloticus L.*) in the two tanks. The exact number of fish in each of the two tanks was uncertain and each tank would receive 100 g of feed once a day. Furthermore, the hydroponic compartment went through a fallow period during which the deep water grow beds were entirely emptied, cleaned and bleached. At the end of February 2019, 1000 litres of tap water were added to the hydroponic compartment which was then re-joined to the system. The day before the beginning of the experiment, on March 10, half of the fish were weighed, resulting in a mean weight per fish of 0.754 kg. They were then redistributed evenly in the two tanks and were fed thrice a day with an average feed weight per tank per day of 156.5 grams throughout the entire experiment. The feed (Trouw Nutrition, Putten, Netherlands) was plant based and animal based (processed animal protein from poultry and fish meal) and contained 30% of crude protein, 6% of crude fat, 6% of ash, 4% of crude fibre, 1% of calcium, 0.3% of sodium and 0.9% of phosphorus. It also contained the following micronutrients: E1 iron (42 mg/kg), E2 iodine (2.1 mg/kg), E4 copper (5mg/kg), E5 manganese (16 mg/kg), E6 zinc (100 mg/kg).

3.2.1 The first 6 weeks of the experiment

The experiment started on March 11, 2019, as soon as lettuce seedlings were introduced into the system. Hence, 100 seeds of Butterhead lettuce (*Lactuca sativa L., 1753 var. Lucrecia,* Rijk Zwaan) were sowed on February 27 in a climate and light controlled greenhouse, 11 days before the start of the experiment. As Resh (2013) recommends, the 100 seeds were placed in rockwool plugs (36 x 40 x 40 mm, Grodan ROCKWOOL B.V., Roermond, Netherlands), one seed being placed in one plug. In order to soak, the plugs were disposed in shallow boxes, halfway filled with tap water.

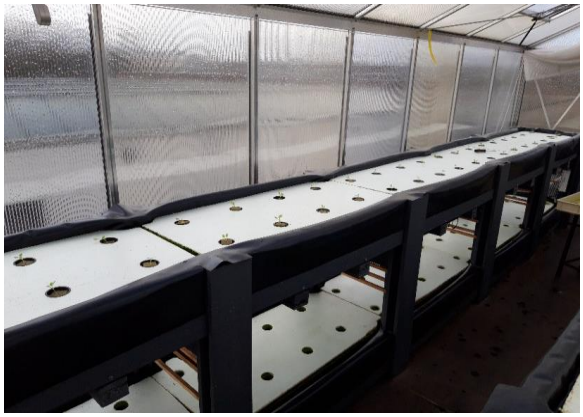


***Figure 7**: Introduction of 80 lettuce seedlings in the top grow beds of the hydroponic compartment of the PAFF Box, on March 11, 2019*

On March 11, after 11 days of germination, 80 seedlings at their 3 leaf-stage were transferred with their rockwool plugs in the two top grow beds of the hydroponic compartment, receiving the most sunlight, to begin the experiment (Fig. 7). Then, 10 of the remaining seedlings were used for the first root sampling for which the preparation is described hereafter. On the same day, the water and biofilter samples were also collected and prepared as explained below. In this way, the 4 types of sample were collected and prepared 9 times throughout the first 6 weeks of the experiment: on March 11, March 15, March 19, March 22, March 26, March 29 (twice a week) and on April 5, April 13 and April 19 (once a week). The samples were taken at higher frequency during the first 3 weeks as the system was expected to undergo important changes right after the introduction of plants, transitioning from a RAS to an aquaponic system. On the last sampling, on April 19, the shoot fresh biomasses of the sampled lettuces were weighed.

As the root samplings were progressively eliminating lettuces of the system, starting from 80 plants in the PAFF Box on the first day to zero on the last day of the first 6 weeks, other lettuces were added once a week to the deep water grow beds throughout the experiment. Those lettuces were not supposed to be sampled but were maintaining a general aquaponic system. Indeed, 14 seedlings at their 3 leaf-stage from the same variety were added to the system on March 11. Likewise, 30 plants were added on March 18, 30 plants on March 25, 30 plants on April 3 and 12 plants on April 9. Those lettuces were sowed and germinated in the same manner as the sampled group of plants.

3.2.2 The last 3 weeks of the experiment

The experiment was supposed to end after 6 weeks in order to fully reach the lettuce maturity (Resh, 2013). However, the initial monitored group of plants (Group 1) started to look weak and diseased after 5 weeks in the system (Fig. 8 and Fig. 9). Thus, the rhizoplane and root endosphere samples from April 13 and April 19 (week 5 and 6) were not studied. In order to still have data for mature lettuces, the 30 plants that had been placed in the PAFF Box on April 3 were followed and sampled. Indeed, at the end of April, this group of lettuces (Group 2) had the same age as the initial group, right before the lettuces started to look diseased, i.e. 4 weeks old. This 2nd group was thus sampled in its 4th, 5th and 6th week of growth cycle, that is, on April 29, May 7 and May 13, 2019. The shoot fresh biomasses of the sampled lettuces were also weighed on May 13. The 4th week sample acted as a comparative element with the sample of the initial group of plant. The following timeline summarizes those roots samplings for the rhizoplane and endosphere samples, according to the age of the plants (Fig. 10).

***Figure 8**: Shoot of a diseased-looking plant of the initial group of lettuces, on March 19, 2019*

***Figure 9**: Roots of a diseased-looking plant of the initial group of lettuces, on March 19, 2019*

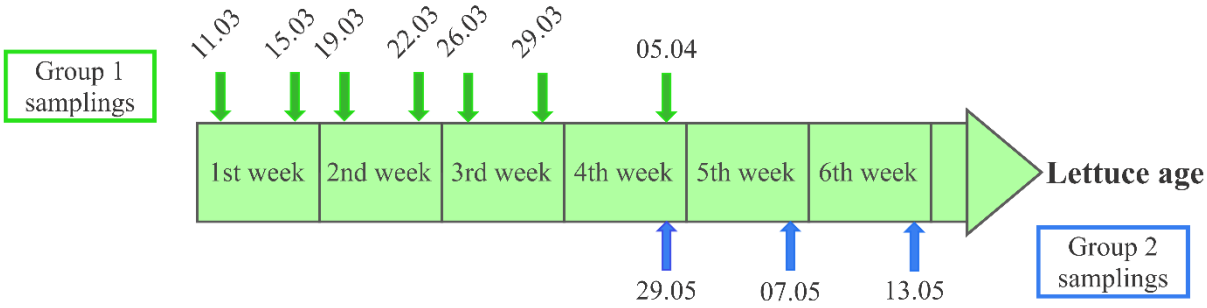


***Figure 10**: Diagram summing the samplings of the lettuce roots according to the age of the plants*

Samples of the water and biofilter were also taken on April 29, May 7 and May 13, 2019. In order to check the representativeness of the samples, i.e. assess if one single sample is representative of the compartment, 4 samples of water as well as 4 samples of biofilter were collected and prepared on April 29.

A timeline in Fig. 12 summarizes the chronology of all the sample collections as well as additional measurements and events.

3.2.3 Comparison of microbiota of lettuces roots at different ages

On April 29, the hydroponic compartment of the PAFF Box contained 5 groups of lettuces at different ages: a 3-weeks-old group (12 lettuces), a 4-weeks-old group (30 lettuces), a 5-weeks-old group (30 lettuces), a 6-week-old group (30 lettuces) and a 7-week-old group (14 lettuces). Thus, a root sample of each age was taken on April 29, in order to compare the microbiota of the rhizoplane and endosphere from lettuces of different ages at a given time. In the aim of testing the representativeness of those two types of samples, 4 root samples of the 7-week-old lettuces were taken and prepared for both of them. The shoot fresh biomasses of all the sampled lettuces were also weighed.

3.3 Samples preparation

The water, biofilter, rhizoplane and endosphere samples were prepared on the same days as their collection, in the IUPPL of Gembloux Agro-Bio Tech, situated right next to the PAFF Box.

3.3.1 Lettuce rhizoplane sample

The method used in order to characterise the microbiota present on the surface of lettuce roots was based on unpublished protocol intern to the laboratory. With a target root weight of 2 grams per sample, on March 11, the entire young root systems of 10 seedlings were taken entirely for the first sample in the aim of having the most biomass, the seedlings being very light weighted. It resulted in a total biomass of 0.30 grams. This bulking of 10 lettuces into one sample was then maintained for all the other samples.

Figure 11 *Shattered rockwool plugs with a 3-week-old lettuce, on April 29, 2019*

In detail, for each sample, 10 rockwool plugs were brought to the laboratory and carefully shattered under a sterile bench (Fig. 11). When the lettuces were young with lightweight root systems, all the roots and root hairs present inside or outside the rockwool plugs were cut with a sterile scalpel and weighed. When the lettuces were older with bigger root systems likely exceeding 2 grams, roots of different kinds that were inside or outside the rockwool were taken in order to have 0.2 grams per plant, resulting in a total weight of 2 grams. This method was used with the aim of getting close to the collection of entire root systems which was done when the lettuces were young, and not simply take root parts that were external to the rockwool support. Indeed, this last process would have brought a likely significant bias.

Once the roots were taken and weighed, they were then put in a Falcon tube of 50 mL containing 30 mL of sterile 0.05 M Potassium Phosphate Buffer, pH 6.5, containing 0.005% of Tween-80 (KPBT 0.005%, tw 80). The tube was then put in an ultrasonic bath (Ultrasonic cleaner, model USC600T, VWR, Leuven, Belgium) for 10 min. After removal of all the roots with a sterile pliers, the solution was filtered through a sterile cheesecloth in another sterile Falcon tube of 50 mL, in order to get rid of rockwool residues and small pieces of roots. The roots were then placed in a third Falcon tube of 50 mL containing 10 mL of sterile KPBT (0.005 %, tw 80) which was vortexed for 30 sec with the aim of rinsing the roots one last time. After the removal of all the roots, the 10 mL solution was filtered through another sterile cheesecloth and transferred to the Falcon tube already containing the 30 mL of washed solution.

In the aim of conserving this 40 ml sample for later DNA extraction, the Falcon tube was centrifuged 20 min at 4053 g. A volume of 6 ml of washed solution including the pellet were then taken and put into a 15 ml sterile Falcon tube. A volume of 2.5 ml of glycerol were then added to the solution in order to have 30% of glycerol. The Falcon tube was then rapidly vortexed before being instant frozen with liquid nitrogen and kept at -80° Celsius.

3.3.2 Root endosphere sample

For each roots sample, the roots and root hairs set aside during the preparation of the rhizoplane sample were directly put in a 15 mL sterile Falcon tube which was then instant frozen with liquid nitrogen before being kept at -80° Celsius, in order to be preserve until further preparation.

With the aim of efficiently extract DNA, the roots were rapidly defrosted by being placed in a 55° Celsius chamber (Thermoshake, Gerhardt GmbH & Co., Königswinter, Germany) for 5 min. The roots were then placed in a mesh bag (Linaris Biologische Produkte GmbH, Dosenheim, Germany) with a certain volume of KPBT (0.005 %, tw 80) according to the ratio of 1 g of roots for 9 ml of buffer (Ioannidis *et al.*, 2018). The roots were grinded and homogenised in the mesh bag using a tissue grinder. The mashed solution was then collected and filtered through a sterile cheesecloth in a 50 ml Falcon tube, in order to get rid of leftover root pieces. Glycerol was then added to the solution at a ratio of 30% in order to be conserved at -20° Celsius until DNA extraction.

The roots were not sterilised beforehand to make sure that the remaining microorganisms on the roots surface that had not been collected in the washing solution were not eliminated. Keeping a broad spectrum, this allowed not to lose data. Thus, the so-called "endosphere sample" included some rhizoplane microorganisms.

3.3.3 Water sample

The method used was based on Eck *et al.* (2019) experiment. For each sample, two litres of water were collected from the sump in sterile Pyrex bottles. "In order to concentrate the microorganisms quantity, the samples were filtered through 0.2 µm filters (PALL Life Science Super ® - 200 47 mm diameter) with a vacuum pump and vacuum flask" (Eck *et al.,* 2019, p 262). Once saturated, each filter was placed in a 50 ml sterile Falcon tube containing 30 ml of sterile KPBT (0.005 %, tw 80). This buffer was used instead of sterile water in order to employ the same buffer as the rhizoplane sample. The falcon was then vortexed for about 4 min to detach particles and microorganisms from the filters which could then be removed with a sterile pliers.

This biofilter sample then underwent the same preparation as the rhizoplane sample in order to be conserved and preserved at -80° Celsius.

3.3.4 Biofilter sample

The method used was also based on Eck *et al.* (2019) experiment. Indeed, for each sample of the biofilter, 15 grams of beads were placed in a 50 mL sterile Falcon tube containing 30 mL of KPBT (0.005 %, tw 80). The tube was then vortexed for 2 min before being placed in the ultrasonic bath for 5 min to detach microorganisms. The solution could then be transferred into another Falcon tube of 50 mL without the beads.

This biofilter sample then underwent the same preparation as the rhizoplane sample and water sample in order to be conserved and preserved at -80° Celsius.

3.3.5 Contamination test

In order to assess the potential contamination of the samples that could occur during later DNA extraction and sequencing, an external "pure culture" sterility test was carried out. Hence, a suspension of *Pseudomonas chlororaphis* (ATCC® 55670 ™) at 10^4 cfu/mL in $MgSO_4$ 0.1M with 25% of glycerol was firstly conserved at -20°C. Then, the suspension was rapidly defrosted by being placed 5 min in a 55° C chamber (Thermoshake, Gerhardt GmbH & Co., Königswinter, Germany) before being inoculated in TSB (Tryptic Soy Broth), a liquid enrichment medium. After 72h of incubation at 25°C, the liquid was centrifuged at 3000g for 10 min. The pellet was retrieved and washed twice

with $MgSO_4$. The DNA concentration of this *P. chlororaphis* – $MgSO_4$ suspension was then estimated using the Nanodrop (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA). A dilution was made in order to attain a concentration of 10^8 cfu/mL. The suspension could then be kept at -20°C until DNA extraction.

3.4 Water and nutrient management

Although the system is a closed loop, daily water loses occur via evapotranspiration, evaporation, spillage and water exchanges. For instance, each biofilter sampling required to empty some water in order to get access to the beads. Tap water was thus added regularly into the sump, the amount being measured with a flow meter. In order to partially clean the biofilter, a backwash was made on March 14 and May 10, which corresponds to an important water withdrawal followed by a refill in tap or rainwater. With the aim of increasing the iron and nitrate concentrations in the system, 1L of FeEDTA 13% (12g of iron) was added to the water both on April 5 and April 26 and 50g of KNO_3 were added on April 25. As the pH was too high, a total volume of 1L of HNO_3 (13%) was added over 3 days, starting on April 30. Likewise, another 1L of HNO_3 (13%) was added over 4 days, from May 3 to May 7. The timeline in Fig. 12 summarizes those supplements.

Water exchanges, nutrients additions as well as events and remarks are shown in the 7.1 Annex 1.

3.5 Parameters monitoring

Several parameters were recorded and analysed throughout the experiment in order to follow the general evolution of the PAFF Box and potentially correlate microbiota variations to parameters variations.

The temperature (T), pH, dissolved oxygen (DO) and electro-conductivity (EC) of the circulating water were measured with an aquarium probe (IKS Aquastar Industrial Version 2.28, IKS ComputerSysteme GmbH, Karlsbad, Germany). From March 8 to April 4, the four parameters were manually recorded in a heterogeneous frequency, ranging from two times per day to one time in 5 days, as a problem occurred and the probe didn't save the data. From April 5 to April 25, the T, pH and EC were measured every 15 min. During that period, the probe didn't save the DO data. From April 26 to the end of the experiment, on May 13, the four parameters were measured two times per day, at 12 am and 12 pm.

In order to follow the macronutrients content in the recirculating water throughout the experiment, water was collected and analysed on the same day or a day before each sampling, before the fish were fed. Measurements were also made before the introduction of lettuces in the system, on March 6 and March 8. The macronutrients concentrations were measured with a multiparameter spectrophotometer (HI 83200, HANNA instruments, Woonsocket, RI, USA) using the following reagents: HI 93700 for the TAN (NH_3, NH_4^+, N-NH_3), HI 93700-01 for the nitrite (NO_2^-); HI 93713 for the phosphate (PO_4^{3-}), HI 93751 for the sulphate (SO_4^{2-}), HI 93750 for the potassium (K^+), HI 93752 for the calcium (Ca^{2+}), HI 93752 for the magnesium (Mg^{2+}). On May 13, a repeatability test was also made on the recirculating water for each reagent (3 repetitions).

Nitrate (NO_3^-) on the other hand, was measured every 15 min with an optical sensor (TriOS Optical sensor, TriOS Messund Datentechnik GmbH, Rastede, Germany) throughout the entire experiment, from March 8 to May 13, 2019.

The air humidity and temperature in the greenhouse of the hydroponic compartment were measured every 30 min with a USB datalogger (MOINEAU Instruments, Chef-Boutonne, France).

The following figure shows the general timeline of the experiment, starting from the seedling of the group 1 to the harvesting of the group 2 lettuces (Fig. 12).

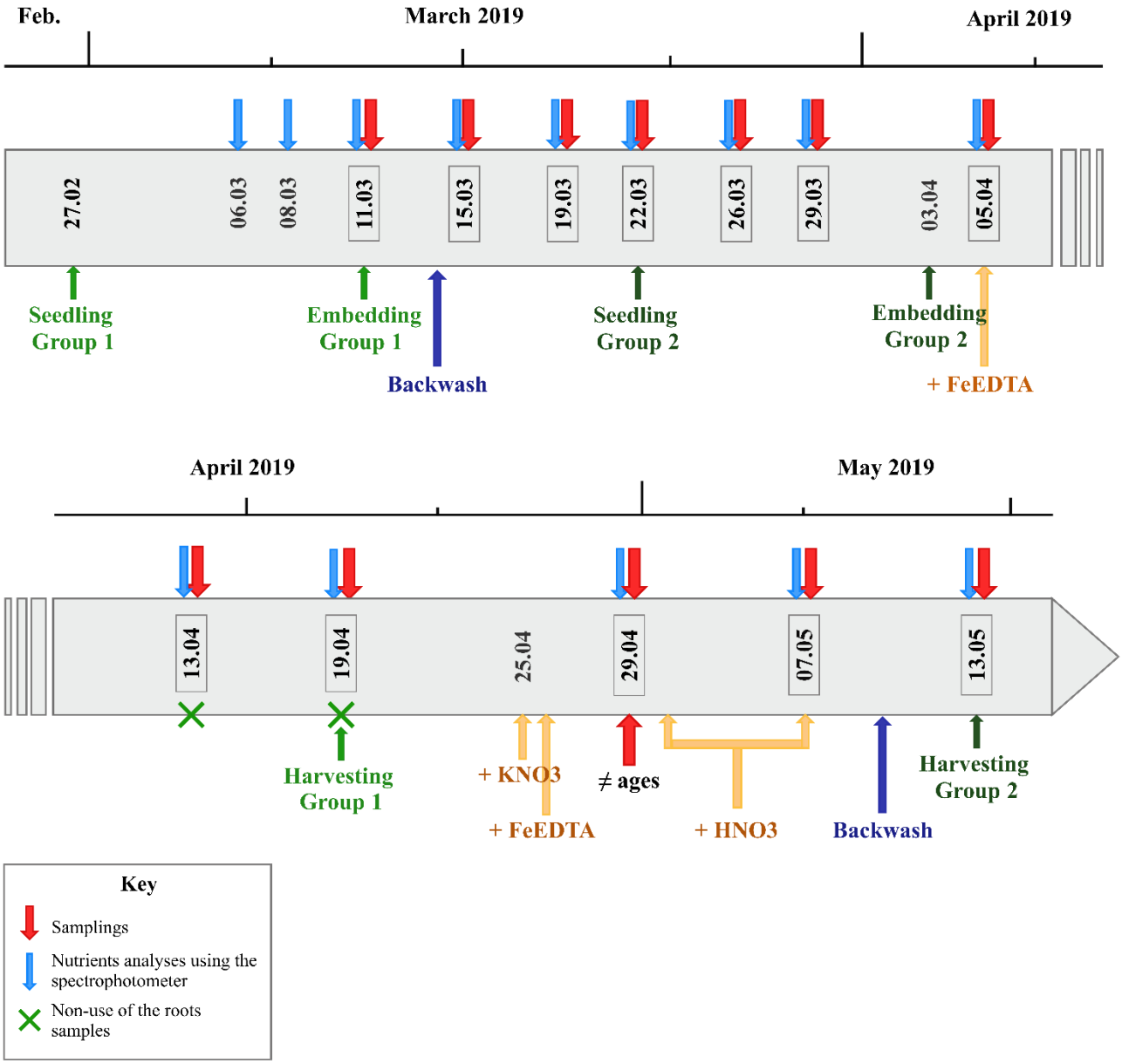


Figure 12 *Timeline of the experiment*

3.6 Parameters statistical analysis

With the purpose of potentially correlate parameters variations to microbiota variations, physicochemical data of the circulating water that were measured every day or at high frequency throughout the experiment, i.e. T, pH, EC and nitrate, were statistically analysed, having enough data for robust tests. Since all those parameters impacted the microbiota simultaneously, analysing the variations of each parameter independently seemed improper. Thus, the aim of the statistical analysis was to combine the four parameters into a more general "environment", i.e. constituted of the T, pH, EC and nitrate factors, and to analyse the potential variations of this environment. To do so, a hierarchical clustering analysis followed by a principal component analysis (PCA) were made, using the RStudio software (version 1.2.1335) and FactoMineR package (version 1.41), via R v3.3.2 language. This enabled to reduce the number of variables, i.e. parameters, while differentiate each date of the experiment depending on the physicochemical parameters and create clusters of highly homogenous dates. Those physicochemical clusters could then be characterised and used as a factor in the microbiota analysis since each sampling date could be associated to a certain physicochemical group. The detailed procedure of the clusters' identification, their validation and the PCA is described in 7.2 Annex 2.

3.7 DNA extraction

In order to prepare the 66 samples and the "pure culture" suspension that were being kept at -80° C or -20° C for DNA extraction, a rapid defrosting was made by placing them in a 55° Celsius chamber (Thermoshake, Gerhardt GmbH & Co., Königswinter, Germany) for 5 min. The water, biofilter, rhizoplane samples and "pure culture" suspension were firstly vortexed for 15 sec and centrifuged 20 min at 2350 g in order to use the pellet for DNA extraction. On the other hand, the endosphere sample, i.e. grinded roots with buffer, was used as is, with no centrifugation. Concerning the "pure culture" suspension, three repetitions were carried out.

The extractions were conducted following the method of Eck *et al.* (2019) using the Fast DNA Spin Kit (MP Biomedicals) utilizing Cell Lysis Solution TC (protocol in 7.3 Annex 3). Before being kept at -20° C, the samples' DNA concentrations were estimated using the Nanodrop (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA), which also provided the 260/280 and 260/280 ratios of absorbance. Those data were used for the quality control required for the sequencing process, as explained in the following section.

3.8 Sequencing

The bacteria and fungi sequencing of each sample were conducted following the DNA Vision's protocol (7.4 Annex 4) (DNA Vision S.A. company, Gosselies, Belgium) that uses the MiSeq Illumina technology. The first two steps, which were the quality control and the amplicons preparation, were carried out in the IUPPL of Gembloux Agro-Bio Tech while the rest of the process was directly performed at DNA Vision S.A.

The first two steps were conducted as follow:

Step 1: Quality control
The quality of the samples was already checked with the Nanodrop directly after the DNA extraction. DNA Vision's protocol required a 260/280 ratio between 1.7 and 2.1.

Step 2: Amplicons preparation

- DNA normalisation

The protocol required a total DNA per sample of 2.5 to 5 ng/µl. Since the samples had heterogeneous DNA concentrations and the Nanodrop did not seem precise enough, all the DNA samples were

diluted 10 times concerning the bacteria sequencing. This also allowed the quality to be improved as the contaminants and enzymatic inhibitors were diluted too (Sharma *et al.*, 2002). Concerning the fungi sequencing, DNA samples were not diluted as PCR tests followed by agarose gel electrophoresis showed amplification occurred or was improved with undiluted samples.

- DNA amplification with PCR

During this step, target DNA region for both bacteria and fungi were amplified. More particularly, the hypervariable regions V1-V3 of the 16S ribosomal RNA gene were targeted for the bacteria sequencing, as used in Schmautz *et al.* (2017) and Eck *et al.* (2019) experiments. On the other hand, the nuclear ribosomal internal transcribed spacer region 1 (ITS1) was chosen for the fungi sequencing as it has been effectively used in a lot of fungal ecology studies (Taylor *et al.*, 2016). In order to amplify those regions, specific primers with Illumina overhang adapters were used:

- 16S ribosomal RNA gene regions V1-V3:

Amplicon PCR primer 27F (Forward) V1-V3 with the adapter (in blue):
5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGCTCAG-3'

Amplicon PCR primer 534R (Reverse) V1-V3 with the adapter (in blue):
5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG-3'

- ITS1 region:

Amplicon PCR primer Forward ITS1 KYO 2 with the adapter (in blue):
5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGAGGAAGTAAAAGTCGTAA-3'

Amplicon PCR primer Reverse ITS2 KYO 2 with the adapter (in blue):
5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTYRCTRCGTTCTTCATC-3'

PCRs were performed using the 2X KAPA HiFi HotStart ReadyMixPCR kit (KAPA Biosystem). Reactions of 25 µl were carried out, containing 5 µl of primer forward and 5 µl of primer reverse both concentrated at 1 µM, 12.5 µl of 2X KAPA HiFi HotStart ReadyMix, a high-fidelity enzyme and 2.5 µl of DNA sample. Concerning the bacteria sequencing, PCRs were performed with the following cycling protocol: a pre-heating process of the lid at 110°C; a denaturation step at 95°C for 3 min; 25 cycles of 30 sec at 95°C; an annealing step of 30 sec at 55°C; an extension step of 30 sec at 72°C; a final extension step of 5 min at 72°C. The samples were then kept at 4°C until further proceeding. Concerning the fungi sequencing, 30 cycles of 30 sec at 95°C were made instead of 25 cycles. All the other steps remained the same. As mentioned in the technical data sheet of the kit, 30 cycles can be performed in cases of low yields due to low template concentrations or low reaction efficiency, in order to have sufficient PCR products. The PCR products of the 16S rRNA region had a size of about 500 bp, and the PCR products of the ITS 1 amplification had a size of about 250 bp. The ITS amplification failed for 3 samples, i.e. the biofilter sample of March 19 and the roots samples of March 11 and March 22, resulting in a total of 66 amplified DNA samples for the 16S and 63 amplified DNA samples for the ITS.

In that manner, the DNA samples were amplified in two phases. The water, biofilter, rhizoplane and endosphere DNA samples of March 11, March 15, March 19, March 22 were amplified both for bacteria and fungi on April 5. The rhizoplane and endosphere DNA samples of March 26 and March 29 were also amplified for bacteria and fungi that same day. These samples will be referred as the "1st amplification group". These samples were brought the next day to DNA Vision S.A. for the rest of the sequencing. However, only the ITS amplified samples and the "pure culture" samples were sequenced at that time. This will be referred as the "1st run".
The rest of all DNA samples were amplified for bacteria and fungi on May 20, referring as "the 2nd amplification group". They were brought to DNA Vision S.A. the next day and sequenced at that time, referring as the "2nd run". The bacteria amplified samples that had not been sequenced during the 1st run were also sequenced during that 2nd run.

In total, 129 amplified DNA samples were sequenced by DNA Vision S.A. The different steps are briefly described hereafter while the details of the DNA Vision's protocol can be found in 7.4 Annex 4.

Step 3: PCR purification
AmpureXP beads were used to purify the PCR products by removing residual 16S or ITS primers.

Step 4: Indexing
During this step, PCRs were used to add indexes to the amplicons. In order to have a unique identifier for each amplicon, two indexes were added per amplicon, forming a unique combination. To that end, the Nextera Index kit v2 (Illumina, San Diego, CA, USA) was used. More specifically, Nextera XT Index Primer 1 and Nextera XT Index Primer 2 were mixed with 2X KAPA HiFi HotStart ReadyMix and normalised purified DNA samples before being amplified with the same cycling protocol as for the previous 16S amplification.

Step 5: Purification of the PCR products
AmpureXP beads were once again used to purify the PCR products by removing the Nextera XT Index residual primers.

Step 6: Validation of the libraries (optional)
The size of the amplicons could be verified by using a bioanalyzer DNA 1000 with diluted DNA samples.

Step 7: Quantification, normalisation and assembly of the libraries
The libraries were quantified using PicoGreen dsDNA quantitation reagent, a fluorescent nucleic acid stain that binds to double-stranded DNA (Singer *et al.*, 1997). The concentrations were determined based on the size of the amplicons measured with the bioanalyzer, before being normalised.

Step 8: Denaturation of the library and loading on the MiSeq
Before the sequencing, the libraries were denatured with NaOH, diluted with hybridization buffer and denatured with heat in order to have single-stranded DNA. The libraries were then loaded on the Illumina MiSeq (2 x 250 bp) (Illumina Inc., San Diego, CA, USA) with the MiSeq reagent kit V3 (600-cycles, Illumina, San Diego, CA, USA).

3.9 Bioinformatics

The sequencing data obtained from DNA Vision S.A. were analysed on QIIME pipeline v1.9.1 (http://qiime.org/). This open-source bioinformatics pipeline allows to process the large amount of raw DNA sequencing data while summarizing and analysing the information into quality graphics and statistics (Caporaso *et al.,* 2010).

3.9.1 Preparation of the data

All the sequencing data from the 1st run and the 2nd run were processed together at the same time, separating bacteria and fungi.

As forward and reverse sequences were stored into two different fastq files for each sample, they firstly needed to be merged into one file thanks to the command multiple_join_paired_ends.py. During that process, forward and reverse sequences that could not be merged were set aside in different files. In order to avoid an important loss of data, the content of the rejected forward file was copy-and-pasted into the "combined file", i.e. the file containing the merged forward and reverse sequences.

To enable the processing of the data by QIIME pipeline, the final "combined files" needed to be converted from fastq files, which contain both the sequences and the quality information, into fasta

files, which only contain the sequence. This was achieved with the command convert_fastaqual_fastq.py.

With the aim of identifying and differentiate each sample, a mapping file was constituted with the samples identifiers (ID) and different factors, i.e. the date, the type of compartment and the physicochemical group for instance. The command add_qiime_labels.py then allocated a QIIME label to each sample based on the samples' IDs of the mapping file in order to connect each fasta file to its identifier.

During sequencing process, artefacts known as chimeras can occur. These hybrids products of multiple parent sequences can be interpreted as new organisms which falsify the downstream analyses (Haas *et al.*, 2011). To remove noisy sequences and chimeras from fasta files, operational taxonomic units (OTU) were picked from the sequences with the command pick_otus.py using the usearch quality filter based on GOLD reference database (version 20110519) for the bacteria and UTAX reference database (02.02.2019 version) for the fungi, having a common similarity threshold of 97%. A single sequence then needed to be selected for each OTU as a representative sequence and this was achieved with the script pick_rep_set.py. In this way, the representative sequence received an annotation and all the sequences within an OTU inherited the same annotation (Nguyen *et al.*, 2016).

A taxonomy is then assigned to each representative sequence with uclust based on SILVA reference database (release 132) for the bacteria and with BLAST based on UNITE reference database (version 8.0) for the fungi. This was made with the script assign_taxonomy.py.

The OTUs were then aligned based on the representative sequences in order to make a phylogenetic tree with the script align_seqs.py. The obtained alignment was filtered to remove the important gaps with the command filter_aligment.py. The phylogenetic tree, useful for further description analyses, was finally generated thanks to the make_phylogeny.py script.

Finally, an OTU table was generated with the make_otu_table.py command. However, this table needed additional cleaning steps in order to be used for analyses. Firstly, OTUs that were observed less than 2 times, called "singletons", were removed from the table with the script filter_otus_from_otu_table.py. Secondly, sequences coming from chloroplasts and mitochondria were also discharged using the script filter_taxa_from_otu_table.py.

3.9.2 Microbiota of the PAFF box in general

In order to only analyse and compare the samples that traced the microbiota evolution over time, the OTU table was filtered thanks to filter_samples_from_OTU_table.py script to remove the samples related to lettuces of different ages at a given time. Concerning the representativeness tests, only one of the repetitions was kept for each type of sample at random to avoid an over-representation of the date. This new OTU table was visualised in text format thanks to the command biom convert. A summary of its information was also given with the biom summarize-table command. The workflow script core_diversity_analyses.py then gave a full set of QIIME diversity analyses, i.e. the commands alpha_rarefaction.py, beta_diversity_through_plots.py and summarize_taxa_through_plots.py. More particularly, the alpha_rarefaction.py gave the rarefaction curves, i.e. reflecting the species richness from each sample; and several alpha diversity indices, i.e. observed OTUs, phylogeny diversity (PD), chao 1, Shannon, Simpson and Equitability index that reflects the evenness. The beta_diversity_through_plots.py command gave beta-diversity measures, i.e. boxplots and PCoA of both unweighted UniFrac and weighed UniFrac. The summarize_taxa_through_plots.py provided bar charts representing the relative abundance of each OTU in each sample depending on each factor, e.g. compartment and physicochemical groups. These analyses were rarefied on the minimal number of sequences in order to properly compare the samples.

The compartments and physicochemical groups were compared using the Adonis test through the command compare_categories.py, based on beta-diversity data. This test is a permutational multivariate analysis of variance for nonparametric data that uses a distance matrix, a mapping file and a category, i.e. factor, to determine whether or not the sampling grouping is significant (Zapala & Schork, 2006; Caporaso *et al.,* 2010). It calculates a p-value to establish the statistical significance

as well as an R^2 value that shows the percentage of variation explained by the grouping factor (Caporaso *et al.*, 2010). This test is typically used for genetic data with a limited number of samples but thousands of columns of gene expression data (Zapala & Schork, 2006).

3.9.3 Microbiota evolution over time in each compartment

After analysing the microbiota of the PAFF Box in general, all compartments combined, a more stringent approach was made for each compartment. To do so, the general OTU table was filtered with the command filter_samples_from_otu_table.py according to the factor "compartment", in order to have one OTU table for each compartment. The workflow script core_diversity_analyses.py was then generated for each sub-OTU-table, each table having its own rarefaction with its own minimal number of sequences.

3.9.4 Description of the microbiota of lettuce roots at different ages

In order to only analyse and compare the samples that came from lettuces of different age at a given time, the general OTU table was filtered once again with the command filter_samples_from_OTU_table.py to remove the samples related to the microbiota evolution over time or related to repeatability tests. The core_diversity_analyses.py workflow script then gave the taxa plots and general diversity analyses.

3.9.5 Representativeness of the samples

The representativeness of each samples was determined thanks to the 4 representativeness tests, i.e. 4 repetitions of the water, biofilter, endosphere and rhizoplane samples. They were analysed in 4 separate OTU tables with the script filter_samples_from_OTU_table.py with the general core_diversity_analyses.py script.

4. Results and discussion

4.1 Root samples' data

At each sampling date, the roots taken from the grouping of 10 lettuces were weighed with a target value of 2 grams per sample. At harvesting (6 weeks after introduction in the PAFF Box), the fresh shoot biomasses of all ten lettuces were weighed. On March 29, at the sampling of lettuce groups of different ages, the shoot fresh biomasses were also weighed. Table 2 shows the date of collection and preparation for each sample, its identifier (ID), its root weight, the age of the lettuces, the average shoot fresh biomass and the belonging of the sampled lettuce to the Group 1 or 2. Indeed, the group of lettuces that started to look diseased is the Group 1 and the healthy group of lettuces that was used afterwards, is the Group 2.

Tableau 2 Table of root samples data showing the sample ID, its date of collection and preparation, the lettuce age, the average shoot fresh biomass and the Group in which the lettuces belonged. The two grey lines correspond to the root samples that were not studied.

Collection & preparation date	Sample ID	Root weight (gram)	Lettuce age, after transplantation in the PAFF Box (week and day)	Average shoot fresh biomass (grams)	Group of lettuce
11.03.2019	1	0.30	1 week - 1 day	/	1
15.03.2019	2	0.36	1 week - 5 days	/	1
19.03.2019	3	0.37	2 weeks - 9 days	/	1
22.03.2019	4	0.54	2 weeks - 12 days	/	1
26.03.2019	5	0.89	3 weeks - 16 days	/	1
29.03.2019	6	1.10	3 weeks - 19 days	/	1
05.04.2019	7	1.35	4 weeks - 26 days	/	1
13.04.2019	8	2	5 weeks – 34 days	/	1
19.04.2019	9	2	6 weeks – 40 days	20.2	1
29.04.2019	10	2	4 weeks - 26 days	19.7	2
29.04.2019	10.3	1.45	3 weeks	3.8	/
29.04.2019	10.5	2	5 weeks	66.7	/
29.04.2019	10.6	2	6 weeks	171	/
29.04.2019	10.7.A	2	7 weeks – repetition A	215	/
29.04.2019	10.7.B	2	7 weeks – repetition B		/
29.04.2019	10.7.C	2	7 weeks – repetition C		/
29.04.2019	10.7.D	2	7 weeks – repetition D		/
07.05.2019	12	2.00	5 weeks – 34 days	/	2
13.05.2019	13	2.00	6 weeks – 40 days	154.6	2

The data of the two root samples that were not studied due to diseased looking lettuces (April 13 and April 19) are also shown in the table, in grey (Group 1 lettuce). It can be noticed that at 6 weeks old, the shoot fresh biomass only weighed 20.2 g on average, while it weighed 154.6 g on the 6 weeks old sample of the healthy Group 2 lettuce.

4.2 Parameters monitoring and analyses

4.2.1 Parameters evolution in the PAFF Box over time

The evolution of pH, temperature, dissolved oxygen and electroconductivity are shown in Fig. 13, Fig. 14, Fig. 15, Fig. 16 respectively. During the first 4 weeks of the experiment, measures were taken punctually by reading the sensor's screen, at irregular frequency. Although less precise, this monitored period of 4 weeks is not considered to be biased since no trends or seasonality occur for the circulating water. Thus, each punctual measure can be considered to be representative of the day. However, not all days were monitored during that period. For instance, from March 29 to April 4, no data were recorded. During the next 3 weeks of the experiment, the three parameters were measured every 15 min. For the last 3 weeks, parameters were measured twice a day. For these two periods, the graphs thus considerer the average value per day.

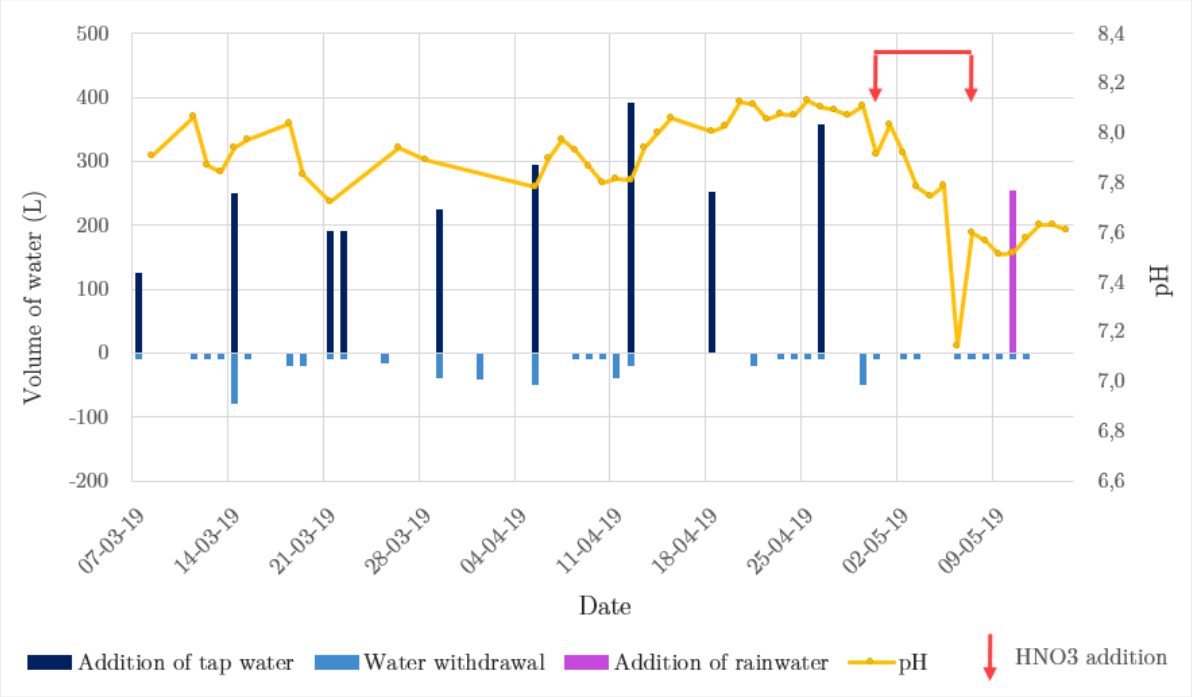


***Figure 13**: pH evolution, water exchanges and nutrient addition from March 8, 2019, to May 13, 2019*

The average pH throughout the entire experiment was 7.87 ± 0.2. This average pH was higher than the optimal range of value recommended for the compromise of bacteria, fish and plants in AP, i.e. between 6 and 7 (Somerville *et al.*, 2014). From March 8 to March 29, the average pH was 7.95 ± 0.11 which led to the addition of acid (HNO_3) in the water from April 30 to May 7, in order to lower the pH. This induced an important drop, going from 8.11 on April 29 to 7.14 on May 7. The pH then seemed to have stabilised at 7.6 on average, to the end of the experiment. While the pH tends to decrease in AP due to the nitrifying bacteria's activity, this tendency did not seem to be observed in this case. As the system was losing water via evapotranspiration, evaporation and spillage, tap water were regularly added to the system, at an average of 253L every week. According to the SWDE, tap water had a pH of 7.4 in Gembloux during that period. However, those additions did not seem to lower the pH. In parallel with tap water additions, water withdrawals were made in order to clean the lamella settler and the biofilter, i.e. backwash.

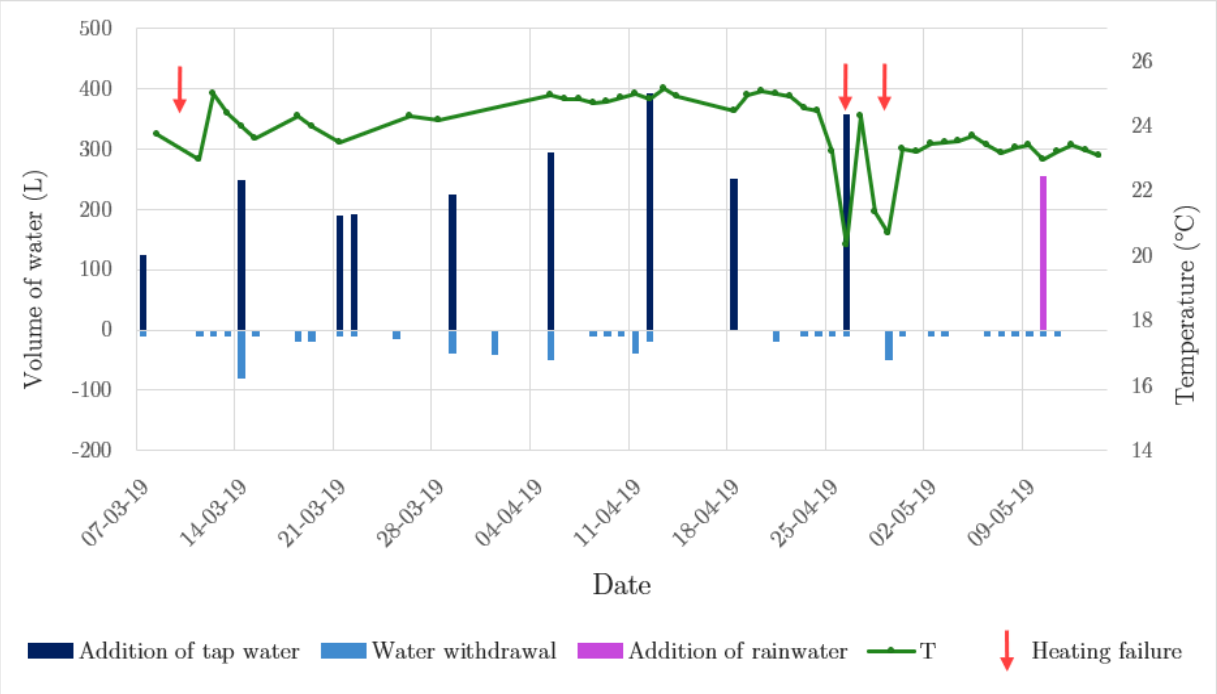


***Figure 14**: Temperature evolution (T), water exchanges and heating failure from March 8, 2019, to May 13, 2019*

The average temperature throughout the entire experiment was 23.87°C ± 1.07°C. This value is lower than the optimal range of temperature recommended for this aquaponic system, i.e. 27-30°C according to Somerville *et al.* (2014) and 25°C according to Delaide *et al.* (2017). Three heating failures occurred during the experiment: one on March 10; one from April 25 to April 26; one on April 29. The two last heating failures induced sharp drops of temperature, going from 24.5°C to 20°C. Apart from these drops, the temperature seemed fairly stable over time. The additions of tap water or rainwater did not seem to impact the temperature of the water.

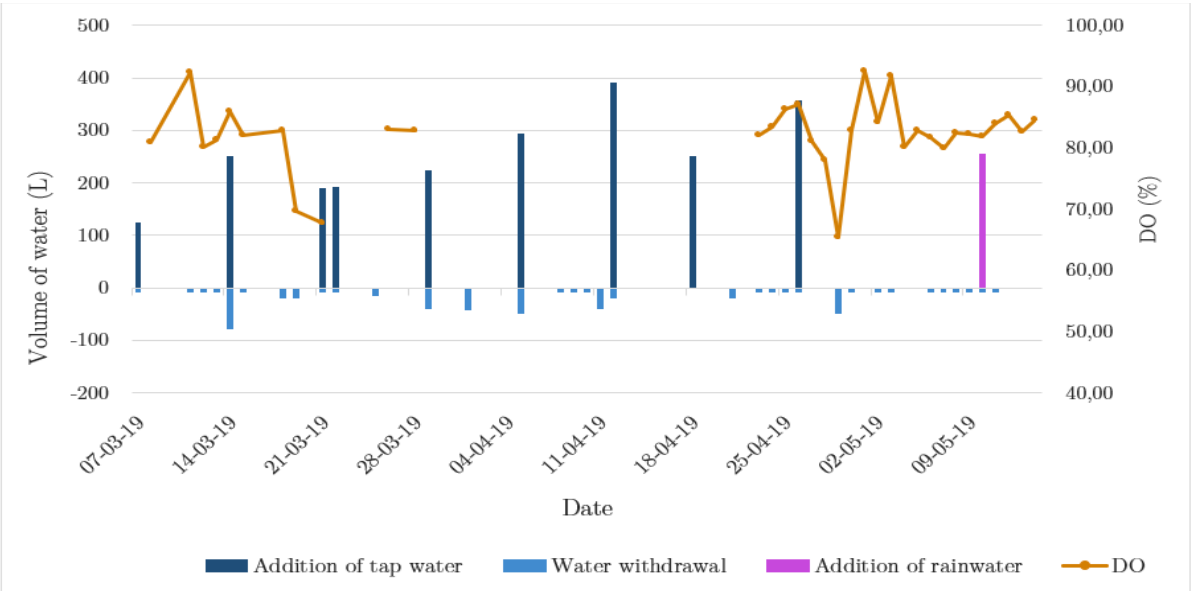


***Figure 15**: Dissolved oxygen (DO) evolution and water exchanges from March 8, 2019, to May 13, 2019*

The average dissolved oxygen level throughout the entire experiment was 82.13% ± 5.7%. From March 29 to April 24, the probe didn't save the data. Important variations occurred, including a drop on March 20 and a drop on April 29, both reaching approximatively 65%. It is rather unsure if those

data are accurate, considering the general problems that occurred with this DO probe. However, if we consider those data, the DO levels seem to have fluctuated a lot during the experiment.

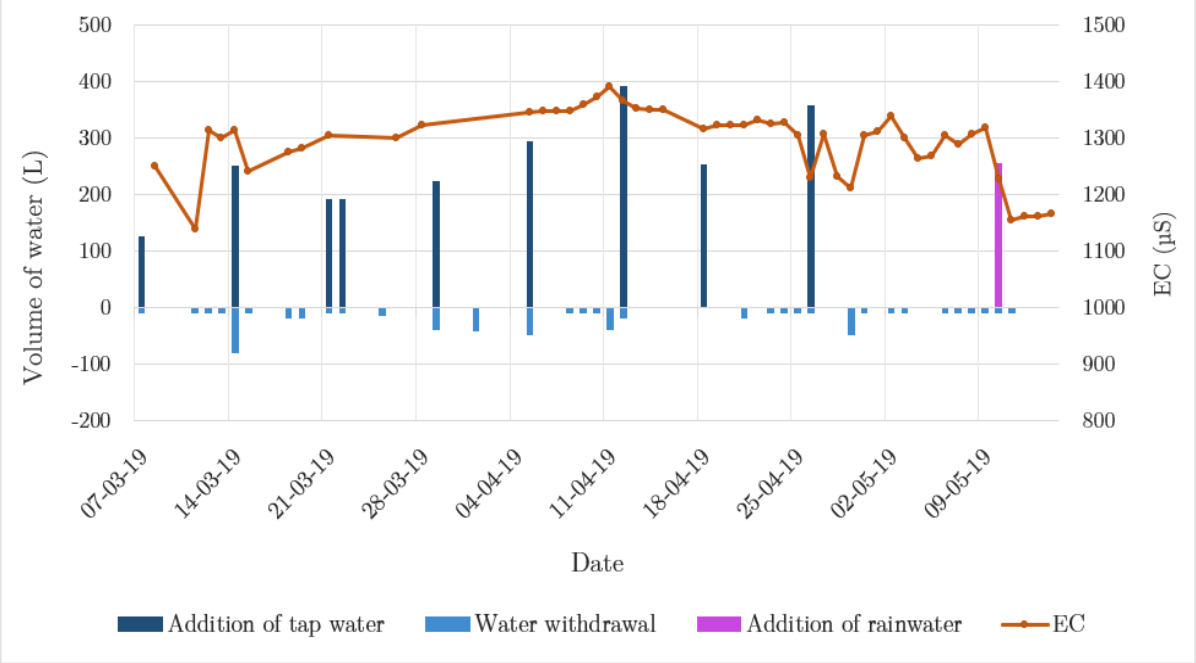


***Figure 16**: Electroconductivity (EC) evolution and water exchanges from March 8 to May 13*

Electroconductivity reflects the concentration of ions in the water, i.e. the nutrient content. The average EC throughout the entire experiment was 1293.1 µS ± 61 µS. Important variations seem to have occurred at the beginning of the experiment, and from April 24 to the end of the experiment. From March 15 to April 11, EC seemed to have generally increased. It then gradually decreased until April 25. After this, several significant drops occurred, i.e. on April 26, April 29, May 5 and May 11. Some EC declines appear to have occurred after important tap water or rainwater additions. However, it was not always the case. According to SWDE, tap water contained 131.7 mg/L of Ca^{2+}, 84 mg/L of SO_4^{2-}, 30.9 mg/L of Cl^-, 19 mg/L of Mg^{2+}, 17.4 mg/L of Na^+, 2.4 mg/L of K^+ and 39.7 mg/L of N-NO_3.

The nutrient content of the circulating water was also directly measured with a probe for the nitrate every 15 min throughout the entire experiment, and with a spectrophotometer at each sampling day for the other macronutrients, i.e. PO_4^{3-}, SO_4^{2-}, K^+, Ca^{2+}, Mg^{2+}, NH_3 and NO_2^-. The evolution of those micronutrients is shown in the following graphs (Fig. 17 and Fig. 18). For the nitrate, the average value per day was considered.

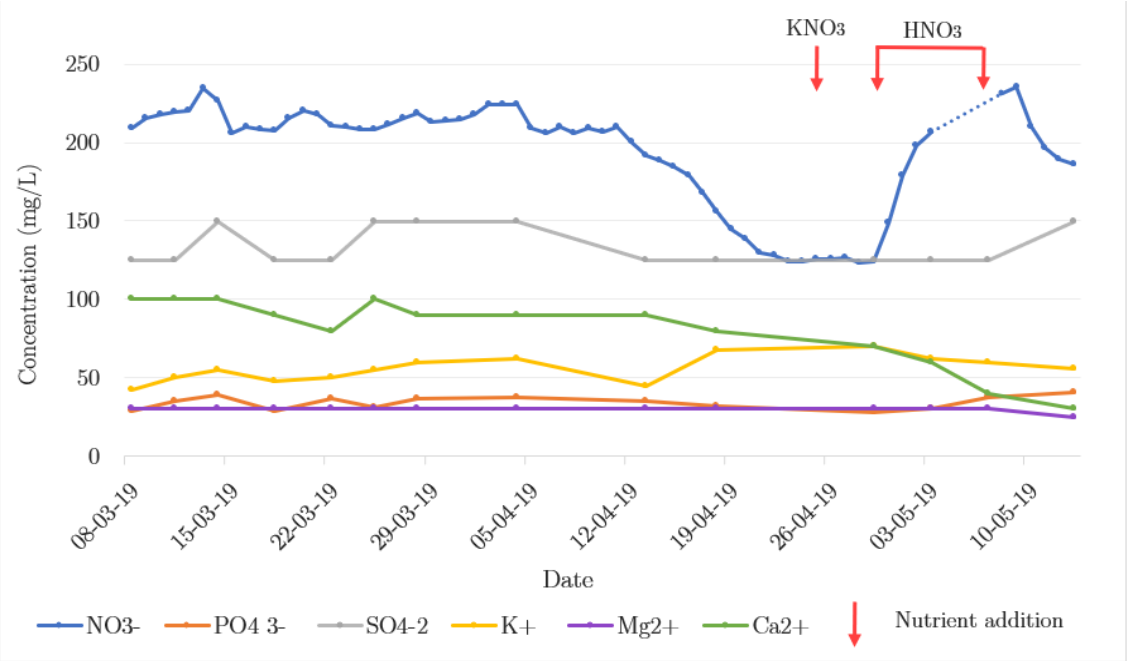


***Figure 17**: Nitrate, phosphate, sulphate, potassium, magnesium, calcium ions evolutions from March 8 to May 13*

The average nitrate concentration throughout the experiment was 43.6 mg/L ± 7.7 mg/L. From May 4 to May 7, the probe did not save the data. Considering the general evolution, it appears that the concentration was relatively stable from March 8 to April 11. The nitrate then gradually decreased, going from 210 mg/L on April 11 to 124 mg/L on April 23. This correlates with the EC decrease observed during this period. It led to the addition of nitrate in the form of KNO_3 on April 25 and in the form of HNO_3 from April 30 to May 7. The nitrate concentration then strongly increased, reaching 235 mg/L on May 9. It then decreased again but at lower extent, after the interruption of nitrate additions. The repeated additions of nitrate might explain the strong fluctuations of EC observed during that period.

Concerning the other macronutrients, the magnesium, potassium, phosphate and sulphate were generally stable throughout the experiment with an average of 29.6 mg/L ± 1.3, 55.9 mg/L ± 8.3, 34 mg/L ± 4.3, 133 mg/L ± 12 respectively. On its part, calcium seemed to have gradually decreased, going from 11 mg/L on March 8 to 30 mg/L on May 15.

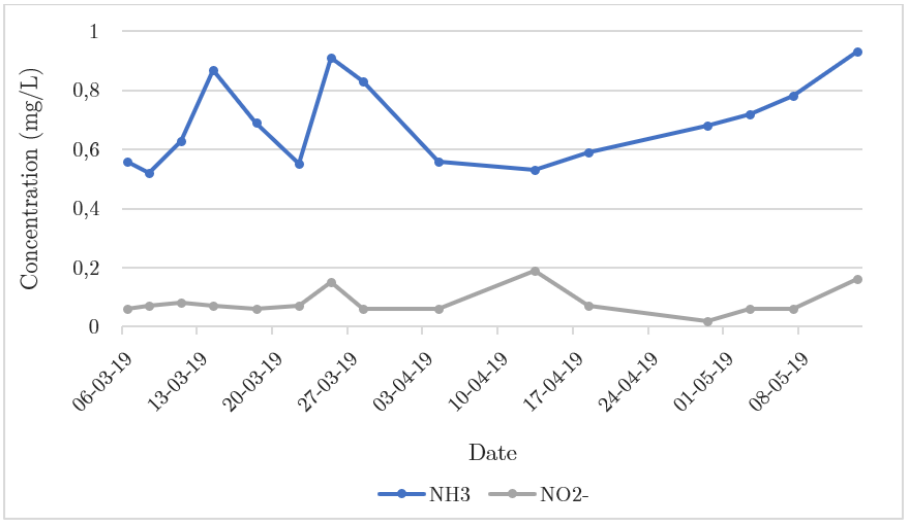
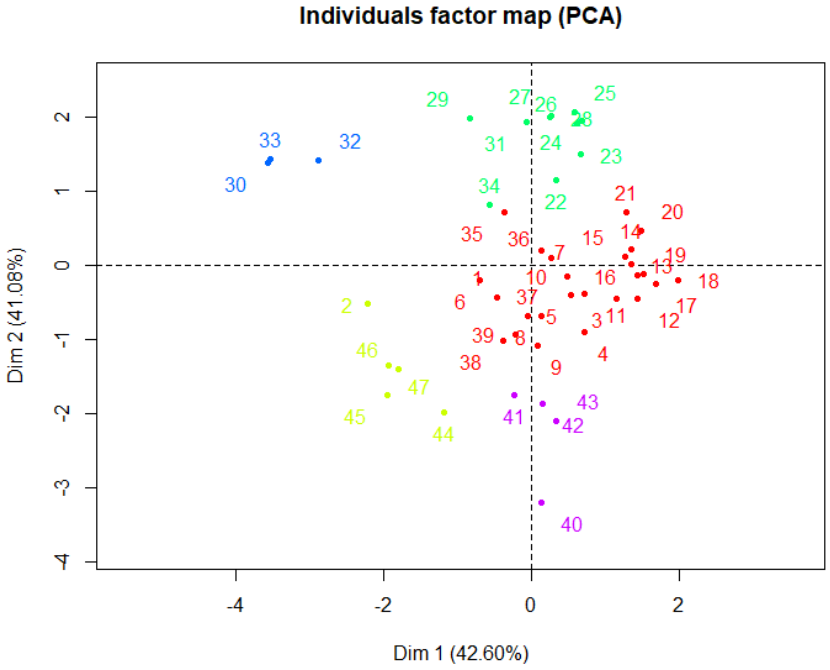


***Figure 18**: Ammoniac and nitrite evolution from March 11 to May 13*

Focusing on Fig. 18, the average ammoniac concentration was 0.69 mg/L ± 0.14 throughout the entire experiment. It seemed to have fluctuated a lot between March 8 and April 4, reaching 0.91 mg/L. It then gradually increased April 13 to May 13, going from 0.53 to 0.93 mg/L. The nitrite concentration was lower than the ammoniac, with an average of 0.077 mg/L ± 0.046 throughout the experiment. The nitrite fluctuated in minor extent and seemed to be pretty stable over time. Both nutrients stayed under the limit of 1 mg/L, beyond which the ammoniac and nitrite can be toxic to Nile tilapia (Somerville *et al.*, 2014).

4.2.2 Physicochemical groups

In order to characterise the parameters variations in a more global view, without considering each parameter independently, physicochemical groups were made with a principal component and a hierarchical clustering analyses on the dates, considering the temperature, the pH, the electroconductivity and the nitrate concentration over time. Thus, dates of the experiment that were highly homogenous in term of the 4 parameters, were clustered together. The 5 resulting groups can be visualized on the first factorial plan of the principal component analysis, explaining the large majority of the total variability, i.e. 83.68% (Fig. 19). Thus, this graph represents very well the variability observed in between the dates, in terms of parameters.



N°	Date	16	09.04	32	28.04
1	08.03	17	10.04	33	29.04
2	11.03	18	11.04	34	30.04
3	12.03	19	12.04	35	01.05
4	13.03	20	13.04	36	02.05
5	14.03	21	14.04	37	03.05
6	15.03	22	18.04	38	04.05
7	18.03	23	19.04	39	05.05
8	19.03	24	20.04	40	06.05
9	21.03	25	21.04	41	07.05
10	26.03	26	22.04	42	08.05
11	28.03	27	23.04	43	09.05
12	05.04	28	24.04	44	10.05
13	06.04	29	25.04	45	11.05
14	07.04	30	26.04	46	12.05
15	08.04	31	27.04	47	13.05

***Figure 19**: First factorial plan of the principal component analysis made on each day of the experiment, with the temperature, pH, EC and nitrate concentration factors. Each number correspond to a date as shown in the table.*

On the factorial plan, the EC and temperature are strongly positively correlated to the dimension 1 while the pH and nitrate concentration are strongly correlated to the dimension 2, the pH being positively correlated and the nitrate being negatively correlated. In that way, the green group is characterised by a high pH whereas the violet group is characterised by a low pH for instance. Extending to all groups and all parameters, Table 3 shows the clusters' characteristics. Additionally, timeline at Fig. 20 allows to visualize those physicochemical groups throughout the experiment. More generally, it allows to see the important variations and shifts in terms of parameters that occurred during the experiment. Those shifts can then be compared with potential microbiota shifts.

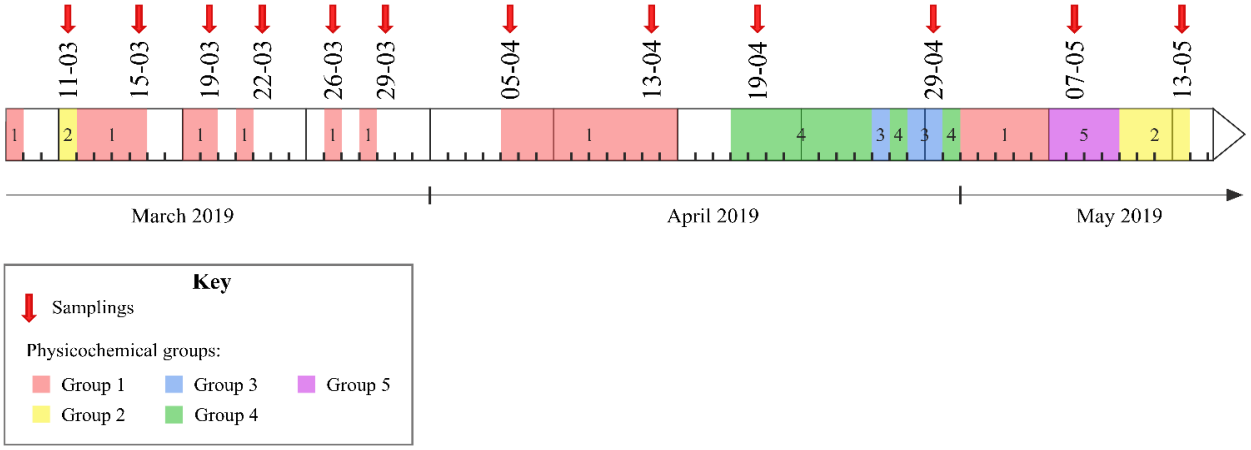


***Figure 20**: Timeline showing the physicochemical groups throughout the experiment and the samplings. The blank days correspond to days in which all the parameters were not monitored.*

Tableau 3: Physicochemical groups' characteristics. Key: $\bar{x}$: mean; σ: standard deviation; CV: coefficient of variation; Min: minimum; Max: maximum

Group	Individuals		EC	pH	T	NO_3^-
Group 1	25	$\bar{x}$	1317,83	7,88	24,29	208,83
		σ	40,02	0,08	0,61	11,63
		CV	0,03	0,01	0,03	0,06
		Min	1241,00	7,72	23,20	179,21
		Max	1389,46	8,04	25,15	234,62
Group 2	5	$\bar{x}$	1168,10	7,68	24,15	200,35
		σ	34,27	0,21	0,19	14,04
		CV	0,03	0,03	0,01	0,07
		Min	1137,50	7,52	22,95	186,24
		Max	1227,00	8,07	23,40	219,20
Group 3	3	$\bar{x}$	1223,23	8,09	20,80	124,49
		σ	10,72	0,02	0,52	1,32
		CV	0,01	0,002	0,03	0,01
		Min	1211	8,07	20,33	123,57
		Max	1231,00	8,11	21,35	126,00
Group 4	10	$\bar{x}$	1317,47	8,06	24,41	134,82
		σ	9,92	0,06	0,66	11,37
		CV	0,01	0,01	0,03	0,08
		Min	1302,97	7,91	23,20	124,21
		Max	1330,24	8,13	25,06	155,58
Group 5	4	$\bar{x}$	1304,12	7,45	23,32	228,96
		σ	11,99	0,21	0,12	5,84
		CV	0,01	0,03	0,01	0,03
		Min	1288,50	7,14	23,15	222,00
		Max	1317,50	7,60	23,40	235,66

Considering Table 3, the coefficients of variation are relatively similar and low, ranging from 0.01 to 0.08, meaning that the clusters were properly established, with highly homogenous dates.

Group 1 covered the most dates with 25 days out of 47. It dominated the first part of the experiment, from March 8 to April 14. This group was characterised by the highest EC, i.e. 1317.83 µS. Its mean temperature was close to the optimal temperature of 25°C (Delaide *et al.*, 2017), i.e. 24.29°C, and had average levels of pH and nitrate concentrations comparing to the other groups, respectively 7.88 and 208.83 mg/L.

Following the Group 1 on the timeline, Group 4 mostly dominated the period going from April 18 to April 30. This group was characterised by a high EC (1317 µS), a high pH (8.06), a nearly optimal temperature (24.42°C) and a low concentration of nitrate (134.82 mg/L). This period represents an important shift in the parameters.

Late April-early May, an alternance of group 4 and 3 occurred. Covering only 3 days, group 3 was defined by an average-to-low EC (1223.23 µS), a high pH (8.09), a very low temperature (20°C) and a very low nitrate concentration (124.34 mg/l). Indeed, this "extreme" group reflects the 2 heating failures that occurred on April 25 – April 26 and on April 29 as well as the steady decline of nitrate concentration.

Following this period, Group 1 covered the dates from May 1 to May 5. This reflects a period of "return" to normal conditions, in which the temperature went back to the optimal one and nitrate were added in the water.

Then, it switched to the Group 5, characterised by an average EC (1304 µS), a low-to-average temperature (23.3°C), a very low pH comparing to the average pH observed during the experiment (7,45) and a very high nitrate concentration (228.9 mg/L). This group reflects the impact of HNO_3 additions, that reduced the pH and increased even more the nitrate concentration. During that period, the pH was actually the closest to the optimal pH, i.e. between 6 and 7 (Somerville *et al.*, 2014).

Finally, the last days of the experiment were covered by the Group 2, from May 10 to May 13. This period was characterised by the lowest EC observed throughout the experiment (1168.10 µS), a temperature close to the optimal (24.15°C), a relatively low pH (7.68) comparing to the other periods and an average nitrate concentration (200.35 mg/L). This group may reflect a stabilisation of the parameters, i.e. the pH and nitrate that were both drastically modified during the previous period (Group 5). It can be noticed that this Group 2 also covered the first sampling day, i.e. March 11, bordered by the Group 1. Since the data were taken manually and the preceding days did not have any parameters data, it is difficult to say if this short period at the beginning of the experiment really had the characteristics of the Group 2.

As a general conclusion, the first part of the experiment, i.e. from March 8 to April 15, seemed pretty constant and homogenous in term of temperature, EC, pH and nitrate concentration. Then, the second part of the experiment, i.e. from April 18 to May 15, was way more heterogenous, undergoing various parameters variations such as drops in temperature, pH and nitrate concentration.

4.3 Microbiota analysis

4.3.1 Contamination evaluation

An external sterility test was made in order to evaluate the potential contamination of the samples, with three repetitions of a pure culture of *Pseudomonas chlororaphis*. As a result, 96.26% of the total reads were assigned to *Pseudomonas chlororaphis* on average and 2.31% to the *Bacteria* kingdom. At minor extent, 0.60% were unassigned, 0.22% corresponded to the *Archae*, 0.13% to *Lactobacillus delbrueckii spp*, 0.11% to *Streptococcus*, 0.1% to *Proteobacteria*, 0.08% to *Sphingomonas*, 0.03% to *Propionibacteriaceae*, and 0.03% to *Oxalobacteriaceae*. The sum of all the other minor OTUs represented 0.1% of the total reads on average. According to this test, we can consider that our studied samples were "pure".

4.3.2 General information

General information concerning the sequences found in the samples and the samples' alpha diversity are found in Table 3. Firstly, the accuracy of the sequences was assessed via Phred Quality score (Q), a common measure that "indicates the probability that a given base is called incorrectly by the sequencer" (Illumina, 2011, p. 1). More particularly, Q30 score corresponds to a probability of incorrect base call of 1 in 1000, giving a base call accuracy of 99.9% (Illumina, 2011). In that way, Table 3 gives for each sample, the percentage of bases with Q30 score or higher. It appears that nearly all samples had a percentage higher than 80% with an average of 84% in the case of bacteria samples and 85% in the case of fungi samples, the minimum being 79%. This indicates a good sequences' accuracy (Eck *et al.*, 2019). Secondly, the table gives the number of reads, i.e. sequences, obtained from DNA Visions' sequencing, before the filtering process. Then, it gives the number of reads after the filtering process completed on QIIME pipeline, that removed the chimera, singletons, chloroplasts and mitochondria reads. In that way, bacteria samples went from an average of 101.697 reads before filtering to an average of 29 178 reads after filtering. For the fungi samples, it went from an average of 92 667 reads to an average of 19 275 reads. Besides, the bacteria endosphere samples had the biggest changes, losing 90% on average of their reads. This might be due to a higher proportion of reads assigned to chloroplasts and mitochondria, since roots were grinded and directly used for DNA extraction.

Thirdly, table 3 gives an indication of the samples OTUs' richness by giving the number of observed OTUs and the samples OTU's evenness by giving the Equitability index. The Shannon index then provides a general overview of the samples' alpha diversity by combining the richness and evenness criteria. Hence, bacteria samples generally had a more diverse community, with an average number of OTUs of 867.8 compared to 301.3 OTUs for the fungi samples; an equitability of 0.62 on average compared to 0.37 for the fungi samples; a Shannon index of 6 on average whereas 2.95 in the case of fungi samples. Within bacteria samples, the biofilter, rhizoplane and endosphere samples share similar Shannon and equitability indices ($p > 0.05$) and seem to have more diverse communities than the water samples as their Shannon and equitability indices are significantly higher than the ones of the water samples ($0.001<p<0.01$). Considering the fungi sequencing, samples appear to have similar alpha diversity indices, except for the endosphere samples that have Shannon and equitability indices significantly smaller than the biofilter samples ($0.001<p<0.01$).

Tableau 4: Table showing general information of the samples, i.e. the sample ID, the Q30 score, the number of reads before filtering and after filtering, the number of observed OTUs, the Shannon index and the Equitability. The blue colour and « S » letter refer to the sump water samples. The pink colour and « B » letter refer to the biofilter samples. The green colour and "E" letter refer to the endosphere samples. The yellow colour and "R" letter refer to the rhizoplane samples. In the sample ID column, the number associated to each sample corresponds to the sampling date: (1) 11.03; (2) 15.03; (3) 19.03; (4) 22.03; (5) 26.03; (6) 29.03; (7) 05.04; (8) 13.04; (9) 19.04; (10) 29.04; (12) 07.05; (13) 13.05. The A, B, C, D letters associated to some samples refer to representative tests, with the four repetitions.

Sample ID	%>=Q30		N° of reads before filtering		N° of reads after filtering		N° of obs. OTUs		Shannon index		Equitability	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
S1	85	84.5	77 826	91 540	34 313	41 641	1 017	856	4.18	3.73	0.42	0.38
S2	84.3	84.4	111 332	105 906	48 736	49 171	839	659	3.63	3.23	0.37	0.34
S3	83.7	84.3	25 202	76 588	11 794	35 459	532	641	3.79	2.92	0.42	0.31
S4	84.1	84.2	16 660	45 528	7 491	19 949	657	623	6.39	5.54	0.68	0.6
S5	84.7	84.5	82 230	94 354	36 238	16 840	690	146	2.97	1.94	0.31	0.27
S6	84.7	85	71 122	119 964	30 369	23 962	850	148	4.26	2.16	0.44	0.3
S7	84.1	79.4	129 040	46 156	55 728	9 902	1 224	125	4.68	2.48	0.46	0.36
S8	82.8	84.1	84 060	53 096	34 386	9 506	1 230	122	6.42	2.35	0.63	0.34
S9	83.2	79.0	97 806	37 682	43 229	7 673	1 057	118	5.26	2.45	0.52	0.36
S10A	83.8	83	137 656	40 928	61 195	8 835	901	113	3.62	1.73	0.37	0.25
S10B	81.6	82.7	82 448	40 846	36 493	7 901	694	104	3.81	1.99	0.40	0.3
S10C	84.2	81.3	96 994	47 090	43 559	8 631	699	125	3.64	2.28	0.38	0.33
S10D	85.2	84	82 840	48 858	36 615	8 395	758	123	3.92	2.41	0.41	0.35
S12	85.6	85	153 252	49 776	67 682	9 454	996	111	4.09	1.77	0.41	0.26
S13	85.2	84.6	217 204	34 464	93 837	6 499	1 591	112	5.78	2.49	0.54	0.37
B1	83.7	83.4	19 940	185 024	8 699	69 917	611	854	6.66	5.84	0.72	0.6
B2	82.4	82.5	19 576	62 002	8 490	23 891	640	700	6.75	5.82	0.72	0.62
B3	84.7	/	23 990	/	10 561	/	706	/	6.84	/	0.72	/
B4	84.4	85	19 968	122 276	8 599	43 890	637	895	6.98	6.61	0.75	0.67
B5	83.3	85.1	76 392	35 874	26 038	5 653	875	108	6.3	2.64	0.64	0.39
B6	84.4	86.1	55 046	126 090	19 428	23 830	711	122	6.03	2.84	0.64	0.41
B7	83.7	86.7	99 900	104 086	34 734	17 157	831	149	5.79	3.19	0.6	0.44
B8	84.6	84.6	47 158	88 866	17 119	18 547	678	123	6.02	2.79	0.64	0.4
B9	82.5	84.7	91 126	95 282	32 244	17 913	879	133	6.18	2.5	0.63	0.35
B10A	84.5	88.6	75 662	203 520	25 586	21 418	858	141	6.43	2.37	0.66	0.33
B10B	84.8	87.6	92 586	146 650	31 274	17 705	890	191	6.35	2.86	0.65	0.38
B10C	83.5	87.9	71 282	113 308	24 477	12 268	838	136	6.25	2.9	0.64	0.41
B10D	81.9	88.6	70 352	96 362	23 482	7 407	797	123	6.14	2.93	0.64	0.42
B12	81.6	87.4	40 142	44 926	14 162	6 407	657	65	6.19	1.58	0.66	0.26
B13	83.6	84	50 278	80 276	16 921	8 349	765	110	6.35	2.35	0.72	0.34

E1	85.2	/	270 096	/	29 749	/	385	/	4.04	/	0.47	/
E2	84.4	83.1	120 960	151 774	9 748	33 645	430	435	4.54	2.39	0.52	0.27
E3	84.8	83.9	131 778	66 434	9 393	12 772	458	196	4.62	1.78	0.52	0.23
E4	84.3	/	77 914	/	34 208	/	131	/	2.76	/	0.39	/
E5	84.4	83.7	79 684	44 760	4 206	11 355	391	241	5.55	1.83	0.64	0.23
E6	83.5	82.2	112 912	147 084	6 953	38 630	386	396	4.8	1.95	0.56	0.23
E7	84.8	85.7	68 562	95 386	3478	8 955	500	155	6.76	2.63	0.75	0.36
E10	84.8	86.9	83 246	98 452	4 241	14 644	512	124	7.1	1.59	0.79	0.29
E12	83.7	89.1	79 528	173 688	4 940	13 480	528	119	6.85	1.91	0.76	0.28
E13	83.9	89.2	85 132	91 982	5 420	8 709	571	85	7.15	1.67	0.78	0.26
E.3.	84.1	82.8	89 696	76 580	3 737	9 204	551	150	7.37	2.12	0.81	0.29
E.5.	83.8	88.2	78 102	81 786	5 780	9 462	566	131	6.77	1.85	0.74	0.26
E.6.	85.2	88.9	95 098	133 356	5 978	11 952	551	158	6.74	2.4	0.74	0.33
E.7.A	84.7	86.4	96 638	91 052	10 235	9 590	706	165	6.8	2.7	0.72	0.37
E.7.B	84.6	87.5	127 552	113 052	14 603	12 107	825	171	7.03	2.57	0.73	0.35
E.7.C	84.9	85.3	158 668	87 706	17 347	11 731	935	195	7.05	2.63	0.71	0.35
E.7.D	84.3	86.8	121 972	82 576	13 380	9 664	819	166	7.11	2.5	0.73	0.34
R1	84.7	86	143 324	213 082	53 289	65 176	526	774	4.31	5.92	0.48	0.62
R2	85.1	85.4	159 118	75 468	53 772	24 751	941	816	5.72	6.46	0.58	0.67
R3	84.7	85	119 604	181 318	39 126	57 019	865	1 055	5.65	6.4	0.58	0.64
R4	83.6	84.6	121 968	72 746	40 014	24 060	1 086	989	6.34	7.16	0.63	0.72
R5	84.4	83.6	136 800	145 440	46 768	46 408	1 165	1 223	6.14	7	0.6	0.68
R6	84.4	84.1	304 398	147 566	96 088	50 916	1 743	1 161	7.1	6.86	0.66	0.67
R7	85.1	85.6	43 760	53 306	12 372	7 794	988	118	7.33	1.75	0.74	0.25
R10	84.1	86.3	187 160	70 470	57 497	13 582	1 656	95	7.71	1.68	0.72	0.26
R12	85.3	87.3	105 696	138 918	34 997	16 854	1 296	155	7.41	1.95	0.72	0.27
R13	85.3	86.3	92 348	76 248	30 879	8 546	1 197	166	7.57	2.43	0.74	0.33
R.3.	84.6	82.8	70 282	33 366	17 813	7 483	1206	88	8.13	1.36	0.79	0.21
R.5.	83.8	85.9	154 996	83 584	51 892	12 964	1 621	176	7.65	2.08	0.72	0.28
R.6.	83.4	86.2	138 758	57 476	44 009	8 507	1 458	136	7.72	2.38	0.73	0.34
R.7.A	81.1	83.1	171 522	63 008	53 830	12 605	1 479	124	7.6	1.8	0.72	0.26
R.7.B	82.7	84.3	88 190	70 036	26 943	11 167	1 166	132	7.62	2	0.75	0.29
R.7.C	82.7	84.3	117 566	94 764	34 593	15 376	1 319	162	7.61	2.11	0.73	0.29
R.7.D	83.5	84.6	156 538	102 922	46 626	18 521	1 474	146	7.72	1.81	0.73	0.25

4.3.3 General microbiota according to system compartments

The PAFF Box is a complex aquaponic system in which each compartment most likely developed separate microbiota as shown in Schmautz *et al.* (2017) and Eck *et al.* (2019) experiments in the case of bacterial communities for instance. Thus, we will now focus on the general microbiota found in each compartment, without considering the evolution over time. This will provide us an overall idea of the microorganisms found in each location while highlighting the differences between the actual compartments.

- Bacterial communities

The bar chart at Figure 21 shows the relative abundance of each OTU according to each compartment, at the phylum level. For each compartment, all the samples taken throughout the experiment were thus combined.

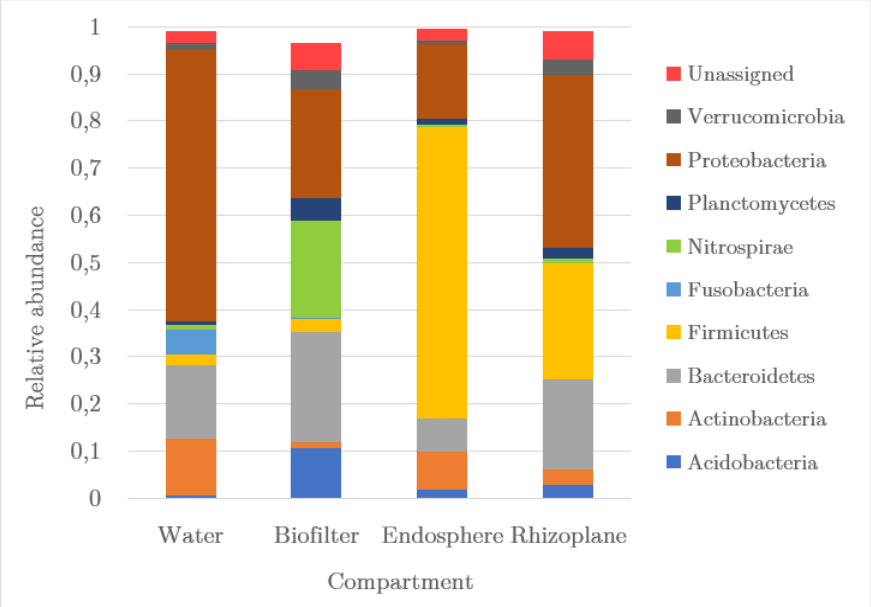


***Figure 21**: Bar chart representing the relative abundance of each bacterial OTU according to each compartment, at the phyla level. Only the OTUs representing more than 0.5% of the total reads on average are shown*

According to Figure 21, each compartment developed specific bacterial communities, and this can be confirmed by the Adonis test, showing that the grouping of samples by compartment is highly statistically significant ($p<0.001$). In addition, the R^2 value indicated that more than 54% of the variation observed between the samples was explained by this grouping, i.e. type of compartment.

This can also be visualized on the principal coordinates analysis (PCoA) achieved on all samples, that used weighed UniFrac distances (Figure 22). As already mentioned in the 1.5.4.3 Introduction, weighed UniFrac is based on the fraction of branch length shared between two samples within the general phylogenetic tree, i.e. that summed all tree branch lengths, considering the relative abundances of the OTUs.

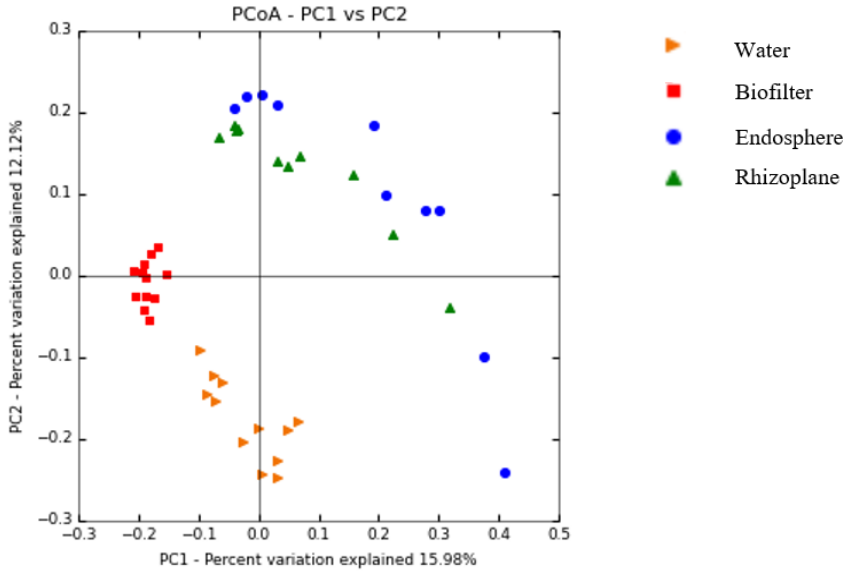


***Figure 22**: Weighted UniFrac principal coordinates analysis showing the grouping of the biofilter, water, endosphere and rhizoplane bacterial samples. Axis 1 and 2 explain 28.1 % of the total variability*

The endosphere and rhizoplane samples are overall situated on very different graph's frames than the biofilter and water samples, indicating the strong microbiota differences between those plants-related samples and the ones from the two AP compartments. While the biofilter and water samples created two very separate clusters, the rhizoplane and endosphere samples were more intertwined and dispersed, respectively showing that the two types of samples were similar an that important microbiota variations could be observed within those compartments (Fig. 22). Moreover, the biofilter samples formed a narrower cluster than the water samples as seen in Eck *et al.* (2019) experiment, indicating a more homogenous and stable compartment in term of microbiota.

Focusing on Fig. 21, all compartments shared the same 9 major phyla, but at different proportions, i.e. *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Nitrospirae*, *Verrucomicrobia*, *Plantomycetes* and *Fusobacteria*. On average, 4.2% ± 1.2% of the reads were unassigned. Even though the endosphere samples contained rhizoplane organisms, important differences in term of relative abundance do seem to appear between the two types of samples. Their separate analyses thus seem useful.

As a general overview, the water, rhizoplane and endosphere samples showed less than 1% ± 0.2% of *Nitrospirae* on average while the biofilter samples showed more than 20%. This showcases the primary role of the biofilter, that is, being the centre of the nitrification process. For its part, the water compartment appears to have a higher proportion of reads assigned to *Fusobacteria* phylum, i.e. 5.4% of the total reads compared to 0.11% ± 0.07% for the three other types of compartments.

The *Firmicutes* phylum seemed to be present in all compartments but was represented at very high proportion in the root samples, with a majority of this group in the endosphere, i.e. more than 60% of the total reads. Considering other microbiota studies in AP, the water and biofilter samples of Eck *et al.* (2019) experiment as well as the biofilter, periphyton and root samples of Schmautz *et al.* (2017) experiment showed very low proportion of *Firmicutes* reads. However, they represented approximatively 5% of the total reads in the fish faeces sample of Schmautz *et al.* (2017). Bartelme *et al.* (2019) study did not show the presence of *Firmicutes* in the AP systems or RAS. On the other hand, Schreier *et al.* (2010) and Sugita *et al.* (2008) identified *Firmicutes* in RAS biofilters at lower extent.

Focusing at the genus level, the *Firmicutes* phylum was mostly constituted of the *Lactobacillus* and the *Streptococcus* genera, representing respectively 60.7% ± 0.09% and 38% ± 0.4% of the total *Firmicutes* reads in the biofilter, endosphere and rhizoplane compartments. In the water, those two genera represented 40% of the *Firmicutes*. Being very important in the root samples and a lower extent, in the biofilter samples, it seems like very little is known about those two genera in AP. Both

members of the *Lactobacillales* order, they are considered as "lactic acid bacteria" (LAB), consuming carbohydrates and producing lactic acid as a major product of their fermentative metabolism (Ringø & Gatesoupe, 1998). Slow growing with complex nutritional requirements, they are usually found in rich habitats such as gastrointestinal tract, dairy products or on plant surface (Ringø & Gatesoupe, 1998). Indeed, LABs are a part of the normal fish microbiota which could explain the high proportion of *Firmicutes* found in the faeces sample of Schmautz *et al.* (2017) and the presence of LABs in the water compartment of our experiment (Villamil *et al.*, 2003). Unlike other members of *Firmicutes*, they do not reduce nitrate (Ringø & Gatesoupe, 1998).

Focusing on their presence in fish, LABs are mostly considered as non-pathogenic. However, some *Streptococcus* members have been identified as fish pathogen such as *S. agalactiae* on Nile tilapia (Ortega Ascencios *et al.,* 2016). Similarly to the use of *Lactobacilli* in human probiotics, more and more studies are made towards the use of *Lactobacilli* and *Streptococcus* as fish probiotics in aquaculture. Supplemented in their feed or directly poured in the water, LABs are used as an alternative to control fish diseases and they also seem to improve growth performance (Gupta *et al.*, 2019; Al Dohail *et al.*, 2009; Verschuere *et al.,* 2000). In Lara-Flores *et al.* (2003) experiment for instance, *Lactobacillus acidophilus* and *Streptococcus faecium* were used as probiotics on Nile tilapia, leading to greater growth performance and feed efficiency. Their presence tends to limit the development of other microorganisms since *Lactobacilli* are known to be capable of producing compounds that inhibit the growth of microorganisms, e.g. bacteriocins, H_2O_2 and organic acids (Zacharof & Lovitt, 2010; Ringø & Gatesoupe, 1998).

- Fungal communities

Considering the fungi OTU table, all the samples from the 1st run showcased a large majority of OTUs with a "No blast hit" taxonomy (> 98%). Those samples corresponded to sample dates of 11.03, 15.03, 19.03 and 22.03 for the water and biofilter and to the sample dates of 11.03, 15.03, 19.03, 22.03, 26.03 and 29.03 for the endosphere and rhizoplane samples. Although no explanation seems to be found on QIIME pipeline, we can say that no satisfactory match was found between the OTUs and the pre-assigned reference sequences of the database (Caporaso *et al.,* 2010). This may be due to the fact that no hit met the minimum length, e-value and percent of ID requirements in order to be assigned. Even though the sequences had sufficient quality to be assigned to OTUs during the OTU picking step, they might not have had the quality requirements for the taxonomy assignment. Looking back to the Table 3, all the fungi samples shared similar Q30 scores but the samples from the 1st run seemed to have more reads after filtering and more observed OTUs than the ones from the 2nd run, although having similar number of reads before the filtering. However, this observation does not explain the differences in taxonomy assignment between the samples from the 1st run and the ones from the 2nd run, considering that the same method was used for all samples.

Hence, only the samples of the 2nd run will be studied. In order to visualise the general fungal communities found in each type of compartment throughout the experiment, a bar chart at Figure 23 shows the relative abundance of each OTU according to each compartment, at the genus level. In this graph, the biofilter and water compartments are thus only composed of the 26.03, 29.03, 05.04, 13.04, 19.04, 29.04, 07.05 and 13.05 samples while the root samples are composed of the 05.04, 29.04, 07.05 and 13.05 samples.

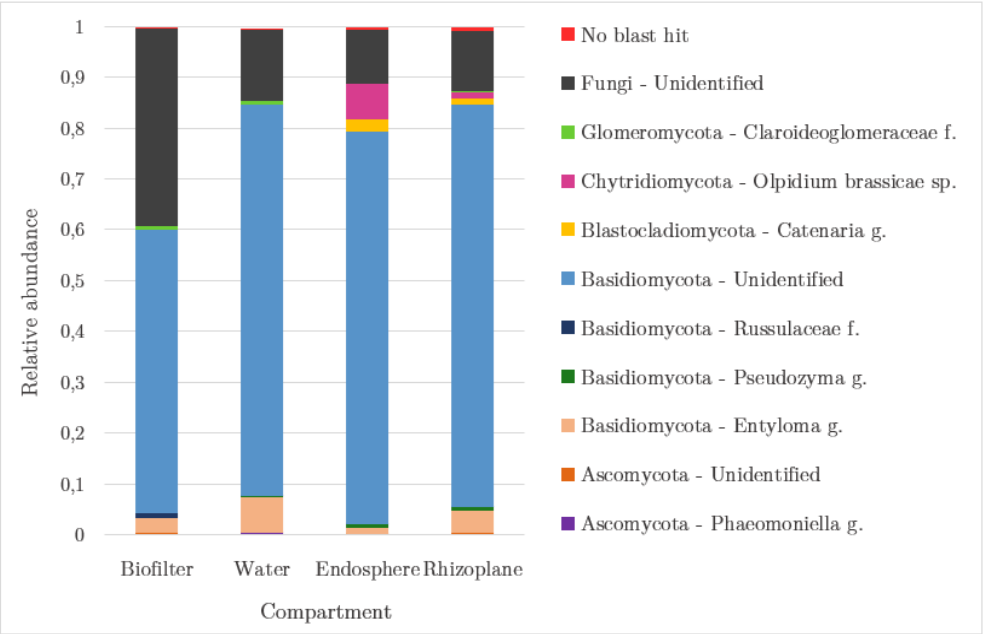


***Figure 23**: Bar chart representing the relative abundance of each fungal OTU according to each compartment, at the genus level. Only the OTUs representing more than 0.1% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family or phyla level, in which case the level is indicated.*

According to Fig. 23, each compartment developed similar fungal communities, and this can be confirmed by the Adonis test, showing that the grouping of samples by compartment is not significant ($p>0.05$). In addition, the R^2 value indicated that only 16% of the variation observed between the samples was explained by this grouping, i.e. type of compartment. This can also be visualized on the principal coordinates analysis (PCoA) achieved on all samples, based on weighed UniFrac (Fig. 24).

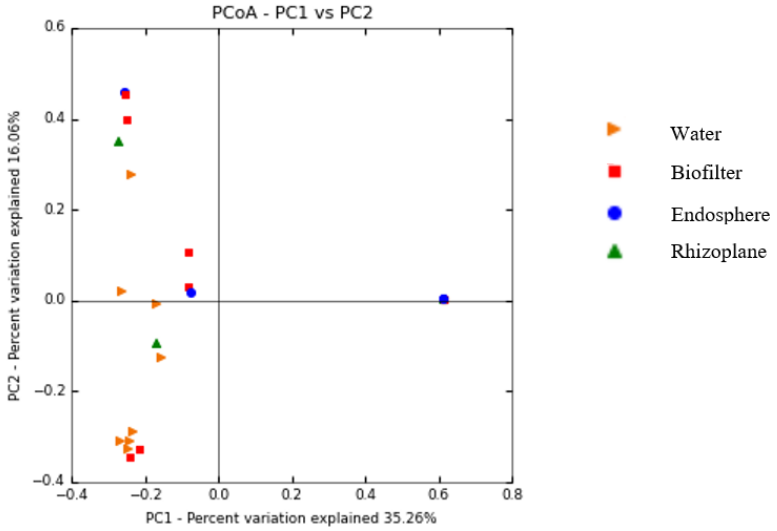


***Figure 24**: Weighted UniFrac principal coordinates analysis showing the grouping of the biofilter, water, endosphere and rhizoplane fungal samples. Axis 1 and 2 explain 51.32 % of the total variability*

Focusing on Fig. 24, no clear distinct compartment clusters are visualized, and most samples are found in the same graph's frames, showing that the microbiota of all compartments are similar. Combining all the information, this can be explained by the large majority of "unidentified *Fungi*" and "unidentified *Basidiomycota*", i.e. more than 90% of the total reads. Major differences could have occurred within those groups, but the reads could not be further identified with the actual reference database. Indeed, the *Basidiomycota* phylum comprises about 37% of all fungal described species,

with a variety of morphologies and ecological roles (Roehl, 2017). A lot of them are saprophytic and decompose organic matter but many of them also form mycorrhizas with plants or parasitize them, which can explain their large presence in both aquaponic compartments and plants-related compartments (Roehl, 2017). Besides, while *Ascomycota* is the largest group in the kingdom with more than 60 000 species, having a variety of morphology and biology, it is interesting to notice that they represent less than 1% of the total reads in each compartment (Fig. 23) (Desprez-Loustau *et al.,* 2010). However, some of them might be included in the "unidentified *fungi*".

Even though statistically, no significant differences were observed between the compartments, a few visible differences can be discussed. On Figure 23, the biofilter seems to have developed a smaller proportion of *Basidiomycota*, compared to the three other compartments, i.e. 55% for the biofilter whereas 77% on average for the other compartments. It is also the only compartment to have developed a small but significant proportion of the *Russulaceae* family. On the other hand, all compartments shared a common specific genus: the *Entyloma* genus. The water and biofilter compartments seem similar in term of identified OTUs, but with different proportions. In contrast, the endosphere and rhizoplane samples appear to have developed relatively high proportions of phyla that are not found in the biofilter and water samples, i.e. *Chytridiomycota* and *Blastocladiomycota.* Likewise, only the biofilter and water samples developed a small but significant proportion of *Glomeromycota.* Hence, the root samples can be qualitatively distinguished from the biofilter and water samples when focusing on minor OTUs. Those OTUs will be discussed in the following section.

The evolution of the bacterial and fungal communities in each compartment over time can now be studied.

4.3.4 Microbiota evolution in each compartment

4.3.4.1 Water

- Bacterial communities

The evolution of the bacterial communities found in the circulating water of the aquaponic system throughout the experiment is visualised with the following graphs (Fig. 25 and Fig. 26).

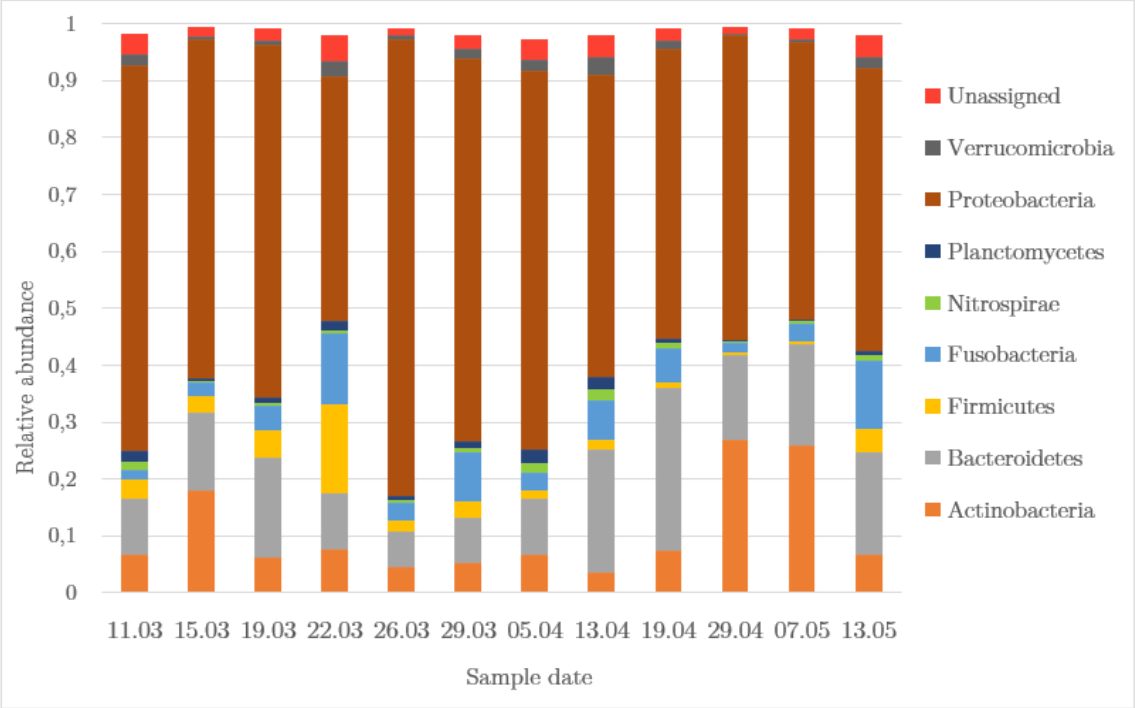


***Figure 25**: Bar chart showing the relative abundance of each bacterial OTU according to each sample date, at the phyla level, in the water compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown*

Concerning the water samples, *Proteobacteria* was the most abundant phylum with 58.5% ± 10.6% of the total reads, followed by *Bacteroidetes* (14.6% ± 5.6%), *Actinobacteria* (10.5% ± 8.3%), *Fusobacteria* (5.5% ± 3.8%) and *Firmicutes* (3.3% ± 4.1%). These major phyla are quite similar to the ones observed in the PAFF Box water sample studied in Eck *et al.* (2019) experiment. However, B*acteroidetes* was the major phylum, followed by *Proteobacteria* and *Actinobacteria*.

No visible "breakdowns" or strong variations in the microbiota seem to be observed, the relative abundances fluctuating in a more gradual way. The sample of March 22, however, appears to show a more specific profile, with more reads assigned to the *Firmicutes* phylum, i.e. 15.7% instead of the general mean of 3.3%. Likewise, more reads were assigned to the *Fusobacteria* phylum with 12.4% of the total reads instead of the general mean of 5.5%. On the next sampling date, on April 26, the sample reads showed a drop in *Firmicutes* and *Fusobacteria* phyla. *Firmicutes* then remained at very low proportions (1.3% ± 0.8% of the total reads), except for the last sample, on May 13 (4%). This last sample also showed a higher proportion in reads assigned to *Fusobacteria* (12.2%). Two noticeable variations in the microbiota at the phyla level could thus be highlighted: a variation between March 22 and March 26 and a 2nd variation between May 7 and May 13.

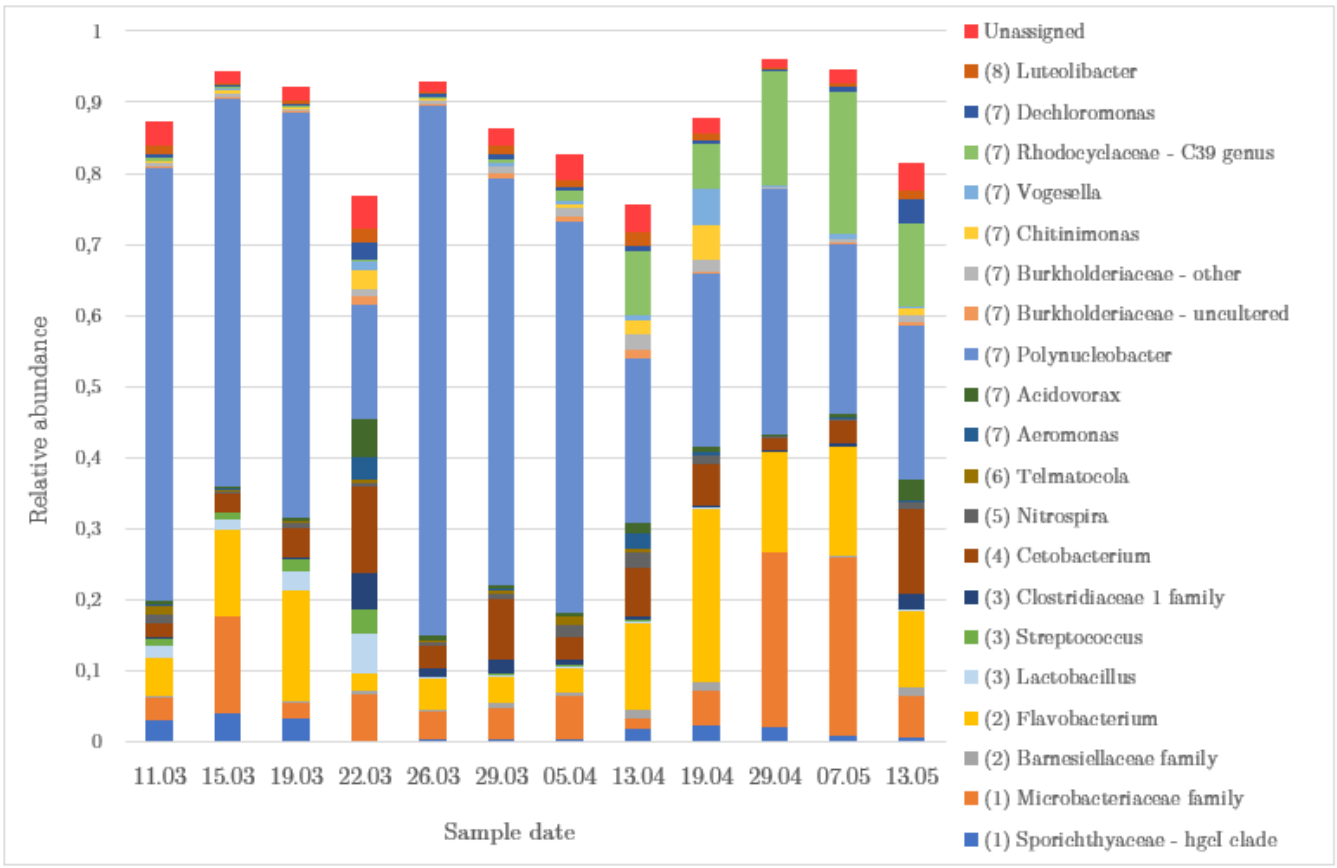


***Figure 26**: bar charts showing the relative abundance of each bacterial OTU according to each sample date, at the genus level, in the water compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family or class levels, in which case the level is indicated. The number in front of each genus corresponds to its phyla, namely: (1) Acidobacteria; (2) Bacteroidetes; (3) Firmicutes; (4) Fusobacteria; (5) Nitrospirae; (6) Planctomycetes; (7) Proteobacteria; (8) Verrucomicrobia*

Focusing at the genus level (Fig. 26), the samples were dominated by the *Polynucleobacter* genus, the most abundant genus of the *Proteobacteria* phylum. This observation correlates with Bartelme *et al.* (2019) experiment in which AP systems had *Polynucleobacter* as the most abundant phylum, while equivalent RAS did not showcase them. This genus is a common heterotrophic member of freshwater microbiota that might be found in AP systems since the water may contain nutrients deriving from plants or phytoplankton commonly found in rivers or lakes, such as glycolate, i.e. a phytoplankton exudate utilized by *Polynucleobacter* (Bartelme *et al.*, 2019; Paver & Kent, 2010). While representing 85% of the *Proteobacteria* from March 11 to April 5, *Polynucleobacter* then represented less than 50% of the *Proteobacteria* from April 13 to the end of the experiment, with a strong increase of the C39 genus instead (*Rodhocyclaceae* family).

With a high proportion of reads assigned to C39 genus in the PAFF Box water sample of Eck *et al.* (2019) experiment, this OTU can also be found in freshwater such as river (Cannon *et al.,* 2017). According to Gao *et al.* (2017), he could be linked to the denitrification process associated to acetate. Its metabolism may be similar to the *Dechloromonas* genus (0.9% of the total reads), a denitrifying bacterium that uses acetate under anoxic conditions (Gao *et al.*, 2017). According to Gao *et al.* (2017) experiment, C39 and *Dechloromonas* development are correlated to high C/N ratio, high pH and high NH_4^+-N concentration. Focusing on the parameters' evolution in the PAFF Box, pH did increase on April 12 while no apparent changes occurred for the NH_4^+ concentration. However, this observation is not sufficient to explain the increase in C39 relative abundance, since the pH fluctuated a lot during the experiment.

Another major OTU is the *Flavobacterium* genus from the *Bacteroidetes* phylum, representing more than 10% of the total reads on average. His relative abundance fluctuated a lot throughout the

experiment, reaching its pick on April 19 with nearly 25% of the total reads. However, this percentage is much lower than the one obtained in the PAFF Box water sample of Eck *et al.* (2019) experiment in which *Flavobacterium* represented 50% of the total reads. This genus, as well as the *Acidovorax* genus which represented more than 1% of the total reads, were mostly found in the root sample of Schmautz *et al.* (2017) experiment and are known to be rhizosphere bacteria. Since the water is constantly flowing from the hydroponic compartment to the fish compartment, it seems plausible to find these rhizosphere bacteria in the sump water. However, *Flavobacterium* was present in the circulating water even prior to the introduction of lettuces in the system, on March 11 (5% of the total reads). Thus, *Flavobacterium* does not seem to be necessarily correlated to plant roots. Indeed, Bartelme *et al.* (2019) and Rurangwa & Verdegem (2013) pointed its presence in all their studied RAS. Bartelme *et al.* (2019) suggested that they may proliferate in compartments with abundant complex organic carbon such as mechanical filters. This genus is also associated to opportunistic fish pathogens (Munguia-Fragozo *et al.,* 2015; Bartelme *et al.*, 2019). However, the fish were supposedly healthy during the experiment.

Another relatively abundant OTU is the *Microbacteriaceae* family, representing 8.5% ± 8.2% of the total reads and reaching nearly 25% of the reads on April 29 and May 7. This family seems to be associated with plants, but it was also found in RAS, wastewater or freshwater such as lakes (Zachow *et al.*, 2014; Munguia-Fragozo *et al.*, 2015; Wang *et al.*, 2012; Cannon *et al.,* 2017).

Cetobacterium, a genus of the *Fusobacteria* phylum, represented 5.5% ± 3.8% of the total reads during the experiment. The members of this genus are mostly described as freshwater fish gut bacteria, representing more than 75% of the total reads in the fish faeces sample of Schmautz *et al.* (2017) experiment (Tsuchiya *et al.*, 2007; Bartelme *et al.,* 2019). Eck *et al.* (2017) suggested that high proportion of this genus in the water may be correlated to ineffective faeces removal from the system, which does not seem to be the case here. *Cetobacterium* might also be involved in the loss of nitrogen via denitrification process (Wongkiev *et al.*, 2018).

The hgcI genus of the *Sporichthyaceae* family (*Acidobacteria* phylum) represented 1.5% ± 1.3% of the total reads during the experiment. Very few studies have documented this genus, but it was found in freshwater lakes (Jiao *et al.*, 2018). The *Sporichthyaceae* family involve bacteria with slow growth rates, tedious growth requirements and was mostly found wastewater (Wang *et al.*, 2012; Wei *et al.*, 2015).

Lactobacillus and *Streptococcus* that dominated the *Firmicutes* in the root and biofilter samples and had some of the highest relative abundances in the root samples, appear as more minor genera in the sump water compartment. In the water samples, their reads proportions dropped in between March 22 and March 26, going from 5.7% and 1.6% of the total reads respectively on March 22, to 0.1% and 0.09% on March 26. Their presence in the water might be due to their potential presence in the fish intestine and thus, in the fish faeces. For instance, in Schmidt *et al.* (2016) experiment on a RAS, fish intestine microbiotas were dominated by both *Lactobacillus* and *Streptococcus* in some cases, i.e. more than 90% of the total OTUs. However, in Schmidt *et al.* (2016) particular study, these highly abundant intestine organisms did not seem to influence the microbiota of the surrounding environment since none of those OTUs were found in the water or biofilter. This might not be the case in our experiment.

Going back to Fig. 26, it can be noticed that *Firmicutes* also included *Clostridiaceae*, a family that was only observed at significant proportion in the water samples and that reached 5% of the total reads on March 22. As for the *Lactobacilli* and *Streptococcus* genera, this family can be found on plant surface and in fish gut, being used as fish probiotics as well (Martínez Cruz *et al.,* 2012; Goel *et al.,* 2017). This might explain the presence of this family and more particularly the *Clostridium* genus in the fish faeces sample of Schmautz *et al.* (2017).

Finally, it can be noticed that a potential fish pathogen genus was found in the water samples, even though the fish were presumably healthy during the experiment. Indeed, *Aeromonas* constituted 0.6% of the total reads, potentially causing cutaneous haemorrhages on fish (CABI, no date). This genus was also found at 0.25% in Schmautz *et al.* (2017) fish faeces sample.

In conclusion, the bacterial communities of the water samples appear to have fluctuated and variated a lot throughout the experiment but did not show drastic breakdowns. However, two samples showcased important variations at the phyla level comparing to their surrounded samples: the March 22 and the May 13 samples. At the genus level, the general microbiota observed in between April 13 and May 13 seems different from the microbiota observed in between March 11 and April 5, with the strong increase of C39 for instance. Thus, we can suppose that events or important parameters variations inducing those modifications might have occurred several times: in between March 19 and March 22; in between March 22 and March 26; in between April 5 and April 13 and in between May 7 and May 13. The potential causes and factors will be discussed later on.

- Fungal communities

The evolution of the fungal communities found in the circulating water of the AP system from March 26 to May 13 is visualised with the following graph (Fig. 27). The samples of March 11, March 15, March 19 and March 22 are not considered in this study since the majority of their reads were "no blast hit", as already explained.

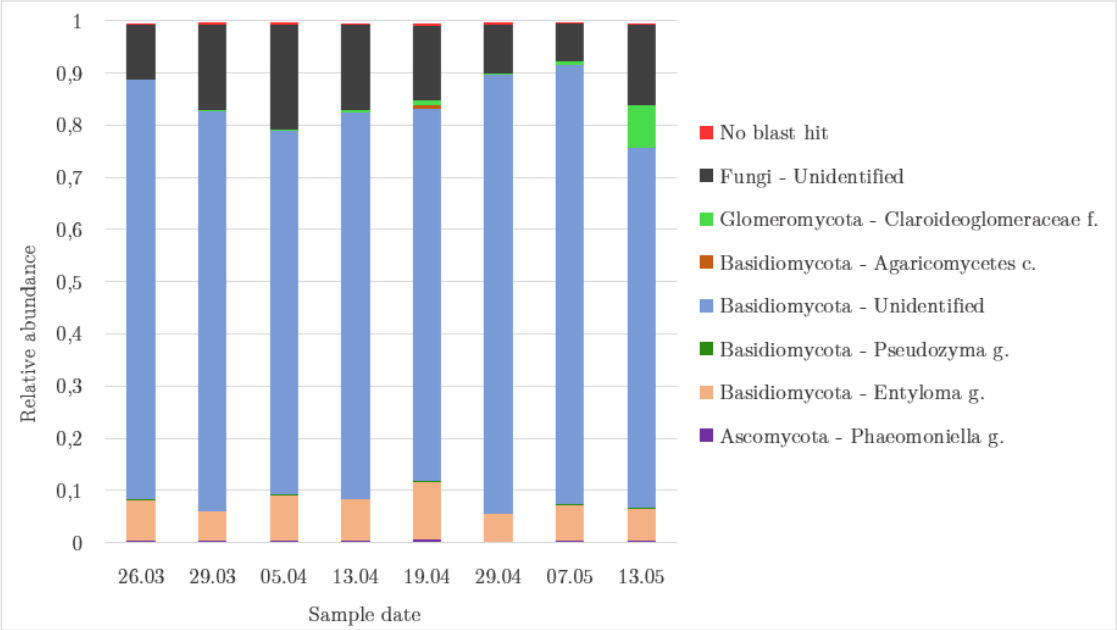


***Figure 27**: Bar chart representing the relative abundance of each fungal OTU according to each compartment, at the fungal genus level. Only the OTUs representing more than 0.1% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family, class or phyla level, in which case the level is indicated.*

Concerning the fungal water samples, unidentified *Basidiomycota* was the most abundant group with 76.2% ± 6.1% of the total reads, followed by unidentified *Fungi* (13.7% ± 4.4%) and the *Entyloma* genus (7.4% ± 1.8%). While this last genus remained relatively stable over time, the unidentified *Basidiomycota* and unidentified *Fungi* fluctuated gradually, in opposite ways. The proportion of *Basidiomycota* slowly decreased from March 26 to April 5, going from 80.2% to 69.7% of the total reads. It then gradually increased, reaching 84.2% on May 7. The next sampling date, i.e. May 13, appears as a relative shift in the microbiota, with a sudden lower proportion of *Basidiomycota*, a higher proportion of unidentified *Fungi* and a surprisingly high proportion of *Claroideoglomeraceae* (*Glomeromycota* phylum). While representing less than 0.5% of the total reads from March 26 to May 7, this family represented more than 8% on May 13. According to these observations, events or parameters variations inducing these modifications might have occurred three times during the sampled period: prior to March 26; in between April 5 and April 13; and in between May 7 and May 13, this last sample being a relative "shift" in the fungal microbiota. Those potential causes and factors will be discussed later on.

The *Claroideoglomeraceae* present at high proportion in May 13 sample, is a part of the *Glomeromycota* phylum. This group is constituted of fungi generally called "arbuscular mycorrhizal fungi" (AMF), i.e. obligate biotrophs that form a symbiosis with most land plants (Ferrol *et al.*, 2004). Presently, the only known genus of the *Claroideoglomeraceae* family is the *Claroideoglomus*, described as AMF (Schüßler & Walker, 2010). Surprisingly, this family of potential PGPM was mostly found in the water samples and biofilter, i.e. 0.02% in the endosphere and 0.06% in the rhizoplane samples on average. Thus, some members of this group might not be obligate biotrophs. Indeed, Schüßler & Walker (2010) mentioned that all *Glomeromycota* were described as AMF based on an analogy with species for which the biology was studied and known.

The *Entyloma* genus from the *Exobasidiomycetes* class, present in all compartments but at higher proportion in the water samples (7.4%), seemed relatively stable over time. This genus is mostly known for parasitizing a wide range of plants and more specifically the *Asteraceae* family (Begerow *et al.*, 2002). Growing and sporulating inside the hosts' cells, it is surprising that this genus had the largest proportion in the water samples (7.4%), and not in the lettuce root samples (*Lactuca sativa* – *Asteraceae* family), i.e. 2.9% on average in the endosphere and rhizoplane samples. Considering that the sampled lettuces were supposedly healthy, the *Entyloma* species found in this compartment might not be plant parasites. Indeed, this genus was also found in activated sludge of a sewage treatment plant (Liu *et al.*, 2007).

Focusing on minor OTUs, the *Phaemoniella* genus was only present at noticeable proportions in the water and biofilter samples and seemed relatively stable over time, representing 0.3% ± 0.1% of the total reads in the water samples. Members of this genus seem to be mostly involved in plant pathogenicity (Damm *et al.*, 2010; Crous & Gams, 2000). Likewise, the *Agaricomycetes* class was only found in noticeable proportions in the water and biofilter samples, representing 0.1% ± 0.2% of the reads in the water samples. This large group contains "fungi that function as decayers, pathogens, and mutualists in both terrestrial and aquatic habitats" (Hibbett *et al.*, 2014, p. 373).

Lastly, the *Pseudozyma* genus present in all compartments except for the biofilter, seemed relatively stable in the water over time, representing 0.16% ± 0.08% of the total reads. Rapidly expanding, this genus is of industrial importance as it produces compounds used as biosurfactants and antimycotics (Boekhout, 2011). Some species are also used for biodiesel production and plastic degradation for instance (Boekhout, 2011).

In conclusion, no drastic breakdowns seem to have occurred in the fungal communities from March 26 to May 13, the relative abundances fluctuating gradually. However, most of the OTUs taxonomy stopped at the phyla or kingdom level, which hid the potential variations within those groups.

4.3.4.2 Biofilter

- Bacterial communities

The evolution of the bacterial communities found in the biofilter of the AP system throughout the experiment is visualised with the following graphs (Fig. 28 and Fig. 29).

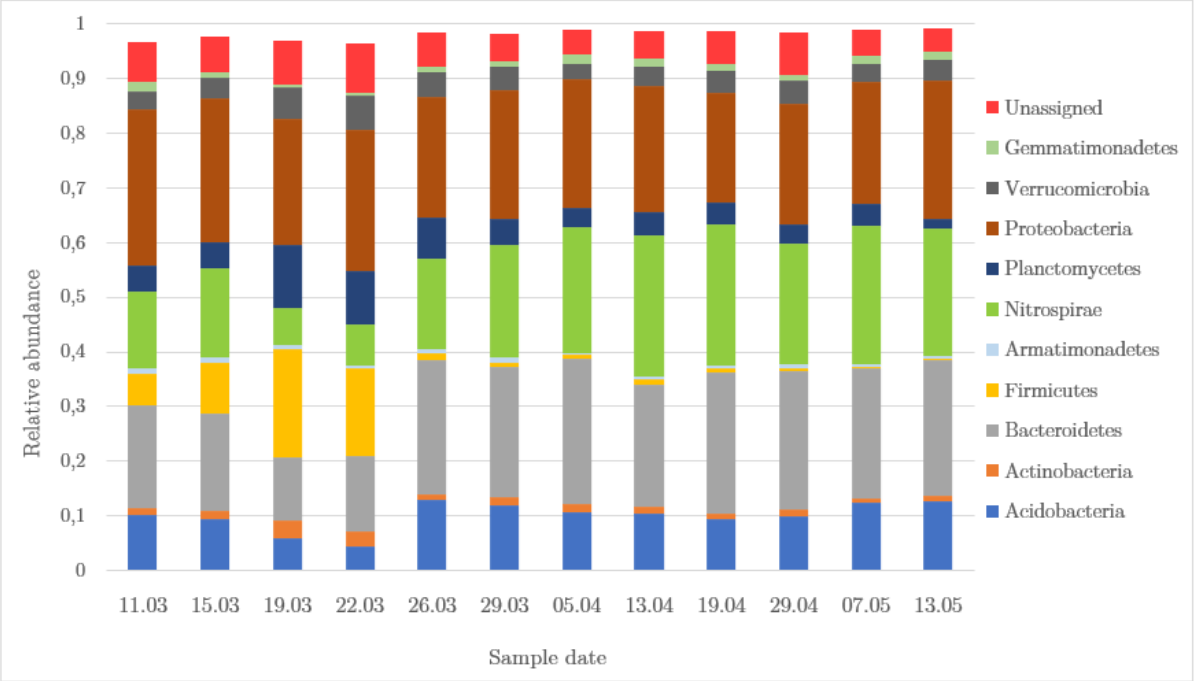


***Figure 28**: Bar chart showing the relative abundance of each bacterial OTU according to each sample date, at the phyla level, in the biofilter compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown*

Proteobacteria and *Bacteroidetes* were the most abundant phyla with 23.8% ± 2.4% and 21.5% ± 5% of the total reads respectively, followed by *Nitrospirae* (18.9% ± 6.7%), *Acidobacteria* (10% ± 2.6%), *Planctomycetes* (5.3% ± 2.8%), *Firmicutes* (4.7% ± 6.8%) and *Verrucomicrobia* (4.1% ± 1%). The major phyla are quite similar to the ones observed in the PAFF Box biofilter sample of Eck *et al.* (2019) experiment as well as the biofilter sample of Schmautz *et al.* (2017) and Wongkiev *et al.* (2018). However, the proportion of *Nitrospirae* reads in their experiments was much lower, i.e. around 5%, and no significant proportion of *Firmicutes* was spotted. In the biofilter, *Firmicutes* reads' relative abundance dropped in between March 22 and March 26 which correlates to the observation made in the water samples, at a lower extent. Here, *Firmicutes* went from an average of 12.8% ± 6.4% between March 11 and March 22 (1st period) to an average of 0.7% ± 0.3% between March 26 and the end of the experiment, on March 13 (2nd period). This marked an important shift towards the biofilter bacterial communities, as the samples of the 1st period are highly significantly different from the samples of the 2nd period, according to the Adonis test made on those two timeframes ($p<0.001$). The R^2 value showed that more than 55% of the variation observed between the samples was explained by this grouping, i.e. the two periods. After the disappearing of *Firmicutes* reads from the major phyla, the microbiota samples seemed to be stable in time, with highly similar OTUs and relative abundances.

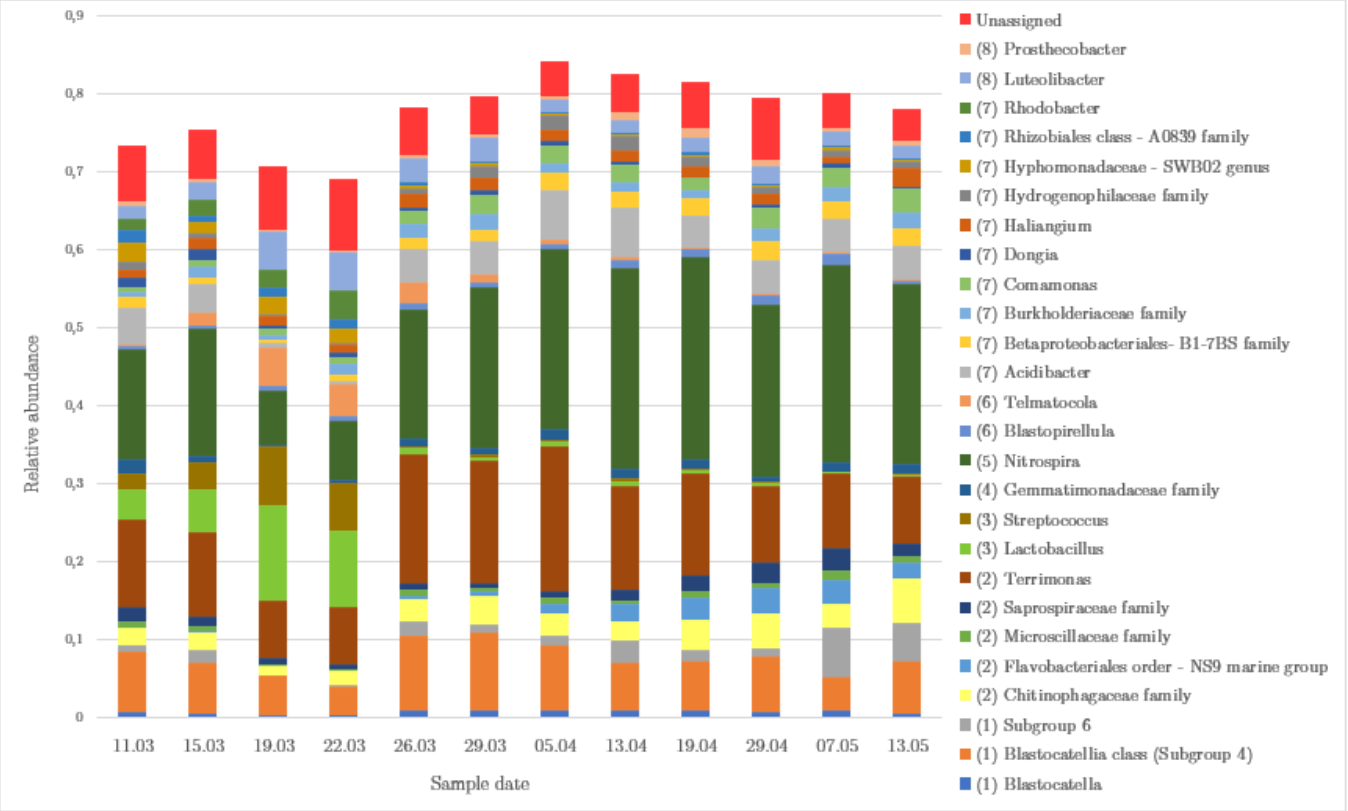


***Figure 29**: Bar charts showing the relative abundance of each bacterial OTU according to each sample date, at the genus level, in the biofilter compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family or class levels, in which case the level is indicated. The numbers in front of each genus corresponds to its phyla, namely: (1) Acidobacteria; (2) Bacteroidetes; (3) Firmicutes; (4) Gemmatimonadete; (5) Nitrospirae; (6) Planctomycetes; (7) Proteobacteria; (8) Verrucomicrobia*

Focusing on the genus level (Fig. 29), *Nitrospira* was the most represented one with 18.9% ± 6.7% of the total reads. In fact, the *Nitrospirae* phylum was only composed of this single genus. *Nitrospira* has recently been described as a complete ammonia oxidizer (COMAMMOX), being capable of doing the entire ammonia to nitrate transformation (Pinto *et al.*, 2016). This nitrification process is in fact the key role of the biofilter. It appears that in a lot of RAS and AP reviews, this crucial process is described as being mainly performed by *Nitrosomonas (Nitrosomonadaceae* family), an ammonia oxidizing bacteria (AOB), and *Nitrobacter (Rhizobiales* order), a nitrite oxidizing bacteria (NOB) (Wongkiew *et al.*, 2017; Rurangwa & Verdegem, 2013; Munguia-Fragozo *et al.*, 2015). However, none of those two genera were found in the data. At a broader level, both *Nitrosomonadaceae* and *Rhizobiales,* the *Nitrobacter* order, had very low relative abundance, i.e. about 0.02% of the total reads. Likewise, Eck *et al.* (2019), Schmautz *et al.* (2017) and Bartleme *et al.* (2019) experiments reported very low percentage of reads to no presence at all of those two genera in their RAS and/or AP biofilters, compared to the *Nitrospira* genus. This observation supports Bartelme *et al.* (2019)' point, saying that *Nitrospira* is actually the most common nitrifying group across RAS and freshwater aquaria and that aquaculture organizations should better inform the system operators in choosing starter cultures and substrates for biofilters. Besides, *Nitrospira* presence in biofilters might be explained by their potential presence in drinking water systems (Bartelme *et al.*, 2019; Pinto *et al.*, 2016). Since about 250 L of tap water were added weekly in the PAFF Box, this might be a plausible hypothesis. A microbiota analysis could perhaps be performed on tap water. With all that being said, it should be noted that a high relative abundance or a greater presence is not necessarily linked to important metabolic activities.

Terrimonas from the *Chitinophagaceae* family, was the second most abundant genus throughout the experiment, representing 11.9% ± 3.7% of the total reads. Found in wastewater and freshwater such as lakes, their presence in RAS or aquaponic biofilter seem to be very little documented (Wang *et al.*, 2012). Strictly aerobic, Luo *et al.* (2017) found a high proportion of this genus in a biofilm sample of

an aquaculture system rearing Nile tilapia. At a broader level, uncultured members of *Chitinophagaceae* family represented more than 3% of the total reads in the biofilter. Also found at similar proportion in Eck *et al.* (2019)'s biofilter sample, this family includes members that are known to reduce nitrate or achieve complete denitrification (Chutivisut *et al.,* 2018). Likewise, *Comamonas* (1.8% ± 0.08%) and *Rhodobacter* (1.1% ± 1.4%) have been described as being capable of denitrification (Chutivisut *et al.,* 2018; Zhao *et al.*, 2017). Their presence was correlated to a low C/NO_3^- environment in Chutivisut *et al.* (2018) experiment, which could explain their presence in the biofilter since higher concentrations of nitrate might be found in this compartment.

Blastocatellia class from the *Acidobacteria* phylum represented a relatively important part of the reads throughout the experiment, i.e. 6.8% ± 1.9%. However, this class is very little documented. Apparently, it can be found in freshwater such as lakes and wastewater (Chaya *et al.,* 2019; Guo *et al.,* 2019).

The *Firmicutes* that strongly decreased in between March 22 and March 26, were represented at 61% by *Lactobacilli* and at 37.7% by *Streptococcus* on average. While showing some potential advantages in the water compartment via fish probiotics for instance, their possible impacts on the biofilter are rather unknown. However, the primary role of the biofilter is the nitrification process meaning that nitrifying bacteria development should be emphasized. In this case, the important presence of *Lactobacilli* and *Streptococcus* could inhibit their development via competition or production of compounds that inhibit the growth of microorganisms, as already explained. Their presence in the biofilter might be explained by the fact they were already found in small proportion in the circulating water, and then in very high proportion in/on the plant roots. The constant water flow from the hydroponic compartment to the biofilter could have participated to their relatively high proportion in the biofilter after March 11. At much lower relative abundance in the water, those organisms might have found a more suitable environment in the biofilter until March 22. They are described as being aerotolerant or anaerobic as well as aciduric or acidophilic (Tannock, 2004). In other words, these lactic acid bacteria (LABs) tend to develop in more acid and more poorly oxygenated environment. While the pH was rather neutral in the water (7.5 on average), the biofilter might have had a more acid and less oxygenated environment than the circulating water. Indeed, the nitrification process that occurs mainly in the biofilter tends to lower the pH via H^+ production, as already explained. However, the presence of those LABs in the biofilter is rather surprising since none of the previously mentioned studies specified their presence in AP or RAS biofilters, e.g. in Schmautz *et al.* (2017), Eck *et al.* (2019), Bartelme *et al.* (2019), Ruranga & Verdeghem (2015) and Schmidt *et al.* (2016) experiments for instance. However, those experiments were not made at early stages of a system transitioning from a RAS to an AP system.

It can be noticed that *Lactobacilli* and *Streptococcus* were not the only genera to undergo a strong decline in between March 22 and March 26. Indeed, the *Hyphomonadaceae* reads proportion was divided by 10 between those two periods (2% ± 0.4% during March 11-March 22 and 0.28% ± 0.09% during March 26 - May13), *Rhodobacter* proportion was divided by 100 (2.4% ± 0.9% to 0.02% ± $8.6.10^{-5}$), and the A0839 family (*Rhizobiales* class) went from 1.3% ± 0.2% to 0.2% ± 0.07% of the total reads.

In conclusion, the bacterial communities of the biofilter samples underwent a significant shift in between March 22 and March 26 with a drop of *Lactobacilli, Streptococci* and other minor taxa. After this change, the biofilter microbiota seemed relatively stable in time, having the same OTUs with similar proportions.

- Fungal communities

The evolution of the fungal communities found in the biofilter of the AP system from March 26 to May 13 is visualised with the following graph (Fig. 30). The samples of March 11, March 15, March 19 and March 22 are not considered in this study since the majority of their reads were "no blast hit".

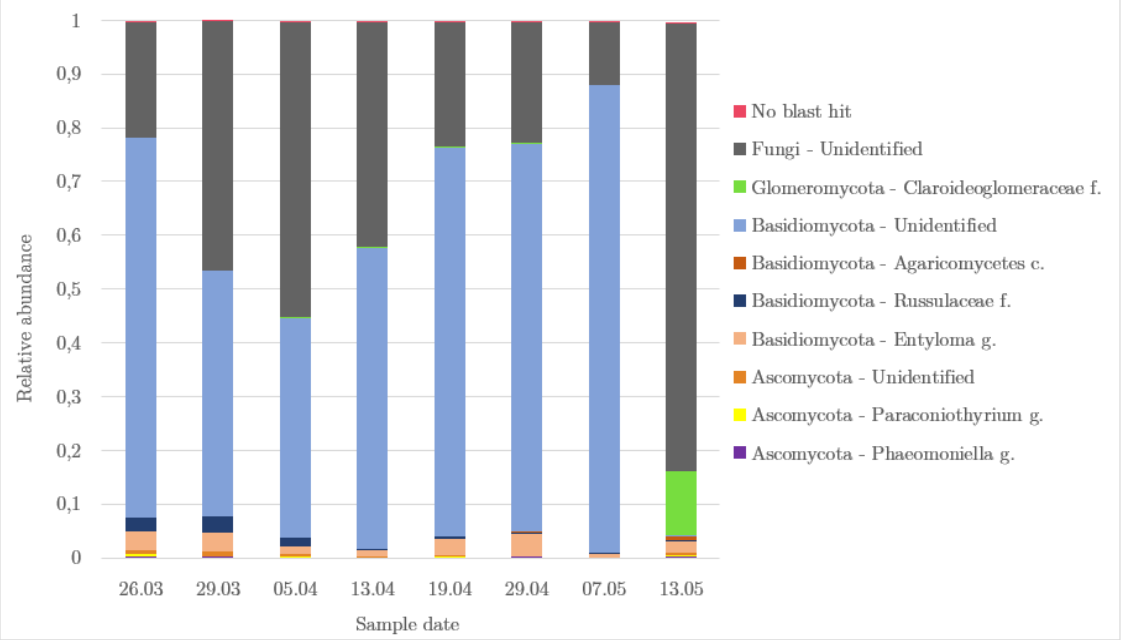


***Figure 30**: Bar chart representing the relative abundance of each fungal OTU according to each compartment, at the fungal genus level. Only the OTUs representing more than 0.1% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family, class or phylum levels, in which case the level is indicated.*

Unidentified *Basidiomycota* was the most abundant group with 55.6% ± 27% of the total reads, followed by unidentified *Fungi* (38% ± 23%) and the *Entyloma* genus (2.5% ± 1.3%). The general microbiota evolution is very similar to the one observed in the water compartment, but in a more "intense" way, with broader variations. Indeed, the unidentified *Basidiomycota* decreased from March 26 to April 5, going from 70.6% to 41% of the total reads. It then strongly increased, reaching 87% on May 7. The next sampling date, i.e. May 13, appears as a drastic shift in the microbiota, with a complete disappearance of the *Basidiomycota* (0.2%), a majority of unidentified *Fungi* (83%) and a sudden high proportion of *Claroideoglomeraceae*, i.e. 12% on May 13 instead of 0.07% on average from March 26 to May 7. The unidentified *Basidiomycota* might have been constituted of similar fungi, being less competitive to the unidentified *Fungi* and the *Claroideoglomeraceae* in the environment found during the period May 7 – May 13. The same hypotheses made for the water compartment could be done for the biofilter: events inducing important fungal microbiota modifications might have occurred three times during the sampled period: prior to March 26; in between April 5 and April 13; and in between May 7 and May 13, this last sample being considered as a "breakdown" in the fungal microbiota.

Considering other OTUs, the *Russulaceae* family was only found in noticeable proportions in the biofilter, i.e. more than 1% on average whereas less than 0.05% for the other compartments. This family was mostly present from March 26 to April 5 (2.4%), disappearing from the important reads from April 13 to the end of the experiment (0.26%). Thus, the potential event that occurred in between April 5 and April 13 and reduced the *Basidiomycota* fungi proportion might also have impacted the *Russulaceae* members. Those are mostly described as mushroom-forming *Basidiomycota* and as obligate ectotrophic mycorrhiza fungi, forming a symbiosis with forest trees and seed plants to a lesser extent, by extracellularly colonizing their roots (Shimono *et al.*, 2004). Since this family is very diverse, all members of *Russulaceae* might not be obligate ectomycorrhiza fungi or their identification might also be due to an incorrect OTU assignation.

Likewise, *Paraconiothyrium* was only found at small but noticeable proportion in the biofilter (0.15% ± 0.17%) while the *Phaemoniella* (0.12% ± 0.05%) and *Agaricomycetes* (0.11% ± 0.2%) were found at similar proportions in the water samples. The *Paraconiothyrium* is a relatively new genus, with

some species described as potential biocontrol agents, producers of antibiotics and potential bioremediator, i.e. organisms that can degrade target pollutants (Verkley *et al.*, 2004).

In conclusion, the biofilter fungal microbiota generally followed the same tendency as the fungal water samples but with broader variations. In particular, the May 13 sample represents a drastic breakdown in the fungal microbiota with a drop of *Basiodiomycota*. This shift was not observed in the bacterial microbiota of the May 13 biofilter sample. Besides, the bacterial communities seemed pretty stable in the biofilter during the March 26 – May 13 period which was not the case for the fungi. However, the bacterial microbiota underwent an important shift in between March 22 and March 26 with the drop of *Firmicutes* and other minor taxa which could correlate with the fungal modifications observed from March 26 to April 5. An event in between March 22 and March 26 could have initiate those fungal and bacterial variations.

4.3.4.3 Endosphere

- Bacterial communities

The evolution over time of the bacterial communities found in the lettuce endosphere of the hydroponic compartment of the PAFF Box is visualised with the following graphs (Fig. 31 and Fig. 32).

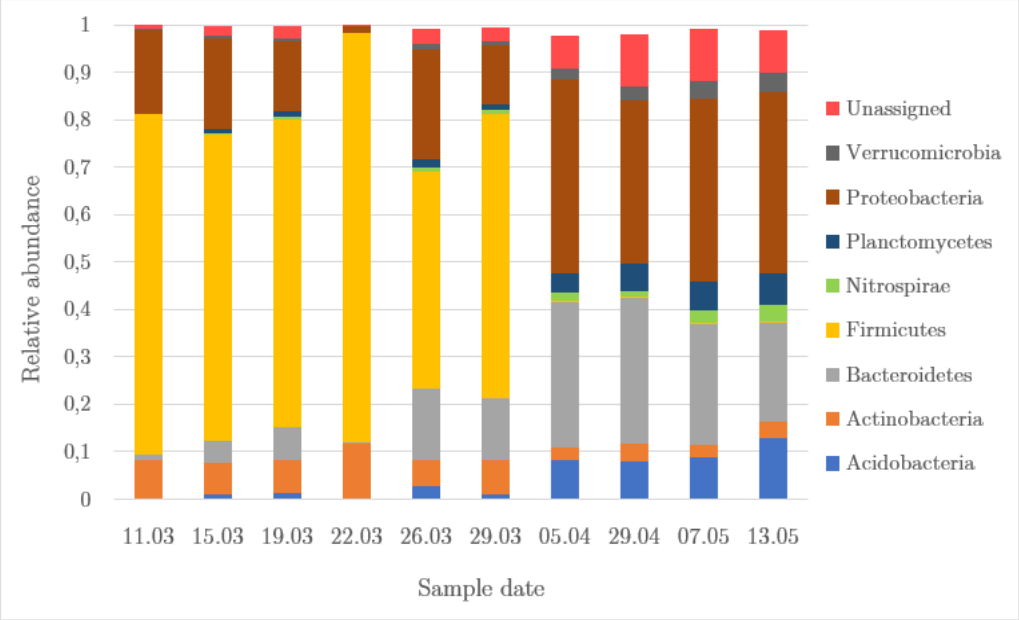


***Figure 31**: Bar chart showing the relative abundance of each bacterial OTU according to each sample date, at the phyla level, in the endosphere compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown.*

As a reminder, the samples from March 11 to April 5 were made on the Group 1 lettuces while the samples from April 29 to May 13 were made on the Group 2 lettuces, since the lettuces from the 1st group were looking diseased after April 5. It thus appears that the two groups of lettuces could be compared and used in the same experiment since the April 5 sample (Group 1 lettuces) is very similar to the April 29 sample (Group 2 lettuces).

Focusing at the phylum level (Fig. 31), two completely different microbiota profiles are observed: the one from March 11 to March 29 (1st period) and the one from April 5 to the end of the experiment (2nd period). During the 1st period, *Firmicutes* largely dominated with 65.6% ± 13.3% of the total reads while they only represented 0.1% ± 0.09% during the 2nd period. *Proteobacteria* was the second most abundant phylum during the 1st period with 14.7% ± 7.4% of the total reads, while it was the most abundant one during the 2nd period, with 38% ± 2.6%. For its part, *Bacteroidetes* appeared as a minor phylum in the 1st period (6.68% ± 6%) but represented a major phylum during the 2nd period

(27.5% ± 4.7%). At minor extent, *Acidobacteria*, *Planctomyctes*, *Nitrospirae* and *Verrucomicrobia* also gained importance during the 2nd period. *Actinobacteria* on another hand, followed the *Firmicutes* phylum tendency by going from 7.7% ± 2% during the 1st period to 3% ± 0.6% during the 2nd period. It can be noticed that the March 22 sample seems to have a different microbiota than the other samples of the 1st period, being dominated at 86.3% by the *Firmicutes*.

Hence, it appears that the 1st group of lettuces underwent a drastic microbiota change between March 29 and April 5, more than a week after the *Firmicutes* change observed in the biofilter samples. This shift is confirmed by an Adonis test showing that the samples from the 1st period were highly significantly different from the samples of the 2nd period ($0.001<p<0.01$). The R^2 value showed that more than 85% of the variation observed between the samples was explained by this grouping, i.e. the two periods.

Comparing our results to other AP studies, the general microbiota observed during the 1st period is very different from what was observed in the plant root samples of Schmautz *et al.* (2017) and Wongkiev *et al.* (2018) experiments for instance. However, the root sample was taken after one month of operating AP system in the case of Schmautz *et al.* (2017) and at the lettuce harvesting in the case of Wongkiev *et al.* (2018). Thus, they do not represent root microbiota of early stages in an AP system. Following the general bacterial breakdown in our experiment, the microbiota is much more comparable to their experiments during this 2nd period, both studies having a predominance of *Proteobacteria*, *Bacteroidetes* and *Acidobacteria*. Focusing on soil-based lettuce culture, Cardinale *et al.* (2015) experiment on lettuce root microbiota also reported a predominance of *Proteobacteria* and *Bacteroidetes* phyla on fully matured lettuces. Erlacher *et al.* (2014) experiment found a majority of *Proteobacteria* as well, on both young lettuce roots (2 weeks) and mature lettuces roots (4 weeks). On its side, Schreiter et al. (2014) study showed a predominance of *Proteobacteria* (around 50%), followed by *Actinobacteria* (around 15%) and *Firmicutes* (around 10%) on lettuce roots of 3 weeks old and 7 weeks old. The results can thus variate between the surveys, however *Proteobacteria* seem to generally dominate the lettuce roots microbiota.

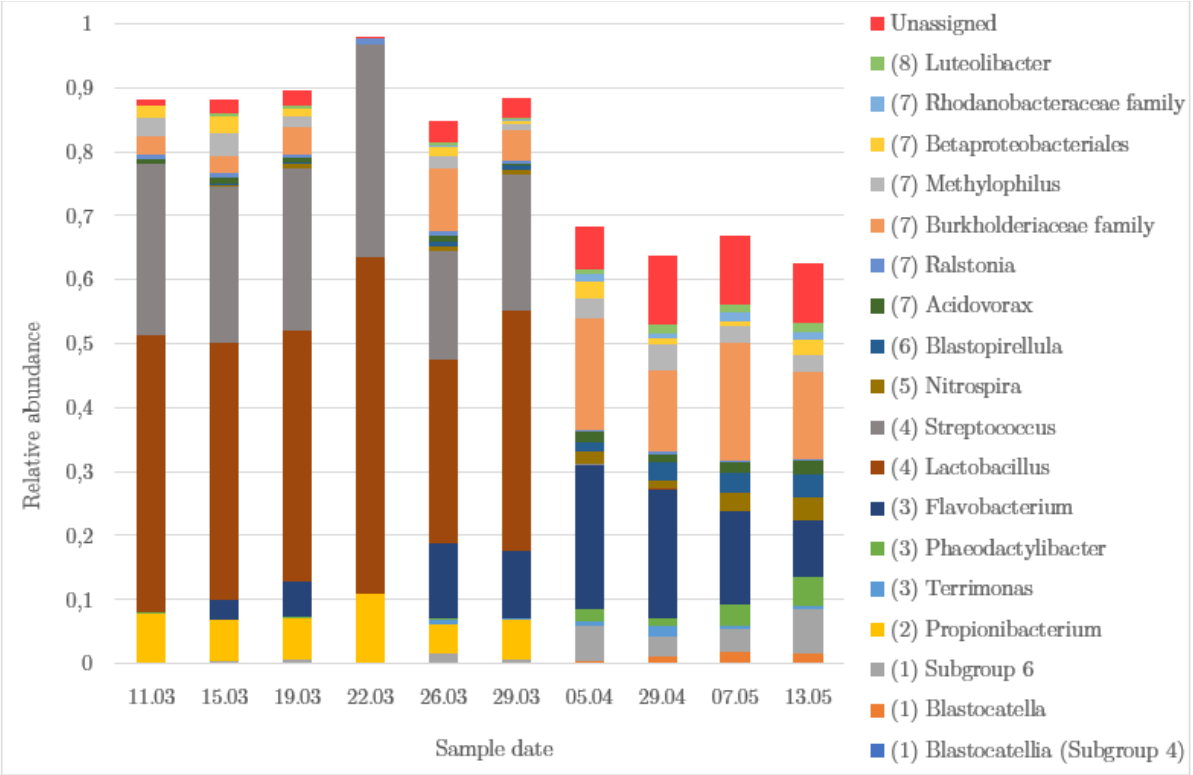


***Figure 32**: Bar charts showing the relative abundance of each bacterial OTU according to each sample date, at the genus level, in the biofilter compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family or class levels, in which case the level is indicated. The numbers in front of each genus corresponds to its phylum, namely: (1) Acidobacteria; (2) Actinobacteria; (3) Bacteroidetes; (4) Firmicutes; (5) Nitrospirae; (6) Planctomyctes; (7) Proteobacteria; (8) Verrucomicrobia*

Focusing at the genus level (Fig. 32), the *Firmicutes* that dominated the 1st period was represented at 61.28% by *Lactobacilli* and at 37.5% by *Streptococci*, as seen in the biofilter. Both genera almost disappeared from the proportions during the 2nd period. The *Actinobacteria* phylum that also declined in between March 29 and April 5 was represented by the *Propionibacterium* genus. The *Propionibacteria* are anaerobic and produce propionic acid from biomass through fermentation process (Suwannakham & Yang, 2005). They do not seem to be usually found in plant roots. On their parts, the *Lactobacilli* and *Streptococci* can be found on plants or plant-derived materials, but they do not seem to be commonly found in/on roots microbiome either (Tannock, 2004; Kolton *et al.,* 2016). In Schreiter *et al.* (2014) experiment in which 10% of *Firmicutes* were found on lettuce roots for instance, this phylum was not characterised by those two genera. However, some *Lactobacilli* are used as "plant" probiotics in soil agriculture and hydroponics, being able to stimulate the germination, the plant growth as well as improve the plant responses to drought (Limanska *et al.,* 2013; Limanska *et al.,* 2018; Yarullina *et al.*, 2014). We can wonder why these two genera were largely dominating the microbiota of the early growth stages of the lettuces, while they do not seem to be commonly found in the rhizosphere. As already said, these LABs have a fermentative metabolism, being aerotolerant or anaerobic as well as aciduric and acidophilic (Tannock, 2004). Hence, they tend to develop in acid and poorly oxygenated environments. These organisms might have found these specific conditions in the shallow boxes in which the lettuces seeds were put to germinate for 11 days, from February 27 to March 11. The boxes were simply filled with tap water, with no particular aeration process or water exchanges. Thus, a strong fermentative environment may have developed in these boxes. This could explain the major presence of *Lactobacilli* and *Streptococci*, as well as the *Propionibacterium*, in the first root sample on March 11, right before the seedlings were transplanted in the PAFF Box. Indeed, the *Propionibacterium* also have a fermentative metabolism. After the transplantation, during the following days and weeks, these organisms might have maintained a relative acid micro-environment in/on the plant roots via lactic and propionic acids production for instance, which might have inhibited the development of other bacteria. Then, the general surrounding environment, i.e. the circulating water and the grow beds, may have supplanted this micro-environment, with a more neutral pH for instance. The LABs and *Propionibacteria* communities then dropped, being supplemented by the other bacteria that were probably more competitive in the AP environment. In Zhang *et al.* (2018) study on rice, the root microbiota underwent a drastic shift after transplantation, showing that the rhizospheric microorganisms could be strongly determined by the geographic location. In our case, it is however only a hypothesis since the drop occurred after 18 days in the PAFF Box, in between March 29 and April 5, which seems like a relatively long period. In Zhang *et al.* (2018) experiment for instance, the microbial shift occurred in the 24h after transplantation. In addition, the biofilter and water compartments also underwent this decline, at much lesser extent. Thus, a particular event or strong parameters variation might also have induced this drop. The two hypotheses can also be combined. Indeed, the LABs and *Propionibacteria* could have dominated the root microbiota in the shallow boxes during germination, these boxes having developed a fermentative environment. Then, after transplantation of the seedlings in the PAFF Box, those communities were able to maintain themselves in/on the roots, but an event or parameters modifications induced their breakdown, both in the roots and biofilter samples.

Going back to Fig. 32, all the other minor genera of the 1st period are generally found in higher proportion during the 2nd period. Nevertheless, the proportions of minor groups such as *Methylophylus* or *Betaproteobacteriales* remained fairly stable throughout the entire experiment, i.e. 2.3% ± 1.2% and 1.4% ± 0.9% of the total reads respectively.

During that 2nd period, the *Flavobacterium* genus and the *Burkholderiaceae* family were the most abundant organisms with respectively 16.54% ± 6.2% and 15.53% ± 2.9% of the total reads. Both types of organisms were found at significant proportions in the water samples which may explain their presence in the root endosphere-rhizoplane samples, since the "root-associated microorganisms are actively recruited by plants from surrounding soil", and in our case, surrounding water (Kolton *et al.*, 2016, p. 190). *Flavobacterium* is known to be a common rhizosphere bacteria genus, being also a major component of the lettuce rhizosphere microbiome in particular (Kolton *et al.*, 2016). While some members are capable of denitrification and sulphate reduction, *Flavobacterium* was shown to be correlated with increased plant biomass and better resistance to pathogens (Wongkiew *et al.*, 2017; Munguia-Fragozo *et al.*, 2015; Kolton *et al.*, 2016). Their strong presence on lettuce roots could thus

be interesting. On another hand, the *Burkholderiaceae* family also found at high proportion in the root samples of Wongkiev *et al.* (2018) and Schmautz *et al.* (2017) experiments, are also known as root-associated bacteria. The *Burkholderia* genera is capable of solubilizing insoluble minerals into soluble forms which can then be used by the plants (D'Amico *et al.,* 2018).

The Subgroup 6 from *Acidobacteria* phylum was an important taxon of the 2nd period microbiota, representing 4.7% ± 1.8% of the total reads. Little documented, this subgroup was positively correlated to pH levels on plant roots and was the most abundant taxon of *Acidobacteria* phylum in Wongkiev *et al.* (2018) experiment.

The *Methylophilus* genus whose proportion remained fairly stable throughout the entire experiment, is constituted of restricted facultative methylotrophs, i.e. bacteria that can metabolize C1 compounds as well as more complex organic compounds in aerobic conditions (Madhaiyan *et al.*, 2009). Little documented, most of the few *Methylophilus'* species seem to be related to plants and roots. In particular, the *Methylophilus methylotrophus* and *Methylophilus rhizosphaerae sp. nov* species isolated from rice rhizosphere, showed multiple PGPM characteristics, as well as an ability to reduce nitrate (Madhaiyan *et al.*, 2009).

Regarding nitrate, *Nitrospira* represented 2.4% ± 0.9% of the total reads during the 2nd period. This shows that the hydroponic compartment, via plant roots, can participate to the nitrification process that mainly occur in the biofilter. *Nitrospira* were also found in the root samples of Schmautz *et al.* (2017) and Wongkiev *et al.* (2018) experiments, at similar proportions.

Other minor genera that were by now, only found in significant proportion in the endosphere samples, can also be mentioned such as *Blastopirellula* (2.8% ± 0.8%), *Phaeodactylibacter* (2.7% ± 1.5%) and *Luteolibacter* (1.2% ± 0.4%), during the 2nd period. Members of those bacteria have been identified as rhizosphere organisms and could also be found in sludge (Nunes da Rocha *et al.,* 2013; Zachow *et al.*, 2014; Shin *et al.,* 2019).

In conclusion, a drastic bacterial shift occurred in the Group 1 lettuces, in between March 29 and April 5, less than 3 weeks after the introduction of the lettuces in the system. Then, the microbiota seems to have remained relatively stable over time, sharing a lot of similarities with other AP studies in which root samples were taken at least 4 weeks after the introduction of the plants in the system.

- Fungal communities

The evolution of the fungal communities found in the lettuce endosphere from April 5 to May 13 is visualised with the following graph (Fig. 33). Indeed, the first 3 weeks of the experiment are missing since all of the 1st run samples contained a majority of "no blast hit" OTUs, i.e. the March 11, March 15, March 19, March 22, March 26 and March 29 samples.

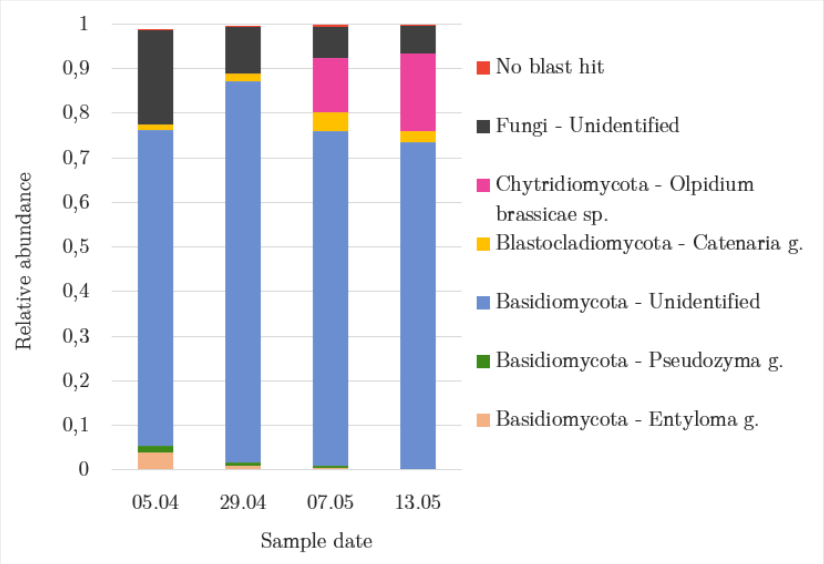


***Figure 33**: Bar chart representing the relative abundance of each fungal OTU according to each compartment, at the fungal genus level. Only the OTUs representing more than 0.1% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family, class or phylum levels, in which case the level is indicated*

Concerning the fungal endosphere samples, unidentified *Basidiomycota* was once again the most abundant group with 76.2% ± 6.4% of the total reads followed by unidentified *Fungi* (11% ± 6.8%). Compared to the water and biofilter compartments, the *Basiodiomycota* seem to have remained fairly stable from April 5 to May 13 while the unidentified *Fungi* gradually decreased, going from 20.9% on April 5 to 6.2% on May 13. Likewise, the *Entyloma* and *Pseudozyma* genera were only represented at relatively high proportion in the April 5 sample (4% and 1.6% respectively). Then, they gradually decreased, reaching less than 0.1% of the total reads on May 13.

In the two last samples, more than 15% of the reads were assigned to the *Olpidium brassicae* species from the *Chytridiomycota* phylum, while it represented less than 0.04% in the previous samples. Only found in the endosphere and rhizoplane samples, this species is described as an obligate parasite living on plant roots (Moreno & Fereres, 2012). In *Lactuca sativa*, it is well known for transmitting lettuce big-vein viruses, responsible of the lettuce big-vein disease (LBVD). Transmitted through viruliferous zoospores, the disease usually induces chlorotic veins, leaves deformations and reduced lettuces size (Moreno & Fereres, 2012). This species was observed at high proportion on the group 2 lettuce, from May 7, but this group was supposedly healthy. Hence, the *Olpidium brassicae* might have not been viruliferous or the symptoms were not visible at that time. Since the zoospores are motile, the arrival and development of this species might have originated from other parasitized lettuces that were in the PAFF Box at the same time. This hypothesis will be discussed later on.

Another OTU that was only found in relatively high proportion in the root samples, was the *Catenaria* genus, from the *Blastocladiomycota* phylum (2.5% ± 1.2%). This genus is mostly described as parasites of nematodes, of other *Blastocladiomycota*, insect eggs and other microinvertebrates, both in aquatic and terrestrial habitats (Strullu-Derrien *et al.*, 2018; Powell, 2017). Throughout the experiment, various nematodes and microinvertebrates were observed in the deep water grow beds, close or on the surface of the plant roots. Some of them might have been analysed as a part of the samples, which could explain their presence in the root rhizoplane and endosphere compartments.

In conclusion, the main fungal variation observed in the endosphere samples was the sudden abundance of the *Olpidium brassicae* species on May 7 and May 13. Considering the bacterial communities, no important microbiota modification was observed at those dates. Apart from that fungal shift, the general fungal microbiota evolution seemed fairly stable in between April 5 and May 7. However, this observation needs to be balanced since most of the samples were not analysable and the majority of the OTUs were only identified at the kingdom of phyla level.

4.3.4.4 Rhizoplane

- Bacterial communities

The evolution over time of the bacterial communities found in the lettuce rhizoplane of the hydroponic compartment of the PAFF Box is visualised with the following graphs (Fig. 34 and Fig. 35).

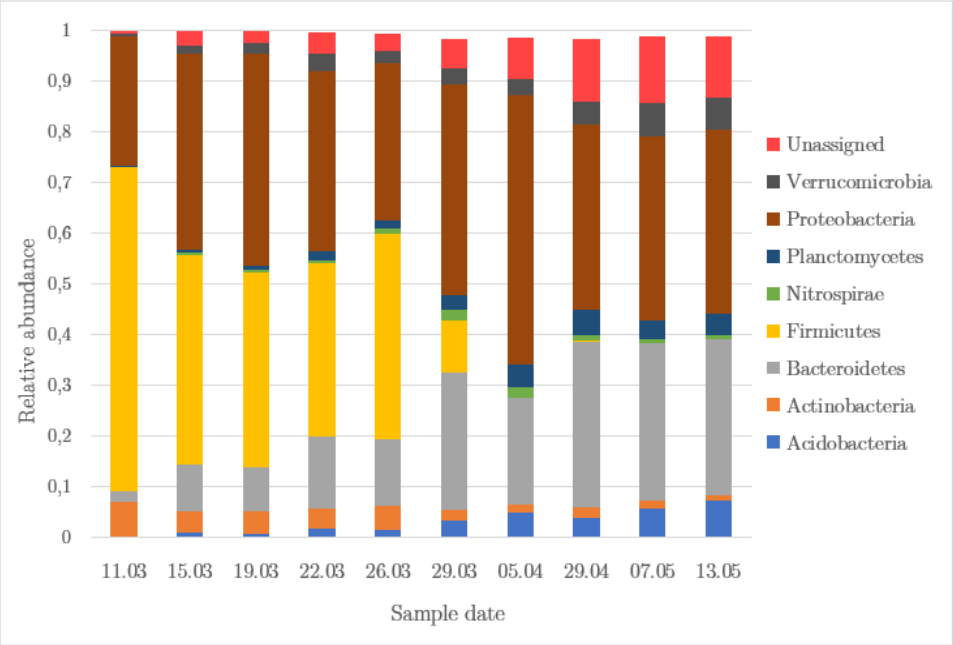


***Figure 34**: Bar chart showing the relative abundance of each bacterial OTU according to each sample date, at the phyla level, in the endosphere compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown.*

Focusing at the phylum level (Fig. 34), the microbiota evolution of the rhizoplane samples is similar to the endosphere samples. The same two separate periods can be observed: the 1st one going from March 11 to March 29 and the 2nd one going from April 5 to end of the experiment. However, the general microbiota of the 1st period appears to have a smaller proportion of *Firmicutes* than the endosphere samples, i.e. 38% ± 17% whereas 65.6% ± 13.3%. In addition, the March 29 sample has a much smaller proportion of *Firmicutes* than the other samples of the 1st period, i.e. 10.2%. Focusing on the 2nd period, the phyla and their proportions were very similar to the ones of the 2nd period in the endosphere samples. Thus, the same important shift was also visualised in the rhizoplane samples but at lesser extent. However, this microbiota shift seemed to have started a bit earlier in the rhizoplane compartment.

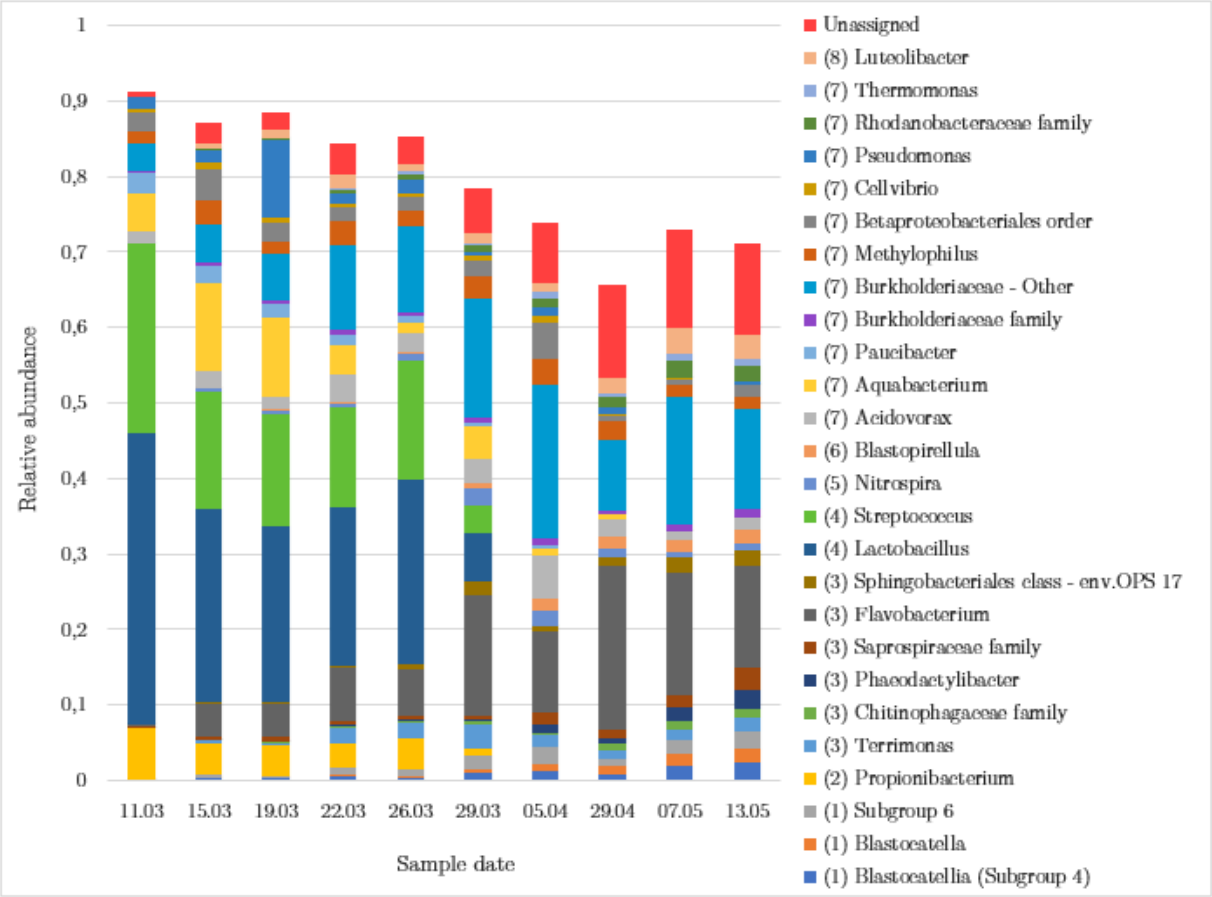


***Figure 35**: Bar charts showing the relative abundance of each bacterial OTU according to each sample date, at the genus level, in the lettuce rhizoplane compartment. Only the OTUs representing more than 0.5% of the total reads on average are shown. The taxonomic assignment sometimes stopped at the class or family levels, in which case the level is indicated. The numbers in front of each genus correspond to its phylum, namely: (1) Acidobacteria; (2) Actinobacteria; (3) Bacteroidetes; (4) Firmicutes; (5) Nitrospirae; (6) Planctomycetes; (7) Proteobacteria; (8) Verrucomicrobia*

Focusing at the genus level (Fig. 35), the *Firmicutes* that dominated the 1st period was represented at 60.8% by *Lactobacilli* and at 38.6% by *Streptococci,* as for the endosphere and biofilter samples. Both genera almost disappeared from the proportions during the 2nd period. Following the same *Lactobacilli* and *Streptococcus* decline tendency, the *Aquabacterium* and *Propionobacterium* genera went from an average of 6.1% ± 3.9% and 3.8% ± 1.8% during the 1st period respectively, to 0.3% ± 0.03% and 0% during the 2nd period. While the same observation was already made on *Propionobacterium* in the endosphere samples, the *Aquabacterium* genus has only been observed at significant proportion in these rhizoplane samples. This genus seems to be found in various environment, such as plant rhizosphere, sludge and even fish intestine (Wang *et al.*, 2014; Shin *et al.*, 2019; Gupta *et al.*, 2019).

The *Pseudomonas* genus was only observed at significant proportion in the first samples, reaching a peak on March 19 with 10.2% of the total reads, even though *Lactobacilli* and *Streptococci* were taken the most relative abundance. Including both PGPR and plant pathogens, this genus was also observed in the endosphere samples during the same period, but at much lower extent (David *et al.*, 2018). It is interesting to wonder why the presence of this genus nearly disappeared from the relative abundance in the latter samples.

Focusing on the 2nd period, the rhizoplane samples essentially shared the same OTUs and the same relative abundances than the endosphere samples. *Flavobacterium* and *Burkholderiaceae* family were also the two major taxa, representing 15.5% ± 4.5% and 15% ± 4.6% of the total reads respectively. Those proportions are nearly identical to the proportions found in the endosphere. The minor taxa that were found in the endosphere samples were also found at relatively similar proportions in the

rhizoplane, e.g. *Luteolibacter* (2.5% ± 1%), *Methylophilus* (2.3% ± 0.9%), Subgroup 6 (1.8% ± 0.7%) *Blastopirelulla* (1.5% ±), *Phaeodactylibacter* (1.5% ± 0.7%) and *Nitrospira* (1.3% ± 0.6%). Small differences can however be noticed. For instance, while Subgroup 6 was the third most abundant taxon in the endosphere samples (2.4% ± 0.9%), *Acidovorax* is the third most abundant genus in the rhizoplane samples (2.71% ± 2%) during the 2nd period. Also found in the water compartment, this genus was also represented at similar proportion during the 1st period, i.e. 2.5% ± 1.3% throughout the entire experiment in the water samples.

In conclusion, the rhizoplane and endosphere samples shared similar OTUs and similar relative abundances. A more in-depth study could be made in order to differentiate organisms that were strictly endospheric and the ones that were only found on the root surface. By now, it can be said that if those obligate endospheric organisms were present in the roots, they were represented at very low proportions. Focusing on the microbiota evolution, the rhizoplane samples seemed to have had lower proportions of *Firmicutes* in general and the shift that brutally occurred between March 29 and April 5 in the endosphere appears to have occurred in a more gradual way in the rhizoplane. With a peak of *Lactobacilli* and *Streptococcus* on the first day of the experiment, their proportions then decreased to attain the almost zero level on April 5.

- Fungal communities

The evolution of the fungal communities found in the lettuce rhizoplane from April 5 to May 13 is visualised with the following graph (Fig. 36). The first 3 weeks of the experiment are missing since all of the 1st run samples contained a majority of "no blast hit" OTUs i.e. the March 11, March 15, March 19, March 22, March 26 and March 29 samples.

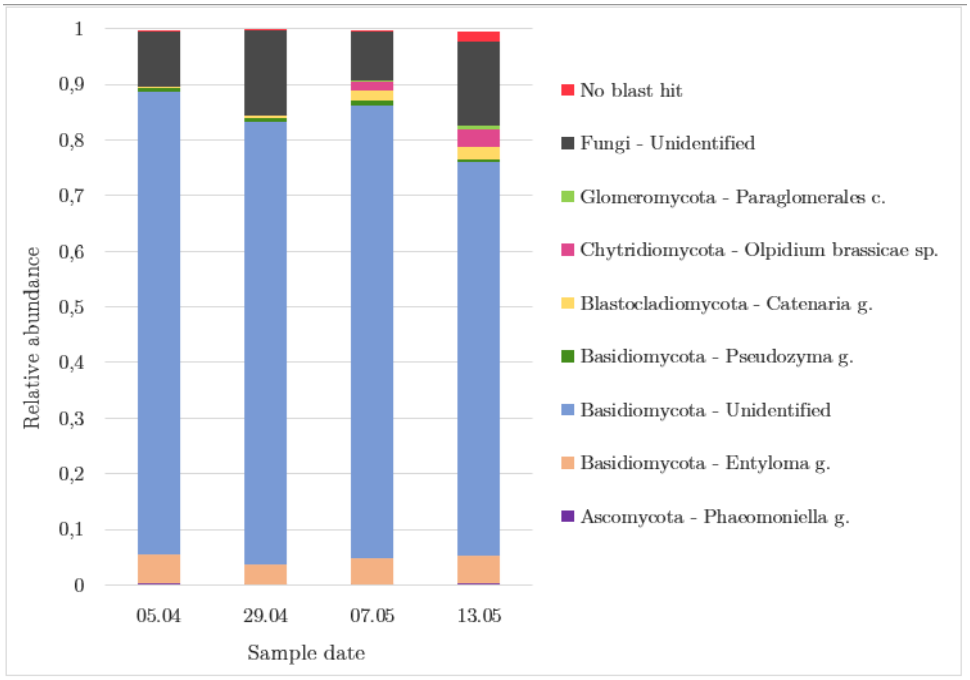


***Figure 36**: Bar chart representing the relative abundance of each fungal OTU according to each compartment, at the fungal genus level. Only the OTUs representing more than 0.1% of the total reads on average are shown. The taxonomic assignment sometimes stopped at family, class or phylum level, in which case the level is indicated*

The fungal rhizoplane samples can directly be compared to the endosphere samples. In the rhizoplane, the unidentified *Basidiomycota* and unidentified *Fungi* had similar proportions, i.e. 78.7% ± 5% and 12.2% ± 3% respectively. However, while the unidentified *Fungi* gradually decreased in the endosphere, they fluctuated around the average value in the rhizoplane samples. The *Olpidium brassicae* appeared in the two last samples, as seen in the endopshere but at much lower extent, i.e.

2.5% in the rhizoplane whereas more than 15% in the endosphere. This could be due to the ability of the *Olpidium* zoospores to encyst on root cells (Hull, 2014). Thus, the washing process whose aim was to uptake the rhizoplane microorganisms might not have been strong enough to remove efficiently the *O. brassicae*, which could then be found in the endosphere samples.

Compared to the endosphere samples, the *Catenaria* and *Pseudozyma* genera were also represented in the rhizoplane but at lower proportion, i.e. 1.1% and 0.6% respectively. On another hand, the *Entyloma* genus was relatively more abundant in the rhizoplane samples and remained stable over time (4.5% $\pm$ 0.7%). The rhizoplane also contained a noticeable proportion of the *Paraglomerales* class, mostly on May 13 (0.7% of the total reads), which was not the case in the endosphere compartment. As for the *Claroideoglomeraceae* found in the water and biofilter compartments, this class is a member of the *Glomeromycota* phylum, mostly described as AMF. In this case, the identified organisms might indeed form a mutualistic symbiosis with the lettuces, representing a potential PGPM.

In conclusion, the rhizoplane fungal microbiota is comparable to the endosphere microbiota but with variations of minor scales, appearing as a more stable compartment. This observation correlates with the one made on the bacterial communities in which the variations were more gradual, and the shifts were less marked.

Considering the fungi and bacteria for both the endosphere and rhizoplane compartments, we might wonder what could induce or influence the variations observed between the samples. Two major factors appear. The first one could be the general "surrounding environment" with events or parameters modifications that might have led to variations and shifts in the microbiota. The second factor could be the actual plants. Throughout the experiment, the lettuces aged and went through different growth stages. Some endophytic organisms may be directly inherited from the seeds but most of the root-associated organisms are recruited by the plants from the surrounding environment through the production of exudates and these compounds differ from physiological stages (Kolton *et al.*, 2016). It can thus be interesting to compare the root microbiota of lettuces of different ages at a given time. In this way, while the first factor will be discussed in Section 4.2.4.4, we will now focus on the "age plant" factor that could have influenced the root microbiota over time.

4.3.5 Rhizoplane and endosphere microbiota according to the lettuce age

As the root samplings were progressively eliminating lettuces of the system, other lettuces were added regularly to the deep water grow beds throughout the experiment in order to maintain a general AP system. Originally, those lettuces were not supposed to be sampled. On April 29, the hydroponic compartment of the PAFF Box thus contained 5 groups of lettuces of different ages: a 3-weeks-old group, a 4-weeks-old group, a 5-weeks-old group, a 6-week-old group and a 7-week-old group. In order to assess the potential impact of growth stages on root microorganisms, we decided to sample those different age groups at a given time, on April 29.

- Bacterial communities

The comparison of the bacterial communities found in the rhizoplane and endosphere at a given time (on April 29) between the different age groups is shown at the genus level in following bar chart (Fig. 37).

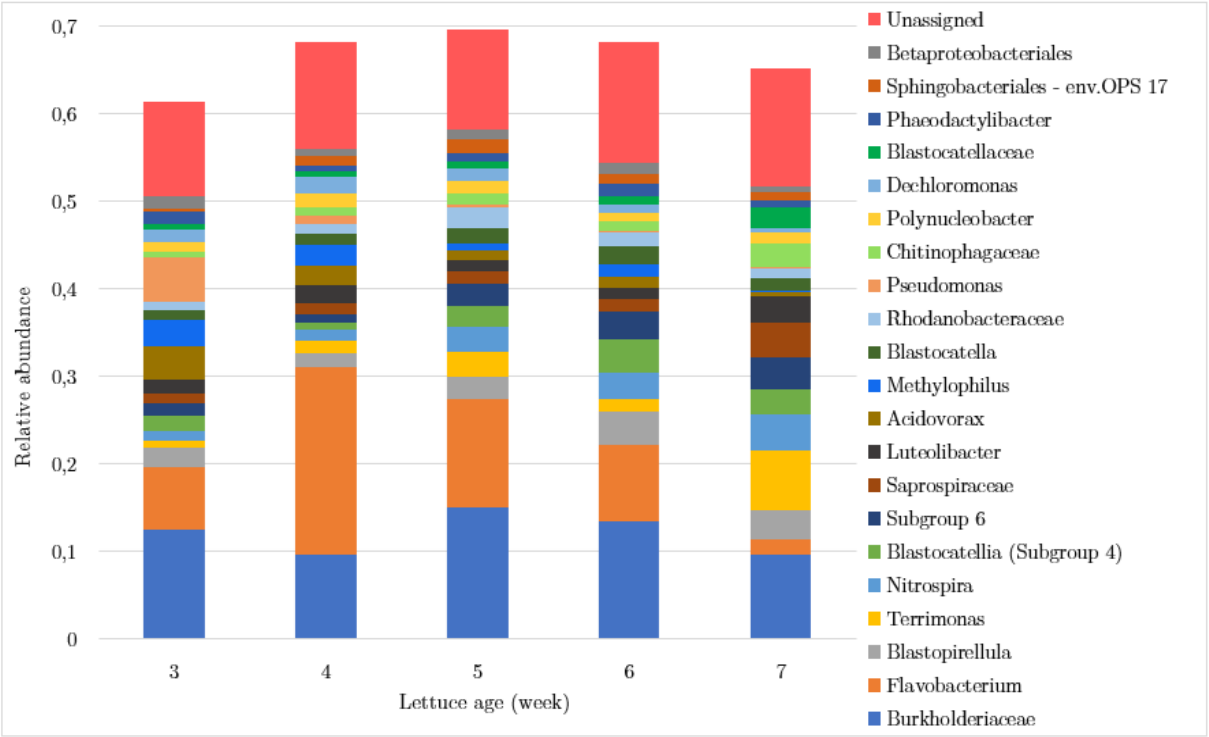


***Figure 37**: Bar charts showing the relative abundance of each bacterial OTU according to each lettuce age, at the genus level, in the lettuce rhizoplane and endosphere compartments. Only the OTUs representing more than 0.5% of the total reads on average are shown..*

The first thing we can notice, is the total absence of *Lactobacilli* and *Streptococci* from the samples as well as the *Propionibacterium* and *Aquabacterium* genera. While these genera were major when the first group of lettuce (Group 1) was 3 weeks old, i.e. March 26 and March 29 samples, the 3-weeks-old group of lettuce studied here does not showcase their presence. In this way, the major proportion of *Firmicutes* as well as the *Propionibacterium* and *Aquabacterium* at lesser extent in the March 26 and March 29 samples, does not seem to be explained by the age of the plants. Instead, the surrounding environment might have induced their presence and later on, their disappearance. Indeed, the 3-weeks-old group of lettuces studied here was sampled on April 29 with all the other age groups, a month after the Group 1 was sampled at 3 weeks old. With a one-month lag, the surrounding environment might have been different. However, no lettuces of 1 week and 2 weeks old were present in the PAFF Box on April 29, so we cannot confirm that those microorganisms were not related to early stages of lettuce. Re-considering the hypothesis of a fermentative environment found during germination, which could explain the presence of those organisms in the first root sample, the general germination process was the same for all the lettuces. Thus, this 3-weeks-old group sampled on March 29 was put in germination in the same manner as our experimental sampled lettuces. Hence, these lettuces could also have developed those fermentative organisms, but it does not seem to be the case here. However, the surrounding environments might have not been the same in both the greenhouse in which the shallow box was put and in the AP system. In other words, the box in which the 3-weeks-old group were germinating may have not developed a fermentative environment. Even if it was the case, the fermentative organisms might have not been the same and they might have disappeared prior to April 29, when arriving in the PAFF Box. Thus, the hypothesis of the *Lactobacilli*, *Streptococci*, *Propionibacterium* and potentially *Aquabacterium* being present due to fermentative conditions during germination process and then dropping 2 weeks later in the PAFF Box due to an event is still plausible.

Focusing on the two major taxa observed in the bar chart (Fig. 37), *Burkholderiaceae* proportion seemed pretty similar between the different age groups, i.e. 12% ± 2.3%, while *Flavobacterium* fluctuated a lot. Its peak was found in the 4-weeks-old group (21.5% of the total reads) and its proportion then gradually decreased with the plant age, attaining 1.6% in the 7 weeks old group. In Qin *et al.* (2016) experiment on wheat, *Flavobacterium* strong presence was correlated to early growth

stages. Here, *Flavobacterium* proportion did gradually decrease with later growth stages but the first 3 weeks old group did not have the most abundant proportion. It would have been interesting to have a 1 and a 2 weeks old groups. In the endosphere samples of our experiment from April 5 to May 13, the decrease observed in *Flavobacterium* over time thus might be explained by the age of the plants.

Another genus that seemed to be correlated with early growth stages was *Pseudomonas*, only represented at high proportion in the 3-weeks-old group (5.1% of the total reads). Comparing to our microbiota evolution study, this genus was observed at high proportion only in the first samples of the rhizoplane, reaching a peak on March 19 when the lettuces were 2 weeks old. It then disappeared from the significant proportion from March 29 to the end of the experiment. In the endosphere samples, this genus was not observed in significant proportion at early stages of the lettuces. However, the *Firmicutes* were occupying most of the microbiota during the 1st period (March 11 to March 29), meaning that *Pseudomonas* relative abundance might have gone unnoticed in the general taxa description of the endosphere. During the 1st period, *Pseudomonas* actually represented 0.35% ± 0.1% of the total reads, which was actually a significant proportion when considering the major proportions of *Lactobacilli* and *Streptococcus*. During the 2nd period, with no reads assigned to *Lactobacilli* and *Streptococci,* the *Pseudomonas* only represented 0.02% of the total reads in the endosphere samples. This genus thus might be correlated to early growth stages. The same observation could be made for the *Methylophylus* and *Acidovorax* genera. Decreasing with the lettuce age on Fig. 37, these genera relatively kept the same proportions throughout the 1st and 2nd period in the endosphere and rhizoplane, meaning that they might have had a bigger importance during the 1st period, i.e. early stages of the lettuces.

Some genera seemed to increase in relative abundance with the age of the plants, e.g. *Nitrospira*, *Terrimonas*, Subgroup 6, *Blastopirellula*, *Blastocatellia* (Subgroup 4), *Saprospiraceae*, *Chitinophagaceae* (Fig. 37). Most of those genera also increased in relative abundance over time in the 2nd period samples (April 5 to May 13), for the endosphere or rhizoplane samples. These variations could thus be due to the plant age. On the other side, some OTUs showed similar proportions in between the age groups, e.g *Betaproteobacteriales*. This order also showed similar proportion throughout the entire experiment in endosphere or rhizoplane samples. Thus, they might not highly depend on the age of the plant or the surrounding environment.

Based on these observations, the general root microbiota observed in between March 11 and March 29 as well as the major bacterial breakdown that occurred in between March 29 and April 5, do not seem to be correlated with the age of the plants. Hence, the root bacterial communities might be highly influenced by the surrounding environment, i.e. the box in which the seedlings were germinating and the AP system in which they were transferred on March 11, and its modifications. In the meantime, the age of the plants appears to have influenced the proportions of more minor OTUs throughout the experiment. This can mostly be observed after the breakdown, from April 5 to the end of the experiment. At lesser extent, some genera also seem to be independent of those factors, being present at similar proportion throughout the experiment or in between the lettuce age groups.

- Fungal communities

The comparison of the fungal communities found in the rhizoplane and endosphere at a given time (on April 29) between lettuces of five different ages, i.e. 3 weeks, 4 weeks, 5 weeks, 6 weeks and 7 weeks, is shown at the genus level in following bar chart (Fig. 38).

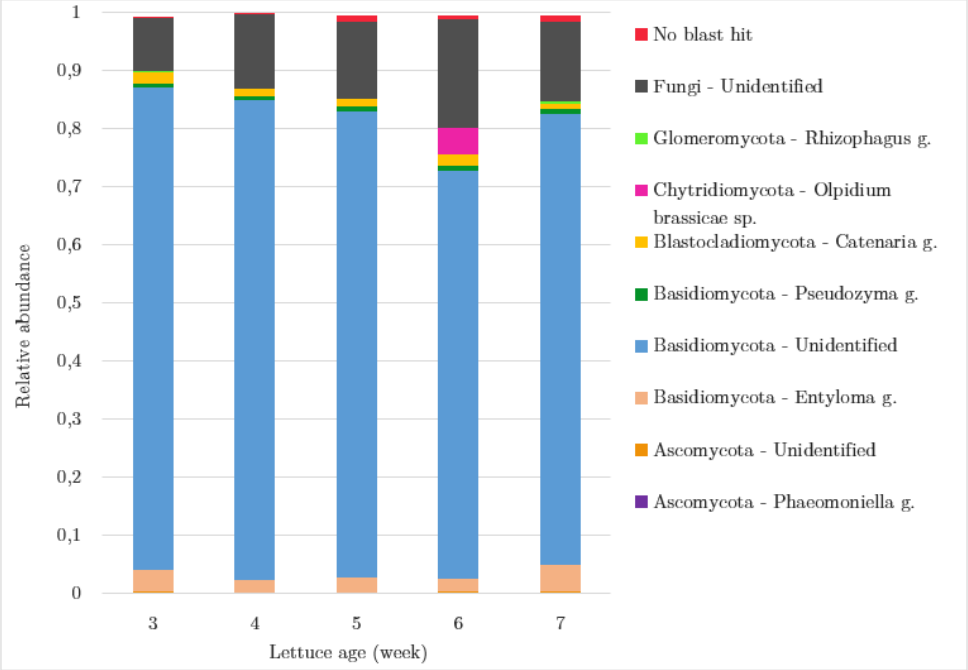


***Figure 38**: Bar charts showing the relative abundance of each fungal OTU according to each lettuce age, at the genus level, in the lettuce rhizoplane and endosphere compartments. Only the OTUs representing more than 0.1% of the total reads on average are shown.*

The first thing we can notice is the high similarities between the age groups of 3 weeks, 4 weeks, 5 weeks and 7 weeks. All of them shared very similar proportions of OTUs, i.e. 80.6% ± 2.5% of unidentified *Basidiomycota*, 12.2% ± 2.1% of unidentified *Fungi*, 3.2% ± 1% of *Entyloma* genus, 1.3% ± 0.3% of *Catenaria* and 0.7% ± 0.07% of *Pseudozyma*. The 6-weeks-old group showed a quite different microbiota, with a high proportion of *Olpidium Brassicae*, i.e. 4.5% of the total reads, while the other groups had less than 0.05%. This group also showed a higher proportion of unidentified *Fungi*, i.e. 18%, and thus a lower proportion of unidentified *Basiodiomycota*, i.e. 70%. On another hand, the relative abundances of *Entyloma*, *Catenaria* and *Pseudozyma* were similar to the other groups.

It appears that the 6-weeks-old group lettuces were probably parasitized by *Olpidium brassicae*, potentially being infected by lettuce big-vein viruses but with no visible symptoms at that time. The development of this parasite may have impacted the other fungi present in and on the roots. This 6-weeks-old group was sampled on April 29 and at that same day, the Group 2 lettuces followed in our experiment did not showcase the presence of this parasite. However, *O. brassicae* suddenly appeared at high proportion a week later, in May 7 and on May 13 samples. Since *O. brassicae* have motile zoospores, we may think that the parasite present in the 6-weeks-old group on April 29 propagated in the deep water grow beds and infected the sampled lettuces of our experiment, explaining its sudden presence on May 7 and May 13. Hence, the main fungal microbiota shift that was observed in the sampled lettuces of our experiment, i.e. appearance of *O. brassicae* on May 7 and May 13, may be due to an infection from other lettuces and not from the lettuce age or parameters variations factors. From that point of view, the fungal communities can be seen as relatively stable over time in the roots if the lettuces are healthy. However, this observation needs once again to be balanced, since most of the samples were not analysable and the majority of the OTUs were only identified at the kingdom of phyla level.

We can now study the potential influence of events and parameters on the microbiota evolution.

4.3.6 Microbiota and parameters variations over time

Considering the general AP system, a major bacterial shift seems to have occurred at the end of April with the disappearance of *Lactobacilli* and *Streptococci* from the root samples at a major extent, and

from the biofilter and water samples at a lesser extent. Focusing on each compartment, this change occurred in the biofilter and in the water samples between March 22 and March 26. In the rhizoplane and endosphere samples, this change seems to have occurred only a week later, between March 29 and April 5. Other minor OTUs underwent the same decline evolution, each compartment having its specific OTUs. The bacterial shift was thus broadened to several taxonomic groups. As seen in the previous section, the age of the plant does not seem to be the main factor of this shift, in the root samples. From this perspective, an important variation in the surrounding environment might have occurred in between March 22 and March 26, but the roots, that may be seen as a more specific micro-environment, more isolated from the surrounding variations than the water and the biofilter, might have undergo the shift with a one week delay. Indeed, roots can modify the physicochemical characteristics around the root zone such as the pH and oxygen levels (Kolton *et al.*, 2016). In addition, this bacterial breakdown seemed to have started a bit earlier in the rhizoplane compartment than in the endosphere, with less "intense" variations. This could be explained by the endosphere being even more isolated from the surrounding environment, and thus "reacting" to important variations with a certain delay. Focusing on the fungal communities, the water and biofilter samples that preceded March 26 could not be analysed. However, their general microbiota was gradually changing in between March 26 and April 5. Thus, the same potential important variation in the surrounding environment might have induced this fungal community's modification, that initiated after March 22. Concerning the root samples however, the fungal communities seemed pretty stable.

Going back to the parameter's evolution, no data of the temperature, pH, EC and DO were recorded from March 22 to March 26. However, the surrounding dates, i.e. March 21 and March 26, and the general 1st half of the experiment were covered by the physicochemical Group 1, having average-to-normal parameters values. Considering the other parameters, i.e. macronutrients and DO, no significant changes seem to have occurred during that period. Likewise, no backwash or specific procedure were made in between those dates. Thus, the cause of the shift is rather unknown, even though we could suppose that the period between March 22 and March 26 was also covered by the Group 1, with no significant changes in T, pH, EC and nitrate. Multiple other parameters could have induced this microbiota variations, but they were not monitored in this experiment. The major OTUs observed from March 11 to March 22 or March 26, might have been correlated to the early stages of an AP system, considering that the other AP studies made on more "mature" systems had different general microbiota, i.e. mostly in the case of root samples. For instance, in the root samples, the *Lactococci*, *Streptococci* and *Propionobacterium* might have been replaced by other more competitive OTUs when the AP system was more "mature" and stabilized. The same observation could be made for the *Lactococci*, *Streptococci*, *Hyphomonadaceae* and *Rhodobacter* taxa observed from March 11 to March 22 in the biofilter for instance. After the general breakdown, the endosphere and rhizoplane bacterial communities seemed relatively stable in time. No drastic change in term of OTUs or relative abundances was observed in the two types of samples, despite the relatively strong parameters variations that were recorded during that 2nd half of experiment, switching between the 5 physicochemical groups (PC groups). The small variations that were observed in between April 5 and the end of the experiment could mostly be explained by the aging of the lettuces. The same conclusion could be made for the fungal communities in the root samples. The main fungal variation that occurred on the two last sampling dates, might mostly be explained by a potential parasitizing of the lettuces.

Focusing on the biofilter after the general breakdown, the bacterial communities seemed fairly stable over time, with similar relative abundances from March 26 to the end of the experiment. Despite the relatively strong parameters variations that were recorded during that 2nd half of experiment, no strong bacterial variations were observed at that time. However, the fungal communities on their side seemed to have underwent important modifications in the biofilter at that time. A hypothesis was made on a potential event that occurred in between April 5 and April 13, inducing a gradual but strong fungal variation from April 5 to May 7, in term of relative abundances. The same observation was made for the fungal and bacterial communities in the water compartment, at lower extent. Considering the parameters, the period in between those dates was covered by the same PC Group 1, i.e. having average-to-normal parameters values. No important changes were observed in the macronutrients' evolution either. However, 1L of FeEDTA (13%) were added on April 5. In addition, the days preceding April 5 were not recorded in term of T, pH, EC and DO, meaning that an event

might have happened just before. The potential cause of the gradual fungal and bacterial variations observed in between April 5 and May 7 in the biofilter and water compartments is rather unknown.

Another important microbial shift was observed on the last sampling date, i.e. May 13. Indeed, the fungal communities of the biofilter drastically changed, mainly by the potential complete disappearance of *Basidiomycota.* In the water compartment, this fungal shift was also observed but at lower extent. In that same water compartment, the bacterial communities in May 13 sample were also very different from the previous sampling date. A hypothesis was thus made on a potential event that occurred in between May 7 and May 13. Focusing on recorded parameters, the two last weeks of the experiment went through important variations, going from the PC group 1 (May 1 – May 5) to the Group 5 (May 6 – May 9), to the Group 2 (May 10 – May 13). The PC Group 5 was characterised by a very low pH comparing to the average pH observed during the experiment and a very high nitrate concentration, reflecting the important HNO_3 additions. These pH and nitrate concentration modifications were relatively strong and sudden. Then, from May 10 to May 13 (Group 2), the pH re-increased and the nitrate concentration re-decreased, showing the relative stabilization of those parameters. An important drop of EC was also observed during that short period. Those rapid and relatively important changes might have impacted both the fungal and bacterial communities in the water and biofilter, some organisms being rapidly dominated by other more competitive organisms in these conditions.

In conclusion, the potential event(s) that initiated the main bacterial breakdown observed in the root samples at a major extent, and in the biofilter and water samples at lower extent, is rather unknown. While the 1st half of the experiment lacked parameters monitoring, the 2nd half of the experiment was more closely followed and relatively important parameters variations were observed, including the transition between several PC groups. Even though some microbiota variations were potentially explained by those changing PC groups, i.e. the May 13 microbiota shifts observed in the biofilter and water samples, an actual correlation between those PC groups and the microbiota of the different sampling dates is complicated. The Adonis test made on the bacterial samples with the "PC group" factor, was significant ($0.01<p<0.05$), meaning that the bacterial communities observed during the different PC groups were significantly different. However, the R^2 value showed that less than 7% of the bacterial variation observed between the PC groups were explained by this grouping. Thus, even though the bacteria were significantly different in between the PC groups, this actual factor do not seem to have directly induce those bacterial variations. The same general observation can be made for the fungi. In this case, the fungal communities observed during the different PC groups were not significantly different ($p>0.05$). However, most of the OTUs were only identified at the kingdom of phyla level, which hid the potential variations at more specific levels.

Directly correlating parameters variations to microbial variations seems complicated since the bacterial and fungal communities might "react" to those variations with a certain delay. In our case, this delay is unknown, and it might not be homogenous for each variation since each organism may react in its own way, with its own time laps. Hence, a certain microbiota that was observed at a certain day which was characterised by a certain PC Group, does not necessarily mean that this microbiota resulted from this particular environment or/and PC group.

5 Conclusion and perspectives

5.1 The microbiota kinetic evolution

This study was, to our knowledge, the first detailed survey of the microbiota observed in an AP system over time, both for bacterial and fungal communities. The experiment lasted 9 weeks and started at the introduction of lettuces in the system on March 11, 2019, thus following the transition of a recirculating aquaculture system to an aquaponic system. Four different "compartments" were sampled twice and then once a week during the experiment in order to have a general overview of the microbiotas found in the system, i.e. the water, the biofilter, the rhizoplane and the endosphere of the lettuces. Several parameters were also monitored in the aim of potentially correlate microbiota variations to parameter variations.

Considering those parameters, the 1st part of the experiment, i.e. from March 11 to April 15, seemed fairly stable and homogenous in term of temperature, EC, pH and nitrate concentration. Nonetheless, this period lacked regularity in the measures. Then, the 2nd part of the experiment, i.e. from April 18 to May 13, was more heterogenous, undergoing various parameters variations such as drops in temperature, pH and nitrate concentration. On their side, the other macronutrients seemed to have remained rather stable throughout the experiment. Even though relatively important variations have been observed, the average value for each parameter were generally in the optimal ranges recommended for this AP system. The pH was the only monitored parameter to globally be outside of the optimal range, i.e. higher than recommended with a mean of 7.87 instead of 6-7 (Somerville *et al.*, 2014).

Now focusing on the microbiota observed in the biofilter, the bacterial communities were mostly composed of *Proteobacteria*, *Bacteroidetes*, *Nitrospirae* and *Acidobacteria* (in descending order). Both *Proteobacteria* and *Bacteroidetes* were also the most abundant phyla in other AP studies (Schmautz *et al.*, 2017, Eck *et al.*, 2019, Bartelme *et al.,* 2019, Mungui-Fragozo *et al.*, 2015). However, the other most important phyla differ between the surveys. At the genus level, the biofilter samples contained high proportions of *Nitrospira*, *Terrimonas* and *Blastocatellia*. At this lower taxonomic level, microbiotas differ a lot in between AP and RAS studies. It can be noticed that our biofilter samples had high relative abundances of *Nitrospira*, comparing to other studies, e.g. less than 5% in Eck *et al.* (2019) and Schmautz *et al.* (2017) experiments, whereas 19% on average in our survey. Concerning the fungal communities in the biofilter, *Basidiomycota* seemed to be the most abundant phylum. However, the other most abundant group were unidentified fungi, which limits the general microbiota characterisation. Concerning the microbiota evolution over time in the biofilter, the bacterial communities underwent a relative shift in between March 22 and March 26, i.e. 11 to 15 days after the introduction of lettuces in the system, mostly by the disappearance of *Lactobacilli* and *Streptococci*. Those lactic acid bacteria can be found in high proportion in fish intestine, however their presence in RAS or AP biofilters at significant proportion did not seem to be reported (Schmidt *et al.*, 2016). After the relative shift, the bacterial communities remained relatively stable until the end of the experiment, even though the 2nd half of the experiment underwent several parameters variations. On the other hand, fungal communities in the biofilter seemed to have fluctuated a lot during that same period, including a drastic shift on the last sampling day with the potential disappearance of *Basidiomycota*. Thus, the fungi in the biofilter might be more affected by the surrounding environment than the bacterial communities.

In the circulating water, the bacterial communities were mostly composed of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* (in descending order). This was generally comparable to water analyses of other AP and RAS studies (Eck *et al.*, 2019; Bartelme *et al.*, 2019). At the genus level, *Polynucleobacter* was the most abundant genus, which correlates with Bartelme *et al.* (2019) study, followed by *Flavobacterium*, *Microbacteriaceae*, C39 (*Rhodocyclaceae*) and *Cetobacterium*. These organisms were also found at high proportion in the PAFF Box water sample of Eck *et al* (2019) experiment for instance. Concerning the fungal communities, *Basidiomycota* was the most abundant phylum from March 26 to the end of experiment. Focusing on the microbiota evolution over time, the bacterial communities fluctuated a lot more in the water than in the biofilter, but in a gradual way. No shift or sudden important variations were observed. In this perspective, the water appears

as a more changeable environment over time. No bacterial stabilization seemed to have occurred in 9 weeks of experiment. On the other hand, the fungal communities seemed more stable over time, showing gradual variations of much lesser extent. However, most of the OTUs taxonomy stopped at the phyla or kingdom level, which hid the potential variations within those groups.

Focusing on lettuce endosphere, the 1st root sample made on seedlings right before they were transplanted in the PAFF Box, showed a large majority of *Firmicutes*, i.e. more than 70% of the total reads, followed by *Proteobacteria* and *Actinobacteria*. At the genus level, *Lactobacilli*, *Streptococci* (*Firmicutes*) and *Propionibacteria* (*Actinobacteria*) were the most abundant genera, all three having a fermentative metabolism. As a hypothesis, the shallow boxes filled with tap water in which the lettuces seeds were put to germinate for 11 days, might have developed a fermentative environment. This could explain the presence of those organisms at high proportion in/on the young roots. This general bacterial profile was maintained in the following samples until March 29, i.e. 18 days after the introduction of the seedlings in the AP system, after which all three genera disappeared from the noticeable proportions (less than 0.5%). This marked a drastic shift in the bacterial communities of the root samples. This breakdown could be paralleled with the relative shift observed one week earlier in the biofilter, which was mainly characterised by the disappearance of *Lactobacilli* and *Streptococci* too. Potentially caused by an event that occurred at that period, the root microbiota might have "reacted" with a certain delay, comparing to the biofilter compartment. After the root bacterial shift, from April 5 to the end of the experiment, *Proteobacteria* and *Bacteroidetes* were the most abundant phyla. This correlates with other AP surveys or soil-based lettuce culture in which root microbiota was studied on lettuces ranging from 2 weeks old to fully matured lettuces (Schmautz *et al.*, 2017; Cardinale *et al.*, 2015; Erlacher *et al.*, 2014; Schreiter *et al.*, 2014). At the genus and family levels, *Flavobacterium* and *Burkholderiaceae* were the most abundant groups, followed by Subgroup 6 (*Acidobacteria*), *Methylophylus* and *Nitrospira* at lesser extent. Those bacterial communities remained fairly stable until the end of the experiment, undergoing small variations that could mostly be explained by the plant growth stages. Focusing on fungal communities during that same period, i.e. from April 5 to the end of the experiment, *Basidiomycota* was the most abundant phylum. On the two last samples, results indicated a high presence of *Olpidium brassicae*, a parasite that can potentially induce the lettuce big-vein disease. Even though no apparent signs of disease were observed, this contamination most likely came from other contaminated lettuces, present in the system at the same time. Indeed, another group of lettuces that was sampled in order to compare the microbiota of lettuces of different age at a given time, showcased the presence of *O. brassicae* one week prior to the appearance of this parasite in our sampled group of lettuces.

In the rhizoplane, results were comparable to the endosphere ones, with similar identified organisms, proportions and general microbiota evolution. However, the variations were usually of more minor scale in the rhizoplane. Focusing on the bacterial communities, the drastic shift that occurred in the endosphere was less marked in the rhizoplane, the *Lactobacilli*, *Streptococci* and *Propionibacterium* proportions decreasing in a more gradual way. Having the same general OTUs, more minor OTUs were found at higher proportions in the rhizoplane than in the endosphere such as *Aquabacterium*, which also declined at the bacterial shift, *Acidovorax, Luteolibacter and Pseudomonas.*

Considering all compartments, we can thus say that the biofilter and lettuce roots reached a relative bacterial stability 18 to 25 days after the introduction of plants in the system. On another hand, the water compartment did not showcase any bacterial stabilization in 9 weeks of experiment. Focusing on fungal communities, the first sample dates were missing and most OTUs were only identified at the phyla or kingdom levels, but the water and roots compartments seemed fairly stable over time. On the other hand, the biofilter appeared as unstable and no fungal stabilization seem to have occurred throughout the experiment.

Considering both the microbiotas evolutions and the parameters evolutions, the potential event(s) that initiated the main bacterial breakdown observed in the root samples at a major extent, and in the biofilter at lower extent, is rather unknown. More generally, no clear correlation was established between the microbiota variations and the monitored parameter variations. The PAFF Box was conceived with the aim of providing the most stable environment for the fish, bacteria and plants, in order not to disturb this tripartite symbiosis. Hence, most parameters changes were unexpected and unintended. Considering the relatively long duration of the experiment, this made the monitoring of

parameters and the potential correlation with the microbiota complicated, since several changes could occur at the same time, with different intensity and different duration. This leads us to the next point, focusing on the perspectives of this study.

5.2 Perspectives

This study gave an insight in the stability of microbiotas observed in a system transitioning from a RAS to an AP system. Results showed that important microbial shifts could occur in the days-weeks following the introduction of plants in the system. Thus, other kinetic studies should be made on other transitioning AP systems in order to see if these shifts are usual and recurrent, and after how many days-weeks they occur. Kinetic studies could also be made on non-transitioning AP systems, that have been running for months-years, in order to see if these systems also go through microbial shifts. In our study, a general stabilization occurred 18 to 25 days after the introduction of plants, in the roots and biofilter compartments. The first stages of the transitioning system are thus crucial. Thus, further research and special attention could be made during this period with more samples and a better parameter monitoring. In our study, the 1st part of the experiment lacked regularity in the measures and one important parameter was left unmonitored for several weeks, i.e. the dissolved oxygen levels. A better parameter monitoring during the first stages of the system could lead to a better understanding of the microbial shifts.

The primary objective of the survey was to assess the stability of the microbiota observed in an AP system and to do so, the experiment was made in a relatively stable AP system, in which monitored parameters were maintained constant and in the range of optimal values as much as possible. However, some relatively important parameters variations occurred, being unexpected and unintended. Those changes could have impacted the microbiota since several microbial shifts and variations occurred during the experiment. Hence, studying the potential correlation between those microbial changes and the parameters changes was very interesting, which led to the constitution of physicochemical groups and their consideration as a factor in the microbial analyses for instance. However, as already mentioned, most of the changes occurred at the same time, with different intensity and different duration which resulted in a complicated interpretation of their potential impacts on the microbiota. Hence, other experiments could be made in which parameters changes are intentionally induced and controlled, one modification at a time for instance. Thus, potential correlations between microbiotas and parameters variations could be more easily assessed and those correlations could help understanding the general microbiota stability of an AP system.

In our study, *Lactobacilli* and *Streptococci* played a significant role in the microbial shifts observed in the roots and biofilter. They were also observed at lesser extent in the water. However, those compartments developed very different micro niches and comparing to other AP or lettuce rhizosphere studies, their presence in those areas was rather surprising. Thus, further research could be made on those two genera in order to understand why they were present in such compartments, and why they suddenly disappeared from the general microbial profiles. It could be interesting to see if their presence was beneficial, potentially acting as probiotics for both plants and fish, or deleterious for the system by inhibiting the development of other crucial microorganisms for instance, e.g. *Nitrospira* in the biofilter.

This study was also an insight in the fungal communities present in an AP system. However, an important part of the samples had a large majority of OTUs assigned as “no blast hit” and the other part of samples mostly had OTUs’ assignments that stopped at the kingdom or phyla levels. Thus, other bioinformatics methods could be made in order to improve the results. Moreover, the sequencing of both bacteria and fungi were made with the same protocol at DNA Vision S.A. However, this protocol was optimized for bacteria sequencing. Hence, a special method for fungi sequencing could be used in order to improve the general results.

The analyses of the lettuce roots in our study was interesting, however the study of both endosphere and rhizoplane was perhaps not necessary in our case. Indeed, the results were very similar, and the separate analysis of both compartments were money and time-consuming. A general “root sample”,

combining both the endosphere and rhizoplane could thus be made if the objective of the survey is to study the overall microbiota of an AP system.

The survey was focused on *Eubacteria* and *Fungi*, however, research could be made on the *Archae* domain since very little is known about their presence and potential roles in AP.

Finally, in order to better understand the potential roles of the bacteria and fungi observed in the AP system, it could be interesting to study the most expressed genes via metatranscriptomic techniques.

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7 Annexes

7.1 Annex 1: Water exchanges, nutrients additions and events

Date	Water additions	Water withdrawals	Nutrients additions	Events
March 7	125 L tap water	10 L		
March 10				Heating failure
March 11		10 L		
March 12		10 L		
March 13		10 L		
March 14	250 L tap water	80 L		Backwash + lamella separator cleaning
March 15		10 L		
March 18		20 L		
March 19		20 L		
March 20		10L		
March 21	191 L tap water	10 L		
March 22	192 L tap water	10 L		
March 25		15L		
March 29	224 L tap water	40 L		
April 1		42 L		
April 2		50L		
April 3		10L		
April 4		10L		
April 5	294 L tap water	50 L	1L FeEDTA 13%	
April 8		10L		
April 9		10L		
April 10		10L		
April 11		40L		
April 12	392 L tap water	20L		
April 18	252 L tap water			
April 21		20L		
April 23		10L		
April 24		10L		
April 25		10L	KNO3 (50g)	
April 26	358 L tap water	10L	1L FeEDTA 13%	Heating failure
April 29		50L		"
April 30		10L	Start : 1L HNO3 13%	
May 2		10L	End	
May 3		10L	Start : 1L HNO3 13%	
May 4				Nitrate probe failure
May 6		10L		"
May 7		10L	End	"
May 8		10L		
May 9		10L		
May 10	255 L rainwater	10L		Backwash
May 13		10L		

7.2 Annex 2: Parameters statistical analysis

1. Pre-treatment of the data

For each parameter, the average of the data was calculated for each day. Those mean values per day were then used for the rest of the analysis after being standardised, i.e. a mean of zero and a variance of 1, with the command scale().

2. Hierarchical clustering analysis

This part aimed to differentiate each date of the experiment depending on physicochemical parameters and to create clusters of highly homogeneous dates (Kazi *et al.*, 2009). To do so, the first step was to measure the Euclidian distances matric with the command dist(), which characterises the dissimilarity between two dates in the variable space (Kazi *et al.*, 2009).

The dates were then clustered with the hclust() command using the complete linkage method, which calculated the distance between the two most distant dates of two separate clusters, for all clusters. The resulting classification was visualised in the form of a dendrogram with the command plot(), which provides an image of the groups and their proximity (Fig. 39) (Kazi *et al.*, 2009).

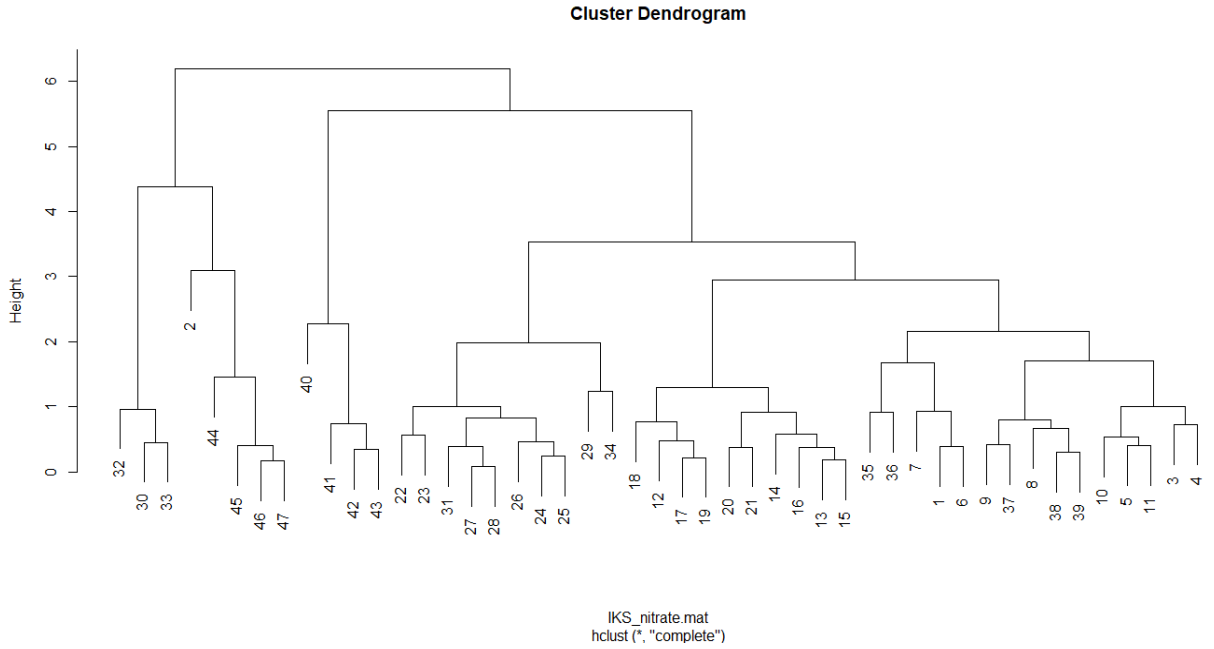

Figure 39 *Cluster dendogram generated by the command plot(), each number representing a day of the experiment (n° 1: March 8; n°48: May 13)*

A partition then needed to be established, corresponding to the number of retained clusters. To do so, the R^2 criteria provided by the command hclust.rsq() and the prsq plot() was used (Fig. 40). R^2 is the result of the sum of squared deviations between the groups divided by the sum of squared deviations of all parameters. The higher this value, the more initial information is retained after the clustering. Thus, the partition was chosen by looking at the successive clustering. The clustering that resulted in a strong R^2 decreasing and therefore, a big loss of information between the groups was spotted and the fusions were interrupted before this clustering. In this way, a partition made of 5 groups was retained.

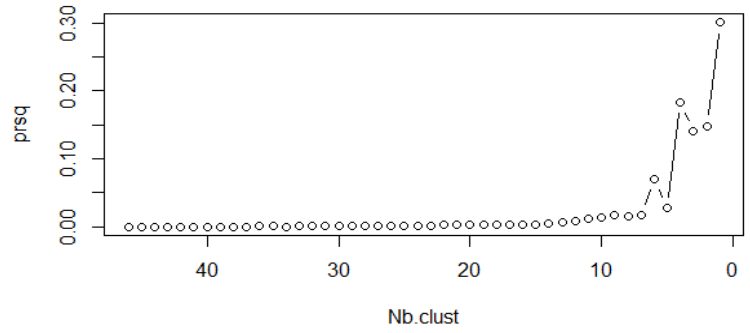

Figure 40 *Graph generated by the command prsq plot(), representing the R2 decrease at each individuals fusion, i.e. dates fusion*

The robustness of the partition was checked using an alternative partition method, i.e. the centroid method. Hence, the gravity centre of each group was calculated with the command aggregate() using the standardised data. The centroid classification was then made with the command kmeans() using the calculated centroids, which aimed to transfer some individuals between groups in order to improve the classification. The two obtained classifications were then compared thanks to the command table() (Fig. 41). Since the two classifications didn't barely differ, we concluded that the chosen partition made of 5 clusters was robust and thus, retained.

group	1	2	3	4	5
1	25	0	0	0	0
2	0	4	0	0	1
3	0	0	10	0	0
4	0	0	0	3	0
5	0	0	0	0	4

Figure 41 *Table showing the comparison of the 5 clusters generated by the two classifications*

The chosen partition was visualised on the dendogram with the command cutree() (Fig. 42) and characterised in term of composition with the sort() function, in term of individuals with table() and in term of mean and standard deviation with aggregate() command.

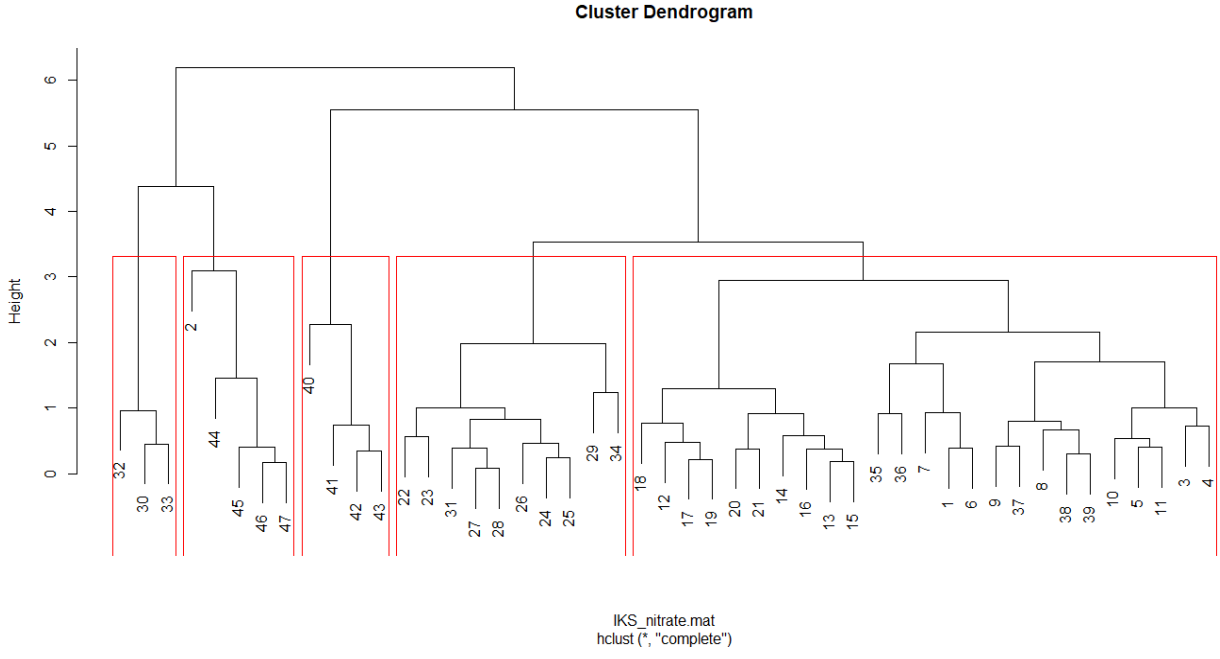

Figure 42 *Cluster dendogram showing the partition made of the 5 physicochemical groups*

3. Visualisation of the clusters via PCA

In order to describe the identified physicochemical groups in a multivariate space, the data were subjected to a principal component analysis (PCA). This enabled to reduce the number of variables, i.e. parameters, and to visualise the clusters in the factorial plans retained by the PCA. To do so, the FactoMineR package was loaded and the PCA() command was carried out on standardised data. In order to determine the number of retained factorial plans, the eigen value graph was generated with the command plot() (Fig. 43).

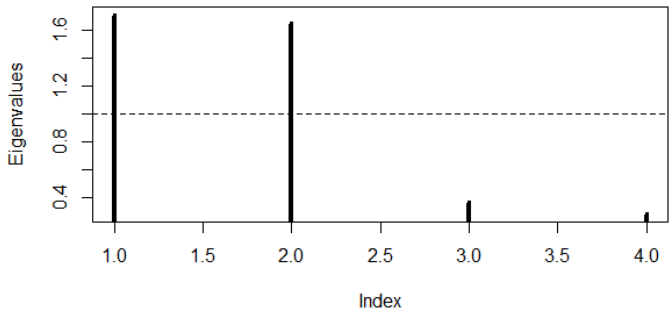

Figure 43 *Eigen values graph*

According to this graph, only the first factorial plan made of the principal components 1 and 2 was retained, as both of them were highly superior to the mean, i.e. 1 since the data were standardised, which was not the case for the components 3 and 4.

The variable factor map showing the correlations between each parameter to the two axes, i.e. principal components 1 and 2, was generated thanks to the plot() command (Fig. 44).

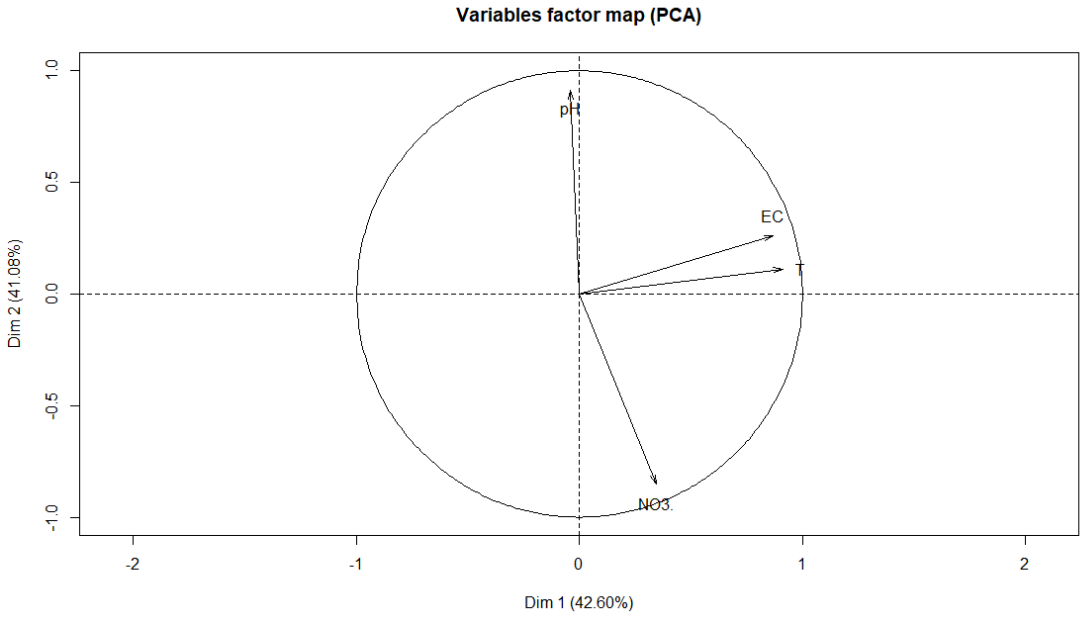

Figure 44 *Variables factor map of the first factorial plan*

The physicochemical clusters were then visualised on the first factorial plan using the command rainbow(), that assigns a colour to each cluster, and the plot() function. The centroids of the groups

were also added to the plan by using the function PCA() on another data set made of the clusters' identifiers and the standardised data.

In order to interpret the data in the factorial map and to understand the two principal components, the correlations of each parameter to the 2 new axes were extracted from the PCA object. The command dimdesc() facilitate the understanding by sorting the correlations in descendent order for each principal component. The quality of the representation of the parameters in the factorial map was also extracted from the PCA object.

7.3 Annex 3: DNA extraction protocol using the FASTDNA Spin kit MpBiomedicals

1. Add sample to Lysing Matrix A tube with maximum suspension volume of 200µl.

2. Add 1.0ml of Cell Lysis Solution CLS-TC.

3. Homogenise in a Vortex (Power-Mix Model L46 (Labinco, Breda, The NetherlandsLabinco, level 7)) for 40 seconds, incubate in ice for 2 minutes and homogenise again for 40 seconds.

4. Centrifuge at 14 000 rcf for 10 minutes to pellet debris.

5. Transfer 800µl of supernatant to a 2.0ml Eppendorf with round bottom and add an equal volume (800µl) of Binding Matrix. Invert to mix.

6. Incubate with gentle agitation (Power-Mix Model L46 (Labinco, Breda, The NetherlandsLabinco, level 1)) for 5 minutes at room temperature.

7. Transfer half (approximately 800µl) of the suspension to a Spin™ filter and centrifuge at 14 000 rcf for 1 minute. Empty the catch tube (tube below the filter) and add the remaining suspension to the Spin™ filter and centrifuge as before. Empty the catch tube again.

8. Add 500µl of prepared SEWS-M and gently suspend the pellet using the force of the liquid from the pipet tip.

9. Centrifuge at 14 000 rcf for 1 minute. Discard content of the catch tube below and put it back.

10. Without any addition of liquid, centrifuge a second time at 14 000 rcf for 2 minutes and replace with a new, clean tube.

11. Elute DNA by gently suspending Binding Matrix above the Spin™ filter in 100µl of DES. Incubate for 5 minutes at 55°C in a heat chamber or water bath.

12. Centrifuge at 14 000 rcf for 1 minute to bring eluted DNA into the clean catch tube.

13. Discard the Spin™ filter. DNA is now ready for downstream applications Store at -20°C for extended periods or 4°C until use.

7.4 Annex 4: DNA Vision's detailed protocol

1) PRINCIPE

Ce protocole a été spécialement développé et optimisé pour préparer des librairies d'amplicons d'ADN 16S qui seront séquencés sur le Miseq Illumina.

2) MATERIEL ET REACTIFS

2.1 Equipement

- 96 puits thermocycleur (avec couvercle chauffant)
- Centrifugeuse de plaques 96 (…)
- Fluoromètre pour quantifier AND double brin ( )
- Truseq index plate fixture kit (Illumina, ref : FC-130-1005)
- Bioanalyzer Agilent 2100 (Agilent, SN : DE72902871)
- Gants non-poudrés: Kimtech Satin Plus
- Tubes 1,5 ml DNA LoBind Eppendorf (VWR, n° 525-0130)
- Strips, tubes PCR et plaques
    - plaque 96 puits (ABI N801-0560)
    - strip PCR (Sarstedt 72.985.992)
    - film adhésif (Greiner 676090)
    - Strip couvercle (Sarstedt 65.989.002)
    - Tube 1.5 ml (Sarstedt 72.706.200)
- Tips filtrés (Greiner FT1000/200/20/10)
- Film aluminium adhésif (Greiner SN :676090)
- Dynal DynaMag2- support magnétique (Invitrogen p/n 123-21D)
- Speedvac (Eppendorf concentrator 5301 SN :530103715)
- Bain-marie (Gesellschaft fur labortechnik mbh SN :11274803K)
- Bloc froid (Biosmith)
- Agitateur (Vortex genie 2 Scientific industries SN :2-145953)

2.2 Réactifs

- Agencourt AMPure XP kit (Analis, p/n A63881 60 ml)
- Eau nuclease-free (non traitée DEPC) (Gibco ref :10977-035 500ml)
- 100% Ethanol (Sigma-Aldrich p/n E7023)
- KAPA Hifi Hotstart ReadyMix (2X) (Sopachem, ref : KK2602)
- Phix control V3 (Illumina ref : 15017666)
- Picogreen (…)
- Miseq reagent kit V3 (Illumina , ref:MS-102-3003)
- Nextera XT Index Kit (Illumina, ref : FC-131-1002)

3. METHODE

3.1 Contrôle qualité

Avant d'utiliser l'ADN génomique, il faut contrôler sa qualité et le doser en suivant les 2 MOP suivantes:

- MOP-SPE-003 : nanodrop
- MOP-SPE-005 : picogreen

Les critères d'acceptation sont les suivants :

- le ratio 260/280 : 1,7 < x < 2,1
- quantité totale : 5ng/µl → 2.5µl

3.2 Préparation des amplicons

3.2.1 Normalisation de l'ADN

Après dosage au picogreen, on normalise l'ADN à 5 ng/µl :
- Soit on dilue l'Adn pour arriver à une concentration de 5 ng/µl
- Soit on speedvaque le volume total de l'extrait jusqu'à lyophilisation et on resuspend l'ADN dans un volume calculé pour au final arriver à 5 ng/µl.

3.2.2 Amplification de l'ADN par PCR

Cette étape utilise le principe de la PCR pour amplifier à partir de l'ADN la région d'intérêt. Pour cela, on utilise des primers spécifiques auxquels sont « attachés » les adaptateurs. 2 régions peuvent être ciblées : V3-V4 ou V5-V6. Voici les séquences des primers + adaptateurs :

Amplicon PCR primer Fwd V1-V3 :
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGCTCAG
Amplicon PCR primer Rvs V1-V3 :
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG

Procédure:

- Préparer le mix suivant par échantillon + Contrôle positif + blanc :

Amplicon PCR primer Fwd 1 µM	5 µl
Amplicon PCR primer Rvs 1 µM	5µl
2x KAPA HiFi HotStart ReadyMix	12,5 µl
Total	22,5 µl

- Distribuer 22,5 µl de ce mix dans une plaque 96
- Ajouter 2,5 µl d'ADN dilué à 5 ng/µl
- Seller la plaque avec un film alu adhésif et la placer dans le thermocycleur
- Lancer le cycle suivant :

	95°C	3 min
25 X	95°C	30 sec
	55°C	30 sec
	72°C	30 sec
	72°C	5 min
	4°C	Infini

3.2.3 Purification des produits PCR

Cette étape utilise les billes AmpureXP pour purifier les amplicons 16S des primers résiduels. Procédure :
- Amener les billes à température ambiante
- Centrifuger la plaque contenant les produits PCR
- Vortexer les billes environs 30 secondes pour être sûr que toutes les billes soient bien resuspendues
- Ajouter 20µl de billes à chaque puits de la plaque
- Mélanger en pipetant up and down environ 10 fois

- Incuber environ 5 minutes à température ambiante
- Placer la plaque sur un support aimanté environ 2 minutes ou jusqu'à ce que le surnageant soit clair
- Laisser la plaque sur le support aimanté, et éliminer le surnageant ; ensuite, laver les billes avec de l'éthanol 80% fraîchement préparé :
    - Ajouter 200µl d'éthanol 80%
    - Incuber 30 secondes en laissant la plaque sur le support aimanté
    - Eliminer délicatement le surnageant
- Tout en laissant la plaque sur le support aimanté, laisser sécher les billes pendant 10 min à l'air libre
- Retirer la plaque du support aimanté et ajouter 27.5 µl de Tris HCl 10 mM pH 8.5 à chaque puits
- Mélanger en pipetant up and down environ 10 fois
- Incuber à température ambiante pendant environ 2 minutes
- Placer la plaque sur le support aimanté 2 minutes ou jusqu'à ce que le liquide soit clair
- Transférer délicatement 25µl de surnageant de la plaque des produits PCR amplifiées vers une nouvelle plaque.

3.2.4 Indexage

Cette étape utilise le principe de la PCR pour ajouter les index aux amplicons. Il s'agit d'un double indexage. Chaque combinaison de 2 index est propre à son amplicon.

Indexage des amplicons :

Préparer le mix suivant par échantillon + Contrôle positif + blanc :

Nextera XT Index Primer1	5 µl
Nextera XT Index Primer2	5µl
2x KAPA HiFi HotStart ReadyMix	12,5 µl
Total	22,5 µl

- Distribuer 22,5 µl de ce mix dans une plaque 96
- Ajouter 2,5 µl d'ADN dilué à 5 ng/µl
- Seller la plaque avec un film alu adhésif et la placer dans le thermocycleur
- Lancer le cycle suivant :

	93°C	3 min
25 X	95°C	30 sec
	55°C	30 sec
	72°C	30 sec

3.2.5 Purification des produits PCR

Cette étape utilise les billes AmpureXP pour purifier les librairies finales avant quantification. Procédure :

- Amener les billes à température ambiante
- Centrifuger la plaque contenant les produits PCR
- Vortexer les billes environs 30 secondes pour être sûr que toutes les billes soient bien resuspendues
- Ajouter 56µl de billes à chaque puits de la plaque
- Mélanger en pipetant up and down environ 10 fois
- Incuber environ 5 minutes à température ambiante

- Placer la plaque sur un support aimanté environ 2 minutes ou jusqu'à ce que le surnageant soit clair
- Laisser la plaque sur le support aimanté, et éliminer le surnageant ; ensuite, laver les billes avec de l'éthanol 80% fraîchement préparé :
    - Ajouter 200µl d'éthanol 80%
    - Incuber 30 secondes en laissant la plaque sur le support aimanté
    - Eliminer délicatement le surnageant
- Tout en laissant la plaque sur le support aimanté, laisser sécher les billes pendant 10 min à l'air libre
- Retirer la plaque du support aimanté et ajouter 27.5 µl de Tris HCl 10 mM pH 8.5 à chaque puits
- Mélanger en pipetant up and down environ 10 fois
- Incuber à température ambiante pendant environ 2 minutes
- Placer la plaque sur le support aimanté 2 minutes ou jusqu'à ce que le liquide soit clair
- Transférer délicatement 25µl de surnageant de la plaque des produits PCR amplifiées vers une nouvelle plaque.

3.2.6 Validation des librairies (optionnel)

Passer 1µl de la dilution 50x sur une puce bioanalyzer DNA 1000 pour vérifier la taille de l'amplicon.

3.2.7 Quantification, normalisation et assemblage des librairies

Il est recommandé de quantifier les librairies en utilisant une méthode fluorimétrique qui utilise des dyes qui se lient à l'ADN double brins (picogreen).

Calcul de la concentration en nM, basée sur la taille de l'amplicon déterminée par le profil sur bioanalyzer : (concentration en ng/µl) * 106 = concentration en nM (660 g/mol * taille moyenne de librairie)

Diluer la librairie à la concentration finale de 4nM avec du Resuspension Buffer (RSB) ou du Tris pH 8.5 10 mM. Pooler 5µl de chaque librairie avec un index unique (4nM) et mélanger.

3.2.8 Dénaturation de la librairie et chargement sur le MiSeq

Avant séquençage, le pool de librairies est dénaturé avec du NaOH, dilué avec du buffer d'hybridation et ensuite dénaturé par la chaleur avant d'être chargé sur le Miseq. Chaque run doit inclure au moins 5% de PhiX (25% pour les runs n'incluant qu'une seule taille d'amplicon) pour servir de contrôle interne dans le cas de faible diversité de librairies. Il est recommandé d'utiliser les kits V3.

Préparation :

- Allumer le bloc chauffant à 96°c
- Sortir une cartouche de réactifs Miseq du congélateur et la laisser dégeler à température ambiante

Dénaturation de l'ADN :

- Combiner dans un tube les volumes suivants de Pools de librairies et de NaOH fraichement dilué à 0.2N :
    - Pool de librairires à 4 nM (5µl)
    - NaOH 0.2N (5µl)
- Garder de côté la dilution NaOH 0.2N pour préparer le contrôle PhiX dans les 12 heures à venir.
- Vortexer le tube pool-NaOH et centrifuger la solution à 280g pendant 1 min
- Incuber 5 minutes à température ambiante pour dénaturer l'ADN en simple brin
- Ajouter 990µl de HT1 froid au tube contenant l'ADN dénaturé (10µl)

→ On obtient ainsi un pool de librairie à 20pM dans du NaOH 1 mM.

- Placer l'ADN dénaturé sur glace jusqu'à la dilution final Rem : Le PhiX contrôle est préparé de la même façon pour arriver à la concentration de 20 pM

Dilution de l'ADN dénaturé :

- Diluer l'ADN dénaturé à la concentration désirée en utilisant le tableau suivant :

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denaturated library	60 µl	120 µl	180 µl	240 µl	300 µl
Pre-chilled HT1	540 µl	480 µl	420 µl	360 µl	300 µl

- Inverser le tube plusieurs fois pour mélanger et centrifuger rapidement
- Placer l'ADN dénaturé et dilué sur glace

Combinaison de la librairie d'amplicons et du PhiX contrôle :

- Combiner dans un tube les volumes suivants de librairies et de Phix 20pM :
    - PhiX 20 pM : 50µl
    - Librairies d'amplicon : 550µl
- Garder la solution sur glace jusqu'à être prêt pour chauffer le mix avant de placer la cartouche dans le Miseq
- Utiliser un bloc chauffant pour dénaturer le mix à 96° pendant 2 minutes
- Après incubation, inverser le tube 1 ou 2 fois pour mélanger et placer sur glace pendant 5 minutes