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TAXONOMIC CHARACTERISATION OF BACTERIA COMMUNITIES FROM WATER OF DIVERSIFIED AQUAPONIC SYSTEMS

MATHILDE ECK

TRAVAIL DE FIN D'ETUDES PRESENTE EN VUE DE L'OBTENTION DU DIPLOME DE MASTER BIOINGENIEUR EN SCIENCES AGRONOMIQUES

ANNÉE ACADÉMIQUE 2016-2017

PROMOTEUR: H. JIJAKLI

[1] Dans ce cas, l'autorité académique est représentée par le(s) promoteur(s) membre du personnel(s) enseignant de GxABT

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Abstract

In 2030, the world's population should reach 8.3 billion people and 60% of them should live in urban areas. It is thus necessary to develop intensive yet sustainable urban production systems in order to increase cities' resilience. Aquaponics is defined as a combination of hydroponic and aquaculture techniques and seems to be a promising technology to meet this resilience. It functions with plants, fish and microorganisms which play a key role in nitrification and mineralisation of fish wastes into nutrients absorbable by plants. Herein we aim at characterising the bacteria present in diversified systems to better understand the composition and role of their communities in aquaponics. To this end, nine diversified aquaponic systems were sampled. The DNA from each bacteria community was extracted and sequenced with Illumina MiSeq technology by targeting the V1-V3 16S rDNA region. The sequences were then analysed with the QIIME bioinformatic software. Results show that *Proteobacteria* and *Bacteroidetes* are the dominant phyla for all the aquaponic systems. Depending on each system, different proportions of other phyla are also present among the bacterial community. The genera which compose all the identified phyla are more diverse and an important proportion of them are usually found in soils and rhizosphere. One of the roles that could be linked to these genera is the breaking down of complex organic compounds which could be related to the mineralisation phenomenon observed in aquaponic systems. Further studies should be undertaken to identify the exact species present in aquaponic systems and to understand their specific functions.

Keywords: aquaponics, bacteria communities, bacteria's functions, NGS, 16S rDNA

Résumé

En 2030, la population mondiale atteindra 8.3 milliards d'habitants et 60% d'entre eux vivront en zone urbaine. Il est donc nécessaire de développer de nouveaux systèmes de production urbaine intensifs et durables dans le but d'augmenter la résilience des villes. L'aquaponie semble être une bonne opportunité. L'aquaponie est la combinaison de l'hydroponie et de l'aquaculture et fonctionne avec des plantes, des poissons et des microorganismes qui jouent un rôle clé dans les processus de nitrification et de minéralisation des déjections de poissons en nutriments absorbables par les plantes. Dans cette étude, nous cherchons à caractériser les bactéries présentes dans des systèmes diversifiés afin de mieux comprendre la composition et le rôle des communautés bactériennes dans un système aquaponique. Dans ce but, neuf systèmes aquaponiques diversifiés ont été échantillonnés. L'ADN de chaque communauté bactérienne a été extrait et séquencé via la technologie Illumina MiSeq en ciblant les régions V1-V3 de l'ADNr 16S. Les séquences obtenues ont été analysées grâce au logiciel QIIME. Les résultats montrent que les *Proteobacteria* et les *Bacteroidetes* sont les deux phyla dominants dans les échantillons. S'ensuivent différentes proportions d'autres phyla en fonction des systèmes. Les genres qui composent ces phyla sont plus divers et sont souvent retrouvés également dans le sol et la rhizosphère. Un des rôles attribué à certains genres est le démantèlement de molécules organiques complexes qui pourrait être lié au phénomène de minéralisation observé en aquaponie. De plus amples études devront être menées afin d'identifier les espèces présentes en aquaponie et de comprendre leur rôle exact dans un système aquaponique.

Mots-clés : aquaponie, communautés bactériennes, fonction des bactéries, NGS, ADNr 16S

TABLE OF CONTENTS

List of figures

List of tables

List of abbreviations

ADN: acide désoxyribonucléique
DNA: deoxyribonucleic acid
QIIME: Quantitative Insight Into Microbial Ecology
NGS: Next Generation Sequencing
RNA: ribonucleic acid
PCR: polymerase chain reaction
rDNA: ribosomal deoxyribonucleic acid
rRNA: ribosomal ribonucleic acid
FAO: Food and Agriculture Arganisation of the United Nations
PCG: Provinciaal Proefcentrum voor de Groenteteelt Oost-Vlaanderen
IGB: Leibnitz-Institute of freshwater ecology and inland fisheries
OTU: operation taxonomic unit
PAFF Box: Plant and Fish Farming Box
RAS: recirculating aquaculture system
BQF: Belgian Quality Fish
WHO: world health organization
USDA: United State Department of Agriculture
L: litre
Kg: kilogram
INAPRO: Innovative Aquaponics for Professional Application
COST: European collaboration in science and technology
IUPPL: Integrated and Urban Plant Pathology Laboratory
DO: dissolved oxygen
NFT: nutrient film technique
pH: potential of hydrogen
mg: milligram
dS: deciSiemens
cm: centimetre
EC: electro-conductivity
TAN: total ammoniacal nitrogen
AOB: ammonia oxidizing bacteria
NOB: nitrite oxidizing bacteria
ANAMOX: anaerobic ammonia oxidation
PGPR: plant growth promoting rhizobacteria
HTR: hydraulic retention time
SBS: sequencing by synthesis
BLAST: basic local alignment search tool
UV: ultra-violet
µm: micrometre
ml: millilitre
µl: microlitre
Guanidine HCL: guanidine hydrochloride
mM:millimolar
dNTP: deoxyribonucleoside triphosphate
ng: nanogram
PCoA: principal component analysis

1. INTRODUCTION

1.1. Context

In 2030, the world's population should reach 8.3 billion people (FAO, 2002) and 60% of them should live in urban areas (United Nations, no date). This rise in the urban population is partly due to rural exodus, for in the last 60 years, more than 800 million rural residents have left the countryside in search of higher incomes (Matthews, 2006) and better life quality (Matuschke, 2009). Furthermore, the changes occurring in basic alimentation around the world are leading to an increasing demand for animal protein (WHO, 2008; FAO, 2011; Goddek *et al.*, 2015) which causes additional pressure on agriculture and land allocation. It is therefore necessary to find new and sustainable ways to provide food for these growing cities' populations. According to Lehman *et al*. (1993) sustainable agriculture should not drain the natural resources which cannot be renewed and which are of prime importance in agriculture

However, before finding an adequate solution for a sustainable future, it is imperative to distinguish the consequences of the growing population and its increasing needs in Northern and in Southern countries. Indeed, even though Northern cities keep on growing, their rate of expansion is way slower than in Southern cities and the new megalopolises which are supposed to develop in the coming years are mostly located in the South (United Nations Population Division, 2001). Thanks to this slower growth, Northern cities can be better prepared and the needs of the population are mostly social (Santo *et al.*, 2016) compared to a need of access to fresh and healthy food in the South (FAO, 2014). Therefore, urban agriculture, even though existent in both cases does not answer the same issues (see 1.2).

Although the conjectural situations appear to be very different, cities tend to have the same aim in view, i.e. to be more resilient and less dependent on rural production and transports. This requires fostering the development of intensive yet sustainable production systems which would enable to produce food close to urban consumers. In such a context, urban agriculture could be an efficient answer. In the following section we will look further into what urban agriculture exactly is.

1.2. What is urban agriculture?

Urban agriculture has many definitions which encompass several aspects such as the localisation, its function in the urban zone or the stakeholders involved. Nahmías and Le Caro (2012, p.13) have tried to take all these aspects into account in the following definition: "Urban agriculture is the agriculture practiced and lived in an agglomeration, by farmers and inhabitants, at the daily life scale and territory application of the urban regulation scale. In that space, agricultures – whether professional or not, orientated towards long or short food supply chains or even self-consumption – maintain reciprocal and functional links with the city (food supply, landscape, entertainment, ecology) thus giving birth to a diversity of noticeable agri-urban forms in the urban cores, suburbs, urban fringe and peri-urban space."

1.2.1. Urban agriculture in the North

If we focus on the various functions of urban agriculture, we have to distinguish the Northern cities from the ones located in the South. In the North, urban agriculture has more of a social function. Indeed, it is mostly represented by collective or community gardens in which people work next to each other or even together on the same plot. This can help recreate bonds between neighbours, rehabilitate unsecure areas and create a help system between citizens. Urban gardens can also serve as a support to educate children to understand the importance of a healthy nutrition and to help them reconnect with nature. This leads to the second aspect of urban gardens which is the economical function. Indeed, urban gardens can often lead to community markets where fresh and locally grown products are sold to local consumers (Santo *et al.*, 2016). This is an effective way to participate in a transition effort from the actual agro-industrial production system to a more sustainable way of production and consumption. It is also a good way to fight against "food deserts" which are defined by the United State Department of Agriculture (USDA, 2011) as "a low-income census tract where either a substantial number or share of residents has low access to a supermarket or large grocery store" and thus fight against overweight which is a recurrent problem in Northern countries (Vallianatos *et al*., 2004).

If we extend the concept of urban agriculture to all the green spots in a city, including green roofs and parks we can say that urban agriculture enables to clean the air, reduce urban heat and floods which are the consequence of waterproofing the grounds (Zimmerman *et al.*, 2016). It also permits to foster biodiversity and to produce with fewer pesticides as vegetables are grown on a smaller scale (Santo *et al*., 2016). What's more, recent studies of a new concept called "biophilia" tend to show that being in touch with nature can improve living conditions in cities whether on the physical and mental health angle or on the socializing angle (Keniger *et al.*, 2013).

Next to this "social urban agriculture", a more productive type of urban agriculture emerges in cities, based on state-of-the-art techniques such as hydroponics and aquaponics. Hydroponics is a precision agriculture technique defined by Sheikh (2006, p. 1) as "the growing of plants in a water and fertilizer solution containing necessary nutrients for plant growth" and aquaponics is a combination between hydroponics and recirculating aquaculture. The crops fostered are high-value crops such as herbs, microgreens and edible flowers but also specific varieties of strawberries for example. Indeed, one of the advantages of producing directly next to the consumers is that fragile cultivars which cannot be transported can be cultivated.

1.2.2. Urban agriculture in developing countries

The goals of urban agriculture are however different in the South. Indeed, in Southern cities urban agriculture enables poor people to grow their own nutritious food and to earn an income by selling their surpluses. At city level, it can also help creating more resilient cities, adapted to climate change. It can "stimulat[e] regional economies and reduce dependency on the global food market" (FAO, 2014, p.5).

As well as in the North, urban agriculture can come in several forms such as community gardens, school gardens and more intensive production techniques even though the first ones are more widely spread. Small livestock farming is also present in Southern cities (FAO, 2014).

The main advantages of urban agriculture in the South are the improvement of the quality of the diet, especially the increased consumption of fruits and vegetables (FAO, 2014) and the greater independence of poor urban households from food prices fluctuations (FAO, 2017).

1.2.3. Urban agriculture and transition

With its multiple functions and impacts both in the developed and developing countries, urban agriculture falls within the transition movement in which more and more cities are involved throughout the world (Transition Network, no date). Urban agriculture can help enhancing cities' resilience to climate change and to the fluctuation of fossil fuels' prices. Indeed, the production and transformation of food inside the cities enable them to be less dependent on their hinterlands and thus less dependent on transport using fossil fuels (Deelstra and Girardet, 1999). In Tokyo for example, the urban vegetables production could potentially feed 700 000 urbanites (Moreno-Penaranda, 2011). In 2000, the Dakar region could provide for 60% of its vegetables consumption (Mbaye and Moustier, 2000).

This increased resilience falls within the "cities in transition" movement initiated by Rob Hopkins in the United Kingdom in which inhabitants form communities aiming at transforming the economy, the agricultural system and the overall way of life (*Transition Network*, no date).

1.3. Fish production and consumption

With urbanisation and globalisation, eating habits change and the consumption of animal protein increases, especially in developing countries (FAO, 2017). Fish is an excellent source of animal protein, it contains healthy fats such as omega-3 fatty acids (Smith *et al.*, 2010) and is a great feed converter (the feed conversion ratio for most fish is 1.4-1.8) (Somerville *et al*., 2014). However, intensive fishing has caused the depletion of wild fish populations in the seas and oceans and the world is more and more turned towards aquaculture which provides for approximately 43% of fish production (Figure 1). Aquaponics could provide healthy, fresh fish and would require less than 100 L of water per kg of fish contrary to conventional aquaculture which consumes between 2 500 and 375 000 L per kg (Goddek *et al.*, 2015). It would then prove to be a solution to the increasing world and cities' demography with a limited impact on the environment (Junge *et al.*, 2017).

Further research is needed in order to better understand its working principle and therefore assure a well-controlled production.

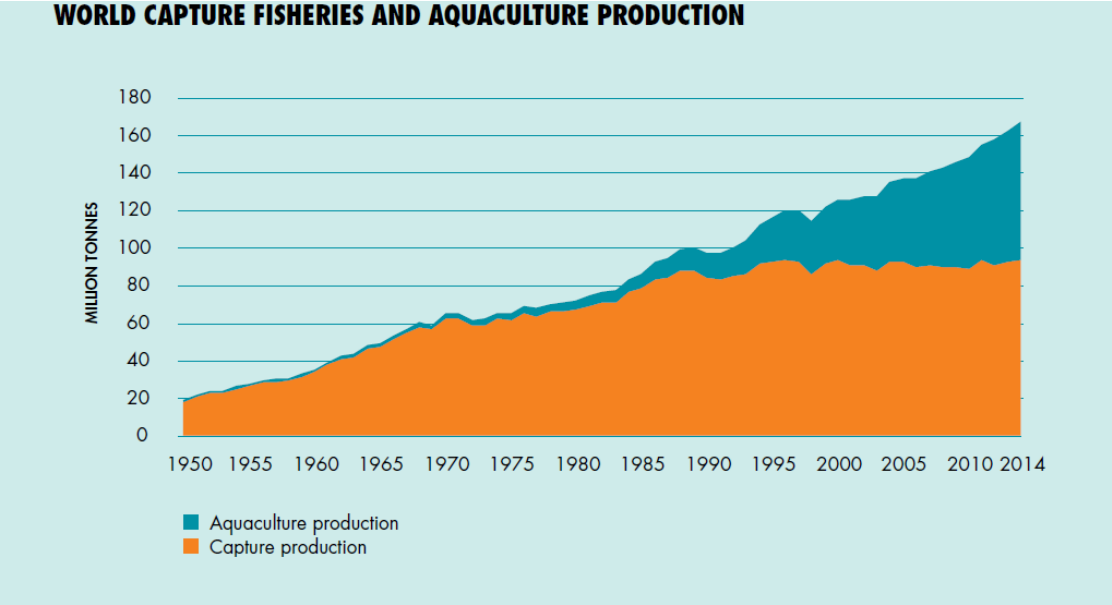

Figure 1. Evolution of aquaculture and capture fisheries from 1950 to 2014 (FAO, 2016)

1.4. What is aquaponics?

Aquaponics is a combination of hydroponics and recirculating aquaculture techniques (Figure 2) (Delaide *et al.*, 2016a). It offers the recycling of nutrient-rich waste water from fish into organic fertilizers for the plants grown in the system (Rakocy *et al.*, 2006), thus reducing the use of chemical fertilizers and the environmental impact of both fish and plant production (Delaide *et al.*, 2016b). The vegetables thus produced can be considered as safer and healthier as almost no chemicals or antibiotics can be used as it would perturb either the plants or the fish (Somerville *et al.*, 2014).

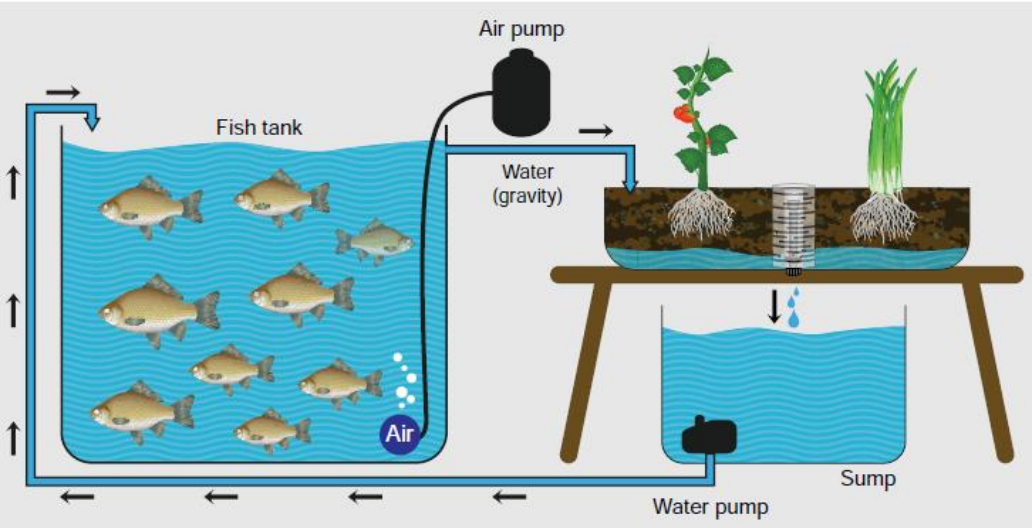

Figure 2. Simple aquaponic unit (Somerville *et al.*, 2014)

Even though aquaponics systems are starting to spread (Villarroel *et al.*, 2016), its inception apparently dates back to the 6th century. Indeed, according to Jones (2002), the idea of combining fish farming and crops appeared some 1500 years ago when a Chinese farmer

decided to save time by feeding only his ducks, whose dejections and uneaten food were eaten by a first species of fish and then a second species of fish. The rest of the food and the fish dejections finally served as fertilizers for a rice field a little further downstream. Even before that, Incas used to build small islands at the centre of ponds to allow birds to rest on it, defecate on it and in the water in order to feed the fish. The birds' dejections would then also fertilize the small islands, making them fit for growing crops. In 1929, Dr. W. Gericke started developing hydroponic systems at the University of California and in the seventies, some experiments began on aquaponics based on a coupling between hydroponics and recirculating aquaculture systems (RAS). These experiments firstly aimed at finding a sustainable and easy way to clean the water before it flew back to the fish tank. In 1980, researchers started thorough researches on aquaponics in order to ensure that it was a viable agricultural activity (Love *et al.*, 2014) and especially in the University of the Virgin Islands by James Rakocy (Goddek *et al.*, 2015).

According to the survey conducted by Love *et al.* (2014), most practitioners consider aquaponics as a sustainable way to produce healthy food for self-consumption. However, more and more companies are being created to develop aquaponics on a commercial scale such as the UrbanFarmers in Switzerland and in The Netherlands or ECF in Germany. These companies' main customers are urban dwellers and restaurants owners but they also sell farm prototypes in order to spread the aquaponic movement further and further. The research sector is also active in this field and several European projects are currently functioning such as the INAPRO project (Innovative Aquaponics for Professional Application) which is developing a system with two separate loops for plants and fish, thus enabling optimal conditions for both types of organisms (Inapro, no date). The COST group (European collaboration in science and technology) also fosters the creation of a hub of scientists and experts in the fields to promote the development of aquaponics in cities, in rural environments and on a commercial scale (COST, 2013). The Integrated and Urban Plant Pathology Laboratory (IUPPL) in Gembloux is currently implementing an aquaponic system in a gastronomic restaurant. The chef will serve locally grown trouts and the fish water will be used to fertilize hydroponic microgreens and herbs which will also be added to the menu.

- **Advantages and drawbacks of aquaponics:**

The advantages of aquaponics are the following:

- The productivity of the system can be as important as in a hydroponic system and even 39% higher if the aquaponic water is complemented with external nutrients (Delaide *et al.*, 2016b)
- Aquaponic systems can be used where little land is available or when the land is either infertile or polluted (Somerville *et al*., 2014; Wortman, 2015)
- Aquaponic systems are more water efficient (Delaide *et al.*, 2016b) (no need to use as much fresh water to change the fish water as in conventional aquaculture) because the water is first cleaned by the bacteria and then by the plants (Rakocy *et al*., 2006)
- Aquaponic systems reduce the discharge of waste nutrients in the environment as they are used by plants (Rakocy *et al*., 2006)

The drawbacks of aquaponics are the following:

- Farmers have to master more diverse skills than in other agricultural techniques (Goddek *et al.*, 2015)
- The launching costs of an aquaponic system are higher than in hydroponics or conventional agriculture (Somerville *et al*., 2014)

- Aquaponics can be energy demanding and further research is needed to improve the systems designs in order to lower this demand (Delaide *et al.*, 2016b)
- A compromise has to be found between the optimal growth conditions of plants, fish and bacteria (Somerville *et al*., 2014)
- Nutrient quantities are less optimal than in hydroponics and some exterior nutrients such as iron may need to be added regularly (Goddek *et al.*, 2015)

NB: it is necessary to nuance these last drawbacks. Indeed, they are true in one-loop aquaponic systems (also called coupled aquaponics) but not in decoupled aquaponics in which the plants and fish each evolve in their own loop, thus enabling a better optimisation of conditions (see 1.4.2.).

1.4.1. Description of an aquaponic system

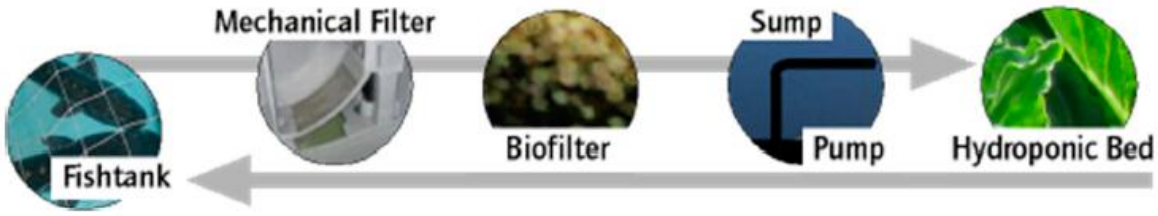

Figure 3. Basic aquaponic system layout (Goddek *et al.*, 2015)

An aquaponic system consists in a closed loop, multi-trophic system composed of a recirculating aquaculture system and a hydroponic production unit (Goddek *et al.*, 2016). More precisely, the system contains a fish tank, a mechanical filter which will retain the solid wastes, a biological filter, and the hydroponic unit (Figure 3). Most of the time the water from the hydroponic unit then goes into a sump before being pumped back to the fish tank (Somerville *et al*., 2014).

- **Mechanical filter**

The role of the mechanical filter is to retain the major part of the solid wastes in order to prevent the plumbing from clogging but also to maintain beneficial bacteria in the system instead of anaerobic bacteria which would produce toxic gases. Removing the solid wastes also prevent the organic matter to rot in the fish tank and thus consume the entire dissolved oxygen (DO) which is vital for the living organisms of an aquaponic system (see 1.4.3) (Somerville *et al*., 2014). Moreover, removing the solid wastes ensures that no film of organic matter forms around the plants' roots, thus creating an anaerobic zone which would lower the nutrient absorption (Rakocy *et al*., 2006).

Several types of mechanical filter exist such as clarifiers with create water movement to force solid waste to deposit itself on the bottom settling basins, tube or plate separators and drum filters (Somerville *et al*., 2014).

In most systems, an important part of the solid wastes is filtered out through the mechanical filtration and therefore does not fertilize the plants. It is however possible to

digest the sludge further and thus to conduct a complete mineralisation so as to complement the water (Goddek *et al.*, 2015).

- **Biological filter**

The biological filter, or biofilter, is usually located after the mechanical filter and helps processing the wastes that went through the first filter because they were already dissolved in the solution. This waste is mainly composed of ammonia and nitrite excreted by fish and yet toxic for the fish if they accumulate in the water. In order to convert these compounds into nitrate, nitrifying bacteria are used (Somerville *et al*., 2014).

In order to ensure a maximum conversion into nitrate, the biofilter is meant to present a high surface/volume ratio to welcome as many bacteria as possible and offer them the possibility to form biofilms. Plastic chips or porous ceramic plates are therefore used as inert biofilter media. The biofilter has to be large enough to host a sufficient quantity of bacteria. The minimum proportion is one sixth of the fish tank but biofilters are usually oversized for safety measures (Somerville *et al*., 2014).

The biofilter also needs to be regularly oxidized for the nitrification process to function properly. The optimal water parameters for the growth and nitrifying function of the bacteria are a pH between 7,0 and 9,0, a temperature comprised between 25°C and 30°C and a maximum quantity of DO (Rakocy *et al*., 2006). Further information concerning the water parameters will be given in further sections.

Different types of biofilters exist, the two main types being moving bed (a high number of beads or coins in constant movement in the water) or fixed bed on which the water from the RAS trickles (Suhr and Pedersen, 2010).

- **Hydroponic unit**

As said before, Sheikh (2006, p.1) defined hydroponics as "the growing of plants in a water and fertilizer solution containing necessary nutrients for plant growth" and "growing plants in a nutrient solution without soil". According to Somerville *et al*. (2014) hydroponics allow a better control of diseases and pests, a better efficiency when it comes to water and fertilizers management, higher yields thanks to growth conditions optimally adapted to the needs of each plant at each phenological state and eventually allows to grow food where land and water are scarce.

Several types of hydroponics can be associated with aquaculture:

- With the ebb and flow media-bed technique, plants are fixed in an inert support media such as rockwool, coco peat, limestone, expanded clay or gravel. This inert media has a role of support and also lodgings for the bacteria (Goddek *et al.*, 2015). The water coming from the fish tank trickles through the media and thus waters and fertilizes the plants. It is considered the easiest way to start a small aquaponic unit.
- With the nutrient-film technique (NFT) plants are usually grown in plastic tubes in which a thin stream of nutrient enriched water flows (Resh, 2013). Thus, the roots of the plants are constantly in contact with nutrient and oxygen is in sufficient supply (Resh, 2013).

- The deep-water culture technique is mostly used in important commercial units because of its more complex implementation. Plants are fixed in an inert support or raft and their roots float in the nutrient enriched water.
- With the drip irrigation technique, plants are grown in inert media such as rockwool while the aquaponic solution is provided drop by drop at the foot of each plant. This technique has been studied by Schmautz *et al.* (2016b) and has given slightly better results than the NFT.

1.4.2. Coupled vs decoupled aquaponics

Just like urban agriculture, aquaponics has different forms and can be classified based on several criteria such as "main stakeholder, size, operational mode of the aquaculture compartment (RAS, flow through), water cycle management (coupled, de-coupled), type of implemented hydroponic system and the use of space." (Junge *et al.*, 2017, p.3).

Most aquaponic systems are composed of one water loop that connects fish and plants and this unique cycle compels us to find a trade-off between the fish and plants' optimal growth conditions (see more details in 1.4.3). However, Goddek *et al.* (2016) brought forward the concept of decoupled aquaponics in which the aquaculture part and hydroponic part would function in separate loops, thus enabling a more acute adaptation of both loops to the needs of fish and plants. As the plant water is complemented it cannot be returned to the fish and thus needs to be completely "evapotranspired". Therefore, it is necessary to size the aquaculture and hydroponic parts in parallel to have enough surface dedicated to vegetal growth in order to evapotranspire all the water coming from the fish.

With the concept of decoupled aquaponics, it becomes possible to consider classic recirculating aquaculture systems as possible sources of nutrient enriched water for hydroponic systems.

1.4.3. Important parameters of an aquaponic system

- **pH**

pH is one of the most critical parameter of water quality in an aquaponic system. The value of the pH has a direct impact on the capacity of plants to uptake macro- and micronutrients, the comfort of fish and the nitrifying capacity of the biofilter's bacteria. It is therefore necessary to dedicate a particular attention to its monitoring. Several processes and especially respiration and nitrification cause a regular decrease in pH. Indeed, when breathing out, the fish release carbone dioxide into the water which converts into carbonic acid H_2CO_3. Nitrification, which transforms ammonia NH_3 into nitrate NO_3^- releases hydrogen ions and this contributes to lowering the water pH as well (Rakocy *et al.*, 2006; Somerville *et al.*, 2014).

- **Nitrogen forms**

Nitrogen can be found under several forms in an aquaponic system. Fish excrete ammonia (NH_3) which can stay as it is or transform into ammonium (NH_4^+) cation when the water pH is between 2 and 7 (Trejo-Téllez and Gómez-Merino, 2012). Ammonia and ammonium enter the

nitrification process during which they are transformed first into nitrite (NO_2^-) and then into nitrate (NO_3^-) by the bacteria mainly present in the biofilter (Somerville *et al*., 2014). This conversion process is crucial because ammonia and nitrite can become ichtyotoxic if over concentrated (more than 1 mg/L of ammonia and 0.1 mg/L of nitrite) (Somerville *et al*., 2014; Goddek *et al.*, 2015).

Exceeding levels of ammonia can damage the fish' central nervous system and can lead to death (Somerville *et al*., 2014). Moreover, high levels of ammonia inhibit the nitrification process thus enabling even higher accumulation of this toxic form of nitrogen. Ammonia toxicity also depends on pH and temperature. Indeed, as already explained, in acidic conditions, ammonia can catch free H^+ and transform into ammonium which is less toxic. High temperature leads to more ammonia than ammonium (Somerville *et al*., 2014).

- **Temperature**

The optimal temperature depends on the fish and plants species selected for the aquaponic system. However, a consensus amongst researchers exists and favours temperatures between 18°C and 30°C (Somerville *et al*., 2014).

- **Dissolved oxygen**

Dissolved oxygen is needed by all living organisms in the aquaponic system and can thus be considered as the most critical parameter. In non-intensive aquaponic systems, oxygen that dissolves itself at the surface between water and air is enough to satisfy the needs of all the water inhabitants but in intensive systems where the fish density is higher it is necessary to introduce more oxygen into the system through the creation of water movement or via oxygenators (Somerville *et al*., 2014)

- **Electro-conductivity (EC) or salinity**

Electro-conductivity is measured in hydroponics as well as in aquaponics and gives a precise idea of the water concentration in nutrients present in the form of ions. The measure is given in dS/cm (Somerville *et al*., 2014). In most aquaponic systems, the conductivity is situated between 0.3 and 1.1 dS/cm (Wortman, 2015).*

- **Optimal conditions in an aquaponic system**

Table 1. Optimal water parameters for plants, fish and bacteria. Optimal compromise water parameters for aquaponic systems.

Parameter	**Optimal value**				**References**
	Plants	**Fish** (Tilapia)	**Bacteria**	**Compromise**	
pH	6-6,5	7-9	5-9	6,8-7	(Goddek *et al.*, 2015)
				6,5	(Schmautz *et al.*, 2016b)
	5.5-7.5		6-8.5	6-7	(Somerville *et al*., 2014)
	5,8 – 6				(Cervantes, 2012)
		7-8			(Graber and Junge, 2009)
			7-8		(Al-Hafedh *et al*., 2008)
				7,2	(Wortman, 2015)
Temperature (C°)	16-30	Warm water 22-32 Cold water 1018	14-34	18 - 30	(Somerville *et al*., 2014)
DO (mg/l)	4-8			>5	(Somerville *et al*., 2014)
	>3				(Trejo-Téllez and Gómez-Merino, 2012)
		>6			(Graber and Junge, 2009)
			>2		(Masser *et al*., 1992)
EC (dS/cm)				0.003-0.011	(Wortman, 2015)
				0.002 -0.004	(Lennard and Leonard, 2004)
	0.015-0.025				(Trejo-Téllez and Gómez-Merino, 2012)
Ammonia (mg/L)	<30		<3	<1	(Somerville *et al*., 2014)
Ammonium (mg/L)				N-NH4 0	(Schmautz *et al.*, 2016b)
		N-NH4 <1			(Graber and Junge, 2009)
Nitrite (N-NO_2) (mg/L)	<1		<1	<1	(Somerville *et al*., 2014)
		<0.2			(Graber and Junge, 2009)
Nitrate (N-NO_3) (mg/L)				5 – 150	(Somerville *et al*., 2014)
				120	(Schmautz *et al.*, 2016)
		<150			(Graber and Junge, 2009)

1.4.4. Description of the three living groups in an aquaponic system

In an aquaponic system, three main groups of living organisms can be found (Figure 4), namely the plants, the fish and the microorganisms. In this section we will discuss the living conditions of these groups of organisms to understand how a compromise between the three can be found.

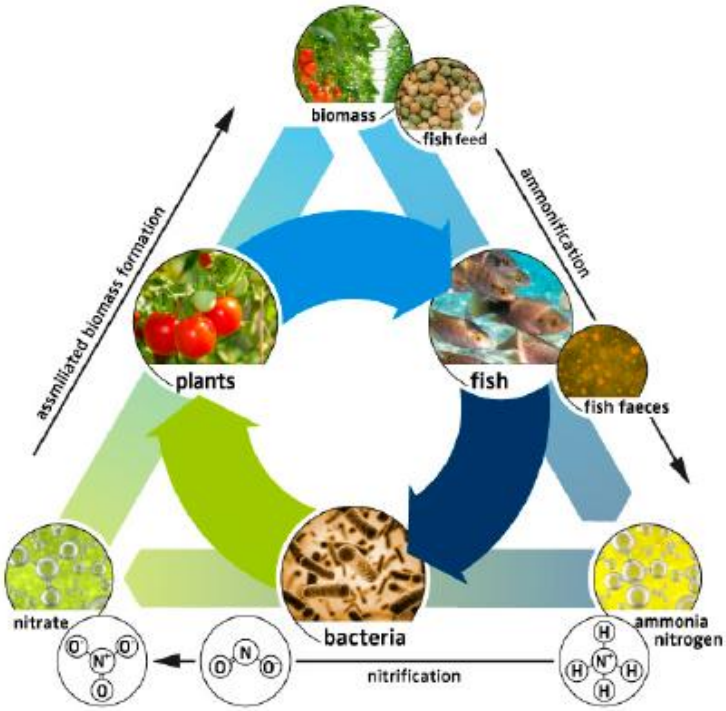

Figure 4. Symbiotic aquaponic cycle (Goddek *et al.*, 2015)

- **Plants**

The species used in aquaponics are mainly the same as those grown in hydroponic systems i. e., mostly leafy greens and fruity vegetables such as tomatoes and peppers. Root vegetables can be grown in aquaponics as well but only with the media bed technique (Somerville *et al.*, 2014). Leafy greens such as lettuce and herbs are considered as nutrients undemanding plants whereas fruity vegetables such as the Solanaceae family are considered as nutrients demanding (Somerville *et al*, 2014).

Most plants require a pH of 5.8 to 6.2 to thrive in hydroponic conditions. Indeed, if the pH reaches levels higher than 7, some micronutrients such as iron, manganese, copper, zinc and boron become less available. Conversely, if the pH drops below 6, the solubility of phosphorus, magnesium, calcium and molybdenum decreases (Rakocy *et al.*, 2006).

According to Jones (2002), it is highly recommended to grow plants which have the same nutritional needs throughout their whole growth because, as the nutrients come mainly from the fish feed, it is complicated to adapt it to the different phenological stages of the plants. The other solution would be to complement the water with mineral nutrients.

- **Fish**

Aquaponics permits to rear freshwater species (Jones, 2002). Among them, the most common species is the Tilapia. because of their short growing cycle (6 to 9 months) and their capacity to survive to rapid changes in water conditions such as a drop in the pH values or in the dissolved oxygen contents. However, Tilapias are not commonly eaten in Europe and the market is still small. Moreover, as Tilapias are a tropical fish, the water in which they live needs to be heated to 28°C which is a supplementary cost (Somerville *et al*., 2014). Other species used in aquaponics are highlighted by (Somerville *et al*., 2014) such as carps, catfish, rainbow trout, jade perch, pike perch or sander and barramundi.

To be adapted for intensive rearing, fish species have to answer to several criteria : they must support crowding of course but also high concentration of potassium due to the complementation of the system (Rakocy *et al*., 2006). All in all, the selected species must tolerate wide ranges of pH, temperature, dissolved oxygen, conductivity and total ammonia nitrogen (TAN). They should also be able to feed on various diets and to grow fast.

According to Jones (2002), it is possible and even recommended to mix several species together in order to secure a better income first but also to ensure resilience of the system against diseases.

- **Microorganisms**

As mentioned before, an aquaponic system could not function properly without its microbiota composed of bacteria and fungi (Somerville *et al*., 2014). The microorganisms have several roles and are the key actors of the mineralisation of solid wastes (uneaten feed, fish faeces, diverse organic matter) into nutrients absorbable by the plants (Rakocy *et al*., 2006) and of the nitrification.

1.5. Focus on bacteria

Bacteria and microorganisms in general are a key component in aquaponic systems because they enable the transformation of rough organic matter into molecules absorbable by the plants (Somerville *et al*., 2014). Microorganisms also clean the water from the accumulation of compounds such as ammonia which, when reaching high concentration levels, can become ichtyotoxic. If the bacteria communities are not healthy and balanced, the whole aquaponic system can crash.

Both autotrophic and heterotrophic bacteria can be found in aquaponic systems (Blancheton *et al.*, 2013), their spatial repartition depending on the oxygen and nutrients availability (Figure 5) (Munguia-Fragozo *et al.*, 2015). According to Rurangwa and Verdegem (2013), the largest reservoir of microorganisms (involved in the nitrification process or not) is the biofilter thanks to the possibility it offers to create biofilms which are bacteria community in which the members are divided up in different layers based on their nutritional needs (Schreier *et al*., 2010). Free suspending microorganisms also exist and finally the walls of the fish tank can also serve as a support for the formation of biofilms. Fungi and algae may also be found on these walls.

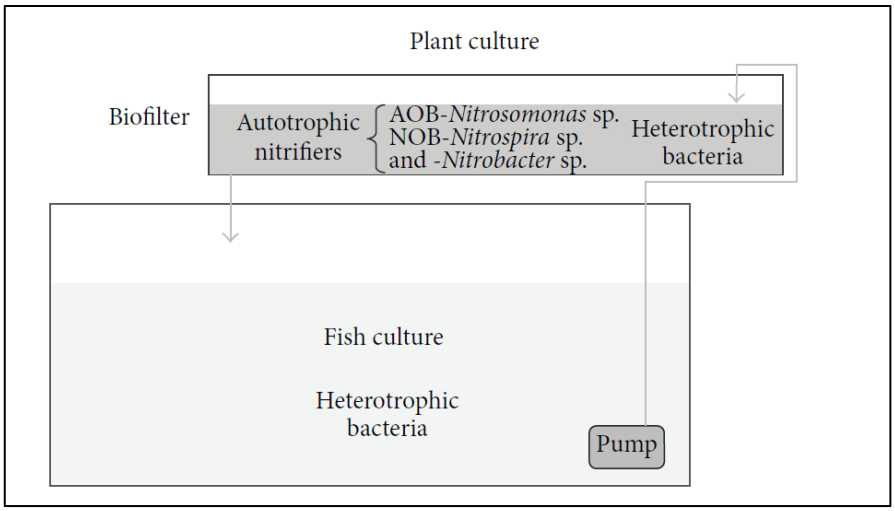

Figure 5. General distribution of microbial populations in aquaculture systems (Munguia-Fragozo *et al.*, 2015)

Additionally, each aquaponic system has its own particular composition in bacteria. Indeed, Schreier *et al*., (2010) observed that "every fish species introduces its own unique microbial flora".

- **What are bacteria?**

Bacteria are "procaryotes, usually unicellular and most of the time with a cell wall containing peptidoglycane […]. Even though some bacteria cause diseases, numerous are those with benefic roles such as biosphere elements recycling, plant and animal material degradation and vitamin production" (Prescott *et al.*, 2010, p. 2).

The classification of bacteria has been under discussion for many years and several more or less similar versions exist. For the study of the bacteria living in aquaponics systems, we will use Bergey's classification used by Prescott *et al*. (2010).

1.5.1. Nitrifying bacteria

Nitrifying bacteria, also called autotrophic bacteria (in the case of aquaponics) are the most known and characterised bacteria in aquaponic systems (Michaud *et al.*, 2006; Schmautz *et al.*, 2016b). Their role is mainly to transform the ammonia excreted by the fish into nitrite and then nitrate which are less toxic to fish (Somerville *et al*., 2014). These bacteria are most commonly found in the biofilter (Munguia-Fragozo *et al.*, 2015) except when the system contains a media bed hydroponic system in which case the inert media of the bed serve as support for the bacteria and thus as biofilter (Somerville *et al*., 2014).

As already discussed, the nitrification is a two steps process. The first one, the conversion of ammonia to nitrite, is carried out by ammonia oxydizing bacteria (AOB) such as *Nitrosococcus* (phylum *Proteobacteria*, class γ-*Proteobacteria*), *Nitrosospira* (phylum *Proteobacteria*, class β-*Proteobacteria*) and *Nitrosomonas* (phylum *Proteobacteria*, class β-*Proteobacteria*). Nitrite is then transformed into nitrate by nitrite oxydizing bacteria (NOB) such as *Nitrobacter* (phylum *Proteobacteria*, class α-*Proteobacteria*), *Nitrospira* (phylum *Nitrospirea*, class *Nitrospira*) (Rurangwa and Verdegem, 2013) *Nitrococcus* (phylum *Proteobacteria*, class γ-*Proteobacteria*) and *Nitrospina* (phylum *Proteobacteria*, class δ-*Proteobacteria*) (Itoi *et al.*, 2007). The genera mostly observed are *Nitrospira*, *Nitrobacter*

and *Nitrosomonas* (Munguia-Fragozo *et al.*, 2015). When it comes to aquaponic systems, Schmautz *et al.* (2016b) realized that the *Nitrospira* genus was mostly found and that it could perform the complete transformation from ammonium to nitrate (Daims *et al.*, 2015).

Archeabacteria such as the *Nitrosopumilus* genus can also be involved in the nitrification process (Rurangwa and Verdegem, 2013).

Nitrification is a process which needs oxygen to convert ammonia NH_3 into nitrate NO_3^- (Somerville *et al*., 2014). It is therefore necessary to ensure maximum available DO to the nitrifying bacteria.

The various genus of nitrifying bacteria have different affinity to oxygen. The richer zones are mostly inhabited by AOB like *Nitrosomonas*, *Nitrobacter* and *Nitrosospira*. The *Nitrospira* genus itself is often present at the limit between "oxic and anoxic" zones (Rurangwa and Verdegem, 2013).

If the C/N ratio is too high, then the heterotrophic bacteria are favoured and tend to stifle the development of nitrifying bacteria (Rurangwa and Verdegem, 2013).

1.5.2. Non-nitrifying bacteria

Few studies have been conducted on non-nitrifying bacteria in aquaponics for now but we can base our reflexion on the phyla already observed in aquaculture (Munguia-Fragozo *et al.*, 2015).

- **Generalities**

Besides nitrifying bacteria, other groups with various roles can be found in aquaculture and aquaponic systems. These groups are often gathered together under the name of heterotrophic bacteria and their main known role is the mineralisation of the organic matter originating from the uneaten fish feed and the fish excrements. These bacteria can be found heterogeneously distributed throughout the system, in suspension in the water (Rurangwa and Verdegem, 2013) but also in the biofilter (Sugita *et al*., 2005). Indeed, according to Rurangwa and Verdegem (2013), microniches can be found in RAS (and thus in the fish part of aquaponic systems) in which specific communities of bacteria thrive and the majority of these bacteria are represented by heterotrophic groups.

Most heterotrophic bacteria multiply faster than bacteria involved in nitrification. They are also situated where "oxygen and substrate concentrations are highest" (Rurangwa and Verdegem, 2013, p.120). The nitrifying bacteria are in deeper layers of biofilms which can become problematic if not enough oxygen is supplied in order to reach them (Rurangwa and Verdegem, 2013; Somerville *et al*., 2014).

Furthermore, the number of heterotrophic bacteria can be correlated to the quantity of organic matter present in the system (Rurangwa and Verdegem, 2013). Indeed, the composition of the bacteria communities can vary based on the nutrient ratio and nutrient availability. A high C/N ratio can also favour the development of heterotrophic bacteria to the detriment of the nitrifying ones.

- **Diversity of bacteria**

Schmautz *et al.* (2016b) carried out a sampling of bacteria in several compartments of an aquaponic system and characterised the bacteria thus found with metagenomic techniques (Figure 6). In most compartments, the phylum of *Proteobacteria* was predominant. The other major phyla were *Fusobacteria*, *Bacteriodetes* and *Firmicutes*. At the family level, *Xanthomonadales* and *Pseudomonadales* were highly present.

Sugita *et al*. (2005) conducted a study in two RAS containing carps and goldfish. The major bacteria groups found there were: the *Proteobacteria* phylum with the α, β and γ *Proteobacteria* classes, the *Nitrospira* phylum, the *Actinobacteria* phylum, the *Firmicutes* phylum with the *Bacilli* class, the *Planctomycetes* phylum with the *Planctomycetacia* class and the *Bacteroidetes* phylum with the *Sphingobacteria* class. The same phyla are highlighted by Munguia-Fragozo *et al.* (2015) in their study on freshwater aquaculture systems/

Another type of bacteria is the ANAMOX ones, which means ammonia oxydizing in anaerobic conditions (Rurangwa and Verdegem, 2013). These transform ammonia, ammonium and nitrite into N_2 gas.

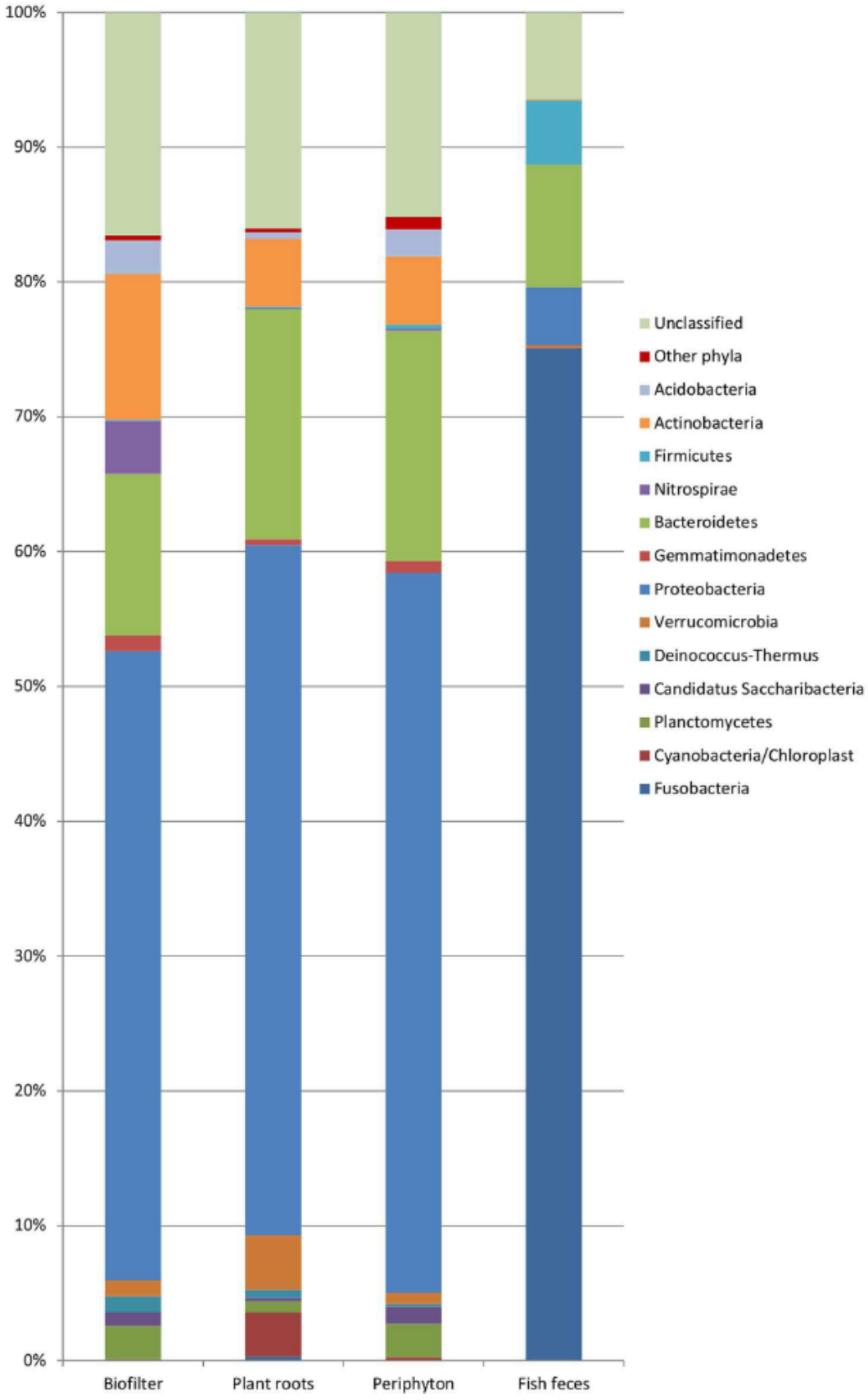

Figure 6. Classification of reads from biofilter, plant roots, periphyton and fish faeces to level phylum indicated as percentage of the population (Schmautz *et al.*, 2016b)

- **Mineralisation process**

Mineralisation is the breaking down of complex polymers such as proteins, carbohydrates and lipids into smaller molecules like amino acids, sugars, fatty acids or alcohol first and then into minerals in order to close the elemental cycles (van Lier *et al*., 2008). In the case of aquaponics, mineralisation is carried out by heterotrophic bacteria which transform the polymers contained in fish feed leftovers and fish dejections into molecules that the plants can easily absorb (Somerville *et al*., 2014). Indeed, as only 30 to 40% of the feed given is eaten and retained by the fish, 60 to 70% is excreted as waste and is available for the bacteria to decompose (Somerville *et al.*, 2014).

- **Conclusion**

Heterotrophic bacteria in aquaponic systems are highly diversified and hence could have various roles involved in the growth of aquaponic plants. These roles could be similar to those of Plant Growth Promoting Rhizobacteria (PGPR) such as "biological N_2 fixation, phytopathogen biocontrol, phosphate solubilisation, production of phytohormones and enzymes" (Lagos *et al.*, 2015, p.505). Heterotrophic bacteria can also serve as a protector against pathogens as they occupy niches leaving no room for harmful species (Blancheton *et al.*, 2013).

1.5.3. Pathogenic bacteria and off-odour

Of course, not all the bacteria present in aquaponic systems are beneficial and some can even be pathogenic. Munguia-Fragozo *et al.* (2015, p.6) found fish pathogens such as certain strains of "*Bacillus sp. (B. Mycoides*), *Aeromonas sp*., *Acinetobacter sp*., *Pseudomonas sp*., *Edwardsiella sp*., *Comamonas sp*. and *Flavobacterium sp*.". They also found bacteria which can be toxic for humans as well as for fish such as "*Vibrio*, *Erwinia*, *Coxiella* and *Aeromonas*".

Escherichia coli and *Salmonella spp*. are used as food safety indicators (Munguia-Fragozo *et al.*, 2015) because of their usual presence in case of faecal contamination. Based on these food safety indicators, it seems that there is less coliforms in aquaponic lettuces than in conventional ones (Munguia-Fragozo *et al.*, 2015).

According to Rurangwa and Verdegem (2013), the presence of pathogenic bacteria could be linked to the C/N ratio of the aquaponic (or aquaculture) system. Indeed, it seems that a high C/N ratio could help the *Vibrio* to develop (Michaud *et al.*, 2006). Other parameters affect the composition of the heterotrophic communities such as the nutrient type (i.e. composition of the fish feed) and the hydraulic retention time (HTR) (Schneider *et al.*, 2007).

Some bacteria, although not pathogenic, can still be problematic as they are responsible for off-flavours in fish. Indeed, the *Streptomyces* bacteria produce a molecule called geosmin which alters the taste of the fish produced in RAS and in aquaponics (Schmautz *et al.*, 2016b). Another problematic compound is the 2-isomethylisoborneol produced by *cyanobacteria* (Tucker, 2000).

1.5.4. Colonisation strategies

Based on their colonisation strategies or demographic strategies, living organisms can be separated into two distinct groups: the "r-strategist" (r being the symbol representing the slope of a population growth's curve) and the "K-strategist" (K representing the carrying capacity of the environment). Logically enough, "r-strategists" produce a lot of offspring in a short period of time whereas "K-strategists" are slower in reproducing but tend to ensure a better survivable probability to their progeny (Paugy *et al.*, 2006).

In a context where bacteria and especially heterotrophs and autotrophs compete for space but mostly for nutrients and oxygen, these demographic strategies become all the more significant. Indeed, in biofilters the two types of bacteria compete for oxygen in the superposed layers of biofilm and K-strategist such as *Nitrospira* or *Nitrosospira* (Van Kessel *et al.*, 2010) could be advantaged (Rurangwa and Verdegem, 2013). Even though heterotrophs and autotrophs are said to be in competition, beneficial relations can also take place and outer layers of heterotrophs could protect the nitrifying bacteria from pathogens or grazing (Blancheton *et al.*, 2013) provided they still let enough oxygen and nutrients pass to the inner layers.

1.6. Diversity indexes

Several diversity indexes exist and it is not always easy to decide which ones are the most pertinent to study a microbial community (Hill *et al.*, 2002). Some of the most commonly used tools are the α- and β- diversities and the Shannon index.

- **α and β diversities**

α-diversity represents the local diversity. It takes into account the "diversity in a uniform habitat of a fixed size" (Marcon, 2016, p.7).

β-diversity compares the diversities of samples taken on a same site but in different locations (Marcon, 2016). There are still some debates on the definition of this measure but most of the time is obtained through the ratio (number of taxa missing in the second sample/total number of taxa in the first sample) (Whittaker, 1972).

- **Shannon index:**

The Shannon index enables the measure of the species diversity. Species diversity takes into account the species richness which is the number of species in a given sample (Marcon, 2016) and the relative abundance which represents the "regularity of distribution of the species" in a given sample (Marcon, 2016, p.6).

The Shannon index is "the negative sum of each Operational Taxonomic Unit's [OTU] proportional abundance multiplied by the log of its proportional abundance" (Hill *et al.*, 2002, p.1). It measures "the amount of information (entropy) in the system and hence is a measure of the difficulty in predicting the identity of the next individual sampled" (Hill *et al.*, 2002, p.1). The Shannon index gives an idea of the species richness and evenness thanks to their positive correlation. It is however necessary to underline the fact that it "gives more weight per individual to rare than common species" (Hill *et al.*, 2002, p.1).

1.7. Next Generation Sequencing techniques

1.7.1. What are Next Generation Sequencing techniques? Advantages and drawbacks

For a long time, the study of bacteria was conducted through their cultivation in Petri dishes on man-made media. However, it is now widely acknowledged that this culture technique is highly limiting and that very few bacteria will actually grow in laboratories (Rodríguez-Valera, 2004; Cruaud *et al.*, 2014; Lagos *et al.*, 2015). The alternative to bacteria plating is to sequence certain distinctive regions of their genetic material in order to classify them (Lagos *et al.*, 2015).

The sequencing techniques have evolved over the years and what is nowadays called "next or second-generation sequencing" (NGS) (Heather and Chain, 2016) has brought a massive advance with the process of parallel sequencing which allows to go much faster in the sequencing process than before (Heather and Chain, 2016). Indeed, the core principle remains the same, i.e. "DNA polymerase catalyses incorporation of fluorescently labelled deoxyribonucleotide triphosphates into a 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 difference between the first and second generations of sequencing techniques is then that NGS simultaneously sequences millions of fragments instead of just one (Illumina, 2016).

Thanks to NGS technologies, it is now possible to analyse the collective genome of whole bacteria communities, called metagenome, without any *a priori* (Adams *et al.*, 2009). This advance in sequencing technologies will thus allow the characterisation of entire communities at once.

Moreover, after using these techniques for several years, databases for 16S rDNA have become more and more detailed and available (Petrosino *et al.*, 2009). DNA sequencing is also more precise, less time-consuming than culturing (Salipante *et al.*, 2013).

1.7.2. How does it work?

The sequencing market is nowadays almost totally controlled by the Illumina technology (Heather and Chain, 2016).

The Illumina technology follows four major steps (Illumina, 2016) (Figure 7):

1) Library preparation: DNA is fragmented and adaptors are ligated to the 5' and 3' ends of each fragment. The adapted fragments are then amplified by PCR (Goodwin *et al.*, 2016).

2) Cluster generation: the fragments are then loaded onto a flow-cell covered with oligonucleotides which are complementary to the adaptors fixed on the fragments. Once the fragments are blocked on the flow-cell, they are amplified through bridge amplification thus creating "clone clusters".

3) Sequencing: Illumina uses the "sequencing-by-synthesis" (SBS) method. A mix containing labelled reversible terminators, primers and DNA polymerase enzyme passes on the flow cell and based on their affinity, the correct base fixes itself in front of its corresponding base on the template DNA strand. The fixation on a base is detected and registered thanks to the emission of fluorescence.

4) Data analysis: the reads are quality proofed to check for sequencing errors etc. They are then assembled together to form longer fragments called "contigs". The contigs are then mapped to a reference sequence with a BLAST (Petrosino *et al.*, 2009), thanks to bioinformatic softwares such as QIIME (Quantitative Insight into Microbial Ecology).

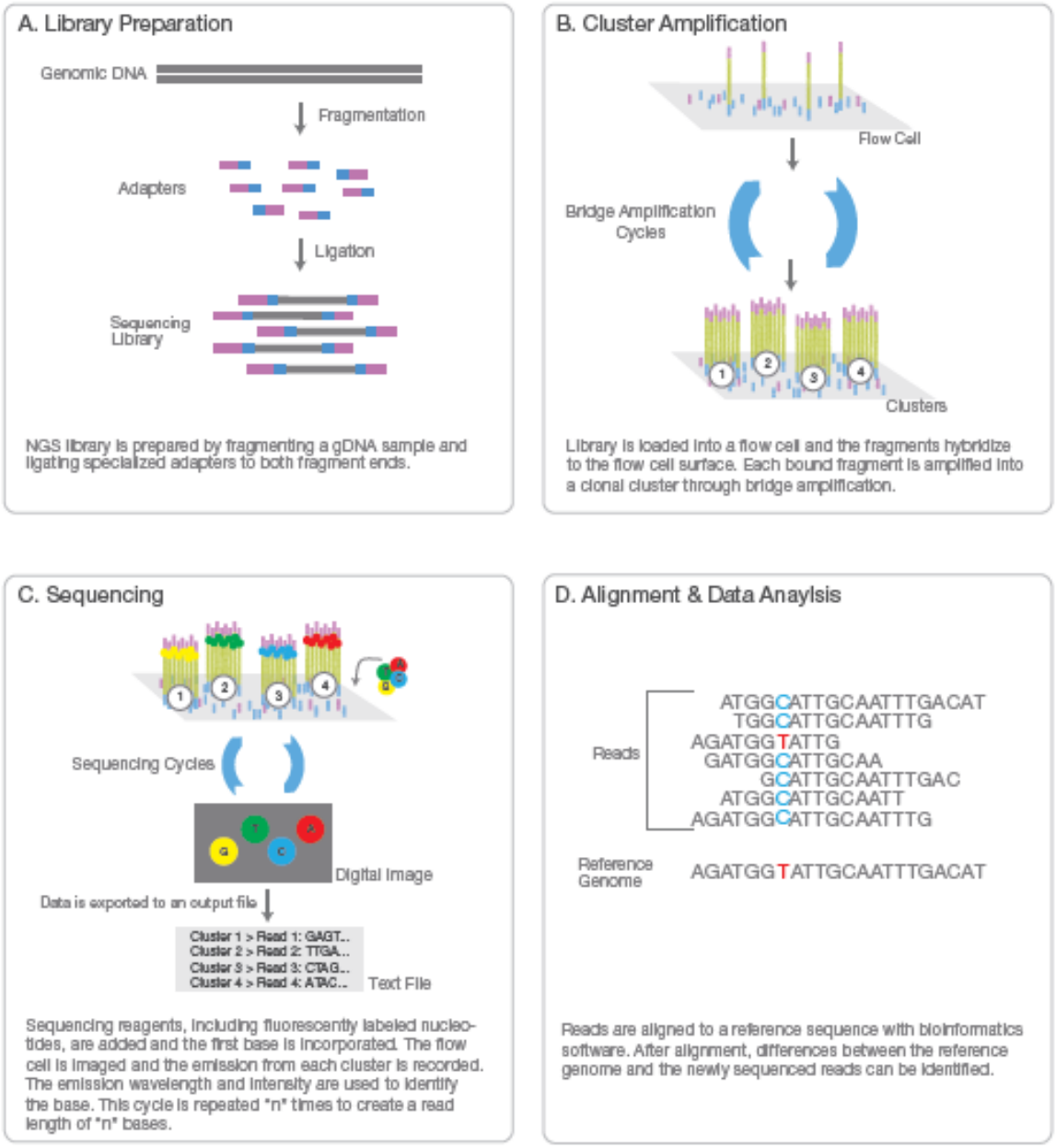

Figure 7. Schematic representation of the Illumina process (Illumina, 2016)

1.7.3. Focus on 16S rDNA/rRNA and the nine hypervariable regions

In order to use NGS we have seen the necessity of selecting primers to amplify specific fragments of the targeted DNA. The ribosomal 16S chromosome is very often chosen for amplification as it is stable in time and as it contains nine conserved and nine hypervariable regions (Figure 8) (Nikolaki and Tsiamis, 2013; Cruaud *et al.*, 2014; Yang *et al*., 2016a). This alternation enables to detect previously unknown organisms as the primers will bind to the conserved regions shared by most of the microorganisms and thus permit the sequencing of yet unobserved hypervariable regions typical of this unknown species (Cruaud *et al.*, 2014).

However, as there are only nine variable regions of various degrees of variability, it is not possible to discriminate all species based on only one region (Nikolaki and Tsiamis, 2013; Cruaud *et al.*, 2014). The choice of primers and targeted hypervariable regions can thus dramatically influence the results of a NGS metagenomic analysis (Cruaud *et al.*, 2014; Yang *et al*., 2016a).

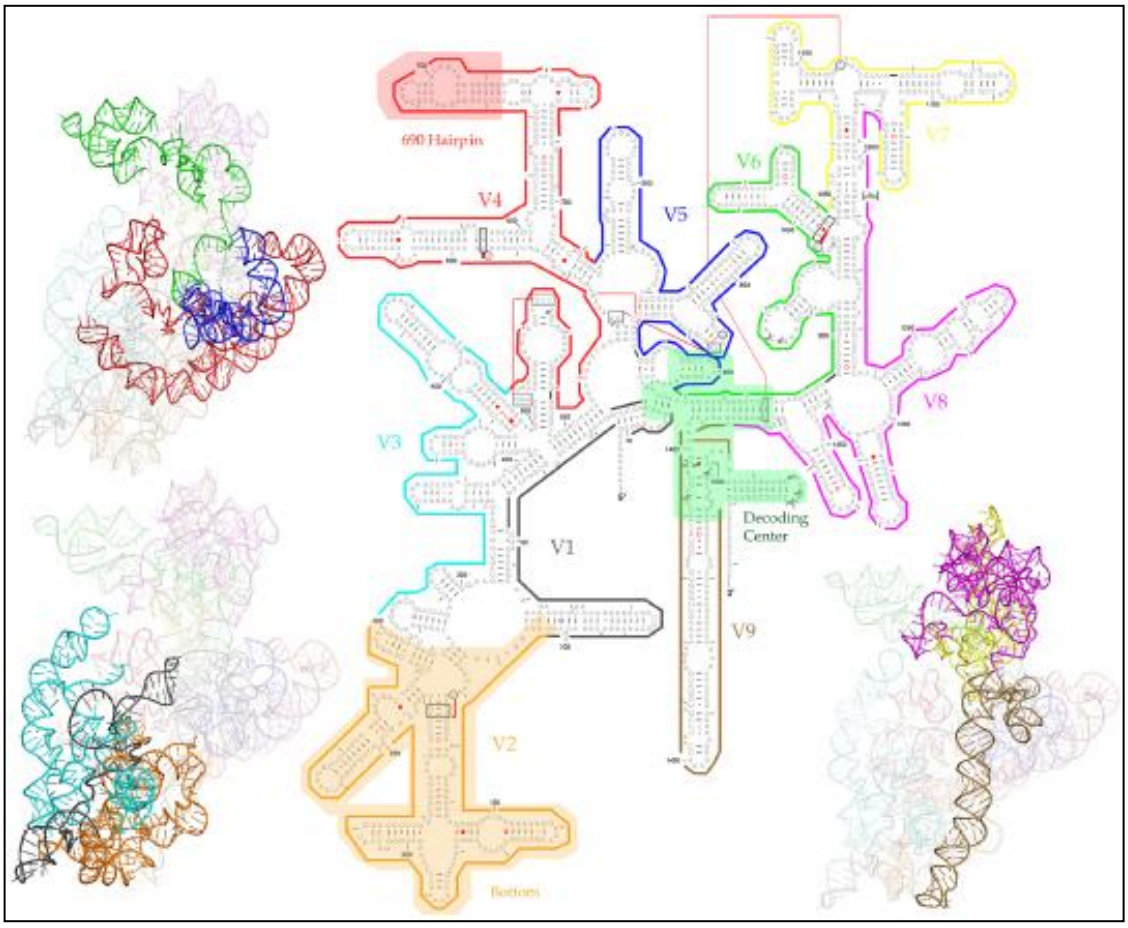

Figure 8. 2D and 3D representations of the 16S rRNA gene. Each region is delimited by a specific colour which are found on both the 2D and 3D strucutres (Yang *et al*., 2016a)

2. PROBLEMATIC AND OBJECTIVES

The bacteria communities in aquaponic systems are of outstanding importance. Indeed, they carry the transformation of the ammonia into nitrate which is less toxic for the fish. They are also responsible for the mineralisation of the organic particles (fish feed leftovers and fish faeces mainly) into molecules and ions absorbable by the plants. They eventually can also have a plant growth promoting effect.

The aim of this study is to analyse the bacteria communities present in various aquaponic systems using NGS technology and the QIIME bioinformatics software in order to taxonomically characterise the communities, compare them and to draw tendencies which could link the characteristics of the communities with the particulars of the diverse aquaponic systems.

In this view, a global study will be conducted to identify the taxa mostly present in diversified aquaponic systems. On the one hand, the predominant taxa observed in various diverse samples will be analysed. On the other hand, the core microbiota, i.e. the bacteria community always associated with a given host or ecosystem (Lemanceau *et al.*, 2017) will be studied.

In order to further understand how the elements characterising an aquaponic system (e.g. system's layout, fish feed type, fish species, fish density, compartment) can influence the composition of the bacteria community, comparisons between contrasted groups of samples will be conducted and hypothesis will be drawn.

3. MATERIAL AND METHOD

3.1. Analysed aquaponic systems and their characteristics

This work was conducted in partnership with six aquaponic/aquaculture systems and on the recirculating aquaculture system (RAS) and Plant and Fish Farming Box (PAFF Box) of the Integrated and Urban Plant Pathology Laboratory (IUPPL) of Gembloux Agro-Bio Tech.

- **UrbanFarmers, The Hague (23/03/2017)**

The first system visited was the UrbanFarmers farm in The Hague. This rooftop greenhouse launched in 2016 is located on the top of a six-storey building and has been designed to resist strong winds and harsh weather conditions. The Tilapias (Nile Tilapias, Red Naturally Male Tilapias, supplied by the Til-Aqua company) are located on the last floor, grown in 20 water tanks and fed with an omnivorous diet. The system can be called "coupled". Indeed, the water flows out of the fish tanks and is directed towards a drum filter which rids the water of most of its solid waste that is collected in a sedimentation basin. The filtered water then goes to four biofilters placed in series which use the moving bed principle. In those biofilters, billions of plastic biochips (Kaldnes media, supplied with the aquaculture system by Fleuren and Noijeen) (Figure 9) serve as support for the formation of bacteria biofilms. The ammonia-purified water is collected into a sump before being directed either back to the fish tanks or to two tanks in which the water will be complemented for different plant types. Indeed, the cleaned fish water is collected into two tanks, one dedicated to leafy green vegetables (lettuces, herbs and microgreens) and the second dedicated to fruity vegetables (tomatoes, peppers, eggplants and cucumbers). Each tank is complemented to reach the exact nutrient concentration optimal for each type of vegetable. After passing through the NFT systems, the water is drained back into the aquaculture system. A volume of approximately 100-150 L/kg of feed/day is added.

Figure 9. Kaldnes media (www.teichhandel-24.de)

Three samples were taken from this system:

- Two litres of water were taken from the sump
- 24 biochips were collected. Six biochips were taken in each biofilter. We dealt with a maximum of the heterogeneity by taking biochips from different places in the filter and at different depths.
- Biofilm present on the walls of the fish tanks was scraped with a sterile scalpel in order to try to find an explanation to the muddy taste of certain fish

The samples taken at 10 a.m. were then transported back to Gembloux Agro-Bio Tech in order to proceed to the water sample preparation at 3 p.m. and DNA extraction at 5 p.m. (see 3.2 and 3.3).

- **Provinciaal Proefcentrum voor de Groenteteelt Oost-Vlaanderen, PCG, Kruishoutem (29/03/2017)**

The second visit took place in the Provincial trial center for vegetable production (PCG) in Kruishoutem, Belgium. PCG rears Jade Perch (*Scortum barcoo,* supplied by Aqua4C) in nine round fish tanks of 1.8 m^3 each. The fish are fed with a vegetarian diet developed by the Aqua4C company (3.2mm Omegabaars Grower, AQUA4C, Kruishoutem, Belgium). In a decoupled aquaponic system, the water from each tank goes through the tank's own small drum filter and small moving-bed biofilter. The biofilters contain Eco Pondchip Filtermedium as biomedia (Figure 10). Each tank therefore has its own filtering system. The hydroponic system is composed of nine rows with tomatoes grown in a rockwool slab and irrigated through a dripping system. Each row of tomatoes is connected to one specific fish tank. The ferti-irrigation water is composed of drain water from the hydroponic system, aquaculture water from the corresponding tank, nutrient complementation and sometimes rain water when needed.

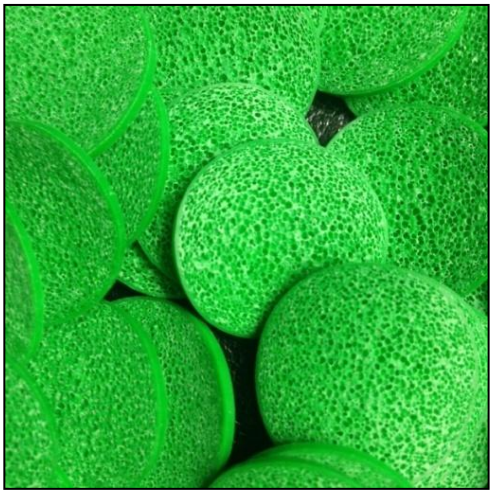

Figure 10. PCG biomedia (www.eco-pondchip.de)

The particularity of this system is that half of the fish tanks contain a density of 60 fish per tank and the other half a density of 100 fish per tank.

Four samples were taken from this system:
- Two litres of water from the sump of a "low fish density" tank
- Biochips from the biofilter of a "low fish density" tank
- Two litres of water from the sump of a "high fish density" tank
- Biochips from the biofilter of a "high fish density" tank

The samples taken at 11 a.m. were then transported back to Gembloux Agro-Bio Tech in order to proceed to the water sample preparation at 4 p.m. and DNA extraction at 7 p.m. (see 3.2 and 3.3).

- **Belgian Quality Fish (BQF), Dottignies (29/03/2017)**

Belgian Quality Fish is an aquaculture company located in Dottignies, Belgium, which rears several species of sturgeons such as Siberian sturgeons (*Acipenser baerii*), Russian sturgeons (*Acipenser gueldenstaedtii*), European sturgeons (*Huso huso*), Sterlets (*Acipenser ruthenus*) and various hybrids. These are all fed on an omnivorous diet. The system is a recirculating one (RAS) composed of four identical systems working in parallel. In each system, the water from the sturgeons' tanks is collected in a canal which goes through

disinfection via ozone and UV light. The water is then directed towards a drum filter and a moving bed biofilter. After this, the water also flows through a denitrification filter. Indeed, as the BQF system is not turned towards aquaponics, they eliminate the nitrate thanks to denitrifying bacteria to be able to reuse their water as much as possible and to discharge less polluted water into the environment. After having been thoroughly cleaned, the water goes back to the fish tanks. A volume of approximately 265 L/kg of feed/day is added.

Three samples were taken from this system:

- Two litres of water for the canal which brought the cleaned water back to the fish tanks. This water had gone through a nitrification process, a denitrification process and ozone and UV disinfection.
- Biochips from the moving bed biofilter where the nitrification should take place
- Biochips from the denitrification filter where the denitrification should take place

The samples taken at 1 p.m. were then transported back to Gembloux Agro-Bio Tech in order to proceed to the water sample preparation at 4 p.m. and DNA extraction at 7 p.m. (see 3.2 and 3.3).

- **Leibnitz-Institute of freshwater ecology and inland fisheries (IGB), Berlin (07/04/2017)**

The IGB is a research centre located in Berlin where the *tomatofish* project is being developed. In this aquaponic project, Tilapias (*Oreochromis niloticus*) are fed on an omnivorous diet based on plants and pellets of fly maggots. The rearing tanks are located in a greenhouse where hydroponic tomatoes are also being grown with NFT (Figure 11). The simultaneous presence of fish and tomatoes in the same greenhouse enables the plants to take advantage of the CO_2 exhaled by the fish. The water coming out of the fish tanks is directed through a drum filter and then through a moving bed biofilter. It is then conveyed either back to the fish or to the tomatoes which evolve in two separate loops. Indeed, when needed, aquaculture water having gone through the mechanical and biological filters can be directed to the tomatoes after being slightly complemented with the required nutrients. The water does not flow back directly from the plants to the fish. Only the water evapotranspired by the tomatoes is collected in “cold traps”, condensed and brought back into the aquaculture system (Figure 12) (IGB, 2014).

Two samples were taken from this system:

- Two litres of the water going back to the fish which was the same as the one going to the tomatoes but more accessible.
- Biochips from the biofilter

The samples taken at 10.30 a.m. were analysed in the IGB laboratory. The water samples preparation took place at 11 a.m. and DNA extraction at 1 p.m. (see 3.2 and 3.3 DNA).

Figure 11. Inside the Tomato fish greenhouse (IGB, 2014)

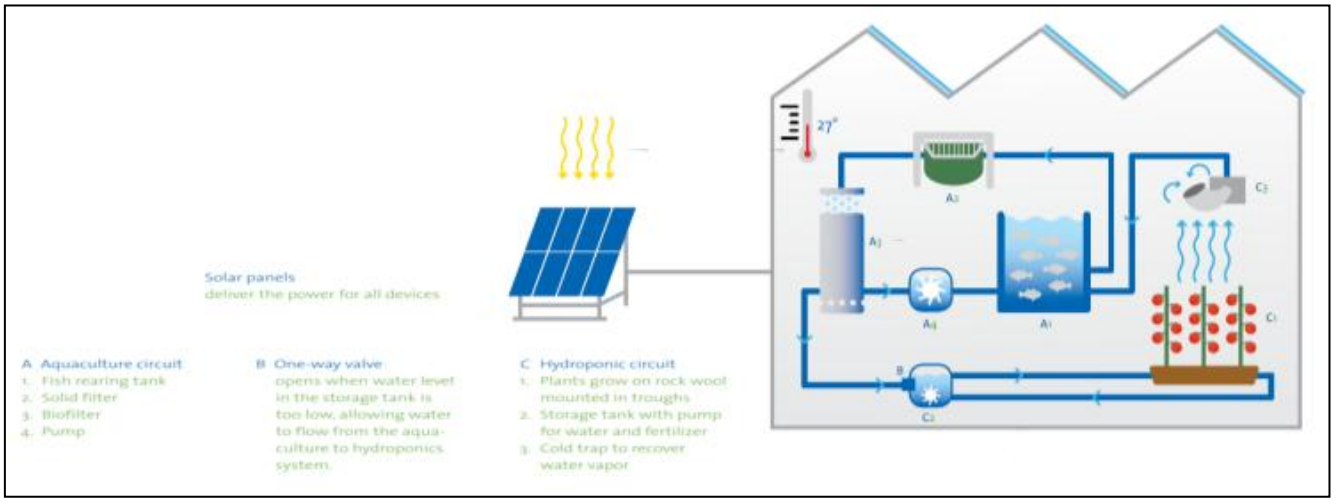

Figure 12. Technology of the Tomato fish system (IGB, 2014)

- **University of Wageningen (12/04/2017)**

Wageningen University possesses several aquaculture systems from which they can collect water to irrigate hydroponic crops situated in Rotterdam. Two systems were visited, one containing catfish and the other one eels. The catfish system was composed of a fish tank, a mechanical filter and a fixed, trickling biofilter. The water was then conducted back to the catfish. In the eel system, the water from the fish tank was directed to a mechanical filter, to a moving bed biofilter and then back to the eels.

Three samples were taken from those systems:

- Two litres of water from the catfish system. The water collected was the one flowing back to the fish after having passed through a mechanical filter and a biofilter. No biofilter sample could be collected this time because the system used a trickling biofilter thus rendering impossible the collect of biomedia.

- Two litres of water from the eel system. The water collected was the one going back to the fish after having passed through a mechanical filter and a biofilter.
- Biochips from the moving-bed biofilter of the eel system.

The samples taken at 11 a.m. were then transported back to Gembloux Agro-Bio Tech in order to proceed to the water sample preparation at 3 p.m. and DNA extraction at 7 p.m. (the filters got clogged very quickly which slowed down the whole sample preparation process) (see 3.2 and 3.3).

- **Inagro, research center, Rumbeke-Beitem, Belgium (18/04/2017)**

The Inagro research center is located in Rumbeke-Beitem, Belgium and rears Pike perch (*Sander lucioperca)* which are fed on an omnivorous diet. The system is a RAS which can also be used in a decoupled aquaponic system since 2015. The system is indeed composed of a classical RAS with fish tanks, a drum filter and a moving bed biofilter. For the sake of the aquaponic experimentation, the RAS water used for the cleaning of the drum filter can be deviated from this loop and directed towards a sedimenter in order to take out most of the solid wastes before being stored in a tank outside the greenhouses. The water is then complemented with the required nutrients before being sent to the hydroponic parts. There, tomatoes are being grown in rockwool slabs with a drip irrigation system similar to the ones used by PCG and IGB. 12 to 16% of the total volume of water is added into the system each day.

Three samples were taken in this system:
- Two litres of water having flowed through the mechanical filter and biofilter and heading back to the fish
- Two litres of water collected from the fish system and having gone through the sedimenter.
- Biochips from the biofilter

The samples taken at 11 a.m. were then transported back to Gembloux Agro-Bio Tech in order to proceed to the water sample preparation at 2 p.m. and DNA extraction at 4 p.m. (see 3.2 and 3.3 DNA).

- **Integrated and Urban Plant Pathology Laboratory, Gembloux, RAS**

The IUPPL has two systems in which Tilapias (*Oreochromis Niloticus* from the CEFRA) are reared and fed on a vegetarian diet supplied by the aquaculture company Aqua4C (3.2mm Omegabaars Grower, AQUA4C, Kruishoutem, Belgium).

The first one is a RAS from which the aquaculture water is used to regularly clean the drum filter. The cleaning water is then collected in a sedimenter where the supernatant is sent back to the system and the sludge is removed. Water is occasionally collected from this system for decoupled aquaponics experiments on plants growing in nearby AeroFlo.

The second one is called the PAFF box (Plant and Fish Farming box) and is a coupled system in which the fish water is pumped to the raft hydroponics system above after having flown through a mechanical filter and a floating bead biofilter. The water is slightly

complemented in iron to ensure full vegetables growth. It then flows back to the fish tanks below.

Several samplings have been conducted on these two systems.

Recirculating aquaculture system:

On the 03/04/2017 two samples were taken:
- Two litres from the sump (after drum filter and biofilter)
- Pieces of the ceramic biofilter media

PAFF box:

On the 27/04/2017, a test for repeatability was carried out in the PAFF box. Eight samples were taken at the same moment:
- Four repetitions of two litres of water arriving in the hydroponic beds (i.e. same water as the one arriving in the sump)
- Four repetitions of bead samples from the pressurised biofilter.

- **Comparison table of water parameters at sampling time**

Table 2. Water quality parameters. Blue: goal values, black: data from the week the sampling was done. N.D.: no data. S. sump, Bio. : biofilter, D. bio : denitrification biofilter

System	UF	PCG 60 fish/tank	PCG 100 fish/tank	BQF	IGB	Wageningen	Inagro	RAS	PAFF box
pH	6.7	7.2	6.8	7.92	N.D.	N.D.	8.56	7.8	7
T° (°C)	27.7	28	28	18.2	N.D.	N.D.	23.5	27	25
DO (ppm)	5.3	>4.5	>4.5	6.8	N.D.	N.D.	>7	N.D.	N.D.
EC (dS/cm)	0.015	0.006	0.005	N.D.	N.D.	N.D.	0.024	N.D.	0.012
N (ppm)	$N\text{-}NH_4$: 0.08	$N\text{-}NH_4$: 0.09	$N\text{-}NH_4$: 0.09	TAN: 0.21	N.D.	N.D.	N.D.	$N\text{-}NH_3$: <0.2	TAN <2
$N\text{-}NO_2^-$ (ppm)	0.37	0.03	0.03	0.23	N.D.	N.D.	N.D.	N.D.	< 1
$N\text{-}NO_3^-$ (ppm)	22.6	28	36.5	10-15	N.D.	N.D.	N.D.	N.D.	30-120
Sampling zone	S. Bio Biofilm	S. Bio	S. Bio	S. Bio D.bio	S. Bio	S. catfish S. eel Bio eel	S. fish loop S. plant loop Bio	S. Bio.	S. Bio.

3.2. Samples preparation

- **Water samples**

For each sample, two litres of water were collected in sterile pyrex bottles. In order to concentrate the bacteria 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. The filters were then placed in a 50 ml sterile Falcon containing 30 ml of sterile water. After vortexing the Falcon for 4 minutes (enough time for the filters to look clean), the filters where removed and the Falcon centrifuged at 7607 G for ten minutes (Yildiz *et al.*, 2017). The centrifuging mass was then used for extraction with the FastDNA Spin Kit, MP Biomedicals.

- **Biofilter samples**

The biochips used in the different systems varied in shape and size. Therefore, a constant number of biochips per sample could not be kept as it would not always fit in a 50 ml Falcon. Instead, a maximum quantity of biochips was inserted in a 50 ml sterile Falcon containing 30 ml of sterile water in order to ensure the harvest of a maximum quantity of bacteria. The treatment of the biochips was inspired by Schmautz *et al.* (2016b). The 50 ml Falcon were hence passed in the vortex for 2 minutes before spending 5 minutes in an ultrasonic bath (VWR ultrasane cleaner) to scrap the bacteria of the biomedia. The Falcons were then centrifuged at 7607 G for ten minutes and the pellet was collected for extraction with the FastDNA Spin Kit, MP Biomedicals.

3.3. DNA extraction and PCR check

DNA extraction is a crucial step in metagenomic analysis. Indeed, it is important to obtain DNA of satisfactory quality in order to be able to sequence it (Cruaud *et al.*, 2014).

The DNA was extracted following the protocol of the Fast DNA Spin Kit, MP Biomedicals:

- The collected pellet was inserted into a tube with a mechanical lysing matrix (grains of silica and one ceramic bead) and a lysing solution to combine physical and chemical destruction of the bacteria cells.
- After homogenisation and centrifugation, the supernatant was collected and mixed with a solution which binds itself to the DNA.
- The mix was then centrifuged several times in a SPIN filter to isolate the solid matrix containing the DNA from the rest of the solution.
- The solid pellet thus obtained was suspended in a washing solution and centrifuged again to obtain a purified solid pellet containing the DNA.
- The DNA was then eluted in 100 µl of a last solution in order to obtain 100 µl of DNA solution.
- The DNA solution was then tested for DNA concentration with the Nanodrop (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) before being stored at – 20°C.

With the Nanodrop, two specific ratios of absorbance were checked, namely 260/280nm and 260/230nm. The reference values for these ratios are respectively 1.8 and 2.0 (De Clerck *et al.*, 2014). The 260/280 ratio enables to detect “proteins, phenol or other contaminants that

absorb strongly at 280 nm". The 260/230 ratio can indicate the presence of carbohydrates or guanidine HCL which is often used for DNA isolation (Wilfinger *et al*., 1997, p.1).

To ensure that nothing in the DNA solution would inhibit the first step of the sequencing process, i.e. the DNA amplification, a PCR was carried out. Indeed, the presence of enzymatic inhibitors such as humic substances is very likely, knowing that the samples were taken in an environment containing decomposing organic matter. Those inhibitors could impede the amplification and thus the sequencing (Cruaud *et al.*, 2014).

The PCR was conducted following the laboratory protocol developed by Mr. Sare, using the Bioline, Mango Taq DNA Polymerase kit and the forward primer 16S A1 (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 16S B1 (5'-TACGGYTACCTTGTTACGACTT-3') (Fukatsu *et al*., 2000). PCR was performed in 50 µl reactions containing 10 µl of colored reaction buffer (Bioline), 1,5 µl of $MgCl_2$ (50 mM) (Bioline), 1 µl of dNTP mix (10 mM) (Eurogentec), 1 µl of each primer (25 mM each) (Eurogentec), 1 µl of Mango Taq polymerase (Bioline, 1000U/µl) and 32,5 µl of water. In the thermocycler, the lid was first heated at 110°C. The temperature cycle was composed of a denaturation step at 94°C for 2 min followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 70°C (Fukatsu *et al*., 2000). An extension step of 10 min at 72°C finished the cycle and then the samples were kept at 4°C for as long as needed.

3.4. Sequencing

The sequencing step was carried out by DNAVision, a company located in Charleroi, Belgium. 28 samples (7.1. Annex 1) collected in the previously presented systems were sent on May 10th. Sequencing data was received on June 13th.

The sequencing was focused on the hypervariable regions V1-V3 of the 16S ribosomal DNA as recommended by Munguia-Fragozo *et al.* (2015) and as already used in their study of the bacterial communities in aquaponics by Schmautz *et al.* (2016b). Three regions were selected as, according to Nikolaki and Tsiamis (2013), it is necessary to use at least two regions in order to obtain a proper characterisation of diversified communities.

- **DNAVision's protocol**

DNAVision uses the MiSeq Illumina technology. A summarised version of their protocol is presented hereafter and the details of the procedure are available in 7.2. Annex 2.

1) Before sequencing the DNA, the first step consists in checking the DNA quality with a Nanodrop and to quantify it with the Picrogreen DNA quantification kit. With the Nanodrop, the 260/280 ratio is controlled and needs to stand between 1.7 and 2.1. The minimum concentration in a sample is $5 ng.\mu l^{-1}$.

2) The amplicons are then prepared for sequencing. Firstly, the DNA is normalised at $5 ng.\mu l^{-1}$ and then it is amplified through a PCR using the following primers:
    - Forward V1-V3 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGCTCAG

- Reverse V1-V3
  GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG

3) The PCR products are then purified with AmpureXP beads in order to remove residual 16S primers. Indeed, discarding "residual adapters, primers and low-quality bases" is primordial to conduct efficient analysis of NGS libraries (Jovel *et al.*, 2016).

4) After being purified, the PCR products need to be indexed. During this step, a PCR is used once again to add indexes to the amplicons previously obtained. Each amplicon will have two indexes and each combination of indexes is unique. To do so the Nextera XT Index Primer 1 and 2 are used in a MasterMix with normalized DNA.

5) The products of this PCR are here again purified with AmpureXP beads.

6) Before going further into the sequencing process, it is possible to validate the libraries with a bioanalyser DNA 1000 to check the size of the amplicon.

7) After all this, the libraries need to be quantified, normalized and assembled. The quantification uses the Picrogreen tool. The normalization is based on the size of the amplicon given by the bioanalyser.

8) The libraries are then denatured with sodium hydroxide (NaOH) and with heat before being charged on the MiSeq which is faster than HiSeq and works with reads of 300 base pairs instead of 150 to 250 base pairs (Lagos *et al.*, 2015).

3.5. Bioinformatics

The analysis of the data obtained thanks to DNAVision is conducted with the QIIME software (Quantitative Insight Into Microbial Ecology) (Caporaso *et al.*, 2010). Indeed as NGS generate a huge quantity of information, a bioinformatics software is necessary to deal with all the data. The QIIME software enables to process these data and to sum it up in easily understandable graphs (Caporaso *et al.*, 2010). For taxonomy assignment, QIIME uses the Greengenes database by default.

DNAVision sent raw data which needed to be pre-treated so as to be useable to work upon. In the following section we shall explain how this data was dealt with.

- **Preparation of the data**

1. The forward and reverse sequences were stocked in two different fastq files for each sample. It was thus needed to merge them in one file per sample with the following script: multiple_join_paired_ends.py
2. Fastq files contain the sequence and the quality information. As the QIIME commands only function with files containing only the sequence it was necessary to convert the fastq files into fasta files with the script: convert_fastaqual_fastq.py
3. In order to identify correctly each sample and to match it with its specific parameters, a mapping file was created and proof-checked for errors with the script : check_id_map.py

4. Then each sample was allotted to a QIIME label based on the mapping file samples' ID with the script: add_qiime_labels.py. This enables to associate each fasta file with the name of the corresponding sample in the mapping file.

Once data were usable, the following step was the picking of the operational taxonomic units (OTUs) present in the sample. For this, the script pick_de_novo_otus.py was used in order to compare the sequences with the Greengenes database. This workflow clusters sequences with 97% similarities.

The next task was to deal with sequencing artefacts called chimeras present in the OTUs table. Indeed, during the sequencing process, hybrids can originate from the sequencing of several parent sequences (Haas *et al.*, 2011). The Chimera Slayer tool in QIIME is able to detect these chimeras and is used with the script: identify_chimeric_seqs.py.

The chimera are then removed from the OTUs table with the script: filter_fasta.py

After removing the chimera, it is necessary to reassign a taxonomy to the OTUs with the script: assign_taxonomy.py

Once the taxonomy has been corrected, a new OTUs table is made without the chimera, the sequences are realigned and the alignment is filtered:

- make_otu_table.py
- align_seqs.py
- filter_alignment.py

After having dealt with the chimera, several other cleaning steps are required before obtaining a ready-for-work OTUs table. The singletons OTUs i.e. the OTUs which are observed less than twice but also the sequences originating from chloroplast and mitochondria DNA need to be discarded:

- filter_otus_from_otu_table.py is used with the precision that all OTUs observed less than twice need to be discarded.
- filter_taxa_from_otu_table.py is used directly on the OTUs table to discard the OTUs which have been assigned to chloroplasts and mitochondrias during the taxonomy assignment process.

A new phylogenetic tree is also needed for further analysis based on the phylogeny. The script make_phylogeny.py is thus used.

To have an overview of the quantity of OTUs in each sample, rarefaction curves are generated with a limit of 50 000 sequences to make the graph more readable with the script: alpha_rarefaction.py. Rarefaction curves are, in our case, a representation of the richness of the samples in function of the sequencing depth that could be reached for each sample.

- **Description of the taxa observed**

In order to evaluate the representativeness of our samples, the composition of the bacteria communities in the four PAFF Box sump samples and in the four PAFF Box biofilter samples were analysed thanks to bar charts obtained with the summarize_taxa_through_plots.py script.

To avoid an over-representation of the taxa in the PAFF Box, all the following analysis were conducted on a OTUs table keeping only one sample for the PAFF Box sump and one sample for the PAFF Box biofilter. The table was modified with the filter_samples_from_otu_table.py script**.**

The first goal of our study being the broad taxonomic characterisation of the bacteria composing the communities of diversified samples, bar charts at every taxa level until genus were generated in order to study the predominant taxa in our samples with the summarize_taxa_through_plots.py script.

To highlight the major taxa composing each sample, the OTUs table was filtered in order to discard all samples representing less than 0.5% of the total reads with the filter_otus_from_otu_table.py script. Bar charts were then again generated to visualise the remaining taxa.

To form an idea of the diversity of the bacteria communities in the samples, the Shannon index of each sample was calculated with the alpha_diversity.py script.

The core microbiota of our samples was also studied. We obtained a list of the taxa present in 100% of our samples and of the taxa common to 90% of our samples thanks to the compute_core_microbiome.py script.

In order to compare our results with the literature, the fastq files from Schmautz *et al.* (2016b) were downloaded from the European Bioinformatics Institute (EBI) website and analysed with the summarize_taxa_through_plots.py script. It is however important to note that, in their study, they have used the Ribosomal Database Project instead of the Greengenes database. This might lead to differences between our interpretation of their results and their paper.

- **Comparison of the composition of the bacteria communities between groups of samples**

After gaining knowledge of the taxa composing the bacteria communities of our diversified systems, the second goal of our study is to determinate how several factors can influence on the composition of the communities. The factors studied are the following:

- The partners i.e. the global system studied
- The fish species
- The sampling compartment
- The fish density
- The feed type
- The type of system (i.e. coupled or decoupled)

Bar charts per factor were then generated from the unfiltered and/or filtered OTUs tables. The samples were grouped by object (7.1. Annex 1) with the –c Factor option of the summarize_taxa_through_plots.py.

The study of the species factor can be looked at from a different angle as several partners rear Tilapias. A OTUs table containing only the Tilapias samples was generated to highlight eventual similarities between these samples and bar charts were built from this table.

The next step is the comparison of the diversity between the objects of one factor. Firstly, several subsamples were generated and rarefied with the multiple_rarefactions.py script.

Then the alpha diversity of these subsamples was calculated with the alpha_diversity.py script.

These values were then collated and rarefaction plots generated:

- collate_alpha.py
- make_rarefaction_plots.py

Once the collated values were obtained, it was possible to compare the Shannon index between the objects of one factor with the script compare_alpha_diversity.py

Afterwards, principal component analyses (PCoA) were also generated to check for eventual clusters which might be able to guide the reflection concerning the comparisons. These PCoA are based on the beta diversity which compares the diversity from one sample to the other and are obtained with the beta_diversity_through_plots.py. A maximum sequencing depth of 5359 sequences was used for all samples to avoid bias linked to samples were the sequencing would have gone deeper and which would thus be richer.

Finally, Kruskal-Wallis tests were conducted to see which OTUs had significantly different abundances between the objects of one factor with the group_significance.py script.

The 100% and 90% core microbiota per object of factor were also generated with the compute_core_microbiome.py script.

4. RESULTS AND DISCUSSION

4.1. General information on the sequencing data

- **Quality of the DNA samples**

A Nanodrop spectrophotometer was used to evaluate the quality of the DNA samples. The 260/280 and 260/230 ratios were determined and the reference values are respectively 1.8 and between 2.0 and 2.2. Most of the times, the 260/280 ratio was correct but the 260/230 ratio was close to zero (Table 3) thus indicating a possible contamination by carbohydrates and phenol (Wilfinger *et al*., 1997). However, as the DNA quantities were quite small, this might also have impeded the instrument in its measures (Thermo Scientific, 2012). Moreover, the reliability of the device could be questioned as two consecutive measures taken on the same drop of sample could be very different.

Table 3. DNA concentration and Nanodrop ratios. D. biofilter: denitrification biofilter, sump F.: sample taken in the fish loop, sump P.: sample taken in the plant loop

Sample ID	Partner	Sampling zone	Concentration ($ng.\mu L^{-1}$)	260/280 ratio	260/230 ratio
GBX RAS S	Gembloux-RAS	Sump	33.3	1.90	0.05
GBX RAS B	Gembloux-RAS	Biofilter	16.2	1.99	0.02
UF S	UrbanFarmers	Sump	21.9	2.87	0.04
UF B	UrbanFarmers	Biofilter	13.3	3.87	0.02
UF biofilm	UrbanFarmers	Biofilm	27.2	2.27	0.05
PCG S 60	PCG 60 fish/tank	Sump	/	/	/
PCG B 60	PCG 60 fish/tank	Biofilter	-4.9	1.43	-0.06
PCG S 100	PCG 100 fish/tank	Sump	16.9	2.24	0.03
PCG B 100	PCG 100 fish/tank	Biofilter	6.5	1.9	0.01
BQF S	BQF	Sump	16.5	1.68	0.03
BQF B	BQF	Biofilter	10	3.09	0.01
BQF DB	BQF	D. biofilter	2.9	1.59	1.20
IGB S	IGB	Sump	39.1	1.49	0.12
IGB B	IGB	Biofilter	26.3	1.70	0.08
Wageningen S cat	Wageningen	Sump cat.	51.6	1.55	0.09
Wageningen S eel	Wageningen	Sump eel	213.4	1.65	1.31
Wageningen B eel	Wageningen	Biofilter eel	96.4	1.43	0.13
Inagro S fi	Inagro	Sump fish loop	23.9	1.52	0.05
Inagro S pl	Inagro	Sump plant loop	36.9	1.54	0.06
Inagro B	Inagro	Biofilter	25.6	1.54	0.04
GBX PAFF Box S1	Gbx - PAFF Box	Sump	30.1	1.89	0.07
GBX PAFF Box	Gbx - PAFF Box	Sump	41.7	1.71	0.07

S2					
GBX PAFF Box S3	Gbx - PAFF Box	Sump	53.1	1.11	0.11
GBX PAFF Box S4	Gbx - PAFF Box	Sump	33.8	1.88	0.06
GBX PAFF Box B1	Gbx - PAFF Box	Biofilter	163.5	1.63	0.42
GBX PAFF Box B2	Gbx - PAFF Box	Biofilter	25.1	1.90	0.06
GBX PAFF Box B3	Gbx - PAFF Box	Biofilter	51.5	2.03	0.08
GBX PAFF Box B4	Gbx - PAFF Box	Biofilter	91.6	1.68	0.17

Due to an experimental error, not enough DNA material was gathered for the PCG sump, 60 fish/tank, sample. Therefore, the tiny amount of DNA collected was kept for the sequencing and no Nanodrop measures were performed.

The negative results obtained for the PCG biofilter, 60 fish/tank, sample could be due to a dirty pedestal or a DNA quantity too small to be detected. This does not seem to have impeded the sequencing process but the results will be interpreted cautiously.

NB: all samples were collected either in a sump or a biofilter apart from two samples. UF biofilmis a biofilm sample from the UrbanFarmers' system and BQF DB originates from the denitrification biofilter of the BQF system. The bacterial composition of these two samples will be discussed further on.

- **Quality of the sequences**

The Phred quality score (Q score) measures the "probability that a given base is called incorrectly by the sequencer" (Illumina, 2011, p.1). The Q30 level indicates that the probability that an incorrect base were introduced in the sequence is of 1 in 1000. With such a low level of error, "Q30 is considered a benchmark for quality in next-generation sequencing" (Illumina, 2011, p.1).

The sequencing of the 28 samples gave good quality results with an average Q30 over 80%. Only three samples from the PAFF Box's biofilter got a Q30 quality index under 60% (repetitions 1, 3 and 4) which will have a repercussion on the analysis. Indeed, in each of those three samples more than 50% of the sequences were unassigned.

Globally, 16.8% of the total reads were not assigned to any taxa, which is quite similar to the results obtained by Schmautz *et al.* (2016b) where 23.9% of the sequences were considered as unassigned by QIIME.

- **Number of sequences and diversity analysis**

To increase the accuracy of our results we cleaned our sequences database from chimera with the ChimeraSlayer tool (Haas *et al.*, 2011) and deleted all OTUs with less than two occurrences i.e. singletons. Similarly the chloroplast and mitochondria DNA were discarded. These treatments result in the following OTUs table (Table 4):

Table 4. Statistics of the OTUs table

Total sequences processed	4 567 416
Minimum number of sequences	5359
Maximum number of sequences	127 166
Median	84 021
Mean	81 561
Standard deviation	28 910.861
Total number of OTUs	27 777
Unassigned OTUs	10 958

40% of the OTUs are unassigned which is linked to the very high number of different OTUs detected.

The lowest number of sequences obtained belongs to the third repetition of the PAFF Box biofilter (PAFF Box B3) (Table 3). This can be seen on the rarefaction curves () (encircled) as the curve corresponding to this sample is the shortest one. Because of this low number of sequences, the diversity of this sample is probably not completely explained as we can see that the curve does not reach an asymptote. More OTUs could hence supposedly be found in this sample had the sequencing gone deeper, in which case the relative abundance values of the OTUs actually detected could have been more realistic and accurate (Jovel *et al.*, 2016).

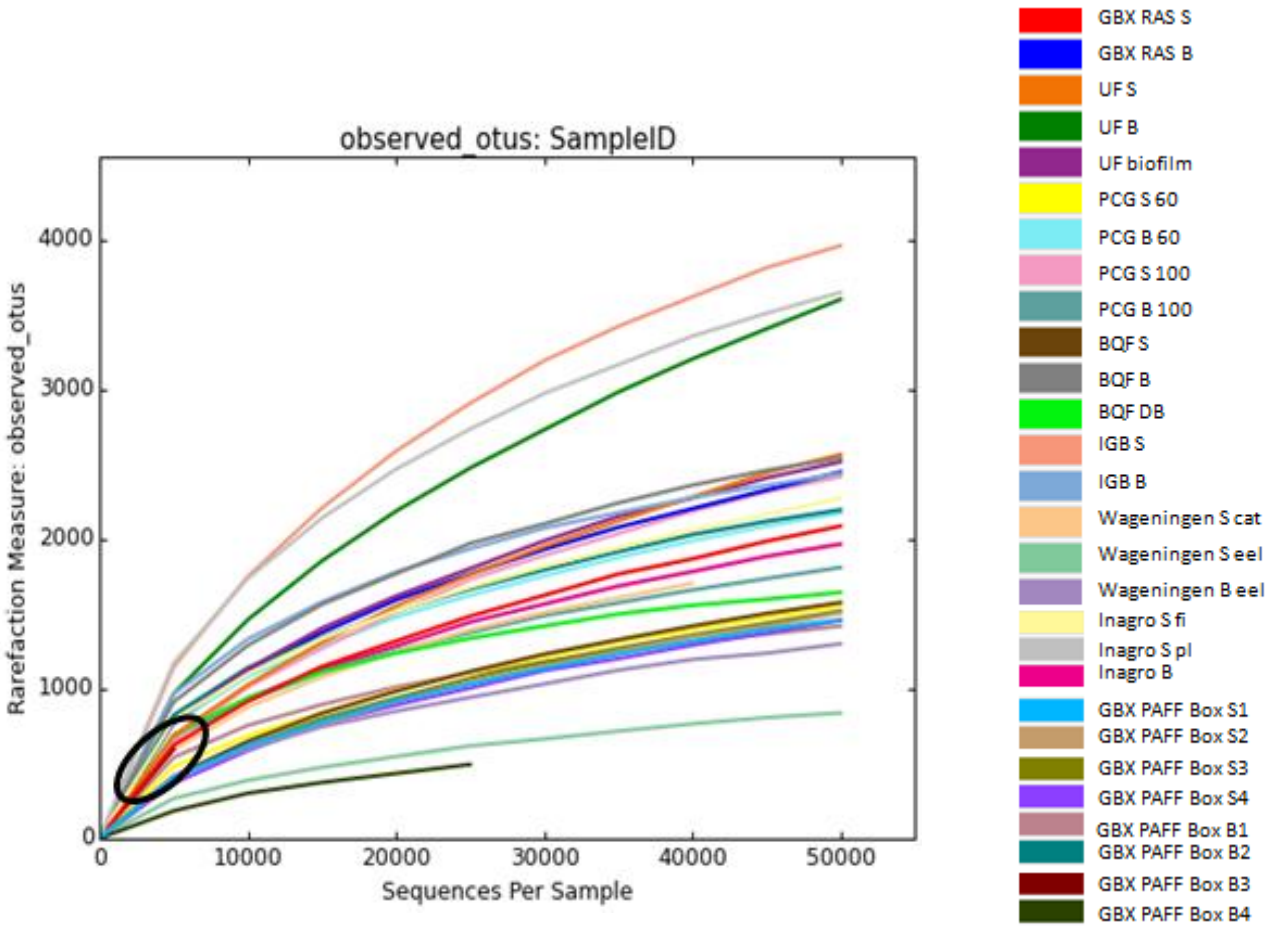

Figure 13. Rarefaction curves of the observed OTUs for all the samples (i.e. richness per sample), maximum depth of 50 000 sequences

On this graph (Figure 13) it is possible to see that the fourth PAFF Box biofilter sample (olive green, PAFF Box B4) and the Wageningen eel sump sample (turquoise) reach their asymptotes quite soon, thus showing that their OTUs richness is low yet well explained. This observation is confirmed by the study of the Shannon index for each sample. Indeed, both samples possess two of the lowest Shannon indexes of respectively 1.4 and 3.2. For the PAFF Box B4 sample this could be explained by the low quality (Q30 of 41.3%) but the Wageningen eel sump sample has a Q30 index of 82.4%. The low number of observed OTUs in this sample could be linked to the fact that the system had recently been implemented. Indeed, the Wageningen eel biofilter sample (mauve) possesses the third lowest number of observed OTUs.

Only three samples detach themselves from the rest of the group, namely the IGB sump sample (orange), the Inagro sump plant loop sample (grey) and the UrbanFarmers biofilter sample (green) and thus seem richer than the others. This is supported by their high Shannon indexes of respectively 7.5, 8.6 and 8.3.

4.2. Description of the taxa present in the samples

This section will seek to describe the different taxa observed in our samples. Most of the time, the identification stops at the genus or family level and very few exact species are identified which is very common with 16S DNA analysis (Jovel *et al.*, 2016).

4.2.1. Representativeness of the collected samples

A representativeness test was conducted on the most accessible of all systems studied, the PAFF Box of Gembloux Agro-Bio Tech, to ensure the validity of the methodology used. In this system, four repetitive sump samples taken at the exit towards the hydroponic system (Figure 14) and four repetitive biofilter samples taken at the top of the pressurised container (Figure 15) were collected at the same moment to allow for comparison.

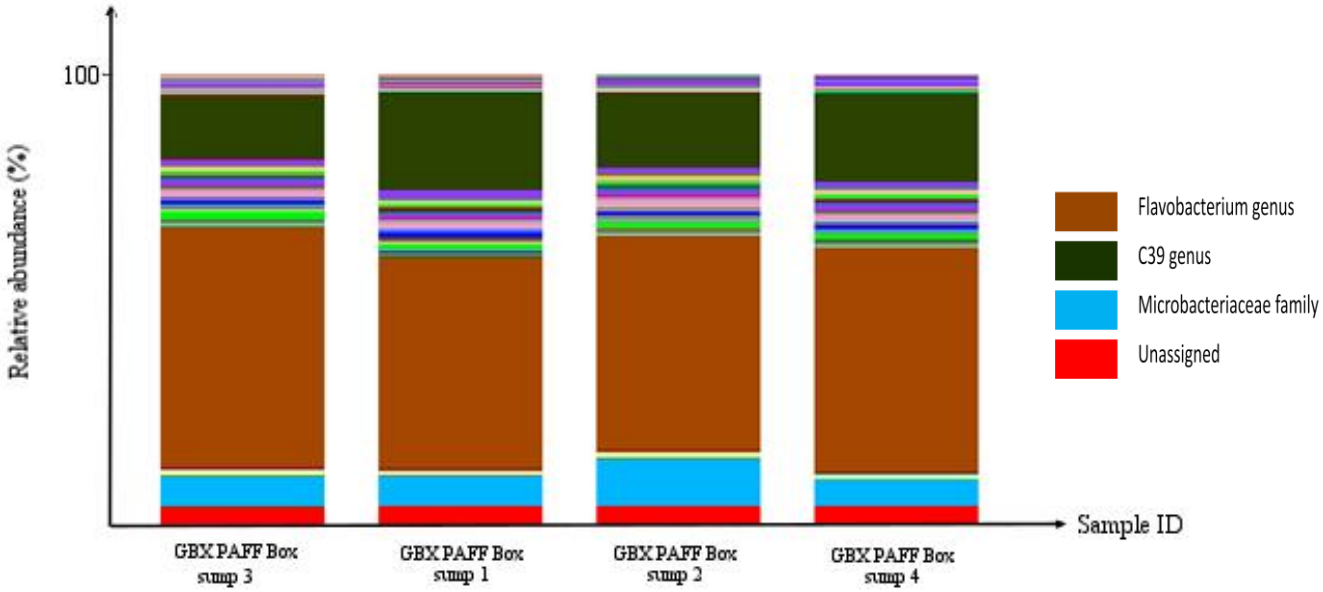

Figure 14. Bar charts representing the relative abundance of the taxa found in the four sump samples of the PAFF Box. Each colour represents one taxon. The taxa representing more than 4% of the total reads have been included in the legend. The size of each colour box is proportional to the relative abundance of the corresponding taxon.

Figure 14 shows the similarity between the sump samples in terms of genera present and relative abundances. Globally a variability of 6 or 7% can be observed in terms of relative abundance for the same taxon between the four repetitions. It is visually clear that the samples present the same taxa abundance and it can thus be concluded that the samples taken in the sumps of all studied systems might be quite representative of the total microbiota of these sumps.

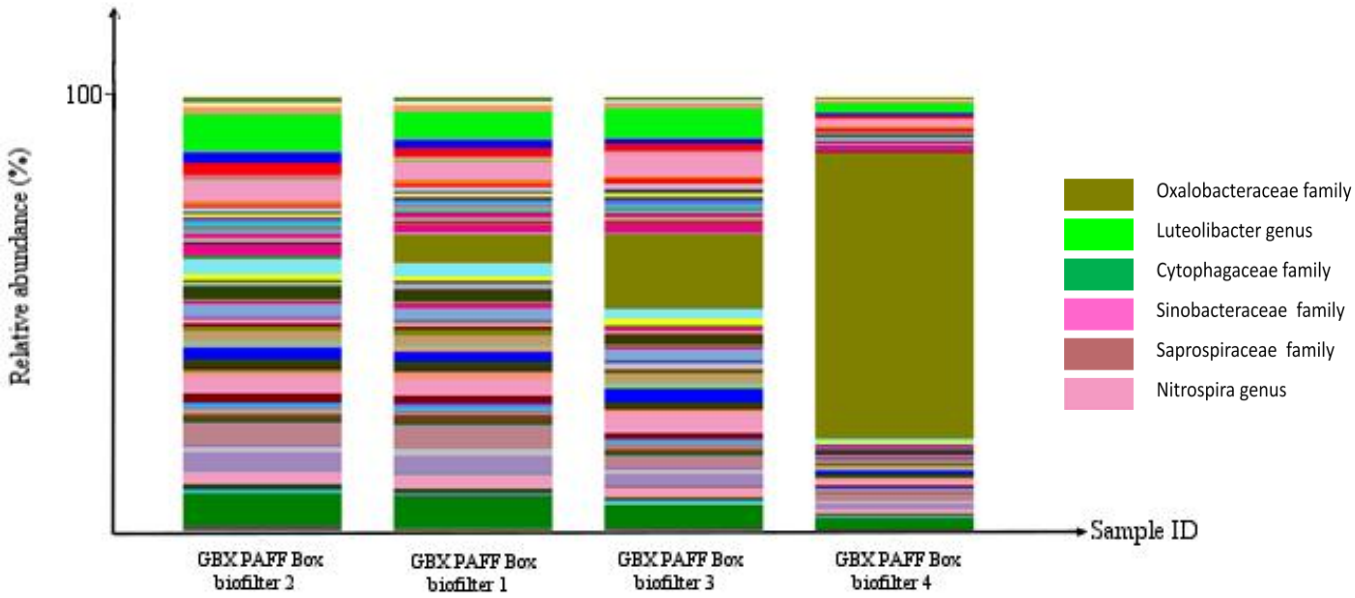

Figure 15. Bar charts representing the relative abundance of the taxa found in the four biofilter samples of the PAFF Box. Each colour represents one taxon. The taxa representing more than 3% of the total reads have been included in the legend. The size of each colour box is proportional to the relative abundance of the corresponding taxon. The unassigned sequences have been discarded for better readability.

In order to compare the four biofilter samples, it was necessary to discard all the unassigned sequences to be able to visualise the identified taxa present. The four samples seem to host similar taxa but with different relative abundances. Indeed, the fourth repetition is dominated (66%) by the *Oxalobacteraceae* family when it is barely present in the second repetition.

The predominance of this family in the fourth repetition of the PAFF Box's biofilter corroborates the low Shannon index and the rapid reach of the asymptote on the rarefaction graph (Figure 13). It might also be linked to the fact that 83.1% of the reads in this sample were unassigned. Indeed, many other taxa could compose this sample but due to DNA quality problems, these could not be identified.

It is nonetheless necessary to outline that this repetitive sampling in the PAFF Box biofilter needs to be nuanced. Indeed, as the PAFF Box biofilter is a small pressurised container, it was only possible to reach the beads at the top of the container. A difference may have been observed if the sampling had been conducted at different depths. Furthermore, it is important to note that all our biofilter samples have been collected at the surface of the biofilters and thus could have captured only a fraction of the total diversity in the biofilters. This is all the more valid for big aquaponic systems with massive biofilters in which the pressure, available oxygen and water flow rate can vary with the depth. Indeed, even though the moving bed technique enables a continuous shuffling of the biomedia, some differences could still be observed depending on the dimensions of the biofilter. Moreover, different biomedia were used in the visited systems (see 3.1) and thus the vortex and ultrasounds steps may not have had the same effect on the scraping of bacteria biofilms from the surface of the biochips.

A correct sampling is primordial to represent the full diversity of a system. Experiments are currently conducted in the IUPPL to develop a systematic method to collect representative samples without bias and to determine whether successive washes of a microbiota enable to collect a representative portion of this microbiota (Sare and Stouvenakers, unpublished). This could apply to the washing of the biofilter beads.

Lastly it is necessary to expand on the unassigned sequences resulting from this experiment. Indeed, unassigned sequences are found in every sample in various proportions but represent respectively 65%, 48% and 83% of the repetition 1, 3 and 4 of the PAFF Box biofilter samples, which is much higher than in the other samples. They have been discarded to correctly study the repetitiveness of this sampling as we do not know the exact reason for the important presence of these unassigned sequences. These unassigned sequences could result from either a DNA extraction problem which could be confirmed by a particular difficulty in the PCR amplification when checking for amplification inhibitors or to species not yet encountered and classified. The DNA extraction problem hypothesis is more likely as the Q30 quality indexes of the DNAVision report are the lowest for repetitions 1, 3 and 4, oscillating between 41.3% for the fourth repetition and 60.0% for the first repetition

.

4.2.2. Study of the predominant taxa in our samples

The first approach to study the composition of the bacteria communities in diversified aquaponic systems is the broad characterisation of the visibly predominant taxa in our 22 samples.

Bar charts representing the relative abundances (%) of the taxa in each samples have been generated from our OTUs table but also from the dataset of Schmautz *et al.* (2016b) to be able to compare our findings without too much bias.

NB: this analysis was conducted on a OTUs table rid of the PAFF Box repetitions to avoid an over-representation of its community. Only the sump's third repetition and the biofilter's second repetition were kept as they were the ones with the lowest amounts of unassigned sequences. This OTUs table with 22 samples will be used for the rest of the analysis.

4.2.2.1. Study of the phyla observed

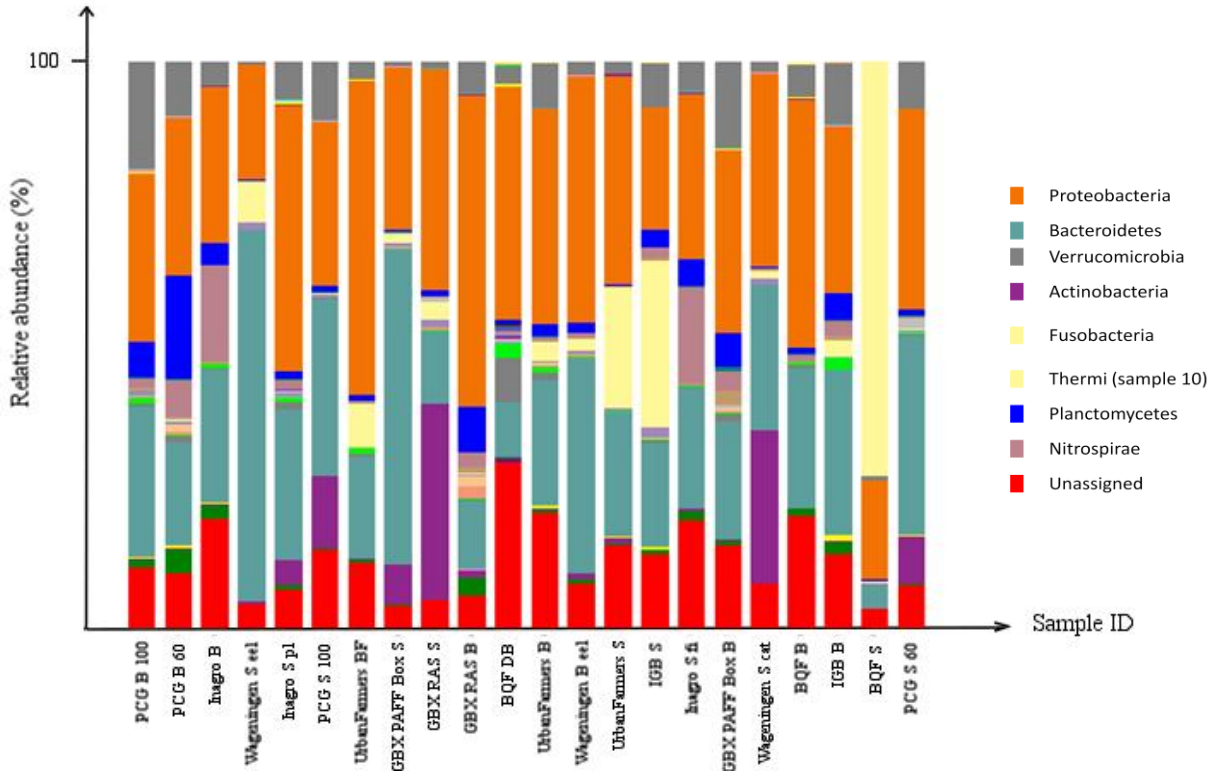

Figure 16. Bar charts representing the relative abundance of the phyla composing each sample. Each colour represents one phylum. The phyla representing more than 2% of the total reads have been included in the legend. The size of each colour box is proportional to the relative abundance of the corresponding phylum. PCG B 100: PCG biofilter 100 fish/tank; PCG B 60: PCG biofilter 60 fish/tank; Inagro B: Inagro biofilter; Wageningen S eel: Wageningen eel sump ; Inagro S pl: Inagro sump plant loop; PCG S 100: PCG sump 100 fish/tank; UrbanFarmers BF: UrbanFarmers biofilm; GBX PAFF Box S: Gembloux PAFF Box sump; GBX RAS S: Gembloux RAS sump; GBX RAS B: Gembloux RAS biofilter; BQF DB: BQF denitrification biofilter; UrbanFarmers B: UrbanFarmers biofilter; Wageningen B eel: Wageningen eel biofilter; UrbanFarmers S: UrbanFarmers sump; IGB S: IGB sump; Inagro S fi: Inagro sump fish loop; GBX PAFF Box B: Gembloux PAFF Box biofilter; Wageningen S cat: Wageningen catfish sump; BQF B: BQF nitrification biofilter; IGB B: IGB biofilter; BQF S: BQF sump; PCG S 60: PCG sump 60 fish/tank

Two major phyla can be found throughout the samples (Figure 16), namely the *Proteobacteria* (34.6% of total reads i.e. the sequences obtained from the DNA molecules (Goodwin *et al.*, 2016)) and the *Bacteroidetes* (25.5% of total reads). Six other phyla are found in smaller proportion but non-negligible, namely the *Verrucomicrobia*, (5.9% of the total reads), the *Actinobacteria* (4.6% of the total reads), the *Fusobacteria* (3.7% of the total reads), the *Thermi* (sample 10, 3.4% of the total reads) the *Planctomycetes* (3.1% of the total reads) and the *Nitrospirae* (2.6% of the total reads).

These results are in agreement with the recent findings and most particularly with Schmautz *et al.* (2016b) and Munguia-Fragozo *et al.* (2015). During this discussion we will compare our results to these two references as they are, to our knowledge, amongst the few whom have focused on bacterial community characterisation in aquaponics. In these two studies, *Proteobacteria* and *Bacteroidetes* were also determined as being the predominant phyla. An important difference to note however is the quasi total absence of the *Firmicutes* phylum in our samples, which are considered important by Schmautz *et al.* (2016b). This

difference is explained by the fact that the *Firmicutes* phylum has been mainly found in a fish faeces sample (Schmautz *et al,*. 2016b) not sampled in our study (Supplementary material, Schmautz *et al.*, 2016b). The *Planctomycetes* have also been observed by Van Kessel *et al.*, (2010) in their recirculating aquaculture system and they brought the hypothesis that these bacteria might be involved in the nitrogen cycle.

The *Thermi* phylum which represents more than 70% of the sample from BQF's sump, (Figure 16) draws the attention. Indeed, the enormous proportion of this phylum probably results from the ozone and UV light treatments in this sturgeon culture system. More details will be supplied in 4.2.2.3.

4.2.2.2. Study of the classes observed

The classes that are mostly observed throughout the samples and amongst the *Proteobacteria* phylum are the α-*Proteobacteria* (9.0% of total reads), the β-*Proteobacteria* (14.9% of total reads) and γ-*Proteobacteria* (6.1% of total reads) which is in accordance with the findings presented by Schmautz *et al.* (2016b) and by Rurangwa and Verdegem (2013) in a review of the microorganisms present in recirculating aquaculture.

Moreover, in our study we can also note the presence of δ-*Proteobacteria* (3.5% of total reads) and ε-*Proteobacteria* (1.1% reads). This last class was also detected by Munguia-Fragozo *et al.* (2015) and especially the genus *Arcobacter* which stands out in our study as well. According to Munguia-Fragozo *et al.* (2015), the species *Arcobacter nitrofigilis* can take part in nitrogen fixation which is the transformation of atmospheric nitrogen into a fixed form such as ammonia for instance. However, as the exact species representing the *Arcobacter* genus in our samples could not be found, it is difficult to affirm that the present *Arcobacter* possess this precise role.

The other visible classes are the *Saprospirae* (10.8%), the *Flavobacteriia* (6.6%), the *Actinobacteriia* (4.5%), *Fusobacteriia* (3.7%), the *Verrucomicrobia* (3.3%), the *Cytophagia* (3.2%), the *Sphingobacteriia* (2.9%), the *Deinococci* (2.7%), the *Nitrospira* (2.6%), the *Planctomycetia* (2.2%), the *Bacteroidia* (1.8%) and the *Pedosphaerae* (1.5%).

4.2.2.3. Study of the families and genera observed

Now that the major phyla and classes have been identified, it is necessary to study the families and genera composing the bacteria communities of the 22 analysed samples. However, more than 700 different genera have been distinguished. To ease the readability and understandability of this study, only the genera representing more than 0.5% of the total reads have been kept (Figure 17). For some taxa, the identification process stopped at the family level and therefore only the family is hereafter presented.

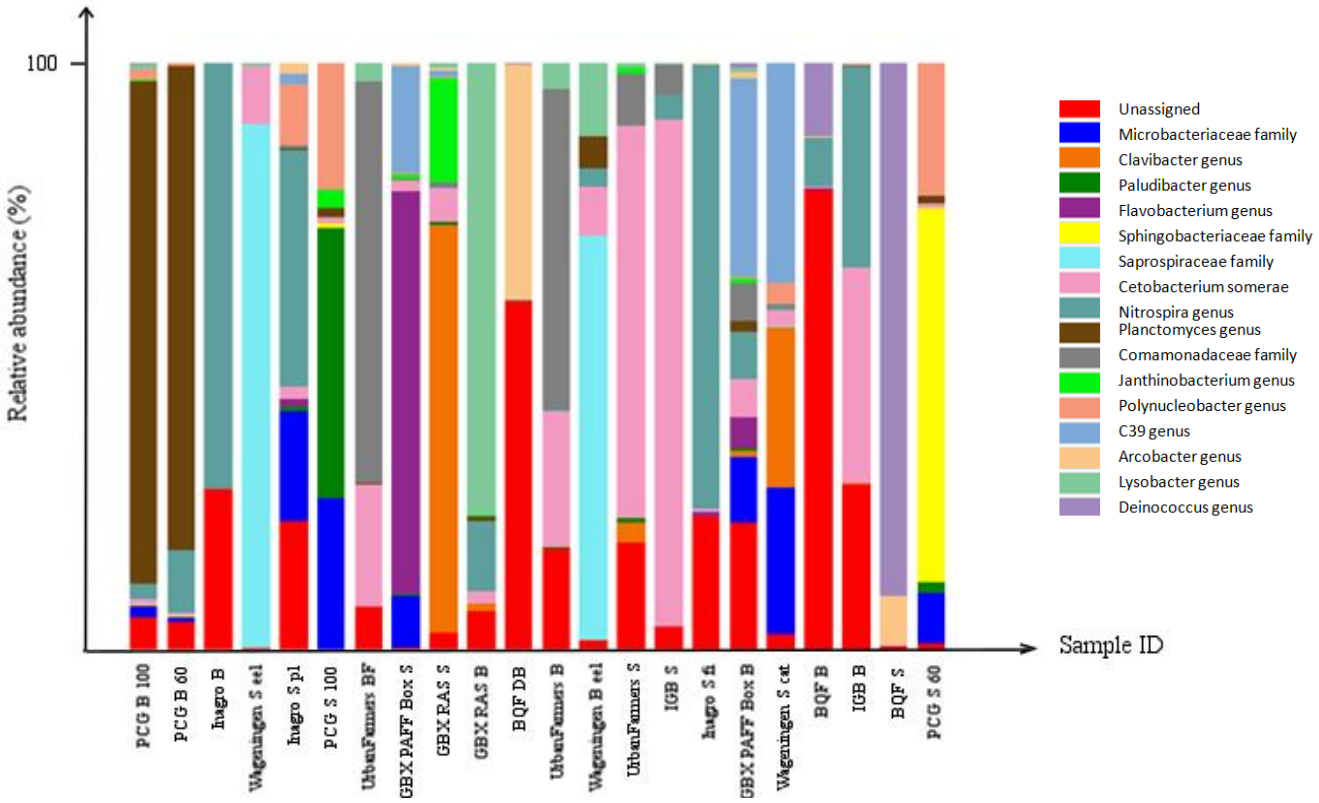

Figure 17. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads. Each color represents one taxon. The size of each colour box is proportional to the relative abundance of the corresponding taxon. PCG B 100: PCG biofilter 100 fish/tank; PCG B 60: PCG biofilter 60 fish/tank; Inagro B: Inagro biofilter; Wageningen S eel: Wageningen eel sump ; Inagro S pl: Inagro sump plant loop; PCG S 100: PCG sump 100 fish/tank; UrbanFarmers BF: UrbanFarmers biofilm; GBX PAFF Box S: Gembloux PAFF Box sump; GBX RAS S: Gembloux RAS sump; GBX RAS B: Gembloux RAS biofilter; BQF DB: BQF denitrification biofilter; UrbanFarmers B: UrbanFarmers biofilter; Wageningen B eel: Wageningen eel biofilter; UrbanFarmers S: UrbanFarmers sump; IGB S: IGB sump; Inagro S fi: Inagro sump fish loop; GBX PAFF Box B: Gembloux PAFF Box biofilter; Wageningen S cat: Wageningen catfish sump; BQF B: BQF nitrification biofilter; IGB B: IGB biofilter; BQF S: BQF sump; PCG S 60: PCG sump 60 fish/tank. Unfiltered graph in 7.3. Annex 3

The first observation after this filtering is that only 16 taxa are left instead of the 700 identified taxa of the unfiltered OTUs table. Most of these discarded taxa did not even represent 0.1% of the total reads as they were only present in tiny proportions in one or two samples.

One specific yet unidentified genus of the ***Saprospiraceae*** family is present in several samples and represents 60% of the bacteria community in the Wageningen eel sump sample. The *Saprospiraceae* are typical aquatic bacteria but can also be found in activated sludge (McIlroy and Nielsen, 2014). It seems like they are capable of hydrolysing complex carbon molecules for their own utilisation. This family is also known for their important role in the "breakdown of complex organic compound in the environment" and especially in sludge wastewater (McIlroy and Nielsen, 2014, p.880).

The ***Flavobacterium*** genus represents 50% of the PAFF Box sump sample (Figure 17) and is a genus widely present in nature and mostly known for their capacity to degrade complex

organic molecules. It seems that the *Flavobacterium* are often involved in association with plant roots and plant leaves which may imply a role in the functioning of plants. *Flavobacterium* might have a role in plant growth and protection (Kolton *et al.*, 2016). *Flavobacterium* are not found only in the PAFF Box's sump (even though this is where they are predominant) but also in several other sumps such as the ones from Wageningen (catfish), Inagro (plant loop), PCG and UrbanFarmers. In their own experiment, Schmautz *et al.* (2016b) found *Flavobacterium* in their samples from root, biofilter and periphyton but they were mostly found in the root sample thus leading to the conclusion that they may have a role in rhizosphere community, their precise role however remains unclear. Munguia-Fragozo *et al.* (2015, p.3) also considered *Flavobacterium* as a "common genera in systems with high richness and diversity" which may be involved in heterotrophic denitrification. Itoi *et al.* (2007) who conducted experiments on the microbiota of filters in RAS detected the presence of *Flavobacterium* both in the inner and outer layers of the biofilms. As the *Flavobacterium* are supposed to be rhizosphere bacteria it seems unexpected to have found them in the sump of the PAFF Box. However, as the PAFF Box is a coupled system with water flowing from the root zone to the fish tanks and then to the sump, it seems possible that bacteria could move from one compartment to another. It is however stranger for a RAS such as Wageningen's catfish system. A simple hypothesis could be that the exact species in the PAFF Box system and catfish systems are different. Further exploration of the *Flavobacterium* species in aquaponics could answer this question and shed a more accurate light as to their role in aquaponics.

One specific yet unidentified genus of the ***Comamonadaceae*** family has been detected. This family belongs to the *Burkholderiales* order and has also been observed by Schmautz *et al.* (2016b) and Itoi *et al.* (2007) in a study of a RAS with goldfish. Munguia-Fragozo *et al.* (2015) confirmed that the *Comamonas sp.* was commonly found in aquaponics. As to their role, Bulgarelli *et al.* (2015) detected this family in the root microbiota of barley.

The ***Cetobacterium somerae*** species has been identified precisely, belongs to the *Fusobacteriaceae* family and has been found in guts of freshwater fish (Itoi *et al.*, 2007; Tsuchiya *et al*., 2008; Schmautz *et al.*, 2016; Yildiz *et al.*, 2017) and human faeces (Finegold *et al.*, 2003). According to Tsuchiya *et al*. (2008), it could be involved in the production of B_{12} vitamin. *C. somerae* has been found in all of our samples which implies that it was present in sumps as well as in biofilters. This could be due to water movements that would transport the bacteria in the whole aquaponic system. However, Schmautz *et al.*, (2016b) did not find *C. somerae* in their biofilter and only a small proportion in their plant root sample.

The ***Deinoccocus*** genus represents 73% of the BQF sump sample. This is probably mainly due to the fact that this genus is composed of extremophile bacteria (Rosenberg, 2006) which are the only ones able to resist to the ozone and UV light treatments applied by BQF. In this sample, the DNA concentration is inferior to the mean value of DNA concentration but these values are highly subject to variations linked to the sampling and the DNA extraction steps. As the *Deinococcus* genus was not observed in our other samples, we could suppose that the ozone treatment and ozone degradation with UV light foster their presence. To our knowledge, this genus is not pathogenic but the dominance of this sole genus in the BQF sump could become dangerous if the *Deinoccocus* mutated and became pathogenic. Indeed, as there does not seem to be any competition they could keep on thriving and affect the whole system.

The ***Nitrospira*** genus is quite present in the Inagro biofilter and in the Inagro sump (fish loop) and is involved in the nitrification process. The fact that it is one of the major genera of our samples is in agreement with the observations of Schmautz *et al.* (2016b) who also considered *Nitrospira* as the major actor of the nitrification process in aquaponics. Indeed, it has been recently discovered that *Nitrospira* could complete a whole nitrification process on its own (Daims *et al.*, 2015) which would then render the presence of AOB redundant.

The genus ***Clavibacter*** represents 34% of Gembloux's RAS's sump's bacteria community and is also present in Wageningen catfish sump sample and Wageningen eel biofilter sample. This genus contains phytopathogenic species (Davis *et al.*, 1984).

The ***Microbacteriaceae*** family can be found in the four PCG samples, in Wageningen's catfish sump sample, in the PAFF Box's sump and biofilter samples and in Inagro sump sample (plant loop). They can form associations with plants and fungi (Evtushenko and Takeuchi, 2006) and have been identified in the microbiota of barley roots (Bulgarelli *et al.*, 2015). Some species could however turn out to be plant pathogens (Evtushenko and Takeuchi, 2006). It was also present in the bar charts obtained from Schmautz's dataset (Schmautz *et al.*, 2016b).

The **C39** genus belongs to the *Rhodocyclaceae* family which was also present in Schmautz *et al.* (2016b) albeit in smaller proportions. According to Gao *et al.* (2017) there is little documentation on this genus but their experiment showed that it could be involved in the denitrification process associated with acetate. This genus is present in the PAFF Box's sump and the Wageningen catfish sump.

One specific yet unidentified genus of the ***Sphingobacteriaceae*** has been detected. This family is often isolated from soil and compost (Lambiase, 2014).

The ***Planctomyces*** genus contains bacterioplankton strains often found in "humic freshwater environment"(Youssef and Elshahed, 2006).

The ***Polynucleobacter*** genus contains heterotrophic freshwater ultramicrobacteria : freshwater ultramicrobacteria, heterotrophic bacteria(Watanabe *et al.*, 2008)

The ***Lysobacter*** genus can be found in soils, freshwater habitats but also in decaying organic matter. They are known for producing antibiotics (Reichenbach, 2006).

The ***Paludibacter*** genus is monospecific and only contains the *Paludibacter propionicigenes* species (Sakamoto, 2014). This species has been isolated from an irrigated rice field in Japan by Ueki *et al*. (2006), is strictly anaerobic and produces propionate (Ueki *et al*., 2006). It could also be involved in the fermentation of the rice residues found in the field.

The ***Janthinobacterium*** genus belongs to the Oxalobacteraceae family and is quite similar to the genera *Herbaspirillum* and *Chromobacterium*. It is strictly aerobic and some species are able to produce gas from nitrate. This genus is often isolated from soil and freshwater habitats. Some species can also be phytopathogenic (Baldani *et al.*, 2014). This genus has also been observed by Itoi *et al.* (2007) in a study of a RAS with goldfish. However, this genus has also been identified from contaminating DNA in DNA extraction kits by Salter *et al.* (2014) and further studies need to be conducted on the kit to qualify and quantify these contaminations.

- **Comparison with the literature**

Some genera such as *Rhizobium*, *Sphingobacterium*, *Acinetobacter*, *Aeromonas*, *Pseudomonas* and *Flexibacter* are considered as common in RAS by Munguia-Fragozo *et al.* (2015) while they are absent in our samples. There can be several ways to understand their absence. The first reason is that the identification often stopped at the family level and even though the *Sphingobacterium* and *Rhizobium* genera have not been spotted in samples, the *Sphingomonadaceae* and *Rhizobiaceae* families have. The second is that, even after the OTUs table cleaning steps and the removing of the three impeding PAFF Box biofilter samples, 11% of the total reads were still unassigned. Finally, we have observed very different communities in our 22 samples, it seems thus understandable that all the genera found by Munguia-Fragozo *et al.* (2015) where not present in our experiment.

If we consider the results of Schmautz *et al.* (2016b), we can note a similarity concerning the abundance of the *Xanthomonadales* order which represents 4.4% of our total reads. On the contrary, we did not find any notable presence of *Pseudomonadales*.

- **Partial conclusion**

The taxa present in our samples often seem to be potentially related to plant rhizosphere or involved in plant-bacteria interaction. In coupled systems such as the Urban Farmers system or the PAFF Box it could be easily understandable as water flowing through the roots of the hydroponic plants then end up in the fish tanks and the sump. A complementary experiment has been conducted in the PAFF Box to study the bacteria present in the rhizosphere of the lettuce grown in the aquaponic system. The results of this study are however not available yet.

In decoupled systems, the presence of rhizosphere bacteria in samples from the aquaculture loop would be more uncommon and could raise the hypothesis that for an unknown reason, plant beneficial bacteria can develop in aquaculture systems. This observation could also be linked to the important yields observed in aquaponics.

However, the taxonomic identification stopped at the genus level and thus we cannot be certain that the present species are the ones related to rhizosphere or involved in plant-bacteria interactions. Indeed, many of the described genera contain very different species. To confirm our hypothesis and to better understand the role of the identified taxa, a species identification should be conducted.

4.2.3. Core microbiota

The first aim of this study being the global taxonomic characterisation of the bacteria communities living in aquaponic systems, the core microbiota of our samples has been identified. According to Lemanceau *et al.* (2017, p.1), "the microbial community that is systematically associated with a given host plant is called the core microbiota". This definition can of course apply to other bacteria communities such as communities in aquaponic systems.

A first restrictive core microbiota was studied, containing only the OTUs present in 100% of our samples.

Regardless of the system and sample location, only six taxa were found for this first core microbiota, namely the *Oxalobacteraceae* family, the *Sphingomonas* genus, the *Cetobacterium somerae* species, the *Comamonadaceae* family, the *Ralstonia* genus and the *Devosia* genus.

The *Comamonadacea* family and *Cetobacterium somerae* species have already appeared as predominant taxa in our samples and thus have already been described in the previous part (see 4.2.2.3).

The ***Oxalobacteraceae*** are heterotrophic bacteria that can be found in water, in soils or in association with plants (Baldani *et al.*, 2014). A few particular species are also considered as slight plant pathogens. The genus *Herbaspirillum* is particularly interesting as it can be considered as plant-growth-promoting bacteria because of its capacity to form root-bacteria associations and its capacity for endophytic nitrogen fixation (Baldani *et al.*, 2014). Some species among this genus already have applications in agriculture as biofertilizer and biocontrol agent. Further studies should be conducted to identify the genera and species present in our samples for a more accurate identification of their potential.

The ***Sphingomonas*** genus contains heterotrophic members (Balkwill *et al*., 2006). Amongst these, some have been identified as a potential antagonist to phytopathogenic fungi. The *Sphingomonas* genus is often found in association with plants and especially with the rhizosphere (White *et al*., 1996) but can also been observed in soils and aqueous environments (Balkwill *et al*., 2006).

The ***Ralstonia*** genus belongs to the *Burkholderiales* order and is known for its nitrogen-fixing capacities and possess genes involved in the nodulation process (Prescott *et al.*, 2010).

The ***Devosia*** genus belongs the *Rhizobiales* order and has been found in the root microbiota of potato plants (Barnett *et al.*, 2014).

It is important to nuance this core microbiota in the light of the paper written by Salter *et al.* (2014). In this article, the authors put forward the fact that most DNA extraction kits contain contaminating DNA from soil and water bacteria, often associated with nitrogen fixation. The kit tested in this work is the FastDNA Spin kit (Mp Biomedicals) i.e. the one we used in our experiment. According to Salter *et al.* (2014), the taxa commonly observed in the kit are notably *Sphingomonas*, *Oxalobacter*, *Comamonas*, *Devosia* and *Ralstonia*. Another experiment conducted with the same kit in the IUPPL obtained quite a similar core microbiota

which may confirm that some genera are present because of contaminations in the DNA extraction kit.

A second, larger microbiota was defined, this time including all the taxa present in at least 90% of our samples. The number of taxa encompassed in this new core microbiota is much more important and the retained taxa are shown in Table 5. In this table, we grouped the taxa for which the identification process stopped at the same level and with the same taxonomy. For example, seven different genus belonging to the *Comamonadaceae* family were identified and grouped under the label "*Comamonadaceae**7". Some OTUs of the core microbiota were unassigned.

Table 5. Core microbiota containing the taxa present in at least 90% of our samples. The classification of each taxa is given as well as their known functions and applications which might be of interest in the bacteria community of an aquaponic system. Alpha: α-*proteobacteria*; beta: β-*proteobacteria*; gamma: γ-*proteobacteria*. **Bold:** taxa part of the 100% core microbiota

Phylum	***Class***	***Order***	***Family***	***Genus***	***Species***	***Role***	***References***
Proteobacteria	*Alpha*	*Rickettsiales*				*Pathogens*	*(Thomas, 2016)*
Proteobacteria	***Beta***	***Burkholderiales***	***Oxalobacteraceae***				
Proteobacteria	***Alpha***	***Sphingomonadales***	***Sphingomonadaceae***	***Sphingomonas*2***			
Verrucomicrobia	*[Pedosphaerae]*	*[Pedosphaerales]*					
Proteobacteria	*Gamma*	*Xanthomonadales*	*Sinobacteraceae*			*Tetracyclin degradation (ability shared with the Comamonadacea)*	*(Yang et al., 2016b)*
Proteobacteria	*Alpha*	*Caulobacterales*	*Caulobacteraceae*	*Phenylobacterium*	*(immobile)*	*Lives in soils Breakdown of chlorizadon, a herbicide molecule*	*(Eberspächer and Lingens, 2006)*
Nitrospirae	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*2*		*Total nitrifier*	*(Daims et al., 2015)*
Fusobacteria	***Fusobacteriia***	***Fusobacteriales***	***Fusobacteriaceae***	***Cetobacterium***	***somerae***		
Proteobacteria	***Beta***	***Burkholderiales***	***Comamonadaceae*7***				
Proteobacteria	*Alpha*	*Sphingomonadales*	*Sphingomonadaceae*				
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Luteolibacter*		*Plant rhizopshere*	*(Nunes Da Rocha et al., 2011; Nunes da Rocha et al., 2013)*
Proteobacteria	***Alpha***	***Rhizobiales***	***Hyphomicrobiaceae***	***Devosia***			
Proteobacteria	*Beta*	*Burkholderiales*	*Comamonadaceae*	*Rhodoferax*		*Photoheterotrophs found in freshwater*	*(Imhoff, 2006)*
Proteobacteria	*Alpha*	*Rhizobiales*3*				*Contributors to crop nitrogen needs*	*(Barnett et al., 2014)*
Proteobacteria	*Alpha*	*Rhodobacterales*	*Rhodobacteraceae*				
Proteobacteria	*Alpha*	*Rhodospirillales*	*Acetobacteraceae*				
Proteobacteria	*Gamma*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*viridiflava*	*Plant pathogen*	*(Almeida et al., 2012; Sarris et al., 2012)*

Proteobacteria	*Alpha*	*Rhodobacterales*	*Rhodobacteraceae*	*Rhodobacter*		*Benzene degrading*	
Proteobacteria	*Alpha*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*		*Nitrate reduction*	*(Takeuchi et al., 2001)*
Proteobacteria	*Gamma*	*Xanthomonadales*	*Xanthomonadaceae*				
Proteobacteria	*Alpha*	*Rhizobiales*	*Phyllobacteriaceae*	*Mesorhizobium*		*Symbiosis with legumes, nodulation*	*(Laranjo et al., 2014)*
Proteobacteria	*Alpha*	*Rhizobiales*	*Rhizobiaceae*				
Proteobacteria	*Alpha*	*Rhodospirillales*	*Rhodospirillaceae*				
Proteobacteria	*Alpha*	*Caulobacterales*	*Caulobacteraceae*				
Proteobacteria	*Beta*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*	*delafieldii*	*Found in wastewater treatment facility*	*(West, 2005)*
Proteobacteria	***Beta***	***Burkholderiales***	***Oxalobacteraceae***	***Ralstonia***			

- **Comparison of our core microbiota with the literature**

We generated the 100% core microbiota of the four samples studied by Schmautz *et al.* (2016b) in order to allow for a comparison. As Schmautz's study only contains four samples, their core microbiota at 100% is already quite rich and therefore we did not generate the 90% microbiota.

The *Comamonadaceae* family is present in both our and their 100% core microbiota thus either strongly establishing this family as part of the core microbiome of aquaponic systems or suggesting a DNA contamination in both the FastDNA Spin kit (Mp Biomedicals) and in the MoBio PowerSoil DNA isolation kit (Mo Bio Laboratories).

The *Novosphingobium* genus, the *Pseudomonas* genus, the *Rhodospirillaceae* family and the *Burkholderiales* order are common between both studies.

The *Burkholderiales* order has been particularly found by Schmautz *et al.* (2016b) in there root sample and they consider that some species could be involved in associations with plants.

Some *Pseudomonas* species can be involved in the mineralisation of organic matter (Ley and De Vos, 1984; Kahlon, 2016).

The *Rhodospirillaceae* family contains the *Azospirillum* genus which himself contains some PGPR (Baldani *et al.*, 2014).

Lastly, a principal component analysis has been conducted on a dataset containing both our data and the ones obtained from Schmautz *et al.* (2016b) (Figure 18). Their samples collected from the plant roots, periphyton and biofilter compartments are clustered with ours while their fish faeces sample is quite isolated on Axis 1. This shows that there is a common pattern concerning the composition of the bacteria community in diversified aquaponic systems regardless of different compartments and extraction methods.

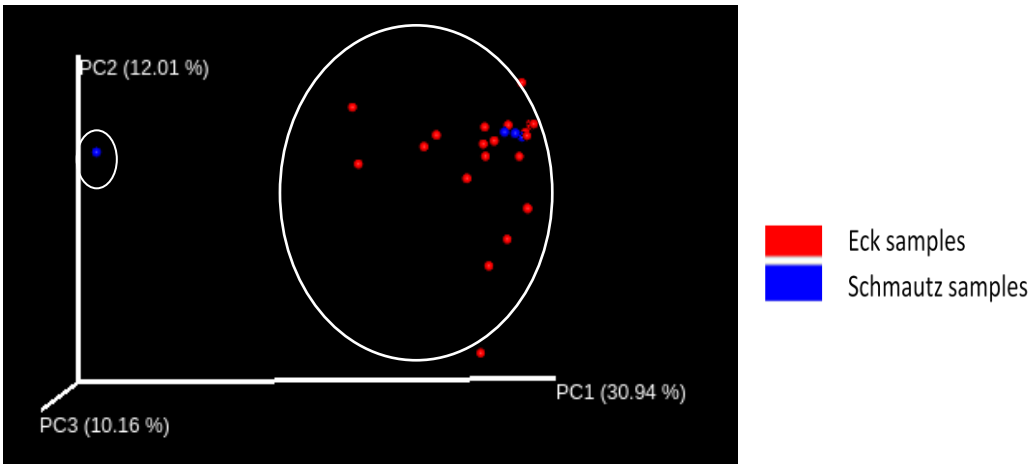

Figure 18. Weigthed UNIFRAC Principal component analysis including our 22 samples plus those from Schmautz's study.

4.2.4. Specific observations

- **Extra samples**

Two samples were collected in unusual compartments, namely the UrbanFarmers biofilms sample that originates from the biofilm located on the walls of the fish tanks in the UF system and the BQF denitrification biofilter sample.

The biofilm sample was collected as a request from the manager of the aquaponic system as he noticed the apparition of off-flavour in the fish and was wondering whether this was due to the fact that the fish often eat the periphyton. However, the literature reveals that the off-flavour in fish in mostly caused by *Streptomyces* which were not found in this sample. This sample contains mostly the *Comamonadaceae* family and the *Cetobacterium* genus which have not, to our knowledge, been linked to off-flavour.

As BQF is an aquaculture system and not an aquaponic system, they have no interest in keeping a high concentration of nitrate in their water. Therefore, the water flows first through a nitrifying biofilter which transforms the TAN into nitrates and then is directed towards a denitrifying biofilter where nitrates are turned into nitrogen (N_2) gaz. It is important to note that both filters were naturally colonised by bacteria. This sample presents a majority of *Arcobacter*, which, as already mentioned, seem to be nitrogen fixator (Munguia-Fragozo *et al.*, 2015). This sample also hosts the *Dokdonella* which is a common denitrifier (Tian *et al.*, 2014).

- **Pathogens**

No *Escherichia* or *Salmonella* were found in our samples even though they are considered by Munguia-Fragozo *et al.* (2015) as common indicators of faecal contamination. However, some *Flavobacterium*, *Comamonas* species were found in our study and could be pathogenic but according to the managers of the systems visited, no particular illnesses were observed. The *Clavibacter* genus which represent 33% of the reads of the RAS' sump sample in Gembloux also contains some pathogenic species (Riley *et al.*, 1988) but no illness was noted in this system.

4.3. Comparison of the bacteria communities' composition between groups of samples

The previous section enabled us to have a detailed view of all the taxa present in our samples. In order to identify elements which could explain the repartition of these taxa between our samples, several comparisons between groups of samples will now be undertaken. Table 6 offers a recapitulative view of all the samples and of the parameters used for the following comparisons.

Table 6. Description of the samples collected for our study. Each column corresponds to a discriminating factor and each factor encompasses several objects used to group the samples. J-perch: Jade-Perch; P-perch: Pike-Perch; bio: biofilter; bioden: denitrification biofilter.

Sample ID	Partner	Species	Fish density	Feed type	Zone	System
GBX RAS S	Gembloux-RAS	Tilapia	High	Vegetarian	Sump	Decoupled
GBX RAS B	Gembloux-RAS	Tilapia	High	Vegetarian	Bio	Decoupled
UF S	UrbanFarmers	Tilapia	High	Omnivorous	Sump	Coupled
UF B	UrbanFarmers	Tilapia	High	Omnivorous	Bio	Coupled
UF biofilm	UrbanFarmers	Tilapia	High	Omnivorous	Biofilm	Coupled
PCG S 60	PCG	J-Perch	Low	Vegetarian	Sump	Decoupled
PCG B 60	PCG	J-Perch	Low	Vegetarian	Bio	Decoupled
PCG S 100	PCG	J-Perch	High	Vegetarian	Sump	Decoupled
PCG B 100	PCG	J-Perch	High	Vegetarian	Bio	Decoupled
BQF S	BQF	Sturgeon	No data	Omnivorous	Sump	Decoupled
BQF B	BQF	Sturgeon	No data	Omnivorous	Bio	Decoupled
BQF DB	BQF	Sturgeon	No data	Omnivorous	Bioden	Decoupled
IGB S	IGB	Tilapia	High	Omnivorous	Sump	Decoupled
IGB B	IGB	Tilapia	High	Omnivorous	Bio	Decoupled
Wageningen S cat	Wageningen	Catfish	No data	No data	Sump	Decoupled
Wageningen S eel	Wageningen	Eel	No data	No data	Sump	Decoupled
Wageningen B eel	Wageningen	Eel	No data	No data	Bio	Decoupled
Inagro S fi	Inagro	P-Perch	Low	Omnivorous	Sump	Decoupled
Inagro S pl	Inagro	P-Perch	Low	Omnivorous	Sump	Decoupled
Inagro B	Inagro	P-Perch	Low	Omnivorous	Bio	Decoupled
GBX PAFF Box S	Gembloux-PB	Tilapia	Low	Vegetarian	Sump	Coupled
GBX PAFF Box B	Gembloux-PB	Tilapia	Low	Vegetarian	Bio	Coupled

We hereby wish to highlight the fact that this thesis aims at exploring the diversity of bacteria communities in diversified aquaponic systems and that no experiment was undertaken to clearly discriminate between factors influencing the composition of the communities. The following paragraphs will thus contain observations based on relative abundances bar charts and hypothesis.

4.3.1. Comparison of the composition of bacteria communities in each partner's system:

- **Sumps**

Figure 19 presents the taxa composing the bacteria communities in each of the partners' sumps. We can see that they are quite different from each other.

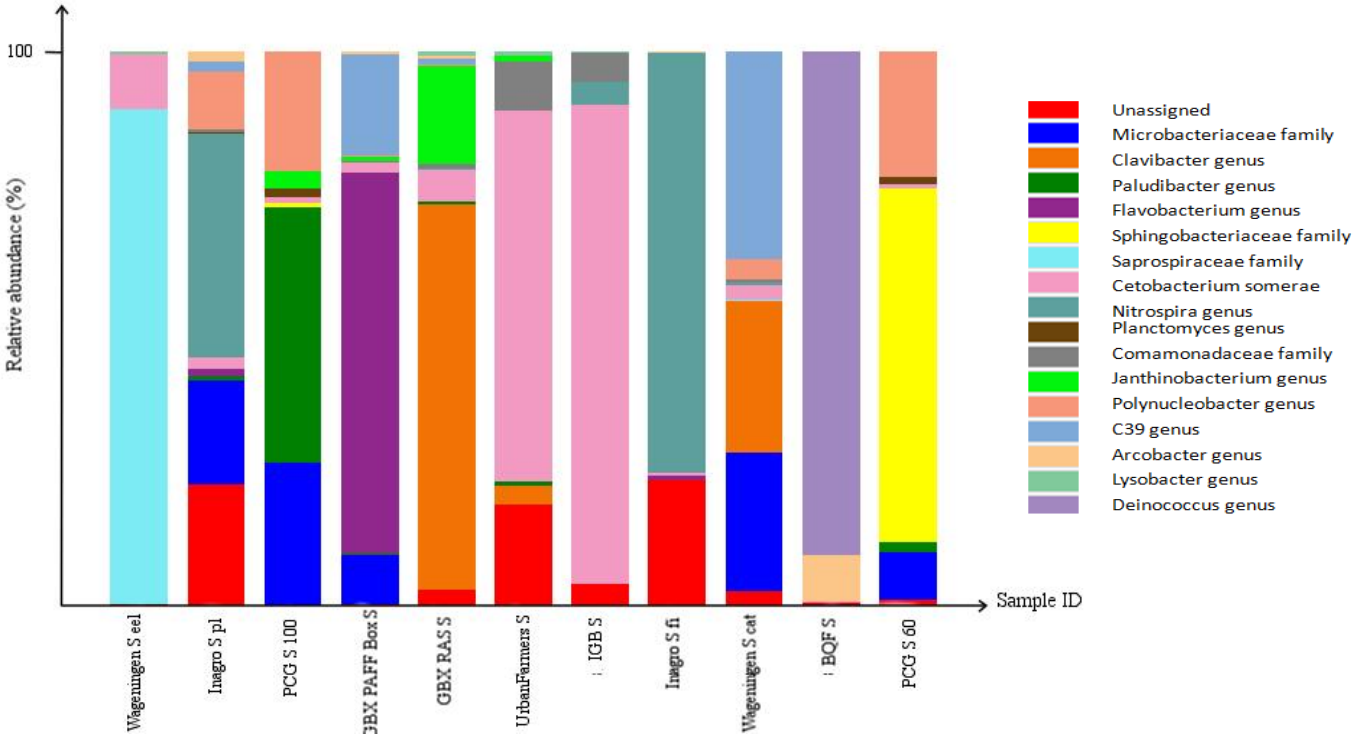

Figure 19. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads. Each bar represents a specific sump. Each colour represents one taxon. The size of each colour box is proportional to the relative abundance of the corresponding taxon. Wageningen S eel: Wageningen eel sump; Inagro S pl: Inagro sump plant loop; PCG S 100: PCG sump 100 fish/tank; GBX PAFF Box S; Gembloux PAFF Box sump; GBX RAS S: Gembloux RAS sump; UrbanFarmers S: UrbanFarmers sump; IGB S: IGB sump; Inagro S fi: Inagro sump fish loop; Wageningen S cat: Wageningen sump catfish; BQF S: BQF sump; PCG S 60: PCG sump 60 fish/tank.

- **Biofilters**

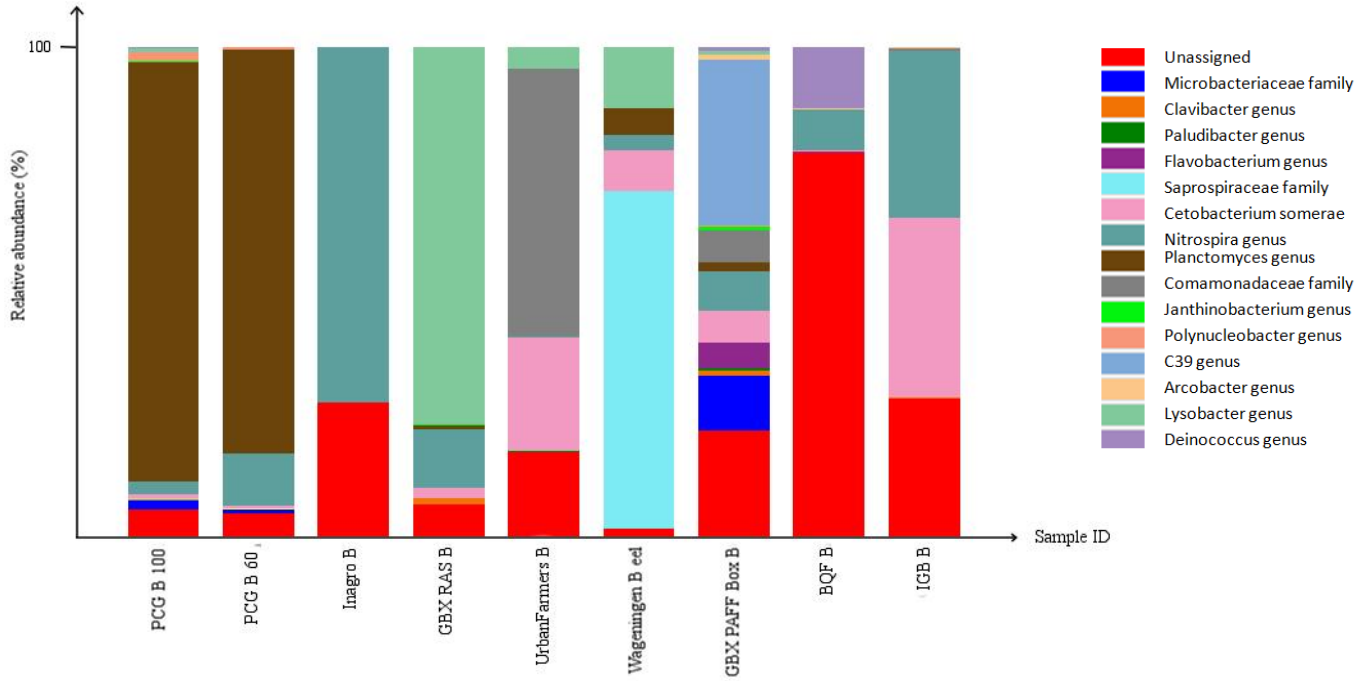

Figure 20. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads. Each bar represents a specific biofilter. Each colour represents one taxon. The size of each colour box is proportional to the relative abundance of the corresponding taxon. PCG B 100: PCG biofilter 100 fish/tank; PCG B 60: PCG biofilter 60 fish/tank; Inagro B: Inagro biofilter; GBX RAS B: Gembloux RAS biofilter; UrbanFarmers B: UrbanFarmers biofilter; Wageningen B eel: Wageningen eel biofilter; GBX PAFF Box B: Gembloux PAFF Box biofilter; BQF B: BQF nitrification biofilter; IGB B: IGB biofilter.

At first sight the bacteria communities composing the biofilter (Figure 20) seem less diversified than those in the sumps (Figure 19) and a common pattern seems more distinguishable between the biofilters. Indeed, the genera seemingly most frequent are the *Planctomyces* genus which dominate the PCG biofilters, the *Nitrospira* genus, the *Lysobacter* genus, the *Comamonadaceae* family and the *Saprospiraceae* family. More details concerning the diversity in the sumps and in the biofilters will be given in 4.3.3.

The two biofilter samples collected from the PCG systems contained EcoPondchip Filtermedium which is quite different from the classic Kaldnes media used in the other visited systems. As the two PCG samples are also quite different from the other biofilter samples with a predominance of the *Planctomyces* genus, we could raise the hypothesis that the biomedia type could influence the composition of the bacteria community.

It appears that there are more unassigned sequences originating from the biofilter samples than from the sump samples. This is in accordance with the observations made in previous sections were we discuss the representativeness of our samples. This could be partly explained by the fact that more PCR inhibitors are present in the biofilter samples as more organic matter clogged inside the biomedia is collected during the sample preparation step.

It is important to note that different time intervals have elapsed for each system between sampling and the beginning of the DNA extraction (see 3.1). This could have led to a slight shift in the composition of the bacteria communities due to a lack of oxygen.

4.3.2. Comparison of the composition of bacteria communities between fish species

As almost each visited system reared a different species of fish, it is not very pertinent to analyse again the differences of bacteria community between fish species. Only the Tilapia species was found in several systems and it is thus interesting to look whether some obvious similarities can be seen.

Cetobacterium somerae and the *Comamonadaceae* family are present in all Tilapia samples (Figure 21) which is coherent as they are part of the 100% core microbiota (see 4.2.3). The Lysobacter genus (greyish-green) is found in 8 Tilapia samples and in the eel samples but not at all in other samples.

Two interesting taxa are found in all Tilapias samples, namely *Thermomonas fusca* species and the genus *Dokdonella*. Both have already been found by Schmautz *et al.* (2016b) and seem to be common denitrifiers (Mergaert *et al.*, 2003; Tian *et al.*, 2014). Further research concerning these taxa would be interesting in order to better understand the complexity of the nitrogen cycle in an aquaponic system.

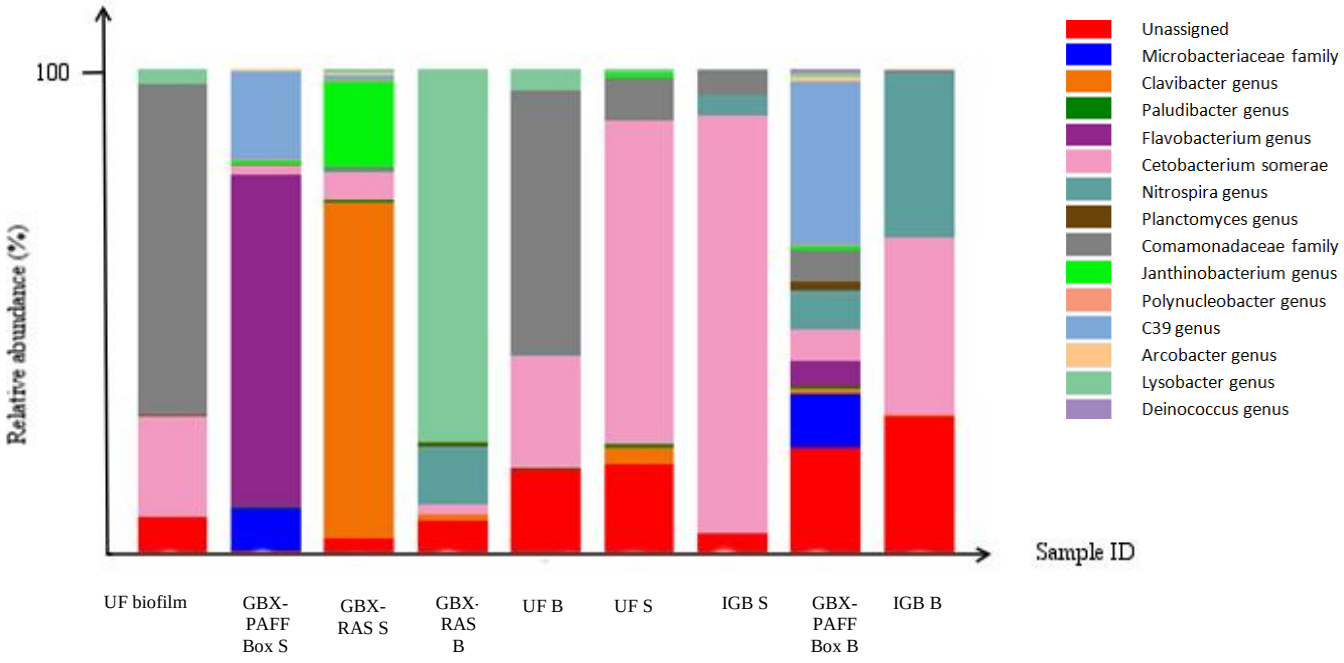

Figure 21. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads, per sample of systems where Tilapia are reared. Each colour represents one taxon. The size of the colour blocks is proportional to the relative abundance of the corresponding taxa. UF biofilms: UrbanFarmers biofilms; GBX PAFF Box S: Gembloux PAFF Box sump; GBX RAS S: Gembloux RAS sump; GBX RAS B: Gembloux RAS biofilter; UF B: UrbanFarmers biofilter; UF S: UrbanFarmers sump; IGB S: IGB sump; GBX PAFF Box B: Gembloux PAFF Box biofilter; IGB B: IGB biofilter.

As no obvious similarities can be observed between all the Tilapias samples (Figure 21), we can emit the hypothesis that despite the fact that each fish species brings its own specific microbiota (Schreier *et al.*, 2010), this is not sufficient to significantly influence the total bacteria community of an aquaponic system. Furthermore, a principal component analysis has been carried out on all our samples and confirms this impression of heterogeneity amongst the Tilapia samples, as it is possible to see that the several Tilapia samples are apparently not particularly clustered (Figure 22).

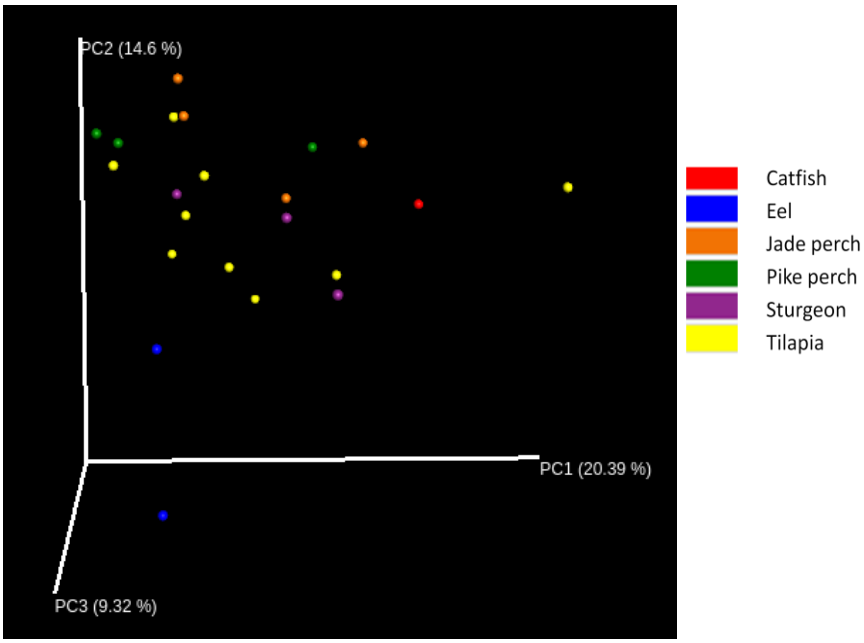

Figure 22. Weighted UNIFRAC Principal component analysis showing the differences in OTUs between the various fish species

4.3.3. Comparison of the composition of bacteria communities between sump and biofilter

The first taxon that draws the attention in this section is the *Nitrospira* genus. Indeed, it has already been observed that it is the major nitrifying genus in our samples. When it comes to the comparison of sump and biofilter, the first arising question is whether there is a significant difference between the amount of *Nitrospira* in the sump and in the biofilter. According to the literature, the biofilter should be the favourite compartment of nitrifying bacteria as they can form biofilms on the biomedia. However, this theory has been recently contested (Silva *et al.*, 2017) arguing that nitrifying bacteria can thrive very well in tank walls and hydroponic media and thus making the use of biofilter redundant and uselessly complicated.

The conducted Kruskal-Wallis test however did not reveal any significant differences between the number of occurrences of the Nitrospira genus in the sumps and in the biofilters. The *Nitrospira* genus is however part of the biofilters' samples 100% core microbiota and not of the sumps samples 100% core microbiota. The *Nitrospira* genus is nonetheless part of the 90% core microbiota of the sumps samples. The *Nitrospira* bacteria are thus found both in the sump and biofilter but we do not know whether free-floating *Nitrospira* are as efficient when it comes to the nitrification process as the ones settled in biofilms in the biofilter.

When it comes to the *Nitrosomonas* and *Nitrobacter* genera we can note the presence of the *Nitrosomonadaceae* and *Bradyrhizobiaceae* families in the biofilters (0,6% and 0,2% of total reads) and very slightly in the sumps (0,1% and 0,0%) of total reads). However the Kruskal-Wallis test did not reveal any significant differences. Moreover these two families are neither part of the biofilter 100% core microbiota of the biofilters samples nor of the 90% core microbiota of the biofilters samples.

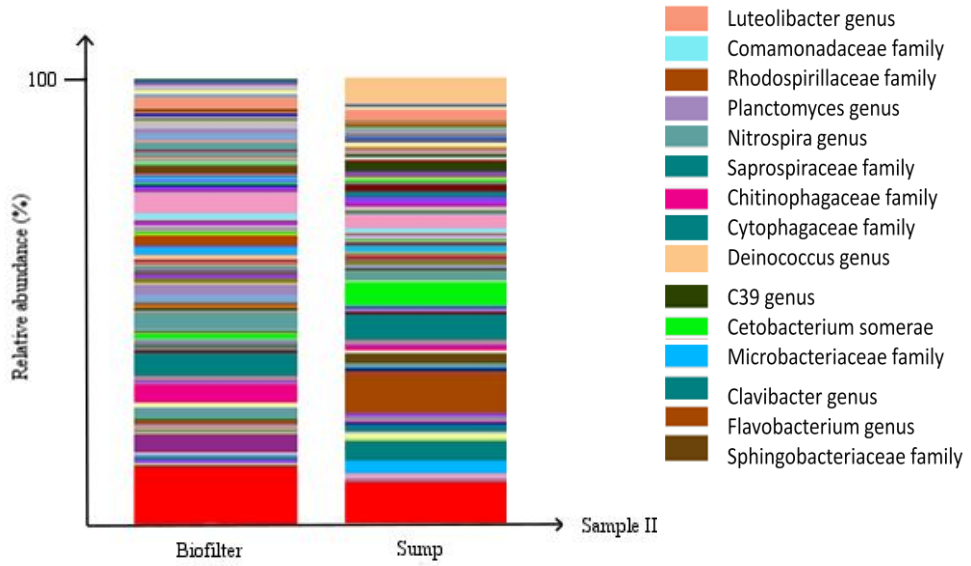

Figure 23. Bar charts of the relative abundances of the taxa present in the biofilters and sumps of all visited systems. Each colour represents one taxon. The size of each colour block is proportional to the relative abundance of the corresponding taxon. The taxa representing more than 2% of either of the two groups of samples were included in the legend.

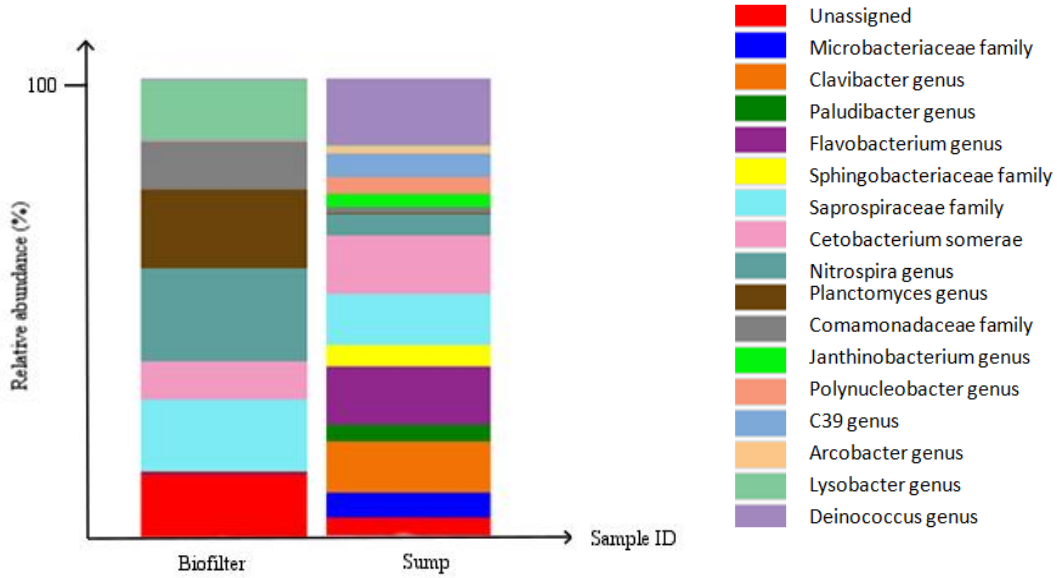

Figure 24. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads and present in the biofilters and sumps of all visited samples. Each colour represents one taxon. The size of each colour block is proportional to the relative abundance of the corresponding taxon.

Figure 23 and Figure 24 present the taxa composing the biofilters and sumps sample in an unfiltered (Figure 23) and 0.5% filtered manner (Figure 24). Before filtering the biofilter group seems more diverse than the sump whereas after filtering we can observe the contrary. The biofilters must then host a wide variety of different taxa in tiny proportions. The Shannon indexes of the filtered and unfiltered data were calculated. Concerning the unfiltered data, there is a statistically significant difference ($p = 0.025$) between the alpha-diversities.

A principal component analysis has been generated to visualise whether the sumps and biofilters samples could be distinguished based on their taxa composition (Figure 25).

Figure 25 shows that the biofilters samples are clustered together on the left of Axis 1 whereas the sumps samples are more disseminated even though more present on the right of Axis 1. Biofilters and sumps thus seem to host different bacteria communities.

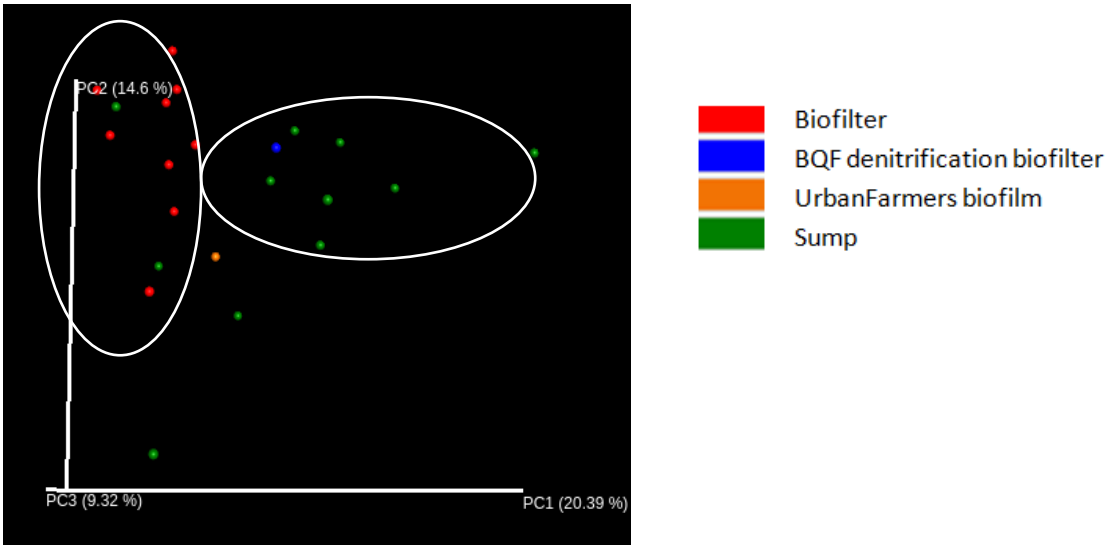

Figure 25. Weighted UNIFRAC Principal component analysis presenting the separation between the sumps and the biofilters samples on Axis 1.

In the sump samples' core microbiota, several genera which are not present in the biofilters can be found such as *Chryseobacterium* and *Hymenobacter*.

Bacteria from the ***Chryseobacterium*** genus used to be part of the *Flavobacterium* genus and still belong to the *Flavobacteriaceae* family. Some species can be found in soils and freshwater. Some strains of *Chryseobacterium* can convert nitrate into gaseous nitrogen and some others have been found in lettuce rhizosphere (Bernardet *et al*., 2006).

The genus ***Hymenobacter*** contains "oligotrophic soil bacteria" and is also phylogenetically linked with the *Flavobacterium* genus. Only three species compose this genus and only one of them has been found in soils, thus having a potential interest in aquaponics (Oren, 2006).

4.3.4. Comparison of the composition of bacteria communities between sumps from the same system

An interesting aspect could be the comparison between the two sump samples originating from the Inagro system. The 'Inagro sump fi' sample was taken from the water going back to the fish in the aquaculture loop and 'Inagro sump pl' from the deviated loop directed towards the tomatoes, after the sedimenter. We can note that 16% of the sample from the aquaculture is occupied by the *Nitrospira* genus when this value is reduced to only 1% in the sample from the plant loop (Figure 26). To elucidate the reason of this difference it would be interesting to study the sludge retained in the sedimenter in order to verify whether bacteria could have settled in it or not. However we can already make the assumption that the fish loop is an environment poorer in organic carbon thus promoting the development of autotrophic bacteria against heterotrophic ones. Inside and after the sedimenter, the environment is richer in organic carbon and poorer in oxygen and the more competitive heterotrophs can thrive again.

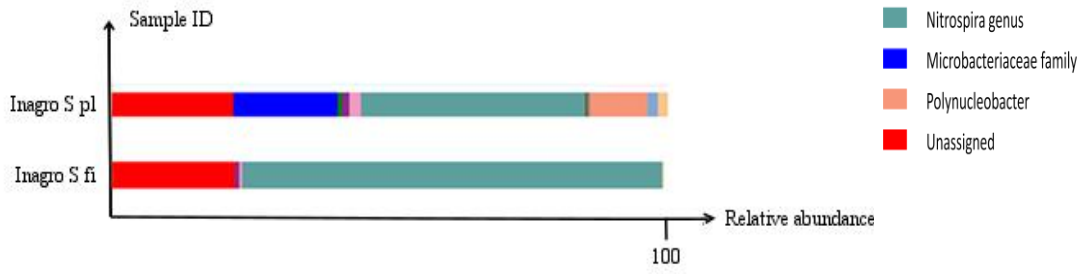

Figure 26. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads in the the samples taken in the Inagro sump in the fish loop and after the sedimenter.. Each colour represents a taxon and the size of the colour blocks represents the relative abundance of the corresponding taxon. The taxa representing more than 10% of any of the two samples have been included in the legend. Inagro S pl: Inagro sump plant loop; Inagro sump fish loop.

4.3.5. Comparison of the composition of bacteria communities between fish densities

In order to analyse the effect of the fish density on the bacteria community we can rely on a more accurate comparison than by grouping samples from diversified systems. Indeed, PCG currently conducts an experiment involving two different densities of fish (see 3.1) and samples were taken from the sumps and biofilters of a "high density" system and a "low density" one.

Both sumps seem quite different (Figure 27) with a predominance of the *Sphingobacteriaceae* family in the "low density" sample and a predominance of the *Paludibacter* genus in the "high density sample". The common taxa are the *Polynucleobacter* genus and the *Microbacteriaceae* family which, contrary to the *Sphingobacteriaceae* and *Paludibacter*, are also quite common in the other samples of our study

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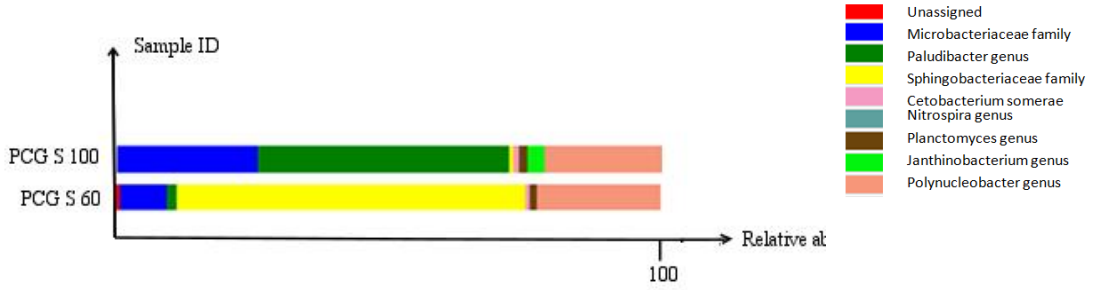

Figure 27. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads in the two sump samples collected from the PCG system. Each colour represents one taxon. The size of each colour block is proportional to the relative abundance of the corresponding taxon. PCG S 100: PCG sump 100 fish/tank (high density); PCG S 60: PCG sump 60 fish/tank (low density).

On the other hand, the two biofilters samples are much more similar (Figure 28) being both highly dominated by the *Planctomyces* genus. The other common genus is the *Nitrospira* which is slightly more present in the "low density" biofilter.

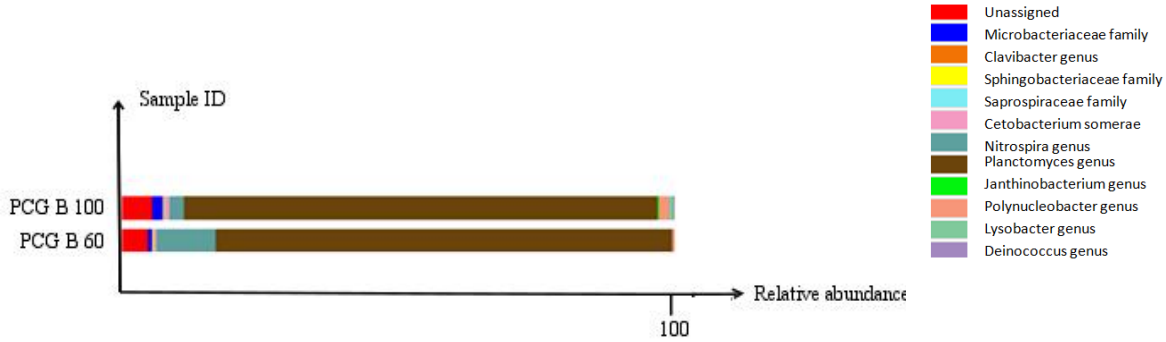

Figure 28. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads in the two biofilter samples collected from the PCG system. Each colour represents one taxon. The size of each colour block is proportional to the relative abundance of the corresponding taxon. PCG B 100: PCG biofilter 100 fish/tank (high density); PCG B 60: PCG biofilter 60 fish/tank (low density).

4.3.6. Comparison of the composition of bacteria communities between feed types

As the fish feed is the principal nutrient input in an aquaponic system, it is interesting to see whether it can influence the composition of the bacteria community. Therefore, our samples were grouped between those using vegetarian feed provided by the Aqua4C company (3.2mm Omegabaars Grower, AQUA4C, Kruishoutem, Belgium) and those using omnivorous feed. Figure 29 shows that both groups seem to host different taxa. Indeed, in the vegetarian feed group can be seen the *Microbacteriaceae* family with notably the *Clavibacter* genus, the *Paludibacter* genus, the *Flavobacterium* genus, the *Sphingobacteriaceae* family, the *Planctomyces* genus, the *Janthinobacterium* genus, the *Polynucleobacter* genus, the *C39* genus and the *Lysobacter* genus. The omnivorous feed type group on the other hand contains *Cetobacterium somerae*, the *Nitrospira* genus, the *Comamonadaceae* family, the *Arcobacter* genus and the *Deinoccocus* genus.

It is interesting to note that even though the vegetarian group is composed of only eight samples instead of eleven, it seems to host more genera than the omnivorous group. The Shannon diversity indexes of both groups are however not statistically different ($p = 1.0$).

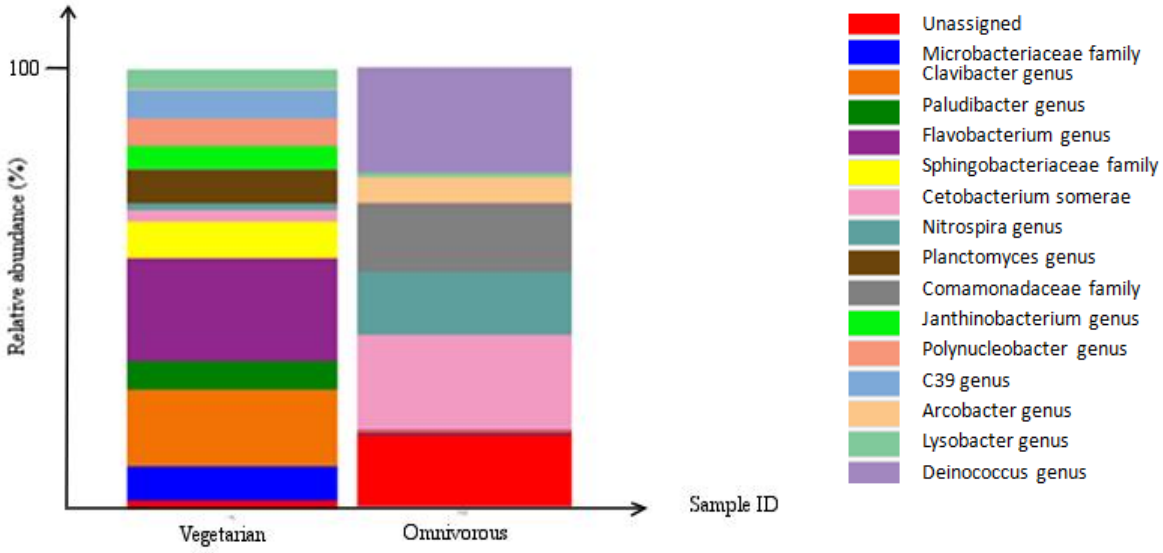

Figure 29. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads and present in the systems fed with vegetarian and omnivorous feed. Each colour represents one taxon. The size of each colour block is proportional to the relative abundance of the corresponding taxon.

4.3.7. Comparison of the composition of bacteria communities between "coupled" and "decoupled" systems

The visited aquaponic systems have been classified between "coupled" and "decoupled" aquaponics. We have considered that systems in which the water flowing through the hydroponic part went back to the fish, even partially, could be called "coupled". The coupled systems include the PAFF Box and the UrbanFarmers system.

However, to allow a better comparison between these two objects we focused on the RAS of Gembloux Agro-Bio Tech and on the PAFF Box . Indeed, these two systems share the same fish, same feed type and are managed by the same people thus rendering a comparison much easier and robust. Figure 30 opposes the two sumps and

Figure 31 the two biofilters. The RAS sump sample is dominated by *Clavibacter* and *Janthinobacterium* whereas the PAFF Box sample presents a majority of *Flavobacterium* and *C39*. The RAS biofilter sample is clearly dominated by *Lysobacter* and also hosts some *Nitrospira* when the PAFF Box biofilter presents *C39*, *Nitrospira*, *Flavobacterium* and the *Microbacteriaceae* family. Each one of these two system layout seem to have a quite distinct bacteria community, common to their sump and biofilter.

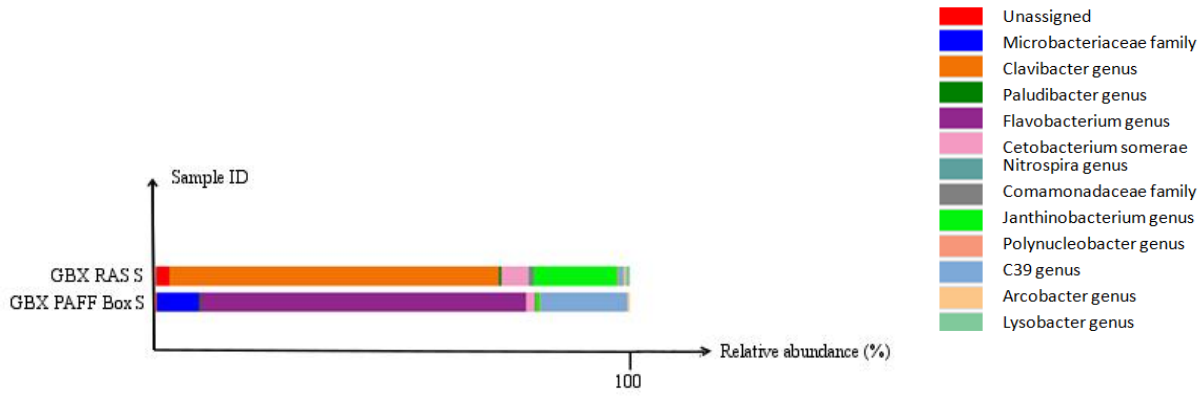

Figure 30. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads in the RAS and PAFF Box's sumps. Each colour represents one taxon. The size of each colour block is proportional to the relative abundance of the corresponding taxon.

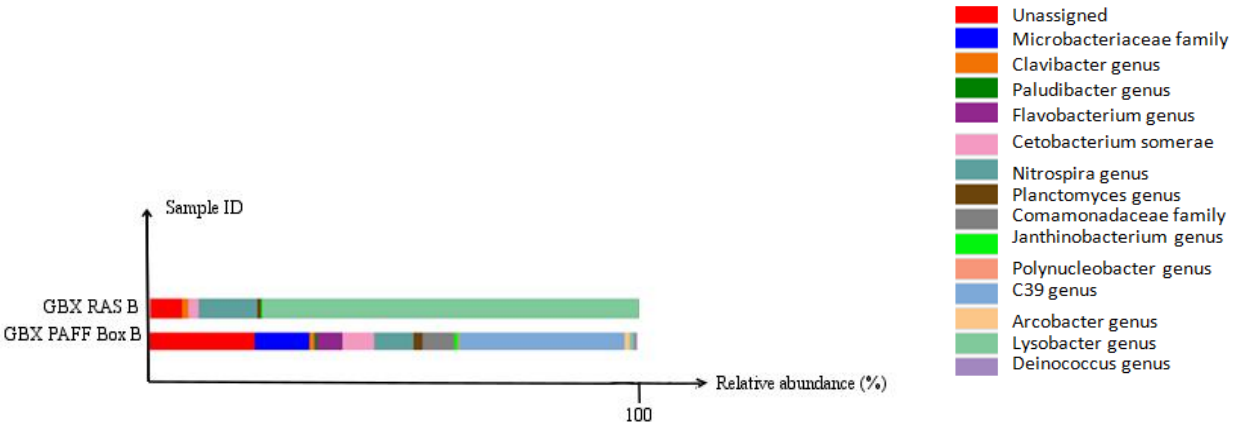

Figure 31. Bar charts of the relative abundances of the taxa representing more than 0.5% of the total reads in the RAS and PAFF Box's biofilters. Each colour represents one taxon. The size of each colour block is proportional to the relative abundance of the corresponding taxon.

5. CONCLUSION AND PERSPECTIVES

5.1. Taxa observed

The first goal of this study was the broad taxonomic characterisation of the bacteria communities in aquaponic systems. In this view, diversified aquaponic systems were sampled in two compartments, namely the biofilter and the sump.

The taxa were studied using two different angles. Firstly, the taxa visibly predominant in our samples were analysed. The major phyla that were found were the *Proteobacteria* and the *Bacteroidetes* which represent almost 50% of the total reads in our samples. The 50% left are shared between the *Fusobacteria*, *Actinobacteria*, *Nitrospirae*, *Planctomycetes*, *Verrucomicrobia* and *Thermi* which were less predominant but visible in most samples. These results correspond to the observations made by Schmautz *et al.* (2016) and Munguia-Fragozo *et al.* (2015) in their own characterisation of microbiota in aquaponic systems.

At the family and genus level, the taxa most present in our study were the *Saprospiraceae* family, the *Flavobacterium* genus, the *Comamonadaceae* family, the *Cetobacterium somerae* species, the *Deinoccocus* genus, the *Nitrospira* genus, the *Clavibacter* genus, the *Microbacteriaceae* family, the C39 genus, the *Sphingobacteriaceae* family, the *Planctomyces* genus, the *Polynucleobacter* genus, the *Lysobacter* genus, the *Paludibacter* genus and the *Janthinobacterium* genus.

Secondly, the core microbiota i.e. the taxa present in 100 or 90% of the samples was studied. In this 100% core microbiota, we could observe taxa such as the *Oxalobacteraceae* family, the *Comamonadaceae* family, the *Sphingomonas* genus and the species *Cetobacterium somerae*. The 90% core microbiota contained more taxa than the 100% core microbiota. The study of this global core microbiota enables us to have an idea of the bacteria present in several diversified aquaponic systems despite differences in fish species, system layout or fish feed. However, these results need to be nuanced as a lot of the genera belonging to the core microbiota could also originate from contaminating DNA present in the DNA extraction kit.

The core microbiota from the biofilter and sump groups were also analysed. The biofilter core microbiota contained notably the *Nitrospira* genus confirming that this complete nitrifier thrives in the biofilter compartment.

The use of these two study angles enables us to have a broad view of the bacteria present in diversified aquaponic systems. Indeed, we now know which bacteria are the most common in aquaponics despite different system layouts, fish species, fish feed type, fish density and in several compartments of the system.

5.2. Roles of these taxa

A secondary aim of this thesis was to understand the potential roles that these bacteria could play in an aquaponic system. In this view, bibliographic research was conducted to highlight the roles of the observed taxa which could suit the most to the functioning of an

aquaponic system. However, as the taxonomic identification did not determine the exact species, it was difficult to assign a specific function per taxa.

Findings show that a lot of the detected taxa such as the *Oxalobacteraceae* family and *Sphingomonas* genus could be involved in associations with plants. Other taxa such as the *Flavobacterium* and *Luteolibacter* genera could also be found in plants' rhizospheres.

Some taxa such as the *Saprospiraceae* family could be involved in the breakdown of organic matter thus going along the idea that bacteria take part in the crucial process of mineralisation of fish feed leftovers and fish faeces into absorbable nutrients for the plant.

Finally, some other genera are involved in the nitrogen cycle. As confirmed by Schmautz *et al*. (2016b), the *Nitrospira* genus is the most present among known nitrifiers and could very well complete the nitrification process on its own (Daims *et al*., 2015). We found several less known genera of bacteria rather involved in atmospheric nitrogen fixation and also denitrification such as the *Novosphingobium* or *Arcobacter*. This observation could lead to the thought that the nitrogen cycle is more complex than expected and that a fragile balance between nitrogen transformations has to be maintained.

5.3. Perspectives

This study was a first investigation into the diversity of bacteria communities present in aquaponic systems and offers a global view of the genera living there. However, in order to better understand the composition and functioning of these bacteria communities, species identification would be interesting.

Moreover, it is very important to be certain that the identified bacteria belong to the aquaponic systems and are not detected because of contaminating DNA in the extraction kit. Therefore, the kit should be used to extract DNA from a pure bacteria culture which would enable to identify and quantify the contaminating DNA.

To better understand the roles of the present bacteria and their impact on the balance and productivity of an aquaponic system, it could also be interesting to identify the most expressed genes through the use of metatranscriptomic techniques.

Because many of the observed taxa seemed to be involved in the rhizosphere interactions with plants, complementary analysis on the rhizosphere of plants in the systems where it is possible would also be interesting. As a first step, lettuce rhizosphere samples were collected from the PAFF Box and its microbiota was washed and collected.

We have tried to discriminate bacteria communities based on descriptive factors such as the visited system, the fish species, the sampling compartment, the fish density, the system layout and the type of fish feed. Indeed, knowing which factor could influence the composition of the bacteria community could enable aquaponics practitioners to foster the presence of useful taxa. However, the systems were too diversified to allow for a robust comparison. Trends could be highlighted but it was not possible to impute the difference observed to the studied factor. Therefore specific experiments should be set into place with only one varying factor in order to be able to allot a difference in bacteria community

composition to this varying factor only. Moreover, repetitions should be carried out to ensure a better representativeness of the observed taxa.

Lastly, the composition of bacteria communities is prone to evolution throughout the time. However, all the visited system have not been launched at the same time, some keep going all year round when others are stopped for a few months before being started up again. An experiment has been conducted to study the evolution of the bacteria community's composition in the PAFF Box during three weeks in order to further look into the variability of these communities with time.

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7. ANNEXES

7.1. Annex 1 : Recapitulative table of the samples' characteristics

DNAVision ID	Sample ID	Partner	Coupled/decoupled	Species	Fish density	Feed type	Sampling zone
DNA17013-001	GBX RAS S	Gembloux-RAS	Decoupled	Tilapia	60kg/m³	Vegetarian	2L sump water
DNA17013-002	GBX RAS B	Gembloux-RAS	Decoupled	Tilapia	60kg/m³	Vegetarian	Ceramic biofilter
DNA17013-003	UF S	UrbanFarmers	Coupled	Tilapia	80kg/m³	Omnivorous	2L sump water
DNA17013-004	UF B	UrbanFarmers	Coupled	Tilapia	80kg/m³	Omnivorous	Biofilter biochips
DNA17013-005	UF biofilm	UrbanFarmers	Coupled	Tilapia	80kg/m³	Omnivorous	Biofilm tank wall
DNA17013-006	PCG S 60	PCG	Decoupled	Jade perch	33kg/m³	Vegetarian	2L sump water
DNA17013-007	PCG B 60	PCG	Decoupled	Jade perch	33kg/m³	Vegetarian	Biofilter biochips
DNA17013-008	PCG S 100	PCG	Decoupled	Jade perch	55kg/m³	Vegetarian	2L sump water
DNA17013-009	PCG B 100	PCG	Decoupled	Jade perch	55kg/m³	Vegetarian	Biofilter biochips
DNA17013-010	BQF S	BQF	No hydroponic part	Sturgeon	No data	Omnivorous	2L canal water
DNA17013-011	BQF B	BQF	No hydroponic part	Sturgeon	No data	Omnivorous	Biofilter biochips
DNA17013-012	BQF DB	BQF	No hydroponic part	Sturgeon	No data	Omnivorous	Denitrification biofilter biochips
DNA17013-013	IGB S	IGB	Decoupled	Tilapia	60kg/m³	Omnivorous	2L sump water
DNA17013-014	IGB B	IGB	Decoupled	Tilapia	60kg/m³	Omnivorous	Biofilter biochips
DNA17013-015	Wageningen S cat	Wageningen	No hydroponic part	Catfish	No data	No data	2L sump water
DNA17013-016	Wageningen S eel	Wageningen	No hydroponic part	Eel	No data	No data	2L sump ater
DNA17013-017	Wageningen B eel	Wageningen	No hydroponic part	Eel	No data	No data	Biofilter biochips
DNA17013-018	Inagro S fi	Inagro	Decoupled	Pike perch	40kg/m³	Omnivorous	2L sump water aquaculture loop
DNA17013-019	Inagro S pl	Inagro	Decoupled	Pike perch	40kg/m³	Omnivorous	2L sump water directed to the plants
DNA17013-020	Inagro B	Inagro	Decoupled	Pike perch	40kg/m³	Omnivorous	Biofilter biochips
DNA17013-021	GBX PAFF Box S1	Gembloux - PAFF Box	Coupled	Tilapia		Vegetarian	2L sump water, repetition 1
DNA17013-022	GBX PAFF Box	Gembloux -	Coupled	Tilapia		Vegetarian	2L sump water, repetition 2

	S2	PAFF Box					
DNA17013-023	GBX PAFF Box S3	Gembloux - PAFF Box	Coupled	Tilapia		Vegetarian	2L sump water, repetition 3
DNA17013-024	GBX PAFF Box S4	Gembloux - PAFF Box	Coupled	Tilapia		Vegetarian	2L sump water, repetition 4
DNA17013-025	GBX PAFF Box B1	Gembloux - PAFF Box	Coupled	Tilapia		Vegetarian	Biofilter beads, repetition 1
DNA17013-026	GBX PAFF Box B2	Gembloux - PAFF Box	Coupled	Tilapia		Vegetarian	Biofilter beads, repetition 2
DNA17013-027	GBX PAFF Box B3	Gembloux - PAFF Box	Coupled	Tilapia		Vegetarian	Biofilter beads, repetition 3
DNA17013-028	GBX PAFF Box B4	Gembloux - PAFF Box	Coupled	Tilapia		Vegetarian	Biofilter beads, repetition 4

7.2. Annex 2: DNAVision'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 V3-V4 : TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Amplicon PCR primer Rvs V3-V4 : GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Amplicon PCR primer Fwd V5-V6 :
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

Amplicon PCR primer Rvs V5-V6 :
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

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
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
25x	95°C	30 sec
	55°C	30 sec
	72°C	30 sec
	72°C	5 min
	4°C	A l'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 :

	95°C	3 min
25x	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 :

$$\frac{\text{(concentration en ng/µl)}}{\text{(660 g/mol * taille moyenne de librairie)}} * 10^6 = \text{concentration en nM}$$

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 denatured 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

7.3. Annex 3: Unfiltered bar chart of the genera present in our study