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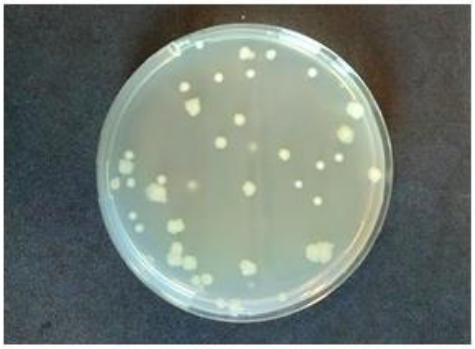
Taxonomical, ecological and functional exploration of aquaponics microbiota in interaction with lettuce growth

Mathilde ECK




COMMUNAUTÉ FRANCAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Taxonomical, ecological and functional exploration of aquaponics microbiota in interaction with lettuce growth

Mathilde ECK

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs : Pr. M. Haïssam Jijakli, Pr. Sébastien Massart

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Abstract

Aquaponics is emerging as an interesting alternative to decrease pressure on planetary resources and to limit the environmental impact of the food production systems. This new technique combines hydroponics i.e. the soilless production of vegetables, with recirculating aquaculture in several types of systems fitted to various environments. Aquaponics, as implemented nowadays, is a fairly novel production system and requires a thorough inquiry of several aspects of its functioning to ensure its viability and reliability. One of these crucial features regards the characterisation of microbial communities and their roles in nutrient cycling. The objective of this thesis is thus to address this huge gap of knowledge regarding microorganisms in aquaponics via the description of the bacterial communities, the study of the potentially plant beneficial functions which could be carried on by the microbiota and their interaction with lettuce growth.

In the first instance, this thesis focuses on the taxonomical description of the bacterial communities which can be found in various aquaponic and aquaculture systems and reports on the evolution of the different communities of one given system over nine weeks, using 16S rRNA gene sequencing. Results show that different systems harboured different microbiota although some common taxa could be found in all samples. It was also observed that within one system each compartment (sump, biofilter, plant roots) hosted specific microbiota and that those communities were relatively stable over time. Indeed, no adaptation period could be noted after the transplantation of seedlings into the system i.e. when the system previously functioning as a recirculating aquaculture system (RAS) was turned into a coupled aquaponic system - which is the main modification that the system underwent during the experiment. No modifications appeared either following the water parameters changes which naturally occur during the functioning of the system. As such it was concluded that the studied bacterial communities were resilient. Eventually, a taxonomic comparison between the lettuce root communities in the aquaponic system with literature regarding soil borne lettuce root communities showed intriguing similarities. This raises new questions regarding the origin of root microorganisms (i.e. seed or system) in aquaponics and its recruitment processes in soilless systems.

However, the use of metabarcoding only provides an overview of the global composition of the communities down to the genus level. Genus identification does not permit to identify specific roles or functions linked with nutrient cycling and plant growth. Consequently, the second part of this thesis focuses directly on the functions present in aquaponics and their potential roles in plant health and care. In this view,

31 bacterial strains were isolated from the sump of the coupled aquaponic system of Gembloux Agro-Bio Tech. Five potentially plant beneficial traits were targeted in these bacteria via the use of *in vitro* biochemical tests: i) phosphorus and ii) potassium solubilisation and the production of iii) indole acetic acid, iv) siderophores and v) ammonia. Three of the most promising strains were then selected for a series of *in vivo* trials to assess their impact on lettuce growth in aquaponics. In these trials, three treatments were compared i.e. a mix of the three strains (AHT), one of the strains alone (T) and a control without any inoculation. The AHT bacterial mix treatment provided encouraging results fostering the production of lettuce leaves in light related and nutritive stress conditions while the T strain alone treatment also impacted lettuce growth in stressful conditions albeit in smaller proportions than AHT. An upscaling of the trials would now be required to confirm these observations.

Overall, this thesis provided a first insight into bacterial communities in aquaponics and constitutes a stepping stone for more in depths research on the ecology of bacterial communities in aquaponics and their roles in interaction with crop growth.

Résumé

L'aquaponie se présente aujourd'hui comme une technique alternative permettant de diminuer la pression sur les ressources naturelles et de limiter l'impact environnemental des systèmes de production alimentaire. Cette technique innovante combine l'hydroponie, ou production végétale hors-sol, avec l'aquaculture recirculée dans différents types de systèmes adaptés à divers environnements. L'aquaponie telle que pratiquée actuellement requiert toutefois une meilleure compréhension de plusieurs aspects de son fonctionnement afin d'assurer sa viabilité et sa fiabilité. Un de ces éléments cruciaux est notamment l'étude des communautés microbiennes et de leurs rôles dans les cycles des nutriments. L'objectif de cette thèse est donc d'aborder cette lacune majeure via la description des communautés bactériennes présentes dans les systèmes aquaponiques en parallèle d'une analyse des fonctions potentiellement bénéfiques aux plantes qui pourraient être présentes dans ces communautés ainsi que de leurs interactions avec la croissance de la laitue.

Cette thèse se focalise en premier lieu sur la description taxonomique des communautés bactériennes observées dans différents systèmes aquaponiques et d'aquaculture ainsi que sur l'analyse de l'évolution des communautés d'un système donné pendant neuf semaines, sur base du séquençage à haut débit du gène 16S. Il a ainsi été montré que des systèmes distincts possédaient des microbiotes différents bien que quelques taxons communs puissent être détectés dans tous les échantillons. Par ailleurs, il a été souligné qu'au sein d'un seul système, chaque compartiment (sump, biofiltre, racines) présentait un microbiote spécifique et que ces communautés étaient relativement stables dans le temps. En effet, aucune phase d'adaptation n'a pu être notée au moment du transfert des plantules dans le système, soit au moment où le système fonctionnant jusqu'alors en aquaculture recirculée a été transformé en système aquaponique couplé – la modification la plus conséquente pratiquée au cours de l'expérience. Aucune modification n'est apparue non plus des suites des changements des paramètres de l'eau se produisant naturellement pendant le fonctionnement d'un système. Il a donc été suggéré que les communautés bactériennes en aquaponie étaient résilientes. Finalement, une comparaison taxonomique entre les communautés bactériennes des racines en aquaponie avec celle des laitues de pleine terre a montré d'importantes similitudes soulevant ainsi de nouvelles questions quant à l'origine des bactéries racinaires en aquaponie (graine ou système) et quant aux processus de recrutement microbiens dans les systèmes hors-sol.

Néanmoins, l'usage du metabarcoding sur base du gène 16S permet uniquement d'obtenir une vue globale de la composition de la communauté bactérienne en termes de genre. L'identification du genre ne fournit pas d'information quant aux rôles précis des bactéries identifiées ou à leurs interactions potentielles avec les plantes. C'est pourquoi le second volet de cette thèse s'intéresse directement aux fonctions présentes au sein des communautés bactériennes aquaponiques et à leurs rôles potentiels dans la croissance des plantes. Dans ce but, 31 souches bactériennes ont été extraites du sump du système aquaponique couplé de Gembloux Agro-Bio Tech et isolées au laboratoire. Cinq traits fonctionnels potentiellement bénéfiques pour la croissance des plantes ont ensuite été ciblés via la mise en place de tests biochimiques *in vitro* : i) la solubilisation du phosphore, ii) la solubilisation du potassium, iii) la production d'acide indole acétique, iv) de sidérophores et v) d'ammoniac. Les trois souches les plus prometteuses ont ensuite été sélectionnées pour une série d'essais *in vivo* dans le but d'évaluer leur impact sur la croissance de la laitue en aquaponie. Trois traitements ont été comparés : un mélange des trois souches bactériennes (AHT), une des souches seules (T) et un contrôle sans aucune inoculation bactérienne. Le mélange bactérien AHT a offert des résultats encourageants en promouvant la production de feuilles de laitue en conditions de stress lumineux et nutritif. Le traitement comprenant uniquement la souche T a également impacté la croissance de la laitue en conditions de stress quoique dans des moindres proportions que le mélange AHT. Il serait maintenant nécessaire de reproduire l'expérience sur une plus grande échelle afin de confirmer ces observations.

En conclusion, cette thèse a fourni un premier jeu de données concernant les communautés bactériennes en aquaponie et constitue un tremplin pour de nouvelles recherches poussées sur l'écologie de ces communautés et leurs rôles en interaction avec la croissance des plantes.

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Table of content

List of figures

List of tables

List of acronyms

UN: United Nations

FAO: Food and Agriculture Organisation of the United Nations

GDP: Gross Domestic Product

IME: Institution of Mechanical Engineers

FCR: Feed Conversion Ratio

GHG: Greenhouse Gas

GAEZ: Global Agro-Ecological Zones

NFT: Nutrient Film Technique

DWC: Deep Water Culture

RAS: Recirculating Aquaculture Systems

UV: Ultra-Violet

IAAS: Integrated Agri-Aquaculture Systems

EU: European Union

LCA: Life Cycle Assessment

UASB: Upflow Anaerobic Sludge Blanket

EGSB: Expanded Granular Sludge Bed

AOB: Ammonia Oxidising Bacteria

NOB: Nitrite Oxidising Bacteria

COMAMMOX: Complete Ammonia Oxidizer

ANAMMOX: Anaerobic Ammonium Oxidizer

PGPM: Plant Growth Promoting Microorganism

ISR: Induced Systemic Resistance

IAA: Indole Acetic Acid

ACC: 1-Aminocyclo-propane-1- Carboxylate

PQQ: Pyrrolquinoline

HCN: Hydrogen Cyanide

AMF: Arbuscular Mycorrhizal Fungi

IAM: Indole-3-Acetamide

Kb: Kilobase

Bp: Base Pair

NGS: Next Generation Sequencing

PCR: Polymerase Chain Reaction

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

rRNA: Ribosomial RNA

AQ: Aquaculture

AP: Aquaponics

ZHAW: Zürich University of Applied Sciences

EBI: European Bioinformatics Institute

SRA: Sequence Read Archive

NCBI: National Centre for Biotechnology Information

OTU: Operational Taxonomic Unit

PCoA: Principal Coordinates Analysis

PGPB: Plant Growth Promoting Bacteria

T: Temperature

EC: Electro-Conductivity

IUPPL: Integrated and Urban Plant Pathology Laboratory

DO: Dissolved Oxygen

KPBT: Kalium Phosphate Buffer and Tween

LB: Luria-Bertani

PDA: Potato Dextrose Agar

TSA: Tryptic Soy Agar

NYDA: Nutrient Yeast Dextrose Agar

PCA: Principal Components Analysis

ASV: Amplicon Sequence Variant

PAR: Photosynthetically Active Radiation

ISO: International Organisation for Standardisation

ANCOVA: Analysis Of Covariance

ANOVA: Analysis Of Variance

1.

Introduction

1.1 The global food challenges aquaponics is willing to address

As we increase the pressure on planetary boundaries (Conijn et al., 2018), Earth is more and more faced with new challenges and the simultaneous combination of these is greater than the sum of their parts. Indeed, with the continuous growth of human population comes an ever-increasing need for food production. This growing demand is also paralleled by an increase of wealth and thus rising consumption per capita and shift of the human diet towards animal proteins, putting even more pressure on already strained resources. At the environmental level, climate change, land degradation, depletion of natural resources and loss of biodiversity raise numerous concerns regarding this growing demand for food (Alexandratos and Bruinsma, 2012; Joyce et al., 2019a; Kahiluoto et al., 2014).

1.1.1 Human aspect

1.1.1.1 Increasing world population

According to the United Nations (UN), the world's population will reach 9.8 billion people in 2050 (UN, 2017) with 68% living in cities (55% in 2018) (UN, 2018). The number of megacities (more than 10 million inhabitants) in the world will rise to 43, mainly located in developing countries (Alexandratos and Bruinsma, 2012; UN, 2018, 2017)). Indeed, 90% of the increasing urbanisation is expected to take place in Asia and Africa. This growing urbanisation will be linked both to population growth in cities and rural exodus (rural population represented 66.4% of the total population in 1960 and dwindled to 45% in 2019 (FAO, 2019; Goddek et al., 2019a) Sustainable urbanisation and urban planning are thus key issues.

The rise in global population will result in a demand for a concomitant increase in food production. Indeed, experts estimate that the global food production will need to increase by 70% between 2005 and 2050 and that it should even double in developing countries (Conijn et al., 2018; FAO, 2009). These estimations can actually vary between 45% and 71% depending on the predictions for biofuels production and global food waste (Gott et al., 2019; Joyce et al., 2019a).

1.1.1.2 Unequal repartition of food and food waste

Waste represents an important factor in the calculations for future food demand and it is necessary to decrease food losses (estimated at 30% of the production all along

the supply chain (Gott et al., 2019) at various levels (Conijn et al., 2018; Joyce et al., 2019a; Kahiluoto et al., 2014). To avoid food waste during storage and transportation, a production system closer to the consumers seems relevant (Joyce et al., 2019a). This double increase in population and food needs will weight even more on already strained food systems, with new challenges such as the supplying of megacities and ensuring that all population has access to fresh, healthy, nutritious food.

In 2005, globally and taking into account the wasted food, there was enough food produced for everyone to have access to a 2,770 kcal/day diet. However, this was not the case due to unequal repartition of the food produced in the world and limited access to food in some countries (Alexandratos and Bruinsma, 2012). The challenge of food production should then not be addressed so much on the global level than on the local and regional level (Alexandratos and Bruinsma, 2012).

1.1.1.3 Income per capita increase and diet shift

Another increase that can be mentioned is the rise in net income or GDP per capita (Alexandratos and Bruinsma, 2012). This increase of income boosts the living standards (Beddington, 2010) which results in an ever swelling demand for energy, land and water and a change in the composition of the human diets (Alexandratos and Bruinsma, 2012; Beddington, 2010; Joyce et al., 2019a). As of now, part of the world's population lives on a diet based on vegetables, legumes, cereals with little meat. Due to population becoming wealthier and the increase of the economic middle class (FAO, 2019) this proportion is now changing with the inclusion of dairy products (consumption of milk has doubled in developing countries (FAO et al., 2012), eggs (increased 5 times in developing countries (FAO et al., 2012)) and poultry. The meat demand is expected to increase by 85% between 2000 and 2030 (Beddington, 2010). This shift towards a more animal based diet represents a supplementary strain on the planet's resources (need for land as well as water and livestock feed) (Garnett, 2011; Goddek et al., 2019a; Joyce et al., 2019a). Indeed, the resources required to produce one kilo of meat are much higher than those needed for the production of cereals that humans could eat directly (e.g. one kilo of beef requires 15,415 L of water while one kilo of rice requires only 2,497 (IME, 2013). Aside from water consumption, the production of animal products also requires more land for grazing and also arable lands to produce livestock feed. For example, the feed conversion ratio (FCR) of beef reaches 25 kg of feed required to produce a kilo of meat (Goddek et al., 2019a). Unfortunately, land and water, amongst others, are not infinite resources.

The assumptions are that food production will have to grow by 1.1% - 1.5% every year until 2050 to meet population growth and change in diets (Alexandratos and Bruinsma, 2012).

1.1.1.4 World fish demand and production

- **Fish demand**

In the context of this dietary shift towards animal protein, fish gain even more importance. Indeed, global fish consumption augmented by 3.1% per year between 1961 and 2017 which is more than the yearly world population growth (1.6%) during the same period and also more than "that of all other animal protein foods (meat, dairy, milk, etc.) which increased by 2.1% per year" (FAO, 2020). If we look at it per person, fish consumption per capita grew by 1.5% per year between 1967 and 2018 reaching a peak in 2018 at 20.5 kg per capita per annum "while total meat consumption grew by 1.1 % per year in the same period" (FAO, 2020) to reach 34.4 kg per capita in 2018 (Statista, 2021). The fish consumption growth rate was however not equal in the world with fish consumption in developed countries peaking at 26.4 kg per person in 2007 to go down again to 24.4kg in 2017. In developing countries, the peak was reached in 2017 at 19.4kg per capita with an average yearly growth rate of 2.4% between 1961 and 2017.

With 38% of the global production being traded in the world in 2018, fishery products have become one of the biggest traded food commodities (FAO, 2020). Most importations are performed by developed countries but the consumption of developing countries is steadily increasing. This expanding demand in developing countries is linked both to a higher income and a diet shift. Indeed, for African populations who mainly depend on a very poor food range, fish imports of low priced species such as pelagics or tilapia have become an even greater part of their nutritional habits (FAO, 2020). Therefore, the increase in global fish consumption is linked to several factors: a growing and wealthier world population more aware of the nutritious importance of fish consumption and a more effective production capacity due to better supply chain (FAO, 2020).

In 2017 fish consumption grew to 17% of the global population's animal proteins intake thus accounting for 7% of the total of proteins consumed. In 2018 fish consumption increased to 20% of the average animal proteins intake for 3.2 billion people and even went up to 50 % in some countries (**Figure 1-1**) (FAO, 2019).

- **Benefits of eating fish**

Amongst the healthy and nutritive benefits of fish consumption are high quality value proteins and "essential amino acids and micronutrients such as vitamins and minerals" (FAO, 2020). Fish thus constitute an important part of the diet of populations suffering from a poor daily protein intake (FAO, 2020) and participate in the diversification of diet especially in countries where other type of staple food is scarce. In 2017 fish consumption only amounted to an average of 35 calories per capita per day (FAO, 2020). Moreover, the production of fish as animal proteins source requires less feed input per kilogram of added growth than other animals (Tilman and Clark, 2014).

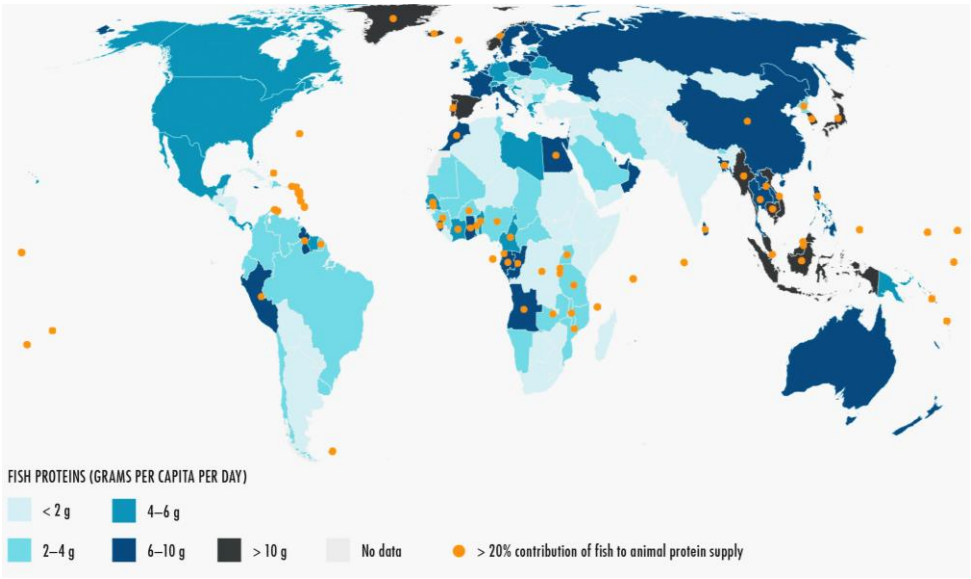


Figure 1-1 Contribution of fish to animal protein supply, average 2015-2017 (FAO, 2020)

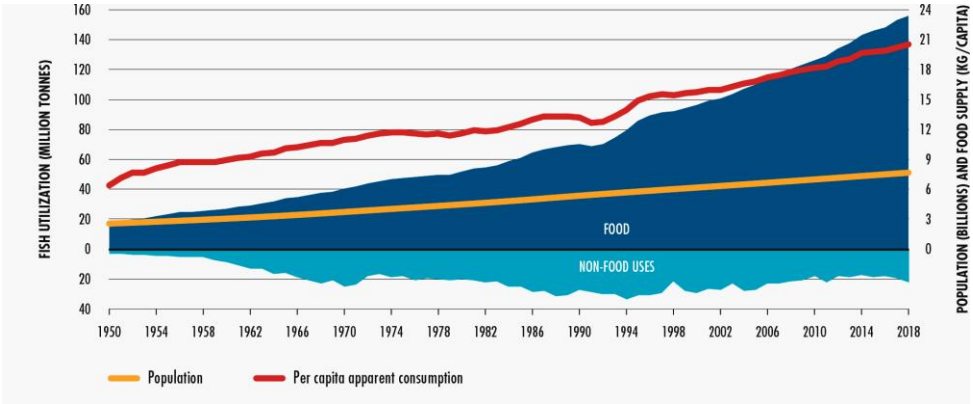


Figure 1-2 World fish utilisation and apparent consumption (FAO, 2020)

- **Fish production**

In 2018, global fish production reached 179 million tons with 156 million tons dedicated to human consumption and 23 million tons destined to non-food use, mainly the production of fish meal and fish oil (**Figure 1-2**). Global fish production has increased greatly during these past decades with the exception of Europe and America, compensated by a most important increase in Africa and Asia. China is nowadays the main fish producer in the world representing 35% of the total fish production while it is fast increasing in other countries such as Indonesia and Ecuador (FAO, 2020). Concerning capture fisheries, a peak was reached in 2018 with 96.4 million tons due mostly to marine fisheries. However, aquaculture production exceeds capture fisheries.

In 2018, aquaculture reached 114.5 million tons and represented 52% of the human consumption (**Figure 1-3**) (FAO, 2020). It has been a steady increase as from 2000 when aquaculture represented only 25.7% of total fish production. Aquaculture production has increased at the important annual rate of 8.8% in the last 30 years (Boxman et al., 2017) with a diversity of more than 345 fish species reared in aquaculture though tilapia itself (*O. niloticus*) accounted for 8.3% of the total production in 2018 thus being the third most important output in aquaculture (FAO, 2020).

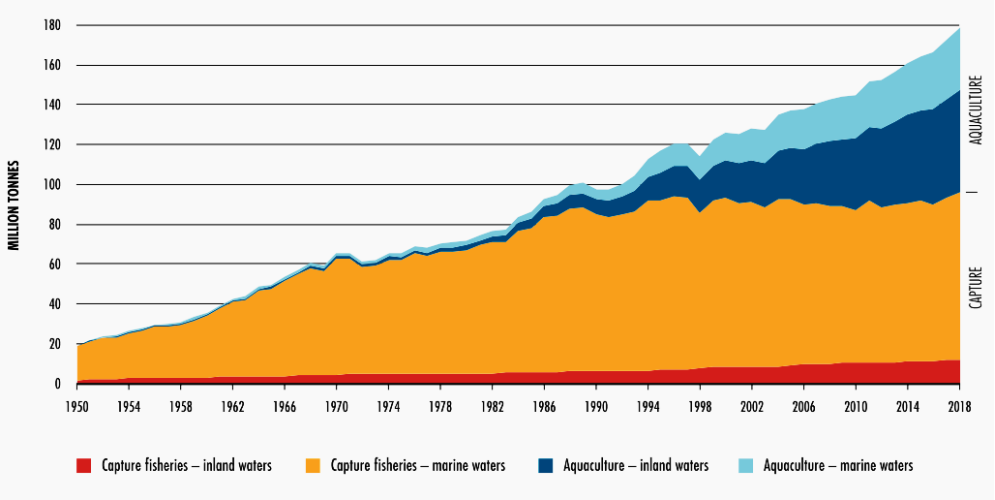


Figure 1-3 World capture fisheries and aquaculture production. Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants. (FAO, 2020)

More specifically: inland aquaculture

The share of inland aquaculture (or freshwater aquaculture) as opposed to marine aquaculture has also steadily increased from 57.9% in 2000 to 62.5% in 2018 of fish production. Inland aquaculture techniques are very diverse though earthen ponds are still the main craftsmanship in use. Improving, it can be coupled with other agricultural activities such as rice production to lead to rice fish co-culture for example, thus not only enhancing the productivity through a better use of resources but also softening the environmental impact (FAO, 2020).

Sustainability problems

A Code of Conduct for Responsible fisheries was published in 1995 to improve the sustainability of fish production (FAO, 2020). Fishing industries have taken a heavy toll on the ocean's resources and fish stocks are shrinking: on average, supplies within biologically sustainable boundaries have dropped from 90% in 1974 to 65.8% in 2017 of which 59.6% are being strained to their maximum (FAO, 2020). Over the same period the rate of fish landings at biologically unsustainable levels increased from 10% to 34.2%. However, it is currently estimated that biologically sustainable stocks actually provide 78.7% of marine fish landings (FAO, 2020).

Nevertheless, intensification of aquaculture also poses threats to the environment as the use of fish meal and fish oil contributes to overfishing (FAO, 2020).

1.1.2 Natural resources aspect

The production of food relies on several pillars: land, water, climate conditions adapted to crops, fertilizers (nutrients) and fossil fuels (Conijn et al., 2018; Goddek et al., 2019a; Van Vuuren et al., 2010). Yet, our current agricultural practices lead to the depletion of the stocks of these natural resources as well as the damage of the environment via nutrient leaching. Indeed, nutrient leaching causes eutrophication of water sources and greenhouse gases (GHG) emissions which contribute to climate change (Conijn et al., 2018).

1.1.2.1 Land

Agriculture takes up more than one third of the terrestrial surface (Goddek et al., 2019a) with 1,600 million of hectares used for crops (excluding pastures) (Alexandratos and Bruinsma, 2012; Beddington, 2010). However, between 1970 and 2013, land devoted to agriculture decreased by 50% and the quality of the land is degrading (Goddek et al., 2019a). Nowadays, 75 million hectares which are used for crop production are in fact classified as non-suitable for this activity by the Global Agro-Ecological Zones study (GAEZ) conducted by the FAO (irrigated deserts for example). Today on the earth's land surface, a total of 1,400 million hectares are actually considered good land for crop production, even though some of them are already in use for pastures for example (Alexandratos and Bruinsma, 2012). It has been calculated that a surplus of approximately 70 million hectares should be required by 2050 (FAO, 2009) to meet the growing food demand and that this increase should take place in developing countries (to provide for the fastest growing population in these regions). In contrast, areas dedicated to crops in developed countries are expected to decrease. These numbers may lead us to believe that land readily available for new crop production would easily be obtainable. Nevertheless, it has to be kept in mind that these lands are often remote, difficult to access and hence far away from infrastructures or markets. The exploitation costs would thus skyrocket. Furthermore, 60% of the 1,400 million hectares of good land are located in only 13 countries which means that land will remain a crucial issue for a majority of countries (Alexandratos and Bruinsma, 2012). Eventually, the increase in food production should mainly be due to an increased harvesting intensity and yields improvements (FAO, 2009) but yield increase is expected to slow down by 2030/2050 (Alexandratos and Bruinsma, 2012).

1.1.2.2 Water

Agriculture currently uses 70% of the total available water supplies (FAO, 2019). Nowadays, 300 million ha are irrigated and this shift from rain fed to irrigated crops has helped promoting crop yields in the last years (FAO, 2019). However, water resources are limited and more specifically in some parts of the world such as the Near East, North Africa and Northern China. Globally 180 million hectares in developing countries could be used in irrigated agriculture, expansion of which 20 million could be pulled into production by 2050. The majority of the irrigated surface currently lies in developing countries (half of it in India and China) (Alexandratos and Bruinsma, 2012). To prove sustainable, a country should not use more than 20% of its water renewable resources for irrigation (Alexandratos and Bruinsma, 2012) defined as "the sum of the annual precipitation and net incoming flows (transfers through rivers from one area to another) minus evapotranspiration, runoff and groundwater recharge". Still, China already uses 20.9% and India 33.9% (FAO, 2019). Additionally, world water supplies are under threat due to the potentially negative impact of climate change (Alexandratos and Bruinsma, 2012).

1.1.2.3 Climate

Climate conditions are changing with higher temperatures in some regions of the world, with more and more frequent extreme weather events such as heavy rains, floods, droughts, changes in temperature and rain patterns all of which will affect food production and food production techniques. The change in climate conditions will also affect pests and diseases development (Beddington, 2010) with dramatic consequences.

1.1.2.4 Nutrient

- **Nutrients stocks in the world**

Conventional agriculture heavily relies on mineral fertilizers, such as phosphorus which stocks are slowly depleting (Goddek et al., 2019a; Van Vuuren et al., 2010). Furthermore, the more the stocks are decreasing the more complicated and expensive it is to extract what is left (Jones et al., 2013). Micronutrient are also becoming scarcer and scarcer with 49% of soils wanting in at least one micronutrient ("33% in B, 12% in Fe and <5% for Cu and Mn") (Jones et al., 2013). Nutrients deficiencies are also unevenly distributed in the world with poorer soils being more often located in developing countries (Jones et al., 2013) what is increasingly worrisome as far as the impact of nutrient resources on worldwide food security is concerned (Jones et al.,

2013). There is also a problem linked to the fact that food is produced in one place (nutrient intake) and consumed in another thus preventing an efficient return to the soils and perturbing the nutrient cycles (Jones et al., 2013).

Nitrogen (N) is provided to plants under the form of ammonia (NH_3) which is obtained via the Haber-Bosch industrial process from the atmospheric N_2 and fossil H_2 (Ciceri et al., 2015). Therefore, the main problem with N fertilisation is not so much the availability of the nutrient but rather its overuse resulting in leaching and pollution of groundwater (De Notaris et al., 2018). Nitrogen can also be provided by animal manure but up to 80% of the total N input into food systems can be lost (Conijn et al., 2018).

The origin of the **phosphorus (P)** used in commercial fertilizers is mined rock phosphate (apatite) which, "could be exhausted in the next 50-100 years" in the worst-case scenario (Cordell et al., 2009; Van Vuuren et al., 2010) or be of a too poor quality or too complicated to extract. Today, with depleting resources, the cost of production is increasing. Before the large mining campaigns, phosphorus was mainly retrieved from the recycling of "animal manure, crushed animal bones, human and bird excreta, city waste and ash" (Van Vuuren et al., 2010). As of today 148 million tons of phosphorus are yearly used for food production (Cordell et al., 2009) and the demand is expected to raise by 50% to 100% to meet food production increase by 2050. The resources of phosphorus in this world have not been equally distributed thus creating tensions in trade and making some regions such as India or Western Europe utterly dependent on importations (Cordell et al., 2009). In Europe and North America, important quantities of phosphorus were added to the soils for 50 years hence only little amounts are now required to compensate for harvest exportations. However, in developing countries the demand is expected to increase (Cordell et al., 2009; Van Vuuren et al., 2010). Eventually, the overuse of phosphorus leads to leaching and pollution of freshwater (Van Vuuren et al., 2010).

Potassium (K) originates from soluble potassium salts (potash) made from sedimentary rocks and is mainly found in countries from the northern hemisphere (Ciceri et al., 2015) while some agricultural regions such as China (¾ of the paddy soils) and Australia (2⁄3 of the wheat belt) are deficient in plant available potassium (Zörb et al., 2014). Potassium stocks in the soil are quite large, thus not making it a subject of tension however they are mostly not directly available for plant uptake (Zörb et al., 2014). Yet, potassium is crucial for plant health and plant quality thus still making it a topic for research in improving plant uptake (Zörb et al., 2014).

Furthermore, potassium availability also depends on soil texture and composition and is a limiting growth factor in organic soils (Zörb et al., 2014).

- **Plant uptake and use of major nutrients for metabolism**

Plants mainly absorb nutrients under the form of ions via their roots. The absorption of ions is compensated by an exchange of protons or hydroxyls to maintain electric balance of charges in the solution. In soilless systems, this process can however alter the solution pH which therefore needs to be precisely monitored and buffered (Maucieri et al., 2019). Macro and micronutrients are all required for the plant to grow with macronutrients required in larger amounts than micronutrients. Nitrogen, phosphorus and potassium are often considered as the most important nutrients for plant growth (Resh, 2013).

Nitrogen is more often absorbed under the form of ammonium and nitrate (Maucieri et al., 2019) and is used by the plant for the production of amino acids and proteins. Nitrate can be easily stored by the plants without deleterious effects which is not the case of ammonium which can interfere with calcium and copper absorption and favour shoot instead of root growth when present in quantities higher than 10 mg/L (Maucieri et al., 2019) . Ammonia in excess can become toxic for the plants. Nitrogen excesses can be observed via an important vegetative growth, intense green colour of the leaves, low fruit production and can lead to nitrate accumulation in the tissues which is dangerous for human consumption. A lack of nitrogen can be noted via pale green colour and reduced growth (Maucieri et al., 2019).

Phosphorus is one of the major macronutrient needed for plant growth and health (Prabhu et al., 2019). It is absorbed by the plants under the form of orthophosphate, from the soil solution, via the roots (Prabhu et al., 2019; Richardson and Simpson, 2011) but its absorption is heavily dependent on the pH of the solution as it precipitates when pH is too high (> 7) (Cerozi and Fitzsimmons, 2016a). Phosphorus takes part in rapid growth of the buds and root development but is mainly known for being a key element of the ATP molecule. More specifically, phosphorus is used for "energy storage and transformation, cell structure component, respiration and photosynthesis, cell enlargement and division, root development" (Prabhu et al., 2019). Phosphorus deficiency can be observed via "reduction in quality of forage, fruit, grain and crop, reduction in leaf expansion and number, decrease in shoot growth, decrease in disease resistance, delayed maturity, reduction in nutrient uptake" (Prabhu et al., 2019) while in excess phosphorus can react with micronutrients and then prevent their uptake by plants (Cerozi and Fitzsimmons, 2017).

Potassium is the third most essential plant macronutrient (after nitrogen and phosphorus) (Etesami et al., 2017) and is mainly up-taken by plants under its cation form K^+ (Sattar et al., 2018) found in the soil solution. It is involved in processes such as cell division and extension, the production of proteins but also photosynthesis. It plays a key role in keeping the balance of osmotic potential in the plant. Potassium deficiency can be observed through yellowish spots and necrosis on leaves. It makes the plants more sensitive to temperature drops in the system or to water stresses (Maucieri et al., 2019).

1.2 Current techniques in use to answer these issues

1.2.1 Hydroponics: what is it? Advantages and drawbacks

Hydroponics is a soilless crop production technique based on the use of a nutrient enriched solution to irrigate and fertilise crops (**Figure 1-4**) thus enabling regions with poor soil and little access to freshwater to develop agriculture (Joyce et al., 2019a).

Soilless cultures exist under different designs (**Table 1-1**) and can be carried out completely without growing medium (nutrient film technique (NFT), deep water culture (DWC)) or with the help of an inert substrate such as rockwool, peat, argex bead etc. (Maucieri et al., 2019). These techniques are nowadays widely used in horticulture in Europe (Maucieri et al., 2019) using readymade commercial solutions (Sonneveld and Voogt, 2009) and are mostly implemented indoors.

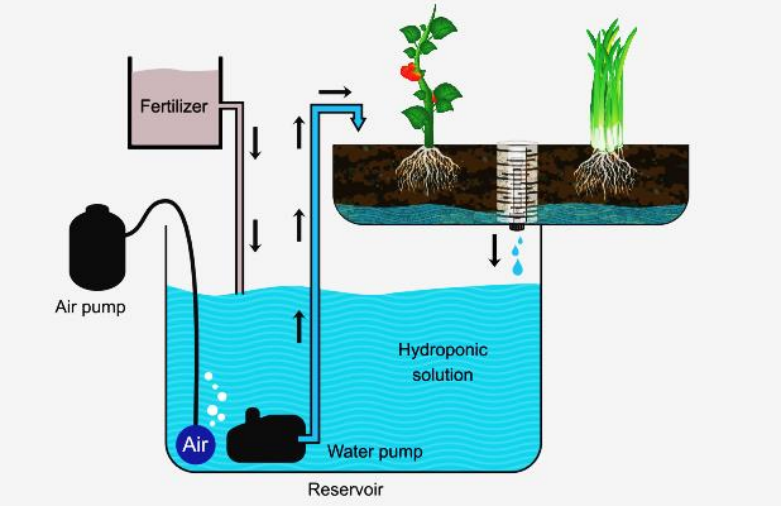


Figure 1-4 Simple hydroponic system (Somerville et al., 2014)

Table 1-1 Classification of hydroponic systems according to different aspects (Maucieri et al., 2019)

Characteristic	Categories	Examples
Soilless system	No substrate	NFT (nutrient film technique)
		Aeroponics
		DFT (deep flow technique)
	With substrate	Organic substrates (peat, coconut fibre, bark, wood fibre, etc.)
		Inorganic substrates (stone wool, pumice, sand, perlite, vermiculite, expanded clay)
		Synthetic substrates (polyurethane, polystyrene)
Open/closed systems	Open or run-to-waste systems	The plants are continuously fed with "fresh" solution without recovering the solution drained from the cultivation modules
	Closed or recirculation systems	The drained nutrient solution is recycled and topped up with lacking nutrients to the right EC level
Water supply	Continuous	NFT (nutrient film technique)
		DFT (deep flow technique)
	Periodical	Drip irrigation, ebb and flow, aeroponics

Hydroponic techniques present several advantages (Maucieri et al., 2019):

- Better yields due to a fertilising solution adapted to every growth stage of the plant
- Maximum use of fertiliser input thus lowering production costs and reducing simultaneously the nutrient solution discharge into the environment. Possibility to reuse fertiliser solution.
- Easier control of “soil borne” pathogen via the use of sterile growth media
- Full control of growth conditions thanks to the use of environmentally controlled greenhouses (temperature, humidity, light intensity, CO_2 concentration)
- No dependency to soil (pests, pathogens, unavailable nutrients…)
- No weeds
- No soil labour required
- Water savings compared to conventional horticulture

However, hydroponics also presents serious drawbacks (Maucieri et al., 2019):

- Reliance on non-renewable resources and mineral fertilisers
- High investment costs
- Reliance on high quality water

- Difficulty to manage a perfectly balanced composition of nutrients in the solution (nutrients ratio, salts accumulation…)
- Risk of extremely fast pathogen dispersion via the water if one plant is contaminated

1.2.2 Recirculating aquaculture: what is it? Advantages and drawbacks

The concept of recirculating aquaculture systems (RAS) was developed in the 1950s to provide an answer to water scarcity and to the discharge of open aquaculture (as opposed to recirculating) wastes into the environment (Joyce et al., 2019a). Recirculating aquaculture systems (**Figure 1-5**) are intensive systems in which the water, instead of flowing steadily through the fish tanks and being directly discharged in the environment, circulates through several treatment units before being returned, cleansed, to the fish. The main components of a recirculating aquaculture system are the following: one or several fish tanks, a mechanical filter to remove suspended solids such as fish faeces, feed leftovers and other particles, a biological filter in which bacteria convert the ammonia excreted by the fish first into nitrite and then into nitrate (less toxic for the fish) and finally gas exchange devices (Espinal and Matulić, 2019). Other optional devices can be added such as ultra-violet (UV) lamps for system disinfection and denitrification filters to transform nitrate into a gaseous form of nitrogen and thus avoid an over accumulation of nitrates in the fish water (Espinal and Matulić, 2019; van Rijn, 2013).

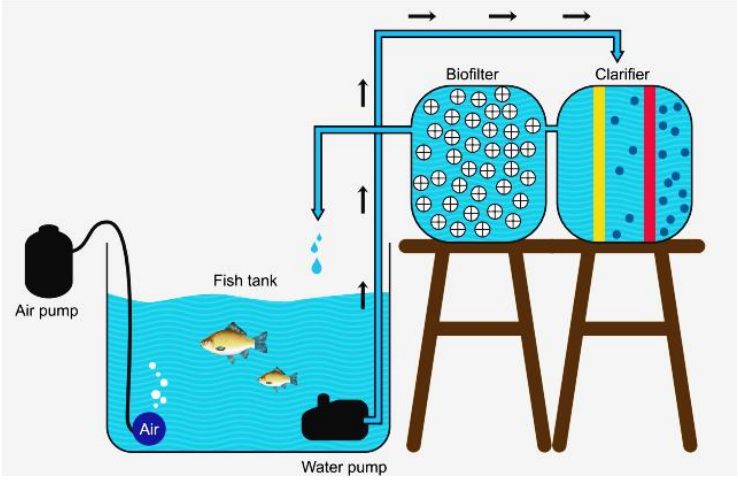


Figure 1-5. Recirculating aquaculture system (Somerville et al., 2014)

The intensive aspect of this technique, coupled with the reuse of the water put a strain on the system parameters monitoring as it is of paramount importance to maintain the quality of the water despite its recycling. Several water parameters are thus regularly monitored: dissolved oxygen, ammonia concentration, biosolids, carbon dioxide, total gas pressure, nitrate concentration and alkalinity are the commonest (Espinal and Matulić, 2019).

Recirculating aquaculture present several advantages:

- 95-99% of the water can be re-used thus reducing to 100 litres the quantity of water necessary to farm 1 kg of fish, instead of 2,500 – 375,000L in conventional aquaculture. It is consequently a more environmentally friendly type of farming (Boxman et al., 2017; Goddek et al., 2015)
- Less aquaculture effluents are discharged in the environment and the ones that are still discarded contain less elements (thanks to denitrification filters for example)

Recirculating aquaculture also present serious drawbacks:

- The water recirculation method requires high maintenance
- Accumulation of toxic compound (either for the environment or for the fish) if not properly treated
- Heavy reliance of fish feed while fed aquaculture poses a threat to the environment as it is most often based on fish meal and fish oil obtained via fishing. Indeed, in 2018 12% of global fish production was used for non-food uses, mainly fish meal and fish oil (FAO, fisheries, 2020)

1.2.3 The answers aquaponics can offer

1.2.3.1 What is aquaponics?

Aquaponics is a technique included in the broader concept of integrated agri-aquaculture systems (IAAS) (Lennard and Goddek, 2019). IAAS can involve different types of aquatic animals combined with several systems of plant production while aquaponic is a specific case combining fish and vegetables and/or herbs. The precise definition of aquaponics has evolved over the years since its ‘redevelopment’ in the USA in the 1970s.

1.2.3.2 History and evolution of the definition

Modern aquaponics was mainly developed in the 1970s-1980s in the USA (Virgin Islands) by John Rakocy, the main objective of his research being the development of more sustainable food production techniques (Goddek et al., 2015; Lennard and Goddek, 2019). Since then, many studies on the topic of aquaponics have been carried out, continually assessing every aspect of it, from the flow of nutrients to the microbial communities to the design of the systems (coupled, decoupled...) and the addition of more units (e.g. desalination units) (Yep and Zheng, 2019). Along with the development of several designs and the wish to improve the understanding of the functioning of aquaponic systems, came the need to define more accurately what exactly is an aquaponic system.

The first definitions of the concept only specified that aquaponics should be a combination of AQUAculture and hydroPONICS (Lennard and Goddek, 2019). A few years after, Rakocy added the precision that the fish and plants compartments should be linked in a closed loop of water (Lennard and Goddek, 2019). In 2009, Graber and Junge (2009) confirmed this definition. The main modification appearing in the definition was the abandonment of the closed loop concept to accept the fact that aquaponic systems could involve separate loops (decoupled aquaponics) (**Figure 1-6** and **Figure 1-7**) (Lennard and Goddek, 2019). This can indeed enhance productivity as it enables to optimise the parameters of both compartments for fish and plants (Lennard and Goddek, 2019; Suhl et al., 2016) and enhance crop productivity via the complementation of the fish water with additional fertilizers (Goddek, 2017; Lennard and Goddek, 2019). Recently, debate around the precise definition of aquaponics occurred within the European (EU) aquaponics hub and it was finally settled on the following: *"A production system of aquatic organisms and plants where the* ***majority (> 50%) of nutrients sustaining the optimal plant growth derives from waste*** *originating from feeding the aquatic organisms*" (Palm et al., 2018). In this definition, it is clear that the focus has shifted from the component and design of the system towards the nutrient cycling aspect (Lennard and Goddek, 2019).

1.2.3.3 General principles

Aquaponics is thus the combination of hydroponics and recirculating aquaculture. It uses the nutrient enriched fish water to grow crops, thus avoiding both the discharge of nitrate laden aquaculture effluents into the environment and the handling of mineral fertilisers (Goddek et al., 2019a). Indeed, contrary to hydroponics where nutrients are manufactured from synthetic fertilizers, the essentials elements for plants in

aquaponics originate from the fish feed. Part of the feed is eaten by the fish which metabolize it while the rest is left to decompose in the fish tanks. Fish faeces and feed leftovers are then ingested and transformed by the microorganisms present in the system. This transformation makes the elements more readily available to the plants which can then easily absorb them, thus also cleaning water before it goes back to the fish tanks (Lennard and Goddek, 2019). Henceforth the same source of input (water and fish feed) is now utilized to produce two crops (Lennard and Goddek, 2019).

To sum up, here are the five highlights of aquaponics (Lennard and Goddek, 2019):

1. Fish wastes as principal nutrient source for plants
2. Optimisation of water and nutrient uses in the choice of design
3. Recycling of possible water and nutrient wastes in other production (e.g. use of the fish sludge for soil-based crops)
4. Appropriate system design to lower or cancel the negative impact of waste water
5. Controlled infrastructures to ensure optimal production

1.2.3.4 Different types of aquaponic systems

The basic elements of an aquaponic system are very similar to a classic RAS with fish tanks, mechanical and biological filtration (**Figure 1-5.** and **Figure 1-6.**). No denitrification unit is included as the nitrate formed in the biofilter is up taken by the plants (Espinal and Matulić, 2019; Lennard and Goddek, 2019; Timmons and Ebeling, 2013). Hydroponic units are then inserted between the biofilter and the return to the fish tanks. Different types of hydroponic units can be added and some of them have proven particularly well-adapted to aquaponics (Maucieri et al., 2018b; Schmautz et al., 2016).

The most reared fish in aquaponics is Nile tilapia (*Oreochromis niloticus*) due to its high tolerance to stress, water parameters changes and crowding and is mostly combined with leafy vegetables (Ghamkhar et al., 2019).

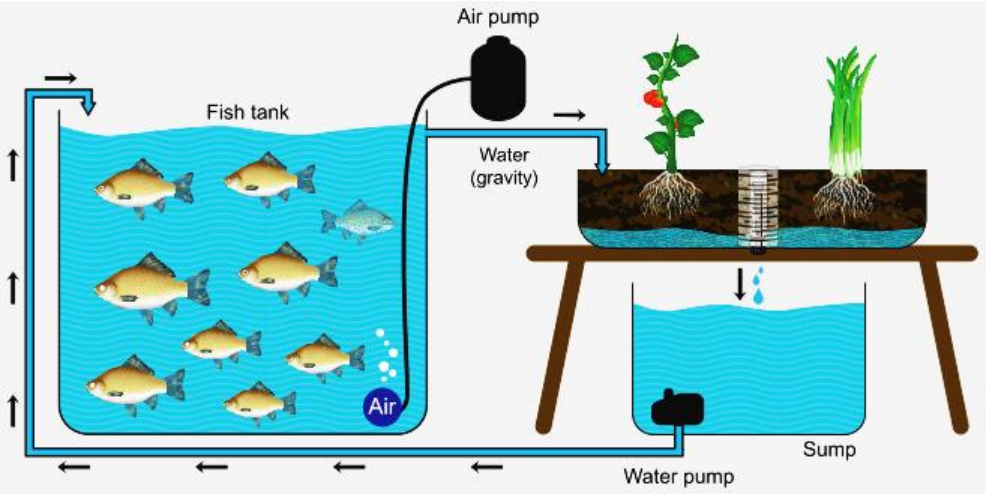


Figure 1-6 Simple aquaponic unit (Somerville et al., 2014)

Figure 1-6 illustrates the simplest aquaponic design which is a 'one-loop' or 'coupled' design where water flows directly from the fish to the plants but production in coupled systems is restrained. Indeed, compromises have to be reached among the optimal living conditions (pH, temperature, DO, EC, nutrient concentrations) of the different groups of living organisms (fish, plants, microorganisms) (**Table 1-2**) thus impeding exploiting the full production potentials of plants and fish (Palm et al., 2018). Therefore, aquaponic systems in which plant and fish compartments are disconnected have been developed, i.e. decoupled systems (Goddek, 2017; Palm et al., 2018). Nowadays, many different designs of aquaponics systems exist with different sizes, scales of production, high or low tech (Konig et al., 2016; Palm et al., 2018).

- **Decoupled systems: improved nutrient use**

Decoupled aquaponics enables to disconnect the fish compartment from the plant compartment with only punctual transfers of water from the fish to the plants (**Figure 1-7**). This design offers several advantages. First, it can provide each organism with optimal growing conditions as fish and plants are both in their own loop of water. A fraction of the fish water can regularly be withdrawn and adapted to the plant requirements in nutrients and pH. This addition of external nutrients is called complementation or supplementation (Palm et al., 2018).

In decoupled aquaponics, other units may be added to improve the functioning of the system and maximize the use of resources such as sludge digesters to extract nutrients stuck in the evacuated solid matter (Goddek et al., 2019a, 2018, 2016) or desalination units. The latter enables to remove excess nutrients from the plant water and still send it back to the fish despite the previous supplementation (Goddek and Keesman, 2018). Sludge digesters are a hot topic in aquaponics nowadays as scientists are increasingly trying to foster nutrients recycling. The aim is to develop the best method to enable microorganisms to convert elements such as phosphorus, magnesium, iron, manganese or sulphur (often lacking in the plant compartment) into a form available for the plants (Goddek et al., 2019a). In decoupled aquaponics, the percentage of nutrients coming from external sources can vary, always bearing in mind that, according to the latest definition, fish waste should provide at least 50% of the plant nutrients (Palm et al., 2018).

1.2.3.5 Important parameters in aquaponics

Table 1-2 summarises the important parameters in aquaponics and their target values.

Table 1-2 Important parameters in aquaponics, their optimal values for fish, plants and microorganisms' welfare and the compromises that have to be reached in aquaponics (Szekely, 2019)

Parameter	Optimal value				Source	Type of system
	Plant	Fish	Bacteria	Compromise		
	Lactuca sativa	*Oreochromis niloticus*				
pH				6.5	Schmautz, 2017	Aquaponics
	6-7	6.5 - 8.5	6-8.5	6-7	Somerville, 2014	Aquaponics
		6.5-8.5			Lund, 2013	RAS
	5.5-6.5				Resh, 2012	Hydroponics
			7-7.8		Licamele, 2009	Aquaponics
				7	Delaide, 2017	Aquaponics
		7-8.0			Graber, 2009	Aquaculture
Electro-conductivity (µS/cm)				< 1500	Schmautz, 2017	Aquaponics
				< 1500	Somerville, 2014	Aquaponics
	1500-2000				Resh, 2012	Hydroponics
	1000-2000				Licamele, 2009	Hydroponics
		< 1200			Graber, 2009	Aquaculture
Dissolved oxygen (mg/L)	> 3	4-6	4-8	> 5	Somerville, 2014	Aquaponics
		4-6	> 1		Goddek, 2016	RAS
		> 5			Lund, 2013	RAS
		> 2	> 2		Licamele, 2009	Aquaponics
		> 6			Graber 2009	Aquaculture
Temperature (°C)				28	Schmautz, 2017	Aquaponics
	16-30	27-30	14-34	27- 30	Somerville, 2014	Aquaponics
		27-30			El-Sayed, 2006	Aquaculture RAS
	21-25	28-35	20-30		Licamele, 2009	Hydroponics/aquaculture
				25	Delaide, 2017	Aquaponics
Nitrate (mg/L)				120 ($N\text{-}NO_3^-$)	Schmautz, 2017	Aquaponics
	/	< 400	< 400	5-150	Somerville, 2014	Aquaponics
		100-200 ($N\text{-}NO_3^-$)			Lund, 2013	RAS
	165 (NO_3^-)				Resh, 2012	Hydroponics
		< 150 ($N\text{-}NO_3^-$)			Graber, 2009	Aquaculture
Nitrite (mg/L)				1 ($N\text{-}NO_2^-$)	Schmautz, 2017	Aquaponics
	< 1	< 1	< 1	< 1	Somerville, 2014	Aquaponics
		< 0.5 ($N\text{-}NO_2^-$)			Al-Hafedh, 2003	RAS
		0.05-1 ($N\text{-}NO_2^-$)			Lund, 2013	RAS
		< 0.2 ($N\text{-}NO_2^-$)			Graber, 2009	Aquaculture
Ammonia nitrogen (mg/L)	< 30 (TAN)	< 3 (TAN)	< 3 (TAN)	< 1 (TAN)	Somerville, 2014	Aquaponics
		< 1 (TAN)			Al-Hafedh, 2003	RAS
		< 0.1 ($N\text{-}NH_3$)			El-Sayed, 2006	RAS
		< 3			Lund, 2013	RAS
				0 ($N\text{-}NH_4^+$)	Schmautz, 2017	Aquaponics
		< 1 ($N\text{-}NH_4^+$)			Graber, 2009	Aquaculture
	25 (NH_4^+)				Resh, 2012	Hydroponics
Phosphate (mg/L)				35 ($P\text{-}PO_4$)	Schmautz, 2017	Aquaponics
	50				Resh, 2012	Hydroponics
	35-80				Licamele, 2009	Hydroponics
Potassium (mg/L)				150	Schmautz, 2017	Aquaponics
	210				Resh, 2012	Hydroponics
Iron (mg/L)				3	Schmautz, 2017	Aquaponics
	5				Resh, 2012	Hydroponics
Sulfate (mg/L)	113 (SO_4^{2-})				Resh, 2012	Hydroponics
Calcium (mg/L)				200	Schmautz, 2017	Aquaponics
				60-140	Somerville, 2014	Aquaponics
	200				Resh, 2012	Hydroponics
Magnesium (mg/L)				40	Schmautz, 2017	Aquaponics
				60-140	Somerville, 2014	Aquaponics
	40				Resh, 2012	Hydroponics

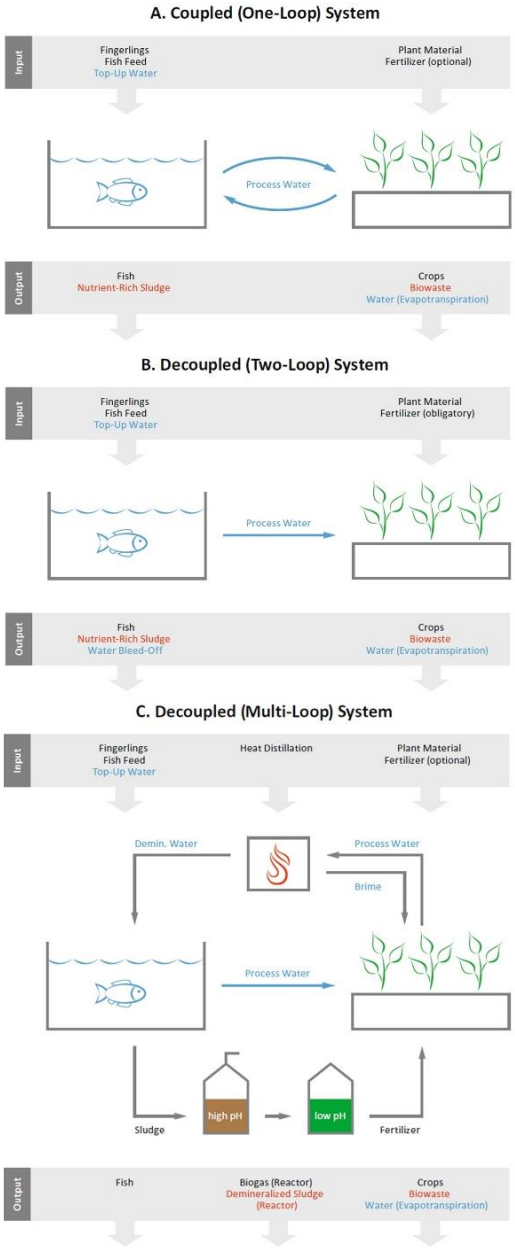


Figure 1-7. The evolution of aquaponics systems. (a) shows a traditional one-loop aquaponics system, (b) a simple decoupled aquaponics system, and (c) a decoupled multi-loop aquaponics system. The blue font stands for water input, output, and flows and the red for waste products (Goddek et al., 2019b)

1.2.3.6 Advantages of aquaponics:

The advantages of aquaponics are the following:

- Production of plants and fish with the same source of inputs (i.e. water and fish feed) is a plus for businesses as it allows the diversification of the production and the inclusion of high-value crops in one system (Espinal and Matulić, 2019).
- Use of nutrients present in dissolved and solid fish waste to provide crops either with their full or part of their nutrient requirements (Lennard and Goddek, 2019) and thus no supply constraints in terms of non-renewable nutrient sources such as rock phosphorus which is mined in a few countries only (Van Vuuren et al., 2010)
- Reduction of environmental impact
    - Life cycle assessment (LCA) studies underline "reduced damages associated to human health, ecosystems, and resources by approximately 84%, 62%, and 48% respectively, compared to separate aquaculture and conventional agriculture systems" (Cohen et al., 2018)
    - Significantly reduced water usage (Ghamkhar et al., 2019) Sludge can be composted and used as fertilizers on soil crop (Konig et al., 2016)
    - On a local level, aquaponics contributes to reducing the loss of nutrients such as nitrogen and phosphorus into the environment and thus avoid eutrophication and pollution (Boxman et al., 2017; Buzby and Lin, 2014)
- Possibility to implement aquaponic facilities virtually anywhere, even in cities, on rooftops, concrete surfaces, industrial wasteland with polluted soils as well as areas with poor soil and scarce water (Joyce et al., 2019a).
- Production in closer proximity to consumer markets and thus less transportation, reduction of CO_2 emission and consumption of fossil fuels, less need to store so less need for fridges, not to mention a better access for city dwellers to fresh, healthy, nutritious food (Goddek et al., 2019a)

1.2.3.7 Drawbacks of aquaponics:

The drawbacks of aquaponics are the following:

- More complex to implement than simple hydroponics or simple RAS
- Requires the skills and knowledge of both a fish expert and a horticulturist

- High investments costs depending on how high the tech (Konig et al., 2016)
- If coupled (i.e. one loop of water), compromises are required for water parameters, leading eventually to sub-optimal production conditions
- If coupled, nutrients concentrations are much lower than in hydroponics and often unbalanced. Nitrate levels are often high enough but K and P levels are usually too low. Iron availability is also often limited (Delaide et al., 2017)
- More complicated to answer the precise needs of the plants as the composition of the fertilising solution is mainly based on fish feed type, feeding rates and fish density (Maucieri et al., 2019)
- High energy consumption which induces a problem of sustainability (Goddek et al., 2019a)

1.2.3.8 The importance of microorganisms in aquaponics

Microorganisms are key players in aquaponics. Bacteria are the most famous for now, particularly due to the bacteria known and thoroughly studied in recirculating aquaculture but also present in aquaponics. Bacteria involved in the nitrification process are paramount as they perform the transformation of toxic ammonia into nitrate which the plants can absorb. However, the focus is more and more shifting on the other microorganisms also present in aquaponics and specifically on those which could serve as plant growth promoting microorganisms and could play a role in nutrient cycling (Bartelme et al., 2019; Maucieri et al., 2019; Sanchez et al., 2019; Schmautz et al., 2017). More details on this fascinating topic will be available in the fourth part of this introduction (see 1.4).

1.2.3.9 Improvements points in aquaponics

Aquaponics is often put forward as the sustainable solution for future horticulture and aquaculture productions (Ghamkhar et al., 2019; Goddek et al., 2015; Junge et al., 2017) as the idea of reusing aquaculture effluents instead of discarding them to produce vegetables without mineral fertilisers is an attractive offer. According to Gott et al. (2019), aquaponics could be part of the new paradigm for sustainable world food production yet this technique is still in its infancy. Admittedly, the reality of the energy costs, of the difficulty to manage such systems and the daily supplementation of the aquaponic water darken this sustainable image.

According to Lehman et al. (1993) sustainable agriculture is “a process that does not deplete any non-renewable resources that are essential to agriculture in order to sustain the agricultural practices”. Aquaponics may avoid effluent discharge and diminish the use of mineral fertilisers but it still consumes an important quantity of

energy whether for the pump, oxygen supply, heating or lighting and the energy source for aquaponic systems is often grid electricity which is not always produced in a renewable way. Several life cycle assessments (i.e. "compilation and evaluation of the inputs, outputs and the potential environmental impacts of a product system throughout its life cycle" (ISO, 2006; Konig et al., 2016) have been recently carried out and now point out the posts where efforts are still needed to significantly improve the sustainability of aquaponic systems but publications tackling the sustainability aspect of aquaponics are still scarce (Konig et al., 2016; König et al., 2018). Therefore, research in aquaponics needs to focus on the "environmental, social and economic challenges" (Gott et al., 2019).

- **Energy and feed: the two most consuming posts**

Studies seem to tally on identifying energy (for heating and electric devices such as water and aeration pumps) and fish feed as the two most consuming posts in aquaponics (Boxman et al., 2017; Ghamkhar et al., 2019; Maucieri et al., 2018a) as they can represent "more than 88% of the environmental impact" of an aquaponic system (Ghamkhar et al., 2019). Energy is such an important post in negative environmental impact that even a slight improvement could contribute to a large change for the better (Boxman et al., 2017). Indeed, according to Delaide et al. (2017), although aquaponics is a true water saving device ("278L of supplementary water needed per kilogram increase in tilapia" in conventional aquaculture) an increase of one kilogram per tilapia fish requires 96.2 kWh. The energy consumption drawback could be compensated with the use of renewable energies such as "solar thermal heat sources" or "solar PV" (Gott et al., 2019) but this would however make the systems more complex and raise investment costs (Kloas et al., 2015). As for the feed post, it could be significantly upgraded by a shift from fishmeal ingredients to plant-based diets (Ghamkhar et al., 2019; Konig et al., 2016) as detailed infra (1.3. nutrient cycling) or better feed conversion ratios in fish (Boxman et al., 2017). Other posts could also be worked on to improve the global sustainability such as the use of non-renewable material like rockwool plugs an inert substrate (Konig et al., 2016).

- **Complexity**

Complexity may hamper aquaponics as the association of aquaculture and hydroponics combines the benefits of the two techniques but also requires more skilful management and higher investments costs (Konig et al., 2016). A system fuelled with renewable sources of energy such as solar panels would then become more sophisticated and might even threaten to undermine an already fragile process. As a

matter of fact, too much complexity may induce aquaponics farmers to champion production and profitability over sustainability (Gott et al., 2019). However, aquaponics can also be kept at a very low-level of technicity and then, prove more sustainable in tropical and sub-tropical countries (Konig et al., 2016).

- **Nutrient cycling**

Except from energy and fish feed, another post to improve would be the management of the nutrient cycles (Aubin et al., 2006) and the lessening of nutrient losses in the aquaponic effluents. To this end, it is essential to focus on the microbial communities present in the aquaponic systems which are deeply involved in "the catabolism of the organic matter contained in the faeces and feed residues as well as for the conversion of the fish-generated ammonia to nitrate" (Bittsánszky et al., 2015; Kloas et al., 2015). Recently, more studies have started to tackle this aspect of aquaponics nutrient cycling (see infra, Kloas et al., 2015) and more specifically the questions of nitrogen cycling (Schmautz, 2021) and phosphorus cycling (Cerozi and Fitzsimmons, 2017).

1.3 Nutrient cycling in aquaponics

This part is adapted from Eck, M., Körner, O. and Jijakli, M.H. 2019. Nutrient cycling in aquaponics. In: *Aquaponics Food Production Systems – Combined Aquaculture and Hydroponic Production Technologies for the Future*. Eds: Goddek, S., Joyce, A., Kotzen, B. and Burnell, G.M. Springer Open

1.3.1 Introduction to nutrient cycling in aquaponics

Despite having two attractive assets (i.e. the recycling of aquaculture effluents and the relying on organic fertilisers for plant growth), the use of aquaculture effluents increases the challenge of monitoring the nutrients within the solution. Indeed, it is harder to control the composition of a solution where the nutrients originate from a biological degradation of organic matter than to follow the evolution of the nutrients' concentration in a precisely dosed hydroponic solution based on mineral compounds (Bittsanszky et al., 2016; Timmons and Ebeling, 2013). Moreover, a plant's nutritional needs vary during the growth period in accordance with physiological stages, and it is necessary to meet these needs to maximise yields (Bugbee, 2004; Zekki et al., 1996). In order to recycle aquaculture effluents to produce plant biomass, it is necessary to optimise the recycling rates of phosphorus and nitrogen (Goddek et al., 2019a, 2016; Graber and Junge, 2009). Several factors can influence this, such as

the fish species, fish density, water temperature, the type of plants and the microbial community. Therefore, it is of prime importance to understand the functioning of the nutrient cycles in aquaponics (Seawright et al., 1998).

1.3.2 Source of nutrients

The major sources of nutrients in an aquaponic system are the fish feed and the water added (containing Mg, Ca, S) into the system (Delaide et al., 2017; Schmautz et al., 2016). With respect to fish feed, there are two main types: fishmeal-based and plant-based feed. Fishmeal is the classic type of feed used in aquaculture where lipids and proteins rely on fish meal and fish oil (Geay et al., 2011). However, for some time now, concerns regarding the sustainability of such feed have been raised and attention drawn towards plant-based diets (Boyd, 2015; Davidson et al., 2013; Hua and Bureau, 2012; Tacon and Metian, 2008). A meta-analysis conducted by (Hua and Bureau, 2012) revealed that the use of plant proteins in fish feed can influence the growth of fish if incorporated in high proportions. Indeed, plant proteins can have an impact on the digestibility and levels of anti-nutritional factors of the feed. In particular, phosphorus originating from plants and thus in the form of phytates does not benefit, for example, salmon, trout and several other fish species (Timmons and Ebeling, 2013). It is not surprising that this observation is highly dependent on the fish species and on the quality of the ingredients (Hua and Bureau, 2012). However, little is known of the impact of varying fish feed composition on crop yields (Yildiz et al., 2017b). Classical fish feed is composed of 6–8 macro ingredients and contains 6–8% organic nitrogen, 1.2% organic phosphorus and 40–45% organic carbon (Timmons and Ebeling, 2013) with around 25% protein for herbivorous or omnivorous fish and around 55% protein for carnivorous fish (Boyd, 2015). Lipids can be fish or plant based as well (Boyd, 2015).

Once fish feed is added into the system, a substantial part of it is eaten by the fish and either used for growth and metabolism or excreted as soluble and solid faeces, while the rest of the given feed decays in the tanks (Goddek et al., 2015; Schneider et al., 2004). In this case, the feed leftovers and metabolic products are partly dissolved in the aquaponic water, thus enabling the plants to uptake nutrients directly from the aquaponic solution (Schmautz et al., 2016). In most cultivation systems (Goddek et al., 2019b; Palm et al., 2019), nutrients can be added to complement the aquaponic solution and ensure a better matching with the plants' needs (Goddek et al., 2015). Indeed, even when the system is coupled, it is possible to add iron or potassium (which are often lacking) without harming the fish (Schmautz et al., 2016).

1.3.2.1 Fish feed leftovers and fish faeces

Ideally, all the given feed should be consumed by the fish. However, a small part (less than 5% (Yogev et al., 2016) is often left to decompose in the system and contributes to the nutrient load of the water (Losordo et al., 1998; Roosta and Hamidpour, 2013; Schmautz et al., 2016), thus consuming dissolved oxygen and releasing carbon dioxide and ammonia (Losordo et al., 1998), amongst other things.

The composition of fish feed leftovers depends on the composition of the feed. Logically enough, the composition of fish faeces depends on the fish's diet which also has an impact on the water quality (Buzby and Lin, 2014; Goddek et al., 2015). However, the nutrient retention in fish biomass is highly dependent on fish species, feeding levels, feed composition, fish size and system temperature (Schneider et al., 2004). At higher temperatures, for example, fish metabolism is accelerated and thus results in more nutrients contained in the solid fraction of the faeces (Turcios and Papenbrock, 2014). The proportion of excreted nutrients also depends on the quality and digestibility of the diet (Buzby and Lin, 2014). The digestibility of the fish feed, the size of the faeces and the settling ratio should be carefully considered to ensure a good balance in the system and to maximise crop yields (Yildiz et al., 2017b). Indeed, while it is a priority that fish feed should carefully be chosen to suit fish needs, the feed components could also be selected to suit plant's requirements when it makes no difference to fish (Goddek et al., 2015; Licamele and David, 2009; Seawright et al., 1998).

1.3.3 Microbiological processes

1.3.3.1 Solubilisation

Solubilisation consists of the breaking down of the complex organic molecules composing fish waste and feed leftovers into nutrients in the form of ionic minerals which plants can absorb (Goddek et al., 2015; Somerville et al., 2014). In both aquaculture (Sugita et al., 2005; Turcios and Papenbrock, 2014) and aquaponics, solubilisation is conducted mainly by heterotrophic bacteria (Joyce et al., 2019b; van Rijn, 2013) which have not yet been fully identified (Goddek et al., 2015). Some studies have started deciphering the complexity of these bacteria communities (Schmautz et al., 2017). In current aquaculture, the most commonly observed bacteria are *Rhizobium sp.*, *Flavobacterium sp.*, *Sphingobacterium sp.*, *Comamonas sp.*, *Acinetobacter sp.*, *Aeromonas sp. and Pseudomonas sp*. (Munguia-Fragozo et al., 2015; Sugita et al., 2005). An example of the major role of bacteria in aquaponics

could be the transformation of insoluble phytates into phosphorus made available for plant uptake through the production of phytases which are particularly present in γ-proteobacteria (Jorquera et al., 2008). Other nutrients than P can also be trapped as solids and evacuated from the system with the sludge. Efforts are thus being made to remineralise this sludge with UASB-EGSB (upflow anaerobic sludge blanket and expanded granular sludge bed) reactors in order to reinject nutrients into the aquaponic system (Delaide et al., 2019, 2017; Goddek et al., 2016). Furthermore, different minerals are not released at the same rate, depending on the composition of the feed (Letelier-Gordo et al., 2015), thus leading to more complicated monitoring of their concentration in the aquaponic solution (Seawright et al., 1998).

1.3.3.2 Nitrification

The main nitrogen source in an aquaponic system is the fish feed and the proteins it contains (Goddek et al., 2015; Ru et al., 2017; Wongkiew et al., 2017; Yildiz et al., 2017a). Ideally, 100% of this feed should be eaten by the fish. However, is has been observed that fish only use about 30% of the nitrogen contained in the given feed (Rafiee and Saad, 2005). The ingested feed is partly used for assimilation and metabolism (Wongkiew et al., 2017), while the rest is excreted either through the gills or as urine and faeces (Ru et al., 2017). The nitrogen which is excreted through the gills is mainly in the form of ammonia, NH_3 (Wongkiew et al., 2017; Yildiz et al., 2017b), while urine and faeces are composed of organic nitrogen (Wongkiew et al., 2017) which is transformed into ammonia by proteases and deaminases (Sugita et al., 2005). In general, the fish excrete nitrogen under the form of TAN, i.e. NH_3 and NH_4^+. The balance between NH_3 and NH_4^+ depends mostly on the pH (ammonium is the favoured form when pH is between 2 and 7 (Trejo-Téllez and Gómez-Merino, 2012) and temperature. Ammonia is the major waste produced by fish catabolism of the feed proteins (Yildiz et al., 2017b). Nitrification is a two-step process during which the ammonia NH_3 or ammonium NH_4^+ excreted by the fish is transformed first into nitrite NO_2^- and then into nitrate NO_3^- by specific aerobic chemosynthetic autotrophic bacteria. A high availability of dissolved oxygen is required as nitrification consumes oxygen (Carsiotis and Khanna, 1989; Madigan and Martinko, 2007; Shoda, 2014). The first step of this transformation is carried out by ammonia-oxidising bacteria (AOB) such as *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*. The second step is conducted by nitrite-oxidising bacteria (NOB) such as *Nitrobacter*, *Nitrococcus*, *Nitrospira* and *Nitrospina* (Rurangwa and Verdegem, 2015; Timmons and Ebeling, 2013; Wongkiew et al., 2017). *Nitrospira* is currently deduced to be a complete nitrifier, i.e. to be involved in the production of both nitrite

and nitrate (Daims et al., 2015). The same bacteria can be found both in aquaculture and aquaponic systems (Wongkiew et al., 2017). These bacteria are mainly found in biofilms fixed to the media composing the biofilter but can also be observed in the other compartments of the system (Timmons and Ebeling, 2013). Nitrification is of prime importance in aquaponics as ammonia and nitrite are quite toxic for fish: 0.02–0.07 mg/L of ammonia–nitrogen is sufficient to observe damage in warm water fish, and nitrite–nitrogen should be kept under 1 mg/L (Losordo et al., 1998; Timmons and Ebeling, 2013). Ammonia affects the central nervous system of the fish (Randall and Tsui, 2002; Timmons and Ebeling, 2013), while nitrite induces problems with oxygen fixation (Losordo et al., 1998). Nitrate– nitrogen is, on the other hand, tolerated by the fish up to 150–300 mg/L (Goddek et al., 2015; Graber and Junge, 2009; Yildiz et al., 2017b). Nitrification mostly takes place in biofilters (Losordo et al., 1998; Timmons and Ebeling, 2013). Therefore, when starting a system, it is recommended to run the system without fish at first in order to allow the slowly growing population of nitrifying bacteria to establish (Timmons and Ebeling, 2013; Wongkiew et al., 2017). It is also necessary to avoid, as far as possible, the presence of organic matter in the biofilters to prevent the growth of highly competitive heterotrophic bacteria (Timmons and Ebeling, 2013). Alternatively, commercial mixes of nitrifying bacteria can be added to the system, prior to stocking, to hasten the colonisation process (Kuhn et al., 2010). Nevertheless, small aquaponic systems without biofilter also exist. In these systems, nitrifying bacteria form biofilms on the available surfaces (e.g. hydroponic compartment walls, inert media when using the media bed technique) (Somerville et al., 2014).

1.3.4 Mass balance: what happens to nutrients once they enter into the aquaponic system?

1.3.4.1 Context

The functioning of aquaponic systems is based on a dynamic equilibrium of the nutrient cycles (Somerville et al., 2014). It is therefore necessary to understand these cycles to optimise the management of the systems. Plants growing hydroponically have specific requirements, which should be met during their various growing stages (Resh, 2013). Therefore, nutrient concentrations in the different compartments of the system must be closely monitored, and nutrients should be supplemented to prevent deficiencies (Resh, 2013; Seawright et al., 1998) either in the system water or via foliar application (Roosta and Hamidpour, 2011).

According to Delaide et al. (2016), in some cases, supplementing an aquaponic solution with mineral nutrients in order to reach the same nutrient concentrations as in hydroponics could lead to higher yields than those achieved in hydroponics. The first step to take towards a balanced system is the correct design and relative sizing of the compartments (Buzby and Lin, 2014). If the hydroponic compartment is too small compared to the fish tanks, then the nutrients will accumulate in the water and could reach toxic levels. The feed rate ratio (i.e. the amount of fish feed in the system based on the plant-growing surface and the plant type) is often used for the first sizing of the system (Rakocy et al., 2006; Somerville et al., 2014). However, according to Seawright et al. (1998), it is not possible to reach a plant/fish ratio which will enable an optimal match of plants' needs if only fish feed is used as an input. To make sure that the system is well balanced and functions properly, monitoring methods are usually based on the nitrogen cycle (Cerozi and Fitzsimmons, 2017; Somerville et al., 2014), but to ensure optimal functioning of the system, it is necessary to monitor more closely the balance of the other macronutrients (P, K, Ca, Mg, S) and micronutrients (Fe, Zn, B, Mn, Mo, Cu) (Resh, 2013; Somerville et al., 2014; Sonneveld and Voogt, 2009) as well. Recent studies (Delaide et al., 2017; Schmautz et al., 2016, 2015) have started tackling this topic. Schmautz *et al.*, (2015, 2016) compared the impact of three different hydroponic layouts (i.e. nutrient film technique (NFT), floating raft and drip irrigation) on the nutrient uptake of aquaponic tomatoes. Drip irrigation was the system which produced slightly better yields with tomatoes. The mineral content of the fruits (P, K, Ca, Mg) was equivalent to the conventional values even though the iron and zinc contents were higher. The leaves however had lower levels of P, K, S, Ca, Mg, Fe, Cu and Zn than in conventional agriculture. Delaide et al. (2016) followed the cycles of macro- and micronutrients in a coupled aquaponic system. They observed that K, P, Fe, Cu, Zn, Mn and Mo were lacking in their aquaponic solution, while N, Ca, B and Na were quickly accumulated. Graber and Junge (2009) noted that their aquaponic solution contained three times less nitrogen and ten times less phosphorus than a hydroponic solution. As for potassium, it was 45 times lower compared to hydroponics. Nevertheless, they obtained yields similar to hydroponics even though the quality of their production was poorer due to a lack of potassium.

1.3.4.2 Factors influencing the nutrient cycles

Light intensity, root zone temperature, air temperature, nutrient availability, growth stage and growth rate all influence a plant's nutrient uptake (Buzby and Lin, 2014). Experiments conducted by Schmautz et al. (2016) and Lennard and Leonard (2006) showed that the hydroponic method could also play a role in a plant's nutrient uptake

capacity, and it is therefore necessary to match the growing system to the type of vegetables being grown. NFT and DWC (deep water culture – raft) are thus suitable for leafy greens, whereas drip irrigation on rockwool slabs is more suitable for fruity vegetables (Resh, 2013).

1.3.4.3 Macronutrient cycles

Carbon (C)

Carbon is provided to the fish via the feed (Timmons and Ebeling, 2013) and to the plants via CO_2 fixation. Fish can use 22% of the carbon contained in the fish feed for biomass increase and metabolism. The rest of the ingested carbon is either exhaled under the form of CO_2 (52%) or excreted in a dissolved (0.7–3%) and solid (25%) form (Timmons and Ebeling, 2013). The expirated CO_2 can be used by plants for their own carbon source as well (Körner et al., 2017). The uneaten part of the feed carbon is left to decompose in the system. The type of carbohydrates found in fish feed (e.g. starch or non-starch polysaccharides) can also influence the digestibility of the feed and the biodegradability of the waste in an aquaculture or aquaponic system (Meriac et al., 2014).

Nitrogen (N)

Nitrogen is absorbed by the plants either in the nitrate or ammonium form (Sonneveld and Voogt, 2009; Xu et al., 2012) depending on the concentration and plant's physiology (Wongkiew et al., 2017 citing Fink and Feller, 1998). Associations between plants and microorganisms should not be overlooked as plants affect the presence of the microorganisms in aquaponics, and microorganisms can play a significant role in the nitrogen uptake capacity of plants (Wongkiew et al., 2017). The uptake of nitrogen by plants is also affected by the ambient carbon dioxide concentration (Wongkiew et al., 2017 citing Zhang et al. 2008).

Phosphorus (P)

Phosphorus is one of the essential elements for plant growth and can be absorbed under its ionic orthophosphate form ($H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}) (Prabhu et al., 2007; Resh, 2013). Little is known about the dynamics of phosphorus in aquaponics. The main input of phosphorus in the system is the fish feed (Cerozi and Fitzsimmons, 2017; Delaide et al., 2017; Schmautz et al., 2015), and in un-supplemented systems (Palm et al., 2019), phosphorus tends to be limiting and thus can impede plant growth (Graber and Junge, 2009; Seawright et al., 1998). According to Rafiee and Saad (2005), fish can use up to 15% of the phosphorus contained in the feed. In a system

growing lettuce, Cerozi and Fitzsimmons (2017) noticed that the amount of phosphorus provided by the fish feed can be sufficient or insufficient depending on the growth stage. Up to 100% of phosphorus present in the fish water can be recycled in the plant biomass, depending on the design of the system. Graber and Junge (2009) observed a 50% recycling, while Schmautz et al. (2015) reported that 32% of the phosphorus could be found in the fruit and 28% in the leaves. The solubility of phosphorus depends on the pH, and a higher pH will foster the precipitation of phosphorus, thus rendering it unavailable for the plants (Yildiz et al., 2017b). Phosphorus can precipitate as struvite (magnesium ammonium phosphate $NH_4MgPO_4 \cdot 6\ H_2O$) (Le Corre et al., 2005) and/or hydroxyapatite ($Ca5(PO4)3(OH)$; inorganic phosphorus) (Cerozi and Fitzsimmons, 2017; Goddek et al., 2015). These insoluble complexes are removed via solid fish sludge from the system. Schneider et al. (2004) reported that 30–65% of the phosphorus contained in the fish feed remains unavailable to plants as it is fixed in the solid excretions which are then removed through mechanical filtration. Yogev et al. (2016) estimated that this loss can be up to 85%. One option to prevent this massive loss of P via solid sludge is to add a microbial digestion compartment to the aquaponic system. During aerobic or anaerobic digestion, the P is released into the digestate and could be re-introduced into the circulating water (Goddek et al., 2016).

Potassium (K)

Delaide et al. (2017) found that the major source of K in their system was the fish feed. Fish can use up to 7% of the K contained in the fish feed (Rafiee and Saad, 2005). However, potassium is not necessary for fish which leads to a low potassium composition of the fish feed and to even lower levels of potassium available for the plants (Graber and Junge, 2009; Seawright et al., 1998; Suhl et al., 2016). To supply potassium, a potassium hydroxide (KOH) pH buffer is often used as the pH often decreases in aquaponics due to nitrification (Graber and Junge, 2009). In an aquaponic system planted with tomatoes, potassium accumulated mainly in the fruits (Schmautz et al., 2016).

Magnesium (Mg), calcium (Ca) and sulphur (S)

The main source for Mg, Ca and S is tap water which facilitates the absorption by the plants as the nutrients are already available (Delaide et al., 2017). Calcium is however present in insufficient levels in aquaponics (Schmautz et al., 2015; Seawright et al., 1998) and is added under the form of calcium hydroxide $Ca(OH)_2$ (Timmons and Ebeling, 2013). According to Rafiee and Saad (2005), fish can use on average

26.8% of the calcium and 20.3% of the magnesium present in the feed. Sulphur is often at low levels in aquaponic systems (Graber and Junge, 2009; Seawright et al., 1998).

1.3.4.4 Micronutrient cycles

Iron (Fe), manganese (Mn) and zinc (Zn) derive mainly from the fish feed, while boron (B) and copper (Cu) derive from the tap water (Delaide et al., 2017). In aquaponics, key micronutrients are often present but at too low levels (Delaide et al., 2017), and supplementation from external sources of nutrients is then necessary (Goddek et al., 2019b). Iron deficiencies occur very often in aquaponics (Licamele and David, 2009 citing Fitzsimmons and Posadas; 1997; Schmautz et al., 2015; Seawright et al., 1998), mostly because of the non-availability of the ferric ion form. This deficiency can be solved by the use of bacterial siderophore (i.e. organic iron-chelating compounds) produced by genera such as *Bacillus* or *Pseudomonas* (Bartelme et al., 2018) or by iron supplementation with chemical chelated iron to avoid iron precipitation.

1.3.4.5 Nutrient losses

Reducing nutrient loss is a constant challenge facing aquaponics practitioners. Nutrient loss occurs in several ways, e.g. the settlement of the sludge (37% of faeces and 18% of uneaten feed) (Neto and Ostrensky, 2015), water losses, denitrification, ammonia volatilisation, etc. (Wongkiew et al., 2017). As an example, Rafiee and Saad (2005) note that 24% of the iron, 86% of the manganese, 47% of the zinc, 22% of the copper, 16% of the calcium, 89% of the magnesium, 6% of the nitrogen, 6% of the potassium and 18% of the phosphorus contained in the fish feed were contained in the sludge. The sludge can hold up to 40% of the nutrients present in the feed input (Yogev et al., 2016). Denitrification can lead to a loss of 25–60% of the nitrogen (Hu et al., 2015; Zou et al., 2016a). Denitrification is also linked to anoxic conditions (Madigan and Martinko, 2007; van Lier et al., 2008) and low carbon levels and is responsible for the transformation of nitrate into nitrite, nitric oxide (NO), nitrous oxide (N_2O) and eventually nitrogen gas (N_2) with flows into the atmosphere. Denitrification is conducted by several facultative heterotrophic bacteria such as *Achromobacter*, *Aerobacter*, *Acinetobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Pseudomonas*, *Proteus* and *Micrococcus* sp. (Gentile et al., 2007; Michaud et al., 2006; Wongkiew et al., 2017). Some bacteria can perform both nitrification and denitrification if dissolved oxygen levels are below 0.3 mg/L (Fitzgerald et al., 2015; Wongkiew et al., 2017). The loss of nitrogen can also occur via anaerobic ammonium oxidation

(ANAMMOX), i.e. the oxidation of ammonium into dinitrogen gas in the presence of nitrite (Hu et al., 2011). Another important loss of nitrogen which should be available for the plants is the consumption of the nitrogen by the heterotrophic aerobic bacteria present in the aquaponic systems. Indeed, the nitrogen used by these bacteria is lost to nitrifying bacteria, and nitrification is thus impeded (Blancheton et al., 2013). These bacteria are particularly present when the C/N ratio increases as they are more competitive and abler to colonise the media than the autotrophic nitrifying bacteria (Blancheton et al., 2013; Wongkiew et al., 2017).

1.3.5 Conclusions

1.3.5.1 Current problems of nutrient cycling in aquaponics

In hydroponics, the nutrient solution is accurately determined and the nutrient input into the system is well understood and controlled. This makes it relatively easy to adapt the nutrient solution for each plant species and for each growth stage. In aquaponics, according to the definition (Palm et al., 2018), the nutrients have to originate at least at 50% from uneaten fish feed, fish solid faeces and fish soluble excretions, thus making the monitoring of the nutrient concentrations available for plant uptake more difficult. A second drawback is the loss of nutrients through several pathways such as sludge removal, water renewal or denitrification. Sludge removal induces a loss of nutrients as several key nutrients such as phosphorus often precipitate and are then trapped in the evacuated solid sludge. Water renewal, which has to take place even if in small proportions, also adds to the loss of nutrients from the aquaponic circuit. Finally, denitrification happens because of the presence of denitrifying bacteria and conditions favourable to their metabolisms.

1.3.5.2 How to improve nutrient cycling?

To conclude, nutrient cycling still needs to be improved in order to optimise plant growth in aquaponics. Several options are therefore currently explored in Goddek et al. (2019b). To avoid losing the nutrients captured in the sludge, sludge remineralisation units have been developed (Delaide et al., 2019). The aim of these units is to extract the nutrients captured in solid form in the sludge and to reinject these into the system under a form which the plants can absorb (Delaide et al., 2017). A further technique to reduce nutrient loss would be to foster plant uptake through the concentration of the aquaponic solution (i.e. the removal of a fraction of the water to keep the same amount of nutrients but in a lesser water volume). Such a concentration could be achieved via the addition of a desalination unit as part of the aquaponic

system (Goddek and Keesman, 2018; Goddek and Körner, 2019). Finally, the use of decoupled/multi-loop systems enables optimal living and growing conditions for all fish, plants and microorganisms. While some research has been undertaken in this field, more research should be conducted to better understand nutrient cycling in aquaponics. Indeed, more information concerning the exact cycles of each macronutrient (what form, how it can be transformed or not by microorganisms, how it is taken up by plants in aquaponics) or the influence of the plant and fish species and water parameters on the nutrient cycles could greatly help the understanding of aquaponic systems.

1.4 Microorganisms in aquaponics: what do we know and why studying them?

1.4.1 Microorganisms in RAS and hydroponics

1.4.1.1 In recirculating aquaculture systems

For years, the focus has been drawn on bacteria involved in the nitrification process (Michaud et al., 2006) such as *Nitrosomonas* and *Nitrobacter*, the two most famous AOB and NOB. Nevertheless, the genus *Nitrospira* has also been singled out for its capacity to perform the complete process of nitrification on its own (Daims et al., 2015) and its important presence in aquaponic systems (Bartelme et al., 2018; Eck et al., 2019b; Schmautz et al., 2017). This unique interest can be easily explained by the fact that nitrification is the most crucial microbial process in RAS to ensure fish welfare. However, recent studies have also started tackling the topic of microorganisms directly involved in fish health and care such as probiotics (Joyce et al., 2019b; Kasozi et al., 2021a, 2021b).

1.4.1.2 In hydroponics

Hydroponic systems are acknowledged to host their own microbial communities which differ in terms of composition, density and diversity between systems designs and choices of substrate (Lee and Lee, 2015), nutrients sources (Lee and Lee, 2015 citing Khalil and Alsanius, 2001) and plant cultivars (Vallance et al., 2011). Hydroponic microbial communities can easily develop in the system solution, in the inert substrates and in the plants' rhizospheres by means of plant exudates and molecules present in the nutrient solution (Lee and Lee, 2015 citing Khalil and Alsanius, 2001; Vallance et al, 2010) with the bacterial inoculum coming from the plant and water source. There has been some evidence though that microbial

communities colonized the roots more easily than the nutrient solution (Vallance et al., 2011). Concerning the substrates, bacteria would mainly take over inorganic substrates while fungi would prefer organic ones (Vallance et al., 2011). It also appears that aerobic bacteria may prevail on roots and in nutrient solutions with a few slight variations however, depending on the type of system ("inorganic and organic media, deep flow technique and nutrient film technique" (Vallance et al., 2011)). Regardless of the system, roots and nutrient solutions often harboured fluorescent *Pseudomonas* carrying possibly anti-pathogens agents (Vallance et al., 2011). In terms of density, concentrations of heterotrophic bacteria of 10^5-10^6 CFU/ml could be detected in the nutrient solution of a hydroponic system "20h after planting tomatoes" (Vallance et al., 2011 citing Berkelmann et al., 1994).

Even in soilless systems, plants are able to select their rhizosphere microorganisms by the secretion of specific root exudates (Vallance et al., 2011). Studies conducted by Chave et al. (2008) showed that the rhizoplane may contain as much bacterial diversity as that of the rhizosphere in soil but further studies are required to confirm this observation. Following their change of physiological stages, plants such as tomatoes can also gradually shift the composition of the root and nutrient solution microbial communities by secreting different exudates (Vallance et al., 2011).

A few microorganisms are naturally present in hydroponics such as *Gliocladium spp., Trichoderma spp., Pseudomonas spp., Bacillus spp*. (Lee and Lee, 2015 citing Khalil and Alsanius, 2009) but most of the time studies report the introduction of known PGPR into the systems. The introduction of PGPR such as *Bacillus spp*. *Pseudomonas spp. Streptomyces griseoviridis* (Raaijmakers et al., 2010), *Pseudomonas chlororaphis, Bacillus cereus,* (Lee and Lee, 2015 citing Liu et al., 2007), *Bacillus amyloliquefaciens* and *Bacillus licheniformis* has been reported to enhance yields (Lee and Lee, 2015). More specifically, *Bacillus subtilis* can be helpful in hydroponics as it can impact the salinity of nutrient solutions (Lee and Lee, 2015). Concerning pathogens, *Fusarium, Phytophtora* and *Pythium* are the most commonly found (Lee and Lee, 2015).

Microorganisms which have already been introduced in hydroponics for their potential plant beneficial effects have been listed in **Table 1-3**.

Table 1-3 List of beneficial microorganisms for plant in hydroponic systems (Lee and Lee, 2015).

Microorganisms		Host plant
Genus	**Species**	
Pseudomonas	*Aeruginosa, aureofaciens, chlororaphis, corrugate, fluorescens, fulva, marginalis, oligandrum, plecoglossicida, putida, syringae*	Bean, carnation, chickpea, cucumber, lettuce, peppers, potato, radish, tomato
Bacillus	*Amyloliquefaciens, cereus, subtilis, thuringiensis*	Carrot, chrysanthemum, cucumber, lettuce, pepper, tomato
Enterobacter	*Aerogenes*	Cucumber
Streptomyces	*Griseoviridis*	Cucumber, tomato
Gliocladium	*Catenulatum*	Cucumber, tomato
Trichoderma	*Asperellum, atroviride, harzianum, virens*	Bean, cotton, cucumber, maize, rice

1.4.2 Current knowledge on aquaponic microorganisms

With the view to improve aquaponics sustainability, more and more studies are now tackling the microbial aspects of aquaponics in order to better understand their roles and more specifically their impact on the nutrient cycles.

Currently, the most famous microorganisms in aquaponics are, thanks to the abundant RAS literature, the nitrifying bacteria. However, slight differences can be noted between the nitrifying bacteria in aquaponics and in aquaculture. Indeed, in aquaponics studies, very few *Nitrosomonas* and *Nitrobacter* have been detected in biofilters whereas important proportions of *Nitrospira* have been observed in several aquaponic systems (Bartelme et al., 2017; Eck et al., 2019b; Schmautz et al., 2017). *Nitrospira* has been recently discovered as a COMMAMOX i.e. a bacteria capable of performing the whole nitrification process on its own (Daims et al., 2015). The predominance of this genus in the biofilter of aquaponic systems could represent a new paradigm for nitrification (Bartelme et al., 2019) and justifies the need for more in depth research on aquaponic microorganisms. Furthermore, *Archaeabacteria* could also be involved in nitrification. Eventually, nitrifying bacteria could also play other roles, in parallel with nitrification (Ajijah et al., 2021). Information regarding aquaponics microbiota will be developed in **chapters 3**, **4** and **5**.

1.4.3 Aquaponic microorganisms and nutrient cycling – bridging the information

Little data is currently available on the various roles that microorganisms could play in aquaponics and more specifically on their roles in nutrient cycling in aquaponics. The following parts will therefore propose a brief summary of the existing information concerning some plant beneficial microbial processes in soils and will also endeavour to adapt it to what we know about nutrient cycling in aquaponics. This will serve as a basis for the further study of microbial processes linked with plant health and care in aquaponics.

1.4.3.1 Plant beneficial functions harboured by microorganisms

The most authoritative source of information regarding plant beneficial functions harboured by microorganisms is soil based systems in which these functions have been studied and classified. Plant growth promoting microorganisms (PGPM) can be classified based on their direct or indirect effects on plants. Direct effects occur in the absence of pathogens and imply a close relationship between microorganisms and plants while indirect effects involve the help that PGPM provide in the fight against pathogens (Lugtenberg and Kamilova, 2009). Another classification splits the PGPM into three groups: biostimulants, biofertilisers and biocontrol agents (du Jardin, 2015). Briefly, biostimulants produce substances that will directly enhance plant growth via hormonal metabolisms and improve the nutrition pathways efficiency or the tolerance to abiotic stress but they do not provide nutrients themselves. Biofertilisers on the other hand increase the availability of the nutrients inside the growing media for the plants to absorb more easily. Biocontrol agents, finally, protect the plant against pathogens via several methods such as competition against the pathogen, predation, parasitism, the production of antibiotics or induced systemic resistance (ISR) (du Jardin, 2015).

- **Biostimulants**

Biostimulants or phytostimulators (Lugtenberg and Kamilova, 2009) are typically the microorganisms that will produce plant hormones (Van Loon, 2007 citing Frankenberg and Arshad 1995), the most significant of which is auxin and its precursor indole acetic acid (IAA). The production of this plant hormone by microorganisms is fostered by the exudation of tryptophan by the plant itself. Examples of auxin producing bacteria are *Pseudomonas fluorescens* (Lugtenberg and Kamilova, 2009) or *Azospirillum brasilense* (Van Loon, 2007). Other hormones such

as gibberellins, cytokinins or 1-Aminocyclo-propane-1- carboxylate (ACC, which is an ethylene precursor) can also be produced by microorganisms (Lugtenberg and Kamilova, 2009; Van Loon, 2007). While auxin is mainly involved in root growth (Lugtenberg and Kamilova, 2009) gibberellins and cytokinins foster shoot development (Van Loon, 2007). Ethylene on the other hand is mainly known for taking part in senescence when produced in high quantities and for inhibiting root and shoot growth at medium levels. However, when present in a small amount it can actually enhance plant growth (Van Loon, 2007). Some microorganisms are also able to interfere with the ethylene pathway by producing ACC deaminase. This enzyme enables them to "degrade ACC and utilize it as a carbon source" (Van Loon, 2007) and thus prevents ethylene to be formed and avoids the inhibition of root elongation (Van Loon, 2007 citing Glick 2005). Nevertheless, biostimulants are not restrained to the production of phytohormones as they can also emit volatile molecules - this is the case of *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Enterobacter cloacae* – such as "the cofactor pyrrolquinoline (PQQ)". "PQQ acts as an antioxidant in plants. However, it cannot be excluded that the effect is indirect because PQQ is a cofactor of several enzymes e.g. involved in antifungal activity and induction of systemic resistance" (Lugtenberg and Kamilova, 2009).

- **Biofertilisers**

Biofertilisers are microorganisms that interact with the nutrients already present in the growing medium (du Jardin, 2015). The most famous are the atmospheric nitrogen fixing bacteria from the *Rhizobium* and *Bradyrhizobium* genera (Lugtenberg and Kamilova, 2009) which form nodules in *Fabaceae's* roots but this capacity is also present in free-living species (Van Loon, 2007). However, biofertilisers can also solubilise nutrients which are little available to the plants such as phosphate. For this, they can exudates "phosphatases, phytases, phosphonatases and C-P lyases. The latter cleaving C-P links in organophosphonates" or produce "organic acids such as gluconic acid to release phosphorus from mineral phosphate" (Lugtenberg and Kamilova, 2009). Biofertilisers can also impact the absorption of iron, zinc and other essential micronutrients (Van Loon, 2007) as for example, microorganisms pertaining to the biofertilisers group can produce siderophores to facilitate the absorption of iron (Van Loon, 2007 citing Vessey, 2003).

- **Biocontrol agents**

Biocontrol agents are involved in the fight against plant pathogens. Their techniques are numerous: competition against the pathogen for food and space, predation and

parasitism (e.g. *Trichoderma* (Lugtenberg and Kamilova, 2009)), production of antibiotics or antimicrobial compounds such as hydrogen cyanide (HCN) (Lugtenberg and Kamilova, 2009) and priming of the plant via ISR which is a way of preparing the plant for stronger resistance against the pathogen via an enhanced expression of defence responses by the plant (Van Loon, 2007). Competition can occur for ferric ions for instance, with the biocontrol agent producing siderophores which bind the Fe_3^+ ions present in the environment and easily absorb it, thus rendering it unavailable for the pathogen and inhibiting its growth. This is all the more striking in iron poor environment (Lugtenberg and Kamilova, 2009) such as aquaponics. Other defence mechanisms are the "reinforcement of plant cell walls, production of anti-microbial phytoalexins and synthesis of pathogenesis-related proteins" (Van Loon, 2007 citing Hammond-Kosack and Jones, 1996). Biocontrol agents can also help plant against insects by modifying the plant secondary metabolism (Van Loon, 2007). The presence of biocontrol agent in a medium makes it suppressive as this has been shown for soils (Lugtenberg and Kamilova, 2009) and aquaponic solution (Stouvenakers et al., 2020).

- **Beneficial bacteria and fungi**

PGPM include both bacteria and fungi of various degree of intimacy with the plant ("free-living, rhizospheric, endosymbiotic") with fungi presenting more of a continuum of relationships between mutualism and parasitism (du Jardin, 2015). In the case of rhizospheric or endosymbiotic relationship, the colonisation capacities of the microorganisms are of prime importance. Indeed, microorganisms beneficiate from the molecules exuded by the plant roots which represent up to 61% of the carbon they fix. Plants can select the rhizosphere microorganisms via the secretion of specific exudates and can also interfere with bacteria development and quorum sensing (van Loon, 2007). It is important to keep in mind that "the concentration of bacteria in the rhizosphere is one hundred times lower than in the average laboratory medium" (Lugtenberg and Kamilova, 2009). Therefore, *in vitro* results are always to be qualified with *in vivo* tests to ensure that the beneficial effect of the microorganisms can still be observed in natural conditions. Beneficial bacteria can be present in the bulk soil, in the rhizosphere, in the rhizoplane or directly inside the plant cells and "association may be transient or permanent, some bacteria being even vertically transmitted via the seed" (du Jardin, 2015).

The most notorious group of beneficial fungi are the arbuscular mycorrhizal fungi (AMF) which are endomycorrhiza i.e. the hyphae penetrates the cell roots. Major taxa from this group are the *Glomeromycota* and *Trichoderma* spp. (du Jardin, 2015). *Trichoderma* spp. has been thoroughly studied as it has long been known for its skills

of mycoparasitism and "inducer of disease resistance". Other effects observed in plants such as "higher tolerance to abiotic stress, nutrient use efficiency, organ growth and morphogenesis" in contact with fungal endophytes can have been induced by these microorganisms which thus can be called biostimulants (du jardin, 2015). Fungi's effects on plants are similar to bacteria's as they can "promote nutrition efficiency, tolerance to stress, crop yield" and can interact with phosphorus as well (du jardin, 2015).

1.4.3.2 Functions in interaction with aquaponics crops

- **Phosphorus solubilisation**

Nutrient sources

In aquaponics, phosphorus mainly originates from fish feed (see 1.3.4) and its form in the system therefore depends on the type of feed. If the fish feed is plant-based, then phosphorus will mainly be found under the form of insoluble phytates (i.e. insoluble organic phosphate) (da Silva Cerozi and Fitzsimmons, 2017). **Figure 1-8** presents a summary of phosphorus flow in aquaponics.

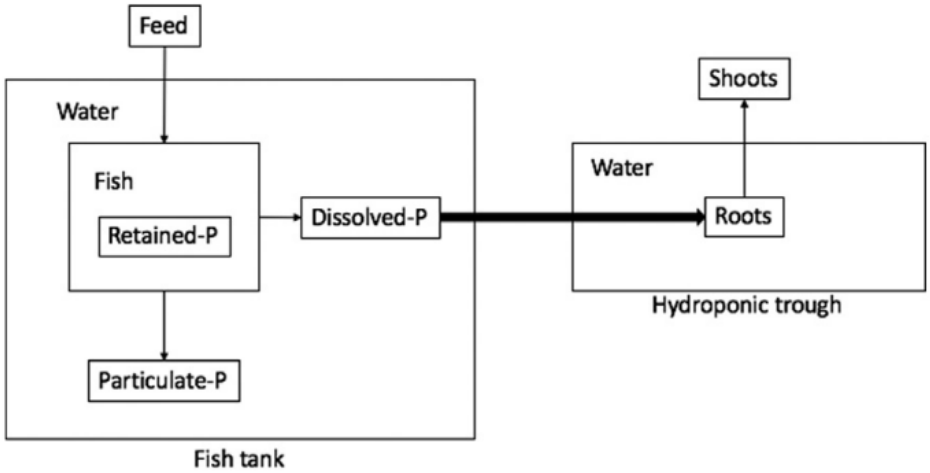


Figure 1-8 Schematic diagram of the phosphorus flow in an aquaponic system (Cerozi and Fitzsimmons, 2017)

For plants to be able to uptake phosphorus, it needs to be under the form of soluble orthophosphate ions which mainly originate from soluble fish faeces. However, orthophosphates are prone to react with other organic or inorganic compounds

(**Figure 1-9**) present in the aquaponic solution and thus become unavailable for plants again (Prabhu et al., 2019). Indeed, the solubility of phosphorus is highly dependent on the pH of the solution with a high pH (>7) leading to phosphorus precipitation under the form of struvite (magnesium ammonium phosphate; $NH_4MgPO_4 – 6\ H_2O$; inorganic phosphate) or hydroxyapatite ($Ca_5(PO_4)_3(OH)$; inorganic phosphorus). Orthophosphates in aquaponics can also reacts with iron (Cerozi and Fitzsimmons, 2017). Regarding the insoluble organic phosphates which enter the system under the form of phytates in plant-based fish feed, they can be solubilised by directly supplementing fish-feed with phytases (i.e. a specific phosphatase enzyme) (Cerozi and Fitzsimmons, 2017). Microorganisms can also produce these enzymes (Prabhu et al., 2019).

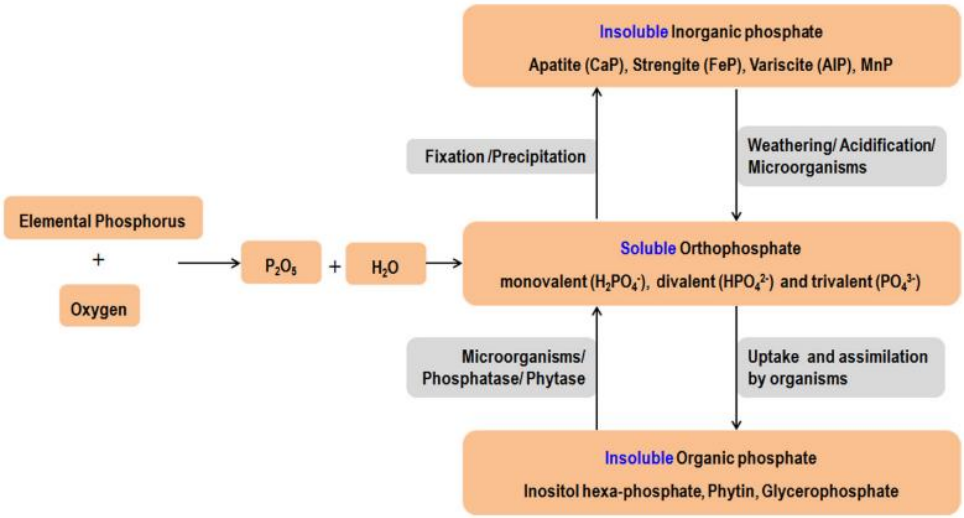


Figure 1-9 Phosphorus cycle in the environment (Prabhu et al., 2019)

Why is P solubilisation interesting in aquaponics?

Phosphorus can be a limiting factor in un-supplemented systems (Graber and Junge, 2009; Seawright et al., 1998) as the high pH necessary for fish welfare often fosters its precipitation under the form of inorganic phosphate salts. With the switch from fishmeal to plant-based aquafeeds, the question of phytates also becomes crucial with a need to solubilise this organic form of phosphorus. Hence, to avoid loss in the environment via solid removal but also to avoid the need to supplement with external

phosphorus, it is essential to focus on ways to enhance phosphorus uptake by fish and plants in aquaponic systems.

Phosphorus solubilising microorganisms

Many microorganisms are known to be involved in phosphorus solubilisation in the soil. They can be separated into inorganic phosphorus solubilising and organic phosphorus mineralising microorganisms and are widespread in nature (Prabhu et al., 2019). Inorganic phosphorus solubilizing bacteria and fungi are listed in **Table 1-4**.

Table 1-4 Inorganic phosphorus solubilizing bacteria and fungi

Bacteria	References	Fungi	References
Bacillus sp.	Richardson and Simpson, 2011; Cao et al., 2018	*Aspergillus sp.*	Prabhu et al., 2019
Bacillus thuringiensis		*Aspergillus japonicus*	
Bacillus megaterium (also for K)			
Enterobacter intermedium	Cao et al., 2018	*Penicillium sp.*	Prabhu et al., 2019
Burkholderia sp.	Cao et al., 2018	*Trichoderma sp.*	Prabhu et al., 2019
Burkholderia caryophylli			
Pseudomonas sp.	Cao et al., 2018; Prabhu et al., 2019	*Rhizoctonia sp.*	Prabhu et al., 2019
Pseudomonas cichorii,			
Pseudomonas syringae			
Actinomycetes	Prabhu et al., 2019		

Organic phosphorus mineralising bacteria often belong to the *Bacillus* genus (*B. cereus* and *B. megaterium*, capable of both types of solubilisation) (Cao *et al.*, 2018). *Bacillus cereus* was detected by Sanchez et al. (2019) in their aquaponic system and they identified some *Pseudomonas* too. The formation of bacterial biofilms could also enhance the phosphate solubilisation effects of bacteria (Prabhu et al., 2019).

Mechanisms for solubilisation

As shown in **Figure 1-10** (Prabhu et al., 2019), microorganisms are deeply involved in phosphorus cycling and transformations (Prabhu et al., 2019). Solubilisation mechanisms are manifold and depend on the phosphorus form (**Figure 1-10**).

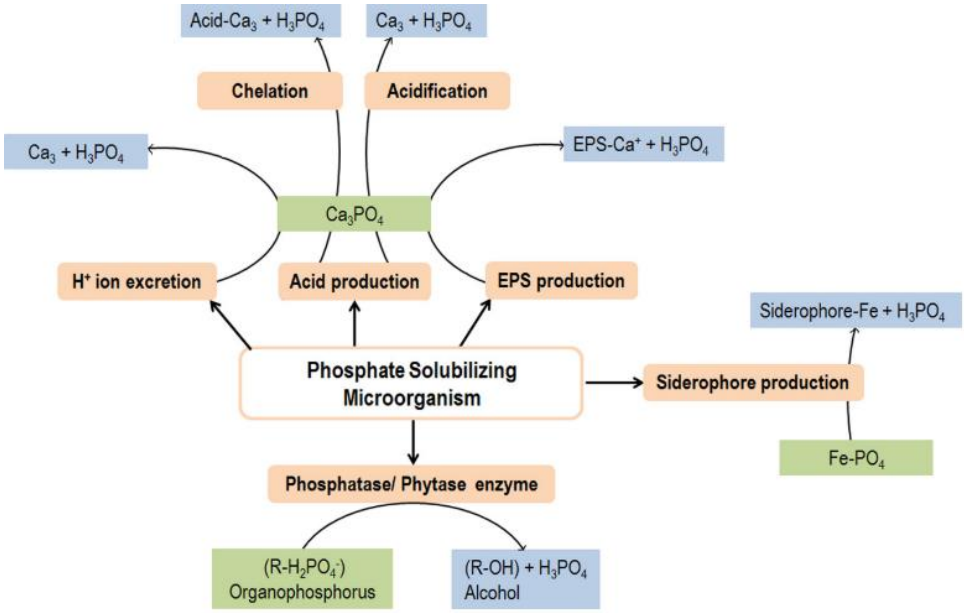


Figure 1-10 Mechanisms of inorganic and organic phosphate solubilisation by microorganisms (Prabhu et al., 2019)

- Mineralisation of organic P:

Organic phosphorus can be mineralised via the production of microbial enzymes such as "phosphatases or phosphohydrolase, phytases, phosphonatases and C-P lyases" (Prabhu et al., 2019). Enzymes cleave by hydrolysis the ester phosphate bonds of molecules to release the phosphate ions (Prabhu et al., 2019). Even though both plants and microorganisms are capable of producing organic P solubilizing enzymes, microbial enzymes are more efficient in this task (Richardson and Simpson, 2011).

- Solubilisation of inorganic P:

Several mechanisms for inorganic P solubilisation exist and most of them are based on acidification of the environment via the excretion of organic acids (Prabhu et al., 2019). Several organic acids can be produced by the microorganisms such as "acetic acid, formic acid, lactic acid, gluconic acid, glycolic acid, oxalic acid, succinic acid, malic acid and citric acid" with gluconic acid being the major acid involved (Prabhu et al., 2019). Briefly, the excretion of these acids lowers the pH of the environment which then leads to the solubilisation of phosphorus from minerals into the soil solution (Prabhu et al., 2019). However, these organic acids can also operate on

minerals via a chelation process. Indeed, some acids such as humic and fulvic acid are able to chelate cations (e.g. calcium, iron, aluminium) and thus detach phosphorus from their mineral form (Prabhu et al., 2019). Humic and fulvic acids originate from plants and are produced during the plant degradation process by microorganisms (Prabhu et al., 2019). However, it must be pointed out that biochemical processes such as phosphorus solubilisation are more straightforward to study *in vitro* that *in vivo* (Richardson and Simpson, 2011).

Some microorganisms such as nitrifying and sulphur-oxidizing bacteria are able to produce inorganic acids like "sulphuric acid, nitric acid and carbonic acid" to proceed to the solubilisation of inorganic phosphates even though this process has been deemed less effective that the action of organic acids (Prabhu et al., 2019). Another possibility to solubilise inorganic phosphorus is the excretion of H^+ ions which also acidifies the environment and thus helps detaching phosphates from minerals. The production of protons is linked to "NH_4 assimilation, respiratory H_2CO_3 production and extrusion of organic acid anions" (Prabhu et al., 2019). Microorganisms can also exude exopolysaccharides which, via an indirect effect not thoroughly understood yet, are able to bond with metal ions and thus release soluble phosphates into the soil solution. The exopolysaccharides are often produced in response to stress (Prabhu et al., 2019). Siderophores can play a role in the solubility of iron phosphates though the precise mechanisms linking siderophores and phosphorus solubilisation are not yet understood (Prabhu et al., 2019).

In addition to phosphate-solubilising abilities, phosphorus solubilising microorganisms may further benefit plant growth as they can also produce plant growth hormones or modify the equilibrium between soil solution and minerals and thus increase transfer of orthophosphate ions into the solution (Richardson and Simpson, 2011).

- **Potassium solubilisation**

Nutrient sources

Potassium in aquaponics originates mainly from fish feed (Delaide et al., 2017) which is quite poor in potassium as fish do not much need it (Graber and Junge, 2009). In fish feed, potassium can be found under the form of KI (potassium iodide) or $KHCO_3$ (potassium bicarbonate (Terpstra, 2015). As it is a vital nutrient for plants it is important to maximise its use in order to avoid complementation. Indeed, Graber and Junge (2009) noted a potassium concentration in their aquaponic system which was 45 times lower than in hydroponics and this impacted the final tomatoes dry mass.

Currently, the easiest way to remedy the potassium deficiency is to add potassium hydroxide (KOH) pH buffer which will also help control the natural acidification of aquaponic systems (Graber and Junge, 2009; Wongkiew et al., 2017).

Why is it interesting in aquaponics?

Some of the potassium brought by fish feed could be evacuated by the system via the uneaten feed in the sludge. Sludge could be re-mineralised via bacterial processes to make nutrients available for plants again (Goddek et al., 2018). Very little information concerning potassium in such remineralisation units is available. Microbial processes could be useful in such units, even more so if the bacteria are naturally present in the water.

Potassium solubilising microorganisms

Several microorganisms are known to possess the abilities to solubilise potassium. It concerns mainly saprophytic bacteria, some fungi and actinomycetes which are naturally present in the soil and in the rhizosphere (Etesami et al., 2017). Some of the bacteria (Etesami et al., 2017) and fungi (Sattar et al., 2018) known for their K solubilising abilities are listed below (**Table 1-5**).

Mechanisms for potassium solubilisation

Potassium solubilisation can be performed by both bacteria and fungi even though bacteria prevail in this process (Sattar et al., 2018). Precise solubilisation mechanisms are however less understood than in the case of phosphorus solubilisation (Etesami et al., 2017). As for phosphorus, the main known mechanisms involve the acidification of the environment via the production of organic acids, inorganic acids and protons (Etesami et al., 2017).

As little information is available as to the form of potassium captured in aquaponic sludge, it is difficult to single out a specific solubilisation process which could be of interest. Still, the formation of microbial biofilm is an important factor in K solubilisation as it can enhance the impact of microorganisms, fostering the contact between organic acids, microbial polymers and the K minerals. Furthermore, the presence of microbial biofilms can also enhance water contact with the minerals and hence foster the weathering of those minerals (Etesami et al., 2017; Sattar et al., 2018). To conclude, the main potassium solubilisation mechanisms used by microorganisms are: “(i) lowering the pH; (ii) enhancing chelation of the cations bound to K; and (iii) acidolysis of the surrounding area of microorganism” (Meena et al., 2016a).

Table 1-5 Potassium solubilising bacteria and fungi (Etesami et al., 2017)

Bacteria	Comments	Fungi
Bacillus edaphicus	particularly efficient (production of carboxylic acids and capsular polysaccharides)	*Aspergillus sp.*
Bacillus mucilaginosus	highly efficient (increase of K solubilisation from 68% to 83% compared to a control according to Sheng and Huang, 2002 cited by Sattar et al., 2018).	*Aspergillus terreus*
Bacillus circulans	able to solubilise both P and K	*Aspergillus niger*
Bacillus megaterium		
Acidothiobacillus ferrooxidans		*Glomas mossea*
Paenibacillus sp.		
Paenibacillus mucilaginosus		
Paenibacillus glucanolyticus		*Glomas intradices*
Pseudomonas sp.	able to solubilise both P and K	
Burkholderia sp.	are able to solubilise both P and K	
Agrobacterium tumefaciens		*Penicillium sp.*
Rhizobium tumefaciens		
Arthrobacter		
Cladosporium		*Penicillium frequentas*
Aminobacter		
Sphingomonas		

- **Indole acetic acid production**

Role of indole acetic acid in plant growth

Indole acetic acid (IAA) is a form of auxin (Hopkins, 2003), the most known plant hormone. It is often produced from L-tryptophan by rhizosphere bacteria and is "one of the most physiologically active auxins" (Mohite, 2013). IAA can enhance plant growth and is particularly influent in root growth, development of lateral roots and root hairs (Mohite, 2013). It is also involved in cell elongation, cell division and differentiation (Hayat et al., 2010) and can contribute to higher shoot biomass (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011). Working upon some conditions such as the increase in cell osmotic contents, in water permeability into

cell, in cell wall synthesis or a decrease in wall pressure and inducing protein synthesis are ways that enable IAA to boost cell elongation (Mohite, 2013).

Sources of IAA

IAA is a metabolite which can be produced either by the plants themselves (Hopkins, 2003) or by microorganisms. Its production is linked to tryptophan-based pathways but can also results from tryptophan independent processes based on other substrates (Mohite, 2013). "In tryptophan dependant pathway, tryptophan is converted to indole-3-acetamide (IAM) by tryptophan-2-monooxigenase and IAM is metabolized to IAA by IAM-hydrolase" (Mohite 2013 citing Matsukawa et al., 2007).

Why is it interesting in aquaponics?

Plant growth promoting bacteria producing IAA have been thoroughly studied in soil as they are a sustainable way of enhancing plant yields. The presence and then the possibility to boost the presence of this kind of microorganisms in aquaponics could help foster plant biomass production despite a lack in nutrients compared to hydroponics. Furthermore, the elongation of plant roots could also enhance the nutrient uptake of the flowing-by nutrients along with the aquaponic solution.

Microorganisms involved

Microorganisms able to produce IAA can be classified in the biostimulants or phytostimulators category (du Jardin, 2015; Lugtenberg and Kamilova, 2009). According to Hayat et al. (2010) ca. 80% of the bacteria present in soil are able to produce IAA. More specifically, *Azospirillum brasilense*, *Bacillus megaterium* (also able to solubilise P and K), *Lactobacillus casei*, *Bacillus subtilis*, *Bacillus cereus* (also able to solubilise phosphorus), *Lactobacillus acidophilus*, *Pseudomonas putida* as well as the fungi genera *Trichoderma* and *Fusarium* have been identified as IAA producers (Mohite, 2013).

- **Siderophores production**

What are siderophores?

Siderophores are molecules which can either be secreted by microorganisms or by some families of plants (Hopkins, 2003) and that can bind iron and more specifically that are able to chelate ferric ions (Fe^{3+}) (Sasirekha and Srividya, 2016).

Role in plant growth

Microbial siderophores can help plant growth and health in several ways. Amongst these ways are the uptake of soil (or aquaponic solution) iron through the chelation of iron ions in the rhizosphere. They can also offer protection against phytopathogens as, when fostering plant uptake of soil iron, pathogens are prevented from absorbing the iron necessary to their development and the colonization of the plant roots (Ahmad et al., 2008; Sasirekha and Srividya, 2016). It has also been noted that microbial siderophores increase the chlorophyll content and plant biomass in cucumber plants (Reddy, 2014). Some siderophores producing rhizobacteria are also known to be able to provoke induced systemic resistance in plants (Sasirekha and Srividya, 2016). Siderophores can also benefit plant growth for the various reasons that have already been mentioned above as a role in P/K solubilisation. Other indirect functions are presented in **Figure 1-11**.

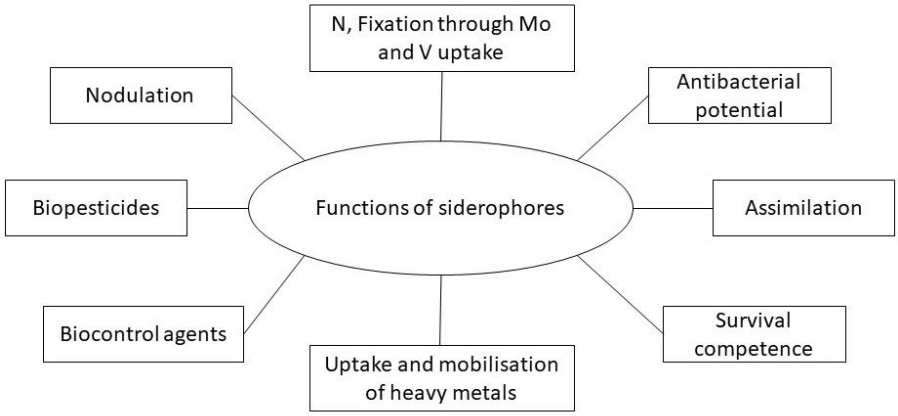


Figure 1-11 Impact of microbial secreted siderophores on plant growth (Reddy, 2014)

Microorganisms involved

Microorganisms known to produce siderophores are the following: *Bacillus*, *Pseudomonas*, *Geobacter*, *Alcaligenes*, *Clostridium*, *Streptomyces* and *Enterobacter* (Kasozi et al., 2019; Radzki et al., 2013; Reddy, 2014; Sasirekha and Srividya, 2016) for the bacteria and *Gliocladium* and *Trichoderma* for the fungi (Cerozi and Fitzsimmons, 2016b; Lee and Lee, 2015; Radzki et al., 2013). Some siderophores producing microorganisms can also have anti-pathogenic effect (Kasozi et al., 2019; Radzki et al., 2013).

Why is it interesting in aquaponics?

Iron is very often lacking in aquaponics as its main source in the system is fish feed and fish only need it in small quantities (Kasozi et al., 2019). Furthermore, the mild pH maintained in aquaponic systems favours the presence of the insoluble ferric ion (Fe^{3+}) form instead of ferrous ion (Fe^{2+}) (Kasozi et al., 2019) and this ferric form can easily react with other elements to form insoluble molecules (Kasozi et al., 2019). The ability to produce siderophores in aquaponics microbial communities would therefore promote iron cycling in aquaponics and enhance plant uptake.

1.5 Recent tools to study microbial communities and the concept of microbiome

1.5.1 Recent sequencing techniques

This part is adapted from Eck, M. 2017. Taxonomical characterisation of bacteria communities from water of diversified aquaponic systems. Master thesis. Gembloux Agro-Bio Tech, University of Liège

1.5.1.1 High throughput sequencing

Sequencing techniques have tremendously evolved over the last seventy years (Heather and Chain, 2016). First-generation DNA sequencing dates back to the sixties with the most known technique being the Sanger sequencing. This technique was based on the incorporation of fluorescently labelled ddNTP of each type which terminated the elongation of the strand, followed by a gel electrophoresis migration (Heather and Chain, 2016). First-generation sequencing was able to produce reads of around 1 kb (Heather and Chain, 2016). The Sanger technique is based on the "sequence-by-synthesis" method i.e. they rely on the use of a DNA polymerase to function (Heather and Chain, 2016).

Second-generation sequencing (or High Throughput Sequencing) was born with pyrosequencing which relies this time on the light emitted when pyrophosphate is turned into ATP which is then used to produce luciferase. Still, the greatest modification to the sequencing world was brought by the parallelization of the sequencing reactions which really made a difference from first-generation sequencing as it allows to go much faster in the sequencing process than before (Heather and Chain, 2016). The length of the fragment thus obtained is however shorter (400-500 bp) (Heather and Chain, 2016). Next generation sequencing (NGS) has allowed an

easier and cheaper access to sequencing techniques which then led to its spread and use in multiple scientific fields (Heather and Chain, 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.

Recently, third-generation sequencing techniques have emerged and regroup techniques bypassing the PCR step and capable of sequencing single molecules which avoid replication biases. Examples of these new devices are the PacBio and MinION (nanopore technology) sequencers (Heather and Chain, 2016).

1.5.1.2 Illumina

Illumina sequencing is a second-generation sequencing technique which almost totally controls the sequencing market (Heather and Chain, 2016). The core principle remains the same as pyrosequencing, 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).

The Illumina technology follows three major steps (Illumina, 2016):

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 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.

1.5.1.3 16S metabarcoding

NGS techniques can be hinged around three different approaches for the study of microbial communities: i) amplicon sequencing, ii) metagenome sequencing and iii) metatranscriptomics, with amplicon sequencing being the most widely used in microbiome studies (Massart et al., 2015). The characteristics of these approaches are described and compared in **Table 1-6**.

Table 1-6 Comparison of the three existing approaches for microbiome study by NGS. PCA: Principal Component Analysis; NMDS: Non Metric Multidimensional Scaling (Massart et al., 2015)

Approach	Amplicon	Metagenome	Metatranscriptome
Nucleic acids target	DNA	DNA	RNA
Laboratory steps	PCR amplification with selected primers	DNA shearing and library preparation	RNA shearing and library preparation
	High throughput sequencing		
Generated sequences	Thousands	Millions	Millions
Basic data analysis	Quality control of the sequences	Alignment of the sequences in contigs or scaffolds	
	Clustering of the sequences in OTUs	Taxonomic and functional assignation of the contigs	
	Taxonomic assignation of the OTUs		
Advanced data analysis	Alpha diversity analysis (richness, diversity…)	Gene and metabolic pathways characterisation and quantification	Sample comparison (genes and metabolic pathways differential expression)
	Sample comparison (taxa presence and abundance, diversity indexes)	Sample comparison (taxa, genes and metabolic pathways presence and abundance)	
	Beta diversity analysis (NMDS, PCA, Box-Plot)		
Output	Taxonomic abundance and diversity of the microbiome	Taxonomic abundance and diversity of the microbiome	Taxonomic abundance and diversity of the living microbiome
Advantages	Simpler bioinformatic analysis is without high computing power	Overview on the functions and pathways present in the microbiome	Overview on the functions and pathways transcribed in the microbiome
	Easy interpretation of the data	Functional analysis of the microbiome at genes and pathway levels	Functional analysis of the living microbiome at genes and pathway levels
	Low cost	More in depths analysis of strain composition and diversity	Better understanding of microbiome functions
Drawbacks	Analysis of dead cells and free DNA	Analysis of dead cells and free DNA	Technically the most challenging in the laboratory
	No functional information on the genes and pathways	The presence and abundance of a gene/pathway not always correlated with transcription level	More challenging bioinformatic analysis than metagenome analysis
		More challenging bioinformatic analysis than amplicon analysis	Higher cost
		Higher cost	

For bacterial communities' study, the 16S rRNA gene is mostly used (Massart et al., 2015).The 16S ribosomal RNA gene is very often chosen for amplification as it is stable in time and contains nine (or eight depending on the source) hypervariable regions with a total length of ca. 1500 bp (Cruaud et al., 2014; Nikolaki and Tsiamis, 2013; Yang et al., 2016). 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) or more simply to use universal primers to discriminate a whole community. However, as there are nine variable regions of various degrees of variability, it is not possible to discriminate all species based on only one region (Cruaud et al., 2014; Nikolaki and Tsiamis, 2013). The choice of primers and targeted hypervariable regions can thus dramatically influence the results of a metabarcoding analysis (Cruaud et al., 2014; Yang et al., 2016) and a sequence of between 500 and 700 bp is required for species identification. The correct choice of hypervariable regions and corresponding primers is a crucial step (Cruaud et al., 2014; Nikolaki and Tsiamis, 2013). Indeed, the regions have varying lengths (from 50 to 100 bases) but also varying polymorphisms. Consequently, some regions are more recommended to discriminate between certain genera (Nikolaki and Tsiamis, 2013). The V1-V3 regions are recommended for work on bacterial communities as the combination of several regions enables a better representation of complex communities (Nikolaki and Tsiamis, 2013).

1.5.2 Concepts of microbiome and microbiota

The development of NGS and metabarcoding has enabled the scientific community to study entire microbial communities without *a priori* i.e. without targeting specific organisms. Two terms emerged in the study of these microbial communities: i) the **microbiota** which is "the ecological community of microorganisms within a defined environment" and ii) the **microbiome** which is "the collective genomes of all microorganisms from a given environmental niche" (Massart et al., 2015). Therefore, the sequencing of a microbiome enables us to better understand the microbiota of a defined environment.

If we focus on plant microbiota, the microbial communities associated with plants are known to provide their hosts with several functions which are "(i) improving nutrient acquisition and growth, (ii) sustaining plant growth under biotic and/or abiotic stress, (iii) inducing resistance against pathogens, (iv) interacting with plant or human pathogens, and (v) interacting with other trophic levels like insects" (Massart et al., 2015). Therefore, the influence of the microbiota in plant health and care is

crucial (Singer et al., 2021) and so is the study of the interactions between plants and associated microbial communities. Indeed, plants are also able to interact with these communities by producing specific exudates or adapting their morphology (Massart et al., 2015). It is important to note that plant microbial communities "have been estimated at 10^6–10^7 cells/cm^2 in the phyllosphere and 10^6–10^9 cells/g in the rhizosphere" (Massart et al., 2015 citing Lindow and Brandl, 2003 and Whitman et al., 1998 respectively). The study of microbiota and, in our case, aquaponic microbiota is complex as many factors influence its composition and behaviour (Fierer, 2017) and many different approaches, angles and technologies would be required to obtain a full picture of this complex network. Whereby the purpose of this thesis is to pave the way to a better understanding of aquaponics microbiota.

2.

Thesis goals

2.1 Gaps to address in aquaponic research

Aquaponics is an ancient crop production technique (Palm et al., 2018) which has been rebranded and modernized by James Rakocy in the seventies (Lennard and Goddek, 2019). Rakocy then implemented his systems worldwide and the technique spread but more research was needed to fully apprehend and improve the systems' processes. Therefore, aquaponics is a relatively new research topic (Yep and Zheng, 2019). The first publications date back to the middle of the seventies and focused on the system design and the types of plants and fish that could be associated therein (Yep and Zheng, 2019). However, aquaponics is a very complex production technique relying on diverse disciplines including aquaculture, microbiology, ecology, horticulture, agriculture, chemistry and engineering (Yep and Zheng, 2019) and more research was thus needed to improve the systems. The publication of aquaponics related research papers was exponential in the last years (Junge et al., 2017). Indeed, around 160 research papers were published between 2015 and 2018 (Yep and Zheng, 2019) and many more challenges are still facing the scientific aquaponic community as highlighted by recent publications listing them (Goddek et al., 2015; Hao et al., 2020; Junge et al., 2017; Turnsek et al., 2020; Yep and Zheng, 2019).

Several reviews focus on the systems' design (Palm et al., 2018), the control of water quality parameters (pH, DO, TSS) and associated technologies such as aeration and filtration (Danaher et al., 2013; Tyson et al., 2011), the association of different types of plants and fish, the type of feed, the nutrient ranges (Delaide et al., 2016; Endut et al., 2010). Once a strong basis on the design and construction on the systems was acquired, it was deemed necessary for research to deepen the understanding of nutrient cycling in aquaponics and associated microbial populations. Indeed, the aquaponic solution's nutrient concentrations are often a limiting factor with respect to recommended thresholds for crop growth in hydroponics (Graber and Junge, 2009). Solutions such as the use of foliar spray or the addition of external fertilizer in the system have been used but have a negative impact on the sustainability of aquaponics given they require additional external inputs (Yep and Zheng, 2019). In their review of aquaponics literature Yep and Zheng (2019) highlighted that the most paramount topic for a sustainable and viable development of aquaponics today was PGPM and their role in nutrient cycles. Indeed, the involvement of PGPM in plant nutrient uptake is supposed to be the reason high yields are obtained in aquaponics despite lower nutrient concentrations than in hydroponics.

A first thesis on aquaponics was pursued in Gembloux Agro-Bio Tech by Delaide (2017) and focused on nutrient cycling and more particularly mineral elements and remineralisation of the sludge evacuated from the system to ensure maximum nutrients availability and uptake for crop production**.** The latter thesis showed that aquaponics can provide yields as high as in hydroponics with lower nutrient concentrations (Bittsanszky et al., 2016; Delaide et al., 2016). Indeed, their aquaponic solution contained only 23% of NO_3^-, 15% of PO_4^{2-}, 27% of K^+ and 8% of Ca^{2+} of their hydroponic solution (Delaide et al., 2016). Such differences were hypothesised to arise from microorganisms and/or organic molecules which could be an undetected source of nutrient in the usual tests performed to measure nutrient concentration in a solution and "promote growth by stimulating natural growing conditions as opposed to sterile hydroponic conditions" (Goddek et al., 2015; Yep and Zheng, 2019 citing Böhme, 1999).

The present thesis focuses on the microbial aspect of aquaponics, their role in nutrient cycling and their interaction with plants, for several reasons. Firstly, the extensive amount of information available regarding PGPM in soils highlights its huge potential for plant growth promotion. In particular, the interactions between PGPM and plants have been thoroughly studied and relevant metabolites and pathways, such as phosphorus solubilisation and absorption for instance, have been highlighted (see 1.4.3.1). Secondly, at the end of the EU Aquaponics Hub COST action (FA 1305), PGPM studies were deemed necessary to improve its current state of knowledge in the field of aquaponics. A better understanding of PGPM related processes would promote the viability, sustainability and predictability of processes occurring in aquaponics, with a reduced reliance on external inputs of nutrient solutions. Moreover, a better understanding of the microbial community could enable to enrich the system with aquaponic beneficial microorganisms cultured artificially. Furthermore, if beneficial microorganisms can be isolated from aquaponic systems and cultured and used as PGPM for enhancing crop yields in aquaponics their use could be generalised for different plant growth support matrices (soilless or soil borne) (Bartelme et al., 2018; Sanchez et al., 2019). PGPM inoculation had already been studied in hydroponics using commercial strains and yielded interesting results thus paving the way for microorganisms' inoculation in soilless systems. A similar reasoning could be applied in aquaponics, the specificity of this thesis being to work with endemic microorganisms. The use of microorganisms originating directly from the targeted aquaponic system could foster the adaptation of the inoculum to the environment and autochthonous microbiota thus easing colonisation (Stouvenakers et

al., 2020). Still, microbial communities are highly complex networks in aquaponics and represents a black box which needs to be opened step by step.

2.2 Thesis outline, conceptual framework and research strategy

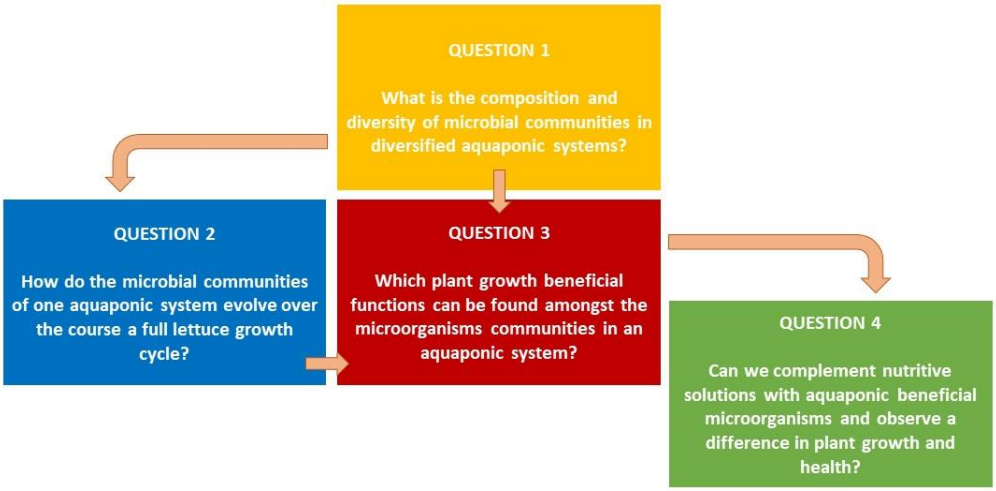


Figure 2-1. Thesis conceptual framework

Three different angles were chosen to tackle this vast topic (taxonomical, ecological, functional) and four key questions were defined to deal with those angles (**Figure 2-1**).

Firstly, we explore the diversity of the microorganisms harboured in various aquaponic and aquaculture systems. In 2017, very little information was available concerning which microorganisms could be found in aquaculture and aquaponic system apart from nitrifying microorganisms. Therefore, having a first idea of which microorganisms are present in aquaponics is the first corner stone of the thesis. The first key question and its underlying questions are detailed below.

Key question 1: what is the composition and diversity of microbial communities in diversified aquaponic and aquaculture systems?

Underlying questions:

- Are there common microorganisms?
- Could trends be distinguished between the systems?
- What elements of the design could participate in shaping the communities?

With this first question we study the composition of several systems but only on one-time point and two compartments (sump and biofilter) (**Chapter 3**). Therefore, we focus our second question on one system only and thoroughly analyse its bacterial communities from 4 locations (sump, biofilter, lettuce rhizoplane and lettuce root) over the course of a lettuce growth cycle (**Chapter 4**). The second key question and its underlying questions are described below.

Key question 2: how do the microbial communities of an aquaponic system evolve over the course of a full lettuce growth cycle?

Underlying questions:

- Is there a microbiota typical from a system which can always be found in this system?
- How does the microbiota settle after the winter fallow period? Is there a transition period between RAS and aquaponics?
- Are there differences in terms of microbiota composition between the compartments?
- Are the microbial communities evolving in time? If yes, how so?
- Are the microbial communities' compositions influenced by water parameters and water parameters modifications?

To answer these first two key questions, the techniques used are total DNA extraction followed by the HTS of the V1-V3 region of the 16S rRNA gene and the analysis of the data QIIME 1 and then QIIME 2. Metabarcoding enables a rapid overview of the global composition of the communities but this technique also bears limitations, the most inconvenient being that the taxonomic assignment usually stops at the genus level. Genus identification does not permit to identify specific roles or functions linked with nutrient cycling and plant growth. Consequently, the second part of this thesis focuses directly on the functions present in aquaponics and their potential roles in plant health and care. Two key questions hinge this part:

Key question 3: which plant growth beneficial functions can be found amongst the microbial communities in an aquaponic system?

Key question 4: can we complement nutritive solutions with aquaponic beneficial microorganisms and observe a difference in plant growth and health?

The third key question tackles roughly the functions which can be detected in an aquaponic system and focuses on bacteria from the sump of the PAFF Box. Biochemical tests are performed *in vitro* on bacteria from the PAFF Box to assess their potential abilities in plant growth promotion (**Chapter 5**). However, the capacity to perform a function *in vitro*, in optimal conditions does not guarantee that the trait will be expressed *in vivo* in a more complex environment or that it will have a significant effect on plant growth. For this reason, the fourth key question is being brought forward. Concentrated suspensions of bacteria are selected in question 3 and inoculated in lettuce rhizosphere growing in a simplified aquaponic system implemented in a controlled growth chamber. Number of leaves, leaves length, root length and final weight are measured and compared between inoculated lettuces and control (**Chapter 5**).

Eventually, **chapter 6** proposes a further discussion of several global points regarding the methodologies chosen throughout this thesis and the limitations of the analyses and obtained results. **Chapter 7** finally summarizes the take-home messages the reader should remember from this work and offers several leads for future research.

3.

Exploring bacterial communities in aquaponic systems

The material presented in this chapter is adapted from:

Eck, M., Sare, A.R., Massart, S., Schmautz, Z., Junge, R., Smits, T.H.M. and Jijakli, M.H., 2019. Exploring bacterial communities in aquaponic systems. Water, 11, 260, *doi:10.3390/w11020260*

Abstract: Aquaponics is a production system based on the dynamic equilibrium between fish, plants and microorganisms. In order to better understand the role of microorganisms in this tripartite relationship, we studied the bacterial communities hosted in eight aquaponic and aquaculture systems. The bacterial communities were analyzed by 16S rRNA gene deep sequencing. At the phylum level, the bacterial communities from all systems were relatively similar with a predominance of Proteobacteria and Bacteroidetes. At the genus level, however, the communities present in the sampled systems were more heterogeneous. The biofilter samples harbored more diverse communities than the corresponding sump samples. The core microbiomes from the coupled and decoupled systems shared more common operational taxonomic units than with the aquaculture systems. Eventually, some of the taxa identified in the systems could have beneficial functions for plant growth and health, but a deeper analysis would be required to identify the precise functions involved in aquaponics.

Keywords: aquaponics; community analysis; next-generation sequencing; 16S rRNA gene

3.1 Introduction

Aquaponics is a combination of hydroponic and recirculating aquaculture technologies (Delaide et al., 2016; Rakocy, 2012). It offers the possibility of recycling 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 fertilizers of mineral origin and the environmental impact of both fish and plant production (Buzby and Lin, 2014; Delaide et al., 2017). The use of the aquaculture wastewater to fertilize the plants can avoid the discharge of phosphorus and nitrogen enriched water into already nitrogen loaded surface and groundwater (Buzby and Lin, 2014; Schmautz et al., 2016).

Along with plants and fish, microorganisms are present in aquaponics. Bacteria are key players in processes which are central for the functioning and equilibrium of an aquaponic system (Schmautz et al., 2017). The best studied process is nitrification, during which ammonia (the main nitrogen form excreted by the fish) is transformed via nitrite to nitrate, which is less toxic for the fish (Graber and Junge, 2009) and preferred by plants (Resh, 2013; Timmons and Ebeling, 2013). The main bacteria involved in this transformation are the ammonia oxidizing bacteria (AOB), such as *Nitrosococcus*, *Nitrosospira*, and *Nitrosomonas*, and the nitrite oxidizing bacteria (NOB), such as *Nitrobacter*, *Nitrospira* , *Nitrococcus*, and *Nitrospina* (Itoi et al.,

2007). Some *Nitrospira* populations are also able to perform the complete ammonia to nitrate transformation -and are known as complete ammonia oxidizers (COMAMMOX)- by themselves (Bartelme et al., 2017; Daims et al., 2015). Archaea, such as the *Thaumarchaeota*, can also be involved in the ammonia-oxidizing process (Bartelme et al., 2017). Finally, the anaerobic ammonium oxidation (ANAMMOX) group, members of the *Planctomycetes* responsible for the anaerobic transformation of ammonium and nitrite into nitrous oxide and N_2 (Hu et al., 2011) may play a role as well where oxygen levels are low.

In addition to nitrification, microorganisms are involved in other important processes. They can contribute to extracting the various macro- and micronutrients from the feed leftovers and solid faeces and make them available for plant uptake (Goddek et al., 2016). Depending on the aquaculture compartment, design, fish species, and feed type, 15-60% of the consumed feed is actually converted into fish biomass and used for fish metabolism. The rest is excreted and is available for the bacteria to decompose (Schneider et al., 2004; Timmons and Ebeling, 2013; Yogev et al., 2017). Besides this, bacteria could also play a role in the solubilisation of nutrients encompassed in solid compounds, such as phytates (Jorquera et al., 2008). Additionally, microorganisms in aquaponics are also involved in various plant growth promotion and protection pathways, such as biocontrol or the enhancement of root growth (Gravel et al., 2015; Schmautz et al., 2017; Sirakov et al., 2016). However, these pathways are not sufficiently elucidated yet.

Here, we compared a set of aquaculture (AQ) and aquaponic (AP) systems, which differ in terms of plant and fish species and/or feed type. AP designs included both "coupled" or closed loop AP systems (one loop containing fish and plants) and "decoupled" or open loop AP systems (two separate loops for fish and plants). The aim of this study was to gain insight into the diversity of the bacterial communities in these systems and, if possible, to link their potential functions to plant growth and plant health. For this, the bacterial communities present in biofilter and sump samples, which were the two common units in all systems, were characterized using 16S rRNA gene deep sequencing.

3.2 Material and methods

3.2.1 Samples collected in this study and samples preparation

Sump and biofilter samples were collected from eight different systems (**Table 3-1**). Three were operated as aquaculture and five as aquaponics cultivating various plant

species. Samples were collected in the period between March and April 2017 as described below. A detailed description of the systems is given in Appendix A (Description of the visited aquaponic and aquaculture systems, **Figure 3-S1** and **Table 3-S1**). For comparison to previously published data (Schmautz et al., 2017), the dataset of the aquaponic system of the Zürich University of Applied Sciences (ZHAW) was downloaded from the European Bioinformatics Institute database (EBI) and analysed similarly to the data generated in this study.

3.2.1.1 Sump samples

For each sample, two litres of water were collected in sterile Pyrex bottles. In order to concentrate the bacteria, the samples were filtered through 0.2 µm filters (Supor®— with a vacuum pump. The filters were then placed in a 50 mL sterile Falcon tube containing 30 mL of sterile water. After vortexing the Falcon tube for 4 min, the filters where removed and the tube centrifuged at 7607 × g for 10 min (Yildiz et al., 2017a). The pellet was then directly used for DNA extraction.

3.2.1.2 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 taken, as this would not always fit in a 50 mL Falcon tube. Instead, as many biochips as possible (between 10 and 30 depending on the size) were inserted in a 50 mL sterile Falcon tube containing 30 mL of sterile water in order to ensure the harvest of a maximum quantity of bacteria. The Falcon tubes were placed on a vortex for 2 min before placing them for 5 min in an ultrasonic bath (Ultrasonic cleaner, model USC600T, VWR, Leuven, Belgium). The Falcon tubes were then centrifuged at 7607 × g for 10 min and the pellet was collected for DNA extraction.

3.2.2 DNA extraction

The Fast DNA Spin Kit using Cell Lysis Solution TC (MP Biomedicals, Santa Ana, CA, USA) was used for the DNA extractions. The manufacturer's protocol was modified as follows: Samples were homogenised with a Power-Mix Model L46 (Labinco, Breda, The Netherlands) at speed setting 7 for 40 s, then incubated on ice for 2 min and again homogenized for 40 s. Subsequently, to remove cell debris, tubes were centrifuged at 14,000 × g for 10 min. All DNA extracts were stored unopened at 4 °C until further analyses.

3.2.3 Sequencing

Library preparation and sequencing were carried out by DNA Vision S.A. (company, Gosselies, Belgium). The library preparation and indexing steps were done using the Nextera Index kit v2 (Illumina, San Diego, CA, USA), while sequencing was conducted on an Illumina Miseq (2 × 250 bp) (Illumina Inc., San Diego, CA, USA) with the Miseq reagent kit v3 (600-cycles, Illumina, San Diego, CA, USA). Sequencing primers were chosen to cover the hypervariable regions V1-V3 of the 16S ribosomal RNA gene as recommended by (Munguia-Fragozo et al., 2015) and Schmautz et al. (2017).

The following primers were used: Forward V1-V3 (27F) 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGC TCAG-3' and Reverse V1-V3 (534R) 5'-GTCTCGTGGGCTCGGAGATGTGTAT-AAGAGACAGATTACCGCGGCTGCTGG-3' (Illumina adapters are underlined).

Data are available under the accession PRJNA513832 on the Sequence Read Archive database (SRA) of the National Centre for Biotechnology Information (NCBI).

3.2.4 Bioinformatics

The analysis of the sequencing data was conducted with the QIIME pipeline v1.9.1 (http://qiime.org/) (Caporaso et al., 2010). Forward and reverse sequences were merged in one file per sample with multiple_join_paired_ends.py. Paired fastq files were converted into fasta files with convert_fastaqual_fastq.py. Sequences were assigned to Operational Taxonomic Units (OTUs) with a cut-off of 97% sequence identity to the reference database Greengenes 13_8 with pick_de_novo_otus.py. Chimeric sequences were identified with the Chimera Slayer tool and then removed with filter_fasta.py. Singletons and sequences originating from chloroplasts and mitochondria were discarded.

For further analysis of the bacteria communities' composition in the 22 samples, the samples were rarefied at 40,000 sequences with single_rarefaction.py (rarefaction curves available in Appendix A, **Figure 3-S2**). Bar charts representing the relative abundances of the various OTUs were obtained using summarize_taxa_through_plots.py. Core microbiomes were generated using compute_core_microbiome.py.

3.2.5 Statistics

The Shannon and equitability indices were calculated via the alpha_diversity.py. The Shannon and equitability indices of biofilter versus sump were compared via the global core_diversity_analyses.py workflow with a nonparametric t-test (using Monte Carlo permutations). Principal coordinates analyses were carried out with the beta_diversity_through_plots.py script in order to compare the communities.

Table 3-1 Description of the samples and systems. N.R.: not relevant

Code	Operator	Location	Design	Fish Species	Feed Type	Biochips Type	Plant Type	Sampling Date	Sump Samples	Biofilter Samples	Extra Samples
PCG	Provincial Trial Centre for Vegetable Production	Kruishoutem, Belgium	Aquaponics—open loop	*Scrotum barco*	Vegetarian	Eco Pondchips	Tomatoes	29-03-17	1 sump 60 (low density) 1 sump 100 (high density)	1 biofilter 60 (low density) 1 biofilter 100 (high density)	
INA	Inagro	Rumbeke-Beitem, Belgium	Aquaponics—open loop	*Sander lucioperca*	Omnivorous	Kaldnes media	Tomatoes	18-04-17	1 sump fish 1 sump hydroponics	1 biofilter	
UF	UrbanFarmers	The Hague, Netherlands	Aquaponics—open loop	*Oreochromis niloticus*	Omnivorous	Kaldnes media	Microgreens Leafy greens Fruity vegetables	23-03-17	1 sump	1 biofilter	1 biofilm
IGB	Leibnitz-Institute of freshwater ecology and inland fisheries	Berlin, Germany	Aquaponics—open loop	*Oreochromis niloticus*	Omnivorous	Kaldnes media	Tomatoes	07-04-17	1 sump	1 biofilter	
GBXP	Gembloux Agro Bio Tech, PAFF Box system	Gembloux, Belgium	Aquaponics—closed loop	*Oreochromis niloticus*	Vegetarian	Microbeads	Leafy greens Fruity vegetables	27-04-17	4 sump	4 biofilter	
GBXR	Gembloux Agro Bio Tech, RAS system	Gembloux, Belgium	Aquaculture	*Oreochromis niloticus*	Vegetarian	Biocerapond	N.R.	03-04-17	1 sump	1 biofilter	
BQF	Belgian Quality Fish	Dottignies, Belgium	Aquaculture	*Acipenser spp. Huso sp.*	Omnivorous	Kaldnes media	N.R.	29-03-17	1 sump	1 biofilter	1 biofilter denitrification
WU	Wageningen University	Wageningen, Netherlands	Aquaculture	Eel, catfish	Omnivorous	Kaldnes media	N.R.	12-04-17	1 sump eel 1 sump catfish	1 biofilter eel	

3.3 Results

3.3.1 Metagenome sequencing

Whereas the previous study on the Wädenswil Aquaponics System (Schmautz et al., 2017) gave a first impression on the bacterial communities in a single setting, a comparison to other systems was not performed. For this reason, we collected 22 samples from various aquaponic and aquaculture systems in Western Europe (**Table 3-1**). Eleven samples were collected from sumps, nine from biofilters, one from an additional denitrification biofilter, and one from the periphyton present on tank walls (**Table 3-1**). The bacterial communities thereof were analysed in this study using 16S rRNA gene deep sequencing. The average Q_{30} of the sequence in the samples was 80%, which indicated samples of good DNA quality. Of the total reads, 11.8% were not assigned at the phylum level.

3.3.2 Taxonomic assignment of reads

Based on the taxonomic assignment of the reads, it can be observed that the bacterial communities in the different systems were highly variable. Of all filtered reads in all samples, an average 11.8% ± 6.7% could not be assigned to any OTU. In general, two major phyla were found throughout the samples (**Figure 3-1**): *Proteobacteria*, representing 34.6% ± 10.1% of the total reads, and *Bacteroidetes*, representing 25.5% ± 14.0%. Other phyla were found in lower quantities in the samples. However, some samples held exceptionally high amounts of individual phyla. An example here is the presence of 73.1% reads representing the *Thermi* phylum, mainly represented by a single OTU (*Deinococcus*) in the sump sample of the Belgian Quality Fish (BQF) system (**Figure 3-1**). Except for the biofilter of the same system, this phylum was only present at very low levels in the other systems.

It could be noted that in almost all systems, the biofilter sample harbored a more diverse community as the Shannon indices of the biofilter samples were significantly higher ($p < 0.05$) than the Shannon indices of the sump group (**Table 3-2**). The equitability was also significantly higher in the biofilter samples than is the sump samples ($p < 0.05$). The only exception was the INA system. A more thorough exploration of this system would be required to explain this difference.

Table 3-2 Summary of metagenomics data

Code	Sampling Zone	Number of Reads before Filtering	Chimeric Reads	Chloroplast and Mitochondrial Reads	Singleton Reads	Number of Reads after Filtering	% of Unassigned Reads	Shannon Index (after Filtering)	Equitability Index (after Filtering)
PCG.S.60	sump low density	75,840	83	9	1016	74,732	7.6%	5.65	0.52
PCG.S.100	sump high density	131,231	443	100	3822	127,166	13.9%	6.55	0.56
PCG.B.60	biofilter low density	104,241	77	10	2661	101,493	9.5%	7.88	0.69
PCG.B.100	biofilter high density	78,392	66	7	1635	76,684	10.6%	7.73	0.70
INA.S.fi	sump fish loop	107,998	146	117	2634	105,101	19.0%	7.68	0.66
INA.S.pl	sump before plant compartment	92,79	708	2	3581	87,985	6.8%	8.63	0.71
INA.B	biofilter	100,948	154	124	2831	97,839	19.2%	7.37	0.65
UF.S	sump	117,695	1122	22	3175	113,376	14.6%	6.77	0.57
UF.B	biofilter	134,73	223	5	7336	126,866	20.3%	8.30	0.67
UF.b	biofilm	99,487	260	56	2407	96,764	11.4%	6.94	0.59
IGB.S	sump	63,482	186	10	2657	57,612	12.9%	7.50	0.62
IGB.B	biofilter	59,923	74	45	2192	58,091	13.1%	8.44	0.75
GBXP.S	sump	97,905	340	864	4	96,697	4.0%	3.79	0.34
GBXP.B	biofilter	69,831	52	0	2233	67,546	14.7%	8.31	0.74
GBXR.S	sump	11,096	1037	100	2318	112,641	4.8%	5.91	0.51
GBXR.B	biofilter	124,569	378	81	3937	120,173	5.7%	7.65	0.65
BQF.S	sump	81,204	50	10	1087	80,057	3.3%	2.83	0.26
BQF.B	biofilter	56,448	85	13	3090	53,26	19.7%	8.42	0.74
BQF.deni	denitrification biofilter	65,693	14	1	1966	63,712	29.5%	6.72	0.63
WU.S.cat	sump catfish system	44,743	119	4	1142	43,478	7.9%	6.20	0.58
WU.S.eel	sump eel system	75,055	32	1	671	74,351	4.2%	3.22	0.32
WU.B.eel	biofilter eel system	101,169	177	8	2059	98,925	7.8%	6.43	0.60

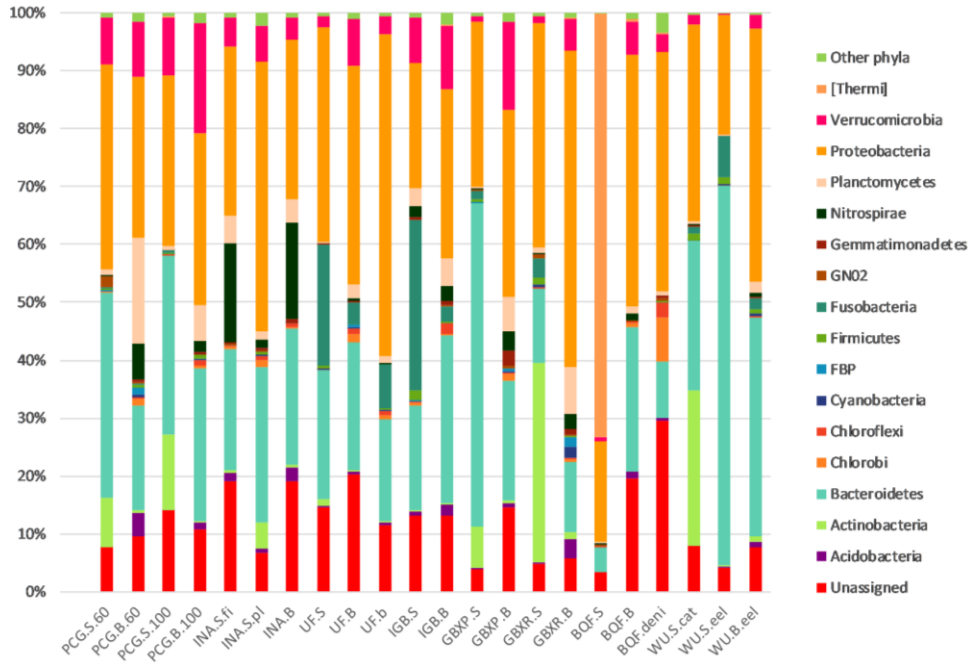


Figure 3-1 Bar charts representing the relative abundances of the phyla in each sample. Phyla which represented less than 0.2% of the total reads are gathered under "other phyla" (*BHI 80139, BRC1, Chlamydia, Elusimicrobia, Fibrobacteras, GN04, GOUTA4, Lentispaerae, NKB19, OP11, OP3, OP8, PAUC34f, SBR1093, SR1, Spirochaetes, Synergistetes, TM6, TM7, Tenericutes, WPS2, WS1, WS2, WS3, WWE1, and Caldithrix*)

At the genus level, reads were assigned to more than 700 different OTUs. To allow for a more in-depth analysis of the genera present in aquaponic systems, it was decided to focus on the OTUs representing more than 1% of the total reads per sample (**Figure 3-2**). For some OTUs, the identification process was only possible at the family level.

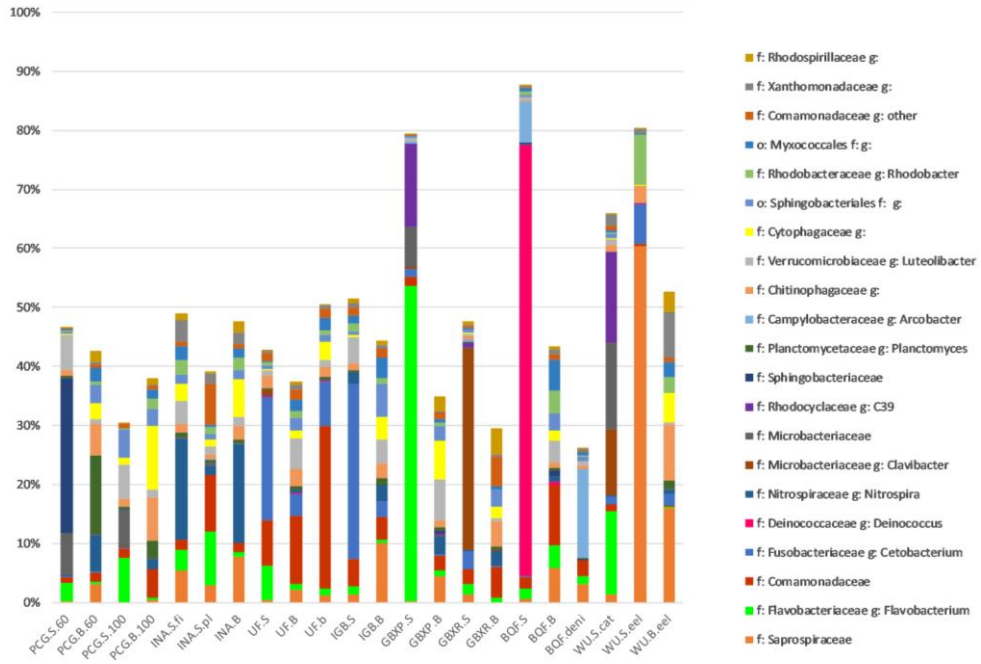


Figure 3-2 Bar charts representing the relative abundances of the families and genera representing more than 1% of the total reads for each sample

One OTU assigned to the genus *Deinoccocus* was mainly present in the BQF system and represented 73% of the BQF sump sample. Members of the genus *Deinoccoccus* are heterotrophic organisms resistant to UV radiation (Rosenberg, 2006) and to our knowledge, there is no link between the rearing of sturgeons and the presence of this genus. As the BQF system was implemented with an ozone plus UV light disinfection treatment (Appendix A, Description of the visited aquaponic and aquaculture systems), it could be expected that this organism was dominating the community based on its resistance to such treatment. However, most aquaponic systems, such as the Wädenswil Aquaponics system (Schmautz et al., 2017), use UV light to prevent the proliferation of undesirable microorganisms and to keep the water clean and clear (Timmons and Ebeling, 2013), without observing such a development of *Deinoccocus*. It could thus be that the dominance of *Deinococcus spp.* in the sump was due to a higher strength of the UV light in combination with the ozone treatment. It was also observed in both biofilter samples, but at levels below 0.5% of the community. This indicated that the *Deinococcus spp.* were more planktonic, while we observed a broader diversity in the biofilms on the carrier in the biofilters.

The species *Cetobacterium somerae* belongs to the *Fusobacteriaceae* family and has been commonly found in guts of freshwater fish (Itoi et al., 2007; Schmautz et al., 2017; Tsuchiya et al., 2008). The discovery of *Cetobacterium* in some of the samples might indicate that the system design in these cases was not sufficiently adapted to remove sufficient amounts of fish faeces from the water of the fish tank. On the day when the Urban Farmers (UF) (Appendix A, Description of the visited aquaponic and aquaculture systems) samples were collected, pipes were clogged with fish sludge in the drum filter compartment and thus sludge may have passed the drum filter towards the biofilter and sump compartments. This might explain the large amounts of reads assigned to the genus *Cetobacterium* in the UF samples (**Figure 3-2**). Additionally, it was observed that *Cetobacterium* were more often detected in the sump of systems than in biofilter samples. It was assumed that the sump in these systems could offer sufficiently anaerobic zones whereas the moving bed biofilters were fully aerobic (Rakocy et al., 2006). However, if the system hosted an important quantity of *Cetobacterium* in the sump, one could also observe their presence in the biofilter albeit at a smaller proportion (**Figure 3-2**).

Conversion of nitrogen compounds is of utmost importance for recirculating systems to avoid toxicity problems of the different nitrogen forms for each species. Of the known nitrifying bacteria, the *Nitrosomonadaceae* family was present in most samples. Even though the relative abundance of this family was quite low (between 0% and 1.7% of reads; average = 0.3%; stdev = 0.5%), the order of magnitude observed in most samples in this study was similar to the one observed in the study of the Wädenswil Aquaponics system (Schmautz et al., 2017). The most abundant nitrifying bacteria were those of the genus *Nitrospira.* This is also in accordance with the study conducted on the Wädenswil Aquaponics system (Schmautz et al., 2017), and may indicate that the COMAMMOX process is more common to aquatic culture systems.

3.3.3 Core Microbiomes

3.3.3.1 General Core Microbiome

Generally, a large diversity of bacteria was observed and all systems hosted different bacterial communities (Figure 3-1). However, in spite of this diversity and the specificities of each system, common bacterial groups were found in all aquaponic and aquaculture systems. A core microbiome containing only the OTUs present in all samples was extracted from the data set and, regardless of the system and sample location, four OTUs were identified. OTUs representing unidentified genera from the

Oxalobacteraceae family and the Comamonadaceae family were identified as being present in all samples. The Oxalobacteraceae family harbours several heterotrophic bacteria that can be found in water, soil and also in association with plants (Baldani et al., 2014). Regarding the Comamonadaceae family, it is also found in aquaculture or aquaponic systems in other studies (Itoi et al., 2007; Schmautz et al., 2017). In their review, Munguia-Fragozo et al. (2015) reported that Comamonas sp. were identified in several bacterial communities of freshwater recirculating aquaculture systems (RAS). At the genus level, OTUs assigned to the genera Cetobacterium were as well part of the general core microbiome. Although Cetobacterium is rather an anaerobe, its presence in all systems could be explained by its common presence in fish guts (Ghanbari et al., 2015; Tsuchiya et al., 2008).

3.3.3.2 System-Specific Core Microbiomes

On the basis of the three basic setups that were sampled in this study, we also calculated core microbiomes for each of these setups (**Figure 3-3**). The AQ group contained nine common OTUs, and the decoupled AP group harboured a core microbiome of 34 OTUs. The coupled AP group contained only the plant and fish farming (PAFF) Box samples and, therefore, harboured a core microbiome of 636 OTUs. Whilst only one and five OTUs were common for aquaculture and the two aquaponics systems, the two aquaponic groups share more OTUs. A total of 17 OTUs at different levels were found, indicating that there are some not yet identified conditions that are specific to aquaponic systems, independently of the setup.

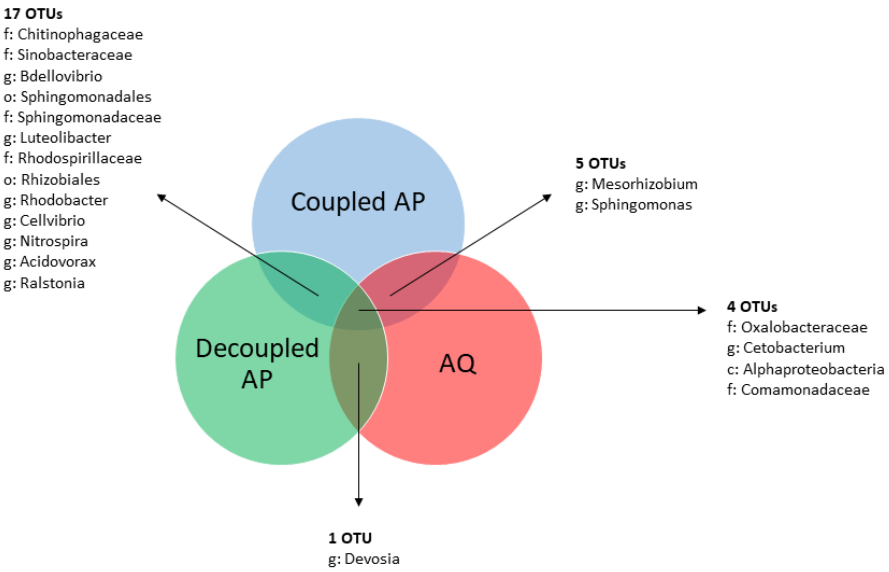


Figure 3-3 Venn diagram representing the intersection of different core microbiomes obtained through the grouping of samples based on the system setup (i.e., coupled aquaponics, decoupled aquaponics, and aquaculture)

3.3.3.3 Sampling site-specific core microbiomes

Based on the sampling strategy, sampling site-specific core microbiomes were generated from biofilter and sump samples. The sump core microbiome was composed of 22 OTUs, while the biofilter core microbiome was composed of 28 OTUs. The larger numbers of OTUs in site-specific core microbiomes indicated that both zones had site-specific bacterial communities. This was confirmed by principal coordinates analysis (PcoA), indicating the presence of two sample clusters: a narrow cluster grouping biofilter samples and a wider cluster grouping the sump samples (**Figure 3-4**). Between these two site-specific core microbiomes, ten OTUs were common (four of them belonging to the global core microbiome). The six additional OTUs found in all biofilters and sumps were *Sphingomonas*, *Devosia*, *Novosphingobium*, *Acidovorax*, *Ralstonia*, and an unidentified OTU from the *Rhizobiaceae* family. *Nitrospira* could be found in all biofilter samples. However, this was not the case for the genera *Nitrosomonas* and *Nitrobacter*.

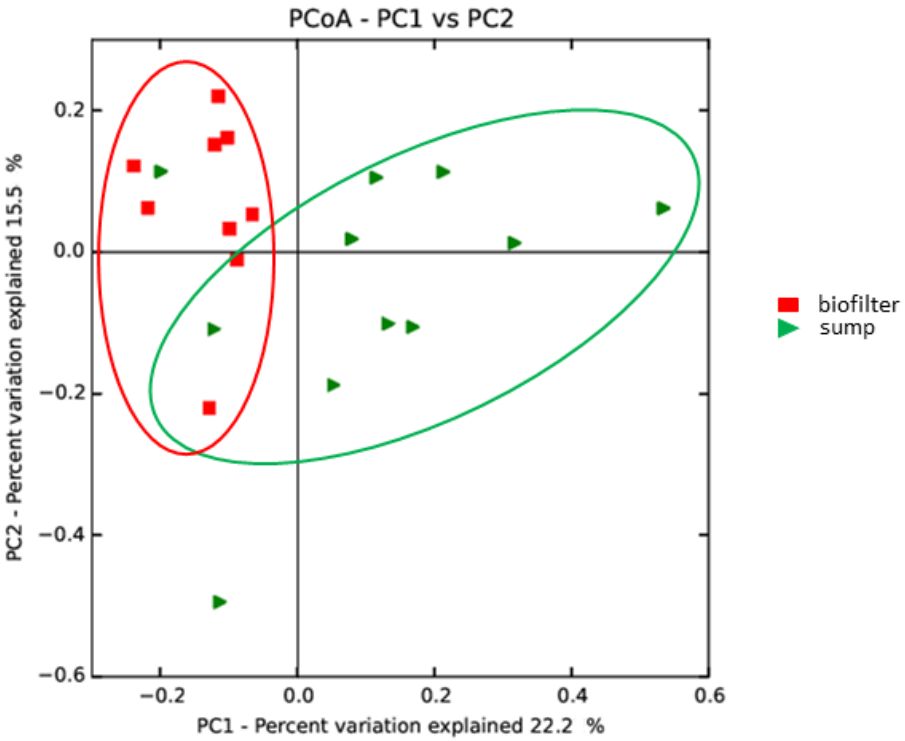


Figure 3-4 Weighted UniFrac principal coordinates analysis presenting the separation between the sumps and the biofilter samples. Axis 1 and 2 explain 37.8% of the total variability

3.3.3.4 Detailed analysis of the microbial communities in the coupled and decoupled Gembloux systems

Up to now, we have compared systems that were highly heterogeneous in their design, size, and operational strategies. This study also included two systems that were highly comparable: the recirculating aquaculture system (RAS) and the Plant and Fish Farming (PAFF) Box of Gembloux Agro–Bio Tech, as both systems shared the same size, fish, feed type and incoming water quality. The main difference was that the PAFF Box is operated as closed circular system, whereas the RAS is an aquaculture system. In order to observe the impact of plants in the system on the composition of the bacterial community, we chose to compare the communities in these two systems in more detail.

The RAS sump sample was dominated by the two genera *Clavibacter* and *Cetobacterium*, whereas the PAFF Box sample contained a majority of the genus *Flavobacterium* and *C39*, an OTU belonging to the *Rhodocyclaceae* family (**Figure 3-5**). The presence of such large numbers of reads assigned to *Clavibacter*, a genus that mainly contains plant pathogenic species (Davis et al., 1984), needs to be examined in more detail to confirm the assignment of all reads to this genus. The RAS biofilter sample was clearly dominated by members of the genus *Lysobacter* and also hosted *Nitrospira* and *Novosphingobium*, while the PAFF Box biofilter contained *C39*, *Nitrospira*, *Flavobacterium*, members of the *Microbacteriaceae* family, and *Cetobacterium* (**Figure 3-5**). This indicated that, despite similarities in the global setup (fish species, feed type, incoming water quality, and size of the fish tanks), each system developed its own specific community. The presence of plants in the aquaculture loop thus had a large influence on the composition of the bacterial community.

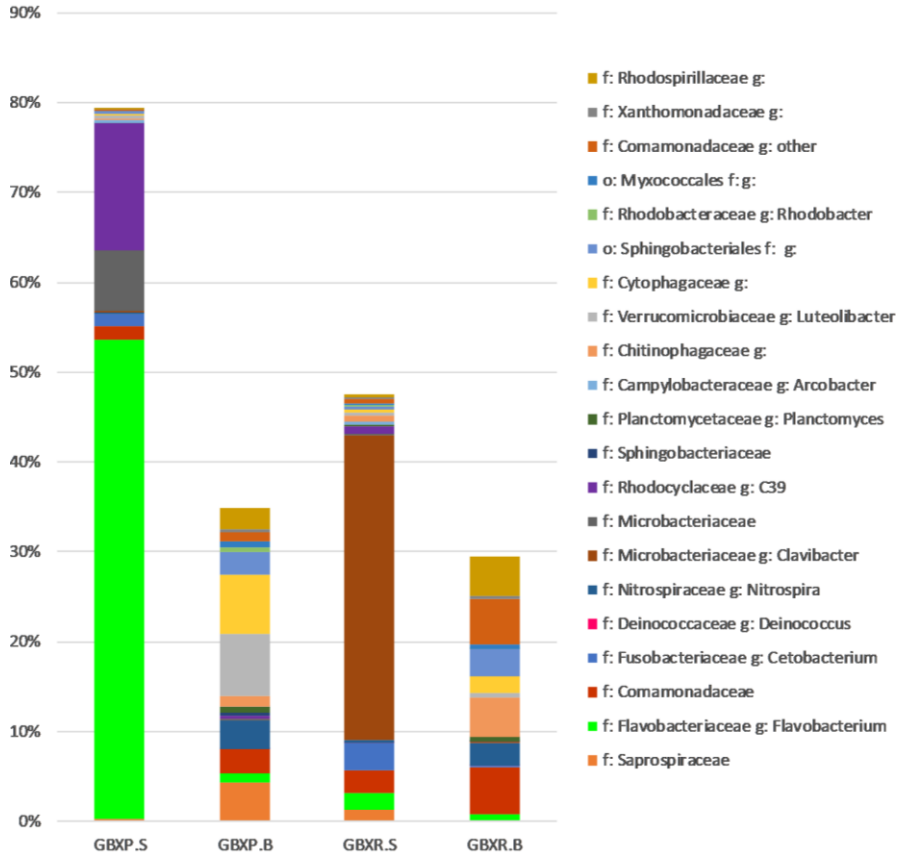


Figure 3-5 Relative abundances of the families and genera representing more than 1% of the total reads within each sample, for the biofilter and sump samples of the recirculating aquaculture system(RAS) and plant and fish farming (PAFF) Box systems of Gembloux Agro-Bio. Tech**.**

3.4 Discussion

3.4.1 Predominant Taxa

In order to work towards better understanding of how bacterial communities function in aquaponics, we decided to focus on the potential impact of the bacteria in the aquaponic solution on plant growth. A few studies have reported that, with aquaponics, they obtained plant yields as good as in hydroponics despite the aquaponic solution containing lower nutrient concentrations (Bittsanszky et al., 2016; Delaide et al., 2016; Graber and Junge, 2009). A first step to take towards the

elucidation of this increased growth would be to check for similarities in the bacterial communities in different aquaponic systems. In order to do so, it was decided to study the core microbiomes of our samples. The concept of core microbiomes has been used and described in several other research fields, such as the plant holobiont (Lemanceau et al., 2017), humans (Huse et al., 2012), or milk microbiomes (Kable et al., 2016). The core microbiome has been defined by Lemanceau et al. (2017) as "the microbial community that is systematically associated with a given host". Until now, the concept of core microbiome has been focused on the taxonomic composition of a community. However, it could also be argued that a core microbiome should have specific functionalities responding to the needs of their associated host (Lemanceau et al., 2017).

At the phylum level, *Proteobacteria* and *Bacteroidetes* were the two major groups representing, respectively, 35% and 26% of the reads obtained from the different aquaponics and aquaculture systems. This was in accordance with the observation from Schmautz et al. (2017), who also found that *Proteobacteria* (approximately 50%) and *Bacteroidetes* (15–20%) were the major phyla in their root zone, biofilter, and periphyton samples. This also corroborated observations made in freshwater aquaculture (Munguia-Fragozo et al., 2015). Other phyla common to the observations by Schmautz et al. (2017), the freshwater data cited by Munguia-Fragozo et al. (2015), and the present study were the phyla *Actinobacteria*, *Planctomycetes* and *Nitrospirae*.

Few reads assigned to the genus *Pseudomonas* were detected in the visited systems. The number of assigned reads to the pseudomonads was in the same order of magnitude as previously observed by Schmautz et al. (2017). *Pseudomonas* are usually found in close proximity to roots and are less prevalent in bulk soil (Dennert et al., 2018). As, in this study, we did not investigate the root zone of the aquaponics systems, this genus may thus rather be represented by planktonic species in the samples taken.

3.4.2 Potential Roles/Functions of the Identified Taxa

In aquaponics, we are interested in the bacteria, which could help us ensure fish welfare and plant care. When it comes to plants, bacteria could help their growth and health through growth stimulation and biocontrol. Taxa, such as the *Microbacteriaceae* family, are known to be able to form associations with plants (Evtushenko and Takeuchi, 2006) and have been detected in the microbiome of barley roots along with members of the *Comamonadaceae* family (Bulgarelli et al., 2015). In the latter family, several species have been detected to possess skills for

siderophores production and protection against *Fusarium* and *Rhizoctonia* (Hynes et al., 2008). The *Microbacteriaceae* family contains species which have aminocyclopropane-1-carboxylate (ACC) deaminase activity, siderophores, and indole production (Hynes et al., 2008). The *Flavobacterium* genus is widely present in nature and mostly known for its capacity to degrade complex organic molecules (Kolton et al., 2016; Liu et al., 2012). *Flavobacterium spp.* are often found in association with plant roots and plant leaves and are believed to be involved in plant growth and protection (Kolton et al., 2016; Liu et al., 2012; Soltani et al., 2010). Several strains were detected to be able to participate in the solubilisation of insoluble phosphate, the production of auxin, and the production of siderophores (Hynes et al., 2008; Kolton et al., 2016; Liu et al., 2012; Soltani et al., 2010). *Flavobacterium* are also used to fight against plant pathogens (*Phytophtora infestans*) in biological control formulation (Kim et al., 2010). In aquaculture and aquaponics, *Flavobacterium* have also been detected and can be considered as a common genus found in such systems (Itoi et al., 2007; Munguia-Fragozo et al., 2015; Schmautz et al., 2017). The *Lysobacter* genus has also been identified as plant growth promoting bacteria (PGPB) and can help fight against plant disease through the production of antibiotics (Folman et al., 2003; Lee et al., 2013; Reichenbach, 2006). The presence of all of those species in most of the samples in this study confirmed the data from the samples from the Wädenswill aquaponics system (Schmautz et al., 2017) and strengthens the statement that the microbiome in aquaponics or aquaculture systems may be able to secure the health and growth of the plants in the first-named type of systems.

A crucial function of the bacteria communities in aquaponics would be the solubilisation of the fish dejections and fish feed leftovers into macro- and micronutrients, which the plants can absorb. The members of the genera *Flavobacterium* and *Sphingobacterium* could participate in the decomposition of organic matter (Liu et al., 2012). The *Saprospiraceae* family is typically found in aquatic environment, such as wastewater treatment plants (Liu et al., 2012; McIlroy and Nielsen, 2014; Xia et al., 2008), and could be involved in the degradation of complex carbon molecules, such as proteins (McIlroy and Nielsen, 2014; Xia et al., 2008). Many other detected genera include heterotrophic organisms able to degrade biomass in the system, as well.

Several of the observed OTUs were related to a role in the nitrogen cycle. The genus *Nitrospira* was detected in all biofilter samples. *Nitrospira* is commonly known as a NOB (Daims et al., 2015; Gao et al., 2017; Itoi et al., 2007; Rurangwa and Verdegem, 2015). Daims et al. (2015) showed that certain strains of the *Nitrospira* genus could

actually be complete nitrifiers, i.e., able to oxidize ammonia to nitrate without the help of AOB, a process now known under the name COMAMMOX. Denitrification has also been often observed in aquaculture and aquaponics (Monsees et al., 2017; Wongkiew et al., 2017). Members of the genus *Arcobacter* are known to perform denitrification (Wang et al., 2017) and have been particularly found in the denitrifying biofilter of the BQF system (**Figure 3-2**). The phylum *Planctomycetes* has already been observed in recirculating aquaculture (Van Kessel et al., 2010) and contains ANAMMOX bacteria (Liu et al., 2012; Van Kessel et al., 2010). However, with the current database used for assignment to genus level, it could not be confirmed that ANAMMOX bacteria were present in the examined systems.

3.4.3 Core Microbiomes

Bacterial populations in aquaponic systems were highly diverse whether between systems or between the different compartments therein. In each sample, all the taxa representing less than 1% of the total number of reads were discarded, and this represented at least 50% of the reads (**Figure 3-2**). Despite this diversity, a core microbiome common to all samples could be identified. Moreover, the core microbiomes were composed of 28 (7.6% of the total reads common to all biofilters) and 22 OTUs (6.1% of the total reads are common to all sumps) for the biofilter and sump samples, respectively. This brought forth the hypothesis that a common bacterial base may exist between all aquaponic systems despite differences in fish species, system layout, or fish feed.

Despite the differences in the bacterial communities due to system specificities, there were still similarities between all examined systems. A principal coordinate analysis combining the data from the tested systems with the data collected by Schmautz et al. (2017) (Appendix A, **Figure 3-S3**) showed that the samples collected from the plant roots, periphyton, and biofilter compartments of the Wädenswil Aquaponic system (ZHAW) clustered closely with the other samples. This showed that there was a common pattern concerning the composition of the bacteria community in diversified aquaponic systems located in Western Europe. It would then be interesting to broaden the study to systems situated worldwide and also on a longer period of time.

3.5 Conclusion and Perspectives

This study was one of the first investigations into the diversity of bacterial communities present in a variety of aquaponic and aquaculture systems. It offered a

global overview of the microbial taxa therein and of the potential roles that microorganisms could play in plant care. Nevertheless, it was shown that the different system setups had a large influence on the bacterial communities, and it needs to be investigated in more detail which species performs what role in such systems.

As the currently available datasets were from a single time point and only limited compartments within single systems were sampled, a more comprehensive sampling of single systems over time would be required to study the influence of sample time and location within a system. This may explain the currently obtained data better within the frame of the operational differences, but helps us also to understand the biological processes taking place in a single system.

4.

Ecological study of aquaponics bacterial microbiota over the course of a lettuce growth cycle

The material presented in this chapter is adapted from:

Eck, M., Szekely, I., Massart, S. and Jijakli, M.H. 2021. Ecological study of aquaponics bacterial microbiota over the course of a lettuce growth cycle. Water, 2021, *doi.org/10.3390/w13152089*

Abstract: The study of microorganisms in aquaponics is an important topic which requires more research before exploiting the full potential of beneficial microorganisms. In this experiment, we focused on the evolution over time of the bacterial communities in four compartments of an aquaponic system i.e., the sump, the biofilter, the lettuce rhizoplane and lettuce root. We studied these communities over the course of a lettuce growth cycle via regular sampling and sequencing of the 16S rRNA gene of the collected bacteria. We also followed the physicochemical parameters of the aquaponic water throughout the experiment. Results show that a different community could be found in each compartment and that all four communities were stable throughout time and resilient to naturally occurring water parameter changes which characterize functioning aquaponic systems. Furthermore, the communities of the sump and biofilter also seem stable over the years as the predominant taxa (*Luteolibacter*, *Flavobacterium*, *Nitrospira*) observed in our study are similar to the ones previously reported for this aquaponic system. Finally, our results provide proof for similarities between aquaponic and soil borne lettuce root communities (gammaproteobacteria, *Flavobacterium*, *Pseudomonadaceae*, *Sphingomonadaceae*) thus showing that aquaponics can be similar to soil production in terms of microbial life.

Keywords: aquaponics; kinetics; microbiota evolution; bacterial communities; 16S rRNA; lettuce

4.1 Introduction

Aquaponics is a combination of hydroponics and recirculating aquaculture, i.e., of plants and fish rearing (Rakocy, 2012) However, plants and fish could not thrive without the help of microorganisms which constitute a bridge between the two main types of living organisms of an aquaponic system (Bartelme et al., 2018; Sanchez et al., 2019; Schmautz et al., 2017). Indeed, in aquaponics, microorganisms are mostly known for their role in the conversion of the potentially toxic ammonia excreted by the fish into nitrite and nitrate, i.e., the nitrification process.

Nevertheless, microorganisms in aquaponics are also assumed to have other beneficial effects on plants such as those observed in soils (Bartelme et al., 2018; Sanchez et al., 2019), but precise knowledge is currently scarce. Indeed, conversely to soil borne plants (Berg, 2009; Berg et al., 2015; Lugtenberg and Kamilova, 2009; Vandenkoornhuyse et al., 2015), the phytobiome of aquaponic crops is barely known while it could prove highly beneficial for plant health and yields in aquaponics. Recent

studies are therefore focusing their efforts on the characterization and understanding of the complex microbial communities present in the different compartments of an aquaponic system and the relationships between those communities and plant health and care.

Schmautz et al. (2017) have focused on the microbial communities present in the different compartments of an aquaponic system, discovering that each compartment (i.e., biofilter, periphyton, plant roots and fish faeces) have quite distinct communities with the fish faeces community being utterly different from the others. This observation has been corroborated by Eck et al. (2020) whom compared the sump and biofilter compartments of a coupled aquaponic system.

Eck et al. (2019b) have also analysed the microbial communities in several diversified aquaponic and aquaculture systems across North Western Europe. According to this study, each aquaponic/aquaculture system has its own microbial community, with an important diversity between systems. This has been confirmed by Bartelme et al. (2019) whom have also investigated different aquaponic and aquaculture systems and noticed that the design and the water source greatly influenced the composition of the microbial communities. However, Eck et al. (2019b) still detected 17 OTUs common to 5 different aquaponic systems (coupled and decoupled) and 34 OTUs common to 4 decoupled systems.

Finally, Sanchez et al. (2019) have started tackling the topic of plant beneficial microorganisms in aquaponics. They have thus discovered that some bacterial strains originating from aquaponic systems were able to produce siderophores and ammonia and to solubilise phosphorus. The genera thus identified were the following: *Dietzia*, *Gordonia*, *Microbacterium*, *Mycobacterium*, *Rhodococcus*, *Bacillus*, *Paenibacillus*, *Myroides*, *Acidovorax*, *Chromobacterium*, *Aeromonas*, *Plesiomonas* and *Pseudomonas*.

However, most of these studies are one-off and do not follow the microbial communities throughout time. To our knowledge, the only time study of aquaponic microbiota was conducted in our laboratory in which the microbial communities of the sump and biofilter of a coupled aquaponic system were studied for 3 consecutive weeks (Eck et al., 2020). Our current study therefore aims at addressing this gap in current knowledge by shedding more light on the kinetics of the microbial communities in one system. Our aquaponic system (described for the first time in Delaide et al. (2017)) has thus been surveyed over the course of 9 weeks, at the beginning of the relaunch of the system after the winter fallow period. On the one

hand, regular samplings of microbial communities were carried out in several compartments (sump, biofilter, rhizoplane and root) to follow the modifications of these communities throughout time, while on the other hand, the system's basic parameters were monitored during the whole experiment (pH, temperature (T°), electroconductivity (EC), nutrients) in order to link potential microbiota modifications with changes in those parameters. To follow the modifications of bacterial communities throughout those 9 weeks, i.e., from the introduction of lettuce in the recirculating system to the final harvest, bacterial communities were characterized with partial 16S rRNA gene high throughput sequencing.

4.1 Material and methods

4.1.1 Description of the aquaponic system

The experiment was conducted in the small scale, closed-loop, aquaponic system of the Integrated and Urban Plant Pathology Laboratory (IUPPL) of Gembloux Agro-Bio Tech (Gembloux, Belgium), named the Plant and Fish Farming box (PAFF Box) and already described in Eck et al. (2019b). **Figure 4-1** summarizes the functioning of the PAFF Box and provides additional technical information.

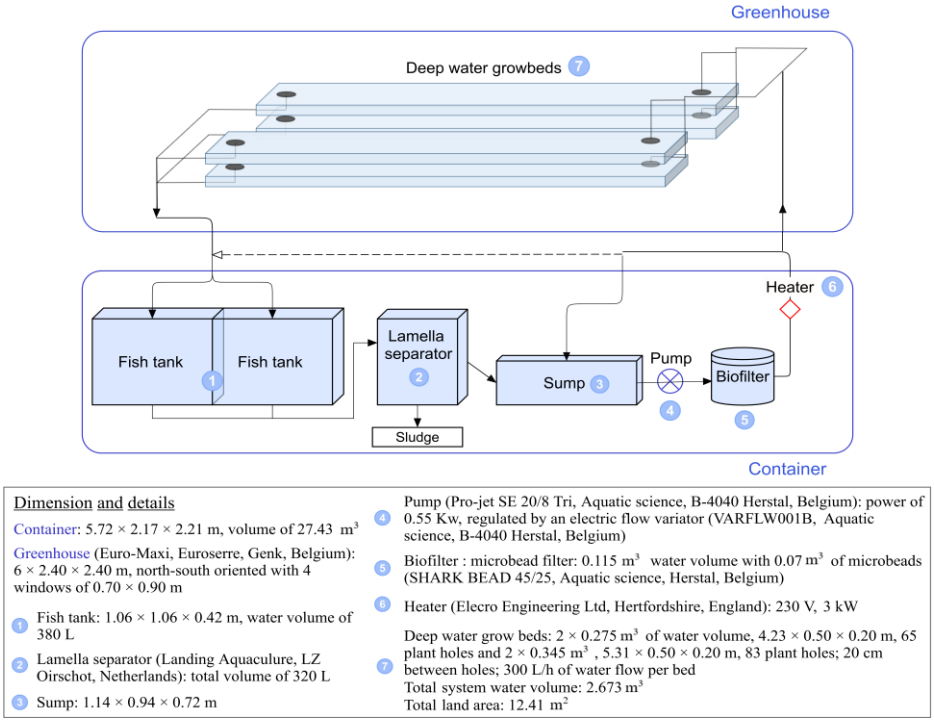


Figure 4-1 Water flow in the closed-loop aquaponic system of Gembloux Agro-Bio Tech, i.e., the PAFF Box**.**

4.1.2 Experimental set-up

Prior to the experiment, the PAFF Box had been running for almost three months without plants, from mid-December 2018 to the end of February 2019 and was operating as a simple recirculating aquaculture system (RAS), using only the compartment containing the fish. The biofilter was maintained by the presence of 34 Nile Tilapia (*Oreochromis niloticus L.*) in both tanks. Furthermore, the hydroponic compartment went through a fallow period during which the deep water grow beds were entirely emptied, cleaned and bleached. At the end of February 2019, 1000 L of tap water were added to the hydroponic compartment which was then re-connected to the system. The feed (Trouw Nutrition, Putten, Netherlands) was plant and animal based (processed animal protein from poultry and fish meal).

On February 27 2019, 90 seeds of Butterhead lettuce (*Lactuca sativa var. Lucrecia rz*, Rijk Zwaan) were sowed in rockwool plugs (Grodan ROCKWOOL B.V.,

Roermond, Netherlands) soaked with tap water in a climate and light controlled greenhouse as Resh (2013) recommends. After 10 days of germination, on March 8, 80 seedlings (Group 1) were transferred with their rockwool plugs in the two top grow beds of the hydroponic compartment. The 10 remaining seedlings were used for the first root sampling for which the preparation is described hereafter. On the same day, the sump and biofilter samples were also collected and prepared as explained below (**Figure 4-2**).

Lettuce seedlings obtained from the same seed lot and germinated in the same conditions than the first group were added once a week starting on the second week of the experiment to the grow beds to counterbalance the loss of plants due to sampling (harvest of 10 lettuces per sampling day). The experiment lasted 9 weeks and ended on May 13 2019 (**Figure 4-2**).

4.1.3 Water and nutrient management and measurements

Several water parameters were monitored throughout the experiment in order to follow the general functioning of the PAFF Box and correlate potential microbiota variations to water parameters' variations. The temperature (T), pH, dissolved oxygen (DO) and electro-conductivity (EC) of the circulating water were measured with an aquarium probe (IKS Aquastar Industrial Version 2.28, IKS Computer System GmbH, Karlsbad, Germany). From March 8 to April 4 , the four parameters were manually recorded in a heterogeneous frequency, ranging from twice a day to once in 5 days, as a problem occurred and the probe did not save the data. From April 5 to April 25, T, pH and EC were measured every 15 min but the probe did not save the DO data. From April 26 to the end of the experiment, on May 13, the four parameters were measured twice a day, at 12 a.m. and 12 p.m. Nitrate (NO_3^-) was measured every 15 min with an optical sensor (TriOS Optical sensor, TriOS Mess- und Datentechnik GmbH, Rastede, Germany) throughout the entire experiment, from March 8 to May 13 2019.

4.1.4 Sampling

Samples collected for the isolation of bacterial communities were taken from four different compartments of the system: i) the water entering the hydroponic compartment, after it went through the biofilter; ii) the biofilter itself; iii) the lettuce rhizoplane, i.e., the root surface and iv) the rest of the lettuce root.

Each water sample was composed of two litres of aquaponic water which were vacuum filtered on 0.2 µm filters (PALL Life Science Supor 200, 47 mm diameter)

(Eck et al., 2019b). The collected microorganisms were then re-suspended in 30 mL of sterile 0.05M KPBT buffer (0.005%, tw 80, pH 6.5) and the suspension was centrifuged to collect the pellet. This pellet was then stored at −80 °C in a 30% glycerol solution for later analysis.

Each of the biofilter samples consisted of 15 g of beads in 30 mL of the KPBT buffer which were vortexed for 2 min before undergoing 5 min of ultrasonic bath (Ultrasonic cleaner, model USC600T, VWR, Leuven, Belgium). The beads were then removed and the remaining bacterial suspension centrifuged and the pellet stored in 30% glycerol at −80 °C.

To collect the rhizoplane microorganisms, the roots of 10 lettuces were pooled together. When the lettuces were still small, the entire root system was used while when they were more developed, only 0.2 g of roots per lettuce were kept to reach a total maximum weight of 2 g. The washing step then consisted in placing the root samples in a 0.05 M KPBT buffer and to sonicate them for 10 min in an ultrasonic bath. Roots were then stored aside and the solution was filtered through a sterile cheesecloth to remove root debris. The roots were then rinsed again by vortexing for 30 s in 10 mL of KPBT buffer. The ensuing 10 mL were then filtered again with a cheesecloth and added to the first wash (Sare et al., 2020). The total suspension was centrifuged to collect a pellet which was stored at −80 °C in a 30% glycerol solution. Only one washing step was performed as advised by Sare et al. (2020) as one wash suffices to collect the majority of the rhizoplane microbiota. The washed roots were then instantly frozen in liquid nitrogen and stored at −80 °C for later collection of the remaining microorganisms tightly attached to the roots and endophytes. This second part of the root microbiota will from here on be named as “root microbiota”.

For the extraction of the root microbiota, roots were quickly defrosted at 55 °C for 5 min then ground in a mesh bag with 0.05M KPBT buffer. The resulting solution was then filtered through sterile cheesecloth and 30% glycerol was added before storing at −20 °C.

Samples were taken at higher frequency during the first 3 weeks as the system was expected to undergo important changes right after the introduction of plants, transitioning from a RAS to an aquaponic system.

Disease symptoms appeared on the group 1 plants during the fifth week, i.e., stunted growth, yellowing and wilting of the older leaves, necrosis of the roots (Appendix B, **Figure 4-S1**). Therefore, the focus of the experiment was shifted to a second group of lettuces aged four weeks also present in the PAFF Box (group 2). Group 1 lettuces

were thus used for the 1st, 2nd and 3rd weeks of sampling, group 1 and 2 were used for the 4th week of sampling to ensure a correct overlap (05/04 corresponding to group 1 and 29/04 to group 2). Group 2 lettuces were then used for the 5th and 6th week of sampling. **Figure 4-2** summarizes the sampling dates as well as the other manipulations performed on the system over the course of the experiment.

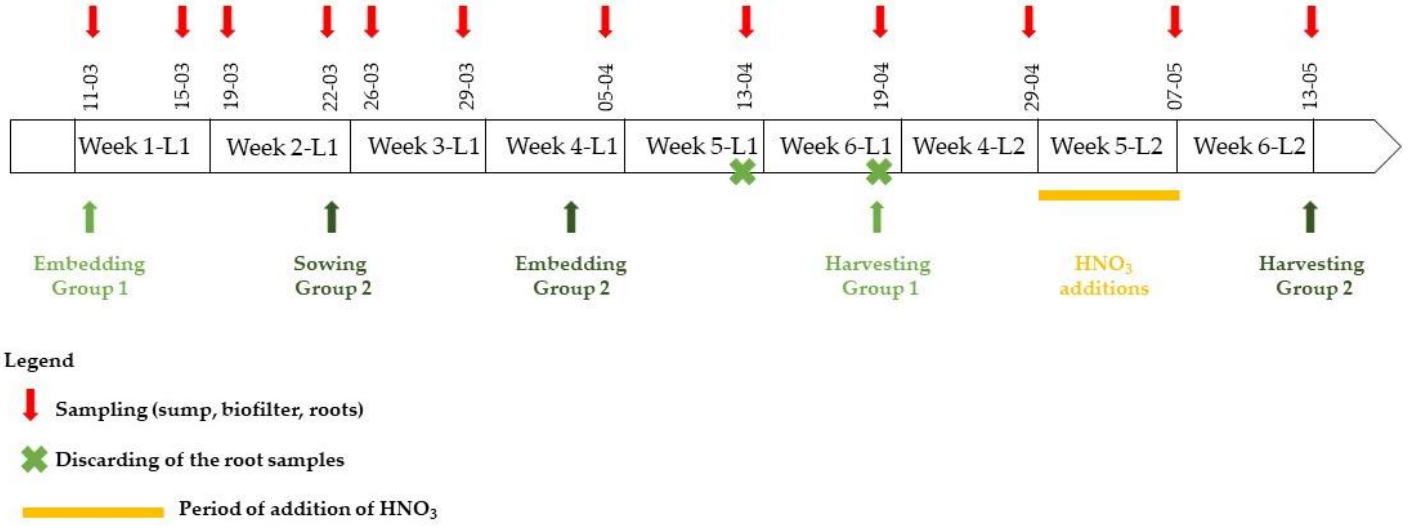


Figure 4-2 Timeline of the experiment. Samplings were conducted in the four compartments simultaneously and are indicated by a red arrow. The first lettuce group was sown on February 28 2019 and embedded on March 11. In parallel, a second group of lettuces was sowed on March 22 and embedded on April 3. Root samples from the first group of lettuce, which were collected on April 13 and 19 were discarded as the lettuces appeared diseased (Supplementary Materials, Figure S1.). All lettuces from group 1 were then discarded and the focus of the study was shifted in group 2 starting from April 29.

4.1.5 DNA extraction

In order to prepare the samples that had been kept at -80 °C or -20 °C for DNA extraction, a rapid defrosting was made by placing them in a 55 °C heat chamber (Thermoshake, Gerhardt GmbH & Co., Königswinter, Germany) for 5 min. After being vortexed for 15 s and centrifuged 20 min at 2350 x *g*, the pellets of the water, biofilter and rhizoplane samples could be used for DNA extraction. The root microbiota samples, i.e., grinded roots with buffer, were used in their present state. The DNA was extracted with the FAST DNA Spin kit (MP Biomedicals, CA, USA) following the steps described in Eck et al. (2019b).

4.1.6 Sequencing

The V1–V3 hypervariable regions of the 16S rRNA gene were amplified with the 2X KAPA HiFi HotStart ReadyMixPCR kit (KAPA Biosystem) and the 27F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGC-TCAG-3′) and 534R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-ATTACCGCGGCTGCTGG-3′) primers (Illumina adapters underlined).

PCRs were performed with the following cycling protocol: a pre-heating process of the lid at 110 °C; a denaturation step at 95 °C for 3 min; then, 25 cycles of 30 s at 95 °C; an annealing step of 30 s at 55 °C, and an extension step of 30 s at 72 °C. These cycles were followed by a final extension step of 5 min at 72 °C. The amplicons were then kept at 4 °C until further proceeding.

Amplicons were sent to the DNAVision company (Gosselies, Belgium) for sequencing on an Illumina MiSeq machine in 250 bp × 2.

Sequencing data are available under the accession PRJNA739097 on the Sequence Read Archive database (SRA) of the National Center for Biotechnology Information (NCBI).

4.1.7 Bioinformatics and statistics

4.1.7.1 Statistics for water parameters data

To avoid analysing the relationships of each water parameter with each variable separately, the four parameters T, pH, EC and nitrate concentration were combined in a principal component analysis using the RStudio software (version 3.3.2). Only those four parameters were kept as the DO data were lost due to a malfunction of the probe. A hierarchical clustering (FactoMineR package, version 1.41 (Husson et al., 2010))

was then conducted to group the different dates of sampling based on the physicochemical parameters (more details in Appendix B). Those physicochemical clusters could then be characterized and used as a factor in the microbiota analysis since each sampling date could be associated to a certain physicochemical group.

4.1.7.2 Bioinformatics

Data were processed with the QIIME 2 (q2) software version 2019-4 (Bolyen et al., 2019) and following the workflow described in Sare et al. (2020). Only the forward sequences were imported under the "Casava One Eight Single Lane Per Sample" format and cleaned with the DADA2 denoise single plug-in with trimming (Callahan et al., 2016). Taxonomy was assigned with the Vsearch classifier implemented in the q2 feature-classifier plug-in with the SILVA_132 database with 99% similarities as a reference. Then the cytoplasmic contaminations (chloroplasts and mitochondria) were removed with the filter-table plug-in. Alpha and beta diversities were then analysed with the q2-diversity core-metrics-phylogenetic plug-in, and principal coordinates analysis (PCoA) based on the UniFrac distance matrices were generated. Pairwise Kruskall–Wallis and Permanova pseudo-F tests were performed to compare alpha- and beta-diversity indices, respectively, using the alpha- and beta-diversity_group_significance plug-ins. Spearman correlation indices were also calculated between alpha-diversity indices and water parameters with the alpha_correlation plug-in.

4.2 Results

4.2.1 General information

In total, 44 samples were collected over the course of 9 weeks (**Figure 4-2**), with 12 samples collected from the sump and biofilter compartments and 10 samples collected from the root microbiota and the rhizoplane of the lettuces' roots (2 root microbiota and 2 rhizoplane samples were discarded due to diseased lettuces). Sampling took place twice a week during the first three weeks and once a week during the last weeks. The bacterial communities composing the samples were analysed using16S rRNA gene deep sequencing with Illumina MiSeq technology. A paired sequencing was conducted on 2 × 250 bp but only the forward reads were kept for the final analysis. The average Q_{30} of the sequences was of 86%.

4.2.2 A different community in each compartment

In our experiment, four different compartments were sampled, i.e., the sump, the biofilter, the lettuce rhizoplane and the lettuce root microbiota. **Figure 4-3** clearly displays that each compartment possesses its own community as already shown by Schmautz et al. (2021a, 2017) with the rhizoplane and root microbiota communities closely intertwined. This might be linked to the fact that no disinfection was conducted on the roots before the grinding and collection of the root microbiota. A continuum of microorganisms might thus have been collected albeit in two steps.

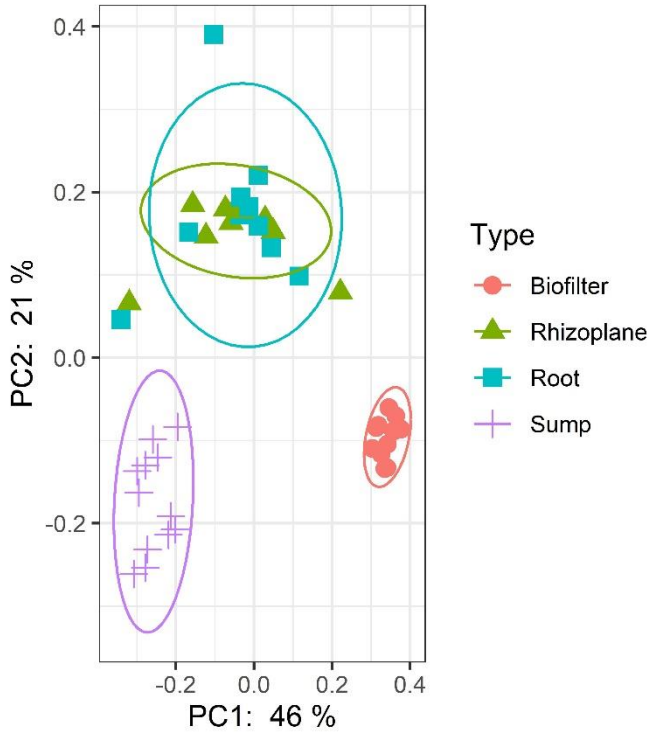


Figure 4-3 Weighted UNIFRAC Principal Coordinates Analysis representing the grouping of samples per compartment

Visual observations have been confirmed by a Permanova, Pseudo_F test on the weighted Unifrac distance matrix (p-value = 0.001). Significant differences are noted between all groups (q-value = 0.0012) except between root microbiota and rhizoplane (q-value = 0.0830).

In terms of diversity, the four compartments are also very distinct (**Table 4-1**). The observed_otus and Shannon indices have been calculated for each compartment and compared with a pairwise Kruskal–Wallis test. The values obtained for both indices are coherent with our previous studies (Eck et al., 2020, 2019b) and higher than those obtained by Schmautz et al. (2017). Concerning the Shannon index, the rhizoplane diversity is significantly higher than in the other compartments, followed by the root and biofilter compartments which have similar diversities. The sump compartment has a diversity significantly lower than the other three compartments. Concerning the observed-otus index, the rhizoplane is the richest compartment (significantly different from the other three compartments), followed by the biofilter (significantly different from the other three compartments) while the root and sump compartments harbour similar richness (Appendix B, **Tables 4-S1** and **4-S2**).

Table 4-1 Observed-otus and Shannon indices for each compartment.

	Biofilter	**Sump**	**Rhizoplane**	**Root**
Observed-otus	528	503	655	223
Shannon	6.6	4.2	7.4	6.3

The four bacterial communities will from here on be mainly studied separately to allow for a clearer interpretation of the evolution of each community.

4.2.3 Water physicochemical parameters in relation with microbial communities: monitoring and analysis

Our main goal aimed at understanding how the bacterial communities in an aquaponic system settled and adapted itself when the system was first launched after a winter fallow period and over the course of a lettuce growth cycle. Our second objective was to be able to link those potential modifications with the changes in the water physicochemical parameters.

4.2.3.1 Global water parameters relationship with bacterial communities

Water parameters (pH, temperature, EC and nitrate concentration) were monitored throughout the experiment with the help of specific probes located in the sump of the aquaponic system. The data obtained were considered valid for the entire system and were thus studied at first on the system scale. The water's main parameters were followed and grouped utilizing a Principal Components Analysis (PCA) (**Figure 4-4** a,b) and a hierarchical clustering (Appendix B, **Figure 4-S8**). This resulted in 5 groups

of sampling dates which, when put onto a timeline give us the following figure (**Figure 4-4** c). Details concerning the parameters' values for each group are given in Appendix B, **Table 4-S3**.

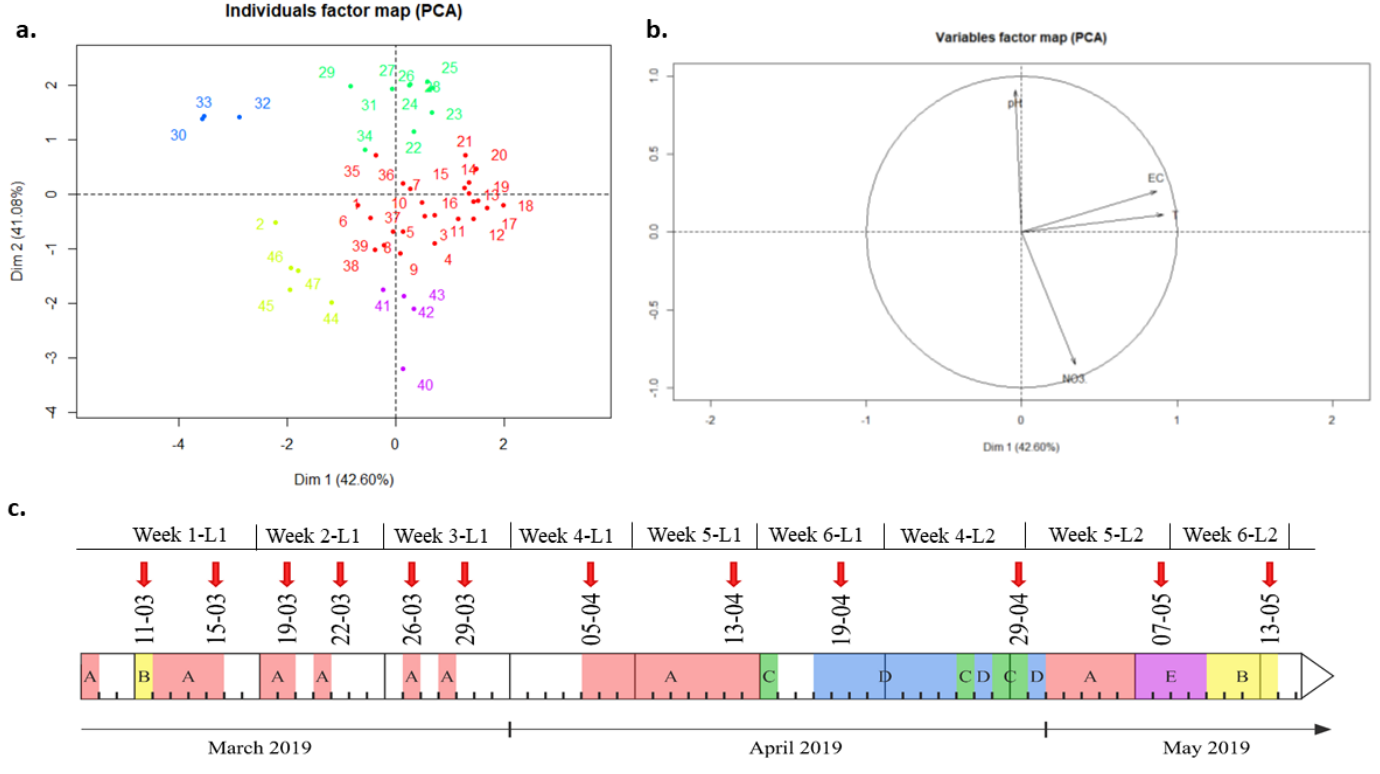


Figure 4-4. *Cont.*.

Figure 4-4 PCA representation of (**a**) the hierarchical clustering of sampling dates based on four water parameters (EC, pH, T, [NO3]). Dimension one represent 42.6% of the variability and distinguishes sampling dates based on EC and T (see variables factor map in (**b**)) while dimension two represents 47.08% of the variability and separates the samples based on pH and [NO3] (see variables factor map in (**b**)). Once put into chronological order, the five physicochemical groups are represented on the timeline (**c**). White intervals on the timeline represent periods where data was lost due to probe problems. Between March 11 and April 5, the lettuce group 1 was sampled. On April 13 and April 19, the lettuce group 1 samples were discarded due to disease symptoms and only the sump and biofilter samples were kept. On April 29, May 5 and May 13, the lettuce group 2 was sampled. Red arrows highlight the sampling dates

The coefficients of variation in each group are relatively similar and low, ranging from 0.01 to 0.08, meaning that the clusters were properly established, with highly homogenous dates (Appendix B, **Table 4-S3** and **Figure 4-S8**). Group A included most dates with 25 days out of 47. It dominated the first part of the experiment, from March 8 to April 14. This group was characterized by the highest EC. Its mean temperature was close to the optimal compromise temperature of 25 °C (Delaide et al., 2017; Somerville et al., 2014) and had average levels of pH and nitrate concentrations comparing to the other groups. Coming right after, Group D mostly dominated the period going from April 18 to April 30. This group was characterized by a high EC, a high pH, a nearly optimal temperature and a low concentration of nitrate. This period represents an important shift in the parameters. Late April to early May, an alternation of group D and C occurred. Covering only 3 days, group C was defined by an average to low EC, a high pH, a low temperature and a very low nitrate concentration. This "extreme" group reflects the two heating failures that occurred on April 25, 26 and 29 as well as a steady decline of nitrate concentration. Then, Group A covered the dates from May 1 to May 5. This reflects a period of "return" to normal conditions, in which the temperature went back to the optimal one and nitrate were added in the water. Then, it switched to Group E, characterized by an average EC, a low-to-average temperature, a very low pH comparing to the average pH observed during the experiment and a very high nitrate concentration. This group reflects the impact of HNO_3 additions, that reduced the pH and increased even more the nitrate concentration. During that period, pH was actually the closest to the optimal compromise pH, i.e., between 6 and 7 (Somerville et al., 2014). Finally, the last days of the experiment were covered by Group B, from May 10 to May 13. This period was characterized by the lowest EC observed throughout the experiment, a temperature close to the optimal, a relatively low pH compared to the other periods and an average nitrate concentration. This group may reflect a stabilization of the parameters, i.e., pH and nitrate that were both drastically modified during the previous

period (Group D). It can be noticed that Group B also covered the first sampling day, i.e., March 11, bordered by the Group A. Since the data were taken manually and the preceding days did not have any parameters' data, it is difficult to say if this short period at the beginning of the experiment really had the characteristics of Group B. As a general conclusion, the first part of the experiment, i.e., from March 8 to April 15, seemed quite constant and homogenous in term of temperature, EC, pH and nitrate concentration. Then, the second part of the experiment, i.e., from April 18 to May 15, was way more heterogeneous, undergoing various parameters' variations such as drops in temperature, pH and nitrate concentration.

The question which now follows is whether the same alternation of groups could be observed in the bacterial communities. All samples were thus grouped based on their water parameters, and a Permanova pseudo-F test was conducted to compare them based on their composition (beta-diversity). No significant difference could be detected based on the physicochemical groups (p-value = 0.921). Considering the timeline, the same reflection was applied to all samples grouped by week of sampling and no significant difference could be noted either (p-value = 0.564). The same results were obtained for each compartment (sump, biofilter, rhizoplane, root microbiota) studied independently and compared by week.

4.2.3.2 Individual water and system parameters' influence on the diversity of the bacterial communities

Spearman correlations between alpha-diversity indices and water and system parameters (days since beginning of the experiment, temperature, pH, EC, nitrate) were calculated for each compartment. In **Table 4-2**, the correlation coefficients (r) are mentioned only when significant and p-value is given. No significant correlation was found between the water and system parameters and the diversity indices in the sump and root microbiota compartments.

Table 4-2 Spearman correlations between alpha-diversity indices and water and system parameters when significant (p-value < 0.05). The correlation coefficients are available in the 'r' column with the corresponding p-value

Alpha-diversity index	Biofilter								Rhizoplane	
	Time		T°		EC		NO_3^-		NO_3^-	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Shannon					-0.64	0.0261			-0.82	0.0038
Observed_OTUs	-0.73	0.0074								
Faith_PD	-0.82	0.0011					0.59	0.0446	-0.81	0.0049
Evenness			-0.67	0.0168						

The biofilter bacterial community seems to be the one most influenced by the water parameters and by time. Indeed, the number of amplicon sequence variants (ASVs) observed decreased significantly throughout the experiment. Furthermore, we can see that the Faith_PD index, which is a phylogenetic diversity index, is negatively correlated with the length of the experiment meaning that throughout the experiment, the phylogenetic diversity decreased as well. Both richness and phylogenetic diversity are much higher during the first four sampling dates and significantly decrease after that. Phylogenetic diversity is also positively correlated with the nitrate concentration. On the other hand, evenness is negatively correlated with the temperature indicating that the higher the temperature, the less even the community. Meanwhile, the Shannon diversity index seems to decrease when EC increases. In the rhizoplane, Shannon diversity and Faith_PD are significantly negatively correlated with the nitrate concentration indicating that when nitrate concentration increases, the diversity of the rhizoplane community decreases, with also a phylogenetic diversity decrease. The augmentation of nitrate in water might thus lead to a selection of specific bacteria in the rhizoplane. The evenness, on the other hand, increases throughout the experiment.

4.2.4 Modifications of the bacterial communities throughout time and composition of the compartments at the genus level

4.2.4.1 General observations

Figure 4-5 corroborates the presence of three distinct bacterial communities i.e. in the sump, the biofilter and the lettuce roots while **Figure 4-6** highlights the shared taxa between the compartments of the aquaponic system.

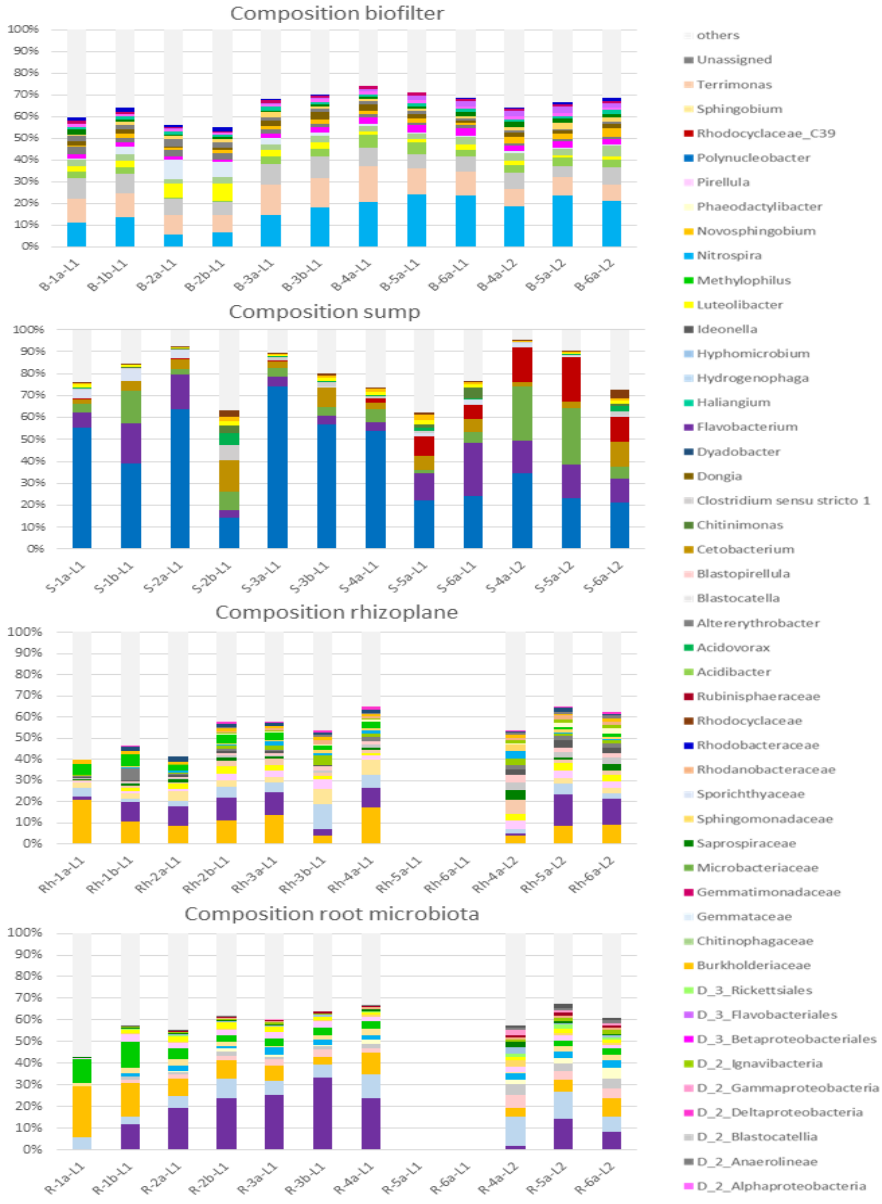


Figure 4-5.Barplots representing the relative abundances of the taxa representing more than 1% (in average) of each of the four compartments. The taxa representing less than 1% are gathered in the "others" group. In the legend, taxa are classified by taxonomic level and alphabetical order. The Rh-5a-L1, Rh-6a-L1, R-5a-L1 and R-6a-L1 were discarded as the lettuces presented disease symptoms.

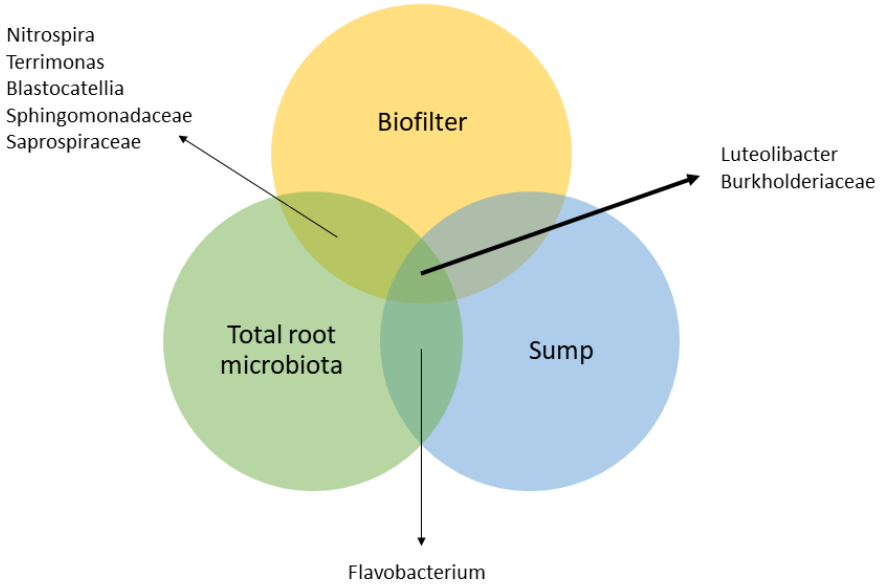


Figure 4-6 Venn diagram representing the shared bacterial taxa between the system's compartments. Rhizoplane and root microbiota are gathered in the total root microbiota circle. Common taxa were identified based on the taxa representing more than 1% (in average) of each compartment

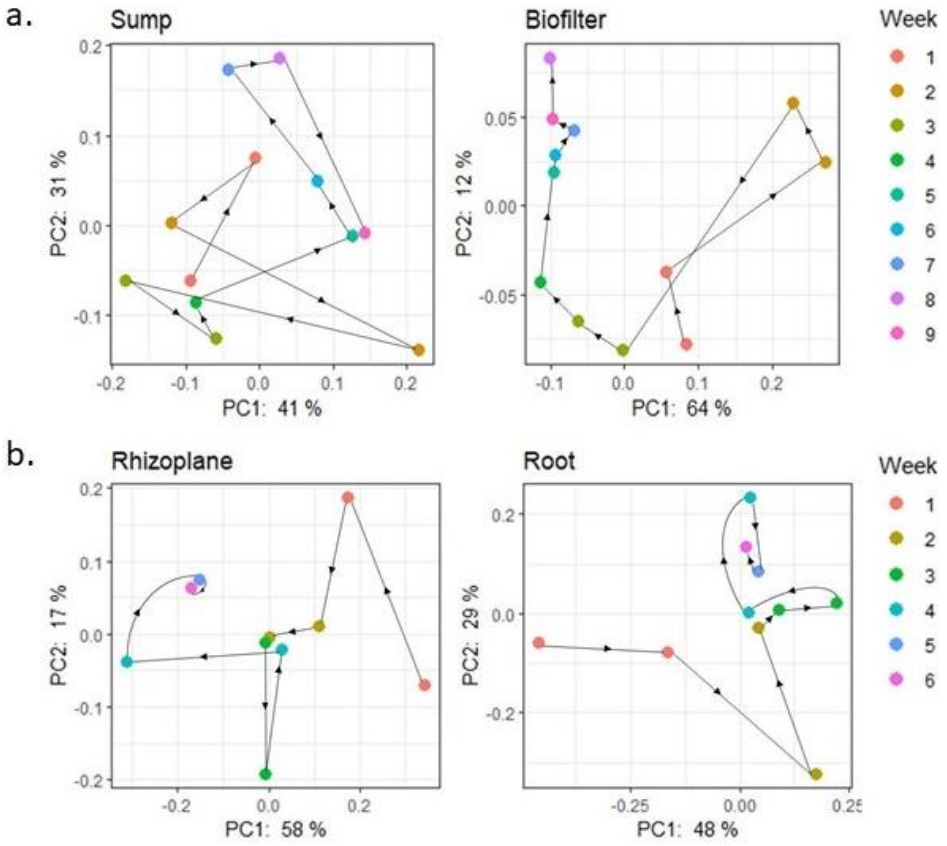


Figure 4-7 Weighted Unifrac PCoA per compartment showing the repartition of the samples week after week in the sump and biofilter (**a**) and in the rhizoplane and root (**b**) compartments. Arrows link the sampling dates in the chronological order. The biofilter, rhizoplane and root compartments seem to present an evolution throughout time.

4.2.4.2 Biofilter

Figure 4-7 clearly displays that, in the biofilter, samples have different composition depending on the week they were collected on, with the first two weeks being clearly separated from the others. However, this change from week to week was not statistically confirmed by a Pseudo F permanova test. Still, week 2 is particularly separated from the other groups and this can be easily observed on the barplot (**Figure 4-5**) in which the B-2a-L1 and B-2b-L1 samples harbour lesser proportions of *Nitrospira*, *Terrimonas* and *Acidobacter* and a higher relative abundance of *Luteolibacter*.

The most important group in the majority of the biofilter samples is the *Nitrospira* genus which shows a steady increase throughout the experiment (**Figure 4-5**). This underlines its crucial role in nitrification as no *Nitrobacter* or *Nitrosomonas* could be detected in the biofilter.

4.2.4.3 Sump

No grouping per week of sampling can be observed in the sump (**Figure 4-7**) and this is confirmed by the barplots (**Figure 4-5**) which are quite similar from one sample to the other. The second sample of the second week is slightly estranged from the others (**Figure 4-7**) and this can also be seen on the barplot (S-2b-L1) with a lesser proportion of *Polynucleobacter* and a greater portion of the community being occupied by diverse ASVs (others). Other than that, the composition of the community is stable throughout time.

Two groups seem distinguishable on **Figure 4-3** though, but none of the factors considered in this experiment are able to explain this separation.

In the sump, samples are highly dominated by the *Polynucleobacter* genus (**Figure 4-5**). This genus is more present at the beginning of the experiment and drops importantly in the S-2b-L1 sample. Nothing specific could be noted in the water parameters on that day (March 22). From the S-5a-L1 sample to the end of the experiment, the *Polynucleobacter* genus represents less than 40% of the community. The second most important taxa in the sump community is the *Flavobacterium* genus. It is less present in the S-2b-L1, S-3a-L1, S-3b-L1 and S-4a-L1 samples. It is to be noted that the *Flavobacterium* genus is also a major taxon in both root compartments. The undetermined genus (C39) from the *Rhodocyclaceae* family presents an increase in relative abundance from the S-5a-L1 sample. To conclude, no major disruption could be observed in the sump during the 9 weeks of the experiment apart from the slight decrease of the *Polynucleobacter's* relative abundance and concomitant increase of the *Flavobacterium* and undetermined C39 presence.

4.2.4.4 Rhizoplane

Figure 4-7 shows that no clear grouping per week is present and that even within the same week, the two collected samples can have a very different composition (i.e., in week 1 and 3). It has to be reminded here that the two samples from week 4 belong to two different groups of lettuces with the sample from the first group being closer to the samples from the previous weeks. On the composition barplot (**Figure 4-5**), we can see that indeed, the first sample from the second group of lettuce (W-4a-L2)

harbours a different and more di-versified bacterial community with a lesser proportion of the *Burkholderiaceae* family and *Flavobacterium*. However, the W-5a-L2 and W-6a-L2 then present a community again coherent with the beginning of the experiment.

The predominant taxon in the rhizoplane is the *Burkholderiaceae* family which represents between 5% and 20% of the samples, with *Flavobacterium* coming as second. However, *Flavobacterium* is quite less present in sample W-1a-L1, i.e., in the lettuce seedling be-fore transplantation. The *Hydrogenophaga* genus was also very present in the rhizoplane compartment.

4.2.4.5 Root microbiota

In the root compartment, **Figure 4-7** shows that the first three samples are quite different (albeit not significantly) from the rest of the experiment. On the composition barplots, however, only the first sample (lettuce seedlings before transplantation) is visibly different from the other with the utter absence of *Flavobacterium* and the importance of the *Burkholderiaceae* family. The *Flavobacterium* genus proportion steadily increases, reaching a peak of more than 30% in R-3b-L1 then decreases. Similarly to the rhizoplane compartment, a strong difference can be noticed between the fourth week sample from lettuce group 1 (R-4a-L1) and lettuce group 2 (R-4a-L2). Here again, the last two samples are more similar to the ones from the beginning of the experiment despite belonging to two different lettuce groups.

Both root microbial communities are quite similar with *Flavobacterium*, *Hydrogenophaga* and the *Burkholderiaceae* family as the three main taxa, albeit with the Flavobacterium being the most important one in the tight root microbiota.

4.3 Discussion

4.3.1 General comment

It is important to start by discussing the choice of shifting the focus of the experiment onto a second group of lettuces due to the appearance of disease symptoms on the first group. Symptoms such as growth stunt, followed by leaf yellowing and wilting as well as root necrosis were indeed observed on the first group of lettuce during the fifth and sixth weeks of growth. In the meantime, batches of lettuces had been regularly embedded in the aquaponic system to compensate for the regular lettuce loss due to sampling. It was therefore decided to keep the experiment running with a focus on a second group of four-week-old lettuce. Indeed, we had kept the

samples of lettuce group 1 until week 4 and starting again with another four-week-old group permitted us to compare the composition of microbial communities of the two groups at this stage and determine whether both groups could be considered similar. Slight differences could be observed between the Rh-4a-L1 and Rh-4a-L2 samples when considering the proportions of the three main taxa of the compartments (*Burkholderiaceae* family, *Flavobacterium* and *Hydrogenophaga* genera) but these differences tended to fade in the following samples. Furthermore, sump and biofilter samples of microbial communities presented no major modifications either during week 5 and 6 of the first lettuce group thus showing that the potential disease affecting the lettuces did not impact the other compartments of the aquaponic system. Finally, no significant modifications appeared throughout time in either compartment confirming that following another lettuce group in the same aquaponic system did not significantly impact the outcome of the study.

4.3.2 Resilience of the bacterial communities to the system's parameters changes and over time

The global aim of this study was to analyse the modifications of aquaponics bacterial communities throughout time and more specifically over the course of a full lettuce growth cycle. The idea behind the experiment was to follow the normal functioning of our aquaponic system (i.e., without provoking any changes in the system) and see whether the bacterial communities would evolve after the winter fallow period, adapt to the presence of plants or be modified by small shocks such as short temperature modifications due to the climate or a short heating failure. Furthermore, it would have been particularly relevant to compare our results to the same type of experiment conducted in hydroponics to be able to assess the impact of the "soilless" element. However, to our knowledge, very little information is available on the natural adaptations of bacterial communities in hydroponic systems and in hydroponic plants' rhizosphere. Therefore, our data were mainly compared to data from soil borne rhizosphere studies.

4.3.2.1 No significant differences between bacterial communities grouping based on physicochemical groups

Microbial communities in our aquaponic system were not influenced by the water's physicochemical parameter changes. However, this conclusion needs to be qualified in the light of certain biases. Indeed, for parameter groups C, D and E, only one sampling date was included (**Figure 4-4**) which means that once separated into compartments, only one sample was left per group. Meanwhile, group A included 7

sampling dates and group B, 2. This could influence the comparison by rendering it less robust than if several samples had composed those groups.

Furthermore, the Permanova pseudo-F test, which was conducted to compare the bacterial communities between physicochemical groups, was performed on the whole system while, when we look at compartments separately, we can notice that in the biofilter, for example, the diversity of the community is still correlated with the water temperature, the EC and the NO_3^- concentration. The correlation with nitrate concentration may seem coherent as this molecule is part of the nitrification process and may thus directly influence nitrifying bacteria's welfare. On the other hand, nitrification is highly dependent on oxygen availability (Schmautz et al., 2021b, 2021a) which is linked to water temperature. Changes in water temperature will thus affect dissolved oxygen levels which would then impact the bacterial community of the biofilter.

Eventually, other water physicochemical parameters might have a stronger influence on the composition of the bacterial communities such as oxygen saturation and total organic carbon (Schmautz et al., 2021a) which we did not measure. Indeed, Schmautz et al. (2021a) recently concluded that oxygen level was a most important parameter and that a major difference in terms of diversity could be noticed between the aerobic and anaerobic compartments of an aquaponic system.

However, Chave et al. (2008) reported that even the addition of a solution of active free chlorine (0.15 mg/L) in their NFT system did not affect roses' rhizoplane communities. The robustness of the root microbial communities in hydroponics has also been confirmed in tomato plants by Calvo-Bado et al. (2006) but it is yet unclear whether this protection effect is linked to a mechanical or chemical sheltering (Chave et al., 2008).

4.3.2.2 Nor between weeks

No significant difference in the composition of the bacterial communities could be noted between the time points sampled during the experiment thus suggesting that the bacterial communities in each compartment are resilient to water parameter changes and are constant over the full lettuce growth cycle and this, even after the winter fallow period. To our knowledge, no other such study has yet been conducted on an aquaponic system thus impeding any strong comparison, especially in the sump and biofilter compartments. However, modifications of the rhizosphere microbial community have been observed and scrutinized in field studies. Depending on the plant species, variations can be observed (Chen et al., 2019; Sugiyama et al., 2014)

albeit not always in terms of diversity of the community (Houlden et al., 2008). Indeed, the variations of microbial communities during the maturation of several plant species observed by Houlden et al. (2008) were mostly observed in terms of functions when analysed through a BIOLOG method even though this technique is limited to cultivable microorganisms. This variation in the functions is justified by the fact that, through their different physiological stages, plants harbour varying needs and therefore recruit different microorganisms via the production of different types of root exudates (Chaparro et al., 2014; Houlden et al., 2008).

This explanation brings forward two hypotheses as to why no variations were observed in our root communities. The first hypothesis is linked to the plant species, namely the lettuce. Indeed, in our experiments, the lettuces were followed from seedlings to vegetative stage but were collected before stemming and flowering as it is the use in lettuce cultivation. Meanwhile, in most studies on this topic, the roots' microbiota is followed from the seedling stage until the flowering or even ripening stages (Chaparro et al., 2014; Chen et al., 2019; Sugiyama et al., 2014). The absence of those contrasting physiological stages might explain the lack of differing needs in the plant and thus lack of variation in our rhizoplane and root communities. To our knowledge, no studies following the modifications over time of the lettuce root microbiota could be found in the available literature. An interesting perspective would therefore be to conduct the same experiment on fruiting vegetables such as tomatoes to assess whether different microbiota can be observed throughout plant growth stages also in aquaponics. Such new information would shed light on the respective influences of aquaponic microbiota on the rhizosphere microbiota and vice versa.

The second hypothesis concerns the very nature of aquaponic systems, i.e., soilless systems in which the roots are in direct contact with flowing water. Indeed, contrary to soil, the flowing water does not constitute strata around the plant roots, i.e., no clear limitation between bulk soil, rhizosphere and rhizoplane. Here, the water is in constant movement and might thus dilute the root exudates which can, in soils, be accessed to by the microorganisms of the solid rhizosphere (Chaparro et al., 2014). However, it has been assessed that even in hydroponic systems, root exudates can influence the composition of the rhizosphere community (Rosberg et al., 2014; Vallance et al., 2011) and Renault et al. (2008) (cited by Vallance et al. (2011)) did find modifications in the composition of the root bacterial communities in hydroponics, albeit in tomato plant, which brings us back to our first hypothesis and the lack of contrasting physiological stages in lettuce. Eventually, an interesting perspective to this study would be to follow the microbiota harboured by the rockwool plugs or other inert

media over time. Indeed, despite being an inert substrate, rockwool can be colonized by microorganisms once introduced in an aquaponic system and intertwined with plant roots (Carlile and Wilson 1991 cited by Calvo-Bado et al. (2006)). It could thus take the place of the physically concrete rhizosphere and could retain plant exudates more easily.

All in all, these findings suggest that the microbial communities of the sump, biofilter and root compartments in aquaponics are stable throughout time but also to small water parameter modifications. It can therefore be assumed that aquaponics microbial communities could also resist modifications brought by the development of external taxa such as pathogens, for example, and therefore constitute a more resilient environment for soilless plant growth.

Eventually, a possible improvement of the experimental design would be to collect a more important number of samples from the system functioning as a RAS to enable a more robust comparison between the RAS state and the aquaponic state of the system. Indeed, we were here limited by the fact that only one sump and biofilter sample were at disposal before the introduction of the lettuce seedlings.

4.3.2.3 Nor between years

The results obtained in this study for the sump and biofilter compartments display the presence of similar predominant families and genera to previous studies conducted on the same system thus highlighting a certain stability throughout experiments and time. This confirms the hypothesis from Eck et al. (2019b) that each system seems to have a specific bacterial community. Indeed, in a previous experience by Eck et al. (2019b) the *Luteolibacter*, C39 (*Rhodocyclaceae* family), *Flavobacterium* and *Cetobacterium* genera as well as the *Microbacteriaceae* family were already detected as predominant taxa of the PAFF Box sump. In the biofilter, the *Luteolibacter* and *Nitrospira* genera as well as the *Saprospiraceae* family were common members of the predominant taxa between 2017 and 2019.

4.3.3 But trends can still be observed and alpha-diversity indexes were correlated with water parameters

4.3.3.1 Day 1 – before transplantation

The first sump and biofilter samples were collected on March 13 before seedlings' transplantation, i.e., when the PAFF Box was still functioning as a RAS. On this first day, the sump was dominated at 55% by the *Polynucleobacter* genus. The biofilter

harbored two main genera, namely *Nitrospira* (11%) and *Terrimonas* (11%). These day 1 samples are not particularly different from the day 2 samples nor from the rest of the sump and biofilter samples thus leading to the hypothesis that both compartments are not strongly influenced by the introduction of plants into the system. For the sump, this could be explained by the fact that such an important volume of water (**Figure 4-1**), with an average weekly renewal of 200 litres may present a stability in front of the addition of 80 lettuce seedlings. For the biofilter, the same argument can be applied as it is constantly supplied with water coming out of the mechanical filter. Moreover, as the fish compartment was kept running during the winter period, the biofilter community was still supplied in fish excreted ammonia thus sustaining a healthy nitrifying community. To allow for a better comparison of the PAFF Box microbiota with and without plants, more samples from the "without plants" period should be collected. This would indeed supply a more robust idea of the "aquaculture microbiota" to be then compared to the "aquaponics microbiota".

Samples were also taken from the lettuce seedlings before their being transferred in the PAFF Box. In the rhizoplane, the predominant taxon was the Burkholderiaceae family representing 21% of the community. The Burkholderiaceae family harbors known plant growth promoting bacteria and is very often found in the rhizosphere of soil cultures (Hassani et al., 2018) as well as in our aquaponics studies (Stouvenakers et al., 2020). This family was also very present in the first seedlings' root microbiota sample (23%). In this first root microbiota sample, the *Flavobacterium* genus which is later very predominant only represents 0.4%.

Flavobacterium are often associated with plants and could prove beneficial for plant health and care (Kolton et al., 2016; Soltani et al., 2010), they have also been identified in aquaculture systems (Itoi et al., 2007; Munguia-Fragozo et al., 2015; Schmautz et al., 2017). Some *Flavobacterium* present functional traits which could prove highly beneficial for crop yields in aquaponics such as phosphate solubilisation, organic matter degradation or auxin and siderophores production (Soltani et al., 2010) or even the fight against pathogens.

4.3.3.2 After transplantation – lettuce growth cycle

- **Biofilter**

Concerning the nitrification process, no *Nitrosomonas* or *Nitrobacter* could be detected in our biofilter throughout the whole experiment thus confirming the observations of Bartelme et al. (2019), Eck et al. (2019b), Schmautz et al. (2017) whom did not observe or, only in very small proportion, the presence of *Nitrosomonas*

and *Nitrobacter* and corroborating Bartelme et al.'s (2017) assumption that *Nitrospira* is a main player in nitrification in freshwater biofilters. The *Nitrospira* taxa maintains itself steadily throughout the experiment and represents the single component of the *Nitrospirea* phylum. *Nitrospira* can be a COMMAMOX (Bartelme et al., 2017; Daims et al., 2015), i.e., a bacteria capable of performing the whole nitrification process on its own. Similarly, the *Terrimonas* taxa keeps relatively stable proportions.

A negative correlation could be observed between the elapsed time and the number of observed ASVs which means that the number of ASVs composing the biofilter community significantly decreases throughout the experiment. This could be the reason why the first two weeks on the PCoA (**Figure 4-7**) are so distant from the samples from the end of the experiment.

We can also note the negative correlations between the evenness and the water temperature, between Shannon and EC and the positive correlation between Faith_PD and NO_3. We can thus state that water parameters such as temperature and EC could influence the composition of the microorganism community in a biofilter. However, the variation ranges of these two parameters were quite restrained. It would thus be interesting to apply more marked temperature and EC modifications on an aquaponic system and see the adaptation of the communities, all the while respecting the fish welfare. According to Schmautz et al. (2021a), the most influencing water parameter is the dissolved oxygen available, clearly distinguishing aerobic and anaerobic bacterial communities. The levels of dissolved oxygen were unfortunately lost in our experiment due to probe failure.

- **Sump**

In the sump, no particular variations nor trends could be observed throughout the experiment. The *Polynucleobacter* genus was always predominant in the samples. This observation correlates with Bartelme et al. (2019), who state that *Polynucleobacter* are common in freshwater aquaponics but also in natural freshwater systems. Their hypothesis explaining the important presence of this genus in aquaponics is that aquaponic systems reproduce natural ecosystems with plant particles being present in the running water (Bartelme et al., 2019). However, we already had a very important proportion of *Polynucleobacter* in the first sump sample when the PAFF Box had actually been running as a simple RAS for three months. According to Bartelme et al. (2019), this taxon is not commonly found in aquaculture albeit other studies (Watanabe et al., 2008; Yildiz et al., 2017a) state that it is a common heterotrophic member of freshwater microbial communities.

No major difference can be observed between the first sample (before transplantation of the lettuce seedling) and the rest of the samples thus leading to think that the bacterial community of the sump is not particularly influenced by the introduction of plants or that the volume of water from the sump is not influenced by the roots. This is different from the soil where the substrate is static and clear zones can be delimited around the roots, i.e., bulk soil, rhizosphere and rhizoplane (Chaparro et al., 2014). The resilience of the sump compartment is also confirmed by the absence of correlation between the water parameters and the alpha-diversity indices.

- **Rhizoplane and endosphere: a typical lettuce root community**

The microbial community observed in our rhizoplane and root microbiota samples is coherent with the common soil cultivated lettuce rhizosphere community with a majority of *Proteobacteria* (Berg et al., 2015) (gammaproteobacteria) and *Bacteroidetes* phyla (Cardinale et al., 2015). In our experiment, the third most important phylum is the *Planctomycetes* conversely from Cardinale et al. (2015) who observed *Chloroflexi* and *Actinobacteria*. If the *Flavobacterium* seem to be common members of the lettuce rhizosphere community (Berg et al., 2015; Schreiter et al., 2014a), the *Burkholderiaceae* family is not mentioned by Cardinale et al, (2015). The *Pseudomonadaceae* family is found both in Cardinale et al. (2015) and our study which is encouraging as the *Pseudomonadaceae* family often contains plant growth promoting bacteria (Hayat et al., 2010). The *Sphingomonadaceae* family is also one of the major family in the rhizoplane and endosphere (Berg et al., 2015) as well as the *Chitinophagaceae* family. To conclude, the aquaponic environment does not seem to disrupt the "classical" lettuce root microbial community found in soil borne lettuces.

Furthermore, the absence of correlation between the aquaponic water parameters and the tight root microbiota alpha-diversity highlights the strong relationship between the lettuce roots and their typical microbiota.

It is of prime importance to dwell on this information. Indeed, despite being in a soilless system, thus widely different from field grown lettuces, the root bacterial communities, i.e., rhizoplane and root microbiota were very similar to classical soil lettuce rhizosphere community. It is nowadays acknowledged that soil type strongly influences the composition of the root microbial communities (Berg and Smalla, 2009). The addition of different designs of soilless systems in studies comparing the effect of soil type on the composition and functions of plant root microbial communities could help expand knowledge and understanding of these communities in production systems.

Yet, a question which might arise is then the origin of these bacteria. Indeed, it seems crucial to understand whether the microorganisms come mainly from the lettuce seed or if the lettuce is able to recruit its usual rhizoplane community in the aquaponic water. This question is even more important as, in a study using the same lettuce seeds as in our experiment and observing the rhizoplane bacterial community in hydroponics, the *Burkholderiaceae* family also represented 50% of the rhizoplane community while the Sphingomonadaceae and Chitinophagaceae family were also part of the predominant taxa (Stouvenakers et al., 2020; supplementary materials).

Reproducing our experiment with other species such as tomatoes would then enable us to distinguish different physiological stages but also to see whether the root microbiota here observed is typical from the lettuce culture or if we can notice the same root microorganisms in tomatoes which would then plead for a more specific "aquaponic microbiota".

Eventually, it is important to nuance the similarities and dissimilarities observed between all the cited studies as most of them targeted different regions of the 16S gene or used different primer sets for a same region, thus highly influencing taxonomic assignment and diversity indices (Cruaud et al., 2014; Darwish et al., 2021). For instance, Cardinale et al. (2015) focused on the V4 region and Schreiter et al. (2014a) on the V6–V8 regions which might skew the comparison of lettuce rhizosphere communities.

4.4 Conclusions and perspectives

This study aimed at offering a first view of the modifications of microbial communities in an aquaponic system over a crop cycle. The plainest answer it can offer is that, throughout a full lettuce growth cycle, no major modifications of the bacterial communities could be observed in the sump, biofilter and root compartments of the aquaponic system. This observation leads to the supposition that the introduction of lettuce seedlings into the aquaponic system does not bring major disruption into the system (sump and biofilter samples). What is more, the lettuce root communities appeared very similar to typical soil borne lettuce root community thus bringing forward the idea that lettuce seedlings carry their own microbiota inherited from the seeds (Barret et al., 2016; Rochefort et al., 2021) and that it is, reciprocally, less influenced by the surrounding aquaponic environment. Another hypothesis would be that lettuces in aquaponics are able to perform a recruitment process similar to soils. Therefore, another key point is the seeming lack of communication between the root and water (sump) compartments as each tends to keep its own specific bacterial

community throughout the experiment. An interesting perspective would be to study concomitantly the community harboured by the rockwool plug which might serve as a proxy for the rhizosphere. Indeed, plants recruit microorganisms via the production of root exudates which attract bacteria and fungi in the root vicinity (Schreiter et al., 2014a). Yet in soilless systems, root exudates may be diluted in the nutritive solution and the absence of surrounding physical support may prevent the formation of microbial strata (Badri and Vivanco, 2009). To dig further into the relationships between plant-brought and aquaponic microbiota, several experiments could be set up such as the monitoring of root microbiota from plants originating from the same seed lot in different aquaponic systems or, conversely, the study of root microbiota of different plant species (e.g., lettuce and tomato) in the same aquaponic system. Further experiments focused on other plant species such as tomatoes or cucumbers also ought to be implemented in order to compare their aquaponics and soil root communities as well.

Finally, a curious observation emerged from the study of the biofilter as its population seemed to evolve over the course of the experiment. These changes may be linked to water parameters such as temperature, EC or nitrate concentration as it was shown that those three parameters are significantly correlated with the alpha-diversity in the biofilter. It is, however, impossible to prove any link between this modification and the introduction of the lettuce seedlings in the system. Still, we can bring forward the hypothesis that the introduction of plants and the ensuing consumption of nitrates by the growing lettuces might play a role in the nitrogen cycle in the system and thus influence the biofilter community. A more in-depth study of this compartment during the RAS period would be necessary to ascertain a potential link

5.

Potentially plant beneficial functions in aquaponics microbiota

5.1 Introduction

Aquaponics is a production technique which combines fish and plants production in order to improve the sustainability of both (Goddek et al., 2019a). The idea behind this merger of two production techniques is the recycling of the nutrients contained in the fish effluents to fertilize the plants which thus leads to the saving of nutrients instead of discarding them in the environment and reduces the dependence on non-renewable sources of nutrients for plants production (Bittsanszky et al., 2016).

However, relying on fish effluents for plant fertilization brings up a new challenge as the nutrient content of the fish effluents fluctuates a lot, both in terms of concentrations and forms of the nutrients. Indeed, the composition of the aquaponic solution totally depends on fish feed inputs and ensuing fish metabolisation (Eck et al., 2019a; Palm et al., 2018). Conversely, in hydroponics, the nutrient solution is highly controlled, all parameters are tightly monitored and the concentrations of nutrients adapted to the plant's varying needs at each physiological stage (Resh, 2013). In aquaponics, the nutrients presence and concentrations are defined by several factors such as the requirements of the fish species, the type of fish feed, the feeding rate – itself linked to fish growth stage, fish density, microbial communities, water parameters (Kasozi et al., 2019) – thus making nutrient cycling much more complicated and the aquaponics practitioner's community still lack information on this topic. It is however crucial to unravel the cycles of nutrients in aquaponics in order to increase its reliability, sustainability and improve its overall functioning.

Nutrient cycling is therefore considered one of the most important research topics in aquaponics (Yep and Zheng, 2019). A review of the current knowledge on nutrient cycling in aquaponics has been published by Eck et al. (2019b), aiming at bridging the information between the form of the major nutrients when inputted into the system, the knowledge about the chemistry of these molecules in aquaponics conditions (pH, nutrient ratios) and the role that microorganisms can play in nutrient cycling (Eck et al., 2019a). The study of the roles of microorganisms in nutrient cycling in aquaponics is indeed of prime importance (Yep and Zheng, 2019) as potentially plant growth beneficial microorganisms could explain the important yields observed in aquaponics despite lower nutrients concentrations than in hydroponics as well as a particular tolerance to diseases (Delaide et al., 2017; Stouvenakers et al., 2020; Yep and Zheng, 2019).

Currently, the most known microbial process in aquaponics is still nitrification but more and more researchers now focus on the many other processes involved in the

correct functioning of an aquaponic system. For instance, microorganisms could play crucial roles in processes linked to nutrient transformation and plant absorption and this arouses general interest both from the scientific and entrepreneur communities as to their impact and potential contribution towards a more efficient and more viable aquaponic production. In this frame we hinged our study on the relationship between nutrients, microorganisms and plants.

Microorganisms in aquaponics have been recently tackled by more and more studies. In 2017, Schmautz et al. (2017) described the specific bacterial communities hosted by the different compartments of their aquaponic system. In 2019, Eck et al. (2019b) explored the microbial communities of various aquaponic and aquaculture systems while Bartelme et al. (2019) published an article also focusing on the composition of microbial communities in aquaponics and the influence of the system design on these communities. This topic has been deepened by Schmautz et al. (2021b, 2021a) who focused on the links between microorganisms and nitrogen as well as the influence of the abiotic parameters on community's diversity. On the other hand, Stouvenakers et al. (2020) searched into the mechanisms of suppressiveness of aquaponic water while others dealt with the health aspect of aquaponic system looking for fish and plant pre- and probiotics (Joyce et al., 2019b; Kasozi et al., 2021a, 2021b).

Beside this descriptive aspect, other studies have focused on the functions and roles microorganisms could play in interaction with plants. In 2018, Bartelme et al. (2018) published a perspective paper highlighting the potential that plant beneficial microorganisms could have in aquaponics, thus being one of the first to make the link between the broad description of aquaponics microbiota and its actual impact on plant health and care. Bartelme et al. (2018) confirmed the hypothesis that microorganisms are involved in nutrient uptake by plants but highlighted that conversely to soil microbiota, microorganisms in aquaponics have been seldom studied yet. Based on soil knowledge, microorganisms belonging to the genera "*Pseudomonas, Bacillus, Enterobacter, Streptomyces, Gliocladium* or *Trichoderma* could increase nutrient availability for plants" (Bartelme et al., 2018). Sanchez et al. (2019) worked both on the characterisation of their aquaponic microbiota and the research of specific functions such as phosphorus solubilisation and ammonia production. They also considered that the presence of potential PGPR in aquaponics could constitute a new reservoir for the production of biofertilisers.

Having already described the microbial communities from the closed-loop aquaponic system of Gembloux Agro-Bio Tech in Eck et al. (2021, 2020, 2019b), this study focuses on the potentially plant beneficial functions harboured in these

communities. It aims at determining whether the presence of these potentially beneficial microorganisms could directly help the growth and health of lettuces in aquaponics and whether their effect could be enhanced by the addition of an important quantity of bacteria.

To this end, biochemical tests were performed *in vitro* on bacterial strains collected from the sump of our aquaponic system to assess their potentially plant beneficial abilities. In addition, as the presence of a functional trait *in vitro* does not always result in its expression or significant impact *in vivo* (Ahmad et al., 2016), *in vivo* trials were set up in deep water culture (DWC) systems in order to evaluate the impact of bacteria inoculations on lettuce yields.

5.2 Material and methods

5.2.1 Microorganisms collection and isolation

The first part of this experiment consisted in the collection, cultivation and isolation of aquaponic microorganisms with the aim of investigating their potential plant beneficial functions. To this end, water samples were collected from the sump of the PAFF Box aquaponic system (described in Eck et al. (2019b)), serial diluted and spread on LB, PDA, TSA and NYDA generalist growing media (Appendix C). The colonies of microorganisms thus obtained were then isolated in individual Petri plates and each strain was purified by repeated streaking and finally stored with 30% glycerol at -80°C. In this way, 31 bacterial strains were collected and stored but no fungi could be retrieved by this method.

5.2.2 Plant growth promotion abilities tests

In order to assess the potential plant growth promoting abilities of the bacterial strains, qualitative biochemical tests were carried out *in vitro* on each of the 31 strains to evaluate five functions highly relevant in aquaponics nutrient cycling: a) solubilise inorganic phosphorus, b) solubilise potassium, c) produce ammoniac, d) produce siderophores and e) produce indole acetic acid (IAA).

- **Phosphorus solubilisation**

Pikovskaya plates (Nautiyal, 1999) were used to detect the ability of the bacterial strains to solubilise inorganic phosphorus. Ten µl of each strain suspension were deposited in a Petri plate and the plates when then incubated in a heated chamber for

5 days at 28°C with natural light entering the chamber. The ability to solubilise phosphorus was observable via the apparition of a translucent halo around the colony.

- **Potassium solubilisation**

Potassium solubilisation was assayed with bromothymol blue modified Aleksandrov media (Rajawat et al., 2016). Ten µl of each strain suspension were deposited in a Petri plate which was then incubated in a heated chamber for 2 days at 38°C with natural light entering the chamber. The solubilisation of potassium could be observed when a yellow hallo appeared around the colony.

- **Siderophores production**

The ability to produce siderophores was detected via the use of the Schwyn and Neilands (1987) Chrome azurol S medium (Louden et al., 2011). Ten µl of each strain were placed in a Petri plate and incubated for 5 days at 28°C in a heated chamber with natural light. The production of siderophores was detected through the apparition of a yellow halo around the colony.

- **Ammonia production**

Ammonia production was assayed using peptone water (1%) and Nessler reagent. 100 µl of each bacterial strain were inoculated in 10 ml of peptone water and incubated for 4 days at 28°C with a 150 rpm agitation. After incubation, 0.5 ml of Nessler reagent was added in each tube. A yellowish/brownish coloration indicated a positive result (Kumar et al., 2012).

- **Indole acetic acid production**

Indole acetic acid production was assayed using tryptophan enriched (0.1%) LB plates and Salkowski reagent (1.2% $FeCl_3$ in 37% sulphuric acid) (Bric et al., 1991). Ten µl of each bacterial strain were deposited in the middle of a Petri plate and covered with a paper filter (90 mm diameter, 5-13 µm, VWR). The strains were then left to grow in a chamber at 22°C. After 7 days, the filters were removed and soaked with Salkowski reagent for 30 minutes. The brownish/red/pink coloration of the filter indicated the presence of IAA. It is of interest to note that the ability to produce IAA can also be detected without providing tryptophan in the growth media thus making it less easy for the bacteria to express this trait and leading to a stricter selection of strains.

5.2.3 Molecular identification of potentially plant beneficial bacterial strains

Strains proving positive for several biochemical tests were considered as potentially beneficial for plant growth in aquaponics and were identified through the sequencing of their 16S rRNA gene with the Sanger technique.

Bacterial DNA was first extracted with the FAST DNA spin kit (MP Biomedicals) following the protocol described in Eck et al. (2019b). The 16S rRNA gene was then amplified with the A1F and B1R primers (Fukatsu et al., 2000) and the Mango Taq PCR kit (Bioline). The thermal cycle protocol was the following: initial denaturation step at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, then annealing at 50°C for 1 min and elongation at 70°C for 2 min. Final elongation was conducted at 72°C for 10 min (De Clerck et al., 2015). The amplicons were then purified using the Qiaquick kit (Quiagen) following the manufacturer's instructions and sent for Sanger sequencing at Macrogen Europe (Amsterdam, The Netherlands) with the same A1F and B1R primers.

Results were analysed with the Geneious Prime software (2020.0.5) for quality trimming and consensus assembly of the forward and reverse reads with default parameters. The consensus sequences were BLASTed on the NCBI database for taxonomy assignment.

Strains assigned to the *Pseudomonas* genus were sequenced again using the PsEG30F and PsEG790R primers targeting the *rpoD* gene of the *Pseudomonas* genus and following the PCR protocol described in Mulet et al. (2009).

5.2.4 **In vivo** *yield tests*

It is acknowledged that what can easily be observed *in vitro* will not always be applicable *in vivo* (Ahmad et al., 2008). The second goal of this study was thus to test whether bacteria presenting potentially plant growth promotion abilities *in vitro* could have a significant impact on lettuce growth in aquaponics. To this end, miniature deep water culture systems filled with aquaponic water from the PAFF Box were implemented and harboured inoculated and un-inoculated lettuces.

5.2.4.1 Bacterial treatments and application method

In this experiment, three treatments were compared namely: a mix of 3 bacterial strains (A, H and T), a bacteria strain alone (T) and a control (C_t) (no addition of bacteria). The selected bacterial treatments were thus the following:

- Aquaponic water + inoculation of a suspension of *Serratia fonticola* (strain T)
- Aquaponic water + inoculation of a mixed suspension of *Serratia fonticola* (strain T), *Chryseobacterium cucumeris* (strain H) and *Pseudomonas aeruginosa* (strain A)
- Aquaponic water + inoculation of isotonic water (control treatment)

Bacteria were cultivated on LB growth media for 5 days before being collected and suspended in isotonic water (0.85% NaCl). In order to normalize the concentration of inoculated bacteria for each trial, the suspensions thus obtained were diluted to reach a DO of 0.6 (± 0.05) at a wavelength of 600 nm (ca. 10^8 CFU/ml) (Spectrophotometer, model Prim 938, Secomam, Domont, France). Ten ml of bacterial suspension per rockwool plug were then inoculated at day 1 (sowing) and at day 11 (transplantation in aquaponic water) to ensure maximum colonization and contact with the seedlings' roots. For the strain T treatment, 10 ml of bacterial suspension were inoculated in each rockwool plug while for the consortium treatment, the three bacterial suspensions (A, H and T) were mixed in equal proportions. The resulting suspension was then used to inoculate 10 ml of bacterial mix per rockwool plug.

5.2.4.2 Phytotron trial outline

Trials took place in a controlled growth chamber (Fitotron® SGC 120 Plant Growth Chamber, Weiss Technik, Liedekerke, Belgium) containing two shelves, with a lighting system composed of two LED rails per shelf (1.9 × 1.1 × 119.75 cm, 27.4 W, Bi-phosphorous white 4000K 24V 2950lm/m CV, VEGELED, Colasse, Seraing, Belgium). The following growth conditions were set up (Resh, 2013):

- Photoperiod of 16 hours of day and 8 hours of night (for the whole experiment)
- A germination period of 11 days with 22°C during day and 18°C during night
- A growing period of 5 weeks with 26°C during day and 22°C during night
- Relative humidity of 65% for the whole experiment

Lettuces (*Lactuca sativa* var. Lucrecia, Rijk Zwann) were sown in sterile rockwool plugs (Grodan B.V., Roermond, Holland) (two seeds per plug) and the germination

step took place in the phytotron, in plant trays filled with tap water during 11 days. After 11 days, the seedlings were transplanted into plastic boxes (30 L Allibert Crownest boxes, Curver Benelux B.V., Rijen, Holland) of 36.3 × 42.5 × 26.3 cm (L ×W× H) each containing 20L of aquaponic water from the PAFF Box which was oxygenated every 6 hours for 15 min with disc diffusers (Hi Oxygen disc, Aquatic Science, Herstal, Belgium) connected to an air pump ((Hi-Blow 40, Aquatic Science, Herstal, Belgium). Three weeks after sowing, only one seedling per plug was kept. Lettuces were harvested five weeks after transplantation.

During the trials pH and EC were regularly monitored with a multimeter (model HQ40d, HACH, Loveland, CO, USA) to ensure that optimal growing conditions were maintained. It was never necessary to adjust pH or EC as they kept within lettuce tolerance boundaries.

5.2.4.3 Two sets of trials in the phytotron: optimal growth conditions and stressful growth conditions

Two types of trials were performed, one set in optimal growth conditions and the other set in slightly stressful light conditions (Hiroki et al., 2014). In the optimal growth conditions trial, the average light received by the lettuces was 163 µmol/m²s (+/- 19 (standard deviation)). In the stressful light conditions trial, the lettuces received in average 120 µmol/m²s (+/- 19 (standard deviation)). This lower light intensity was settled on to assess whether, in case of a failure of lighting equipment in a real life aquaponic system, the inoculation of bacteria could compensate for the ensuing lack of plant growth. The experimental design was slightly different between the two sets of trials as displayed on **Figure 5-1**.

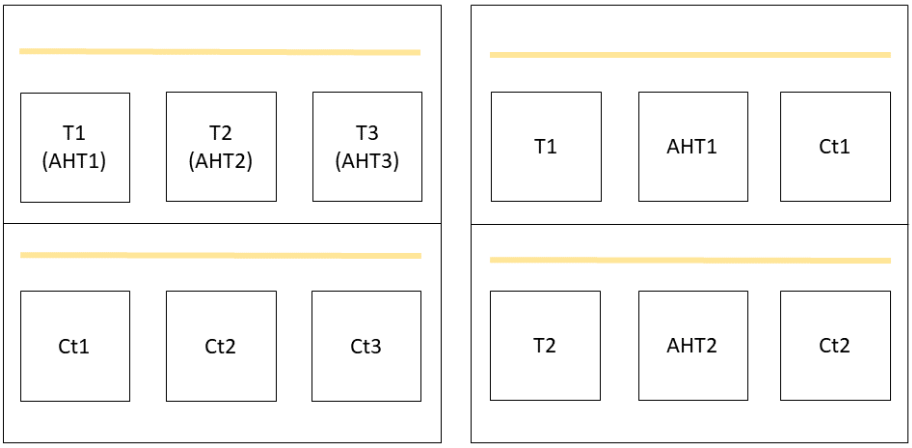


Figure 5-1 Experimental design in the set of trials in optimal growth conditions (left) and in the set of trials in light stress conditions (right). Each square represents a 20L box containing four lettuces. Each yellow lines represent the two LED bars per shelf installed in the phytotron. T: treatment with the T strain alone; AHT: treatment with the bacterial mix of strains A, H and T; Ct: control. In the optimal growth conditions trials, the two treatments were tested one after the other and each time compared with the control. In the light stress conditions both treatments were tested at the same moment and compared with the control.

In the optimal conditions growth set, the two treatments were tested one after the other. A first trial compared the mixed bacterial treatment to the control and afterwards a second trial compared the T treatment with the control. In this design, 12 lettuces per treatments were grown simultaneously (4 lettuces per box) (**Figure 5-1** - left). In the stressful conditions growth set, the two treatments were tested at the same time thus reducing the number of lettuces per treatment to 8 (4 lettuces per box) (**Figure 5-1**- right).

As the light intensity in the phytotron was heterogeneously distributed (border effect) a co-variable correction based on the light intensity received by each lettuce and measured with a parmeter (PAR, µmol/m²s) (HD 2102.2, series 15020628, Delta Ohm, Padova, Italy) was applied during statistical analysis.

5.2.4.4 Sampling in the phytotron trials

Sampling began on day 11 i.e. after germination and transplantation of the seedlings in aquaponic water and went on until lettuce harvest (day 47). Water samples were collected (50 ml per sample) on transplantation day (day 11), during the third week of growth (day 30) and on harvest day (day 47) in each box and were frozen at -30°C to

enable later measure of NPK and iron concentrations. On harvest day, lettuces were collected and the following additional measurements were taken: shoot and root fresh weights and dry weights after 72h of drying at 50°C, root/shoot ratios, NPK and iron contents in the dry plant. It was decided to focus on NPK and iron as first targets in this exploratory study as NPK are the major macronutrients in plant nutrition. Iron was also included as, as for NPK, the selected bacterial strains could have an impact on their cycles due to their identified functional traits. The NPK and iron measurements in the water and lettuce samples were performed by the Centre Provincial de l'Agriculture et de la ruralité. In the water samples total nitrogen (internal protocol) and phosphates (derived from NF EN ISO 6878) were measured via spectrocolorimetry UV-visible while potassium and iron were measured via spectrophotometry (method derived from NF EN ISO 1185). In the lettuce samples total nitrogen was measured following the NF EN ISO 16634-1 protocol. Phosphorus, potassium and iron were measured with a method derived from NF EN ISO 15510.

5.2.4.5 Trial on lettuce seedlings: response to nutrient stress

A final test was performed in greenhouse, on lettuce seedlings growing in various nutritive stress conditions. Indeed, as the selected bacterial strains had been chosen based on their abilities to participate in nutrient cycling (e.g. phosphorus and potassium solubilisation, siderophores production), the aim of this last trial was to assess whether the bacterial treatments would improve lettuce yields when nutrients were poorly available in the environment. The same bacterial treatments i.e. bacterial mix AHT and strain T alone were used in this experiment.

The nutrient stress factor was tested growing the seedlings in demineralized and tap water (nitrate concentration between 6.7 and 24.8 mg/L; potassium concentration 2.4 mg/L; phosphate concentration < 0.05 mg/L (SWDE, 2021)), following at the same time the growth of seedlings in aquaponics and hydroponics solutions (Mills, Nutrients Basis A (8-0-0) and B (0-10-8) set, 5 ml of each in 10 L of water). The demineralized and tap water solutions were selected as they created an extremely stressful environment for plant growth. The aim was thus to assess whether a general nutritive stress in the plant would trigger a better interaction with bacteria than in stress free conditions in which plants would not specifically require the help of the bacteria. The hydroponic and aquaponic solutions were selected as positive controls for plant growth. Samples of the aquaponic solution were collected but could not be analysed due to technical problems. Still, the aquaponic water was collected from the PAFF Box, therefore the order of magnitude of NPK concentrations should be similar to those measured for the phytotron trials.

Regarding the experimental design, the combination of 3 bacterial treatments (T, AHT, control) and 4 nutrient solutions resulted in 12 objects. For each object, 12 lettuces were studied, allocated in 4 plant trays (3 lettuces per plant tray) which were randomized each week for four weeks.

One lettuce seed was sown per rockwool plug before being inoculated with either 10 ml of T, 10 ml of AHT or 10 ml of isotonic water. A germination phase occurred for 11 days in demineralized water first. After 11 days, the rockwool plugs were inoculated again and the different solutions added to the lettuces in plant trays.

During the 4 weeks of the trial, 200 ml of the adapted solution was added to the plant trays every two days. The number of leaves and the length of the longest leaf were measured on each lettuce at the end of the 4 weeks of growth.

5.2.4.6 Data analysis

Data collected during the phytotron and greenhouse trials were analysed with the Rstudio statistic software (version 4.0.3).

Regarding the final yield variables, two ways ANCOVAs were performed on all variables for each trials, the two factors being “treatment” and “box” with a co-variable correction using the light intensity to dim out the border effects. The means were then corrected and structured with the emmeans function (emmeans package).

To analyse the final nutrient concentrations in the lettuce, one way ANCOVAs were performed as, as the four lettuces of each box had to be pooled, only two or three measures (depending on the trial set) were available per bacterial treatment. It is important to note that, based on this pooling and the availability of only two or three measures, the conclusions may not be robust enough.

Concerning the nutrient stress experiment, the lettuces were separated into four groups based on the nutritive solution. One way ANOVAs were performed on the final number of leaves and final leaf length variables to compare the effect of the bacterial treatment.

One way and two ways ANCOVAs and ANOVAs were performed on all variables for each trial using the aov function. Normality and equality of variances were tested beforehand using the Shapiro and Wilkes and Bartlett tests respectively.

5.3 Results

5.3.1 Bacterial isolation and plant growth promoting functions

In total, 31 bacterial strains were isolated from the sump of the PAFF Box. Twelve strains were isolated on LB media, 8 on PDA, 8 on TSA and 3 on NYDA. **Table 5-1** presents a summary of the responses of the 31 strains to the biochemical tests implemented to assess their potential plant growth promoting abilities. The details of the reaction of each strain to each of the five tests are available in Appendix C, **Table 5-S1**.

Table 5-1 Summary of the responses of the 31 bacterial strains to the five biochemical tests

Function	Number of positive strains	Positivity rate (%)
Phosphorus solubilisation	9	29
Potassium solubilisation	13	42
Ammonia production	19	61
Siderophores production	17	55
IAA production	8	26

Comparatively, Sanchez et al. (2019) isolated 61 strains from their aquaponic system out of which 38% were positive for phosphorus solubilisation, 20% were positive for ammonia production and 46% were positive for siderophores production. In soil studies, percentage of isolates positive for P solubilisation varied between 40% and 68% (Ahmad et al., 2008; Kumar et al., 2012). Ahmad et al. (2008), also obtained 12.5% of their isolates positive for siderophores production and 76% positive for IAA production.

Eight strains were highlighted as strains of interest as they proved positive for three or more tests out of five and were then identified via 16S rRNA sequencing. Strain H was positive to only two tests but showed particularly rapid and intense response for siderophores and IAA production and was thus selected as well. Six of these strains were assigned to the *Pseudomonas* genus but could not all be assigned a species as the 16S rRNA gene is not discriminative enough to distinguish between close *Pseudomonas* species (Mulet et al., 2010, 2009). A second Sanger sequencing was thus performed on their *rpoD* gene. Three strains could be assigned to the *P. aeruginosa* group while the three others were assigned to the *P. putida* group (Girard

et al., 2020). Taxonomic assignment and associated functions of each of the 8 selected strains are displayed in **Table 5-2**.

Table 5-2 Taxonomic assignment of the selected strains and associated functions. Positive response: +; negative response: -

Strain	Solubilisation P	Solubilisation K	Production NH_3	Production siderophores	Production IAA	Identification
A	+	+	+	+	-	*Pseudomonas aeruginosa*
E	+	+	+	+	-	*P. aeruginosa*
I	+	+	+	+	-	*P. aeruginosa*
D	+	+	-	+	-	*Pseudomonas putida group*
H	-	-	-	++	++	*Chryseobacterium. cucumeris*
M	+	-	+	+	-	*P. putida group*
Q	-	+	+	+	-	*P. putida group*
T	+	+	-	+	+++	*S. fonticola*

5.3.2 In vivo *yield tests*

After the identification of potentially plant beneficial functions in the bacterial community *in vitro*, it was decided to assess the ability of the selected strains to impact lettuce growth in miniature DWC systems containing aquaponic water. To this end, three of the eight strains of interest were selected namely strain A, assigned to *Pseudomonas aeruginosa* and strain T assigned to *Serratia fonticola* as they proved positive for 4 tests out of 5 and were complementary i.e. T proved highly positive for the IAA test while strain A was negative. Strain H (assigned to *Chryseobacterium cucumeris*) was added to the two previous strains as it belonged to a different genus, also known to harbour PGPR species (Jeong et al., 2017) and proved highly positive for siderophores and IAA production, two key functions to enhance plant growth (Berg, 2009; Mohite, 2013).

Three types of *in vivo* trials were then conducted: i) in optimal growth conditions for lettuce; ii) in stressful growth conditions due to insufficient light intensity, iii) in stressful growth conditions due to insufficient nutrient input.

5.3.2.1 Trials conducted in optimal growth conditions

Two tests were conducted in optimal growth conditions, a first test comparing lettuce inoculated with the AHT bacterial mix treatment and a control and a second test comparing lettuce inoculated with the T treatment and a control. Data were analysed with an ANCOVA taking into account the photosynthetic active radiation (PAR) variability in the phytotron and thus correcting the final yield data accordingly.

- **Comparison between AHT bacterial mix and control**

No significant difference could be observed between the inoculated lettuces and the control in terms of final yield measurements (shoot and root fresh and dry weights, fresh and dry root/shoot ratios, number of leaves and length of the longest leaf on harvest day). The final values of the indicators are displayed in **Table 5-3**.

Table 5-3 Final values for yield indicators measured on harvest day (day 47). Each displayed value is the mean value of the 12 lettuces per treatment ± standard deviation. ShootF (g): shoot fresh weight; RootF (g): root fresh weight; ShootD (g): shoot dry weight; RootD (g): root dry weight; Rf/Sf: fresh root/shoot ratio; Rd/Sd: dry root/shoot ratio; nb leaves: number of leaves; leaf length (cm): length of the longest leaf

	AHT		Ct	
ShootF	73.66	± 7.51	73.16	±7.29
RootF	4.09	± 0.93	3.86	± 0.71
ShootD	2.55	± 0.25	2.46	± 0.29
RootD	0.19	± 0.05	0.22	± 0.06
Rf/Sf	0.06	± 0.01	0.05	± 0.01
Rd/Sd	0.08	± 0.02	0.09	± 0.02
Nb leaves	38.58	±1.78	37.92	± 2.68
Leaf length	18.42	± 1.56	18.42	± 1.38

The final NPK concentrations in the lettuce were also measured with the four lettuces from a same box being pooled to provide enough material for the dosages. It appeared that the treated lettuces proved slightly more concentrated in potassium (p-value = 0.0447) with a concentration of 84.9 g/kg of dry matter versus 80.3 g/kg of dry matter for the control. Still, it has to be kept in mind that due to the pooling of lettuce, only three replicates per treatment were available. The iron concentration both the in the lettuce and in the aquaponic water fell below the analytical range of the performed measures

- **Comparison between T alone and control**

In this trial, slight differences were noted between the inoculated lettuces and the control in terms of root growth. Indeed, the control lettuces showed higher root dry weight (p-value aov = 0.0152; p-value emmeans = 0.0113) and higher dry (p-value aov = 0.0031 p-value emmeans = 0.0041) and fresh (p-value aov = 0.00064, p-value emmeans = 0.0015) root/shoot ratio. The inoculation of strain T also influenced leaf

length (albeit not in box 3) (p-value box 1 = 0.0228 and p-value box 2 = 0.0261) with slightly longer leaves in inoculated lettuces. The final values of the indicators are displayed in **Table 5-4.**

Table 5-4 Final values for yield indicators measured on harvest day (day 47). Each displayed value is the mean value of the 12 lettuces per treatment ± standard deviation. ShootF (g): shoot fresh weight; RootF (g): root fresh weight; ShootD (g): shoot dry weight; RootD (g): root dry weight; Rf/Sf: fresh root/shoot ratio; Rd/Sd: dry root/shoot ratio; nb leaves: number of leaves; leaf length (cm): length of the longest leaf. *: significant difference; **: very significant difference.

	T		Ct	
ShootF	63.51	± 8.47	58.67	± 10.03
RootF	3.04	± 0.69	3.33	± 0.61
ShootD	2.39	± 0.33	2.34	± 0.38
RootD	0.19*	± 0.05	0.23*	± 0.06
Rf/Sf	0.05**	± 0.01	0.06**	± 0.01
Rd/Sd	0.08**	± 0.01	0.10**	± 0.02
Nb leaves	37.50	± 3.21	36.50	± 3.26
Leaf length	18.88	± 1.25	17.54	± 1.44

No difference could be observed concerning the nutrient concentration in the lettuce on harvest day.

For both trials, it has to be noted that after 47 days of growth, the obtained lettuces were still small (Tables 5-3 and 5-4) compared to hydroponic or soil born lettuces (ca. 450 g (unpublished data)). This may be due to the smallness of the phytotron as otherwise the lettuces did not look diseased.

- **NPK concentrations in the aquaponic water during the trials**

During both trials, the evolution of NPK contents in the aquaponic water of the compared treatments were analysed via the dosage of NPK on transplantation day (day 11), day 30 and final day (day 47). Each measure was performed only once, on only one sample chosen randomly between the 3 boxes (replicates).

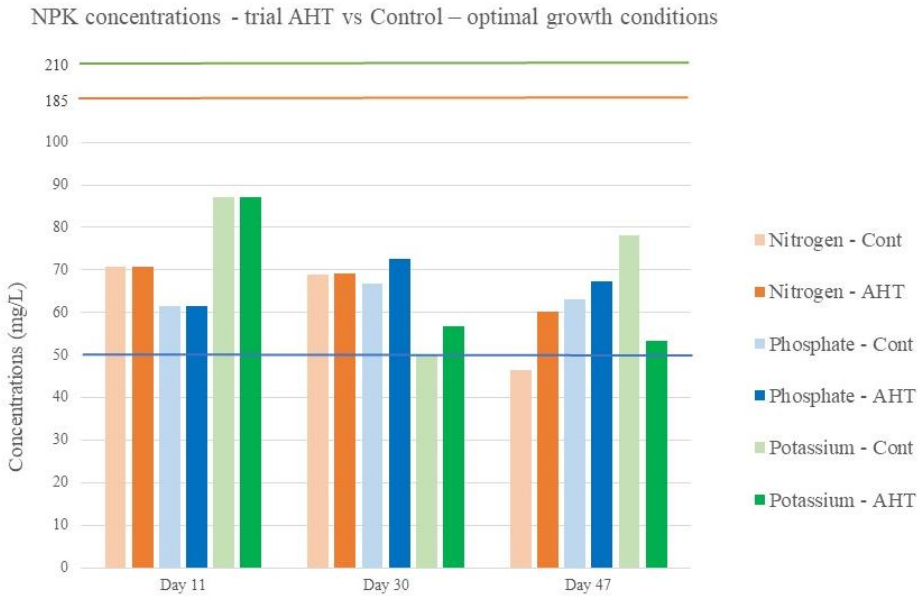


Figure 5-2 NPK concentration in the aquaponic water during the AHT vs Control trial in optimal growth conditions. Samples were collected on day 11 (transplantation of the lettuce seedlings into aquaponic water freshly collected from the PAFF Box), day 30 (middle of the trial) and day 47 (harvest day). Each measure was performed only once and on only one sample chosen randomly between the 3 boxes (treatment replicates). The coloured lines on the graph represent Resh's (2013) minimum concentrations recommendations for lettuce growth in hydroponics. Blue: phosphate, orange: nitrogen, green: potassium. The recommended concentrations for nitrogen and potassium being much higher than the measured values, the vertical scale has been truncated to facilitate reading and visualisation.

Concentrations on day 11 are the same in both treatments as the water sample was collected directly from the PAFF Box, before repartition in the 6 boxes of the phytotron. According to Resh (2013), the recommended NPK concentrations for lettuce are N: 185-195 ppm / P: 50 ppm / K: 210 ppm. On figure 5-2, it can be noted that throughout the whole experiment, the N and K concentrations were far lower while the P concentration kept above the correct range. Still, the phosphate concentrations decreased in the day 47 samples compared to day 30. The trends of potassium concentrations are also curious with the highest K concentration on day 47 being observed in the control (i.e. without the addition of K solubilising bacteria). Regarding the N movements, a more fine-tuned analysis of the concentrations of ammonia, nitrite and nitrates may shed more light on the impact of bacterial treatments

on the nitrogen cycle (Cerozi and Fitzsimmons, 2016b). Eventually, the recommended nutrient ratio of 1/0.2/1 (Resh, 2013) was also not respected with, on day 11 a ratio of ca. 0.8/0.6/1, on day 30 ca. 1/1/0.8 and on day 47 in the control ca. 0.6/0.9/1 and in the AHT treatment ca. 0.9/1/0.8. However, lettuce are tolerant plants which do not require very precise ratios of NPK for specific physiological stages such as flowering and fruiting as they are usually harvested before.

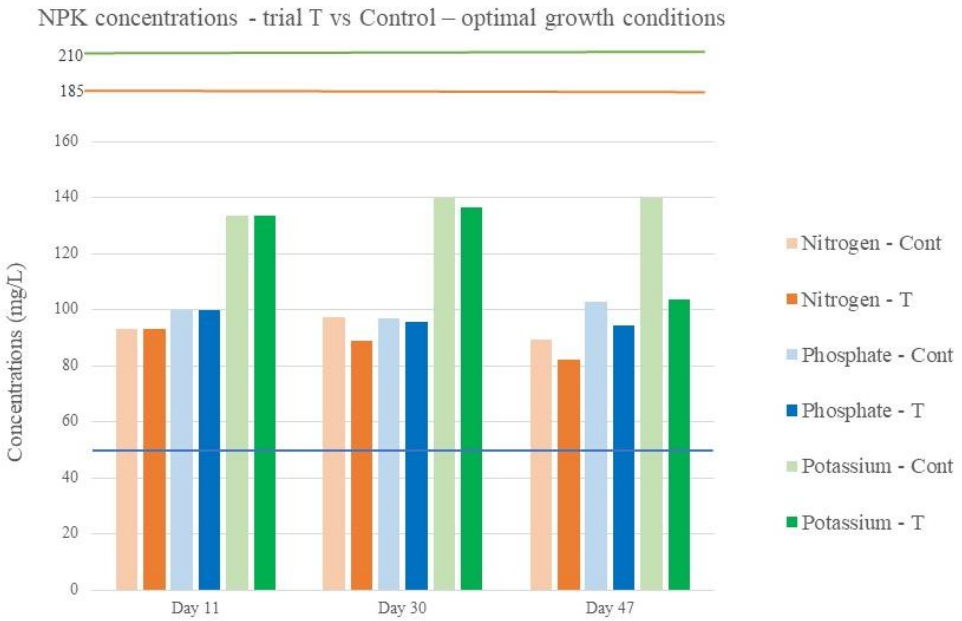


Figure 5-3 NPK concentration in the aquaponic water during the T vs Control trial in optimal growth conditions. Samples were collected on day 11 (*Cont.*) (transplantation of the lettuce seedlings into aquaponic water freshly collected from the PAFF Box), day 30 (middle of the trial) and day 47 (harvest day). Each measure was performed only once and on only one sample chosen randomly between the 3 boxes (treatment replicates). The coloured lines on the graph represent Resh's (2013) minimum concentrations recommendations for lettuce growth in hydroponics. Blue: phosphate, orange: nitrogen, green: potassium. The recommended concentrations for nitrogen and potassium being much higher than the measured values, the vertical scale has been truncated to facilitate reading and visualisation.

NPK concentrations on transplantation day were higher in this trial than in the previous one (**Figure 5-2** and **Figure 5-3**). However, N and K concentrations were still too low compared to Resh's recommendations (Resh, 2013) while P concentration doubled the required amount for optimal lettuce growth. P

concentrations stayed high during the whole experiment with a marked difference between control and T treatment only appearing on day 47. As in the previous trial, on day 47 potassium concentration was higher in the control aquaponic water than in T treatment but this time it is also the case for N and P. The recommended ratios were here again not respected with N and P concentrations being relatively similar during the experiment and a higher K concentration.

More in depth analyses of nitrogen, potassium and phosphorus cycles in the trials should be undertaken to closely monitor the exchanges of nutrients between aquaponic water and plants.

5.3.2.2 Trials conducted in stressful growth conditions due to lower light intensity

For this set of trials, two repetitions of the same experimental design were carried out one after the other (time repetitions) thus using aquaponic water collected from the same aquaponic system at different timings and lettuce seedlings from the same seed lot. Yet, as aquaponic water composition is highly variable, the composition in terms of nutrient concentrations varied between the two trials. Moreover, despite originating from the same seed lot, a difference was noted in the germination power of the seeds between trial 1 and 2 (76% for trial 1 and 51% for trial 2). This difference may be due to the fact that trial 2 took place two months after trial 1 and that the seeds were therefore too old. Indeed, the average shelf life of lettuce seeds is 6 months and the two extra months of trial 2 were too close to the limit date. To smooth out those variabilities, a first analysis was performed merging the data obtained in the two trials. More fined tuned analyses were then conducted on each trial separately. The aquaponic water nutrient contents were further analysed independently for each trial.

- **NPK concentrations in the aquaponic water during the trials: light stress conditions**

As for the optimal conditions trials, the evolution of NPK contents in the compared treatments were analysed via the dosage of NPK on transplantation day (day 11), day 30 and final day (day 47) for both light stress trials. Furthermore, concentrations on day 11 were similar in all three treatments as only one sample was taken directly from the water collected from the aquaponic system for both trials.

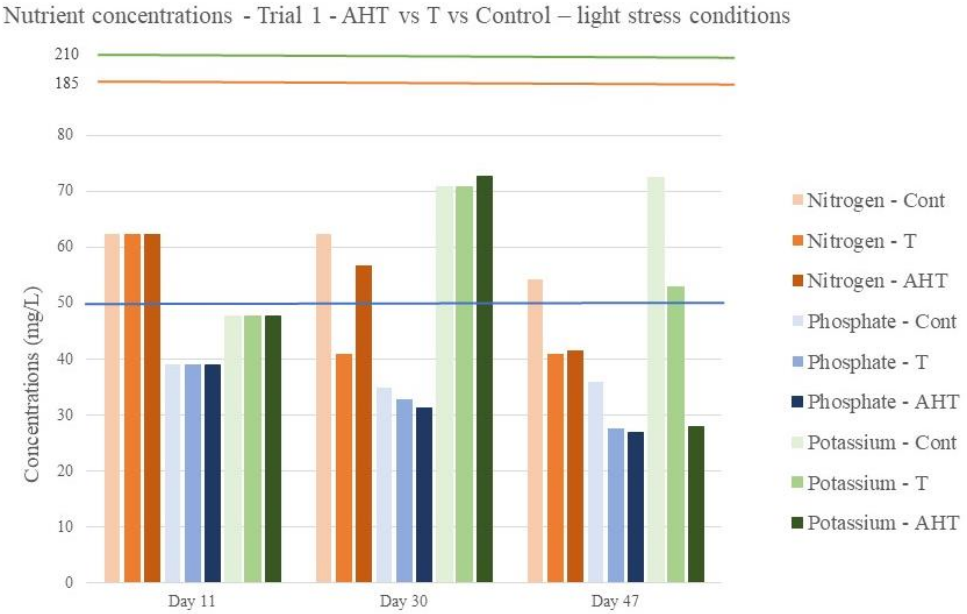


Figure 5-4 NPK concentrations in the aquaponic water during the first repetition of the light stress conditions trial. Samples were collected on day 11 (transplantation of the lettuce seedlings into aquaponic water freshly collected from the PAFF Box), day 30 (middle of the trial) and day 47 (harvest day). Each measure was performed only once and on only one sample chosen randomly between the 3 boxes (treatment replicates). The coloured lines on the graph represent Resh's (2013) minimum concentrations recommendations for lettuce growth in hydroponics. Blue: phosphate, orange: nitrogen, green: potassium. The recommended concentrations for nitrogen and potassium being much higher than the measured values, the vertical scale has been truncated to facilitate reading and visualisation.

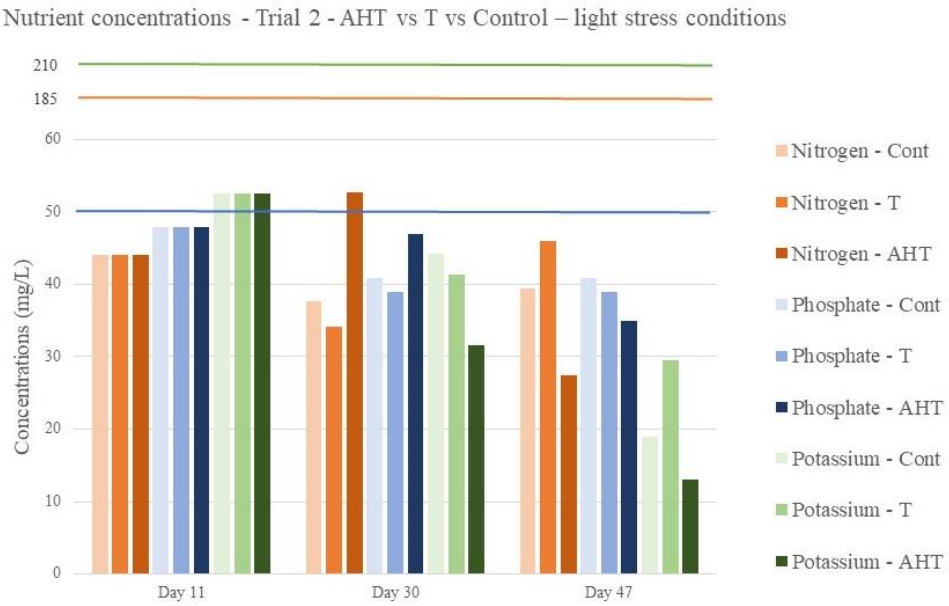


Figure 5-5 NPK concentrations in the aquaponic water during the second repetition of the light stress conditions trial. Samples were collected on day 11 (transplantation of the lettuce seedlings into aquaponic water freshly collected from the PAFF Box), day 30 (middle of the trial) and day 47 (harvest day). Each measure was performed only once and on only one sample chosen randomly between the 3 boxes (treatment replicates). The coloured lines on the graph represent Resh's (2013) minimum concentrations recommendations for lettuce growth in hydroponics. Blue: phosphate, orange: nitrogen, green: potassium. The recommended concentrations for nitrogen and potassium being much higher than the measured values, the vertical scale has been truncated to facilitate reading and visualisation.

NPK concentrations in both trials were particularly low (**Figure 5-4** and **Figure 5-5**) with N and K concentrations being at some point 7 times lower than Resh's (2013) recommendations. N concentrations were also globally lower in the second trial than in the first which might contribute to the smaller yields obtained at the end of the experiment (**Tables 5-3 to 5-6**). Even the phosphate concentrations which were correct in the optimal conditions trials were lower than the required amount in both light stress trials. The obtained shoot fresh weights were consequently smaller in both light stress trials than in the optimal growth conditions trials (**Tables 5-3 to 5-6**). Nevertheless, no deficiency symptoms such as yellowing or necrosis could be observed. This may lead to think once more that the aquaponic water might allow for correct plant growth with lower nutrient concentrations (Delaide et al., 2016).

In the first trial, nitrogen contents decreased throughout the experiment in all treatments. The decrease was the rapidest in treatment T with a drop at day 30 while followed by a stabilisation while in the AHT treatment the drop appeared on day 47. Phosphate concentrations also decreased in all treatments with a more marked drop in AHT. Here again potassium was the nutrient with the most intriguing movements as it started by increasing in all treatments and then stayed stable in the control while it plummeted in T and even more in AHT. However, no significant difference could be noted between the final nutrient contents in the lettuces. In the second trial, all nutrient concentrations decreased on day 30 and day 47 except for N concentration in the AHT treatment which increased in day 30 to then reach its lowest level on day 47. Potassium levels were extremely low on day 47 reaching down to 13 mg/L in the AHT treatment.

- **Analysis of both trials together**

When both trials were studied together results showed that despite the two important sources of biological variability (i.e. seeds and aquaponic water) the final shoot fresh weight was still influenced by bacterial treatment (p-value aov = 0.0022). The highest shoot fresh weight was obtained with the AHT bacterial mix inoculation that was significantly higher than the control (p-value emmeans = 0.0194). The shoot fresh weight was the only yield indicator significantly different when both trials were studied together.

Regarding the final nutrient concentrations in the lettuce, potassium is the only nutrient to stand out as observed in the AHT vs control trial in optimal conditions. Potassium concentration was indeed significantly different between the three treatments with a higher value observed in T treated lettuce, then AHT treated lettuce, then control. However, the emmeans structuration does not return a significant answer.

- **First trial**

Data from the first trial, when analysed independently, showed interesting differences in terms of final yields. Indeed, the shoot and root fresh weights of the lettuces treated with AHT and T treatments were higher than the control (p-values emmeans of 0.0013 and 0.0403 respectively for the shoot fresh weight and < 0.001 and 0.0308 for root fresh weight). Regarding the dry shoot and root final weights, only the lettuces treated with AHT proved significantly heavier than the control. In terms of root/shoot ratios, the same reasoning could be observed with the fresh ratio being higher than control for both treatments while the dry ratio was only influenced by the AHT treatment. The final number of leaves was also impacted by treatment T that

yielded significantly more leaves than the control. This was not the case for the AHT treatment. Conversely to the trial conducted in optimal conditions, the length of leaves was not impacted by the treatments.

Table 5-5 Final values for yield indicators measured on harvest day (day 47) for the first trial in light stress conditions. Each displayed value is the mean value of the 12 lettuces per treatment ± standard deviation. ShootF (g): shoot fresh weight; RootF (g): root fresh weight; ShootD (g): shoot dry weight; RootD (g): root dry weight; Rf/Sf: fresh root/shoot ratio; Rd/Sd: dry root/shoot ratio; nb leaves: number of leaves; leaf length (cm): length of the longest leaf. Letters following the mean indicate significant difference according to emmeans structuration

	AHT		T		Ct	
ShootF	51.70 ^{a}	± 10.98	41.90 ^{a}	± 6.95	30.90 ^{b}	± 5.46
RootF	1.80 ^{a}	± 0.25	1.21 ^{b}	± 0.34	0.830 ^{c}	± 0.19
ShootD	1.82 ^{a}	± 0.37	1.52 ^{ab}	± 0.24	1.16 ^{b}	± 0.21
RootD	0.0696 ^{a}	± 0.02	0.0433 ^{ab}	± 0.02	0.0234 ^{b}	± 0.01
Rf/Sf	0.0356 ^{a}	± 0	0.0267 ^{bc}	± 0	0.0284 ^{c}	± 0
Rd/Sd	0.0371 ^{a}	± 0.01	0.0272 ^{ab}	± 0.01	0.186 ^{b}	± 0.01
Nb leaves	31.10 ^{ab}	± 2.67	31.9 ^{a}	± 2.30	28.70 ^{b}	± 1.98
Leaf length	18.25	± 1.16	18.00	± 0.76	17.50	± 0.93

Regarding the nutrient concentrations in the lettuce, no difference could be noted between the treatments.

- **Second trial**

The data obtained in the second trial showed no differences in terms of final yields, final number of leaves or length of leaves. This may seem odd when compared with the first trial as the same experimental design was used for both tests. The difference with the first trial might be due to a germination problem (only 51% of germination) as after 11 days the lettuce seedlings were much smaller than usual and lacked in vigour. Moreover, the aquaponic water collected for the second trial contained lower NPK concentrations than for the first trial (**Figure 5-4** and **Figure 5-5**).

Table 5-6 Final values for yield indicators measured on harvest day (day 47) for the second trial in light stress conditions. Each displayed value is the mean value of the 12 lettuces per treatment ± standard deviation. ShootF (g): shoot fresh weight; RootF (g): root fresh weight; ShootD (g): shoot dry weight; RootD (g): root dry weight; Rf/Sf: fresh root/shoot ratio; Rd/Sd: dry root/shoot ratio; nb leaves: number of leaves; leaf length (cm): length of the longest leaf.

	AHT		T		Ct	
ShootF	40.93	± 17.01	22.83	± 11.28	31.35	± 10.06
RootF	2.64	± 0.50	1.79	± 0.63	2.25	± 0.91
ShootD	1.66	± 0.57	1.00	± 0.48	1.38	± 0.40
RootD	0.14	± 0.03	0.09	± 0.04	0.12	± 0.05
Rf/Sf	0.07	± 0.02	0.09	± 0.03	0.07	± 0.02
Rd/Sd	0.09	± 0.02	0.09	± 0.02	0.08	± 0.02
Nb leaves	27.75	± 5.01	22.63	± 4.00	25.50	± 4.24
Leaf length	15.75	± 2.49	14.50	± 2.39	15.63	± 1.60

Regarding the final nutrient concentrations in the lettuces, no significant difference could be noted between the treatments. Yet, when looking at the potassium concentration here again, the T treatment lettuces held a higher concentration than the AHT and control lettuces albeit not significantly.

5.3.2.3 Trials conducted in stressful growth conditions due to low nutrients levels

To test the ability of our selected bacterial strains to support plant growth in nutrient deficiencies conditions, four nutrients solutions were used i.e. demineralized water, tap water, aquaponic water and hydroponic solution thus proposing a gradient of nutrients availability. For each solution the three bacterial treatments were tested and the number of leaves and length of the longest leaf were measured after four weeks of growth in the greenhouse. The data collected for each nutrient solution have been studied separately.

- **Demineralized water**

Lettuce growing in demineralized water were the most stressed as their solution did not contain any nutrient. Two one-way ANOVAs were performed on the data i.e. on the number of leaves and on the length of the longest leaf. For both variables, significant differences were noted between the treatments (p-values of 0.0015 and $1.75 * 10^{-9}$ respectively). HSD and LSD multiple comparisons tests were then performed. For the number of leaves, lettuce inoculated with the AHT mix obtained a

higher mean than T and control for both tests. For the length of the longest leaf, the HSD test proposed two groups, AHT and T being higher than control while LSD distinguished the three treatments with AHT being higher than T which was higher than control. The mean values for both variables are displayed in **Table 5-7**.

Table 5-7 Final values for the number of leaves and length of the longest leaf (cm) measured after 4 weeks of growth. Each displayed value is the mean value ± standard deviation of the 1ettuces studied for each combination of solution and bacterial treatment. Letters following the mean indicate significant difference according to LSD test.

	AHT		T		Ct	
Number of leaves	4.20 ^{a}	± 0.79	3.20 ^{b}	± 0.92	2.83 ^{c}	± 0.72
Lenght of the longest leaf	3.01 ^{a}	± 0.67	2.40 ^{b}	± 0.84	0.53 ^{c}	± 0.41

- **Tap water**

The same analysis was performed on the tap water lettuce group. For both variables, significant differences were noted (p-values of 0.0005 and $6.42*10^{-6}$ respectively). HSD and LSD structuration tests were performed with again two groups for the number of leaves (AHT being higher than T and control) and three groups for length of leaf (AHT higher than T higher than control). The mean values for both variables are displayed in **Table 5-8**.

Table 5-8 Final values for the number of leaves and length of the longest leaf (cm) measured after 4 weeks of growth. Each displayed value is the mean value ± standard deviation of the 1ettuces studied for each combination of solution and bacterial treatment. Letters following the mean indicate significant difference according to LSD test.

	AHT		T		Ct	
Number of leaves	4.58 ^{a}	± 0.67	3.75 ^{b}	± 0.45	3.73 ^{b}	± 0.47
Lenght of the longest leaf	4.01 ^{a}	± 0	3.25 ^{b}	± 0.45	2.45 ^{c}	± 0.52

Based on these two groups of lettuce and the results obtained after four weeks of growth we can state that the inoculation of the AHT mix into the rockwool plugs enabled the lettuces to grow more leaves and longest leaves in both solutions. The inoculation of strain T did not seem to influence the number of leaves but had an impact on the leaves length which is coherent with the trials performed in the phytotron in optimal conditions but which was not observed in the light stress conditions trials.

- **Aquaponic solution**

Aquaponic solution was regularly collected from our closed-loop aquaponic system. No significant differences could be noted with one-way ANOVAs either on the number of leaves or on the length of leaves between the three bacterial treatments. The mean values for both variables are displayed in **Table 5-9**.

Table 5-9 Final values for the number of leaves and length of the longest leaf (cm) measured after 4 weeks of growth. Each displayed value is the mean value ± standard deviation of the 1ettuces studied for each combination of solution and bacterial treatment.

	AHT		T		Ct	
Number of leaves	13.55	± 2.11	13.73	± 0.79	12.73	± 1.35
Lenght of the longest leaf	15.45	± 0.82	14.55	± 1.13	14.45	± 1.29

- **Hydroponic solution**

Hydroponic solution was prepared with commercial solutions. In hydroponics, the bacterial treatments did not influence the final number of leaves but a significant difference could still be noted concerning the length (p-value = 0.0286) with the LSD test showing that lettuce treated with T strain alone presented slightly longer leaves than control and AHT treated lettuces. The mean values for both variables are displayed in **Table 5-9**.

Table 5-10 Final values for the number of leaves and length of the longest leaf (cm) measured after 4 weeks of growth. Each displayed value is the mean value ± standard deviation of the 1ettuces studied for each combination of solution and bacterial treatment. Letters following the mean indicate significant difference according to LSD test.

	AHT		T		Ct	
Number of leaves	13.36	± 1.21	13.4	± 1.26	13.64	± 1.12
Lenght of the longest leaf	14.18 [b]	± 0.98	15.4 [a]	± 0.7	14.27 [b]	± 1.42

If the data from the aquaponics and hydroponics groups are analysed together, no difference can be noted either in terms of number of leaves or leaf length which is coherent with Delaide et al. (2016). Still, it could be argued that the absence of difference between the aquaponics and hydroponics groups may be linked to the early growth stage of the lettuces during which the lower nutrient concentrations of the aquaponic solution may not have impacted the growth. This equivalent growth and linked lack of nutritive stress may then explain the absence of difference of yields between the bacterial treatments.

5.4 Discussion and perspectives

5.4.1 In vitro *tests and selection of superstrains*

This study aimed at discovering potentially plant beneficial functions harboured by the aquaponic microbiota and to assess the effect of these potential PGPM on lettuce growth in aquaponic water. To this end, five microbial functional traits relevant for aquaponics crop production were targeted and aquaponics bacteria were then selected based on their abilities as several beneficial traits can often be found in the same microorganism (du Jardin, 2015). Eventually, the impact of the chosen bacterial strains on lettuce yields were assessed *in vivo*.

5.4.1.1 Choice of biochemical tests, plant growth and nutrient cycling

To explore the potential of aquaponics bacteria to improve plant growth in aquaponic systems, bacteria were collected and isolated from the sump of the PAFF Box. Five tests were then selected to assess the abilities of our bacterial strains in terms of i) inorganic phosphorus solubilisation, ii) potassium solubilisation, iii) ammonia production, iv) siderophores production and v) IAA production.

It was decided to focus on phosphorus solubilisation as it is a major macronutrient required for plant growth (Resh, 2013) and is prone to precipitation at a pH between 7 and 8 (Cerozi and Fitzsimmons, 2016a), maintained in aquaponics. Indeed, in aquaponic systems phosphorus can easily react with magnesium or calcium to form inorganic solids such as struvite or hydroxyapatite (Daneshgar et al., 2018) respectively. These solids are then removed from the systems with sludge evacuation and are thus lost to plants. Microbial re-solubilisation or remineralisation (Cerozi and Fitzsimmons, 2016b; Goddek et al., 2016) could avoid this waste and diminish the reliance of aquaponic systems on external phosphorus inputs (Graber and Junge, 2009; Seawright et al., 1998). The inoculation of phosphorus solubilising bacteria in aquaponics system has already been studied by Cerozi and Fitzsimmons (2016b) who inoculated a commercial suspension of *Bacillus* into their system which impacted the phosphorus cycle. In our study, we chose to work directly with bacteria originating from the aquaponic system and therefore selected strains using Pikovskaya plates (Nautiyal, 1999) to detect the ability to solubilise inorganic phosphorus i.e. typically the precipitates of struvite or hydroxyapatite.

Potassium cycling is much less known in aquaponics and little information is available on the subject. Still, it is known that the major source of potassium in

aquaponics is the fish feed (Delaide et al., 2017) but that, as fish feed contains very low concentration of K, it is often lacking in aquaponics despite being crucial for plant growth (Eck et al., 2019a). Still, potassium can be added into the system via the use of KOH buffer to counterbalance acidification linked to nitrification. As part of the fish feed is left to decay into the system, uneaten by fish (Eck et al., 2019a), and is then evacuated in sludge, the sludge remineralisation abilities (Goddek et al., 2018) of bacteria could help render potassium available for plants again.

Iron is acknowledged to be one of the most limiting elements in aquaponics as it is mainly brought in by fish feed which contains very low quantities (Kasozi et al., 2019). Iron supplementation is therefore required most of the time to avoid deprivations in plants and demands careful management (Kasozi et al., 2019). In aquaponic systems, iron can be found under the form of soluble ferrous iron (Fe^{2+}) or insoluble ferric iron (Fe^{3+}) the oxidised form (Andrews et al., 2003; Bartelme et al., 2018). In the aquaponic environment i.e. around a neutral pH and in presence of oxygen, the ferric form prevails and reacts with other elements to "form insoluble oxides or hydroxides" (Kasozi et al., 2019; Radzki et al., 2013). Synthetic chelators can be added into aquaponic systems but these compounds can be easily degraded (Kasozi et al., 2019). The production of siderophores molecules by bacteria present in the system would thus help chelation of the iron ions present in the system and lead to a better uptake by plants.

Indole acetic acid is one of the major plant growth hormone involved in root growth (Mohite, 2013). Numerous bacterial strains are known for their ability to produce IAA within species such as *Azospirillum brasilense*, the *Bacillus* and *Pseudomonas* genera (Mohite, 2013). Bacteria producing IAA were therefore targeted in our experiment as it is a widely known process and could easily impact plant yields by enhancing root growth and thus enabling better nutrient uptake (Mohite, 2013). The combination of wider root systems with more soluble nutrients such as phosphorus, potassium and iron could foster plant yields in aquaponics.

Eventually, ammonia producing bacteria were targeted as they could potentially compensate for a lack of ammonia production by fish in case of behavioural disorder. Ammonia can be produced by bacteria directly via the degradation of high-protein resources such as manure (Weise et al., 2013). Bacterial ammonia production can also be linked to an alkalization of the environment (Weise et al., 2013). This phenomenon is however poorly understood and has never, to our knowledge, been studied in aquaponics. One of the major known mechanisms for the bacterial production of ammonia is the use of ureases to produce ammonia from urea (Romero-Gómez et al.,

2009; Vince et al., 1973). Other processes are the deamination of nitrogen-compounds such as peptone (Vince et al., 1973) "nitrite ammonification, degradation of amino acids, decarboxylation of amino acids, deamination, urease-mediated hydrolytic degradation of urea" (Weise et al., 2013). Bacterial ammonia production can be positively correlated to pH (Vince et al., 1973; Weise et al., 2013). A few genera have been studied so far for their ability to produce ammonia i.e. *Bacteroides, Bifidobacteria, Clostridia, Proteus spp., Klebsiella spp., Pseudomonas spp., Clostridium, Shigella, Enterococcus, Blautia, Serratia, Bacillus* (Vince et al., 1973; Weise et al., 2013).

5.4.1.2 Selection of the strains of interest

- **Only bacteria were retrieved from the sump**

Thirty-one bacterial strains were collected from the sump of the PAFF Box aquaponic system while no fungal strains were retrieved via the same collection method. This could be linked to several factors. Indeed, the microbial samples were collected from flowing water. This mobile environment might be less suited to the development of fungi while fungal strains could have been more easily collected from the periphyton on the tanks walls or from the plants roots as their presence in aquaponic system has already been acknowledged (Stouvenakers et al., 2020). Secondly, the sampled water was plated onto general growing media allowing for both the growth of bacteria and fungi and all grown microorganisms were retrieved from the plates on the same day. As fungi are often slower growing, they might not have been allowed enough time to be detected. Specific plates should have been dedicated to fungi recovery and left to incubate for longer in the growth chamber.

Furthermore, a reverse strategy could have been adopted for the cultivation and isolation of microbial strains. Indeed, instead of using general growth media providing ideal growth conditions, a medium more similar to the complex aquaponic environment could have been designed. In this case, only the most competitive or most adapted strains could have been isolated before checking for their plant beneficial functions. Similarly, a growth medium mimicking rhizoplane conditions and containing root exudates could have been conceived to select the microorganisms most prone to colonise lettuce roots.

- **Selected strains**

Eight strains were selected and were then identified via Sanger sequencing of their whole 16S gene. Out of these eight strains, 3 were considered for the *in vivo* tests as

they belonged to 3 different genera and offered a wide panel of potentially beneficial functions.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is mostly known as a human (Bonneau et al., 2020) and fish (Thomas et al., 2014) pathogen but has also been extensively studied for its plant growth promoting abilities (Adesemoye and Ugoji, 2009; Anjaiah et al., 2007; Audenaert et al., 2002) and antibiotics production capacities (Adesemoye and Ugoji, 2009). While De Meyer et al. (1999) and Tambong and Höfte (2001) focused on several strains of *P. aeruginosa* to assess their abilities as biocontrol agents, Adesemoye and Ugoji (2009) tested the effect of *P. aeruginosa* inoculation on seeds of okra, tomato and amaranth. The *P. aeruginosa* strain that was tested had been previously isolated from a soil similar to the one use in the experimental design, similarly to our extraction of bacteria from our aquaponic system. They obtained better yields for inoculated plants than controls. The yields obtained for the inoculated plants were similar to those of plants which had received NPK fertilizers.

Chryseobacterium cucumeris

The *Chryseobacterium* genus is part of the *Flavobacteriaceae* family and used to be mixed with the *Flavobacterium* genus (Jeong et al., 2017) which has already been found in previous studies of the PAFF Box's bacterial communities (Eck et al., 2021, 2019b). Both genera were later separated due to genotypic, biochemical and phenotypic differences and the *Chryseobacterium* genus now holds 95 species which can be found in various environments such as "soil, water, plants, rhizospheres and fish" (Jeong et al., 2017). Some of the *Chryseobacterium* species present plant growth promoting abilities (Jeong et al., 2017; Kasozi et al., 2019). Indeed, *Chryseobacterium spp.* inoculation has already been studied in hydroponics for its siderophores production abilities (Radzki et al., 2013) and was noted to help iron-deprived tomato plants to grow. *Chryseobacterium cucumeris* has been described by Jeong et al. (2017) whom isolated this endophyte from cucumber roots in Korea and detected genes related to biocontrol, plant colonization and antimicrobial activities such as urease and indole production. In our study, the *Chryseobacterium cucumeris* strain proved indeed positive for IAA and siderophores production but not for ammonia production.

Serratia fonticola

Serratia fonticola can be a human pathogen but has also been studied as a PGPR (Devi et al., 2013) able to solubilise phosphate, produce IAA and act as biocontrol agent (Devi et al., 2013; Jung et al., 2020). Indeed, García et al. (2004) have conducted *in vivo* plants inoculations of a bacterial mix containing a strain of *S. fonticola* able to produce auxin and to solubilise phosphate (which is coherent with our strain) that enhanced plant growth. In 2013, a draft of *S. fonticola*'s genome was proposed by Devi et al. (2013). The studied strain proved positive for phosphate solubilisation, ammonia production, HCN production and siderophores production and presented an antifungal activity against *Fusarium*.

5.4.1.3 Better knowledge of the selected strains to improve the understanding of the *in vivo* trials

To improve the efficiency of the strains as PGPB in aquaponics, a more in depths understanding of their genomes and abilities would be required. In this view, their whole genomes could be sequenced to allow for a broader view of their functions and abilities. Moreover, the experiment could benefit from a thorough characterisation and phenotyping of the selected strains, their relationships within the bacterial mix (synergies or antagonism), with the endemic communities from the aquaponic water and the lettuce roots (Sare et al., 2021). To this end, root samples could be collected at the end of the experiment to assess whether the inoculated bacteria colonised the rhizoplane and whether one of the three strains dominated over the others. Furthermore, complementary analyses could be focused on previously selected pathways such as involved in potassium cycling and closely study the genes expression in the bacterial inoculums (transcriptomics) and in the lettuce involved in potassium uptake. Finally, a thorough monitoring of the adaptation of the inoculated strains could be performed via regular samplings and q-PCR analyses in order to test their abilities to colonize the rhizosphere and maintain their presence in this environment.

5.4.2 T strain alone influences yields although with a smaller effect than the AHT bacterial mix and has a marked effect on leaf length

5.4.2.1 Optimal growth conditions: influence on final yields conversely to AHT and specific impact on leaf length

The T strain treatment had an influence on the yields indicator in optimal conditions with a marked impact on root growth. Indeed, roots proved smaller for the treated lettuces than for the control in terms of final root dry weight but also fresh and dry R/S ratio. These smaller roots in treated lettuce come as a surprise as strain T had been selected mostly for IAA production which can improve root growth (Mohite, 2013; Shahab et al., 2009). However, the growth promotion effect of IAA also depends on the concentration of IAA provided to the plant and a non-adapted amount of IAA can conversely lead to growth inhibition (Spaepen et al., 2007). However, strain T was also able to solubilise phosphorus and the phosphorus availability in the environment might also influence root architecture (Beroueg et al., 2021). A deeper root architecture analysis (Delory et al., 2016) would bring interesting information as to the influence of strain T on root branching and elongation (Delaplace et al., 2015). Otherwise, more specific experimentations could be implemented to disentangle the effects of IAA on root growth from those linked to the varying availability of phosphorus and nitrogen. To this end, a root architecture analysis could be performed in the presence of the beneficial strains added in a sterile hydroponic solution to avoid the complexification linked to the interaction with the indigenous aquaponic microbiota. Furthermore, the T strain treatment seemed to increase leaf length in optimal conditions in the phytotron but also in the greenhouse experiment in the hydroponic treatment (i.e. optimal nutrient input).

5.4.2.2 Stressful conditions: influence on yields but lower effect than AHT

In stressful conditions (both light and nutrient stresses), the T strain treatment seemed to influence yield indicators albeit always in a less marked manner than the AHT bacterial mix treatment.

5.4.3 AHT bacterial mix: no yield differences in optimal growth conditions vs an effect in stressful conditions more marked than with T alone

5.4.3.1 Impact of AHT bacterial mix on lettuce growth

On the one hand, the three strains A, H and T were tested together for the assessment of a potential synergetic effect (du Jardin, 2015) and compared to the impact of T strain alone and to a control. On the other hand, strain T was chosen for the one strain treatment as is was positive for 4 traits out of 5 and was the best IAA producer (based on a colour scale).

In optimal growth conditions, the AHT bacterial mix treatment did not influence the final yields indicators either in the phytotron trial or in the greenhouse experiment with the aquaponic solution. Meanwhile, in stressful conditions, the AHT bacterial mix seemed to provide a growth advantage to the inoculated lettuces. Indeed, in light stress conditions, the lettuce inoculated with the AHT bacterial mix reached a higher shoot fresh weight than the control lettuce and a higher R/S ratio. In nutrient stress conditions (i.e. demineralized and tap water treatments) the AHT treatment led to a more important number of leaves at the end of the experiment and a longer leaf length.

The absence of performance in optimal conditions while marked differences appear in stressful conditions has already been reviewed (Nadeem et al., 2014; Rubin et al., 2017). An interesting perspective to this study would be the repetition of such trials on fruiting vegetables such as tomatoes or cucumber. Indeed, if the AHT bacterial mix treatment provides an advantage in stressful conditions, it could mean that it increases the plant's fitness which could be more easily evaluated via the production of fruits and seeds.

A discussion is also required regarding the variability observed between the time repetitions in the phytotron. Indeed, different results were obtained in the two trials conducted under light stress conditions despite using the same experimental design. These differences could be due to several factors such as the aquaponic water variability. Indeed, if we compare the composition of the aquaponic water between trial 1 and trial 2 we can note that the initial concentrations of N, P and K are quite different and that the variations of nutrient concentrations also follow very different trends. The biological variability of lettuce seeds should also be taken into account as trial 2 obtained a lower germination percentage (51% instead of 76%) than trial 1 after

11 days that may be due to the slightly older lettuce seeds albeit coming from the same lot.

Still, it is acknowledged that producing reproducible results when working with PGPB inoculation is difficult (Cipriano et al., 2016). Indeed, bacteria cultures in the lab can present sources of variability while the transition from *in vitro* to *in vivo* can also prove tricky (Richardson and Simpson, 2011).

5.4.3.2 Bacterial mix or strain alone:

Another difficulty was to assess the difference between the efficiency of the T strain alone treatment and the AHT bacterial mix treatment as, depending on the indicators and on the repetitions, no clear trend could be distinguished.

The comparison between strains alone and put together has already been explored by Kang et al. (2014) and Cipriano et al. (2016) who observed that in field conditions, strains inoculated alone exerted an impact on plant root growth but once inoculated together the effect was dimmed. It brings forward the conclusion that the inoculation in real life aquaponic systems was extremely complex due to the multiple factors and interactions which could influence the development of the strains.

This brings us to the core concept of the inoculation of mixes of bacterial strains. Mixes of bacteria are explored in the aim of obtaining a more robust and synergistic effect combining all the beneficial traits of the strains used. Indeed, it relies on the idea that the strains will reinforce each other, influence each other's behaviour or even phenotype and that different strains will perform the effect depending on the environmental conditions and use various modes of action and pathways (Avis et al., 2008; du Jardin, 2015; Khastini et al., 2019; Raklami et al., 2019; Roy et al., 2014; Sare et al., 2021). However, it increases the complexity of PGPB inoculation as we do not know the mechanisms, the relationships between the bacteria in the treatment. Indeed, these can depend on the taxonomic relationships i.e. same family, genus, species etc. or include help relationships between a producer strain and a helper strain (Sare et al., 2021) and depend on the relationship between inoculum and endemic microbiota (Cipriano et al., 2016; Dutta et al., 2014; Ibañez et al., 2014) or be based on nutrient exchanges (Zengler and Zaramela, 2018). Indeed, some combinations do not show any benefit as the used strains can be antagonists (Couillerot et al., 2011) such as the *Pseudomonas* and *Azospirrilum* species. This antagonism seems to happen often with the *Pseudomonas* species (Cipriano et al., 2016 citing Pierson and Weller, 1994) although it strongly depends on the strains. As we used a *Pseudomonas* species in our bacterial mix, it would be interesting to study more thoroughly the relationships

between the three strains via *in vitro* observations first, followed by *in vivo* trials in which the three strains populations would be closely monitored via q-PCR for example (Massart et al., 2005). To this end, the knowledge of the whole genomes of the strains would be compulsory as within one species strains may differ from a single base (Massart et al., 2005).

In our study, the application of a mix of bacteria seems to have brought better results than the application of the strain alone in terms of final fresh weight which is the most relevant indicator for commercial lettuce production. We can thus suspect that the bacteria worked in a synergy such as has already been reported for a mix of *Bacillus* and *Staphylococcus* or *Bacillus*, *Pseudomonas*, *Rhizobium* (Cipriano et al., 2016; Dutta et al., 2014) but we have no clear idea as to the exact functioning of this enhancing effect. More in depths studies should be performed with transcriptomics focusing on specific pathways for example.

5.4.3.3 Upscaling the experiments to strengthen the results

In order to ensure more robust results and conclusions while also avoiding the problems of aquaponic water variability it would be recommended to proceed to the same experiment on a bigger scale, using a fully functioning decoupled aquaponic system. This would allow for the same fresh aquaponic water to be brought to the plants regularly without perturbing the fish with the addition of bacteria in the system. The increase of the number of lettuces studied would also drown out the variability effect of the seeds. The addition of a hydroponic control would enable to compare the final yields obtained with and without bacterial inoculation such as done by Delaide et al. (2016) for complemented and un-complemented aquaponics. The simultaneous testing of the effect of stain T alone and AHT bacterial mix would also facilitate the comparison of the two treatments. More specifically, it could shed a new light on the impact of the T strain treatment on root growth as it might seem odd that the root fresh weight was not impacted in the optimal conditions trial contrary to the root dry weight. Still, the upscaling of the experimental design would not solve the problem of bacterial suspension and inoculation reproducibility. Furthermore, new trials could be set up on fruiting species such as tomatoes to see whether the bacterial inoculum provides a fitness advantage on the seeds and fruit production in stressful conditions and to see if their impact is significant on the growth of more demanding crops. Finally, regarding the impact of bacterial treatments on lettuce growth in nutrient deficiencies conditions, more fine-tuned trials should be implemented firstly focusing on one nutrient at a time and setting response curves to decreasing concentrations of the observed nutrient. This would permit to find the threshold of nutrient deficiencies for

the bacterial treatments to have a significant impact on yields. In a second stage, the use of controlled concentrations in hydroponic solutions could bring a more global view on poor fertilisation solutions while avoiding the dependence on water from an aquaponic system containing also dissolved organic matter and microorganisms already organized in communities which effects are also complex and not fully understood yet.

5.4.4 Bacterial suspensions: the difficulty of working with living organisms and their complex networks

5.4.4.1 Difficulty to work with living bacterial suspensions instead of chemical fertilizers

This study provides a first idea regarding the potential of inoculating suspensions of endemic bacterial strains with PGP functional traits into an aquaponic system (as opposed to the inoculation of commercial products into aquaponic systems (Cerozi and Fitzsimmons, 2016b; Khastini et al., 2019)). However, working with living organisms both in the treatments and in the targeted environment fosters difficulties (Cipriano et al., 2016). Indeed, contrary to the use of a chemical fertilizer, the inoculated bacteria have to colonize the environment i.e. the roots of the lettuces and the aquaponic water and to thrive in it in order to find the required molecules to produce metabolites such as IAA from tryptophan for instance.

Furthermore, the strains used in this study have been selected for specific traits identified *in vitro* but we do not know if these traits have been expressed *in vivo* or if other traits or functions played a role in the final yields obtained (Cipriano et al., 2016). The various biotic and abiotic factors composing the *in vivo* environment (Allison and Martiny, 2008) such as soil type or type of soilless system in our case (Schreiter et al., 2014b) could indeed impact and shift the inoculum and its functions (Richardson and Simpson, 2011). Amongst other factors, temperature can also influence the efficiency of the inoculum (Allison and Martiny, 2008) but as the bacteria were collected from aquaponic water at 24°C in average, grown *in vitro* in a culture chamber at 23°C and re-inoculated in a growth chamber maintained at 26°C during the days, no radical modification of the temperature should have impacted our strains. Eventually, the effect of the inoculum on lettuce growth can also depend on the plant's physiological stage (Cipriano et al., 2016).

5.4.4.2 Relationships between inoculated bacteria and root microbiota

Another reason for the complexity of the interpretation of the results obtained in the inoculation of PGPB is the interaction they play with the already present rhizosphere microbiota (Cipriano et al., 2016; Sare et al., 2021). Indeed, while the functional traits were identified in perfectly adapted conditions *in vitro* with each studied strain alone in the plate, there is no guarantee that the same trait will be expressed in the presence of other living organisms which could be competing for space and resources. Indeed, once inoculated into the rockwool plug, the selected bacteria have to interact with the native microbiota both in the rhizosphere and in the aquaponic water. Indeed, the host community already in place presents a challenge for the inoculum as it is already a complex network of microorganisms interacting with each other (via commensalism, mutualism, amensalism, competition and/or parasitism), with the nutrient cycles and the abiotic environmental conditions (Sare et al., 2021). In the rhizosphere, the host community will be mainly influenced by the type of soil (or may be influenced by the soilless system here) and crop (Massart et al., 2015) and is shaped by four main processes i.e. selection, transmission (dispersion), speciation (diversification) and ecological drift. These processes may strongly influence the capacity of the inoculum to thrive once in real conditions (Sare et al., 2021). The host community is also very often resilient to external modifications as shown in (Eck et al., 2021). Being able to settle, develop, thrive and on top of it perform specific functions which could prove helpful for the plant is a mighty challenge for the inoculated strain despite being applied at a high concentration (Sare et al., 2021).

To better the odds at impacting plant health and care, several aspects such as "inoculum ratio, nutrient profile and colonisation site" should be taken into account (Sare et al., 2021). Indeed, the reaction of the host community to the inoculation of an external microorganisms may be dependent on a quorum for detection thus requiring an optimal inoculum concentration to ensure an effect on the plant without deterring the host community. Furthermore, various techniques have been tried out to enforce the establishment and positive effect of inoculum such as the co-inoculation with macronutrient to foster the development of the inoculum (Sare et al., 2021).

For all these reasons, we do not know whether the observed effects are directly linked to the previously identified traits or to interactions with the rhizosphere community. For example, Cipriano et al. (2016) suggested that the injection of their *Pseudomonas* strains could have altered the production of plant root exudates and thus modified the composition of the rhizosphere microbiota decreasing the proportions of *Proteobacteria* and *Chloroflexi*. As Sare et al. (2021) concludes: "microbiome

resilience, impact of inoculum on microbiome, ecological processes will define whether a microorganism will be able to establish, survive and grow".

5.4.5 Potassium is the only nutrient in the lettuce which is influenced by the treatments

The only nutrient contained in the lettuce which has been affected by the treatments is potassium. Indeed, the potassium concentration was higher in the AHT treated lettuces than in the corresponding control in optimal conditions while in the same conditions the T treatment did not affect the final nutrient concentrations. In the light stress conditions trial both T and AHT treatment influenced the final potassium concentration in the lettuces. This impact may be linked to the abilities of strains A and T to solubilise potassium from the aquaponic solution. However, the lack of monitoring of the inoculated bacterial populations prevents the clear identification of the accumulation processes involved during the trials. For instance, the uptake of potassium is linked to the uptake of calcium (Resh, 2013) which we did not dose during our trials either in the water or in the plant.

Furthermore, the greater accumulation of potassium in the lettuces treated with AHT and T suspensions could be linked to the higher yields observed in stressful conditions as it was noted by Inthichack et al. (2012) that a more important potassium input could lead to higher lettuce yields. Indeed, potassium is a nutrient involved in photosynthesis, enzyme activation and proteins synthesis (Meena et al., 2016b; Resh, 2013). However, as potassium is not part of any stable molecule within the plant (Goddek et al., 2019a; Meena et al., 2016b; Resh, 2013), a more thorough study of its uptake and presence in plants during the trials should be undergone.

Eventually, this impact of the treatments on potassium concentration within the lettuce is interesting as potassium is one of the most challenging nutrient in aquaponics being inputted in very low quantities which cause deficiencies in plants. Indeed, Delaide et al. (2017) reported potassium concentrations in the PAFF Box to be twice as low as Resh's (2013) recommendations.

6.

General discussion and perspectives

6.1 Objectives of the research

Microorganisms are now acknowledged to be key players in aquaponic systems (Bartelme et al., 2018; Eck et al., 2019a; Yep and Zheng, 2019), involved in the nitrification process but also in various pathways influencing nutrient cycling and plant nutrient uptake as well as plant growth and health. Fostering the development of aquaponic systems should thus comprise research on this topic in order to improve the sustainability and viability of aquaponics (Yep and Zheng, 2019). This thesis therefore aimed at shedding a new light on aquaponics' microbial communities with three focal points: i) the composition and diversity of bacterial communities in aquaponics, ii) their ecology and changes during crop growth and iii) their potentially positive impact on crop yields. In this view, a panel of aquaculture and aquaponic systems were sampled both in their sump and biofilter to assess bacterial diversity and taxonomically characterise the bacterial communities (**chapter 3**). Then, a single system was selected for a more in depth study of its different compartments' communities, their modifications over the course of a lettuce growth cycle and the adaptation skills of the communities to the system's parameters changes (**chapter 4**). To conduct both these studies, metabarcoding focusing of the V1-V3 regions of the 16S rRNA gene was performed. To address the third focal point dealing with bacteria's roles in aquaponics, bacteria were directly collected and isolated from our aquaponic system. Strains were then selected based on their positive responses to *in vitro* biochemical tests focusing on potentially plant beneficial traits. Finally, the impact of these strains on lettuce growth was evaluated via *in vivo* trials (**chapter 5**).

Before we sum up the take-home messages of this thesis in the conclusion, a few more points encompassing all chapters require discussion.

6.2 Assessment of chosen methods

6.2.1 Focus on bacterial communities

Despite both bacteria and fungi being recognized as key players in aquaponics and more generally soilless systems (Lee and Lee, 2015), the latter were not included in this thesis. The focus was drawn on bacteria as, at the beginning of the thesis, virtually no information was available on fungi in aquaponics whereas a research article by Schmautz et al. (2017) had already been published regarding bacteria. As bacterial networks are already a complex topic, it was deemed more relevant to focus on bacteria only and to explore different aspect of their communities in aquaponics. rather than disperse and include fungal studies. Furthermore, ITS databases and

bioinformatics tools for fungal studies are more complex and less developed than for bacterial studies even though they are regularly improving and new algorithms are created (Chandelier et al., 2021). Nevertheless, a master thesis was supervised in 2020, focusing on the development of a bioinformatics pipeline adapted to fungal studies and analysing fungal samples from the closed-loop aquaponic system in Gembloux. To this end, a new pipeline was created using algorithms from existing programs such as QIIME 2 (Bolyen et al., 2019) and PIPITS (Gweon et al., 2015). The application of this pipeline on the aquaponic data provided good quality results with a higher taxonomical identification than when using QIIME 1. Work is still in progress on those data to propose conclusions regarding fungal communities in aquaponics.

6.2.2 Biases linked to metabarcoding

Metabarcoding was the method selected for the description of the bacterial communities both in **chapter 3** and **chapter 4** with a focus on the V1-V3 hypervariable regions of the 16S rRNA gene. In this method, the choice of hypervariable region and set of primers can influence the results of the analysis thus creating a slightly biased picture of the studied community (Cruaud et al., 2014; Yang et al., 2016). We chose to focus on the V1-V3 regions of the 16S rRNA gene as they were the ones used by Schmautz et al. (2017), also utilizing the same set of primers both in chapter 3 and chapter 4. The use of the same regions and primer sets enabled us to conduct a meta-analysis in chapter 3 encompassing Schmautz *et al.*, (2017)'s samples to compare the data obtained during their study, on their system, with ours. Furthermore, the V1-V3 regions were recommended by Kumar et al. (2011) as the most adapted to describe a complex community. Still, important biases remain, due to the differences in sampling method, extraction protocol (Salter et al., 2014), choice of database and choice of bioinformatics analyses (Massart et al., 2015).

Furthermore, the eight bacterial strains selected for their potential plant growth abilities in **chapter 5** and identified via the Sanger sequencing of their whole 16S gene did not belong to the predominant taxa identified in **chapter 3** and **4**. Indeed, one strain was assigned to the *Serratia* genus, another to the *Chryseobacterium* genus while the other six were assigned to the *Pseudomonas* genus. The *Pseudomonas* genus was detected in these analyses but represented a minor proportion of the community. These results highlight once again the differences between holistic analyses conducted with HTS technique and culture methods. Indeed, despite using general media, only a fraction of known microorganisms are actually culturable in the lab. Conversely, some of the lab isolated bacteria were not detected by HTS also showing the limits of these

techniques in terms of accuracy and precision. Indeed, some taxa may be overlooked due to a too low abundance in the community or because of ill-adapted primers. The bacterial strains which we managed to isolate may thus not be representative of the whole sump community. However, the impact of beneficial bacteria is not linked to the quantity even though there might be a quorum.

6.2.3 Representativeness and repeatability

This question of representativeness, as well as the one of repeatability, also deserve discussion regarding the samples on which the conclusions of **chapter 3** and **chapter 4** are based.

6.2.3.1 Representativeness

In **chapter 3**, only one sample per compartment was collected in each system which brings forward the question as to the way these unique samples can represent correctly the bacterial community of their compartment. Sump samples were composed of 2 litres of water which flowed through the system and were thus considered a homogeneous environment. A side study was conducted to compare four water samples taken at the same moment from the PAFF Box sump. Those four samples were considered sufficiently similar for the water samples to be considered representative of their compartment. The representativeness of the root samples in **chapter 4** had been ensured by grouping ten lettuces by samples. Only one washing step was performed to collect the roots' bacteria as recommended by Sare et al. (2020) who had previously studied the representativeness of the collected bacteria in only one washing step compared to four steps. Concerning the biofilter samples, the representativeness of the collected samples can be called into question. Indeed, depending on the design of the biofilter the samples could be taken at very different location i.e. in the middle of the biofilter when it was shallow enough but only on the top of it when the biofilter was several meters high. Despite the generalised use of moving beds in which the biomedia is constantly mixed by the oxygen flow, a moving bed several meters high could still present varying abiotic conditions (pressure, oxygen availability, temperature) depending on the sampling depth (Bartelme et al., 2017).

6.2.3.2 Repeatability

The repeatability of the studies conducted in the various studied systems in **chapter 3** but also on the PAFF Box alone is not ascertained. In **chapter 4**, the sump samples collected during 9 weeks were quite similar. Indeed, no adaptation period was

observed after the winter fallow period, no major modifications were observed throughout the lettuce growth cycle. However, this experiment was only conducted on a single system and we do not know if this can be applied to all the systems studied in **chapter 3** or if more long term cycles could be observed in other systems. Furthermore, **in chapter 5** an important variability regarding the nutrient concentrations in the aquaponic water collected from the PAFF Box over 6 months was observed. Even though the small water parameters modifications studied in **chapter 4** did not seem to influence the composition of the bacterial communities in the PAFF Box, the impact of important NPK concentrations variations should still be taken into account.

6.2.4 Choice of in vitro *and* in vivo *tests*

In **chapter 5**, it was decided to look into the potentially plant growth beneficial functions which could be harboured by the aquaponics microbiota via collection and cultivation of microorganisms and *in vitro* biochemical tests. However, several other options could have brought interesting information regarding this topic. Indeed, a whole genome sequencing analysis of the entire bacterial community could have been performed on the bacterial communities of the PAFF Box to assess the presence or absence of genes of interest in the community. Several bioinformatics tools such as Kraken (Wood and Salzberg, 2014), eggnog (Huerta-Cepas et al., 2019) or antismash (Medema et al., 2011) are nowadays easily available online to conduct such screening analyses (more details in 6.5.3).

Another solution would have been to target a specific process involved in nutrient cycling such as a particular way to solubilise phosphorus for example and to study the expression of the group of genes involved in the process via transcriptomic analysis. However, this method would have required the preselection of specific processes and would thus have missed out on other functions.

A combination of whole genomes sequencing, bacteria isolation and transcriptomics could also have been performed. Indeed, after the analysis of the complete genomes and the selection of bacteria bearing interesting genes or operons, it could have been possible to isolate the bacteria in the lab and then focus on the expression of the targeted genes in various conditions. The difficulty of this method resides in the lab isolation of bacteria detected via HTS as described in 6.2.2.

Finally, metatranscriptomics could have enabled us to study the expressed genes of the whole bacterial community but this method is still very complicated both in the

implementation and downstream bioinformatics analyses. The potential of the 'omics' techniques in the frame of aquaponics microbial communities study is addressed with more details in the perspectives of this thesis (see 6.5.3).

The *in vivo* option was chosen as it was rapid, cheaper and needed less preliminary preparation. An evident drawback of this method however was the restriction of the second part of this thesis to the study of culturable microorganisms. This was considered acceptable as a follow up of the thesis would be to study the impact of the inoculation of PGPM in aquaponics to improve crop yields and this would be forcefully restricted to culturable organisms.

Another possibility to improve the functioning of aquaponic systems via the prism of beneficial microorganisms would be to work on the parameters of the system to foster the development and predominance of strains of interest instead of inoculating bacterial suspensions produced in the lab. This approach would avoid the troubles linked to bacterial cultivation, inoculation and then uncertain colonisation of the environment. However, the complexity of aquaponic systems could render this method quite complicated as many parameters are interlocked and our understanding of the impact of each parameter on the bacterial communities is still scarce. Furthermore, the presence of fish in the system limits range of possible modifications of water parameters thus making it possible to use this approach only in decoupled systems. In this view, modifications could be performed only in the hydroponic compartment and incoming solution.

6.3 Description of the bacterial communities

Chapter 3 and **chapter 4** dealt mostly with the description of bacterial communities in aquaponics. The main conclusions which can be drawn from these two chapters are that a great diversity of genera could be detected across all ten studied systems (**chapter 3**) with each of them harbouring a community quite different from the others. A small core microbiota could still be found between all samples. **Chapter 4** offered that the bacterial communities in each compartment were quite resilient with an absence of adaptation phase in the sump and biofilter communities after the winter fallow period and no specific reactions to the naturally occurring water parameters changes in the system.

6.3.1 Inter systems versus intra system comparisons

Chapter 3 describes various system while **chapter 4** compares several samples from the same system thus the two chapters perform comparisons according to different benchmarks i.e. inter systems versus intra system. Indeed, when all ten studied systems are compared at the family and genus levels in **chapter 3** (**Figure 3-2**), it seems that each system possesses a microbiota quite distinct from the others and that within a system, similarities can be noted in terms of predominant taxa between sump and biofilter. However, when put on an PCA (**Figure 3-4**) it can still be seen that biofilter and sump samples are grouped by type of compartment and not by system. Furthermore, the sumps of the ten systems share 22 OTUs and the biofilters 28 OTUs highlighting the specificity of each compartment. This is coherent with the PCA obtained in **chapter 4** (**Figure 4-3**) in which samples are also grouped by compartment. Eventually we can note that in **chapter 4** the sample groups are much tighter than in **chapter 3**, again showing the variability between sumps or between biofilters of different systems.

6.3.2 Influence of the hydroponic compartment

Another curious comparison can be made between the conclusions of **chapter 3** and **chapter 4** regarding the influence of the plant compartment on the aquaponics microbiota. Indeed, in **chapter 3** the RAS and the closed-loop aquaponic system of Gembloux Agro-Bio tech were compared, arguing that their similarities in terms of design, fish and water could enable a robust comparison between the sumps microbiota. The conclusion of this analysis was that both sumps harboured very different communities and that it could be linked to the presence of a plant loop in the PAFF Box system. However, in **chapter 4**, the same sort of comparison was performed albeit in a more precise way as we focused on only the PAFF Box this time comparing the sump and biofilter communities before and after the introduction of seedlings. This time we concluded that the introduction of seedlings did not alter the previously settled communities. Still in the biofilter we could note a negative correlation between the richness index Observed-otus and the number of days elapsed since the beginning of the experiment. To sum up, both chapters provided different conclusions but those differences can be qualified. Indeed, in the case of **chapter 4** we had only one sample from the sump and biofilter before the introduction of the lettuce seedlings. More samples of the RAS period would be required to ensure a more robust comparison and go more in depth into this hypothetical adaptation phase. Regarding **chapter 3**, we could suggest that the observed differences were linked to

other factors rather that the presence of plant seedlings such as biofilter type of fish behaviour for example. It could also be linked to the fact that the RAS never had any plants and while the PAFF Box periodically does and that it could have an impact despite the bleaching of the hydroponic compartments before the winter fallow period. Once again a more in depth study of this particular aspect of aquaponics microbiota i.e. the influence of the introduction of plants should be conducted with a strong focus on the transition period.

6.3.3 Concept of core microbiota

The very notion of core microbiota requires discussion. Indeed, the core microbiota has been defined in **chapter 3** as "the microbial community that is systematically associated with a given host" (Lemanceau et al., 2017). With hindsight, it seems difficult to associate this definition with the "core microbiota" observed in our chapters and especially in **chapter 3**. Firstly, the taxa compositing the observed core microbiota concerned families or genera. It seems thus difficult to be sure that exactly the same bacterial species or strains were present in the identified families or genera identified in all samples. It would be more correct to write about common taxa between aquaponics system rather than core microbiota as we do not know yet whether the taxa identified in all samples and all systems are systematically associated with aquaponics environment. Indeed, the taxa present in all samples in **chapter 3** are the *Oxalobacteraceae* family, the *Cetobacterium* genus, the *Alphaproteobacteria* and the *Comamonadaceae* family all being either very large groups or very common taxa such as the *Cetobacterium* which is typical from fish guts. Still, 34 OTUs were detected in all decoupled aquaponics system which is still encouraging as to the detection of common points between aquaponics microbiota. This could be compared to core microbiota obtained via the comparison of different types of soils.

In chapters 3 and 4, the focus was drawn on taxonomical identification and therefore only on the taxonomical core microbiota. However, Lemanceau et al. (2017) proposed the novel concept of functional core microbiota defined as "a subset of the microbiota associated with a given host irrespective of the macroenvironment (e.g., soil type) and that encompasses microbial vehicles carrying replicators (genes) with essential functions for holobiont fitness". This definition focuses more on the functions systematically detected in the vicinity of the plants rather than the taxa. The authors argue that the surrounding environment will strongly influence the taxonomical composition of the microbial reservoir from which plants can recruit their rhizosphere communities (Berg and Smalla, 2009) and thus will impact the taxonomical core microbiota. Conversely, they offer that a given plant genotype will always be able to

recruit the functions necessary to its proper functions even though in different microorganisms due to the functional redundancy which "ensures that functions are maintained despite environmental fluctuations". These core functions are most often beneficial for the host plant fitness, health and care and are mostly linked to nutrition. It has indeed been shown in several studies that for a given plant species more similarities could be found in the functional than in the taxonomical core microbiota (Bulgarelli et al., 2015; Lemanceau et al., 2017; Massart et al., 2015). Therefore, as we try to understand the roles of microorganisms in aquaponics nutrient cycling and plant yields, it would be more interesting in the future to focus on the functional core microbiota of each system or crop in aquaponics.

6.4 Crop-bacteria interactions in aquaponics

6.4.1 Sterile growth modules

The first approach at understanding the impact of microorganisms on lettuce growth in aquaponics was attempted via a comparison of lettuce growth in aquaponics water in the presence and the absence of microorganisms. The aim was to distinguish the effect of microorganisms from the effect of organic nutrients or compounds (Delaide, 2017; Yep and Zheng, 2019). To this end, specific growth modules comprising a small hydroponic system enclosed in a Plexiglas chamber were designed to allow for the cultivation of lettuces in sterile conditions. Unfortunately, the modules prototypes were not sufficiently efficient to avoid the recontamination of the sterilised aquaponic water and could thus not be used for the expected comparison. Still, such growth modules could be used for other experimental designs and more specifically via the approach of synthetic microbial ecology which is "a bottom-up approach where two or more defined microbial populations are assembled in a well-characterized and controlled environment" (Roy et al., 2014). Indeed, these modules can creates simplified and controlled ecosystems, "with a low complexity and higher experimental reproducibility" (Sare et al., 2021) with a high level of control of the circulation of microorganisms and could enable tests focusing on the inoculation of PGPR in environment with a varying amount of microorganisms (Fließbach et al., 2009).

6.4.2 Endemic microorganisms to boost aquaponic systems

After the abandonment of the idea of comparing lettuce growth in the presence and in the absence of microorganisms, it was thought of choosing potentially plant beneficial traits in microorganisms and to detect their presence in an aquaponic

system. To this end, several bacterial strains were isolated from the PAFF Box sump and selected based on five biochemical tests focusing on phosphorus and potassium solubilisation and ammonia, IAA and siderophores production. Then, bacterial suspensions containing purposefully selected strains from the PAFF Box collection were inoculated in mock aquaponic systems filled with PAFF Box water and in which lettuces were grown. The idea behind this experiment was to evaluate the possibility of enriching the aquaponic water with endemic strains in order to boost crop yields. Indeed, PGPR inoculation has been thoroughly studied in soil culture while such studies regarding soilless systems are less abundant (Bartelme et al., 2018). Still, inoculations of commercial suspensions of *Bacillus spp*. were tried out in aquaponics to assess its impact on phosphorus cycling (Cerozi and Fitzsimmons, 2016b) while the addition of another commercial product (B103, Biozym) helped Zou et al. (2016b) to boost their lettuce yields. On the other hand, several bacterial and fungal species have already been tried out in hydroponic systems, often positively influencing yields (Lee and Lee, 2015). However, it has been shown in Stouvenakers et al. (2020) that important differences in terms of microbial communities could be observed between a hydroponic system, an aquaponic one and a complemented aquaponic one albeit the aquaponic water for the complemented and un-complemented systems coming from the same system. Hence, different abiotic conditions seem to strongly influence the bacterial communities. Therefore, it would seem safer to inoculate in a soilless system bacteria originating from this same system or at least from a system as close as possible even though it would prove more complicated for the development of commercial bacterial boosters.

Regarding the hypothetical development of our strains into commercial biostimulants, they could be marketed as biostimulants enhancing lettuce tolerance to abiotic stresses such as nutrient deficiency and lack of lighting. The mechanisms underlying this tolerance are suspected to be linked to the production of IAA and the facilitated access to nutrients via solubilisation which may influence the root system. However, the links between enhanced tolerance to stress and the presence of those beneficial traits in the strains require further investigation. Furthermore, prior to the marketing of biostimulants, the selected microorganisms have to validated by European law. Unfortunately, as strains A and T belong to species harbouring potential human pathogens, their chances of appearing on this list seem weak.

6.4.3 Other factors impacting inoculum efficiency

Overcoming the obstacle of adapting to a new environment is a small step towards ensuring the proper development and efficiency of the inoculum. Indeed, as well as

adapting to the abiotic conditions, inoculated bacteria also have to interact with the endemic microbiota from the aquaponic water and from the crops roots (Singer et al., 2021). Another complicating element is the concept of quorum sensing. Quorum sensing is "a process by which bacteria co-ordinately regulate gene expression in response to sensing of diffusible chemical signals" (Jung et al., 2020) and can strongly impact the effect of PGPB (Jung et al., 2020). The bacteria we isolated from the sump of the PAFF Box do not belong to the predominant taxa detected by 16S metabarcoding in **chapter 3** or **chapter 4** and we did not quantify their abundance in our system. Therefore, the absence of knowledge regarding the quorum aspect of our microbial inoculum may also impede our better understanding of the processes under way in aquaponic system.

6.5 Perspectives

This thesis was one of the first of its kind tackling aquaponics microbiota. This is why most experiments and trials were conducted on small scales, with miniaturised systems and a low number of repetitions. Now that interesting leads have been defined, it would be relevant to repeat or upscale the experiments either to provide more stable and robust conclusions or to broaden the range of application of the conclusions.

6.5.1 Broadening the horizon

In the case of the study conducted in **chapter 3**, only 10 aquaculture and aquaponics systems were taken into account, all located in north-western Europe and presenting a relatively equal level of sophistication. It would be tremendously interesting to conduct the same study on a bigger scale englobing all of Europe (north and south), the USA where many systems have been implemented (Love et al., 2014) but also countries with a warmer climate as the design of the system is actually very dependent on the climate of the location (Palm et al., 2018). The inclusion of a broader range of designs and technology levels targeting also low tech systems would lead to a more complete and inclusive picture of aquaponics microbiota. To build this study would require the help of the aquaponic nomenclature developed by Palm et al. (2018) and the prior classification of the targeted systems in well-defined categories to ensure robust comparisons.

Similarly, more studies akin to the one conducted in **chapter 4** should be performed on a variety of other systems to generalise the conclusions that we drew. This implementation of similar studies would shed light on the questions raised at the end

of **chapter 4** regarding the origin of the root microbiota in aquaponics, the recruitment mechanisms available to plants in soilless environments and the potential modifications of root microbiota with the change of plant physiological stages.

Furthermore, in **chapter 5**, only five biochemical tests were performed, focusing on different aspects of plant growth promotion. More functions could be tested or conversely, more tests focusing on a specific part of nutrient cycling could be performed. For example, we now know that some bacteria are able to solubilise inorganic phosphorus but other, more specific tests could be carried out to determine which precise pathway is involved or if other phosphorus related traits are present (**see chapter 1**). For example, the solubilisation of organic phosphorus which could be trapped in fish feed leftovers could also be investigated via the targeting of phosphatases producing bacteria. Additionally, the collection of microorganisms was performed from the sump only while a broader collection involving all compartment would have been more complete as it has been shown that they all harbour a specific community. More specifically, the isolation of root microorganisms and their use to develop potentially plant beneficial inoculums could facilitate the colonisation of the plant roots by the inoculum as the bacteria would originate exactly from that zone. Still, the question of whether the root microbiota of the aquaponic lettuce originate from the lettuce seed or the aquaponic environment has not been solved yet and the isolation of lettuce seed microorganisms would defeat the purpose of studying aquaponics microbiota.

Eventually, fungi should be included in future aquaponics microbiota studies as they represent a key part of plant growth promoting microorganisms and have already been detected in aquaponic systems. Regarding **chapter 5**, the exploration of other compartments than the sump could enable the collection of fungi which are also known to play major roles in plant growth promotion and which presence have been assessed in aquaponics and in our aquaponic system. Several hypotheses could explain this absence: i) only sump water was collected for the constitution of the collection and fungi might be less adapted to flowing water as an environment, ii) fungi are slower growing than bacteria (i.e. might have appeared on the Petri dishes after the bacteria) and require growing media modified with antibacterial molecules.

6.5.2 Deepening what we know

Both in **chapter 3** and **chapter 4**, this thesis tried to link the multiple factors defining an aquaponic system (design, fish and plant species, water parameters) to their influence on the composition and modifications of the bacterial communities.

However, the interlock of these factors prevented us from tying one particular element to a specific community. New experiments using identical systems and making one factor vary at a time should be carried out. The use of identical systems would also enable to assess the "repeatability" of a microbial community. Indeed, nothing ensures us yet that two identical systems should bear the same communities as many uncontrollable factor come into play such as fish behaviour for example. The comparison of similar systems and the controlled variation of one factor after the other is however a very complicated endeavour as most elements in aquaponics are linked. For instance, changing the feed type would influence the fish feeding behaviours and thus digestion and metabolisation and in the end the nutrient availability. How to differentiate the impact of nutrient availability from the presence of fish feed leftovers is complex. The use of a hierarchical clustering and ACP as done in chapter 4 to group factors could ease this type of analyses. Furthermore, a better understanding of the ecology of bacterial communities in aquaponics could maybe be included into aquaponics modelling and may help better understand the links between bacteria, system design and management.

Chapter 4 was a first follow-up of the natural modifications of bacterial communities in a closed-loop aquaponic system and raised new questions which would require complementary experiments to be answered. Indeed, the attention was drawn on the transition phase between the system functioning as a RAS and then as an aquaponic system. Therefore, more samples from the RAS period should be collected before plant transplantation to enable a more robust comparison between the two periods and assess the impact of the addition of the plant compartment as discussed in **chapter 3** as well. The question of the origin of the lettuce root microbiota could also be tackled by a complementary analysis comparing the root microbiota from aquaponic and soil borne lettuces originating from the same seed lot or from contrasted aquaponic and hydroponic systems using the same seeds. Such an experiment would still comprise a very high number of uncontrollable factors.

Regarding the bacterial strains which were selected in **chapter 5,** a more complete description of their functional activities should be conducted with more biochemical tests (e.g. ACC deaminase activity, HCN production, ethylene production, antifungal activity (Ahmad et al., 2008; Kumar et al., 2012)) in order to get a broader picture of their abilities. Moreover, the sequencing and analysis of the complete genomes of the selected bacterial strains could help describe the functions harboured by the strains and also discover new functions via comparisons with the pangenome of the species (Gautreau et al., 2020). A tool like antismash would fit perfectly here to detect

interesting new biosynthesis gene clusters (Medema et al., 2011). Furthermore, the already identified traits which aroused attention in the *in vivo* trials such as potassium and phosphorus solubilisation and IAA production should be studied more thoroughly with a focus on the involved pathways both in the bacteria and in the plant.

Eventually, the several types of *in vivo* trials that were conducted gave a good indication that the inoculation of PGPB suspensions could help plant growth in aquaponics and more specifically in stressful conditions. However, the important water variability and biological variability (lettuce germination and growth, bacteria development and interactions) led to the observation of trends rather than accurate results. To consolidate the observations obtained in our trials, more repetition or an upscaling of the experimental design as discussed in **chapter 6** would be required. Another way to provide more robust comparisons between treated and control lettuces in stressful conditions would be to conduct prior experiments comparing stressed and non-stressed lettuces without any bacterial treatment. This would enable the precise assessment of the impact of the abiotic stress on lettuce yields and thus the alleviation of the stress by the bacterial treatments could be measured with more accuracy. Moreover, these trials provide the beginning of an answer as to the potential impact of the selected strains on yields but does not shed any light on the functioning the bacteria, their relationship within the mix, the pathways involved in the interaction with the nutrients present in the water and with the plants. To help better understand the behaviour of the inoculated strains in the trials, a monitoring of the strains via quantitative PCR could be carried out during the trials.

6.5.3 The prospects offered by 'omics' technologies

Microbial communities in aquaponics, as well as in other environments such as soils or rhizospheres, are complex networks with numerous microorganisms interacting with each other (Singer et al., 2021; Zengler and Zaramela, 2018) but also with other types of organisms such as plants and fish in the case of aquaponics. This tight network complicates the global understanding of its functioning, of the impact of a single organism on plant growth or fish health and of the influence of abiotic parameters on the global behaviours of the microorganisms. Nevertheless, the development of 'omics' technologies could provide a better understanding of the functioning of these networks and thus a potential broadening and deepening of the results obtained in this thesis (Munguia-Fragozo et al., 2015). Still, the more sophisticated the technique, the riskier it seems to induce biases either in the experimental design or in the processing of the samples. Great caution is thus required when working with 'omics'.

6.5.3.1 Genomics and metagenomics

As already mentioned in 6.2.4 and 6.5.2, genomics and metagenomics could provide new information regarding the functions present in our strains of interest or in the whole aquaponic bacterial community respectively. Firstly, sequencing of the whole genomes of our selected strains would provide a better knowledge of these strains and their functions (6.5.2). Secondly, a metagenomics study, which focuses on genome characterisation, conducted on the whole bacterial communities would enable the direct identification of functions of interest present in the community instead of selecting in the lab and then testing *in vitro*.

Several technologies are nowadays available for genome sequencing and the choice of technique can strongly impact the obtained results. Indeed, a most recent technology such as nanopore sequencing (Heather and Chain, 2016) can provide reads hundreds of thousands nucleotides long thus greatly facilitating the assembly step of the bioinformatics processing to obtain a complete genome. Illumina on the other hand provides reads which are 100 to 250 or 300 nucleotides long thus requiring a complex assembly step, especially when working on a community (Heather and Chain, 2016). A choice therefore needs to be made beforehand to ensure the best possible results.

6.5.3.2 Transcriptomics and metatranscriptomics

The presence of the genes of interest in the genomes does not guarantee that the genes are expressed (Carvalhais et al., 2013). Indeed, genomics and metagenomics will focus on the presence of genes and their abundance, still the most abundant genes are not always the most expressed as the expression of genes also depends on the external conditions and thus can vary through time (Bharti et al., 2021; Massart et al., 2015). To address this new challenge, transcriptomics could be used. On the one hand, it could be applied on specific operons involved in pathways of interest such as phosphorus solubilisation or IAA production (Munguia-Fragozo et al., 2015) in isolated strains or, on the other hand, in the whole bacterial community. The genes expression of the targeted crop could also be studied to assess the reaction of the plant to the presence of specific bacteria in aquaponics or varying conditions in aquaponic systems. Still, the use of transcriptomics to study the expression of specific functions requires an important upstream preparation. Indeed, to be able to correctly interpret the results of a transcriptomics analysis it is necessary to understand the selected pathways and known the several genes involved and their precise roles and interactions. It is thus necessary to select key genes in each function. Furthermore, the prior knowledge of the studied genome is required to map the expressed genes.

Another possibility to study the expression of all functions in a bacterial community is the use of metatranscriptomics. Indeed, metatranscriptomics opens up a new era of studies for aquaponics or rhizosphere microbial communities and their interactions with the plants in which it is possible to follow the activity of whole communities via the analysis of the genes' expression. For example, metatranscriptomics could be used to study the interactions between endemic aquaponic community and plants or between inoculated bacteria and endemic community or plants. More specifically, this technique could be used to assess the influence of the bacterial inoculum on the endemic community's gene expression profiles or the influence of the water parameters on this community. The impact of the plants in aquaponics and their roots exudates on the aquaponic bacterial community could also be addressed if the root exudates are closely monitored (see infra limitations and design). Moreover, it is interesting to link metatranscriptomics with the notion of functional core microbiota. Indeed, metatranscriptome study would permit the analysis of functional core microbiota i.e. of the core functions which are systematically associated and expressed with a system or a plant rhizosphere (Carvalhais et al., 2013) .

Conversely to transcriptomics, metatranscriptomics does not require to focus on a specific pathway as it englobes all expressed genes. However, as for transcriptomics, the prior knowledge of the metagenomes facilitates the processing of the data. The knowledge of the metagenome tremendously facilitates the use of metatranscriptomics as it provides a general map of the genes present before analysing their expression and is used as a base for taxonomical and functional annotation (Carvalhais et al., 2013). Indeed, cDNA/DNA reads ratio are calculated for each gene. These expression ratios are useful as they give normalized information (expression of cDNA compared to abundance of the gene in the metagenome) which is more precise than relative frequency analyses (Carvalhais et al., 2013). More specifically, it allows to compare the level of expression of genes in specific conditions, at a precise moment and assess the impact of external factor on gene expression and in our case on the influence that the microbial community can have over the plant and as to the influence of water parameters on the communities (Carvalhais et al., 2013; Massart et al., 2015; Munguia-Fragozo et al., 2015). The basic assumption for metatranscriptomics is that " as the majority of ecosystem functions are related to the more dominant gene transcripts, metatranscriptomics data can be directly linked to active metabolic pathways" (Carvalhais et al., 2013).

Studying the metatranscriptomics profile of an entire bacterial community is a complex endeavour and therefore the experimental design needs to be very precisely

defined. Indeed, to be able to interpret expression profiles it is necessary to understand and monitor all the other factors involved such as metagenome, metaproteome, metabolome. Then links can be drawn between all variables, correlations can be found but not causes. In aquaponics studies, it would consist in a very broad exploration of aquaponic systems. Otherwise, researchers should design experiments as controlled as possible with well-defined treatments to reduce the number of parameters which need to be monitored. This would "facilitates the identification of genes and pathways that are differentially expressed due to a smaller number of parameters" (Carvalhais et al., 2013). For example, the influence of the introduction of plants in the system could be used as treatment and the metratranscriptoms before and after transplantation could be compared. On the other hand, the impact of the inoculation of superstrains in the rockwool plugs could also be used as treatment and see how they influence the expression of genes in the global community.

Aside from the experimental design, metatranscriptomics holds several very sensitive points. The first complication is linked to the handling of RNA which is fragile with a very short half-life which requires to be very attentive, work with ice and store at -80°c or use preservative solutions. When working with soil or rhizosphere communities, another focus point is the presence of humic and fulvic acids which can inhibit enzyme activity as they precipitate during the nucleic acid extraction. However, several solutions and kits are available to solve this either during extraction, before for soil treatment or after for purification. Moreover, metatranscriptomics focus on gene expression, thus gene translation in mRNA. However, mRNA only represent between 1 and 5% of total RNA and samples need to be enriched via several methods or kits. Despite the enrichment in mRNA, rRNA stay very abundant in metatranscriptomics datasets and in the end they need to be removed during the bioinformatics step. Finally, to be exploitable mRNA have most of the time to be used as model for the synthesis of cDNA and many errors can occur during the reverse transcription step and chimeras can be created. A potential solution would be to work directly on RNA sequencing but this is made difficult by the instability of RNA molecules (Carvalhais et al., 2013).

All these challenges linked to the difficulty to work with soil communities or aquaponic rhizosphere or water communities because of humic and fulvic acids renders the use of metatranscriptomics is this field complicated and thus few studies are available. The study of microbial communities in aquaponics is also a new topic and the metagenomes of the bacterial communities of the systems are not yet known thus preventing the use of the metagenomes to map the metatranscriptomes.

6.5.3.3 Metaproteomics and metabolomics

To go even further, other technologies such as proteomics and metabolomics would permit to shift the focus from the bacteria directly to the compounds (proteins and metabolites respectively) (Munguia-Fragozo et al., 2015) present in the water hence providing a complementary vision to the study of the microbial communities. Indeed, the study of the proteome or metabolome of the lettuce in presence of different aquaponics microbiota would shed a new light on the study of their interactions. Furthermore, new tools are currently being developed to enable the large screening of the molecules secreted by single cells and would thus permit to associate specific secretions with the studied bacteria.

7.

Conclusions

The aim of this thesis was to explore the composition, the ecology and the functions of bacteria related to plant growth in aquaponic systems. The take-home messages to be drawn from this work are therefore listed below.

- Ten different aquaculture and aquaponic systems were studied. Each system possessed its own bacterial communities defined by a multitude of factors that were highlighted in our research. Several common taxa could however still be found amongst all systems.

- It was deemed necessary to better understand the potential modifications of the bacterial communities related to time, introduction of plants or changes in the system's water parameters. Such an improved understanding would enable to anticipate the reactions of the bacterial communities and to manage them to ensure a healthy community. Over the course of nine weeks during which lettuces were grown in the system, no major changes in the bacterial communities could be noted either in the sump, biofilter or lettuce root communities. No particular patterns in the composition of communities could be observed, nor an adaptation period after the introduction of plant seedling. Such findings suggest a resilient aquaponic microbiota yet additional thorough studies dealing with larger systems and addressing various plant species are required to ensure the validity of these findings.

- A comparison of the bacterial compositions of the lettuce root communities in aquaponics and in soil rhizosphere literature highlighted strong similarities between the two. This brought new research questions as to the origin of the root microbiota in soilless systems and the recruitment mechanisms for the constitution of a tailor-made root microbial community.

- Regarding the potentially plant beneficial functions of aquaponics microbiota, five functional traits were detected in the sump community i.e. phosphorus and potassium solubilisation, ammonia, siderophores and IAA production. Several strains presented a combination of relevant functional traits and were assigned to the *Pseudomonas*, *Chryseobacterium* and *Serratia* genera.

- The inoculation of concentrated suspensions of these endemic bacteria into small DWC systems containing aquaponic water and growing lettuces led to encouraging results regarding the impact on final yields in lettuce with a

stronger effect noted with the inoculation of a mix of bacteria and more particularly in stressful growth conditions.

APPENDICES

A. Supplementary material – chapter 3

Description of the visited aquaponic and aquaculture systems

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

In the UrbanFarmers farm of The Hague, Nile tilapia (*Oreochromis niloticus*, Red Naturally Male Tilapias, supplied by Til-Aqua Ltd.) are grown and fed with an omnivorous diet. Water, after flowing through a drum filter and a moving-bed biofilter (Kaldnes media) for nitrification, is collected in a sump before being directed either back to the fish tanks or to two headers for supplementation to conventional hydroponic levels [8] with hydroponic solutions prior to delivery to plants in the greenhouse. A fraction of the water containing aquaculture effluents which flows through the nutrient film technique (NFT) systems is then drained back to the aquaculture system.

- **PCG: Provincial Trial Centre for Vegetable Production (29/03/2017)**

The Provincial Trial Centre for Vegetable Production (PCG) in Kruishoutem (Belgium) rears Jade Perch (*Scortum barcoo*, supplied by Aqua4C) in eight fish tanks of 1.8 m^3 each. The fish are omnivorous but are fed on a vegetarian diet developed by the Aqua4C company (3.2 mm Omegabaars Grower, AQUA4C, Kruishoutem, Belgium). This system is considered "decoupled", wherein the water from each tank goes through the tank's own small drum filter and small moving-bed biofilter prior to flowing partly back to the fish tanks and partly to the hydroponic compartments. The biofilters contain Eco Pondchip Filtermedium (Schlangen, Germany) as biomedia. The hydroponic system is composed of eight rows with tomatoes grown in a rockwool slab and irrigated through a dripping system. Each row of tomatoes is connected to a fish tank. Four of the fish tanks contain a density of 60 fish per tank (low density) and the other four a density of 100 fish per tank (high density).

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

Belgian Quality Fish (BQF) is an aquaculture company located in Dottignies, Belgium, which rears several species of sturgeon such as Siberian sturgeon (*Acipenser baerii*), Russian sturgeon (*Acipenser gueldenstaedtii*), European sturgeon (*Huso huso*), Sterlets (*Acipenser ruthenus*) and various hybrids that are given an omnivorous diet (Sturgeon Grow-out, Aquabio, Turnhout, Belgium). The system is a recirculating aquaculture one. The water from the sturgeon tanks is collected in a canal and undergoes disinfection via ozone and UV light (2 KWh lamp per production unit of

4300 m³). The water is then directed towards a drum filter and a moving bed biofilter. After this, the water also flows through a denitrification biofilter. Indeed, as the BQF system is a recirculating aquaculture system (RAS), they eliminate the nitrate from the recirculating water as much as possible to discharge less polluted water into the environment.

- **IGB: Leibnitz-Institute of Freshwater Ecology and Inland Fisheries (07/04/2017)**

The Leibnitz-Institute of Freshwater Ecology and Inland Fisheries (IGB) is a research centre located in Berlin, Germany. In their aquaponic system, tilapia (*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 tomatoes are also being grown with NFT. The effluent from 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 are grown in two separate loops. When needed, aquaculture water having gone through the mechanical and biological filters can be directed to the tomatoes. 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.

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

Wageningen University possesses several aquaculture systems. 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.

- **INA: Inagro (18/04/2017)**

The Inagro research centre is located in Rumbeke–Beitem, Belgium and rears Pike perch (*Sander lucioperca)*, which are fed on an omnivorous diet. The system is a recirculating aquaculture system (RAS) which can also be used in a decoupled aquaponic system since 2015. The system is composed of a RAS with fish tanks, a drum filter, and a moving bed biofilter (Kaldnes media). For the aquaponic experimentation, the RAS water used for the cleaning of the drum filter can be deviated from this loop and directed towards a settler in order to eliminate most of the particles before being stored in a tank outside the greenhouses. The water is then supplemented up to classical hydroponic levels (Resh, 2013) before being directed

towards the hydroponic parts. There, tomato plants are being grown in rockwool slabs with a drip irrigation system similar to the ones used by PCG and IGB.

- **GBX: Integrated and Urban Plant Pathology Laboratory, Gembloux Agro–Bio Tech**

The Integrated and Urban Plant Pathology Laboratory (IUPPL) possesses two systems in which tilapia (*Oreochromis Niloticus* from the CEFRA) are reared and fed with a vegetarian diet supplied by the aquaculture company Aqua4C (3.2mm Omegabaars Grower, AQUA4C, Kruishoutem, Belgium).

GBXR: Recirculating aquaculture system (RAS) (03/04/2017)

The first system is a recirculating aquaculture system composed of two fish tanks (0.380 m^3 each), a drum filter (Ratz Aqua und Polymer Technik, Remscheid, Germany), a biofilter containing Biocerapond media (Aquatic Science, Herstal, Belgium) and a sludge settler (Figure 3.S1). Water temperature is maintained at around 22 °C. Samples were taken from the sump and from the biofilms present on the ceramic plates.

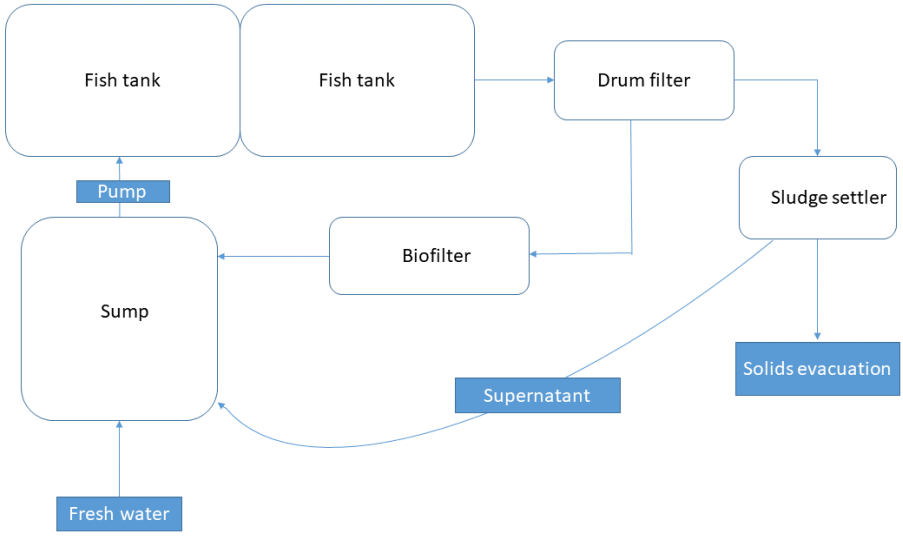


Figure 3-S1 Recirculating aquaculture system of the Integrated and Urban Plant Pathology Laboratory

GBXP: Plant and Fish Farming Box (PAFF Box) (27/04/2017)

The second system is a coupled system composed of a container topped up by a greenhouse. The fish are reared in two tanks in the container. The water from the fish tanks flows through a sieve gravity filter before entering the sump. It is then pumped through a pressurised microbeads biofilter and sent up to the hydroponic compartment. The hydroponic compartment is composed of four floating rafts. Full details are given by Delaide et al. (2017). Water samples were taken before the water enters the floating rafts. Biofilter samples were taken from the top of the pressurised biofilter.

- **ZHAW: Zürich University of Applied Sciences**

The ZHAW aquaponic system is composed of one aquaculture tank connected to three deep water hydroponic systems. Tilapia (Oreochromis niloticus) are reared in the fish tank while lettuces are grown in the hydroponic compartment. Full details concerning the layout of the system are given in Schmautz et al. (2017). Samples were collected for the biofilm present on the fish tank walls, from the roots of the lettuce plants and from biochips from the biofilter.

Table 3-S1 Water quality parameters for the individual systems at or around the time of sampling. DO: dissolved oxygen; EC: electro-conductivity; N: nitrogen (under the ammonium N-NH_4^+, ammonia N-NH_3 or total ammonia nitrogen TAN form); N-NO_2^- : nitrogen under the nitrite form; N-NO_3^-)

	UF	PCG 60	PCG 100	BQF	IGB	WU	INA	GBXR	GBXP
pH	6.7	7.2	6.8	7.92	N.D.	N.D.	8.56	7.8	7
Temperature (°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-NH_4^+ 0.08	N-NH_4^+ 0.09	N-NH_4^+ 0.09	TAN 0.21	N.D.	N.D.	N.D.	N-NH_3 < 0.2	TAN < 2
N-NO2- (ppm)	0.37	0.03	0.03	0.23	N.D.	N.D.	N.D.	N.D.	< 1
N-NO3- (ppm)	22.6	28	36.5	10-15	N.D.	N.D.	N.D.	N.D.	30-120

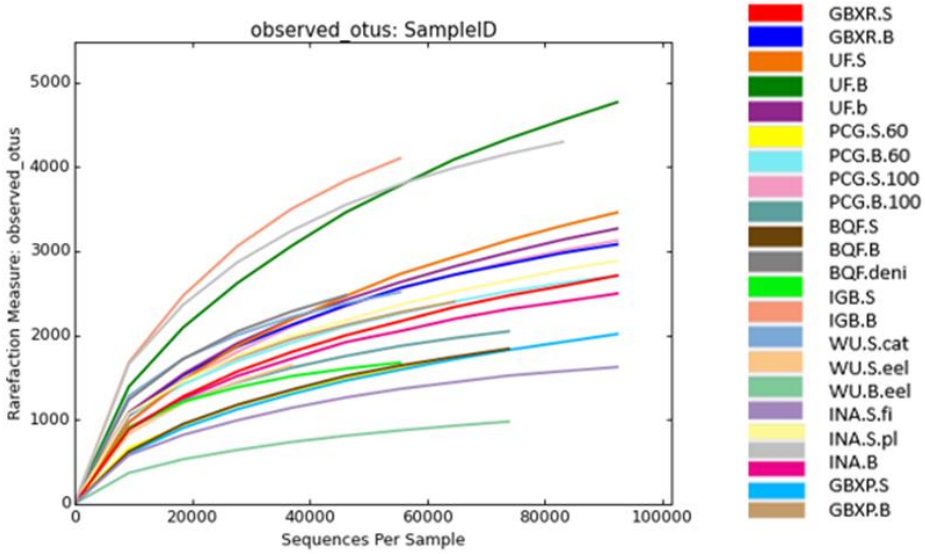


Figure 3-S2 Rarefaction curves of every samples indicating the number of observed OTUs according to sequencing depth. These curves were obtained with the alpha_rarefaction.py script

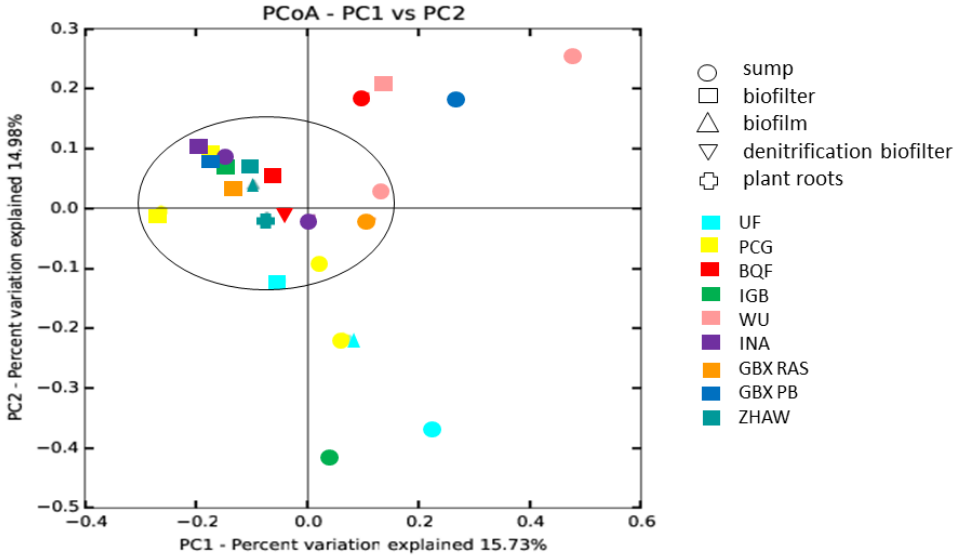


Figure 3-S3 Weighted UniFrac Principal Coordinates Analysis, including the eight visited systems and the Wädenswill Aquaponic System.

B. Supplementary material – chapter 4

Disease symptoms observed on the first group of lettuces during the fifth week of growth

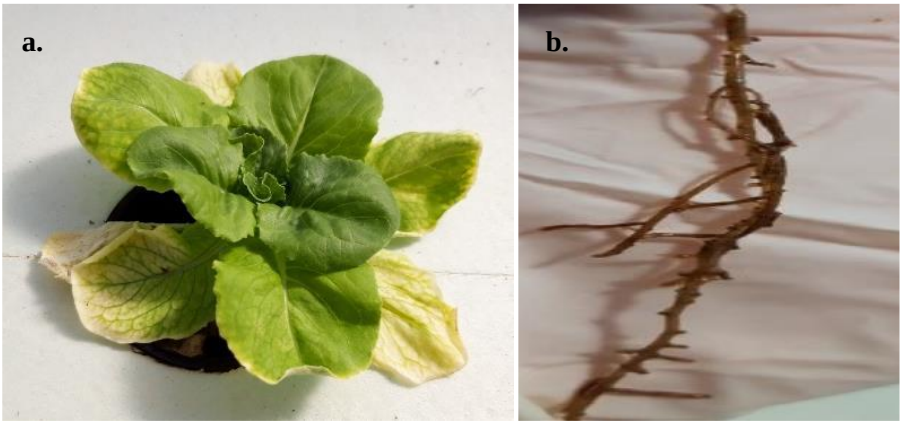


Figure 4-S1 Disease symptoms observed on the shoot (a) and roots (b) parts of the first group of lettuces during the fifth week of growth.

Details on the hierarchical clustering and PCA procedures

- **Pre-treatment of the data**

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

- **Hierarchical clustering analysis**

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

The dates were then clustered with the hclust command using the complete linkage method, which calculated the distance between the two most distant dates of two separate clusters, for all clusters. The resulting classification was visualised in the form of a dendrogram with the command plot, which provides an image of the groups and their proximity (**Figure 4-S2**) (Kazi et al., 2009).

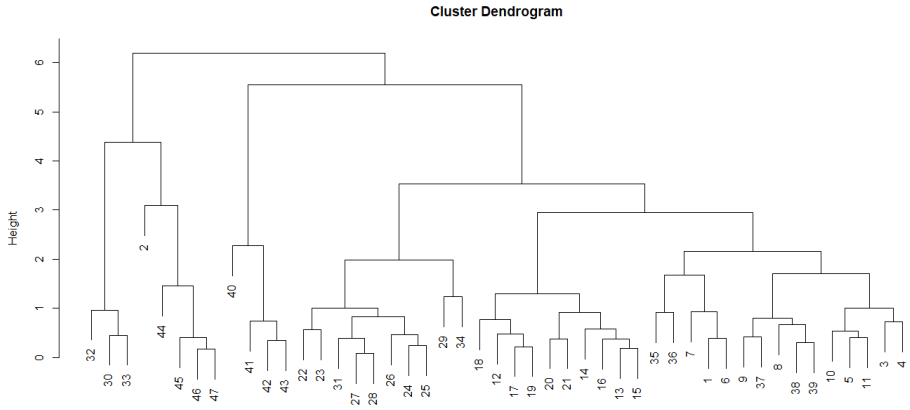


Figure 4-S2 Cluster dendrogram generated by the command plot, each number representing a day of the experiment (n° 1: March 8; n°48: May 13).

A partition then needed to be established, corresponding to the number of retained clusters. To do so, the R2 criteria provided by the command hclust.rsq and the prsq plot was used (**Figure 4-S3**). R2 is the result of the sum of squared deviations between the groups divided by the sum of squared deviations of all parameters. The higher this value, the more initial information is retained after the clustering. Thus, the partition was chosen by looking at the successive clustering. The clustering that resulted in a strong R2 decrease and therefore, a big loss of information between the groups was spotted and the fusions were interrupted before this clustering. In this way, a partition made of 5 groups was retained.

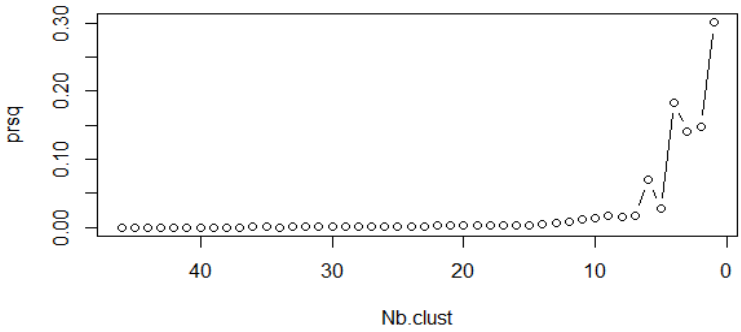


Figure 4-S3 Graph generated by the command prsq plot, representing the R2 decrease at each individual fusion, i.e. dates fusion.

The robustness of the partition was checked using an alternative partition method, i.e. the centroid method. Hence, the gravity centre of each group was calculated with the command aggregate using the standardised data. The centroid classification was then made with the command kmeans using the calculated centroids, which aimed to transfer some individuals between groups in order to improve the classification. The two obtained classifications were then compared thanks to the command table (**Figure 4-S4**). Since the two classifications did not differ much, we concluded that the chosen partition of 5 clusters was robust and it was thus retained.

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

Figure 4-S4 Table showing the comparison of the 5 clusters generated by the two classifications**.**

The chosen partition was visualised on the dendrogram with the command cutree (**Figure 4-S5**) and characterised in term of composition with the sort function, in term of individuals with table and in term of mean and standard deviation with aggregate command.

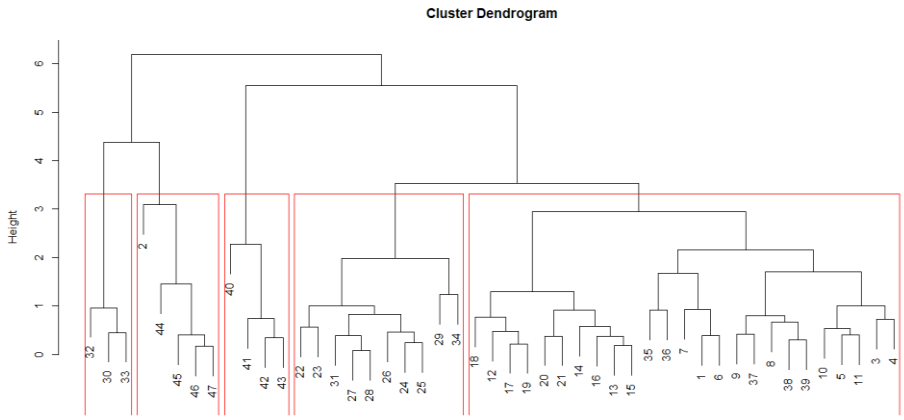


Figure 4-S5 Cluster dendrogram showing the partition made of the 5 physicochemical groups**.**

- **Visualisation of the clusters via PCA**

In order to describe the identified physicochemical groups in a multivariate space, the data were subjected to a principal component analysis (PCA). This enabled to reduce the number of variables, i.e. parameters, and to visualise the clusters in the factorial plans retained by the PCA. To do so, the FactoMineR package was loaded and the PCA command was carried out on standardised data. In order to determine the number of retained factorial plans, the eigen value graph was generated with the command plot (**Figure 4-S6**).

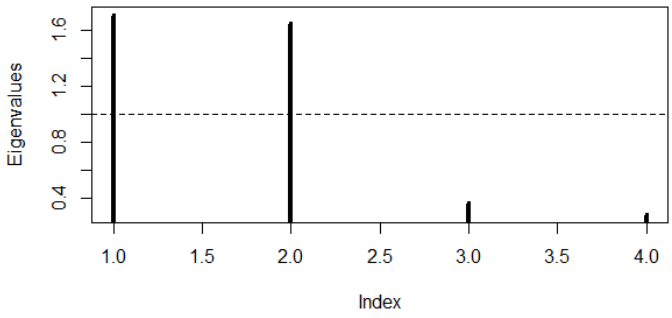


Figure 4-S6 Eigen values graph**.**

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

The variable factor map showing the correlations between each parameter to the two axes, i.e. principal components 1 and 2, was generated thanks to the plot command (**Figure 4-S7**).

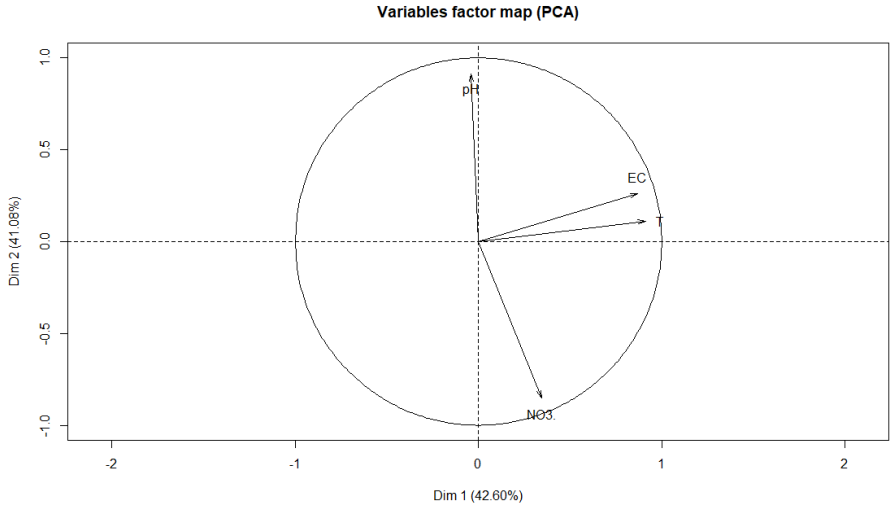


Figure 4-S7 Variables factor map of the first factorial plan.

The physicochemical clusters were then visualised on the first factorial plan using the command rainbow, that assigns a colour to each cluster, and the plot function. The centroids of the groups were also added to the plan by using the function PCA on another data set made of the clusters' identifiers and the standardised data.

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

Q-values of pairwise Kruskal-Wallis test performed to compare the diversity indices (Shannon and observed-otus) of the four compartments

- **Shannon**

Table 4-S1 Q-values of pairwise Kruskal-Wallis test performed on the Shannon indices of the four compartments.

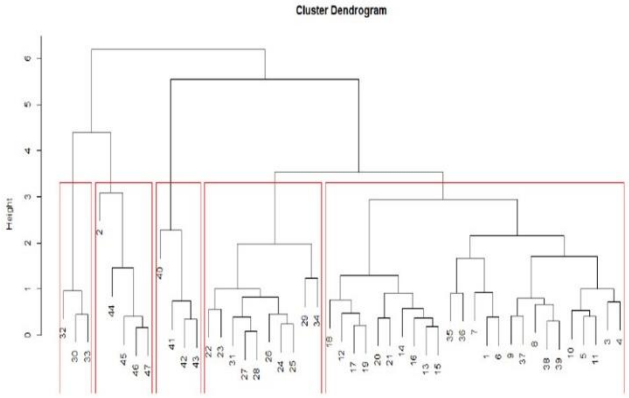
q-values	Biofilter	Sump	Rhizoplane	Root microbiota
Biofilter	/	0.0005	0.0007	0.4288
Sump	/	/	0.0005	0.0007
Rhizoplane	/	/	/	0.0008
Root microbiota	/	/	/	/

- **Observed_otus**

Table 4-S2 Q-values of pairwise Kruskal-Wallis test performed on the Observed-otus indices of the four compartments

q-values	Biofilter	Sump	Rhizoplane	Root microbiota
Biofilter	/	0.0452	0.0152	0.0012
Sump	/	/	0.0012	0.5750
Rhizoplane	/	/	/	0.0012
Root microbiota	/	/	/	/

Hierarchical clustering of water parameters and details of the water parameters for each group



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

Figure 4-S8 Cluster dendrogram showing the repartition in 5 groups of sampling dates based on water parameters and correspondence between date and day number

Table 4-S3 Physicochemical groups' characteristics. $\bar{x}$: mean; σ: standard deviation; CV: coefficient of variation; Min: minimum; Max: maximum

Group	Individuals		EC	pH	T	NO_3^-
Group A	25	$\bar{x}$	1317.83	7.88	24.29	208.83
		σ	40.02	0.08	0.61	11.63
		CV	0.03	0.01	0.03	0.06
		Min	1241.00	7.72	23.20	179.21
		Max	1389.46	8.04	25.15	234.62
Group B	5	$\bar{x}$	1168.10	7.68	24.15	200.35
		σ	34.27	0.21	0.19	14.04
		CV	0.03	0.03	0.01	0.07
		Min	1137.50	7.52	22.95	186.24
		Max	1227.00	8.07	23.40	219.20
Group C	3	$\bar{x}$	1223.23	8.09	20.80	124.49
		σ	10.72	0.02	0.52	1.32
		CV	0.01	0.002	0.03	0.01
		Min	1211	8.07	20.33	123.57
		Max	1231.00	8.11	21.35	126.00
Group D	10	$\bar{x}$	1317.47	8.06	24.41	134.82
		σ	9.92	0.06	0.66	11.37
		CV	0.01	0.01	0.03	0.08
		Min	1302.97	7.91	23.20	124.21
		Max	1330.24	8.13	25.06	155.58
Group E	4	$\bar{x}$	1304.12	7.45	23.32	228.96
		σ	11.99	0.21	0.12	5.84
		CV	0.01	0.03	0.01	0.03
		Min	1288.50	7.14	23.15	222.00
		Max	1317.50	7.60	23.40	235.66

C. Supplementary material – chapter 5

Protocols for growth media preparation

- **Luria-Bertani growth medium**

For 1 litre, pH 7: 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar

Add agar after checking pH

- **Potato Dextrose Agar growth medium**

For 1 litre: 39g potato dextrose agar

- **Tryptic Soy Agar growth medium**

For 1 litre: 30g tryptic soy broth

Boil for 1 min then add 15g agar

- **Nutrient Yeast Dextrose Agar growth medium**

For 1 litre: 7g peptone, 5g yeast extract, 1g glucose, 4g NaCl, 15g agar

Response of each strain to each biochemical test

Table 5-S1 Response of each strain to each biochemical test. Positive response: +; negative response: -

Strain	P solublisation	K solublisation	Ammonia production	Siderophores production	IAA production
A	+	+	+	+	-
B	-	-	+	-	-
C	-	-	+	-	-
D	+	+	-	+	-
E	+	+	+	+	-
F	+	+	-	+	-
G	-	+	-	+	-
H	-	-	-	++	++
I	+	+	+	+	-
J	-	-	+	-	-
K	-	-	-	+	+
L	-	-	-	+	+
M	+	+	+	+	-
N	-	-	+	-	-
O	-	+	-	+	-
P	-	+	-	+	-
Q	-	+	+	+	-
R	-	-	-	-	+
S	-	+	-	+	-
T	+	+	-	+	+++
U	-	-	+	-	-
V	-	+	-	+	+
Wa	-	-	+	-	-
Wb	+	-	+	-	-
Wc	+	-	+	+	-
X	-	-	+	-	-
Y	-	-	+	-	-
Z	-	-	+	-	-
α	-	-	+	-	+
β	-	-	+	-	+
γ	-	-	+	-	-

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