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par **Salam AL DAKDOUKI**

Comprehensive assessment of the effect of fish species on microbial diversity and dynamics in Recirculating Aquaculture Systems (RAS)

Sous la direction de Pascal FONTAINE et Frédérique CHANGEY

Mercredi 10 décembre 2025

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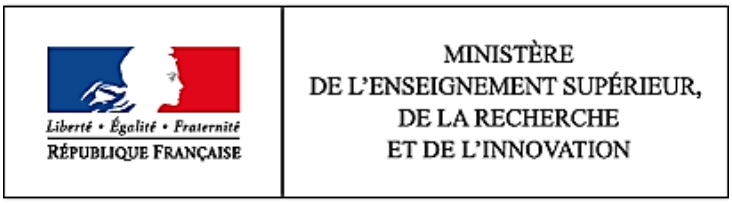

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« Tout est possible à qui rêve, ose, travaille et n'abandonne jamais »

Xavier Dolan

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List of abbreviations

Anammox : Anaerobic ammonium-oxidizing

ANOSIM : ANalysis Of SIMilarity

ANOVA : Analysis of variance

AOA: Ammonia-oxidizing archaea

AOB: Ammonia-oxidizing bacteria

ARGs : Antimicrobial resistance genes

ASVs : Amplicon Sequence Variants

CO_2 : Carbon dioxide

Comammox : Complete ammonia oxidizers

DNA : Deoxyribonucleic acid

DNRA : Dissimilatory nitrate reduction to ammonium

DO : Dissolved Oxygen

DPAO : denitrifying phosphorus-accumulating organisms

Dx : Day

EDG : European Green Deal

EPS : Extracellular polymeric substance

EU : European Union

F2F : Farm to Fork

FAO : Food and Agriculture Organization

FCR : Feed Conversion Ratio

FROGS : Find Rapidly OTU with Galaxy Solution

GHG : Greenhouse gas

H_2O_2 : Hydrogen peroxide

HB : Heterotrophic bacteria

HGT : Horizontal gene transfer

HS^- : hydrogen sulfide form

IFREMER : Institut Français de Recherche pour l'Exploitation de la MER

IMTA : Integrated Multitrophic Aquaculture

INRA : Institut National de la Recherche Agronomique

N : Nitrogen

N_2 : Dinitrogen gas

NaOH : sodium hydroxide

NGS : Next-generation sequencing

NH_3 : Ammoniac

NH_4^+ : Ion ammonium

NMDS : Non-Metric Multidimensional Scaling

NO_2^- : Nitrite

NO_3^- : Nitrate

NOB : Nitrite-oxidizing bacteria

OTU : Operational taxonomic unit

P : Phosphorus

PAOs : Phosphorus-accumulating organisms

PCoA : Principal Coordinates Analysis

PEA : Platform of Experimental Aquaculture

PERMANOVA : Permutational multivariate analysis of variance

PO_4^{3-} : Orthophosphate

RAS : Recirculating Aquaculture Systems

SBE: Communication on Sustainable Blue Economy

SI: Supplementary Information

SO_4^{2-} : Sulfate

SOB : Sulfide oxidizing bacteria

SP : Standard treatment

SRB : Sulfate-reducing bacteria

TAN : Total ammonia nitrogen

TiLV : Tilapia lake virus

UN : United Nations

USD : United States dollar

UV : Ultraviolet

I- State of art

1) Global situation of aquaculture

Aquaculture is the farming of aquatic organisms including fish, mollusks, crustaceans, and aquatic plants under controlled conditions (Ahmad et al., 2021; Troell et al., 2013). This farming involves some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators (*FAO*, 2020). Aquaculture sector plays a critical role in global food security and economic development (Bondad-Reantaso et al., 2023). From 1950, global aquaculture production increased significantly and reached an estimated 94 million tonnes in 2022 meanwhile capture fisheries production has remained largely unchanged for decades (fig. 1) (*FAO*, 2024). According to FAO's 2024 report, aquaculture production hit a new high by contributing to 51% of the total production of aquatic animal products and surpassed capture fisheries for the first time in 2022 (fig. 1) (*FAO*, 2024).

Rising demand for fish products is driving growth in aquaculture, which is steadily increasing its share of total fish production to help meet supply needs. World production of aquatic animals is projected to reach 205 million tonnes in 2032, with a share of 111 million tonnes from aquaculture (*FAO*, 2024). In fact, aquatic animals constitute a significant component of global nutrition due to their high-quality protein content and essential micronutrients, including omega-3 fatty acids, vitamins, and minerals, which are vital for human health and development (Dewals et al., 2024; Kelling et al., 2023).

Geographically, aquaculture production has been unevenly distributed (Guillen et al., 2025). Asia dominates the aquaculture industry providing 83.4 million tonnes of farmed aquatic animals in 2022 driven primarily by China, with a share of 55.4 percent (Li et al., 2016; *FAO*, 2024), which has been the top producer of farmed aquatic products since 1980 (Abdullah et al., 2024). While in Europe, aquaculture production in 2022 provided only 3.5 million tonnes (*FAO*, 2024).

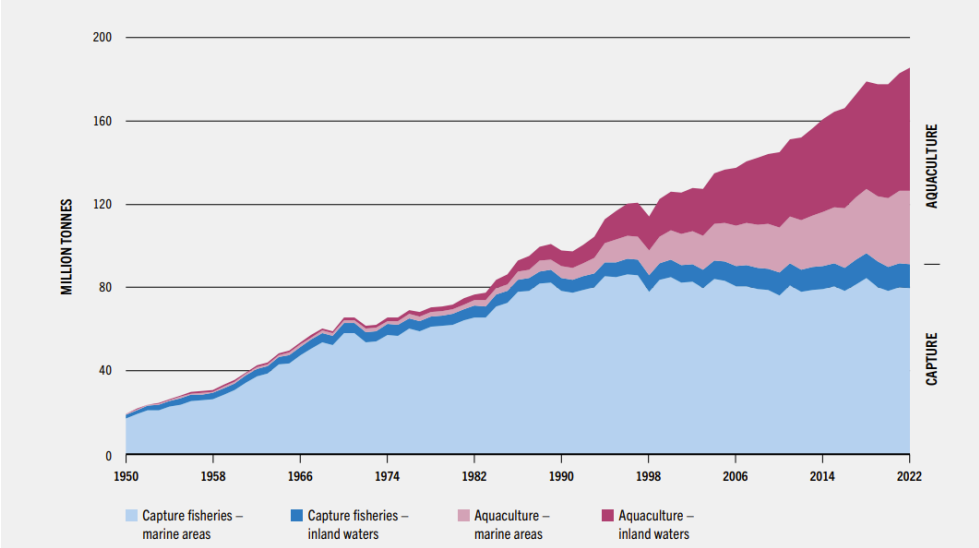

***Figure 1**. World fisheries and aquaculture production of aquatic animals, 1950–2022 (*FAO, *2024).*

2) State of aquaculture in Europe and in France

2.1. Aquaculture in Europe

Aquaculture is a critical sector contributing to food security and economic development in Europe. The European aquaculture sector is dominated by relatively few species (Eurostat, 2025). For instance, land-based freshwater production is dominated by trout and carp while in the marine finfish production salmon, trout, seabass and seabream dominate (Guillen et al., 2019; Svanidze et al., 2023). Since the late 1980s, european aquaculture production gradually decreased with modest signs of recovery emerging in the late 2000s (fig. 2) (*FAO*, 2024). From 2020 to 2022, aquaculture production in EU recorded an increase of 7.1% and surpassed capture fisheries in 2022 (Guillen et al., 2025; FAO , 2024). However, despite the investments and funds of the European Union countries that aimed to boost the sector, it accounted for only approximately 1.7% of global aquaculture by volume in 2014 (Guillen et al., 2019) and for less than 1% of the global aquaculture production in 2020 (EU aquaculture policy, 2023). This maybe because European countries are primarily importing and consumption regions (Heutte et al., 2023; Oglend, 2020; *FAO*, 2022).

The lack of growth in the aquaculture sector of the EU has been partly explained by complex and strict policies (European Commission, 2024; Toni et al., 2019), such as the European Green Deal (EDG) launched in 2019, Farm to Fork (F2F) strategy adopted in May 2020, and the communication on Sustainable Blue Economy (SBE) in May 2021, which prioritize minimizing environmental harms threating Europe and the world (EU aquaculture policy, 2023; European Commission, 2020), by cultivating, for example, low trophic-level species like algae and filter feeders known for their minimal

or even positive environmental impacts, such as nutrient assimilation (nitrogen and phosphorus) and carbon sequestration (Raza et al., 2025; Guillen et al., 2025).

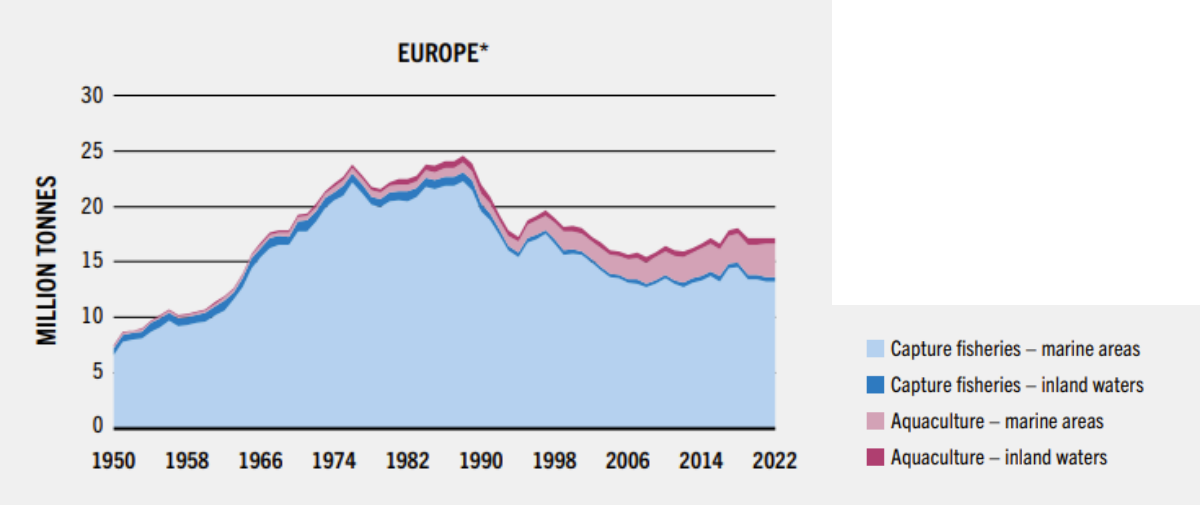

Figure 2. *Fisheries and aquaculture production of aquatic animals in Europe, 1950–2022 (*FAO*, 2024).*

2.2. Aquaculture in France

Historically, France was a global leader in aquaculture development in the 19th century (*FAO*, 2020). It is one of the highest producers in Europe among the EU countries (EU aquaculture policy, 2023; *FAO*, 2024). In 2022, France accounted for 5.7% of Europe's total aquaculture production, highlighting its moderate contribution to the continent's aquaculture output. French aquaculture industry is notably established in the production of farmed shellfish and trout (FranceAgriMer , 2023). However, current aquaculture production accounts for only a fraction of demand for seafood consumption, necessitating imports to meet high demand in France like high imports of Salmon (Govaerts, 2021; Heutte et al., 2023; Svanidze et al., 2023). In order to reduce imports and increase aquaculture production, French policies efforts aimed to improve the efficiency and sustainability of aquaculture production through the diversification of farming practices (Amoussou, 2022) like the implementation of polyculture practices for example (Thomas et al., 2021).

France is distinguished by its focus on sustainable aquaculture, with initiatives aimed at maintaining high-quality standards and reducing the environmental impact of aquaculture farms through innovative practices (Lucas et al., 2020; *FAO*, 2020). Advanced research institutions like IFREMER (Institut Français de Recherche pour l'Exploitation de la MER) and INRA (Institut National de la Recherche Agronomique) provide a good foundation for technological and environmental innovations (Ferlin, 2009). The country has seen progress coping with recommendations for a higher level of sustainability

by reducing levels of feed waste, the use of treatments and environmental impacts (European Commission, 2024; Sarker et al., 2024).

3) Environmental challenges and issues in aquaculture

Aquaculture, as a long-established food production sector, faces growing environmental, social, and economic challenges, threatening its resilience (Jennings et al., 2016; Oglend, 2020; Young et al., 2019).

The world is confronted with the critical challenge of ensuring food security for a rapidly expanding population, projected to reach around 10 billion by 2050 (UN news, 2017). With growing demand for fish, the growth and rapid development of farms with intensive and hyper-intensive production emerged (Boyd et al., 2020; Goddek et al., 2019). But while the industry is expanding to satisfy current demand for seafood, it has also caused major environmental damage worldwide (Ahmad et al., 2021; Young et al., 2019).

Aquaculture practices expansion has increased water pollution, habitat degradation and chemical contamination (Cozzolino et al., 2025; Hua et al., 2024). In intensive farming systems only 25–30 % of the nitrogen in feed is absorbed or utilized by the rearing organisms, while the majority is released into the water (Raza et al., 2025). The release of nutrients into surrounding water through uneaten feed and fish excreta such as nitrogen and phosphorus and significant amounts of organic waste (uneaten feed, feces) trigger algal blooms (Focardi et al., 2005; Gamble et al., 2021; Sarker et al., 2024), eutrophication, oxygen depletion and degradation of many ecosystems beneath open aquaculture systems (e.g. ponds and cages) affecting therefore their biodiversity (Hua et al., 2024; Luo, 2023; Streicher et al., 2021).

This excessive nutrient input, deteriorating water quality, can trigger pathogenic bacteria and viruses (Barange et al., 2018; Raza et al., 2025). Pathogens present in the water can disrupt the microbial balance of the ecosystem by competing with beneficial microorganisms participating in nutrient cycling (Ahmed and Azra, 2022; Espinal and Matulić, 2019). Chemicals, including antibiotics, usually are used to manage these pathogens and disease outbreaks but are another major concern (Boxall, 2004; Martinez-Porchas and Martinez-Cordova, 2012; Pérez-Sánchez et al., 2018). The misuse of antibiotics and the residual concentration of antibiotics persistent in the environment can contribute to antimicrobial resistance acquisition in pathogenic bacteria (Campanati et al., 2022; Focardi et al., 2005; Pérez-Sánchez et al., 2018), posing public health risks (Song et al., 2025). Escaped farmed stocks "Biological pollution" may also disseminate pathogens and resistant bacteria into surrounding ecosystems leading to disease spread but also leading to ecological disruptions, including competition

with native species (Campanati et al., 2022; Paez-Osuna, 2001; Song et al., 2025).

Aquaculture expansion has also raised significant concerns regarding limiting resources like water and space. The high use of water in aquaculture, especially in freshwater pond systems, is a major challenge in aquaculture (Boyd et al., 2022, 2020; Verdegem et al., 2006). For instance, filling a one-acre pond with an average depth of 1.25 m requires about 500,000 L of water, with a similar volume needed each year to offset seepage and evaporation (Roy et al., 2025). The impacts of aquaculture on the quantity and quality of water resources have impacted directly associated aquatic biodiversity (Boyd et al., 2017; Troell et al., 2013).

Moreover, aquaculture is increasingly vulnerable to the impacts of climate change, increasingly stressing aquaculture systems (O'Neill et al., 2022), as the majority of fish species are ectothermic and therefore lack the physiological mechanisms to regulate their internal body temperature (Engelhard et al., 2022; Réalis-Doyelle, 2016). Variations in ambient temperature exert both direct effects by influencing key physiological processes of fish, tightly related to external environmental conditions, such as metabolic rate, growth, and immune function as well as health, and survival of fish (Cascarano et al., 2021, 2021; Lefevre, 2019; Li et al., 2021; Policar et al., 2024; Réalis-Doyelle, 2016; Réalis-Doyelle et al., 2016) and indirect effects by modifying water quality parameters, including dissolved oxygen levels (DO), pH, and ammonia toxicity (Barange et al., 2018; Engelhard et al., 2022; Ficke et al., 2007). Many fish diseases have been reported to arise from fluctuations in water parameters such as temperature, pH, dissolved oxygen, salinity and ammonia concentration (Abdullah et al., 2024). Particularly, elevated temperatures in intensive aquaculture systems have been reported to increase the occurrence and virulence of pathogens leading to disease outbreaks, posing risks to both animal and human health (Barange et al., 2018; Sanches-Fernandes et al., 2022; Stride et al., 2014).

In fact, freshwater aquaculture systems, which dominate global production, are especially vulnerable to temperature warming causing eutrophication (Réalis-Doyelle et al., 2016), reduced oxygen in water (Engelhard et al., 2022), increased disease risks (Gubbins et al., 2013; Sanches-Fernandes et al., 2022), and increased evaporation and altered rainfall which reduce water availability and degrade water quality (Barange et al., 2018). Similarly, marine systems are affected the same way by warming causing also ocean acidification, compromising fish health and shellfish quality (Gubbins et al., 2013; Janmaimool et al., 2025), water salinity changes, oxygen depletion, harmful algal blooms, and habitat degradation (Cascarano et al., 2021; Roessig et al., 2004). Another major consequence of climate change is hydrological changes (Barange et al., 2018). Altered precipitation and extreme weather events further disrupt habitats and fish life cycles affecting therefore livelihoods of fish farmers and fish availability and quality (Barange et al., 2018; Martinez-Porchas and Martinez-Cordova, 2012; Muthoka et al., 2024) and causing a decrease in aquaculture productivity (Li et al., 2016).

As for socio-economic challenges of aquaculture, socially, stakeholders are responsible for impacts of aquaculture on a broad range of issues related to human welfare such as food security, environmental quality, working conditions, health issues, or economic opportunity (Bueno, 2008; Little et al., 2013). This includes for example the takeover of mangroves and disruptions in lands by intensive aquaculture, displacing local communities and reducing their access to fish and resources (Muthoka et al., 2024; Primavera, 1997). Additionally, to address environmental challenges, policymakers are investing in resilient adaptive and innovative strategies (Cappell and Huntington, 2023) such as Polyculture or integrated multitrophic aquaculture (IMTA) of multiple fish species occupying a shared culture system but different ecological niches (Amoussou, 2022; Biswas et al., 2020; Lalramchhani et al., 2019; Thomas et al., 2021) because diversification can increase productivity, resource utilization and ensure ecological sustainability while generating high revenues (Barrington et al., 2010; Blidariu and Grozea, 2011; Campanati et al., 2022; Lima et al., 2026) and Recirculating Aquaculture Systems (RAS), which are indoor controlled systems with limited interaction with natural ecosystems minimizing environmental damages and limiting pollution due to nutrient discharge for example (Badiola et al., 2012; Bartelme et al., 2017). Sustainability initiatives also include certification programs such as geographic and environmental restrictions, and disease control management (Young et al., 2019). However, further advancement and adopting new technologies require investment in research, infrastructure, and policy (Ficke et al., 2007; Little et al., 2013). Meanwhile, economic challenges include all the financial burdens of aquaculture, ranging from covering costs of infrastructure maintenance to market fluctuations such as changes in consumers preferences and tastes (Castillo et al., 2025; Li et al., 2016).

To achieve sustainability, aquaculture has to combine high fish production and mitigation of environmental impacts while integrating social responsibility and economic gain (Lima et al., 2026; Muthoka et al., 2024; Tal et al., 2009).

4) Types of aquaculture practice : monoculture vs polyculture

In aquaculture two rearing approaches are identified: monoculture and polyculture, each with distinct ecological and economic implications (Stryjecki, 2022; Zeng et al., 2021).

Aquaculture intensification (high stocking densities per rearing system) (Engle et al., 2017) has been regarded as a solution to limit the destruction of natural habitats and to decrease the need for new lands for aquaculture practices while increasing productivity, assuring food security and increasing incomes (Ahmed and Azra, 2022; Little et al., 2018; *FAO*, 2022). In this context, intensive monoculture systems were developed, involving the cultivation of a single aquatic species within the farming production

system (Lima et al., 2026). To this day, these monoculture systems largely dominates modern aquaculture and are considered a major contributor to food supplies (Dawood et al., 2025). They are widely used typically by small businesses (Stryjecki, 2022) due to their high production and simplicity to farmers, but the major focus on high commercial value species reliant on fishmeal-based feeds (FAO, 2020), combined with inefficient use of resource, heavy agrochemical use (Aubin et al., 2019), and high disease susceptibility (Kim et al., 2022; Zeng et al., 2021), have led to significant environmental impacts, including water pollution (nutrient, residual feed, fish waste) (Bregnballe, 2015;Aubin et al., 2019) and eutrophication, as well as threats to systems resilience, and sustainability (Medeiros et al., 2017). To meet the growing demand for aquatic food, while minimizing environmental impacts and promoting socio-economic development, a more sustainable approach is essential (FAO, 2025; Muthoka et al., 2024; FAO, 2024).

A sustainable alternative to monoculture practices is polyculture (Hisano et al., 2019), also referred to as coculture (ponds, aquaponics, recirculated aquaculture systems, integrated agriculture–aquaculture, integrated multi-trophic aquaculture), representing the farming simultaneously of two or more noncompetitive species together in a shared culture system (ponds, tanks, cages, pens) (Mehrim et al., 2018; Rohmana et al., 2015; *FAO*, 2020). Polyculture is based on the principle of maximizing resource efficiency by combining complementary species, thereby enhancing the utilization of available water and resources and generating higher overall productivity (Pěnka et al., 2024c; Thomas et al., 2020). In the past, traditional polyculture was developed thousands of years ago in Asia, particularly in China (Aubin et al., 2019; Lecocq et al., 2024) while in Europe, it began in monastic ponds during the Middle Ages with some cyprinids, notably the common carp, which had been introduced earlier by the Romans (Amoussou, 2022; Woynarovich et al., 2010). Polyculture remains a common practice in European aquaculture, combining predatory species such as *Perca fluviatilis*, and *Sander lucioperca* with lower-trophic level species including *Cyprinus carpio*, *Tinca tinca* and *Rutilus rutilus*, due to its multiple ecological and economic advantages (Lecocq et al., 2024).

5) The benefits of the polyculture practice

When the objective is to produce high-value fish that can cover the cost of feeds, monoculture practice offers ease of management (Milstein, 1992; Thomas et al., 2022) and uniformity in production considering the optimal growth parameters of a single species, with higher survival rates recorded of some fish species in monoculture than polyculture. For example, Nile tilapia (*Oreochromis niloticus*) showed higher survival rates in monoculture compared to polyculture with African catfish (*Clarias gariepinus*) in net cages of 4.5 m^3, as predation by fast-growing catfish reduced survival of tilapia in

polyculture (Mansour et al., 2021; Omondi et al., 2017). Intensive monoculture practice is considered as a major contributor to future food supplies (Boyd et al., 2017; Engle et al., 2017). However, high commercial value fish, depending heavily on high amounts of formulated feeds based on fish meal and fish oil, cause significant environmental harm (Medeiros et al., 2017; *FAO*, 2020; Thomas et al., 2021). Polyculture practice has been shown to be more sustainable alternative to monoculture (Can and Can, 2023; Pandit et al., 2023) for maximizing fish production as it may result in diverse beneficial consequences like ecological resilience, economic benefits, and environmental sustainability (Ibrahem et al., 2011; Park et al., 2018; Xu et al., 2020). The interest in traditional polyculture has been promoted through the synergy of interactions between species. Polyculture of species from different trophic levels has long been practiced in Europe and other regions to enhance efficiency, for example through the rearing of various carp species in ponds (Cappell and Huntington, 2023).

One key advantage of polyculture is improved nutrient cycling and water quality (Silknetter et al., 2020; Zijpp et al., 2007). Monocultures of fish have been linked to the risk of excessive nutrient release thereby negatively affecting surrounding waters and causing environmental degradation (e.g. algal bloom, eutrophication) and reduction of production quality (Herath and Satoh, 2015; Park et al., 2018; Shi et al., 2013). Meanwhile, polyculture systems have been reported to enhance upcycle nutrients efficiency, enhancing survival and production of fish (Boyd et al., 2020; Chopin et al., 2012; Shi et al., 2013). For example, monoculture of white shrimp (*Penaeus indicus*) recorded higher concentrations of nitrogen and lower survival of white shrimp compared to polyculture along with milkfish (*Chanos chanos*) which increased the efficiency of production (Lalramchhani et al., 2019). Another example is the polyculture of shrimp (*Penaeus vannamei*) with (*Siganus guttatus*) and (*Selenotoca multifasciata*) that showed lower sediment deposition of Nitrogen N and Phosphorous P in ponds compared with the monoculture of shrimp (Wang et al., 2023). Thus, polyculture of fish occupying different niches can significantly improve the N and P utilization rates, reducing their accumulation in aquaculture systems compared with monoculture, enhancing the survival of the cultured fish (Hoang et al., 2020; Lima et al., 2026; Wang et al., 2022).

Another key to the success of polyculture is mixing diverse species capable of exploiting different parts of the culture system and taking advantage of their interactions (natural behavior, feeding habits, and ecological niches) to help exploit the spatial and trophic resources more efficiently (natural feed, by-products, organic detritus) (Agrawal and Gopal, 2013; Amoussou et al., 2022a; Da Silva et al., 2006; Khalil et al., 2023). Through synergistic interactions, resources not used by one species can be used by the other in a biculture (Costa et al., 2025; Hisano et al., 2019) and in some cases can increase the productivity and growth of fish by increasing the biomass yield (Ng and Chan, 2021; Wahab et al., 2011). For example, in polyculture in ponds or cages of Nile tilapia (*Oreochromis niloticus*) and mullet

(*Mugil cephalus*), uneaten feed and fecal matter of tilapia sink to the bottom and become directly available for mullet consumption contributing to improved mullet growth. In contrast, these natural resources and wastes are not unutilized properly in monoculture systems (El-Sagheer, et al., 2008; Mehrim et al., 2018). This shows that polyculture can also reduce environmental pollution by reducing quantities of required feeds and wastes accumulation. The variety of species that have different spatial and feeding niches means that overall ecological efficiency can be higher than monoculture (Khalil et al., 2023; Little et al., 2018).

Polyculture has also a large effect on animal production (growth) and feed conversion efficiency (Abdel-hakim et al., 2006; Tibihika et al., 2015). This is supported by the findings of Shoko et al., 2016, who showed that when cultured in polyculture, Nile tilapia (*Oreochromis niloticus*) exhibited significantly greater mean weight gain and achieved a significantly higher net annual yield when reared together with African sharptooth catfish (*Clarias gariepinus*) in the same pond, compared to *O. niloticus* monoculture (Shoko et al., 2016). Another example of production benefits of polyculture practices is the high feed conversion ratio (FCR) record of Nile tilapia with Grey mullet and Common carp in polyculture ponds on feeding level of 2.5% (Abdel-hakim et al., 2006).

Additionally, some species combinations can be used as a sustainable biocontrol strategy of pathogens like cleaner shrimp (*Lysmata vittata*), and (*Epinephelus coioides*) polyculture reported to reduce ectoparasite burdens thereby enhancing fish welfare and decreasing the reliance on antibiotics and environmental pollution with chemicals (Vaughan et al., 2018).

Finally, polyculture can contribute to socio-economical sustainability, reflected by the fact that species diversification supports local food security, as more biomasses can be obtained per unit (Mehrim et al., 2018; Shrestha et al., 2018), and generates employment opportunities, but also allows for greater economic resilience and generating higher revenue for farmers while offering diversification of income (Azhari et al., 2026; Gonzalez-Mon et al., 2021; Kaminski et al., 2024). However, there have been only few detailed analyses of the socio-economic effects of different polyculture (Wang et al., 2022).

Overall, a shift from limited monoculture to polyculture helps reduce environmental impacts (Zhen-xiong et al., 2001) while enhancing productivity, resource use and animal welfare making it both an environmentally and socio-economically sustainable practice (Jana et al., 2007; Pandit et al., 2023).

6) Successful polyculture: Requirements and challenges

6.1. Compatibility and advantages of complementarity of species

Successful polyculture systems depend on a fundamental requirement called species compatibility (Stryjecki, 2022). It refers to the cultivation of species with optimal growth and minimal predation and

interspecific competitive behaviors (Trung, 2014; Makvandi et al., 2024; Shyne Anand et al., 2018). When incompatible species are combined, antagonistic interactions such as aggression may arise (Milstein, 1992).

Therefore, the rearing species are selected based on their compatible but also complementary traits and behaviors with the objective of maintaining animal welfare and optimizing overall farming performances (Can and Can, 2023; Thomas et al., 2021; Wang and Lu, 2016). Considering both zootechnical data and behavioral responses such as space occupation, morphological traits (e.g. the rate of development and the species food requirements) (Amoussou et al., 2022a; Mehrim et al., 2018; Thomas et al., 2020), species are selected based on different complementary ecological roles. First, spatial complementarity refers to mixing a water-column feeder such as pikeperch (*Sander lucioperca*) (Kozłowski et al., 2014; Mihailov et al., 2017) with a bottom feeder like sterlet (*Acipenser ruthenus*) (Thomas et al., 2020). This basic complementarity of species that use the same trophic resource in different spatial niches is called monotrophic polyculture (fig. 3B) (Kozłowski et al., 2014; Thomas et al., 2020). Secondly, species can be additionally selected based on trophic complementarity refereeing to associating species that consume different feed in the same environment (Chary et al., 2025).

Combining several species inhabiting different trophic levels of water volume and consuming different trophic sources is called multitrophic polyculture and has been proven to be highly effective for ecological balance and offering environmental and economic benefits (Chopin et al., 2012; Martinez-Porchas and Martinez-Cordova, 2012). As in multi-trophic polyculture, waste or unused feed from one species can for example serve as a valuable trophic resource for the other cultivated aquatic species (e.g. filter-feeding fish), reducing therefore water pollution and eutrophication but also decreasing the need for excessive feed and its cost (Pawar et al., 2020; Wang et al., 2022). This strategy includes cultivating herbivorous species like grass carp (*Ctenopharyngodon idella*) with zooplanktivorous species like bighead carp (*Hypophthalmichthys nobilis*), phytoplanktivorous like silver carp (*Hypophthalmichthys molitrix*) and/or omnivorous like common carp (*Cyprinus carpio*) (fig. 3A) (Milstein, 1992; Thomas et al., 2021).

Additional forms of complementary interactions found in aquaculture include commensalism and mutualism, adding benefits including improved health, water quality, and food availability (Papoutsoglou et al., 2002; Chithambaran, 2019; Felton et al., 2016; Pěnka et al., 2021). Specific behavior in some fish species can resuspend nutrients in sediment layers, benefiting others through enhanced nutrient availability (Wahab et al., 2011). For instance, associating Common carp (*Cyprinus carpio*) with rohu (*Labeo rohita*) is an example of commensalism in which carp behavior foraging and burrowing in the pond bottom releases nutrients into the water, increasing food availability for the rohu and therefore enhancing its growth (Chary et al., 2025; Rahman et al., 2006). Commensalism

refers to an interaction in which only one of the reared species benefits but not the other while in the contrary, in mutualism both reared species gain a net benefit (Silknetter et al., 2020). An example of mutualism is the use of cleaner species capable of consuming ectoparasites causing important problems for fish culture (Abbas et al., 2014) like cleaner shrimp (*Lysmata amboinensis*) who showed a significant consumption capacity of harmful monogenean parasite *Neobenedenia sp.* (eggs and larvae), in polyculture in closed experimental aquaria with (*Pseudanthias squamipinnis*) reducing thereby their infection while the shrimp gain nutrition from the parasite (Militz and Hutson, 2015). This shows that the positive biotic interactions among combined species in polyculture can be an efficient alternative to replace excessive pharmaceutical interventions and treatments (Pradhan et al., 2020; Zeng et al., 2021).

All the insights above highlight the importance and the complexity of maintaining compatibility and complementarity in polyculture systems. A large and variable combination of species may be possible but there is a need for hypothesis-driven research to select the best species match to ensure sustainable productivity (Amoussou, 2022; Lecocq et al., 2019).

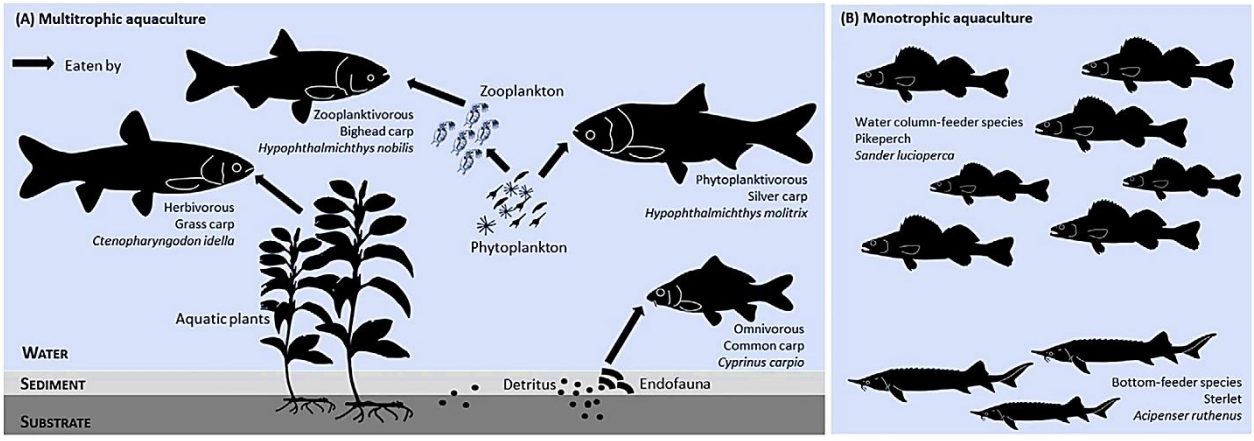

Figure 3. *Example of basic complementarity in: multitrophic polyculture illustrating the traditional polyculture of Chinese carp in pond system (A) and monotrophic polyculture of pikeperch and sterlet illustrating spatial complementary (B) (Thomas et al., 2021).*

6.2. Challenges of polyculture

The choice of species combinations, stocking density and the choice of the optimal species ratio are also essential to prevent increased competition for food and space which can therefore negatively impact fish growth and prevent predation between and among species (Khalil et al., 2023; Marques et al., 2016; Milstein, 1992). Despite its benefits, coculture can sometimes favor one species over the other (Sarturi et al., 2021). For instance, the pond polyculture of mullet (*Mugil platanus*) with marine shrimp (*Litopenaeus vannamei*) revealed positive impact on the growth of the mullet but a negative

effect on the growth of the shrimp in comparison with its monoculture, suggesting being due to food competition between the reared species (Costa et al., 2013). Polyculture can also be a cause of stress for some species. Benni (*Mesopotamichthys sharpeyi*) experienced stress when cultured with Nile tilapia (*Oreochromis niloticus*) impacting negatively its growth indices suggested to be the result of competition ability of Nile tilapia against the Benni (Makvandi et al., 2024).

Different species stocked at varying densities have been reported to modify their environments to differing degrees (e.g. through their feeding activities or excretion) impacting therefore microbial communities' function and/or structure (Yan et al., 2019; Zhao, 2021). To make the system more efficient, pathogen control is also essential in polyculture by designing species combinations (Debnath et al., 2024; Yan et al., 2019; Zijpp, 2007). It was reported that some species combinations may trigger spillovers of interspecific pathogens (Ibrahem et al., 2011; Thomas et al., 2021). For instance, the pond polyculture of prawn (*Penaeus monodon*) with jellyfish and sea cucumber has been reported to increase viruses and pathogens which may cause a significant threat to fish and human health (Zhao, 2021) and can potentially disseminate, affecting negatively the wild populations in the nearby ecosystems in case of open aquaculture system. Moreover, more studies are needed to demonstrate beneficial interspecies interactions that support pathogens and disease control in polyculture (Molloy et al., 2011; Skår and Mortensen, 2007).

Nutrient input monitoring is also a challenge that needs to be considered in polyculture (Ng and Chan, 2021). Polyculture systems can lead to high nutrient inputs, in cases of improper choice of stocking densities, feed management, and species combination (Liu et al., 2023; Zhang et al., 2018). Wrong stocking densities choice in a certain ecosystem is tightly linked to high nutrient levels (Hossain and Islam, 2006; Liu et al., 2023; Yan et al., 2019; Zijpp, 2007) and can affect negatively the production and nearby environment with potential eutrophication risk (Streicher et al., 2021).

Polyculture in open aquaculture system is also vulnerable to environmental changes. Different species have varying environmental growth factors and development rates (Stryjecki, 2022; Thomas et al., 2021). For a successful polyculture, one important consideration is to ensure that water quality meets the requirements of the selected species (Lazartigues et al., 2013, 2012), so fluctuating in physico-chemical variables including temperature, salinity, dissolved oxygen or nutrient compounds level (nitrogen and phosphorus) can influence negatively on the growth, production and health of cultured species raising therefore management complexity (Kim et al., 2022; Yu et al., 2017). Also, fluctuations in water parameters due to climate change (O'Neill et al., 2022), inappropriate species combinations, incorrect stocking densities or any other possible factor, can induce pathogen and diseases outbreaks in the system that can disseminate in the surrounding ecosystems (Abdullah et al., 2024; Martinez-Cordova and Martinez-Porchas, 2006; Ibrahim et al., 2011).

Polyculture appears to be more complex to manage and more demanding for farmers, which raises concern about its adoption by small-scale farmers. Addressing this challenge through stakeholder education and guidance with the need for better multi-disciplinary research is essential to provide additional evidence on polyculture benefits (Cappell and Huntington, 2023; Alexander et al., 2016).
Overall, despite of all polyculture benefits, this practice still raises environmental and management challenges and vulnerabilities particularly in open aquaculture systems. In intensive aquaculture, the demand for more sustainable practices that conserve resources and minimize waste like polyculture is increasing (Campanati et al., 2022). Consumers also nowadays are showing greater awareness of the environmental footprint associated with their food consumption (Vaughan et al., 2018). Hence, closed systems such as Recirculating Aquaculture Systems (RAS) can substantially overcome a number of these challenges of polyculture, particularly off-farm impacts and reducing the dependence on external climatic and environmental conditions (Pyanov, 2014; Wang et al., 2025).

7) Development of Recirculating Aquaculture Systems

7.1. Advantages of RAS

Despite notable progress to ameliorate traditional aquaculture sustainability, through polyculture for example, concerns about environmental footprint and long-term sustainability continue to rise. Major challenges remain, including: climate change impacts, the high demand for land and water resources (Ahmed and Turchini, 2021; Arbour et al., 2024; Badiola et al., 2018; Ranjan et al., 2023), and the pollution of water and surrounding ecosystems with chemicals, antibiotics, and wastes which can lead to aquatic ecosystem degradation and potential risks to fish and human health (Ahmed and Thompson, 2019; Murray et al., 2014; Bregnballe et al., 2015). This environmental vulnerability of open aquaculture systems (cages, tanks, and ponds) has driven more interest in indoor Recirculating Aquaculture Systems (RAS) (fig. 4) (Böpple et al., 2024). These systems are key technology that have gained significant attention in recent years in Europe and in many other parts of the world, as a more sustainable alternative to traditional ponds and open aquaculture systems for the production of high fish stocking densities (Dalsgaard et al., 2013; Espinal and Matulić, 2019; Verdegem et al., 2006; Vielma et al., 2022).
From an environmental perspective, indoor RAS are considered as a more environmentally friendly alternative of open aquaculture production systems (Bregnballe, 2022). They offer several advantages compared to open aquaculture systems, including lower water intake and land use, reduced environmental impacts, greater control over culture conditions, better waste elimination and nutrient recycling, and no risk of escapees (EUMOFA, 2020; Martins et al., 2010; Timmons and Ebeling,

2010). These systems adopt a closed farming approach that enables a remarkable efficient reuse of 95–99% of rearing water through multiple water treatment technologies that rely on using mechanical and biological filters to remove fish waste while constantly supplying the water with oxygen essential for fish survival (Wang et al., 2025; Bregnballe et al., 2015; Timmons and Ebeling, 2010). Meanwhile aquaculture ponds for example were reported to have significant water loss resulting from water exchange, evaporation or seepage (Gephart et al., 2017; Martins et al., 2010). RAS not only reduces water supply and exchange which contributes to the conservation of water resources but also minimizes environmental impact by treating and reducing water within the system (Davidson et al., 2016; Jiang et al., 2022; Kamali et al., 2022; Moschos et al., 2022). This decreases significantly the discharge of pollutants into surrounding ecosystems, preventing environmental hazards pollution and the release of pathogens from intensive culture systems into surrounding waters (Badiola et al., 2018; Böpple et al., 2024; Bregnballe et al., 2015). Additionally, these closed systems allow the isolation of the culture system from the surrounding ecosystems, reducing the dependance on external environmental conditions (Badiola et al., 2017; Rurangwa and Verdegem, 2015). RAS technologies allow a major control over physico-chemical parameters of water including temperature, pH, DO, ammonia, nitrites ensuring ideal growth requirements for each fish species and enabling flexible production timing all year round. The stability of adequate water parameters is essential to improve fish growth, welfare and health (Brown et al., 2025; EUMOFA, 2020; Murray et al., 2014; Losordo et al., 1992). Finally, indoor RAS enhances biosecurity (Brown et al., 2025; Davidovich et al., 2020) by isolating fish stocks from external pathogens and environmental pollutants, significantly lowering the risk of disease outbreaks in RAS. Additionally, diseases can be better controlled and even avoided thanks to better control over water parameters and treatment (EUMOFA, 2020). The system's flexibility allows it to support diverse fish species at high densities within indoors while producing healthier and safer aquatic products (Aich et al., 2020; Lopez et al., 2022; Michaud, 2007).

Operationally, RAS can lower costs in different ways. High levels of control in RAS eliminate predator losses and reduce the need for chemicals such as antibiotics. In addition, transportation costs are reduced due to the proximity of RAS facilities to markets (Aich et al., 2020).

Effective management and success of RAS require a deep understanding of all filtration and water treatment mechanisms.

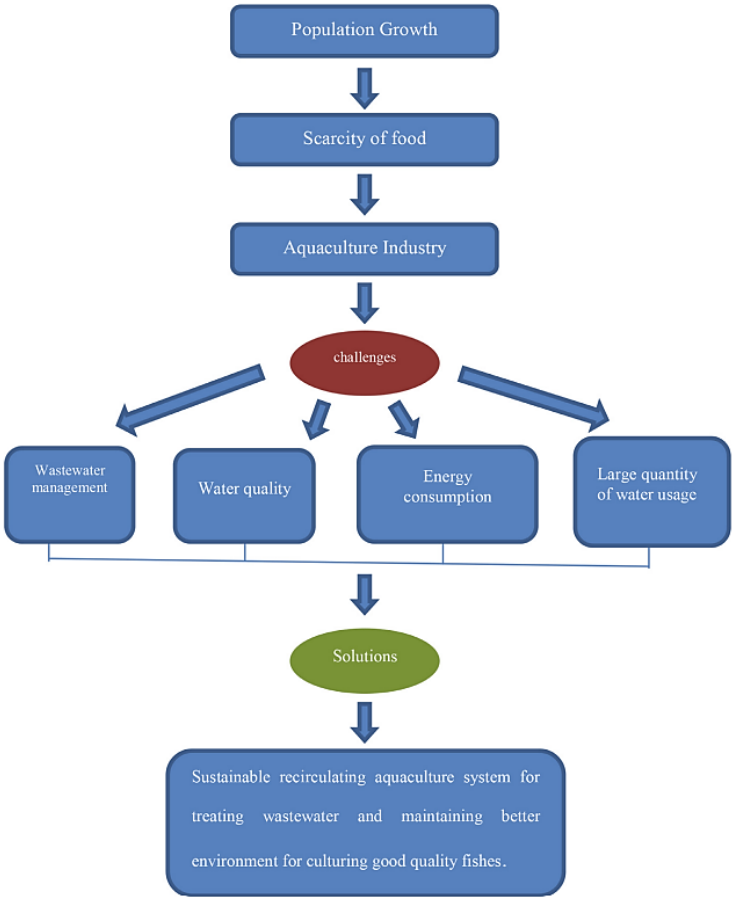

Figure 4. *Challenges of aquaculture industry and Recirculating Aquaculture Systems as a more sustainable alternative to traditional aquaculture (Tom et al., 2021).*

7.2. Water treatment process in RAS

Maintaining good water quality is essential for fish health and growth. A typical RAS is composed of fish tank, pumps, pipes, possible feeding systems, and most importantly water treatment units involving multiple stages to ensure water quality and efficient nutrient management. It is achieved through advanced mechanical and biological filters, to remove solid and dissolved waste respectively (e.g., ammonia, organic matter, and CO_2), possible disinfection units including ozone or Ultraviolet UV sterilization and oxygen supply and degassing unit as shown in fig. 5 (Bregnballe et al., 2015; Almeida et al., 2019; Chen et al., 2019; Fossmark et al., 2020; Lindholm-Lehto et al., 2021).

In its early treatment stages, wastewater must be cleared of residual feed, excrement, and other solid particulates present in large quantities. Mechanical filtration is the first filtration used to remove these large solid particles (Turcios and Papenbrock, 2014; Xiao et al., 2019). The primary water flow passes through mechanical equipment such as drum filters which remove particles approximatively greater than 40-100 µm in diameter resulting primarily from the production of feces and uneaten food

(Summerfelt and Penne, 2005; Timmons et al., 2018). Solid particles are trapped inside the rotating drum, directed to a backwash zone, and transported out of the mechanical filters for further handling (Vielma et al., 2022). The proper remove of solid particles is a major step to avoid blockage of the system, excessive growth of heterotrophic microorganisms or opportunistic bacteria (Aguilar-Alarcón et al., 2020; Jafari et al., 2024a) including fish pathogens and adverse effects on biological filters (Fossmark et al., 2020) and on the reared fish welfare and health, directly damaging fish gills for example (Aguilar-Alarcón et al., 2022; Chen et al., 1993; Lindholm-Lehto et al., 2021).

The residual small suspended solids, in particular those below 20 μm, remain in the system and will be solubilized (Chen et al., 1993; Fernandes et al., 2015) serving as organic matter substrate for heterotrophic bacteria. Derived organic matter rich in carbon are then oxidized by heterotrophic bacteria (HB), by consuming oxygen, producing carbon dioxide and ammonia (Aguilar-Alarcón et al., 2020; EUMOFA, 2020).

Next, water enters biofilters containing bioelements "biofiltration media" colonized by microbial biofilms hosting microbial communities responsible for the removal and conversion of dissolved waste, produced by fish metabolism, into less harmful compounds to sustain water quality, and this biological process is called "biofiltration" (Hagopian and Riley, 1998; Schreier et al., 2010; Timmons et al., 2018). The main nutrients controlled in RAS are dissolved ammonia (NH_3/ NH_4^+) and nitrite (NO_2^-) found at high concentrations in wastewater of the system resulting from uneaten feed and from fish excretion as a result of the metabolism of feeds (Foore, 2016; Guerdat et al., 2013; Schumann and Brinker, 2020; Takeuchi, 2017; Zhang et al., 2023). Ammonia and nitrite are toxic for fish, their toxicity levels vary according to each species and specific rearing conditions in RAS (Gomez Isaza et al., 2021; Laza et al., 2021).

Ammonia conversion and removal in RAS biofilters is called "nitrification" and involve several interconnected microbial pathways (fig. 6) to convert highly toxic ammonia into less toxic nitrate (NO_3^-) (Chary et al., 2025). Traditionally, nitrification has been described as a two-step aerobic process, where ammonia-oxidizing bacteria (AOB) and archaea (AOA), such as *Nitrosomonas* and *Nitrosospira*, oxidize ammonia to nitrite, which is then converted to nitrate by nitrite-oxidizing bacteria (NOB), including *Nitrobacter* and *Nitrospira* (Neissi et al., 2022; Sarosh et al., 2024). More recently, complete ammonia oxidizers (comammox), represented by certain *Nitrospira* species, were identified as capable of performing both steps within a single organism, to convert ammonia to nitrate (Godzieba et al., 2025; Sakoula et al., 2021). In parallel, anaerobic ammonium-oxidizing (anammox) bacteria contribute to nitrogen removal by oxidizing ammonia or directly nitrite to dinitrogen gas N2 in low-oxygen zones of the biofilter (Gottshall et al., 2021; Ismail et al., 2022; Jetten et al., 2009). In addition, denitrification, primarily driven by heterotrophic bacteria such as *Pseudomonas aeruginosa*,

reduces nitrate to nitrogen gas under low-oxygen conditions, preventing nitrate accumulation that can impair fish health at high concentrations (Gupta et al., 2024; Schreier et al., 2010).

Phosphorus (P) is also a nutrient found at high concentrations in RAS wastewater released from feed and fish excretion, which can lead to eutrophication if discharged untreated into the environment. A typical RAS is reported to remove 65–96% of P trapped in solid particles through the effective mechanical filtration, but dissolved phosphorus remains. Dissolved phosphorus can be incorporated by microorganisms in the biofilter into microbial biomass and finally eliminated from the system via sludge removal (Li et al., 2021; Liu et al., 2023; Zhang et al., 2018; Zheng et al., 2023).

Consequently, microbial water quality is essential and includes bacterial abundance and enzymatic activities (Fossmark et al., 2020) thereby the proper management of biofilters and knowledge of their operational requirements are crucial in RAS. Any disruption in biofilter microbial balance in RAS can lead to the emergence of opportunistic pathogenic bacteria menacing the productivity of RAS and the quality of the aquatic products (Blancheton et al., 2013; Li et al., 2023). The structure and performance of microbial communities in aquaculture biofilters are influenced by many factors including the farmed fish species, the type of feed, and physicochemical parameters of water including pH, alkalinity, temperature, salinity, as well as the overall microbial diversity within the system (Chen et al., 2006; Godzieba et al., 2025; Jafari et al., 2024a; Neissi et al., 2022).

Various systems are usually used in RAS to monitor and control physicochemical parameters of water depending on the reared species requirements (Li et al., 2023; Schreier et al., 2010). For instance, pH decreases due to release of carbon dioxide by fish (Hu et al., 2011) so maintaining pH range, adequate with reared species needs and also essential for nitrification process, requires sometimes adding alkaline chemicals like sodium hydroxide (NaOH) (Jafari et al., 2024b, 2024a).

Also, depending on the needs, the system can be supplemented with an oxygenator to increase the oxygen content of the water (Sarosh et al., 2024). Oxygen levels demonstrated significant effects on growth, feed intake and conversion of juveniles Pikeperch (*Sander lucioperca*) cultured in RAS along with water temperature (Policar et al., 2024). Water aeration adjusts dissolved oxygen levels to sufficiently compensate for the oxygen loss used by fish and nitrifying bacteria and to provide the oxygen demand of fish and bacteria breaking down organic waste and dissolved compounds in biofilter (Losordo et al., 1992; Michaud, 2007; Bregnballe, 2015; Vielma et al., 2022). This is often combined with a degassing system to prevent toxic CO_2 accumulation and flows back to the rearing tanks essential for fish welfare especially in intensive production (Espinal and Matulić, 2019; Gupta et al., 2024; Jafari et al., 2024b). It was reported that when CO_2 levels can rise above safe limits, originated from fish gills excretion or released by nitrification and denitrification bioreactors (Steinberg et al., 2017). Therefore, the ability of fish hemoglobin to bind and transport oxygen is greatly reduced, which

can lead to respiratory distress even if the water contains high concentrations of DO (Hu et al., 2011). Some species can be particularly vulnerable to diseases and infections. So, to finish the treatment and enhance biosecurity and quality of water, it may be necessary to implement additional disinfection measures to remove pathogens from the system including bacteria, viruses, fungi and small parasites (Bazyar Lakeh et al., 2013; Davidson et al., 2016; Gonçalves and Gagnon, 2011; Re and Timur, 2016). Cleaning physical methods such as UV light lamps or chemical methods such as ozone generators are usually used for this disinfection step, but over-dosing with ozone can be harmful for fish and farmers(Aguilar-Alarcón et al., 2022; EUMOFA, 2020). In case of sanitary problems diverse chemical disinfectants, including Chloramine-T and H_2O_2, can be used to improve water quality and to treat diseases in RAS, mostly in outdoor RAS. But, if improper doses are used these chemicals can potentially inactivate essential microbes in the biofilter (Bögner et al., 2021; Pedersen et al., 2019; Quezada-Rodriguez et al., 2022).

Finally, the treated water returns to the fish tanks and 1-2% of new water can be added to compensate for water loss but also to dilute the accumulated nitrate level in the system (Vielma et al., 2022).

These series of water treatment and control steps ensure the need to maintain water quality, minimize nutrient discharges, and achieve high sustainability.

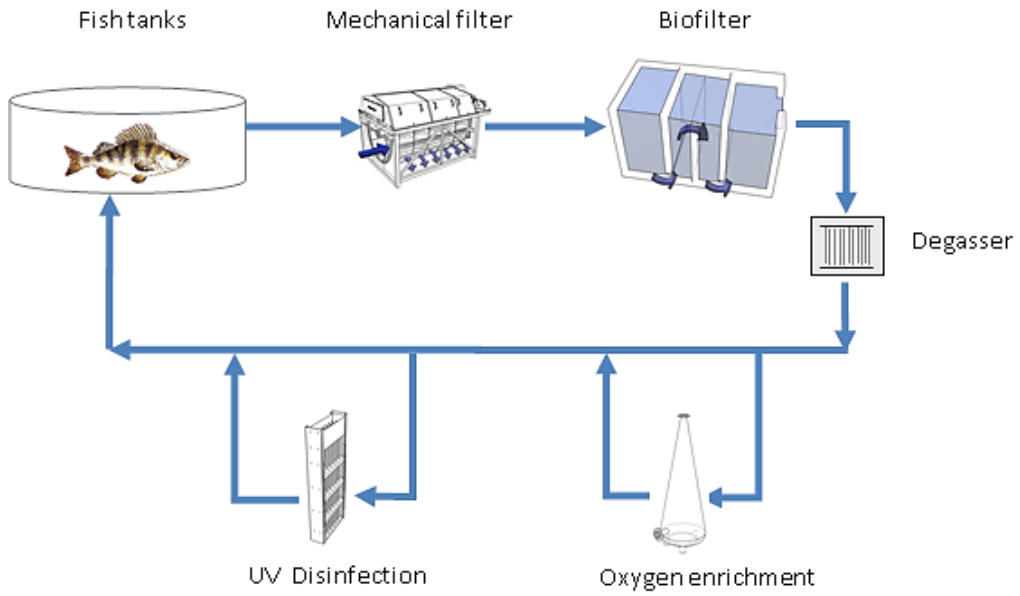

Figure 5. *Schematic of water treatment and recirculation in a Recirculating Aquaculture System (RAS) (Eurofish, 2017).*

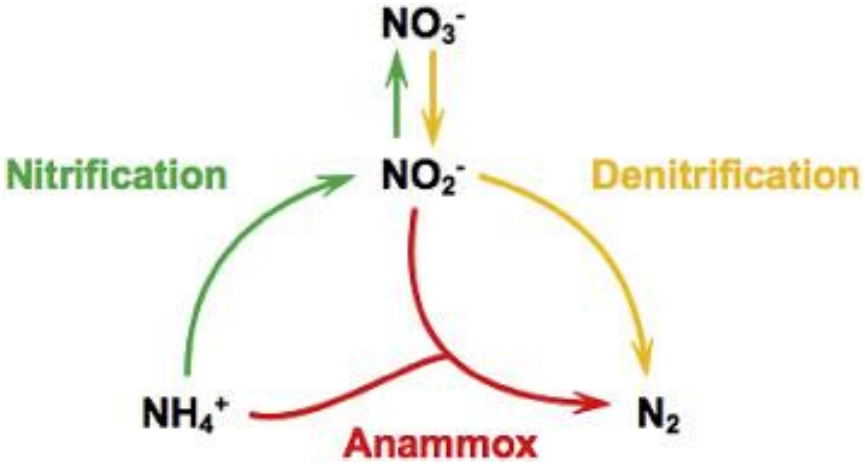

Figure 6. *Schematic representation of nitrogen conversion processes: Nitrification, Denitrification, and Anaerobic Ammonium Oxidation (Anammox) (Dai et al., 2019).*

7.3. Challenges faced by RAS

The number of RAS farms around the world is increasing but despite their sustainable potential and promises, RAS is still not widely used globally and still faces many challenges (Brown et al., 2025; Nie et al., 2020).

RAS main disadvantage is their very expensive construction, operation and input costs (feed and energy) requiring high initial investments and challenging the profitability of RAS (Murray, 2014; Badiola et al., 2018). For instance, an estimated investment of 500,000 USD is needed annually to produce 100,000 pounds of fish in RAS, excluding costs for land or buildings, due to installation expenses of RAS equipment notably due to the elevated water treatment costs (Ahmad et al., 2021; Dalsgaard et al., 2013; Nie et al., 2020). High operating costs in RAS are due to high energy consumption and costs, such as electricity used in RAS, the ventilation and the water cooling along with greenhouse gas (GHG) emissions are also rated as major constraints preventing the widespread application of RAS especially in developing countries (Ahmed and Turchini, 2021; Barange et al., 2018; Brown et al., 2025; Espinal and Matulić, 2019). For instance, the high rearing temperature required for pikeperch (*Sander lucioperca*) rearing significantly increases energy costs associated with heat pumps installation and operation (Dalsgaard et al., 2013). As a result, RAS is economically feasible when the market value of the cultivated fish exceeds the production cost (Bregnballe, 2022; Dalsgaard et al., 2013; Verdegem et al., 2023).

In addition to high initial investment, RAS are technologically advanced and complex and require the need for highly trained operators to carefully manage RAS due to their sensitivity to technical failures such as filtration inefficiencies (EUMOFA, 2020; Martins et al., 2010; Ngoc et al., 2016). While

advancements in filtration reactors have improved waste management, the biofilter in RAS is sensitive to many factors including blockage by suspended solids, fluctuations in temperature, or chemical treatments leading to filtration inefficiencies challenging RAS (Murray et al., 2014). Studies reported that large solid particles have indirect negative effects by triggering the proliferation of bacteria and fast-growing opportunistic heterotrophs microorganisms that can compete with biofiltres nitrifying bacteria for space, oxygen, and nutrients (Pedersen et al., 2017). In addition, trained personnel should have a high knowledge in water parameters control, specific and essential for species growth and survival, especially species sensitive to water parameters change such as Pikeperch (*Sander lucioperca*) (Aich et al., 2020; Pourhosein-Sarameh and Falahatkar, 2024).

Although closed RAS systems offer high levels of control over water quality parameters (Badiola et al., 2012) and disinfection units enhancing biosecurity measures, high stocking densities still pose challenges in preventing disease outbreaks (Rupp et al., 2019). In fact, pathogens can be introduced through external sources like infected fish or contaminated water. If the system is not well managed, pathogens may persist within biofilms and re-enter the system since the water is continuously reused in RAS (Gonçalves and Gagnon, 2011; King, 2001). Consequently, repeated exposure of fish to these pathogens and the potential of the development of asymptomatic carriers of pathogens can lead to rapid diseases spread, affecting water quality, elevating stress levels, impacting reared species welfare and growth, and ultimately increasing mortality rates (Ende et al., 2024; Gonçalves and Gagnon, 2011). An additional, major drawback in RAS is the accumulation of off-flavors compounds in fish (geosmin and 2-methyl-isoborneol) that can be produced by cyanobacteria and actinomycete bacteria in RAS biofilms (Brown et al., 2025). This also highlights the need for additional expertise in microbes and diseases management in RAS (Aich et al., 2020). To limit or prevent pathogens in RAS, application of chemicals including antibiotics is often widely used, thanks to their proven effectiveness and ease of use in preventing the spread of diseases among reared species (Kampouris et al., 2022; Sha et al., 2022; Wang et al., 2019). Antibiotics may accumulate in fish tissues, causing adverse effects on fish health like oxidative stress and negatively affecting growth causing also a risk to human health (Ende et al., 2024; Mei et al., 2024). Approximately 10 million premature deaths caused by antibiotic resistance transmission to humans are expected by 2050. Yet no documented studies mention effective control or removal of antibiotics from RAS, highlighting the need for further research and investigation (O'Neill, 2016; Sha et al., 2022).

Finally, accumulated sludges rich in nutrients N and P, resulting from RAS filtration processes (mainly from drum filter), represent challenges to achieve an eco-friendly system if released directly into the environment (Ende et al., 2024; Luo, 2023). Moreover, huge amounts of nutrients, notably ammonia, present in the wastewater and sludge of RAS (Campanati et al., 2022; Dawood et al., 2025) require

treatment before being released to the environment which increases costs even more and therefore consumer market prices (Chen et al., 1993; Guerdat et al., 2013; Turcios and Papenbrock, 2014).
In order to ensure economic sustainability in a fluctuating market RAS continues to optimize and diversify with the emergence of aquaponic systems, and IMTA systems for example (Chopin et al., 2012; Dalsgaard et al., 2013). Thus, innovative approaches for cost-effective RAS coupled with high production and high quality of aquatic products are essential to move towards global adoption of RAS (Houle et al., 2011; Sitek, 2020; Turcios and Papenbrock, 2014) and one way to do this is through species diversification (Amoussou et al., 2022a; Thomas et al., 2021).

8) Polyculture adoption in RAS

8.1. Polyculture as a strategy to overcome RAS challenges

At present, intensive aquaculture in RAS is predominantly conducted in monoculture (Amoussou, 2022; Badiola et al., 2018; Blancheton et al., 2013). Nevertheless, polyculture can represent a potential strategy to mitigate some of the challenges faced by RAS. Intensive farming in RAS is a costly production system that requires the input of external feed, which represents a significant portion of annual expenses (Bregnballe, 2015; Baki and Yücel, 2017; Liu et al., 2023; Thomas et al., 2021). Culturing different species with complementary exploitation of trophic and spatial resources could enhance feed utilization thereby contributing to lower excessive feed costs and decreasing the need for maintenance operations (Espinal and Matulić, 2019; Mugwanya et al., 2022).
In polyculture systems, different species can utilize various forms of waste produced within the system. For instance, filter-feeding species removing suspended particles and the use of unexploited resources or uneaten feed and debris of one species by secondary species (Chen et al., 2023; Hamilton et al., 2023; Zhang et al., 2018) is a potential strategy to maintain water quality and improve eco-efficiency of the system (Kim et al., 2022; Kuang et al., 2020) which can help reduce the overall cost of cleaning operations and effluent treatment in RAS, and can further help maintain the health of cultured species (Chen et al., 2023; Guerdat et al., 2013; Mihailov et al., 2017) since accumulation of suspended solid waste has been reported to lead to nutrients accumulation (mainly nitrite) and thereby favorizing bacterial growth menacing microbial water quality and reared species in RAS (Aich et al., 2020; Michaud et al., 2014, 2006).
The integration of polyculture within RAS, allows diversification of production to meet fluctuation of market, can maximize production within the same rearing environment and can improve economic sustainability, social benefits and system profitability by selecting species with high market value like shrimp for example (Espinal and Matulić, 2019; Gupta et al., 2024; Mugwanya et al., 2022; Thomas

et al., 2021). However, unlike ponds and/or cages, little knowledge is available on the benefits of polyculture in RAS (Khalil et al., 2023), as few experimental studies have addressed its implementation in these systems, maybe that's why they're not widely adopted yet (Dawood et al., 2025; Kozłowski et al., 2014).

8.2. Polyculture benefits in RAS: Why polyculture outperforms monoculture in RAS systems

While intensive fish monoculture in RAS can achieve high productivity, it is expensive as it necessitates very high feed inputs (Baki and Yücel, 2017). In addition, these systems show low feed efficiency as it is unlikely that a single species will exploit all parts of the water column (surface and bottom layers) (Mihailov et al., 2017). This results in a loss of feed inputs which in turn generate effluents and wastes (notably organic waste and ammonia) deteriorating water quality (Li et al., 2023; Wang et al., 2025, 2022) and reducing health status of reared species (Liu et al., 2015; Wang and Lu, 2016) therefore potentially increasing economic concerns (Amoussou et al., 2022b).

Alternatively, implementation of polyculture in RAS can offer many potential advantages enhancing system resilience (Amoussou, 2022; Thomas et al., 2021). One key advantage is that polyculture allow for better feed utilization and reduce wastage by making better use of feed as in polyculture compatible species with complementary behaviors can make use of water and trophic resources more efficiently, optimizing thereby economic gains and profitability (Amoussou et al., 2022b; Mansour et al., 2021; Pěnka et al., 2024a). For example, the polyculture of common carp (*Cyprinus carpio*) and tilapia (*Oreochromis aureus*) in RAS has demonstrated greater overall biomass gain for both species, thanks to better efficient use of distributed feed and improved control of rearing parameters related to water quality (Papoutsoglou et al., 1992). Another example of efficient resources utilization in RAS is the polyculture of pikeperch (*Sander lucioperca*) with Danube sturgeon (*Acipenser ruthenus*) that enabled better feed utilization, as the sturgeons fed on the bottom and collected the leftover feed not consumed by the pikeperch feeding in the water column and never or rarely retrieves food that sinks to the bottom (Kozłowski et al., 2014; Mihailov et al., 2017). However, choosing the correct combination of fish species is highly recommended to ensure the success of polyculture in RAS, limiting the consequences of detrimental interactions such as feed intake and swimming competition (Chithambaran, 2019; Dawood et al., 2025).

Polyculture systems may also enhance the profitability of cultivating species that are otherwise economically unprofitable in monoculture (Policar et al., 2016; Stickney, 2012). This major benefit is attributable to the more favorable biotic and abiotic conditions established under polyculture

conditions, which can promote growth and performance of some species in comparison with their monoculture (Shoko et al., 2016; Thomas et al., 2020) which help enhance productivity (Dumont et al., 2020). For example, Thomas et al., 2020 showed an increase in the biomass gain of pikeperch when farmed in polyculture in indoor RAS aquariums (100 × 60 × 50 cm) with tench (*Tinca Tinca*), corresponding to an increase in pikeperch weight from 25 % in monoculture to 51 % in polyculture without any significant impact on survival (Thomas et al., 2020).

Polyculture may also contribute to amelioration of fish welfare (Thomas et al., 2021). For instance, the mixed culture of pikeperch and largemouth bass (*Micropterus salmoides*) showed better feed and use of the middle part of RAS tanks by pikeperch who were found less timid, calmer and as suggested by Penka et al., 2021 suffered less stress compared to the monoculture stocks of pikeperch (Pěnka et al., 2021).

Species with various trophic levels and complementary feeding behaviors can optimize nutrient cycling and reduce waste accumulation (Amoussou, 2022; Kim et al., 2022) improving the sustainability and resilience of RAS production systems (Amoussou et al., 2022a; Medeiros et al., 2017).

Polyculture in RAS is gaining traction for sustainable development of these systems, but it is still not as widely adopted as monoculture practices (Thomas et al., 2022, 2021). Combining multiple species, with different environmental needs in RAS can be challenging and requires the careful selection of compatible species with similar physicochemical requirements, that do not compete for food and space, and that are also commercially valuable to maximize economic returns (Amoussou et al., 2022b; Bocek, 2004; Shyne Anand et al., 2018). So, species diversification could increase the complexity of RAS (e.g. maintaining water quality) and necessitate adjustment measures and operational costs (EUMOFA, 2020; Gupta et al., 2024; Luo et al., 2014) to avoid possible adverse effects on behavior (Oladi et al., 2017), survival and growth (Kadir et al., 2007) and health and immunity of fish (Abbas et al., 2014). In addition, selecting the right stocking density and feed ratio is essential for because it can directly affect the growth, survival, and overall production of the reared species and profitability in polyculture systems (Debnath et al., 2024; Hossain and Islam, 2006; Pěnka et al., 2021; Zijpp, 2007).

Disease occurrences remain a main challenge of polyculture (Abbas et al., 2014). For example, infection of common carp (Cyprinus carpio) and rohu (Labeo rohita) by Lernaea cyprinacea, a freshwater copepod, has been reported in pond polyculture (Hemaprasanth et al., 2011). Disease transmission is also a key challenge faced by RAS (Loufi et al., 2026; Xue et al., 2017). In fact, in polyculture pathogens may spread between species, and treatments suitable for one may be harmful to others, complicating therefore biosecurity measures (Amoussou et al., 2022a; Thomas et al., 2021). This highlights the complexity of managing microbial communities (Guan et al., 2020) in the context

of polyculture in RAS which has clearly not been investigated yet.

9) Microbial complexity in RAS

Microbial ecology examines the diversity of microbes within microbial communities and how they interact (i) with one another and (ii) with their environment. A microbial community can be defined as an assemblage of co-occupying and potentially interacting microbes sharing a common living space (Boughner and Singh, 2016; Gray and Head, 2008; Panikov, 2010; Ramette, 2007).

Continuous water recirculation, characteristic of RAS, makes it favorable to microbial growth. These communities consist of a collection of microorganisms, including bacteria, fungi, and viruses (Dahle et al., 2022; Rurangwa and Verdegem, 2015). The recent advances in next-generation sequencing (NGS) analyses have broadened our understanding of the complexity of microbial communities in RAS paving the way for continuous research in order to optimize these systems (Boughner and Singh, 2016; Drønen et al., 2021). Furthermore, numerous studies on microbial ecology have been conducted in RAS, exploring the diversity, dynamics, and interactions of microorganisms (Guan et al., 2020; Rojas-Tirado et al., 2017; Tal et al., 2003). For instance, studies have shown that most bacteria in RAS belong to Proteobacteria phylum followed by Bacteroidetes phylum (Bartelme et al., 2017; Pulkkinen, 2020). Within Proteobacteria group, Rhodobacterales (of Alphaproteobacteria class) has been reported to dominate water and biofilms in RAS as they are known as key surface colonizers in marine environments (Dahle et al., 2022; Pulkkinen, 2020, 2020; Rud et al., 2017).

RAS is a complex microbial ecosystem. It hosts two principal microbial niches: (1) surface-attached sessile microbial communities, colonizing biofilter media and system surfaces (e.g. tanks, pipes), and (2) free-floating or suspended planktonic microorganisms within the water column (Acosta et al., 2021a; Crab et al., 2007; King et al., 2004). These microbial populations of RAS can be categorized into autotrophs and heterotrophs (Bugten et al., 2022). Autotrophic bacteria are typically associated with biofilter compartments, where they play a key role in oxidizing ammonia and nitrite (King, 2001; McKnight and Neufeld, 2024). While HB, on the other hand, are not only fixed within biofilms but also found to adhere to suspended particles, where they contribute to the degradation of organic matter (Bugten et al., 2022).

Microorganisms in RAS can be introduced through different external pathways: make-up water, air, food, stocked fish, non-sterile equipment and human (Bartelme et al., 2019; Llewellyn et al., 2014; Rojas-Tirado et al., 2018; Rud et al., 2017). Microbial interactions, abundance and activities in RAS are crucial for creating optimal operation of RAS and optimal water quality for fish growth, production, welfare and survival (Michaud et al., 2009; Rojas-Tirado et al., 2017; Rurangwa and Verdegem, 2015; Schoina et al., 2022). Through their interactions with each other and with larger organisms, microbial

communities participate directly and indirectly in diverse processes such as waste decomposition and production, highlighting their importance for ecological balance of the system influencing both water quality and fish health (Chhanda et al., 2024; G. M. Osman Et Al., 2021; Preena et al., 2021; Rud et al., 2017).

The most relevant factors influencing microbial ecology in RAS are: (i) system design (e.g. biofilter type, disinfection, water recirculation rate) (ii) management routines (e.g. cleaning frequency), (iii) physicochemical water parameters (pH, temperature, salinity, DO, concentrations of waste), (iv) external sources (mainly make-up water, reared species, feed), and in some cases (v) grazing by eucaryotes (e.g. gastropods) (Almeida et al., 2021; Attramadal et al., 2012; Bakke et al., 2017; Blancheton et al., 2013; Chen et al., 2019; Duarte et al., 2019; Rud et al., 2017; Schreier et al., 2010; Zhang et al., 2023) (fig. 6).

Water quality, as a major factor for optimal operation of RAS, has been particularly well reported to be a major selective pressure affecting significantly microbial communities' structure and composition (Auffret et al., 2013; Chen et al., 2019; Michaud et al., 2009; Navada et al., 2019) and thereby a key factors in disease occurrence (G. M. Osman Et Al., 2021; Guan et al., 2020).

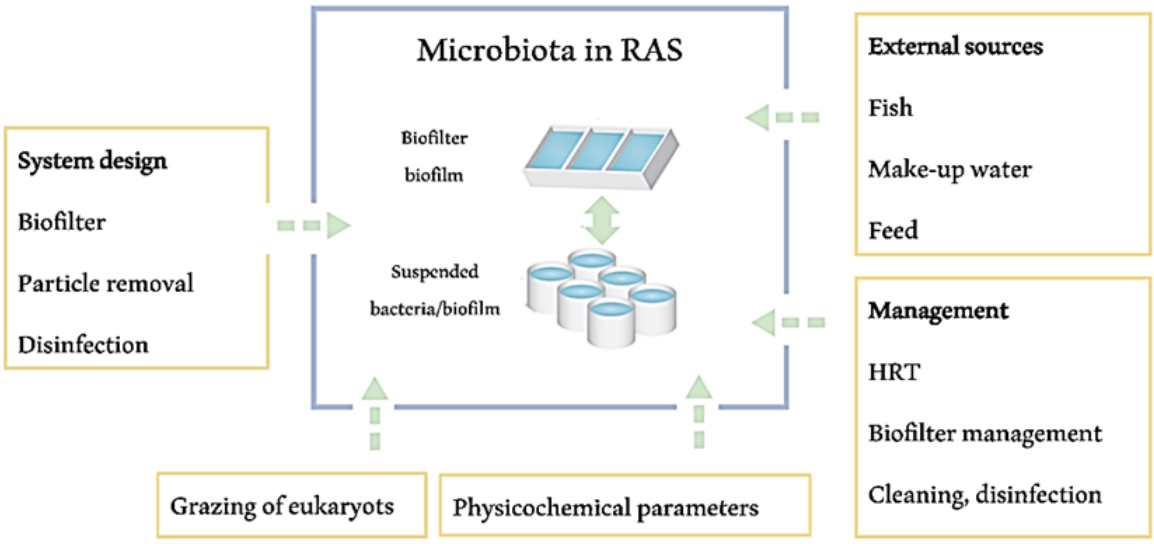

Figure 7*. Factors influencing microbial ecology in Recirculating Aquaculture Systems (RAS). Roles of system design, external inputs, management practices, physicochemical parameters, and grazing by eukaryotes. (Dahle, 2022).*

Furthermore, microbial management in RAS and research on the composition and diversity of microbial communities and their relationship with their environment in RAS are thereby one key challenge that should get special attention.

10) Microbial compartments in RAS

The major compartments hosting microbial communities in RAS are (i) biofilms in biofilters and other RAS surfaces, (ii) microbes in system water and suspended particles and (iii) reared species-associated microbiomes (Bartelme et al., 2019; Bugten et al., 2022; Rojas-Tirado et al., 2018; Rud et al., 2017).

10.1. Water and Suspended particles

In RAS, the water column is a microbial habitat with its own assembly characterized by its own taxonomic diversity and community structure, harboring planktonic microbial communities freely in the water flow including Rhodobacterales for example (Bartelme et al., 2019; Duarte et al., 2019). Functionally, planktonic microbial populations can include microorganisms with beneficial functions such as organic matter decomposition, improving water quality. These microorganisms often include heterotrophic taxa can also be found in suspended particles (feed and fish waste) in the water column (Aguilar-Alarcón et al., 2020; Auffret et al., 2013; EUMOFA, 2020; Michaud et al., 2006). However, water planktonic assemblages can also harbor harmful microorganisms. Consequently, good water quality in RAS, crucial for the growth and survival of reared species (Lindholm-Lehto et al., 2021), is directly linked to microbial water quality parameters including bacterial abundance and enzymatic activities (Gregersen and Joao, 2020; Fossmark et al., 2020; Rojas-Tirado et al., 2018).

10.2. Fish microbiota

Fish themselves constitute a major microbial compartment in RAS, that differs from the surrounding water and biofilms, harboring microbial communities mainly bacteria in their tissues: skin, gills and gastrointestinal tract (Diwan et al., 2023; Lorgen-Ritchie et al., 2021; McMurtrie et al., 2022; Minich et al., 2020), associated with various important host physiological functions notably in immunity and disease resistance (Haque et al., 2022; Lazado et al., 2025; Mes et al., 2023).

These host-associated microbiomes are different and specific for each tissue (De Bruijn et al., 2018; Moschos et al., 2022). For instance, the gut typically harbors communities responsible for digestion and nutrient processing such as Proteobacteria, Firmicutes and Bacteroidetes the most abundant typically found in fish intestinal microbiota (Talwar et al., 2018). Meanwhile skin and gill mucus are reported to be the first barrier to infection, so they harbor taxa mainly implied in protection from pathogens and capable of producing antimicrobial compound (Balasubramanian et al., 2012; Benhamed et al., 2014; Guivier et al., 2020; Yi et al., 2018).

In fact, host-associated microbiotas are highly diverse and are often species-specific (Dai et al., 2021).

In general, fish microbiotas are influenced by numerous intrinsic factors, such as the morphology of the gastrointestinal tract and immune system state (Butt and Volkoff, 2019; Smith et al., 2015), and extrinsic factors, such as the initial captive-state of the environment prior to capture (De Bruijn et al., 2018; Dulski et al., 2020; Egerton et al., 2018).

For instance, The gut microbiome of fish has been highly studied and was reported to be mainly influenced by habitat but also strongly shaped by the gut morphology of fish species (Butt and Volkoff, 2019; Egerton et al., 2018; Kim et al., 2021). Fish digestive systems shape is highly diverse (fig. 7) including species with no true stomach counting for 20% of fish species such the Gobiidae and Blennidae families, species with straight stomach, U-shaped, or Y-shaped stomachs (Wilson and Castro, 2010). Beyond the shape, the length and structure of the intestine, associated with diet, also drive the microbial composition of gut as herbivorous fish often have long intestines harboring Firmicutes as the dominant phylum for example, whereas Proteobacteria and Vibrionaceae were frequent rather than Firmicutes in most carnivores having shorter intestines (Egerton et al., 2018).

In general, fish associated microbial communities are usually divided into two main groups: resident (autochthonous) and transient (allochthonous) communities (Nyholm et al., 2022; Ringø et al., 2003). Firstly, resident microbes are long-term colonizers able to attach and colonize the mucosal surfaces (skin, gills, gut) or even live within epithelial tissues, where they form close associations with the host's cells (Legrand et al., 2020, 2020; Ringø et al., 2016). These resident communities are strongly shaped by the host's genetics and are thought to play important roles in fish health (Banerjee and Ray, 2017; Legrand et al., 2020; Wang et al., 2010). Secondly, transient microbes are short-term free-living and non-adherent microorganisms (Nyholm et al., 2022). For instance, in fish gut, transient microbes mostly found in the digesta are originated from fish food and surrounding water and are eventually eliminated via fish feces (Banerjee and Ray, 2017; Ringø et al., 2016; Viver et al., 2023). Unlike the resident communities, transient microbiota is mainly influenced by external factors such as the environment and diet (Egerton et al., 2018; Yang et al., 2022).

Overall, fish species (e.g. trophic level, sex, feeding habit), their life stage and habitat history (e.g. diet, season, habitat) exert strong selective pressures that shape "core" community structure that are distinct between hosts even when they share the same rearing water (Bano et al., 2007; Clements et al., 2007; Cordero et al., 2015; De Bruijn et al., 2018; Dhanasiri et al., 2011; Zhao et al., 2020). The specification of microbial communities by tissue and host species highlights the importance of fish compartment as an important ecological reservoir for microbes within RAS.

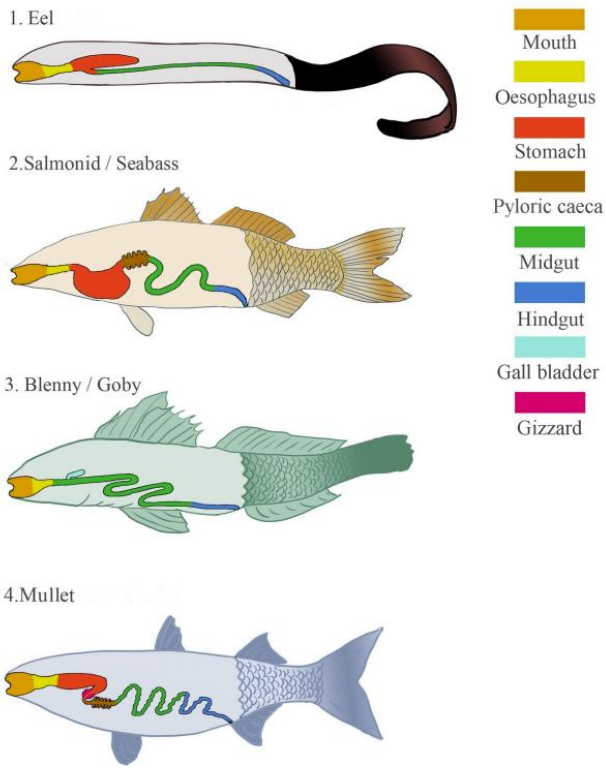

***Figure 8**. Comparative gut morphology across different fish species: Anatomical variation in digestive tract of Eel, Salmonid/Seabass, Blenny/Goby, and Mullet. (Egerton et al., 2018).*

10.3. Biofilm

In RAS, the majority of bacterial cells are found as biofilms, particularly within biofilters, which represent the largest reservoir of complex microbial communities (Bartelme et al., 2019; Blancheton et al., 2013; Schoina et al., 2022). A biofilm is defined as aggregates of microbial cells attached to a surface, including tank walls and pipes in RAS (Mahto and Das, 2022), and embedded in a matrix of biopolymers called extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, nucleic acids, and lipids as the major constituents (Flemming et al., 2023; Flemming and Wingender, 2010).

Biofilm development proceeds in several stages, from initial attachment of water planktonic cells to a surface marking the first step (Flemming and Wingender, 2010). Next, this attachment becomes irreversible allowing the firm adherence of microbial cells through the action of adhesins and the synthesis of the EPS matrix. At this stage, the cells proliferate and form a microcolony followed by maturation into a structured community embedded in EPS called “mature biofilm” capable of resisting stressors. Finally, potential detachment can allow the microbial cells to disperse from the matured biofilm, enabling thereby the recolonization of a new surface (fig. 8) (Akter et al., 2025; Gebreyohannes et al., 2019; Liu et al., 2024).

However, it is not a universal trait. The ability of a bacterial species to form biofilms depends on genetic (adherence genes expression) and phenotypic traits (like motility and flagellar structure) of bacteria as not all strains have the capacity to form biofilms and even the biofilm forming ability can vary among strains of the same species (Di Martino et al., 2002; Martinez-Medina et al., 2009; Sung et al., 2006). Biofilm formation is viewed as a survival strategy that helps microbial community to withstand environmental stresses (Penesyan et al., 2021). The capacity of microorganisms to form biofilms is driven by adaptive benefits ensured by the EPS playing multiple roles in providing stability, adherence and protection of microorganisms from harsh conditions such as toxic compounds exposure, improved nutrient access and trapping capacity, and increased survival of microbes compared to planktonic species (Akter et al., 2025; Cai et al., 2013; Flemming and Wingender, 2010). For instance, in case of contaminated wastewater passing through the biofilms in the biofilter, the EPS matrix acts as a sorption agent for wastes so the cells can detoxify the water (Mahto and Das, 2022; Raj Deena et al., 2022; Verderosa et al., 2019).

Microbial biofilms can harbor a diversity of microorganisms of the same species or different species. They are central to the functioning of RAS especially in biofilters as they contribute beneficially to water quality maintenance, playing important roles in nutrient cycling (Eck et al., 2019; Godzieba et al., 2025; Kasozi et al., 2021; Somerville, 2015). However, they can also host detrimental taxa such as off-flavors, opportunistic pathogens and pathogenic microorganisms. These biofilms have been reported to show increase resistance against antibiotics, disinfectants and detergents that threaten system resilience, fish health and product quality (Auffret et al., 2013; Schreier et al., 2010). Thus, biofilm-associated microbial communities can either enhance system resilience (Mahto and Das, 2022) or, under certain conditions, act as sources of disease (Jamal et al., 2018; Satpathy et al., 2016).

11) Insights into microbial ecology of RAS

Microorganisms, planktonic and sessile, in aquaculture environment have various kinds of functions and participate in all aspects of activities. In RAS, microbial diversity includes both beneficial and negative and harmful microbes, each affecting distinctly the operation of the system and fish health (Blancheton et al., 2013; Moschos et al., 2022; Sergaliev et al., 2021).

11.1. Beneficial bacteria

Stable rearing conditions in RAS, including pH, temperature, DO, and continuous nutrient supply (e.g. nitrogenous waste), promote the growth of diverse beneficial bacteria, forming biofilms on biofilter

media (Timmons et al., 2018; Vadstein et al., 2018). In return, maintaining good water quality in RAS depends on these diverse microbial populations, largely mediated by bacterial biofilms in biofilters (Blancheton et al., 2013) playing crucial roles in main biological filtration processes summarized in fig. 8, essential for the removal of toxic metabolites resulting mainly from the breakdown of feed, feces and microbial growth and activities (Jiang et al., 2019; Sakoula et al., 2021; Sugita et al., 2005; Tal et al., 2003; Gregersen and Joao, 2020; Wang et al., 2022).

11.1.1. Organic matter (OM) degradation

Heterotrophic bacteria (e.g. Proteobacteria, Bacteroidetes) found in both the biofilter and in the water (Sugita et al., 2005) play an important role in the degradation of organic matter, derived from uneaten feeds, dead bodies and excreta of fish, producing CO_2 and ammonia as final byproducts (Ebeling et al., 2006; Gupta et al., 2024; Moschos et al., 2022).

11.1.2. Nitrogen (N) cycle

Nitrogenous wastes are excreted by fish through gills, urine, feces and by uneaten feed in the system. In salmonids, it was reported that the maximum nitrogen retention recorded in the body was only about 40 to 50%, meaning that the majority of ingested nitrogen is excreted rather than retained (Foore, 2016). Nitrogenous waste accumulation, especially ammonia, causes a serious challenge to RAS. Ammonia is the main form of nitrogen excreted by aquatic species and generated as a metabolic by-product of fish protein catabolism excreted mainly through the gills but also generated by heterotrophic bacterial decomposition of organic matter in RAS (Bishop, 1976; Dewi et al., 2024; Takeuchi, 2017) and can be found in rearing water in two forms: ionized ammonium (NH_4^+) and unionized ammonia (NH_3) depending on pH, temperature and salinity of the system (Timmons and Ebling, 2010; Chen et al., 2006; Dosdat, 2003). The concentration of both ammonia forms is referred to as total ammonia nitrogen (TAN) or simply ammonia and is usually measured as a water quality parameter in RAS (Ebeling et al., 2006).

Microorganisms present in RAS biofilter play relevant roles in preventing the accumulation of nitrogenous wastes. Nitrogen cycling involves various processes: nitrification, anaerobic ammonium oxidation (anammox), anaerobic denitrification of nitrate, sulfur-dependent denitrification of nitrate and dissimilatory nitrate reduction to ammonium (DNRA) (Liu et al., 2023; Preena et al., 2021).

Among the key processes for the removal of toxic ammonia and the most reported is nitrification essential in RAS functioning (Gupta et al., 2024; Liu et al., 2023; Sakoula et al., 2021; Schreier et al.,

2010; Zhang et al., 2023). Nitrification refers to the process of conversion of ammonia, highly toxic even at low concentrations (limit concentration of 0.5 mg/L) (Dewi et al., 2024; Michaud, 2007), into nitrite, toxic when above 0.5 mg/L (Gomez Isaza et al., 2021), and finally to nitrate that could accumulate in the culture water as non-toxic form up to a safe limit depending on reared species (Davidson et al., 2017; Flemming et al., 2016; Losordo et al., 2009; Summerfelt and Penne, 2005). Ammonia and nitrite as the most discharged nutrients in wastewater, are a major cause of water quality degradation posing menacing the health and survival of reared species in RAS.

Traditionally, the main and the most common nitrification was known as a two-step process catalyzed by two functionally different microorganisms co-existing in the same system. First, ammonia is oxidized to nitrite by AOB and AOA (e.g. *Nitrosomonas, Nitrosococcus, Nitrosospira and Nitrosovibrio*) (Auffret et al., 2013; Bartelme et al., 2017; Hüpeden et al., 2020; Navada et al., 2019; Sarosh et al., 2024; Shitu et al., 2020) and then nitrite-oxidizing bacteria (NOB) (e.g. *Nitrospira, Nitrobacter, Nitrotoga*, and *Nitrospina*) are responsible for the conversion of toxic nitrite to nitrate stable form of nitrogen (Dewi et al., 2024; Hüpeden et al., 2020; Neissi et al., 2022; Wang et al., 2022; Yao and Peng, 2017). Then comammox (complete ammonia-oxidizing) bacteria, including *Nitrospira* species, that can perform both steps of nitrification within a single organism was discovered, and have been found to have a greater affinity for ammonia than AOB (Bartelme et al., 2017; Godzieba et al., 2025). These bacteria utilize ammonia monooxygenase to catalyze the initial oxidation of ammonia to hydroxylamine, which is further oxidized to nitrite by hydroxylamine dehydrogenase. Finally, nitrite is oxidized to nitrate through nitrite oxidoreductase (Godzieba et al., 2025; Sakoula et al., 2021; Schreier et al., 2010). In addition, some heterotrophic bacteria in RAS biofilters including *Vibrio, Marinobacte,* and *Pseudomonas* genus are able to perform nitrification (Gao et al., 2012; Zhang et al., 2023).

In parallel, anammox bacteria (e.g. *Brocadia anammoxidans*) can also oxidize anaerobically ammonia along with the ability to oxidize nitrite directly to dinitrogen gas form (Gottshall et al., 2021; Jetten et al., 2009; Van Duc et al., 2018). This process offers advantages over nitrification cycle, including lower oxygen need, the absence of external carbon requirement and reduced sludge production (Mahto and Das, 2022).

Also, heterotrophic bacteria play essential roles in RAS. Denitrification is another process of biological filtration occurring under low-oxygen conditions in biofilter and carried out by HB using an organic carbon source (Preena et al., 2021; Xiao et al., 2019) (e.g. *Pseudomonas aeruginosa*) (Jafari et al., 2024a; Schreier et al., 2010). It was reported that nitrate accumulated up to 400–500 mg/L, can induce adverse effects on the growth and health of farmed species. Thereby, denitrifying heterotrophic bacteria are responsible for complete anaerobic denitrification of nitrate to atmospheric nitrogen N2

(Davidson et al., 2017; Laza et al., 2021; Müller-Belecke et al., 2013; Yogev et al., 2017). Heterotrophic denitrifying microorganisms (e.g. Proteobacteria, Bacteroidetes, Furmicutes) are also capable of nitrate-dependent sulfide oxidation referring to sulfur-based oxidation/reduction cycle established in anaerobic conditions that allow nitrate reduction using inorganic sulfide as an electron donor and organic carbon to produce N2 gas and sulfate (SO_4^{2-}) (Rios-Del Toro and Cervantes, 2016; Schreier et al., 2010).
In addition, heterotrophic bacteria participate in dissimilatory nitrate reduction to ammonia (DNRA) (e.g. Proteobacteria, Firmicutes) converting nitrite effluent to ammonia (Almeida et al., 2021).

11.1.3. Sulfide cycle

Sulfide, notably hydrogen sulfide form can be generated in RAS from decomposition of organic matter and other sources of organic debris or from the breakdown process of products such as the reduction of sulfate (SO_4^{2-}) (Smith and Oseid, 1972). In fact, sulfate in RAS is reduced to sulfide in hydrogen sulfide form (HS^-) by sulfate-reducing bacteria (SRB). Moreover, excessive production of sulfide can be harmful in RAS because sulfide is a toxic compound to fish. But, toxic hydrogen sulfide is oxidized by aerobic sulfide oxidizing bacteria (SOB) to sulfate (He et al., 2020; Van Duc et al., 2018; Zhang et al., 2025). Sulfide is also removed by nitrate-dependent sulfide oxidation also called sulfur-mediated denitrification described above (Rios-Del Toro and Cervantes, 2016).

11.1.4. Phosphorus removal

In RAS, phosphorus is nontoxic for fish, but it accumulates at high concentrations mainly in the form of dissolved orthophosphate (PO_4^{3-}) associated with deterioration of water quality. Beneficial microbial communities provide sustainable biological processes for phosphorus removal which is then removed when microbial biomass (sludge) is harvested. Phosphorus-accumulating organisms (PAOs), such as *Pseudomonas putida* and *Candidatus Accumulibacter*, mainly store excess phosphate as intracellular polyphosphate granules under alternating anaerobic and aerobic conditions. Other microorganisms include denitrifying phosphorus-accumulating organisms (DPAO) that can use NO_3^- and NO_2^- as electron acceptor for phosphorus uptake into microbial biomass under aerobic conditions (Barak et al., 2003; Luo, 2023; Turcios and Papenbrock, 2014; Zheng et al., 2023).

Overall, the dominance, abundance and associated activity of these microbes is related to nutrients availability, environmental conditions and/or competition between these microbial species leading to

new and changing conditions for microbial growth. Moreover, biofilter failures are commonly linked to inadequate or imbalanced microbial communities, poor maintenance practices, and insufficient water quality management (Blancheton et al., 2013; Rojas-Tirado et al., 2017; Rurangwa and Verdegem, 2015).

Process	Reaction
Nitrification	
Ammonium oxidation	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$
Nitrite oxidation	$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$
Denitrification	
Autotrophic (sulfide-dependent)	$S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 0.8N_2(g) + 0.8H_2O$
Heterotrophic	$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3^- + 4N_2(g) + 4H_2O$
Dissimilatory nitrate reduction to ammonia (DNRA)	$NO_3^- + 2H^+ + 4H_2 \rightarrow NH_4^+ + 3H_2O$
Anaerobic ammonium oxidation (Anammox)	$NH_4^+ + NO_2^- \rightarrow N_2(g) + 2H_2O$
Sulfate reduction	$SO_4^{2-} + CH_3COO^- + 3H^+ \rightarrow HS^- + 2HCO_3^- + 3H^+$
Sulfide oxidation	$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$

Figure 9. *Key microbial metabolic processes and associated reactions in biofilters: Nitrification, Denitrification, Anammox, Sulfate Reduction, and Sulfide Oxidation. (Schreier et al., 2010).*

11.1.5. Importance for fish health

Removing excess organic matter, nitrogen, sulfur and phosphorus waste from RAS protects fish health by lowering stressors, pathogen risk, disease susceptibility and acute toxic events.

Excess dissolved and particulate organic matter can result in unfavorable conditions increasing the growth of heterotrophic bacteria (Attramadal et al., 2012). These HB exhibit faster growth rates than nitrifiers, they could compete with nitrifying bacteria for space and oxygen dominating thereby in the biofilters, leading to increased demand for biological oxygen, disruptions to the microbial equilibrium in RAS and inhibiting nitrification process leading to toxic ammonia accumulation in the system (Aguilar-Alarcón et al., 2020; Fossmark et al., 2020; Losordo et al., 2009; Michaud et al., 2014; Rojas-Tirado et al., 2017; Summerfelt and Penne, 2005).

The removal of ammonia is critical to avoid its toxic impacts on fish, which include severe tissue damage, particularly to the gills and kidneys, reduced growth performance, increased stress, immunosuppression and increase mortality (Al-Hazmi et al., 2023; Badiola et al., 2012; Blidariu and Grozea, 2011; Dosdat, 2003).

The incomplete ammonia removal can contribute to nitrite accumulation which is highly toxic to aquatic organisms at high levels, causing disruptions to energy metabolism, tissue damage and brown blood disease in fish which is reduced oxygen-carrying capacity of the blood (He et al., 2020; Kim et al., 2019; Xiang et al., 2010). Nitrate can also reach high toxic levels for fish, especially in RAS with low water exchange rate, causing immunosuppression, deformities, endocrine system disruption, the oxidation of hemoglobin to methemoglobin incapable of carrying oxygen "methemoglobinemia" and in some cases fish mortality (Davidson et al., 2017; Steinberg et al., 2018; Van Bussel et al., 2012). Moreover, ammonia, nitrite, and nitrate have been reported to cause oxidative stress in aquatic animals (Kim et al., 2019). The removal of these nitrogenous compounds allows to create healthy rearing conditions for fish growth (Sarosh et al., 2024).

Also, high OM increases oxygen consumption resulting in higher activity of sulfate-reducing bacteria and production of high levels of sulfide. Removing sulfide is crucial to prevent toxic effects on fish such as liver malfunction, lower food consumption and conversion and notably fish mortality at high levels (Bagarinao and Lantin-Olaguer, 1999).

11.2. Detrimental microbial communities

While RAS provide significant operational advantages thanks to nutrient removal by beneficial bacteria, they also present inherent risks related to detrimental microorganisms including off-flavor microorganisms (Burr et al., 2012), pathogens (Guan et al., 2020; Stride et al., 2014) and opportunistic pathogens (Samsing and Barnes, 2024) showing a strong ability to form biofilms on a variety of surfaces in RAS facilities, posing quality and health risks for the reared species (Harrison et al., 2020; King et al., 2004; Schoina et al., 2022; Sergaliev et al., 2021).

11.2.1. Off-flavors microorganisms

Many off-flavors microorganisms, mainly planktonic and benthic cyanobacteria and actinomycetes, have been increasingly reported in RAS causing poor quality of marketed aquatic products and therefore enormous economic losses (Auffret et al., 2013; Burr et al., 2012; Moschos et al., 2022; Nam-Koong et al., 2016). The Off-flavor microorganisms' growth and the release of their metabolites

is favorized by aerobic organic-rich conditions of water (Bedner and Saito, 2020; Nam-Koong et al., 2016). In indoor RAS, Streptomyces are believed to be the main producers of off-flavor compounds such as geosmin and 2-methylisoborneol (MIB), which are then absorbed from water into fish flesh in lipid-rich tissues (Davidson et al., 2020; Houle et al., 2011; Pedersen et al., 2017), causing "musty" and "earthy" flavors and odors based on consumers description (Burr et al., 2012; Lindholm-Lehto et al., 2021). Notably, aquatic species eliminate off-flavor compounds much more slowly than they absorb them, with the process being even slower in cold water conditions and in species with high fat content (Houle et al., 2011) making this a serious challenge in aquaculture.

11.2.2. Pathogens

Recirculating aquaculture systems are designed to provide high water quality and tight biosecurity, yet their closed design and water circulation makes these systems susceptible to pathogen occurrence and persistence, especially in biofilters in biofilm form, increasing thereby the risk of disease outbreaks, causing mortality events and high economic losses (Gonçalves and Gagnon, 2011; Krishna et al., 2026). Studies have documented that RAS can harbor a variety of pathogens ranging from bacteria, viruses, fungi, and parasites (Mota et al., 2022) particularly favorized by high fish densities and nutrient availability and able to cause the break of microbial balance in RAS (Xue et al., 2017).

Pathogens use various virulence factors such as toxins, adhesins, pili, products involved in motility (flagella produced by diverse bacteria, including vibrios) and enzymes to colonize and evade hosts' immune defenses (Olymon et al., 2024). The infectious cycle of pathogenic bacteria begins with their entry into the host, followed by their establishment and multiplication, during which they evade host defenses while inflicting damage on their tissues and cells. Finally, the cycle concludes with the exit of the pathogen, enabling its transmission to new hosts (Defoirdt, 2014).

For instance, *Flavobacterium columnare* is a recognized bacterial pathogen reported to affect many freshwater fish causing columnaris disease in closed aquaculture systems and to persist in the system in biofilm forms (Cai et al., 2013). In addition, it is established that one pathogen can increase the invasiveness of other ones by suppressing host immunity for example, as demonstrated in tilapia (*Oreochromis spp.*) where infection with tilapia lake virus (TiLV) led to markedly higher mortality after previous infection by *Aeromonas hydrophila* (Wang et al., 2025).

Particularly, the presence in RAS of fish and human pathogens such as *Aeromonas hydrophila, Vibrio cholerae* and *Mycobacterium marinum*, cause serious public health risk (King et al., 2004; Volpe et al., 2019). *Mycobacterium marinum* for example is recognized as a fish pathogen in aquaculture, responsible for mycobacteriosis, a chronic disease in fish species (Mondal and Thomas, 2022). This

pathogen has been reported to cause significant mortality in pikeperch (*Sander lucioperca*) reared in RAS, highlighting its capacity to cause severe disease and economic losses in fish populations (Gjurčević et al., 2020). It's also capable of causing extrapulmonary mycobacterial infection in human and skin lesions, posing occupational health risks especially to farmers (Canetti et al., 2022).
The diversity and abundance of detrimental microbial communities in RAS have been correlated with disease outbreaks and low quality of aquatic products, highlighting the importance of managing pathogens and maintaining biosecurity in RAS (Guan et al., 2020; Krishna et al., 2026; Stride et al., 2014).

11.2.3. Opportunistic pathogens

In addition to fish pathogens in RAS, opportunistic pathogens can be found in conditions that favor their proliferation and increase their abundance. Common opportunistic pathogens include *Vibrio, Aeromonas, and Pseudomonas*, which are often found in RAS niches such as biofilms, water columns, and fish-associated microbiomes (Gupta et al., 2024; Moschos et al., 2022; Rieder et al., 2023; Sergaliev et al., 2021). In fact, these microorganisms are normally found at low abundance, in RAS microbiome or in biofilms, existing as a part of the system's harmless microbiome and only causing disease when environmental or host stressors occur such as fluctuations in temperature, high stocking densities, compromised water quality, or weakened fish immune systems (Samsing and Barnes, 2024; Xue et al., 2017). For example, *Pseudomonas aeruginosa* is a common heterotrophic bacteria participating in denitrification process and removal of toxic nitrogen compounds but reported as an opportunistic pathogenic heterotrophic bacteria causing infection in freshwater fish (Clols-Fuentes et al., 2023; Gupta et al., 2024; Schreier et al., 2010).
Under unfavorable conditions, mostly fluctuations in water quality physico-chemical parameters, opportunistic pathogens can form biofilms. The external matrix of these biofilms gives them protection but most importantly allows them to produce virulence factors to enhance their survival and pathogenicity, which increase significantly the proportion of disease occurrence and mortality rate to 50-70% (Aich et al., 2020; Akter et al., 2025; Chhanda et al., 2024; Michaud, 2007; Pedersen et al., 2017; Sergaliev et al., 2021) posing serious threats in closed RAS (Drønen et al., 2022). This is often the case in RAS with high stocking densities and high feeding inputs resulting in high loads of metabolic waste, especially high load of organic matter, creating an environment favorable to the overgrowing of opportunistic bacteria (Asmani et al., 2017; Bugten et al., 2022; Michaud et al., 2014; Sanches-Fernandes et al., 2022) which could compromise the health of reared species, if opportunistic pathogens were allowed to grow (Roalkvam et al., 2019). Healthy water environment requires more

balanced bacterial population which suppresses the bloom of opportunistic pathogenic bacteria.

11.2.4. Antibiotic resistant communities

In RAS, because of pathogens and opportunistic pathogens growth, certain diseases may persist and disseminate especially in intensive culture. Antibiotic therapy continues to be the most used effective treatment method to overcome this challenge (Cabello and Godfrey, 2019; De Bruijn et al., 2018; González et al., 2010; Sherif and Kassab, 2023).

A large proportion of used antibiotics, most commonly: sulfonamides, quinolones, tetra-cyclines and diverse group of β-lactams (penicillins, cephalosporins and carbapenems) have been reported to leave residues in the closed-loop water of RAS (Kampouris et al., 2022; King, 2001; Muziasari et al., 2017; Shi et al., 2022). This residual concentration of antibiotics can alter the structure and function of the system's microbial communities reducing for example the abundance and/or activity of key functional groups such as nitrifying bacteria, with serious impacts on nitrogen cycling and water quality in RAS (Collins et al., 1976; Klaver and Matthews, 1994; Zhou et al., 2024). This microbial shift will therefore create an opportunity for opportunistic pathogens to proliferate (Sanches-Fernandes et al., 2022).

In particular, pathogens and opportunistic pathogens in biofilms (e.g. *Flavobacterium columnare, Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda,* and *Vibrio anguillarum*) display increased antimicrobial tolerance to high doses of antibiotics thanks to EPS protection and due to the complex nature of biofilms, via a multitude of strategies (Gebreyohannes et al., 2019; Romero et al., 2012). One main strategy is the development and spread of antimicrobial resistance genes ARGs between bacterial cells (Domínguez et al., 2019). In fact, microbes in biofilms are in close contact thanks to the EPS, which can promote horizontal gene transfer HGT accelerating the dissemination of ARGs across bacteria in biofilms (Abe et al., 2020; Cooper et al., 2017; Lécuyer et al., 2018; Olymon et al., 2024). Antibiotic resistance genes can be transferred between bacteria by three main DNA transfer processes : (i) transformation, occurs when bacteria can obtain resistance genes from the uptake of DNA from their external environment which may be released from lysed cells; allowing bacteria to acquire resistance without direct contact with donor cell; (ii) transduction, involves bacteriophages and viruses that infect bacteria, transferring thereby ARGs from one host to another; allowing to transfer resistance genes even distantly and (iii) conjugation, often considered the most efficient route, and refers to the direct transfer when a copy of plasmid containing ARG or multiple resistance genes from one bacteria to another through cell-to-cell contact (Cedeño-Muñoz et al., 2024; Nesse et al., 2019; Romero et al., 2012; Vernikos and Medini, 2014).

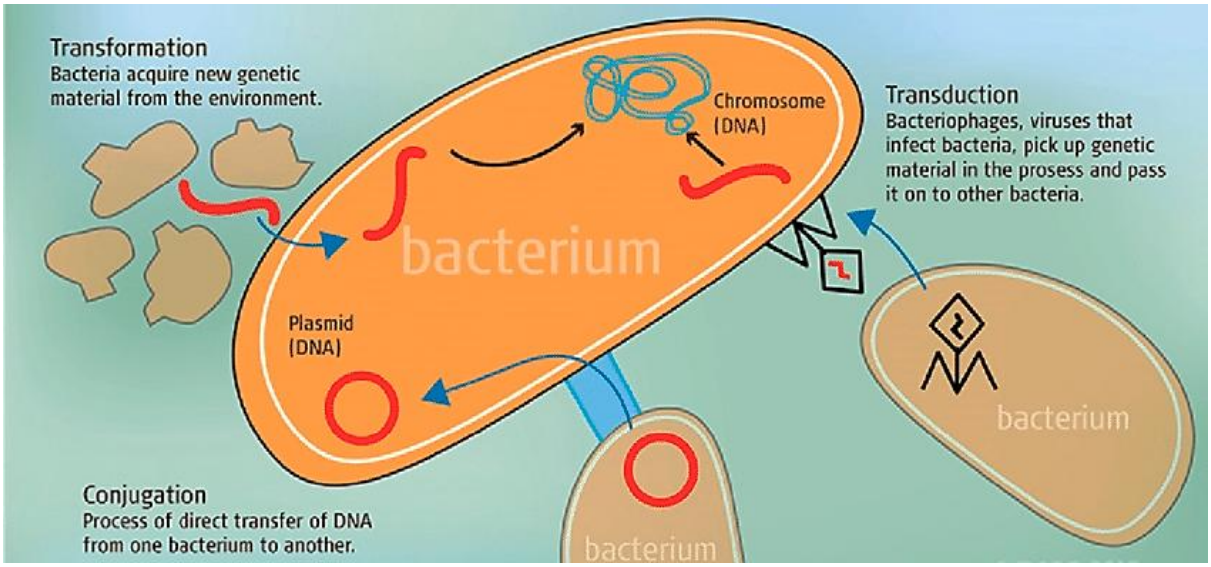

Figure 10. *Mechanisms of Horizontal Gene Transfer (HGT) in Bacteria: Transformation, Transduction, and Conjugation as pathways for dissemination of antibiotic resistance genes. (Nesse et al., 2019).*

Taken together, ARGs can persist (Lin et al., 2023), accumulate, and spread among commensal bacteria, fish and human pathogenic bacteria and even fish associated microbiota (Chowdhury et al., 2022; Li et al., 2024; Lin et al., 2023; Pamanji et al., 2024; Zhou et al., 2024), thereby posing risks for RAS microbial balance and sustainability (Sha et al., 2022) and for human (Sanches-Fernandes et al., 2022) and fish health (Cai et al., 2013; Cedeño-Muñoz et al., 2024; Guan et al., 2020; Hong et al., 2018) particularly in immune-compromised species (Sherif and Kassab, 2023). For instance, it has been shown that antimicrobials use can alter microbiota composition and increase the abundance of pathogenic bacteria and ARGs gut microbiomes of zebrafish (*Danio rerio*) (Pamanji et al., 2024).

The presence of antibiotic residues and ARGs in aquatic products can enter the human food chain. Human consumption of fish containing antibiotic resistant microbiota result in antibiotic resistance in the human gastrointestinal tract (Sanches-Fernandes et al., 2022) posing significant public health risk including chronic organ dysfunction and disruption of the beneficial intestinal microbiota, ultimately weakening the human immune system (Ende et al., 2024; Okocha et al., 2018). World health leaders have identified the transfer and proliferation of antibiotic resistance genes as a significant threat (Cooper et al., 2017; Fu et al., 2017; Pruden et al., 2013) especially with the growing consumption of raw, rare, and cold-smoked seafood (King, 2001; Mulder et al., 2020).

Since use of antibiotics seem to be less effective when pathogens become resistant to commonly used antibiotics (Mondal and Thomas, 2022). Developing disinfection strategies to control pathogen outbreaks in RAS is challenging as they must be effective against pathogens without harming fish health, welfare, or beneficial bacteria in biofilters (Gebreyohannes et al., 2019; Mota et al., 2022; Shi et al., 2022). One promising way to prevent pathogen infection is through commensal microbes of fish

mucosal tissues that could potentially inhibit pathogen growth (Caruffo et al., 2015). Studies have reported that fish mucus showed inhibitory effect on some bacteria via proteins that have antibacterial activity for example. For instance, the cell-free supernatant of more than 70% of the lactic acid isolates obtained from the gut of Atlantic salmon showed inhibitory ability on the growth of the fish pathogen *Aeromonas salmonicida* (Ringø and Holzapfe, 2000). Another interesting example is the decrease of the growth of luminous vibriosis *Vibrio harveyi*, a major cause of shrimp mortality, thanks to the presence of tilapia (*Tilapia hornorum*) in polyculture with shrimp (*Penaeus monodon*) in outdoor tanks (3x 1x 1.25 m). However, the mechanism of how the polyculture system efficiently reduced the bacterial count of luminous vibriosis in the water was not studied (Tendencia et al., 2004).

Thus, the importance of microbial communities' management in RAS remains a vast topic of ongoing scientific research. The development of efficient, productive, and disease-preventive RAS requires comprehensive knowledge of overall RAS factors and selective pressures influencing dynamics of microbial populations ranging from physico-chemical control (e.g., temperature, DO, pH, salinity, nutrients levels) to biological processes including complex interactions among microbial communities and their environment which is more difficult to control.

12) The dynamics of microbial colonization in RAS

The dynamics of microbial colonization in RAS is how microbial communities establish, change, and interact over time within different compartments of the system (mainly water column, biofilms and fish) and is directly linked to the diversity of microorganisms in RAS (Attramadal et al., 2012; Murray, 2014). It includes the processes from the start-up and attachment of microbes, growth, competition, succession (which species dominate at different times), to stabilization and changes in response to environmental change (e.g. feed, water chemistry, temperature), and microbial detachment from biofilms that form on pipes, filters and tank surfaces for example (Abe et al., 2020; Bakke et al., 2017; Dahle et al., 2023, 2022; Emmanuel et al., 2025; Jiang et al., 2019).

During the start-up of RAS, the microbial structure of the system is not yet stable and is still functionally developing because of imbalances and fluctuations in initial nutrients levels due to uneaten feed, fish excreta, and dissolved organic carbon accumulation (Blancheton et al., 2013; Navada et al., 2019; Rojas-Tirado et al., 2017; Steinberg et al., 2018). Notably, the inoculation of biofilters at the start-up is crucial for the successful operation of RAS and is divided into three phases: incubation, growth and stability (Jiang et al., 2019). Eventually, microbial communities become dynamically robust and stable (Honjo et al., 2024).

As shown previously, microbial assemblages are specific for each compartment in RAS and differences has been notably reported especially between biofilter media and the rearing water (Almeida et al., 2021; Bartelme et al., 2017, 2019; Drønen et al., 2021; Duarte et al., 2019; Kuang et al., 2020; Roalkvam et al., 2019; Rud et al., 2017; S. Zeng et al., 2020) as water column harbor planktonic microbes, in contrast biofilters predominately harbor bacterial biofilms (e.g., classical nitrifiers and other biofilms for nutrient cycling), while fish hosts maintain their host-associated core that are specific for each species (Bakke et al., 2017; Michaud et al., 2009; Minich et al., 2020). However, studies using 16S rRNA profiling showed some considerable shared microbial resembles between the culture water and other compartments feed, fish (mucus/gut/skin) and attached biofilms of the system (Mes et al., 2023; Sadeghi et al., 2023).

Water in RAS is highly connected to other microbial compartment, both receiving and redistributing microorganisms, while hosting taxa that persist as free-living plankton in the water column. The interconnections between microbial compartments in RAS is generally assumed to be due to the water circulation and reuse in RAS, explaining the shared taxa found between the different microbial compartments (Gonçalves and Gagnon, 2011; King et al., 2004; Moschos et al., 2022; Rojas-Tirado et al., 2017).

Biofilms and water communities are dynamically interconnected, with constant microbial exchange in both directions. For example, even though the microbial composition of water and biofilms were significantly different (Vadstein et al., 2018), shared abundant bacterial orders Rhodobacterales, Burkholderiales and Sphingomonadales were found in a start feeding RAS of Atlantic salmon (Gaarden, 2020). The bidirectional transfer between water and biofilm is driven by the nature of biofilms, as reservoirs of microbial communities, as the EPS matrix enhances microbial retention but also provides niches that harbor both sessile and temporarily planktonic cells (Flemming and Wingender, 2010) but can also undergo detachment and dispersal processes of microbial cells; as they revert to the planktonic state under various conditions including nutrient limitation and microbial competition, and are able to colonize new surfaces such as fish mucosa for example (Akter et al., 2025; Flemming et al., 2016; Gebreyohannes et al., 2019).

Correlations between microbial communities of tank water and biofilms with fish tissues microbiota have been reported in RAS (Bugten et al., 2022; Giatsis et al., 2015; Lazado et al., 2025; Loufi et al., 2026). In general, water is continuously in direct contact with fish and is believed to have a potential shaping effect on host associated microbiota (Chen et al., 2022; Kim et al., 2021; Lazado et al., 2025; Muziasari et al., 2017; A. Zeng et al., 2020). For instance, Minich et al., 2020 reported correlations between microbial communities of RAS water and biofilms with fish tissues microbiota gill, skin, and digesta of juvenile Atlantic Salmon (*Salmo salar*) corresponding to the established resident microbiota

development (Minich et al., 2020). Moreover, a study on yellowtail kingfish (*Seriola lalandi*) reared in RAS found many Amplicon Sequence Variants (ASVs) shared between fish mucosa and the water and biofilm and suggested that fish mucosal microbiomes are differentially influenced by RAS environment with gill communities being particularly the most influenced by the surrounding water. While studies reported important correlation between water and gut microbiota (Giatsis et al., 2015; Loufi et al., 2026), Minich et al., 2021 found gill and skin microbial communities to be more influenced by RAS environment than the gut microbiome which was more stable (Minich et al., 2021), which can be due to the gut "allochthonous" microbiome differentiated in later stages of life of fish species (Nyholm et al., 2022). Despite the importance of the external mucosa of skin and gills as the first line of defense against infectious pathogens (Lorgen-Ritchie et al., 2022), the number of studies related to skin and gill microbiomes in RAS is limited (Wang et al., 2010).

In addition, being an important input in RAS (Pěnka et al., 2024b; Ringø et al., 2016), feed often introduces a pool of transient organisms that can transiently colonize gut microbiota of fish and then get excreted in the water via fish feces (Karlsen et al., 2022). Yet, Minich et al., 2020 found that feed had a relatively low impact on microbial communities on mucosal tissues (Minich et al., 2020). Suggestively, the microbial impact of feed on microbial communities of fish and RAS system may be related to specific conditions such as the type of feed and its content and life stage of fish for example. In parallel, microorganisms in RAS are notably influenced by stocked fish (Bartelme et al., 2019; Llewellyn et al., 2014; Rojas-Tirado et al., 2017; Rud et al., 2017; Rurangwa and Verdegem, 2015). For instance, Giatsis et al., 2015 showed numerous Operational taxonomic unit (OTUs) shared between larval tilapia guts and tank water suggesting that gut-derived microbes are abundant in the tank water (Giatsis et al., 2015). These similarities can be explained by different processes: (i) mucus sloughing due to shedding of epithelium from skin and gills since mucus contains abundant bacteria (Krishna et al., 2026; Loufi et al., 2026), (ii) gut transient microbial communities can be expelled into the water via digesta/feces (Duarte et al., 2019; Nyholm et al., 2022; Steiner et al., 2021), and (iii) gill-associated bacteria maybe released into the water column during vital functions such as gas transfer as water flows directly over the gills (when the fish operculum opens and closes) (Chen et al., 2023). These processes can potentially release both free planktonic cells and particle-attached cells (e.g., fish feces) explaining different inoculation of RAS water and biofilms related to stocked species.

Even after stabilization of each compartment, uncontrollable fluctuations continue to occur within RAS due to selective pressures (Almeida et al., 2021; Rojas-Tirado et al., 2018, 2017). Every variation in selective pressure on a daily basis such as fish biomass, nutrient levels or other changes over time during the culture period can lead to changes in the microbial community composition in RAS (Bakke et al., 2017; Bartelme et al., 2019, 2017; Blancheton et al., 2013; Drønen et al., 2021; Duarte et al.,

2019, 2019). Additionally, microbial communities' changes in RAS can be due to different microbial interactions with each other, such as mutualism and competition. Different microbial species are able to compete for space and resources until more resilient species dominate, leading to shaping of microbial communities' structure (Jiang et al., 2019). For instance, certain resilient bacteria can produce inhibitory compounds called "bacteriocins" that can help suppress or eliminate pathogens influencing the shaping dynamics of microbial communities while offering operational benefits to RAS (Wang et al., 2020).

Biofilms are the most dynamic compartment, changing their composition over time (Drønen et al., 2021; Gebreyohannes et al., 2019; Moschos et al., 2022; Schoina et al., 2022; Zheng et al., 2017). The evolution and shifts of biofilms microbial composition could be linked with changes of RAS physico-chemical conditions, such as level of nutrient and wastes in the water during the rearing cycle, disease occurrence, biomass changes or in some cases technical failures. Continuous water flow in RAS implies that it may trigger microbial cells detachment from the biofilms (Akter et al., 2025; Liu et al., 2024; Schreier et al., 2010; Sergaliev et al., 2021). Another potential aspect that could drive the bacterial dynamic in RAS is the possible loss of microbial biomass caused by grazing by protozoan for example (Huws et al., 2005; Michaud, 2007) which is poorly understood in RAS (Saengsuk et al., 2024).

In general, each RAS has a unique design and operation conditions including: biofilter type, rate of supply water, physicochemical parameters of water, disinfection methods, farmed fish species, feed type and nutrient concentrations and wastes (Acosta et al., 2021b; Giatsis et al., 2015; Gonçalves and Gagnon, 2011; Kuang et al., 2020; Zhang et al., 2023). The conditions of each RAS facility are the main factors that shape a specialized associated microbial communities community composition (Moschos et al., 2022).

While considerable progress has been made to investigate microbial exchanges between RAS compartments, important gaps remain especially concerning host-to-water inoculation as the impact of fish-driven shifts in RAS is still poorly studied. Particularly, to our knowledge, microbial dynamics in the presence of multiple fish species in the same RAS facility remain unexplored. So, driven by the major shaping and inoculation role of fish-specific associated microbiomes, we showed interest in investigating the influence of diversification of fish species on the structure and on temporal changes of the planktonic and sessile communities of RAS under stable rearing conditions. Understanding these aspects will help optimizing polyculture practice in RAS.

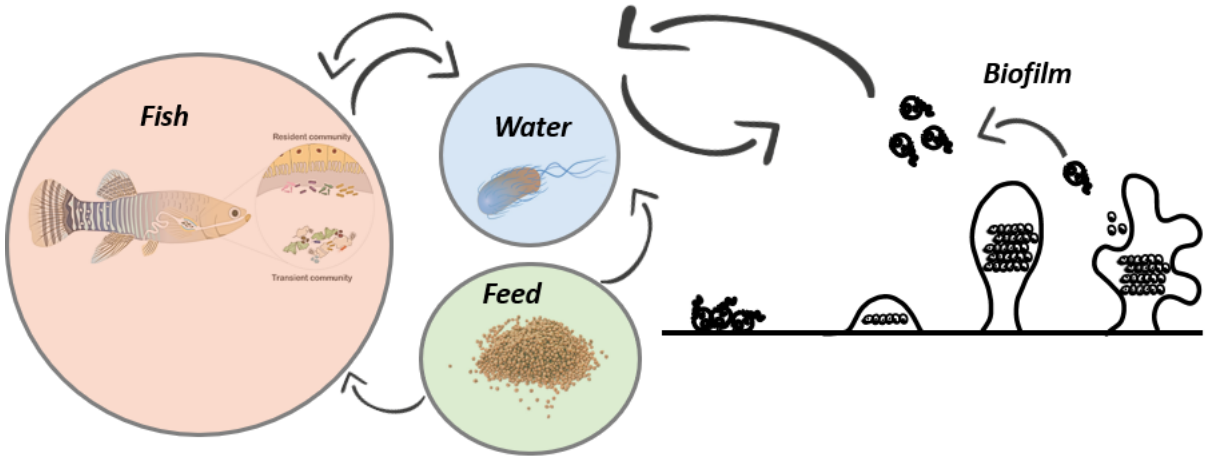

Figure 11.*Dynamics of microbial communities in Recirculating Aquaculture Systems (RAS). Interactions Between water, biofilms, feed and fish-associated microbial communities. .*

13) Objectives and hypothesis of the thesis

13.1. Effects of biofilter management modalities on the microbiological status in RAS water to avoid recording capacities of biofilms

As described previously, microorganisms in RAS can form biofilms on a variety of surfaces, including biofilters, pipes, tank walls and other substrates in RAS (Dahle et al., 2022; Mahto and Das, 2022). These biofilms harbor microorganisms playing crucial roles in RAS functioning, such as nutrient cycling and waste removal (Akange et al., 2024; Dewi et al., 2024) but can also harbor pathogens (Drønen et al., 2023; King et al., 2004) exhibiting enhanced abilities to resist antimicrobial therapies and disinfections via the EPS protecting the biofilm or through development of resistance genes to antimicrobial compounds (Cooper et al., 2017). However, some aspects in RAS remain poorly understood or insufficiently explored. One important aspect is the residual microorganisms persisting in RAS from the past rearing conditions at the startup of a new culture. Since biofilms are reservoirs for microbial diversity (Schreier et al., 2010), we believe that they exhibit a unique capability "memory" to record the microbiological diversity of previous farming conditions that has been practiced within the system "tanks' history" through the accumulation of bacterial species and their genetic information within the biofilms.

Moreover, after refilling of the tank at the startup of a new farming cycle, biofilms can release the bacteria of the recorded "memory" into the water system (Akter et al., 2025; Liu et al., 2024; Mahto and Das, 2022; Ramírez-Larrota and Eckhard, 2022). We believe that the effect of the persistent microbiome due to "tank's history" makes it unreliable to conduct studies on microbial compartment in 2 or more RAS that had different past farming conditions as each RAS design, notably the choice

of farmed fish species, was previously shown to impact microbial communities in the system (Moschos et al., 2022; Muziasari et al., 2017). To our knowledge, studies on microbial flora in RAS in literature do not mention specific protocols to obtain a similar initial microbial state in their systems before conducting their studies on the microbial compartment in RAS. In this context, we believe that it is essential to control the initial RAS microbial status (before starting a new breeding cycle) by having a reliable, standardizing protocol aiming to obtain a uniform microbial state in all our RAS facilities in a reproducible way. Preliminary studies in our laboratory, conducted by Fourrier et al., 2022, aimed to investigate the impact of a polyculture of pikeperch (*Sander lucioperca*) and tench (*Tinca tinca*) on microbial communities in comparison with monoculture of pikeperch alone in order to investigate the effect of rearing modality on the microbial compartment of water in RAS. A significant effect of fish species on the structure of water microbial communities was observed (Fourrier et al., 2022) but they found a high inter-tank variability that persist even though a raw cleaning of the tanks was performed before conducting the study which interfered with the interpretation of the results. These findings allowed us to develop a hypothesis about the presence of an effect of "tank's history", most probably linked to the recording "memory" of biofilms and their ability to reintroduce microbial diversity into the system influencing therefore the water microbiome even after a raw cleaning of the system.

13.2. Impact of fish species and polyculture on microbial communities in RAS

We detailed previously that polyculture offers many advantages compared to monoculture which can facilitate the transition to more sustainable aquaculture in RAS and thereby enhance the resilience of the system (Dawood et al., 2025; Pěnka et al., 2024a; Thomas et al., 2021). Species-specific associated fish microbiota has been studied in RAS along with microbial transfer between fish microbiota and the surrounding water and biofilms but only in monoculture systems (Minich et al., 2021, 2020). To our knowledge fish microbiota has been characterized in polyculture (Elsheshtawy et al., 2021) but there is no information on the effects of reared species in polyculture on microbial communities in RAS. The specification of microbiota for each fish species (De Bruijn et al., 2018; Moschos et al., 2022; Muziasari et al., 2017) allow us to assume in a co-culture in the same RAS, reared fish species will inoculate the system through their microbiotas, but the microbial interactions and dynamics of this inoculation are unknown along with the impact of the resulting microbial diversity from a co-culture, including both planktonic and sessile microbes harboring beneficial and detrimental species, on fish health. This highlights the need to sharpen our understanding of the microbial ecology of RAS in context of polyculture of different fish species (Sergaliev et al., 2021).

Based on the results of Thomas et al. 2020 and Fourrier et al., 2022, we have chosen to study : the

assessment of microbial communities in RAS in a polyculture of pikeperch with tench in comparison with monoculture of each species alone in order to understand the impact of reared fish species on the shaping of microbial communities in RAS.

These two species were chosen for several reasons:

- Pikeperch was selected as our species of interest due to its high commercial value, delicate white flesh with few intermuscular bones, excellent growth performance, and strong market demand (Pourhosein-Sarameh and Falahatkar, 2024). This valuable fish species is highly consumed in Europe and increasingly farmed in RAS (Dalsgaard et al., 2013; Rieder et al., 2023).
- Considerable knowledge about pikeperch in monoculture in RAS (Pěnka et al., 2024b; Steinberg et al., 2018; Tran et al., 2021; Yanes-Roca et al., 2024) and pikeperch polyculture with different fish species in different rearing systems (Kozłowski et al., 2014; Mihailov et al., 2017; Pěnka et al., 2024a, 2021b).
- Selected species have similar rearing abiotic requirements (i.e. pH, temperature, salinity, dissolved oxygen, and light parameters (Baekelandt et al., 2018; Pula et al., 2018).
- These species notably show compatibility in their feeding habits as they feed in different areas in their living environments (Lecocq et al., 2019; Thomas et al., 2020). Pikeperch feeds in the water column and never or rarely retrieves food that sinks to the bottom which results in a direct loss of feed and can lead to the deterioration of water quality, potentially causing health problems for fish (Mihailov et al., 2017). Meanwhile, tench allows the recirculation of minerals and nutrients that settle at the bottom (Thomas et al., 2020).
- Finally, because the combination of pikeperch polyculture with tench is already studied (Thomas et al., 2020; Fourrier et al., 2022).

13.2.1 How fish biodiversity in RAS affects sessile and planktonic microbial communities of water and biofilms

The functionality of RAS critically depends on microbial communities, particularly biofilms in the biofilter (Bartelme et al., 2019; Moschos et al., 2022). The microbial composition and diversity in RAS water and biofilters are highly influenced by various factors including stocking density, organic contamination, physicochemical parameters of water such as pH, temperature, oxygen and nitrogenous compounds concentrations (Sergaliev et al., 2021) and the farmed species (De Bruijn et al., 2018; Godzieba et al., 2025; Moschos et al., 2022). It has been reported that microbial diversity in filters

differs from one RAS to another suggestively linked to fish species-specific microbiota introduction in the system (Liu et al., 2021; Schreier et al., 2010; Steiner et al., 2021). Despite the knowledge on microbial exchanges between RAS compartments, the microbial dynamics in RAS farming multiple fish species in the same RAS facility have yet to be explored. Given the major role of fish-associated microbiomes in shaping and inoculating water and biofilm, we aimed to investigate how different fish species and diversification influences the structure of both planktonic and sessile microbial communities in RAS under stable rearing conditions.

The resulting microbiome in polyculture in RAS could end up being more diverse which can strengthen system stability by outcompeting opportunistic pathogens and thereby improving RAS resilience (Harrison et al., 2020) while an unbalanced microbial composition and/or reduced microbial diversity can have detrimental effects on the growth of beneficial microbes and potentially trigger disease outbreaks and occurrence of opportunistic pathogens such as Aeromonas or Pseudomonas (Akange et al., 2024; Godzieba et al., 2025). A diverse and resilient microbiome is therefore essential to prevent disease outbreaks and support water quality (Gupta et al., 2024; Harrison et al., 2020).

This study aims to evaluate how polyculture of two ecologically distinct fish species, pikeperch and tench, influences microbial communities in RAS water and biofilms, compared to their respective monocultures. Given differences in feeding strategy, gut morphology, and previous farming conditions, we hypothesize that polyculture leads to more complex microbial assemblages through microbial interactions, addition, or competition. Using metabarcoding tool, the first aim of this thesis was to characterize how microbial communities colonize and evolve within RAS under different rearing conditions: monoculture of pikeperch, monoculture of tench, and their biculture (polyculture).

13.2.2. The main vectorization contribution of fish-associated microbiota of skin, gills and gut to the microbial colonization of water and biofilms in RAS in a pikeperch/tench polyculture

Studies have shown that fish can act as vectors of microbial communities via the microbiota of their skin, gills, gut (De Bruijn et al., 2018) contributing to the inoculation of RAS, which impacts the microbial communities of water and biofilms (Minich et al., 2021, 2020; Moschos et al., 2022; Rurangwa and Verdegem, 2015). For example, pathogens can be shed from the mucus layers of the skin and gills (Krishna et al., 2026; Loufi et al., 2026). Krishna et al., 2026 reported a direct link between gill epithelial shedding and pathogen levels in RAS water, resulting from the active shedding of gill epithelium containing a virus during infection into the water column, which can be exacerbated by physical stressors such as handling during farming and high fish activity in the tank (Krishna et al.,

2026). While in the gastrointestinal system, pathogens can be expelled with feces (Benhamed et al., 2014; Fu et al., 2017; Muziasari et al., 2017).

However, the dynamic and exact mechanisms of microbial transfer of fish associated microbiota and the pathways of colonization in RAS and the long-term stability of these communities remain poorly understood, in particular, in a polyculture practice in RAS. Fish microbiome is influenced by both intrinsic factors (e.g., digestive tract morphology, immune system) and extrinsic factors (e.g., water microbiota, rearing conditions) (Egerton et al., 2018; Smith et al., 2015) which make it specific for each species even when reared in the same environment. Existing research often focuses on monocultures or does not address how fish interspecies interactions might promote beneficial microbial synergies or increase the risk of pathogen spillover. While polyculture is known to offer ecological and economic advantages by combining species with complementary feeding habits or spatial niches (Thomas et al., 2022, 2021), it also raises concerns about microbial competition, pathogen transfer, and system imbalance (Kim et al., 2022; Makvandi et al., 2024).

This is why in this chapter, we aimed to (1) understand potential microbial transfers between fish species and between the different compartments in RAS specifically identifying the primary vector of fish responsible for the system inoculation (digestive tract, gills, skin), and (2) assessing microbial specification between fish tissues and fish species in polyculture and monoculture while assessing temporal inoculation and (3) examines whether the stabilized microbial diversity and final microbiota composition in polyculture are associated with fish-specific zootechnical traits like weight and size or physiological traits including stress level.

Together, these questions aim to elucidate how species diversification influences microbial ecology and dynamics and whether it offers microbial health-related benefits over monoculture practices in RAS hypothetically by suppression opportunistic pathogens and favoring beneficial bacteria.

Ultimately, this research contributes to improving the microbial management of RAS while diversifying productivity, potentially optimizing biosecurity and maintaining fish welfare.

II- CHAPITRE 1

Within RAS, biofilms act as reservoirs of microorganisms that can persist and reinoculate the system after their detachment from the surfaces. We believe that biofilms in RAS exhibit a “memory” capacity harboring residual microbes from across all past farming practices, creating a potential “tank’s history” effect. These residual communities are suggested to influence microbial dynamics in new cultures even after system cleaning by reinoculating the water at the start-up of the system after the refill of the tanks, thereby compromising microbial homogeneity between RAS units with different rearing backgrounds. We can’t ensure reliable results about species diversification effect on RAS microbiome using RAS units with different initial microbial state. So, to address this challenge, our study aimed to confirm the effect of the “tank’s history” on water microbiome in RAS and to develop a protocol to standardize the initial microbial structure between our indoor RAS facilities “ecotrons”, producing a similar starting microbial state.

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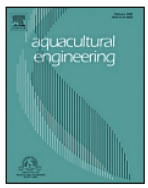

Effects of biofilter management modalities on the microbiological status in RAS water to avoid recording capacities of biofilms

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Abstract

Recirculating Aquaculture Systems (RAS) are increasingly seen as a breeding system of the future. The development of RAS requires understanding the microbial communities, as they play a significant role in the system functioning, water quality and therefore fish welfare and quality. However, some aspects remain poorly understood or insufficiently explored, such as information regarding the residual microorganisms persisting in the system and originating from the past rearing conditions. To our knowledge, any studies on microbial flora in RAS mention specific protocols to obtain a similar initial microbial state in their systems before conducting their new studies. In this context, controlling the initial RAS microbial status (before starting a breeding cycle) and having a reliable, standardized protocol for obtaining this state in a reproducible way is essential. In contrast to a standard protocol (i.e. only raw cleaning), this study aimed to evaluate the effects of two treatments that differ in the way of management of biofilter media. The water microbial communities' structure was assessed through the 16S rRNA gene over two weeks' post-treatment. The results show that the standard protocol does not shift the microbial past of the tank and is responsible for strong inter-tank heterogeneity. The testing protocol number T1 is the most reliable for homogenizing initial microbial composition of tanks.

Keywords: RAS, microbial communities, biofilm, cleaning and disinfection protocols

1) Introduction

Driven by the increased demand for protein, aquaculture has become one of the fastest-growing food industries worldwide. According to FAO (2020), aquaculture production is expected to increase from

77 million tons in 2018 to 109 million tons in 2030, corresponding to a 32% increase. Recirculating aquaculture systems (RAS) are a type of aquaculture systems based on innovative technology used for intensive fish production that offers the greatest control of rearing parameters and recirculate and reuse 90–99% of the water used in rearing tanks after treatment (Kamali et al., 2022; Moschos et al., 2022) to limit the supply of water in the system. The water treatment in RAS involves multiple steps through advanced mechanical and biological filtration systems, which remove solid and dissolved waste, ammonia, and carbon dioxide, ensuring optimal water quality for fish health (Aich et al., 2020). Faced with new challenges (climate change, food security, and sovereignty), these farming systems are increasingly seen as a future solution for developing sustainable aquaculture. In continental environments such as Central Asia and Europe, this farming system is used for the production of various fish species, including percids, pikeperch *Stizostedion lucioperca*, and Eurasian perch *Perca fluviatilis* (Dalsgaard et al., 2013; Rieder et al., 2023).

Microorganisms play a significant role in RAS, particularly regarding nutrient cycling, water quality, disease control, and the environmental quality of the effluent (Rurangwa and Verdegem, 2015). For example, the nitrifying bacteria of the biofilter transformed toxic ammonia excreted by fish into nitrate, which is less toxic for fish. Biofilters media, responsible for biological filtration, provide a surface for biofilm development which makes it the largest reservoir of microorganisms in RAS are, and therefore, essential to ensuring the well-being of fish. (Moschos et al., 2022; Rurangwa and Verdegem, 2015). However, some microorganisms present in RAS could have undesirable effects. Certain pathogens, especially the opportunistic bacteria of the Aeromonas or Flavobacterium genus, can cause health risks (fish diseases) (Rieder et al., 2023), while other bacteria are responsible for off-flavors and the otherwise poor quality of marketed products (Moschos et al., 2022). The pathogenic components of microbial communities in RAS constitute a significant challenge for the fish farm industry (Rieder et al., 2023). Microorganisms in RAS can be introduced through different pathways: make-up water, air, animal and insect exposure, feed, stocked fish, equipment used and staff (Blancheton et al., 2013). Moreover, the structure of the microbial community in RAS is affected by many factors including water contamination with excreted material of fish and food residues, water pH and chemical parameters (the level of nitrates, nitrites, etc.) and the population density of aquatic organisms (Sergaliev et al., 2021). In RAS, bacteria organized in biofilms are considered a potential reservoir for pathogenic bacteria. Once released from the biofilms, these pathogenic bacteria can cause recurring diseases (King et al., 2004). As the biofilms mature, they become reservoirs for microbial diversity, storing a record of past aquaculture conditions. Different management approaches are currently practiced in RAS to reduce opportunistic pathogen species, including raw cleaning normally applied to clean tanks and chemical disinfection treatments in case of disease outbreaks and sanitary problems

such as the use of chloramine T and hydrogen peroxide (Bigarré et al., 2017; Schreier et al., 2010). In aquaculture, chloramine T is highly used to inactivate bacteria and viruses because of its fast microbicidal action but especially because of its low bioaccumulation potential due to its high solubility in water and great biodegradability (Quezada-Rodriguez et al., 2022). Also, Hydrogen peroxide (H_2O_2) is another effective disinfectant used in aquaculture and regarded as "green" due to its decomposition into non-toxic and environmentally friendly by-products (Bögner et al., 2021). For all these reasons, monitoring the effectiveness of cleaning and disinfection treatments on RAS microbial communities is crucial to the future of this promising aquaculture system.

In that context, a recent prospective study by Fourier et al. (2022) aimed to compare the rearing performance in RAS of pikeperch reared in monoculture or biculture with tench (Tinca tinca) and to assess RAS planktonic microbial communities using a metabarcoding approach. As expected, Fourrier et al., (2022), identified a significant effect of the fish species but also a strong “tank effect”. The authors observed high intra-treatment and inter-tank variabilities that persist even though a raw cleaning of the tanks was performed before conducting the study. This finding indicated that the history of fish tanks, probably linked to the memory of bacterial biofilms formed over the years, may underscore their ability to reintroduce microbial diversity into the system and influence the water microbiome even after a raw cleaning of the system. It’s believed that bacterial biofilms within RAS exhibit a unique capability to record the microbiological diversity of previous farming practices within the system, essentially storing a record of the past aquaculture conditions. This “tank” effect generated a high level of initial residual variability, which interfered with the interpretation of the results of Fourrier et al., 2022. In literature, no information was found regarding the acquisition of an initial identical microbial state across replicates before conducting studies on microbial communities in RAS. Studies on microbial ecology in RAS do not mention any precautions or specific disinfection recommendations before conducting experiments on microbial communities. This observation leads us to consider this issue very seriously. To better study the effects of new farming practices (mixed farming) on the microbial communities in RAS, returning tanks to a similar initial microbial state and limiting variability due to breeding history is essential.

The present study supports the hypothesis of a connection between the tanks’ history and the composition of microbial communities in the water after cleaning and refilling. Therefore, the main purpose was to evaluate and identify a reliable cleaning and standardizing protocol in order to achieve a homogeneous initial tank microbial status with the lowest inter-tanks variability and prevent microbial recontamination of tanks originating from their individual history. To accomplish this three modalities based on cleaning protocols of tanks and biofilter management were tested without being followed by any fish addition to the system. Then the microbial community’s structure was studied

using a 16S rRNA gene metabarcoding approach and by analyzing. α- and β-diversity. Thus, this study's objective was to test and select a cleaning and standardizing procedure reliable enough to avoid tank-dependent microbial recontamination and to smooth out global heterogeneity.

2) Materials and methods

The study was carried out at the Platform of Experimental Aquaculture (PEA) of the Faculty of Sciences and Technologies (University of Lorraine, Nancy, France) (registration number for animal experimentation D54–547-18) from October 24th until (day 1, D1) to November 22th 2022 (day 30, D30).

2.1. Experimental facilities

12 independent temperature-controlled rooms "ecotrons" were used for this experiment. Each ecotron has its own closed system with its own water recirculating system. It contained an experimental unit of fiber and polyester resin (fish tank, 2400 L, L = 2 m; l = 2 m; h = 0.8 m), connected to a water recycling system with a flow rate of 3,000 L/h. The outlet of the fish tank was directed towards the mechanical filter (drum filter, mesh size: 63 µm), then water successively crosses a 300 L buffer tank (recovery tank for pumping), a 55 W Ultraviolet sterilization reactor and finally a fluidized biological bed filter of 450 L filled with media (Helix type). The management of the ecotrons has not changed since the creation of the PEA in 2014 (for all he tanks same origin of water: tape water of Nancy, same equipment and water retention time).

2.2. Experimental design

Taking as a basis the ecotron disinfection protocols used at the PEA (instruction N°AFPA-INS-ANI-23, March 27th, 2020), either routinely in the case of minor risk (standard protocol) or occasionally in the case of viral infection (Bigarré et al., 2017; reinforced protocol), three two cleaning/disinfection treatments were designed by integrating high-pressure physical cleaning of the basins, chemical treatments with hydrogen peroxide and chloramine T, two powerful disinfectants, physical shocks (high temperature and drying of the system) (Arvin and Pedersen, 2015; Linley et al., 2012; Quezada-Rodriguez et al., 2022), and biofilter management. These treatments were applied with 4 replicates (n = 4) randomly defined (fig.12). The standard protocol is applied one day, whereas the reinforced protocol is based on several actions carried out over 2 weeks:

T1 Standard treatment SP (control): 4 tanks will bewere treated one day one (D15) by a simple cleaning

of rearing tank with a high-pressure cleaner (raw cleaning ; no action on biofilter media, no chemical treatment, no thermal shock) followed by water emptying and filling of the tanks (temperature = 25°C) on day 15 (D15).

Treatment T1 (Standard + chemical disinfection + biofiltration media mix): 4 tanks were treated in 15 days. On day 1 (D1) raw cleaning, removal, and homogenization/mixing of biofiltration media of the 4 biofilters in one separate tank and redistribution of the mix in between the 4 tanks; followed by addition of 80 ml/m3 of hydrogen peroxide 30% and 7.5 g/m3 of chloramine T, both left to incubate. On day 3, draining and drying the system then filling and increasing water temperature to 45°C on days 9-10 and finally decreasing the temperature to 30°C (D11-D14) then to 25°C on day 15 (thermal shock).

Treatment T2 (Standard + chemical disinfection + replacement of biofiltration media) T2: 4 tanks were treated in 15 days. This treatment included the same steps as the T1 treatment except for the management of the filtration media. In the T2 treatment, the biofiltration media of the 4 biofilters were removed on day 1 and replaced with new media for each ecotron on day 15 after the thermal shock.

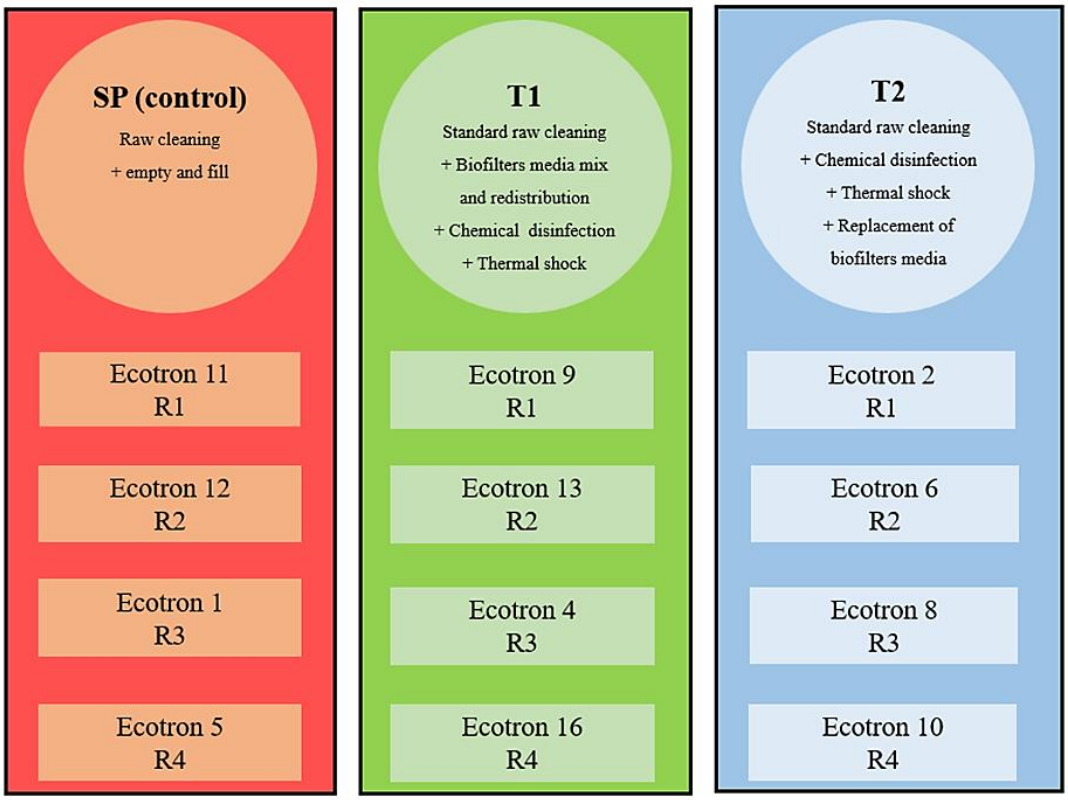

Figure 12. *Experimental design. 12 independent temperature-controlled rooms "ecotrons" including 4 replicates (R1, R2, R3, R4) for each treatment were randomly selected.*

2.3. Sampling strategy

The experiment lasted 30 days with two distinct periods: firstly, the cleaning/disinfection 15 days' period (day 1-day 15) dedicated to the application of the treatments T1 and T2 (only day for T1, day 15) and secondly a post-disinfection 15 days' period focused on the evaluation of the effects of the disinfection treatments on the tank microbial communities. Volumes of 500 ml of water were sampled in triplicates in each tank selected for the experiment. The samples were collected at different times: day 1 (D1, initial time), day 16 (D16, beginning of the post-treatment period), day 23 (D23, one week after the end of the treatment period) and day 30 (D30, two weeks after the end of the treatment period). Water samples were stored at -20°C.

2.4. Water quality

Physico-chemical parameters were measured to ensure that our protocols will not have effects on water quality in this study and therefore on fish welfare in future studies. Over the D15-D30 period, basic physico-chemical parameters of water were measured twice a week, in the morning before any manipulations on the system. Temperature (24.2 – 25.2°C), dissolved oxygen (> 6 mg.L-1) and pH (7.3-8.2) are measured daily with a multi-parameter probes WTW pH/Oxi 340i. The water concentrations of N- NH_4^+ (< 0.01 mg.L-1) and N- NO_2^- (< 0.05 mg.L-1) were measured spectrometrically.

2.5. DNA extraction and amplicon sequencing

Three 100 mL replicates of water were filtered through Whatman® NucleoporeTM Track-Etched membranes of 0.22 µm pore size before extraction. DNA extraction was performed using DNeasy PowerWater kit (Qiagen, Hilden, Germany) according to the manufacturer recommendations and were eluted in 100 µL of DNase-free water. DNA concentration was measured by the QuBit4 Fluorometer (Invitrogen, Waltham, USA) method. In addition, purity of the DNA extractions was assessed by measuring both 260/280 nm and 260/230 nm ratios with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA). DNA was stored at -20°C until further analysis.

2.6. Amplicon sequencing

Metabarcoding targeted the hypervariable V3-V4 region of the bacterial 16S rRNA gene and was carried out by at the GIGA Institute (Liège University, Belgium), using the universal primers 341F and 806R. Briefly, variable regions V3 and V4 of the 16S rRNA gene are amplified using the primer

pair 357F-806R in a dual-barcoding approach according to Kozich et al. (Kozich et al., 2013).

2.7. Bioinformatic analysis

Data were subsequently imported into the FROGS pipeline (Find Rapidly OTU with Galaxy Solution) implemented on a galaxy instance (v.2.3.0) (http:// sigenae-workbench.toulouse.inra.fr/galaxy/) (Escudié et al., 2018). After a quality control, 16S paired-end sequences were merged into contigs with VSEARCH (Rognes et al., 2016). Sequences were dereplicated before being clustered using SWARM algorithm (v.2.1.5) (Mahé et al., 2021) with a first denoising step using an aggregation distance equal to 1 and a second one equal to 3. Chimera were removed using VSEARCH (Rognes et al., 2016). Filters were applied to remove clusters which are not present in at least 3 samples or with an abundance below a 0.005% threshold (Bokulich et al., 2013). The taxonomic assignation of each OTU was performed using the BLAST tools against (Camacho et al., 2009) the database SILVA 132 16S (Pruesse et al., 2007). Phyloseq (1.26.1) R package was used to identify community composition analysis, to normalize and to generate α-diversity indexes (richness and evenness) after a rarefaction curve using the transform counts method (McMurdie and Holmes, 2013).

2.8. Statistical analysis

All statistical analyses were carried out using R software version 3.3.1 (R Development Core Team, http://www.R-project.org). We first compared α-diversity indexes to summarize the structure microbial communities with respect to its richness (number of Amplicon Sequence Variant (ASV)) and Shannon diversity index (distribution of abundances of the ASVs) with Phyloseq (1.26.1) (McMurdie and Holmes, 2013). After normality and homoscedasticity verifications (Shapiro and Bartlett test respectively), ANOVA (significance was declared at $P < 0.05$) were performed on richness and Shannon indexes and Tukey HSD test was used for pairwise comparisons using “agricolae” package. The β-diversity, to assess the degree of community differentiation, was estimated by using the relative abundance matrices and multivariate analyses. The dissimilarities (weighted Unifrac Distance) among treatments were assessed using Non-Metric Multidimensional Scaling, (NMDS) were performed followed by PERMANOVA using “adonis” function on vegan 3.0.2 R package to test for significant differences in sample clustering patterns across sampling point (9999 permutations). Plots were drawn using the R packages ggplot2 (3.1.1). Heatmaps were plotted with R package ampvis2 (2.5.1) (Andersen et al., 2018).

3) Results

3.1. Water quality

Temperature (24-25°C) and pH (8.02-8.04) remained constant and stable in all tanks (Table 1). A higher mean level of dissolved oxygen level was recorded using the standard protocol; however, it remained higher than 7.5 mg O_2/L in all tanks indicating well-oxygenated water in all tanks. Concerning ammonia concentrations, a higher concentration was recorded using treatment T1, only explained by a higher concentration on day 1. After day 1 the ammonia level was zero as in other treatments. Regarding the nitrite level, the concentration was continuously higher in tanks of T2 treatment.

Table 1. Water quality parameters (mean ± SE) of water in RAS (n=4) measured during the post-treatment period from D16 to D30 between the three tested treatments: standard treatment SP, treatment T1 (standard + chemical disinfection + biofiltration media mix) and treatment T2 (standard + chemical disinfection + replacement of biofiltration media).

Water quality parameters	Standard protocol SP (control)	Treatment T1	Treatment T2	Test statistic and p-Value
Temperature (°C)	24.8 ± 0.2 a	24.9 ± 0.2 a	25.0 ± 0.1 a	ANOVA, p-Value = 0.611
O2 (mg.L^{-1})	7.98 ± 0.10 a	7.66 ± 0.10 b	7.72 ± 0.10 b	ANOVA, p-Value = 0.009
pH	8.04 ± 0.02 a	8.03 ± 0.01 a	8.02 ± 0.03 a	ANOVA, p-Value = 0.309
NH^{4+} (mg.L^{-1})	0.000 ± 0.000 a	0.033 ± 0.000 b	0.000 ± 0.000 a	Kruskal-wallis, p-Value = 0.004
NO^{2-} (mg.L^{-1})	0.000 ± 0.000 a	0.001 ± 0.002 a	0.034 ± 0.006 b	Kruskal-wallis, p-Value = 0.009

3.2. Microbial analysis

Sequencing of the amplified rDNA yielded 5.95 E106 sequences over 150 samples, with an average of 39,696 reads per sample.

3.2.1. Alpha-diversity

Bacterial ASV richness was reported as the total number of amplicon sequence variants (ASVs) per sample (fig. 13). There was significant variability between the tanks, regardless of the treatment used, and no significant difference was recorded on Day 1. There were significant differences in the number

of ASV in the tanks treated with T2 compared with the number of ASV in the tanks treated with the other two treatments (SP and T1). Mixing biofiltration media already colonized by bacteria (T1) allowed for a significantly higher richness of planktonic microbial communities in the tanks than new biofiltration media (T2). Furthermore, no significant differences were observed between the different sampling times, except in tanks treated with modality 3, where microbial richness decreased significantly at D16 (when the new biofiltration media was used).

No significant differences in ASV diversity (approximated by Shannon index calculation) (fig. 14) were found with either treatment. Moreover, no significant differences in kinetic alpha diversity measurements were observed throughout the experiment.

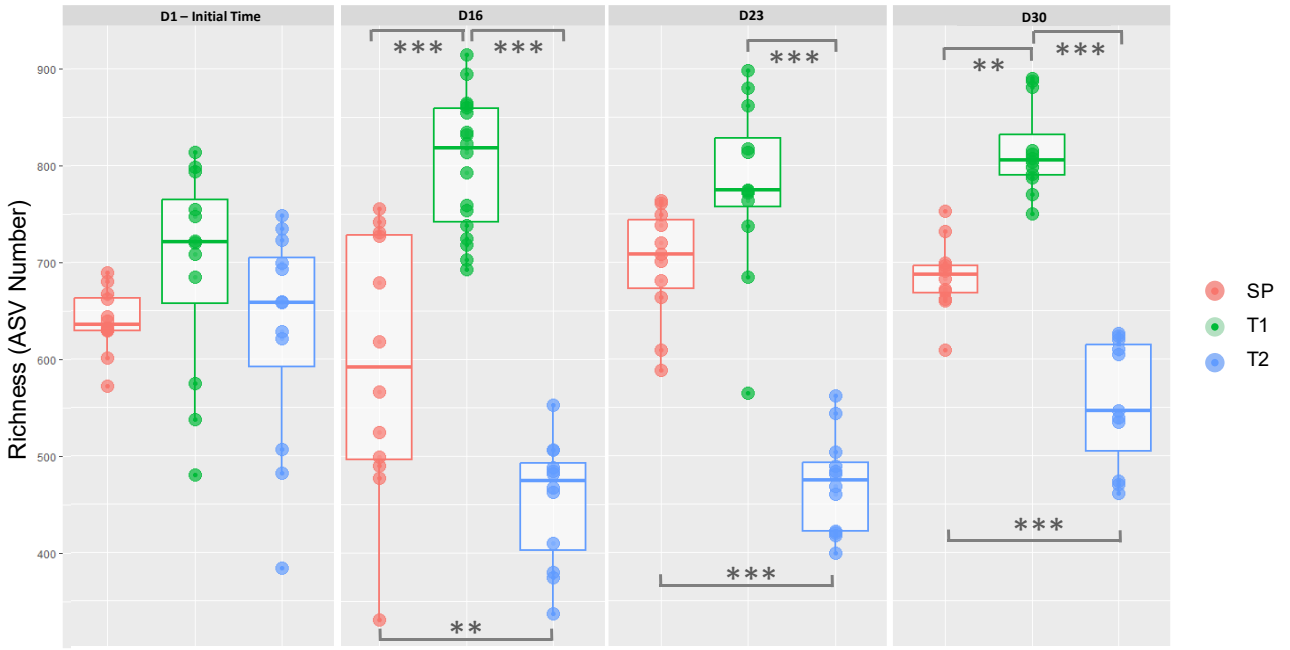

Figure 13. *Microbial alpha-diversity indexes between treatments (Standard protocol SP (control), treatment T1 (Standard + chemical disinfection + biofiltration media mix) and treatment T2 (Standard + chemical disinfection + replacement of biofiltration media) measured at D1(initial time before any treatment), D16 (just after the treatment period), D23 and D30 (one and two weeks after the treatment period). Alpha-diversity is presented as a measure of species richness (number of ASVs). Stars above the box-plots indicate significant differences between compartments; *p < 0.05, **p <0.005 and ***p <0.001*

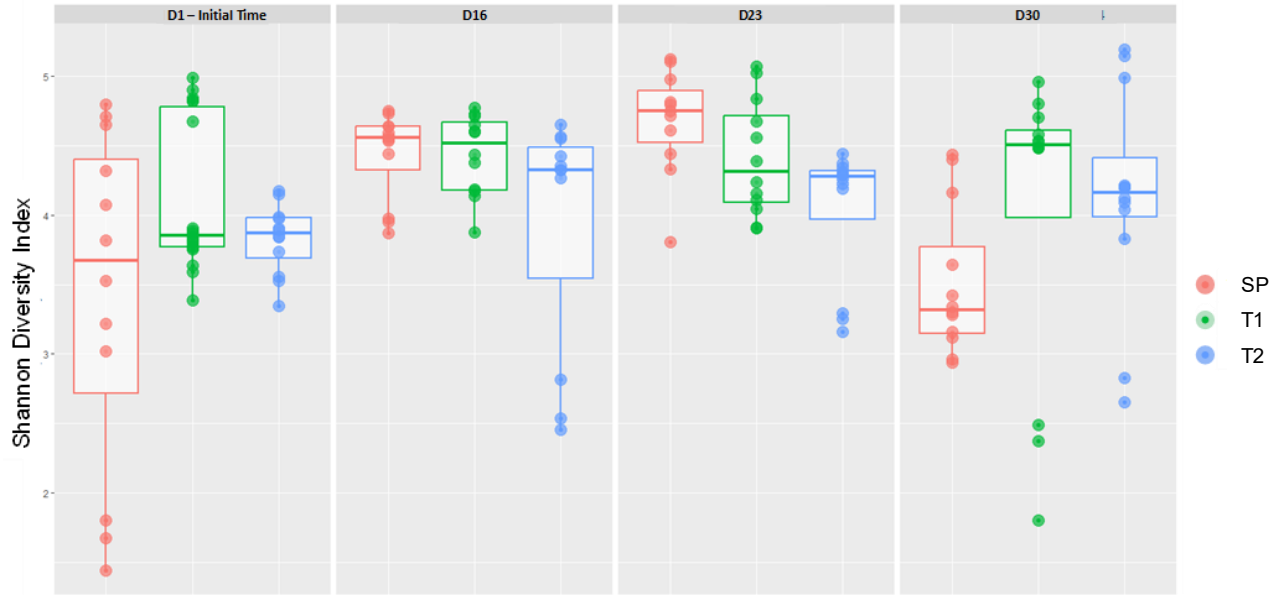

Figure 14. *Alpha-diversity between treatments (Standard protocol SP (control), treatment T1 (Standard + chemical disinfection + biofiltration media mix) and treatment T2 (Standard + chemical disinfection + replacement of biofiltration media)) measured at D1(*initial time before any treatment), D16 (just after the treatment period), D23 and D30 (one and two weeks after the treatment period). The alpha diversity is presented as a measure of Shannon diversity index (biodiversity index). Stars above the box-plots indicate significant differences between compartments; *p < 0.05, **p <0.005 and ***p <0.001.

3.2.2. Beta Diversity

Non-Metric Multidimensional Scaling (NMDS) using weighted Unifrac Distance was performed to make a quantitative beta diversity measurement, established that the water microbiomes clustered by treatment but not by sampling time (fig. 15). A permutational multivariate analysis of variance (PERMANOVA) test indicated that the difference between treatments was statistically significant (P = 0.001). Fig. 15 shows that treatments for T1 and T2 sites clustered separately were away from the SP treatment. This experiment confirmed that the routine washing and filling of water tanks results in the recolonization of the water by biofilm microbial communities. It found that routine washing and filling the water tanks resulted in the recolonization of the water by biofilm microbial communities (since no significant difference was observed between the initial treatment and modality 1) because the tanks shared common microbiomes at the endpoint (P = 0.4) The inter-tank heterogeneity of the microbiome structure was lower in the water sampled in the tanks treated with T1 (disinfection and media mix).

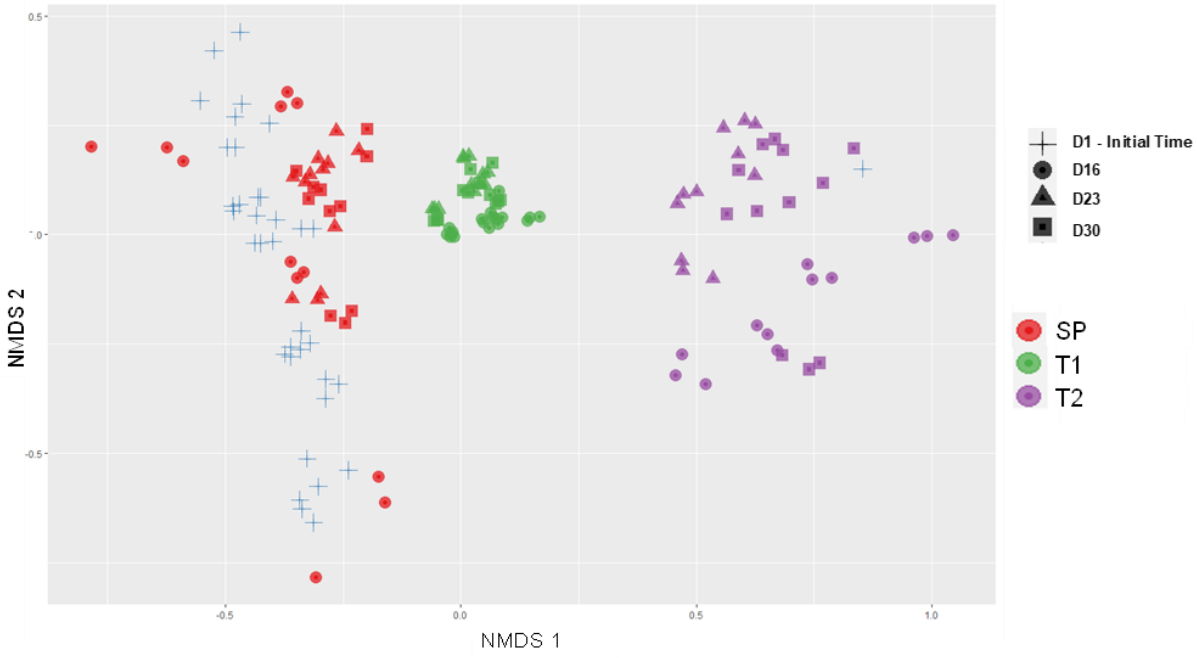

Figure 15. *Non Metric Multidimensional Scaling (NMDS) of microbial communities' structure among treatments (Standard protocol SP (control), treatment T1 (Standard + chemical disinfection + biofiltration media mix) and treatment T2 (Standard + chemical disinfection+ replacement of biofiltration media)) measured at D1(initial time before any treatment), D16 (just after the treatment period), D23 and D30 (one and two weeks after the treatment period) based on weighted Unifrac Distance. The colors of symbols are blue: before any treatment (D1), red: treatment SP, green: treatment T1 and violet: treatment T2.*

3.2.3. Microbial community composition

The distribution of bacterial orders among the three treatments was examined to identify the general composition of the water microbiome and the orders preferentially associated with the treatments and sampling times. Order distributions are shown as a heat map (fig. 16), from which it can be observed that the treatment affects the microbial composition. Water sampled for the T2 and T1 treatments seemed to host microbial orders at different relatives abundances than the SP treatment. The difference in microbial composition between T2 and T1 treatments dealt, for example, with the Gammaproteobacteria order, which was significantly more abundant in the samples taken from the T2 tanks. The results also indicated that T2 treatment removed some bacterial orders by replacing media, such as *Clostridia, Oligoflexia,* and *Planctomycetes*. The percentage of *Clostridia* in the T2 tanks decreased from 2.3 at D1 to 0.4 at D16. However, a greater decrease in the percentage of *Anaerolineae*, from 3.5 at D1 to 0 at D16, was observed.

The same heat map was performed at genus and family level (data not shown) and shows, on the one hand, that a large proportion of reads are unaffiliated, but, on the other hand, that some organisms with positive or negative functions in aquaculture (according to the literature) show different relative abundance profiles depending on the treatments. At the end of the experiment, the *Nitrospira* genus

was significantly less represented in treatment T3 than in SP and T2. Conversely, bacteria from the *Flavobacterium* genus and the Alteromonadaceae family were relatively more abundant in the T3 treatment.

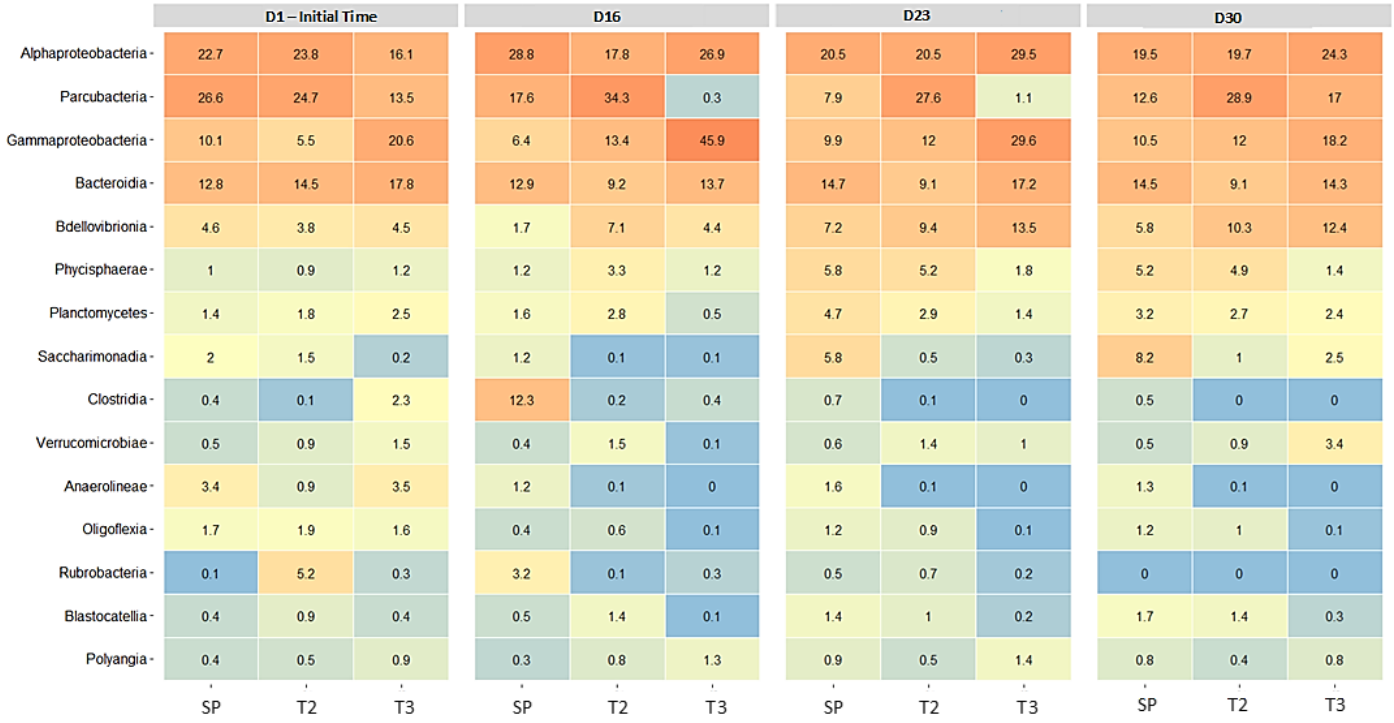

Figure 16. *Heatmap visualizing the microbial community composition and relative abundances of the most abundant bacterial orders (n=15) among treatments (Standard protocol SP (control), treatment T1 (Standard + chemical disinfection + biofiltration media mix) and treatment T2 (Standard + chemical disinfection + replacement of biofiltration media)) measured at D1(initial time before any treatment), D16 (just after the treatment period), D23 and D30 (one and two weeks after the treatment period) among sampling point and lines. Colors are scaled from highest (red) to lowest (blue) values within columns.*

3.2.4. Venn Diagram

The percentage of shared ASVs between the tanks of SP and T1 treatments significantly increased from 5% to 15% at D30. ASV was shared between the three treatments on days 1 and 30 (fig. 17). The percentage of shared ASVs between the tanks of the SP and T1 treatments significantly increased from 5% to 15% at D30. Meanwhile, the core microbiota between the tanks of the three treatments at D30 was 58%, significantly lower than the initial percentage of shared ASVs, which was 72%.

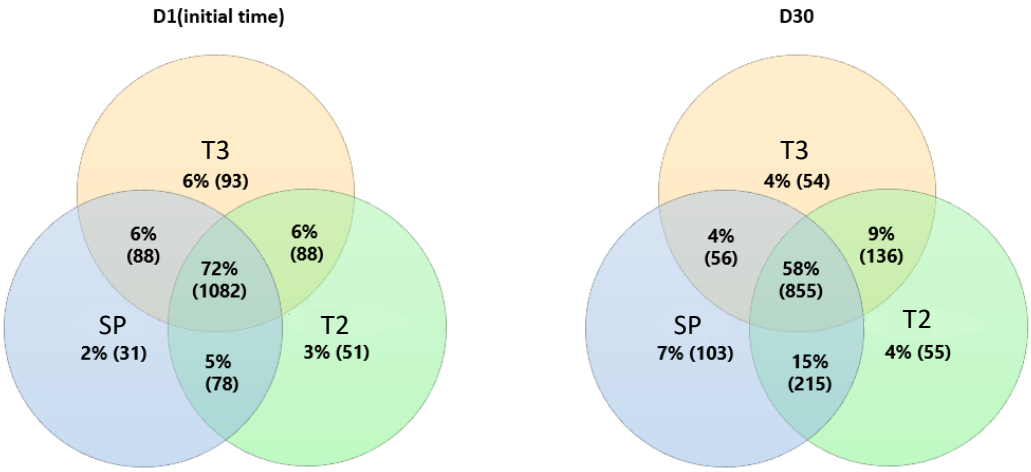

Figure 17. *Venn diagram visualizing the amplicon sequence variant ASV shared between the three modalities D1(initial time before any treatment) and D30 (two weeks after the treatment period). Colors of symbols are blue: Standard protocol SP (control), green: treatment T1 (Standard + chemical disinfection + biofiltration media mix) and beige: treatment T2 (Standard + chemical disinfection + replacement of biofiltration media).*

4) Discussion

First, this study corroborates the hypothesis of an association between the tanks' history and the water microbial communities after refilling. The β-diversity representation permitted highlighting a high degree of heterogeneity in the initial microbial communities' structure, as graphically illustrated by the splitting of the points associated with these samples in the multivariate analysis. In addition, despite performing SP treatment (consisting of routine raw cleaning), this heterogeneity remained unchanged throughout the experiment. Finally, as the PERMANOVA results showed no difference between the communities before and after cleaning, we can conclude that the planktonic communities were inherited from the former sessile communities. Biofilms, the aggregates of microbial layers (Costerton et al., 1999), have been considered to be more resistant to antimicrobial than free-living bacteria (Lewis, 2001). The propensity of biofilms to recontaminate the surrounding environment is a well-known medical phenomenon (Whitehouse et al., 1991) but also in food contamination (Abebe, 2020). Previous studies of microbial diversity assessment in RAS have been performed, but none mention this recolonization phenomenon or the option of using a decontamination protocol.

As previously discussed, SP treatment had a relatively minor effect compared to the difference between the initial time and decontaminated treatments (T2 and T1). A decrease in tank-dependent heterogeneity could be observed on the cloud of points illustrating tanks treated with the T1 modality. A significant decrease in microbial richness was observed in tanks treated with T2. This decrease was linked to the use of new media and the microbial loads being much lower than those of native media.

Specific orders showing a difference in their relative abundance were identified. For example, the Parcubacteria order was underrepresented in T2 treatment. Biofilm plays also a central role in the conversion of nitrogen metabolites in recirculating aquaculture systems, avoiding the accumulation of toxic form for fish (Eddy and Williams, 1987). Thus, even if T2 treatment was reliable for erasing tanks' history (by using new biofiltration media), it could be responsible for a decrease in richness and an imbalance in microbial communities, especially those involved in nitrogen cycle. β-diversity analyses showed a greater phylogenetic and structural similarity between the initial communities of tanks before any treatment and the communities hosted in tanks treated with T1 than those treated with the T2 treatment. Moreover, the Venn diagram analysis indicated that at the end of the experiment, the percentage of shared ASVs between the tanks treated with T1 and SP was the most important of the total microbiome. This treatment was, therefore, likelier to maintain operating conditions (the nitrogen cycle and system resilience) since it was most similar to the initial system from a microbial perspective. This phenomenon can be largely explained by the fact that the particularity of T1 treatment was to mix the media from the different tanks. This allowed the initial bacterial community to be maintained, while making biofilm recontamination more homogeneous.

Samples obtained from the T1 modality showed higher microbial richness than other modalities, meaning that mixing biofiltration media allowed for disseminating many microbial species. The higher the biodiversity of a bacterial community, the more resilient and resistant it is (to invasion, for example (Mallon et al., 2015).The microbial communities of mixed biofilms will converge while adapting to their environment, as has already been reported in the literature in natural ecosystems (Fechner et al., 2012). All these data allowed us to select this modality as the most reliable cleaning and standardizing protocol to overcome the issue of the effect of tanks' history. This modality will be our new routine procedure for treating tanks before performing future studies on microbial communities in RAS.

5) Conclusion

To conclude, our research provides the first detailed description of the effect of a tank's history. Microbial diversity varied from one tank to another, as each fish species introduced a particular microbial flora (linked physiological status and origin) through intimate interaction with its surrounding water. This work is the first to offer preventive measures to avoid the recolonization effects of biofilms, which can be considered recorders of the previous microbial life of rearing tanks.

CRediT authorship contribution statement

Renaud Nivelle and Yannick Ledoré: experimentation setup and sampling. Frédérique Changey and Renaud Nivelle: molecular biology work. Frédérique Changey: Data analysis and production of figures. Frédérique Changey, Salam Al Dakdouki and Pascal Fontaine: writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Declaration of Competing Interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript. No approval from research ethics committees was required to accomplish this study's goals. The datasets generated during the current study are available from the corresponding author upon reasonable request.

Data availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Acknowledgments

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III- CHAPITRE 2

We confirmed that biofilms in recirculating aquaculture systems (RAS) serve as reservoirs of microbial diversity, retaining a “microbial memory” of past rearing conditions that can confound microbial studies influencing the recolonization of planktonic communities after cleaning, leading to high inter-tank variability when disinfection is omitted. The reinforced protocol combining chemical disinfection with biofilter homogenization (T1) effectively reduced this variability and established a reproducible microbial initial state, addressing the challenge of standardizing initial microbial conditions across RAS tanks. Research have begun to increasingly study fish polyculture in RAS as it gained attention as a sustainable strategy to enhance RAS resilience, but they mainly focus on zootechnical and physiological traits (Mihailov et al., 2017; Thomas et al., 2020; Dawood et al., 2025). Despite reported correlations between fish microbiota and water and biofilms microbial communities in RAS (Munich et al., 2020; Mes et al., 2023), yet no study has explicitly characterized the influence of fish species biodiversity in polyculture on the shaping of the structure of sessile and planktonic microbial communities within RAS.

This chapter addresses the question of how fish polyculture, through the combination of different fish species with different phenotypes and distinct associated microbiota, can affect the composition and diversity of microbial communities in both water and tank walls biofilms in RAS using 16S rRNA gene metabarcoding.

Can fish biodiversity affect sessile and planktonic microbial communities of water and biofilms in Recirculating Aquaculture Systems (RAS)?

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Abstract

Recirculating Aquaculture Systems (RAS) offer a sustainable alternative to open fish farming systems, with microbial communities playing a critical role in maintaining water quality and system resilience. However, the influence of fish phenotypic characteristics on the structure and dynamics of planktonic and sessile microbial communities, particularly in polyculture context, remains underexplored. This study investigated the impact of fish species composition on water and biofilm microbiota in RAS using monocultures of pikeperch (*Sander lucioperca*) and tench (*Tinca tinca*), and polyculture of both species. Microbial communities were analyzed at the beginning and the end of the experiment four months later using 16S rRNA gene metabarcoding. Alpha and beta diversity analyses revealed species-specific microbial signatures in both water and biofilms, with polyculture systems generating unique, non-additive microbial profiles. Notably, polyculture reduced the relative abundance of potentially pathogenic taxa (e.g., *Clostridium, Mycobacterium*) and promoted beneficial groups such as Rhodobacteraceae. These findings highlight the significant role of fish phenotypes and species in shaping microbial community structures in RAS, with implications for improving aquaculture system health and sustainability through fish diversity management.

Keywords: Recirculating Aquaculture Systems, microbial communities, fish polyculture, water, biofilm.

1) Introduction

The global aquaculture industry has evolved significantly over the past 50 years, now surpassing

capture fisheries in contributing to the global seafood supply (Botta et al., 2020) and aquaculture production is projected to reach 111 million tons in 2032 (FAO, 2024).

Aquaculture approaches have evolved considerably to address environmental issues such as climate change and water pollution (Cai et al., 2023), notably via the development of Recirculating Aquaculture Systems RAS (Badiola et al., 2018).

RAS are land-based aquaculture productions systems – either open air or indoors – that minimize water consumption by filtering, treating, and reusing the water (90-99%) (Vielma et al., 2022). The functioning of RAS consists of rearing tank, mechanical filter, biofilter, oxygenators and water circulation pump (Aich et al., 2020). Water treatment involves multiple stages including mechanical filtration (to eliminate solid and dissolved waste) to biological filtration for ammonia removing in the biofilter, and further steps including carbon dioxide removing and disinfection; this ensures optimal water quality essential for fish health (Aich et al., 2020). In Europe, this farming system is used to produce a wide variety of fish species including percids, pikeperch (*Sander lucioperca*), and European perch (*Perca fluviatilis*) (Dalsgaard et al., 2013; Rieder et al., 2023).

In RAS, microbial communities play a predominant role (notably in nutrient cycling) and can be found in planktonic or sessile form (Bartelme et al., 2019; Moschos et al., 2022). Among sessile communities, those forming a biofilm in the biofilter are responsible for the oxidation of toxic metabolic by-products, such as ammonia (from fish metabolism) (Moschos et al., 2022). These nitrifying bacteria, such as the genera *Nitrosomonas* and *Nitrobacter*, are the main converters of ammonia and nitrite (both toxic to fish), into the less harmful compound nitrate (Sakoula et al., 2021). Additionally, heterotrophic bacteria contribute to the degradation of organic matter, originating of uneaten fish feed and excrement, which supports the biofilter (Rurangwa and Verdegem, 2015; Moschos et al., 2022). Despite these essential functions, biofilters in RAS are sensitive and can be impacted by many factors like suspended solids, fluctuations in temperature, or chemical treatments such as the use of antibiotics therefore causing the outbreak of diseases. Studies showed that accumulation of organic waste, uneaten feed, and fish feces can cause a degradation of water quality, triggering the proliferation of opportunistic pathogens, including opportunistic heterotrophic bacteria that can compete with biofilters nitrifying bacteria for space, oxygen, and nutrients (Pedersen et al., 2017; Aich et al., 2020). For example, the genus Aeromonas and Pseudomonas are common heterotrophic bacteria in the aquatic environment known as denitrifiers and can play an important role in the removal of toxic nitrogen compounds, but some strains can cause infections in fish, and compete with nitrifying bacteria (Godzieba et al., 2025). Conversely, high-density rearing in RAS can favor the development of pathogenic bacteria (Wang et al., 2020) which contribute significantly to the most proportion of diseases observed in RAS (Sergaliev et al., 2021). For instance, Mycobacteriosis

infection caused by *Mycobacterium marinum* was recorded and associated with high mortality in pikeperch cultured in a recirculating aquaculture system (Gjurčević et al., 2020). Opportunistic pathogens including *Vibrio, Aeromonas*, and *Pseudomonas* are also often found in RAS in biofilms, water columns, and fish-associated microbiomes (Gupta et al., 2024). Finally, water-associated off-flavour bacteria could be unbalanced in aquaculture, altering the organoleptic qualities of products (Pedersen et al., 2017). Microbial biomass and diversity in RAS are subject to imbalances in response to multiple factors: water physico-chemical parameters (pH, temperature, O_2, ammonium, nitrite and nitrate levels) (Sergaliev et al., 2021) and could result in a bloom of pathogens (Gupta et al., 2024). Rich and diverse microbiota is known to enhance the resilience of the system by promoting competition for ecological niche with opportunistic pathogenic microorganisms (Harrison et al., 2020). Microorganisms can colonize systems through various pathways, including water, feed, air, equipment or fish (Rurangwa and Verdegem, 2015). Indeed, fish tissues through their microbiota (gut, mucus or gills) could be considered reservoirs for invasive microbial communities (De Bruijn et al., 2018) that are in direct contact with the surrounding water (Moschos et al., 2022). These microbiotas are influenced by intrinsic factors (genotype, which determines digestive tract morphology, diet and ethology) (Smith et al., 2015; Dai et al., 2021) and extrinsic factors (environment and diet) (Egerton et al., 2018; Dulski et al., 2020). Nevertheless, the impact of fish microbiota on the microbial state of the surrounding environment remains poorly studied (Chen et al., 2022). Additionally, biofilms previously attached to the materials are reservoirs of microbial diversity inoculating the water after the start-up of the system (Schreier et al., 2010; Al Dakdouki et al., 2025). Moreover, the dynamics of microbial colonization linked to the phenotypic characteristics of fish have not been studied in a polyculture context, which is known to benefit from complementary effects between fish. Indeed, different species could occupy different trophic levels and have varying feeding habits (Reynaud, 2019; Thomas et al., 2022) and might increase the chance of pathogen spillovers (Amoussou et al., 2022).

Firstly, this study aims to assess how the phenotype of fish with different life-history traits (e.g. feeding strategies and previous rearing conditions) and different digestive tract morphology influence the structure and dynamics of water microbial communities and biofilms in closed rearing systems. Secondly, this work investigates how fish polyculture could change the inoculation trajectory of microbial communities compared to monoculture of each species alone. The hypothesis is that in polyculture, interactions between fish microbiota can result in a more complex microbial community than in monoculture. This study examined the diversity of sessile and planktonic microbial communities by metabarcoding on the rRNA16S gene in RAS pilots with 2 monocultures: one of pikeperch (*Sander lucioperca*) and one tench (*Tinca tinca*) and a polyculture of these two fish.

2) Materials and methods

The study was carried out at the Platform of Experimental Aquaculture (PEA) of the Faculty of Sciences and Technologies (University of Lorraine, Nancy, France) (registration number for animal experimentation D54–547-18). The study was conducted from 18 September 2023 to 15 January 2024, lasting a total of four months.

2.1. Experimental facilities

12 independent temperature-controlled rooms "ecotrons" were used for this experiment. Each ecotron has its own closed system with its own water recirculating system (fig. 18). It contained an experimental unit of fiber and polyester resin (fish tank, 2400 L, L = 2 m; l = 2 m; h = 0.8 m), connected to a water recycling system with a flow rate of 3,000 L/h. The outlet of the fish tank was directed towards the mechanical filter (drum filter, mesh size: 63 µm), then water successively crosses a 300 L buffer tank (recovery tank for pumping), a 55 W Ultraviolet sterilization reactor and finally a fluidized biological bed filter of 450 L filled with media (Helix type).

2.2. Experimental design

The ecotron were cleaned/disinfected, to avoid recontamination by microorganisms from previous handling (Al Dakdouki et al., 2025), consisting of high-pressure cleaning of the tanks, chemical treatments with hydrogen peroxide and chloramine T, two powerful disinfectants, physical shocks (high temperature and drying of the system) ((Bigarré et al., 2017); reinforced protocol) (Linley et al., 2012; Arvin and Pedersen, 2015; Quezada-Rodriguez et al., 2022), and biofilter media mixing. In addition, a solution of nitrifying bacteria was added in all ecotrons to avoid an increase in ammonium concentration after the introduction of the fish in the system.

In this study, a polyculture between pikeperch (*Sander lucioperca*) and tench (*Tinca tinca*) was selected, based on previous laboratory results published in 2020 (Thomas et al., 2020). During the period of application of the treatment, the fish were acclimated and maintained under optimal rearing conditions for each species. The pikeperch (origin: La Ferme Intégrale) were stocked in two free ecotrons at the PEA, with 60 kg per ecotron, while the tench (origin: BAJCSHAL kft. 9062 KISBACS-HUNGARY) were stocked in 700-liter tanks in a larval rearing room at PEA, with 12 kg of tench per tank. 3 rearing modalities were defined for this experiment, 12 ecotrons were selected with 4 replicates (n = 4) randomly defined to achieve a similar initial total biomass of 8 kg of fish in all ecotron as follow:

i) a monoculture of tench (*Tinca tinca*) (T),

ii) a monoculture of pikeperch (*Sander lucioperca*) (P),

iii) a polyculture of pikeperch with tench (50%-50% in biomass) (P_T).

A standardized daily feed ration of 80 g using Legouessant Percid Grower Floating No. 4 feed was applied. The water' temperature was maintained at 25°C, and the photoperiod was set to 16 hours of light (lights on at 7:00 AM, with 30 minutes of dawn and 30 minutes of dusk) at an intensity of 20 lx.

2.3. Physico-chemical parameters measurement

Physico-chemical parameters of water were measured three times a week. Temperature, dissolved oxygen (> 6 mg.L-1) and pH (7.3-7.9) are measured daily with a multi-parameter probe WTW pH/Oxi 340i. The water concentrations of $N\text{-}NH_4^+$ (< 0.01 mg.L-1) and $N\text{-}NO_2^-$ (< 0.05 mg.L-1) were measured spectrometrically.

2.4. Sampling strategy

Samples of water and biofilms (from the tank walls) were collected in triplicate at different times: T0 (2 days after the distribution of fish among the 12 ecotrons) and Tf at the end of the experiment. Water samples were collected directly from the upper 30 cm of the tanks in 500 ml sterile bottles.

Biofilms were collected by scraping the walls of the rearing tanks and transferring them into sterile 15 ml Falcon tubes.

In addition, snails that have appeared in the last month of the experiment were collected at Tf from the tank's surface and transferred into sterile 15 ml Falcon tubes. Snail's body was removed and separated for further DNA extraction. All samples were stored at -20°C until DNA extraction.

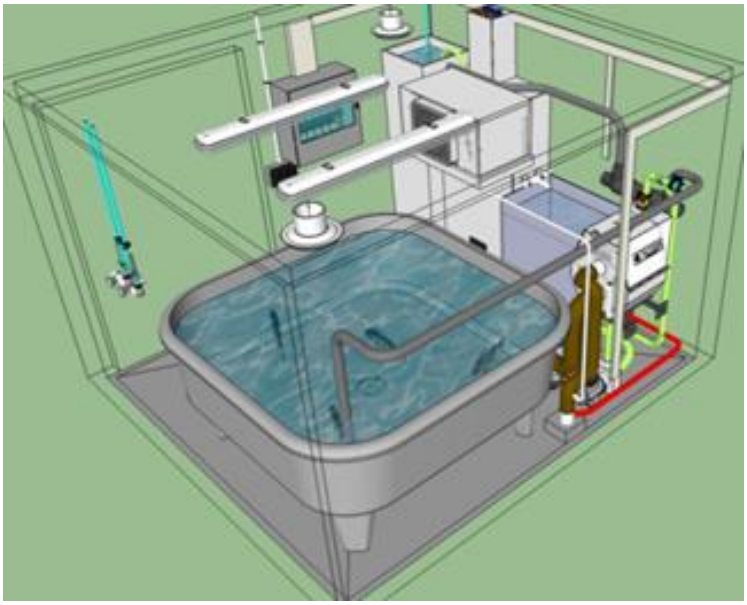

Figure 18. *Diagram of one of the pilots used to reproduce the operation of a small-scale RAS.*

2.5. DNA extraction

100 ml of the collected water were filtered through Whatman® NucleoporeTM Track-Etched membranes of 0.22 µm pore and extraction was performed using DNeasy PowerWater kit (Qiagen, Hilden, Germany). Microbial DNA from biofilm samples were extracted using the NucleoSpin Soil Kit (Macherey-Nagel) following cell disruption with mechanical force with FastPrep-24™ All collected DNA were eluted in 50 µL of DNase-free water. Concentration and purity of the DNA extractions was assessed by measuring both 260/280 nm and 260/230 nm ratios with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA). DNA was stored at -20°C until further analysis.

2.6. Amplicon sequencing

Metabarcoding targeted the hypervariable V3-V4 region of the bacterial 16S rRNA gene Amplicon library preparation, and sequencing services were provided by Novogene Co., Ltd. (Beijing, China). Briefly, variable regions V3 and V4 of the 16S rRNA gene was amplified using the primer pair 357F-806R in a dual-barcoding approach according to (Kozich et al., 2013).

2.7. Bioinformatic analysis

Data were subsequently imported into the FROGS pipeline (Find Rapidly OTU with Galaxy Solution)

implemented on a galaxy instance (v.2.3.0) (http:// sigenae-workbench.toulouse.inra.fr/galaxy/) (Escudié et al., 2018). After a quality control, 16S paired-end sequences were merged into contigs with VSEARCH (Rognes et al., 2022). Sequences were dereplicated before being clustered using SWARM algorithm (v.2.1.5) (Mahé et al., 2022) with a first denoising step using an aggregation distance equal to 1 and a second one equal to 3. Chimera were removed using VSEARCH (Rognes et al., 2022). Filters were applied to remove clusters which are not present in at least 3 samples or with an abundance below a 0.005% threshold (Bokulich et al., 2013). The taxonomic assignation of each OTU was performed using the BLAST tools (Camacho et al., 2009) against the database SILVA 132 16S (Pruesse et al., 2007). "Multi-affiliation" indicates that different taxa are assigned to the same OTU, such that a unique taxonomic assignment cannot be determined.

2.8. Statistical analysis

All statistical analyses were carried out using R software version 4.5.0 (R Development Core Team, http://www.R-project.org) and "Cranberry Hibiscus" RStudio version.

Water quality parameters were compared using the ANOVA test (p-value = 0.05) and Tukey HSD for pairwise comparisons ("agricolae" package, version 1.3-7).

Phyloseq (1.52.0) R package was used to analyze community composition, normalize the data and generate α-diversity indices (richness and evenness) after rarefaction using the transform counts method (McMurdie and Holmes, 2013). It was also used to ordinate the data using Bray-Curtis dissimilarity and perform PCoA representations.

Renyi's diversity profiles were used to compare several alpha indices of microbial diversity among the modalities using "BiodiversityR" (2.17.-2) and "ggplot2" (3.5.2) packages (Kindt, 2020). Renyi simultaneously represents richness, diversity, and evenness across multiple modalities. The curves are plotted along the y-axis according to H(α), defined as follows: , with pi the proportions of each species and α representing the value on the x-axis, ranging from 0 to infinity. The profiles read at α values of 0, 1, 2, and infinity (∞) correspond to the species richness index, the Shannon index, the Simpson index, and the Berger-Parker index, respectively (Kindt and Coe, 2005).

A modality with higher richness will have a higher H(0) value; a modality with greater evenness will result in higher H(α) values for α>0. As α increases, Renyi's diversity representation places more emphasis on the most abundant species, with Hα (the Berger-Parker index) representing the proportion of the most abundant species.

α-diversity indexes were compared from the trues index values calculated with "vegan" (2.6-10) package, to summarize the structure of microbial communities. After normality and homoscedasticity

verifications (Shapiro and Bartlett test respectively), ANOVA (significance was declared at $P < 0.05$) and Kruskal-Wallis test were performed on α-diversity indexes and Tukey HSD or Dunn-test with BH (Benjamini-Hochberg) adjustment were used for pairwise comparisons using "agricolae" package.
Bray-Curtis distances were statistically tested between groups using multivariate PERMANOVA ("pairwiseAdonis" package, version 0.4.1) and ANOSIM ("vegan" package), both performed with 9999 permutations. Pairwise statistical comparisons of the relative abundance of each family subsequently performed using both "DESeq2" package (version 1.48.0) and SIMPER ("vegan" package) to cross-validate the findings.
Hierarchical clustered heatmaps were constructed using the "pheatmap" package (version 1.0.12). OTUs were aggregated at the family level by summing abundances per sample. These family-level abundances were averaged per modality (P, T, or P_T), then normalized by adding a pseudo-count of one, and performing a Centered Log-Ratio (CLR) transformation. Finally, each row (family) was centered and scaled (z-scored) for visualization. Similarities between families were computed using Euclidean distance, while similarities between columns were assessed via Pearson correlation. Clustering of both rows and columns was performed using the Ward.D2 method.
The Venn diagram was constructed using the "MicEco" package (version 0.9.19).

3) Results

3.1. Water quality

Water quality measurement was summarized in (SI). Statistical analysis showed no significant differences in the mean levels of T°, O_2, NH_4^+ and NO_2^- between the three modalities. However, a higher mean level of pH was recorded in pikeperch monoculture 7.51 compared to pikeperch/tench polyculture 7.45 and tench monoculture 7.47. Nevertheless, given the small amplitude of the difference (0.02), we considered its impact on fish physiology and microbial communities to be negligible.

3.2. Microbial analysis

3.2.1. Alpha-diversity

Renyi's diversity index (fig. 19) reflects the microbial α-diversity of water and biofilm samples in the three modalities at D0 and D120. At T0, Renyi's diversity showed no differences in bacterial richness of water and biofilm samples across the different modalities based on shape curve (fig. 19A and 19B). However, differences became apparent with the Simpson index and for the Berger-Parker especially

for monoculture of tench showing lower diversity compared to the other modalities.

At Tf, distinct differences in diversity profiles were observed. In water and biofilm samples, bacterial richness was not significantly different (SI) across the three modalities while, based on the curve shape, P exhibited lower diversity compared to P_T and T which is more pronounced in biofilm samples while T and P_T maintain closer profiles.

Based on the H(α) values plotted on the y-axis, comparison between T0 and Tf in Renyi's diversity profiles for water and biofilm revealed that alpha diversity increased over time in the tench modality (T), whereas is decreased in the pikeperch monoculture (P).

These results were confirmed by statistical analysis approximated by Richness, Shannon, Simpson and Berger parker indexes calculation (SI).

At T0, in water samples, no significant differences were detected for richness or for the Shannon index among modalities. But significant differences were detected only between the monocultures T and P approximated by Simpson index calculation with higher values in P compared to T and Berger–Parker index calculation with significantly lower values in P compared with T. Meanwhile, in biofilm samples no significant differences were revealed among the three modalities at T0.

At Tf, alpha diversity analysis of water communities showed no significant differences in richness between the modalities. However, alpha diversity approximated by Shannon index, Simpson index and Berger Parker index calculation showed significant differences only between monocultures P and T modalities. T modality exhibited significantly higher Simpson and Shannon values compared with P exhibiting the lowest values but also significantly lower Berger-Parker values than P modality. Meanwhile, no significant differences in alpha diversity measurements were observed between modalities for biofilm samples at Tf.

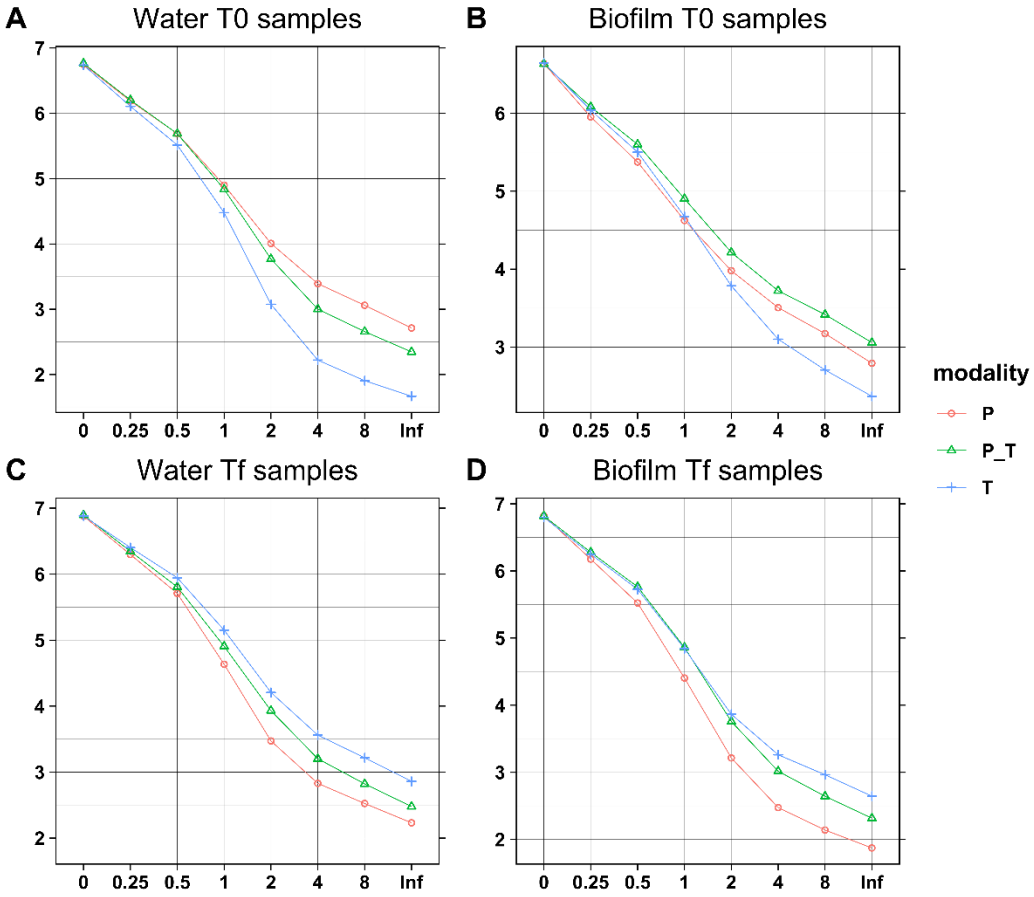

Figure 19. *Renyi diversity performed on ASV tables comparing alpha diversity indices across different modalities P, P_T and T at T0 and Tf. Figures 19A and 19B display the samples in Water and Biofilm at T0, respectively, while Figures 19C and 19D depict those at Tf. The x-axis values 0, 1, 2, and Inf represent species richness, Shannon, Simpson, and Berger-Parker index respectively.*

3.2.2. Beta Diversity

The analysis of beta diversity based on Bray-Curtis dissimilarity revealed significant differences at T0 (i.e., 2 days after fish introduction) between the three modalities (P, T or P_T) in both water (PERMANOVA: R^2=0.354, p=0.0001; ANOSIM: R=0.659, p=0.0001) and biofilm (PERMANOVA: R^2=0.132, p=0.0001; ANOSIM: R=0.159, p=0.0009) samples. Each pairwise comparison revealed a significant group separation (SI) between modalities. Changes in the community structure between modalities at Tf were addressed with a PCoA performed on the metabarcoding results (fig.20). In water samples microbial communities were significantly clustered by modality (PERMANOVA: R^2=0.331, p=0.0001; ANOSIM: R=0.604, p=0.0001), with the PCoA (fig. 20A) projection on the first two axes explaining 41.1% of the variability. Pairwise comparisons, performed on the same data frame, demonstrated similar and significant separations whether comparing P vs T (PERMANOVA: R^2=0.254, p=0.001; ANOSIM: R=0.627, p=0.0003), P vs P_T (R^2=0.289, R=0.600, for both PERMANOVA and ANOSIM p<0,05), or T vs P_T (PERMANOVA R^2=0.269, p=0.001; ANOSIM

R=0.589, p=0.0003). On the biofilm samples, the PCoA analysis (fig. 20B) accounting for 44.3% of the variability revealed that microbial communities from modalities T and P_T were partially clustered together and separated from modality P. In addition, the microbial community structure in the ecotrons of modality P was much more dispersed. PERMANOVA (R^2 = 0.299, p = 0.0001) and ANOSIM (R=0.580, p=0.0001) statistical analyses carried out between the modalities confirmed this result. Considering the pairwise comparisons, this is especially the case for P vs P_T (PERMANOVA R^2 = 0. 281, p = 0. 001; ANOSIM R = 0.786, p = 0.00045) and P vs T (PERMANOVA R^2 = 0.270, p = 0.002; ANOSIM R = 0.615, p = 0.0025), which showed strong differences, while T vs P_T which showed a moderate but still significant separation (PERMANOVA R^2 = 0.191, p = 0.001; ANOSIM R = 0.552, p = 0.0003).

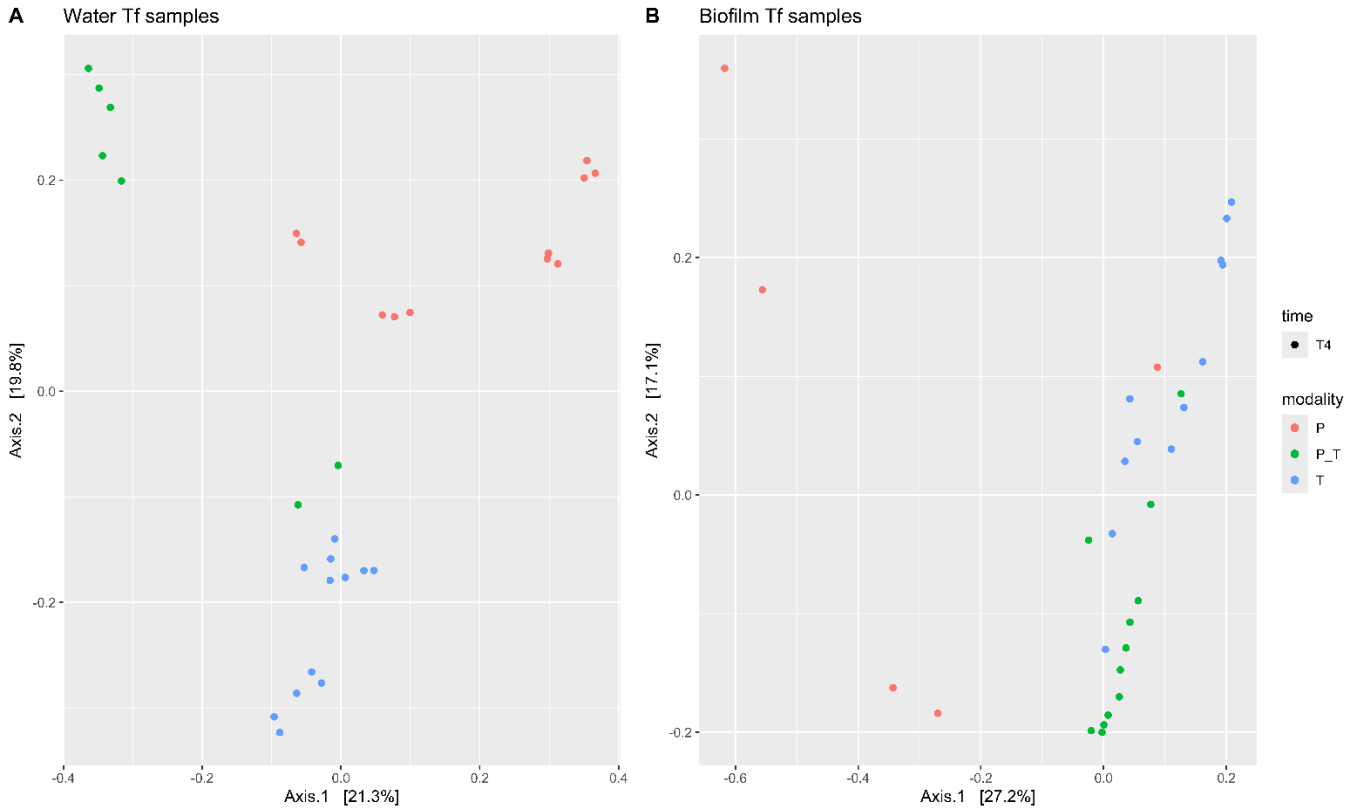

Figure 20. *Principal coordinate analysis (PCoA) of distances between microbial communities based on Bray–Curtis among modalities at Tf. The colors of the symbol are red: P (pikeperch monoculture), green: P_T (polyculture of pikeperch and tench) and blue: T (tench monoculture). Figures 3A and 3B display the samples in Water and Biofilm respectively.*

A comparison of diversity between T0 and Tf reveals a temporal shift in community composition, as evidenced by the opposite disposition of samples from each modality on the PCoA, for both water (SI) and biofilm (SI) samples. The behavior of microbial communities varies between the two environments. In water, communities show increased heterogeneity over time, as indicated by a more

dispersed arrangement of individual samples in the PCoA. In contrast, biofilm communities from modalities T and P_T become more homogeneous over time, with samples clustering more closely together, while modality P remains heterogeneous, similar to the initial time point. Multivariate tests (Tables 3C, 3D, SI) reveal that these changes in bacterial diversity are significant for each modality, in both water and biofilm, between T0 and Tf.

3.2.3. Microbial community composition

The composition of bacterial communities was examined at the family level (fig.4). 184 different categories were found in the totality of the dataset including: 181 identified families, “No data” category, “unknown family” category and “multi-affiliation” category.

This figure shows the relative abundance of the 11 majority families (including “multi-affiliation” and “unknown family”) found at T0 (fig. 21A) and at Tf (fig. 21B) in biofilm and water samples. The 10 majority families representing around 70% of the total relative abundance analyzed in biofilm at Tf while they represent about 50% in biofilm at T0 and in water samples at T0 and Tf. It should be noted that the majority families represented at T0 and Tf in fig. 21A and 21B respectively are not the same, except for the five families: Comamonadaceae, Fusobacteriaceae, Mycobacteriaceae, Rhodobacteraceae, and Xanthomonadaceae, which are found dominant both at T0 and Tf.

For biofilms at T0, the figure shows that P_T has a similar overall profile to P and T but with a higher proportion of Rhodobacteraceae and Flavobacteriaceae and a lower proportion of Comamonadaceae.

As for the composition of water at T0 (fig. 21A), the polyculture P_T was dominated by Fusobacteriaceae and Rhodobacteraceae while tench monoculture T showed the highest abundance of Fusobacteriaceae but the lowest abundance of Rhodobacteraceae, among modalities based on histograms shape.

At Tf, the microbial composition had shifted considerably, with distinct differences among modalities. For biofilm samples, fig. 21B and statistical results obtained with DESeq2 and the adjusted p-value obtained with the SIMPER test revealed that biofilm in P modality represented significantly higher relative abundance of Rhizobiaceae, Fusobacteriaceae and Rhizobiales Incertae Sedis than T and P_T modality. Fig. 21B also showed that biofilms in P_T and T exhibited a closer composition than the P modality. While DESeq2 analysis of biofilms in T showed significantly a higher relative abundance of Fusobacteriaceae but a lower relative abundance of Weeksellaceae compared to P_T.

In the water samples at Tf, notable differences among modalities were again observed. In P modality, there is a very significant high relative abundance of Clostridiaceae and Chromobacteriaceae than T based on DESeq2 test and the adjusted p-value obtained with the SIMPER test (DESeq2, $p<0.05$;

SIMPER, $p<0.05$). The tests also showed that the modality P_T displayed significantly a high representation of Rhodobacteraceae and Fusobacteriaceae than P and T but a significant lower relative abundance of Comamonadaceae in comparison with P modality.

At Tf, the composition of bacterial communities of snails was also examined (SI). Based on the histogram shapes, biofilms and snails exhibited a closer composition than snails and water. Snail-associated microbiota shared similarities with biofilms, being dominated by Rhizobiales Incertae Sedis and contained high relative proportions of Rhizobiaceae. In contrast, water samples were dominated by Comamonadaceae with high relative proportions of Clostridiaceae, while Rhizobiales Incertae Sedis, abundant in biofilms and snails, was comparatively less represented.

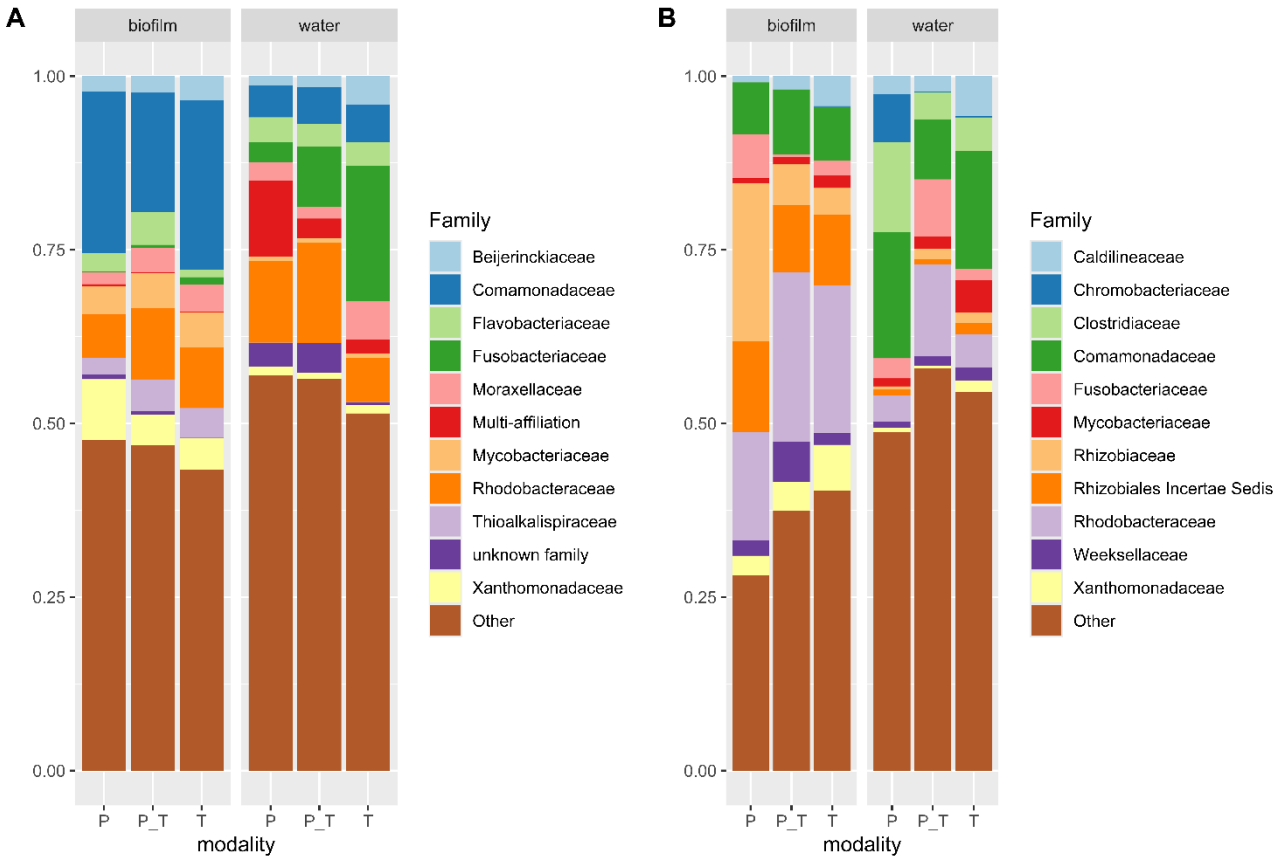

Figure 21. *Microbial composition and relative abundances of the 15 most abundant OTU of bacterial families at T0 (21A on the left) and the 15 most abundant OTU of bacterial families at Tf (21B on the right) in water or biofilm, and among modalities, P (pikeperch monoculture), P_T (polyculture of pikeperch and tench) and T (tench monoculture).*

3.2.4. Heatmap

This heatmap allows to identify differences and similarities between the composition and the relative abundances of the 40 most abundant bacterial families found in water and biofilm samples (fig. 22A

and 22B respectively) in the three rearing modalities P, T and P_T at Tf. The bacterial community composition showed clear differences among the modalities.

As shown in fig. 22A, the clusterisation of water data showed that water samples from T and P_T modalities exhibited a closer composition than with P modality at the family level. Water from Pikeperch monoculture P was dominated by families like Oxobacteraceae, Flavobacteriaceae, Clostridiaceae, Aeromonadaceae and Chromobacteriaceae which were less abundant in the modalities T and P_T. Species from the families Aeromonadaceae and Clostridiaceae have been described as important fish pathogens in aquaculture environments which constitutes a disease-causing limiting factor (Du et al., 2019; Cortés-Sánchez, 2021). Also fig. 5A shows that the families Haliangiaceae, Saprospiraceae, Aquaspirillaceae and Comamonadaceae were highly present in water in both P and T modalities but not high in P_T modality. In contrast, water in P_T was dominated by the families Spirosomaceae, Fusobacteriaceae, Intrasporangiaceae, Microbacteriaceae, Rubrobacteriaceae, Caulobacteraceae, Exiguobacteraceae and Rhodobacteraceae which were weakly represented in P and T modalities. The family Spirosomaceae are well-known for their ability to degrade polymeric organic substances (Yanes-Roca et al., 2024). Also, the family Rhodobacteraceae may play a significant role in nitrogen conversion within RAS (Attramadal et al., 2014).

Water in P_T and T shared more similar family composition than P_T with P. Lachnospiraceae, Rhizobiaceae, Nocardioidaceae, Corynebacteriaceae, Hyphomonadaceae, Hyphomicrobiaceae and Weeksellaceae were highly present in both P_T and T modalities at different levels, but these families were weakly represented in P modality. Meanwhile water in T modality is dominated by different families like Rhizobiales Incertae Sedis, Ilumatobacteraceae and Oscillospiraceae, found in very low abundance in P and P_T modalities.

As for biofilm samples (fig. 22B), each modality was dominated by different bacterial families. The families Rhizobiales Incertae Sedis, Bdellovobrionaceae, Holosporaceae, Rhizobiaceae and Listeriaceae were dominant in P and not P_T or T modality. Some species from Llisteriaceae family can be, in some cases, considered fish pathogen. Meanwhile biofilms of P_T modality were dominated by different families like Rhodobacteraceae, Nitrospiraceae, Pseudomonadaceae, Flavobacteriaceae, Weeksellaceae and Comamonadaceae present at lower abundance in the other modalities especially in P. Similarly, different families dominated in biofilm of T modality like Nocardiaceae, Xanthobacteraceae and Mycobacteriaceae present at lower abundance in P and P_T.

fig. 22B also showed that T and P_T modalities exhibited a closer composition than with P modality. T and P_T showed similar relative abundance of many bacterial families like Microscillaceae, Pseudonocardiaceae, Reynellaceae and Sutterellaceae which are very weakly represented in P modality. Additionally, two families present in P_T appear to derive from P, namely

Hyphomycrobiaceae (associated with biofilm) and Moraxellaceae (associated with water and biofilm).”

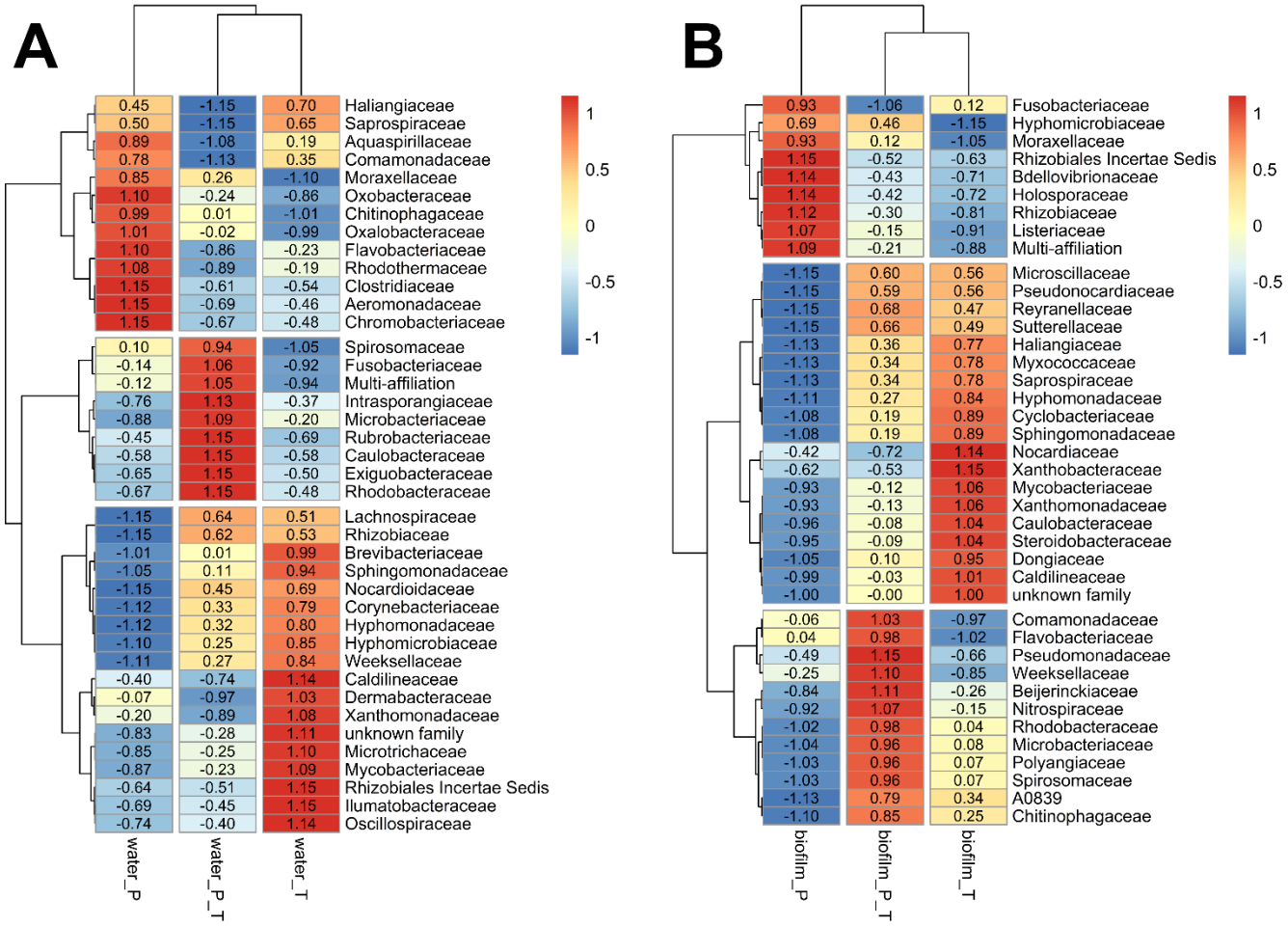

Figure 22. *Hierarchically clustered (Ward.D2 method) heatmap illustrating the microbial community composition and relative abundances of the 40 most abundant bacteria family. Three modalities are compared: P (pikeperch monoculture), P_T (polyculture of pikeperch and tench) and T (tench monoculture). Water samples are shown on the left (22A) and biofilm samples on the right (22B) at Tf (final time). The color scale represents per-row z-scores (i.e., centered and scaled within each family), following addition of a pseudo-count of one and centered log-ratio (CLR) transformation of mean abundances for each modality.*

3.2.5. Venn diagram

The Venn diagram was designed to explore the relationship between snails and biofilms only in pikeperch monoculture (fig. 23), as the emergence of snails at Tf was unexpected and persisted in P modality but was eliminated by tench in T and P_T. The biofilms and the snails showed a percentage of 44% of shared ASVs at Tf in pikeperch monoculture. Meanwhile, 52% of the ASVs were specific to biofilms and not related to the presence of snails.

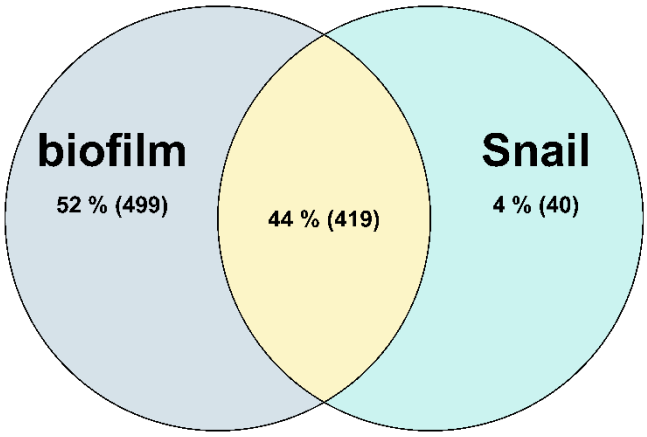

Figure 23. *Venn diagram visualizing the amplicon sequence variant ASV (in yellow) shared between the biofilm samples and snail samples in pikeperch monoculture P on Tf.*

4) Discussion

Microbial communities in RAS play a key role in stability and the quality of the recirculating water and the good management of pathogenic bacteria and friendly bacteria is vital for the resilience of recirculating systems (Moschos et al., 2022). But microbial balance in RAS is delicate and can be disrupted. The microbial composition and diversity in RAS water and biofilters are highly influenced by various factors, including organic contamination, and physicochemical parameters of water such as pH, temperature, oxygen and nitrogenous compounds concentrations, stocking density and particularly the composition of microbial communities in the biofilters is influenced by the farmed fish species (Godzieba et al., 2025). However, the potential effects of polyculture in shaping the structure of microbial communities in RAS remain to be demonstrated. A more diverse microbial community can strengthen system stability by outcompeting opportunistic pathogens and thereby improving overall resilience (Harrison et al., 2020). Meanwhile, reduced microbial diversity can have detrimental effects on the growth of beneficial microbes and potentially trigger disease outbreaks accompanied by an occurrence of opportunistic pathogenic taxa such as Aeromonas or Pseudomonas (Gupta et al., 2024; Godzieba et al., 2025). A diverse and resilient microbiome is therefore essential to prevent disease outbreaks and support water quality (Harrison et al., 2020; Gupta et al., 2024). This is why the objective of this study was to determine whether fish, through the cumulative effects of their phenotype, particularly those related to species, diet, digestive tract morphology, and traits acquired during development (such as feeding practices, life history, antibiotic treatments, ...), influence the structure and composition of the microbiota associated with their environment. The second objective was to test the hypothesis of interaction (interactions, addition, or competition) between two microbiotas in a polyculture context, in order to identify the characteristics of the resulting microbiota,

whether planktonic or sessile (biofilm).

Our results confirm the existence of a species-specific effect of fish on microbial diversity, both in terms of alpha and beta diversity.

For planktonic communities in water, the differences are mainly observed at the level of beta diversity representation. PERMANOVA analyses (SI) combined with PCoA representations (fig. 20) show that each fish species (pikeperch or tench) imprints a distinct microbial signature on planktonic communities. These findings are supported by the hypotheses formulated in a previous study conducted in aquaponics (Changey et al., 2025). They are particularly significant given that they were obtained using strictly identical experimental setups. The composition representation, 120 days after the beginning of the experiment (fig. 21B), permitted highlighting that water at Tf in monoculture of pikeperch appears to be associated with an overrepresentation of the Chromobacteriaceae and Clostridiaceae families found in lower relative abundances in polyculture and monoculture of tench. Clostridium botulinum is an anaerobic, sporulated pathogen commonly found in aquatic environments present in free "planktonic" form, or form aggregates such as "biofilms". It can also be found as part of microbiota in various organs such as fish intestines, skin and gills (Cortés-Sánchez, 2021). Similarly, species of the Mycobacterium genus represent a growing and serious threat to the aquaculture industry, causing chronic and often fatal infections in a wide variety of aquatic organisms. These slow-growing, robust bacteria are ubiquitous in freshwater, brackish, and marine ecosystems, and are particularly difficult to eliminate due to their persistence and ability to form biofilms (Davidovich et al., 2020). Their resistance to standard disinfectants further complicates control efforts, often requiring lengthy and costly decontamination procedures (King, 2001; Gauthier and Rhodes, 2009). Whereas tench monoculture favored the proliferation of Mycobacteriaceae, and Caldilinaceae in water more than P and P_T modalities.

Meanwhile, our results (fig. 21B and 22A) show that in polyculture, few families originating from the P monoculture are detected in water, alongside several families that derive from the T monoculture, which suggests the presence of a partial additive effect, whereby taxa from both source systems are retained. Moreover, the microbial community in polyculture P_T does not simply represent an addition of the communities found in P and T. This indicates that the polyculture of pikeperch and tench results in a unique composite microbial signature in water that does not simply correspond to the additive combination of the two individual species microbiotas likely driven by ecological interspecific interactions between distinct populations. Beta diversity analysis demonstrates that this species combination impacts microbial community composition, notably reducing the presence of Clostridiaceae and Mycobacteriaceae, which is a family of bacteria including some fish pathogens species (Hashish et al., 2018), while increasing the abundance of families such as Fusobacteriaceae,

which are characteristic of oral and intestinal mucosae of animals and considered less problematic for fish from a sanitary standpoint (Olsen, 2014). But also, Rhodobacteraceae family that is reported to play an important role in maintaining water quality by removing sulfur compounds and toxic nitrogen and enhance fish health by colonizing gut microbiota and exerting probiotic effects (Rajeev and Cho, 2024).

As for biofilms, we observed at Tf trends similar to those seen in the water column, although the microbial signatures in biofilms were markedly different. For instance, a proliferation of Listeriaceae family, that include species considered fish pathogens, was detected in biofilms in pikeperch monoculture (fig. 22B). The biofilm associated with pikeperch displayed a microbial signature particularly distinct from those of the other two modalities. This may be explained by the systematic presence of Physa snails (freshwater gastropods; probably arrived by traveling up the wastewater drainage pipes (backwash from mechanical filters)) in the monoculture of pikeperch rearing systems; likely arrived by moving up through the wastewater drainage pipes (backwash from the mechanical filters); while tench is known to be a natural predator of these mollusks. Several hypotheses could explain an influence of these gastropods on biofilm structure observed in fig. 23: (1) a direct contribution of their own microbiota, which would imply strong similarity between the snail and biofilm microbiotas (44%); a pattern that is partially observed but does not account for all the variation; (2) an indirect effect via biofilm grazing, which could lead to niche vacating and allow colonization by opportunistic species.

All these data allowed us to suggest an effect of fish species in RAS, responsible for shifting and structuring microbial communities. The choice of reared fish species and fish combinations create very unique and distinct microbial environments shaped by interspecific interactions in water and biofilm.

5) Conclusion

The presence of different fish species in RAS significantly influences the composition and structure of microbial communities. Taken together, these findings reveal that water and biofilm microbiota in polyculture of pikeperch and tench is characterized by both additive and new emergent community assembly processes shaped by interspecific interactions. This highlights the important impact of species diversity on microbial ecology in RAS.

Author contributions

F. Changey and P. Fontaine conceived and planned the experiments. S. Al Dakdouki, F. Changey and P. Fontaine carried out the experiments and contributed to sample preparation. S. Al Dakdouki carried

out DNA extractions. A. Laprie and F. Changey planned and carried out data analysis. F. Changey, L. P. Fontaine was in charge of overall direction and planning. F. Changey and S. Al Dakdouki took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of interest statement

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript. No approval from research ethics committees was required to accomplish this study as it did not involve any animal experimentation. The datasets generated during the current study are available from the corresponding author upon reasonable request.

Data availability statement

The data that support the findings has been deposited at the following URL: https://ent.univ-lorraine.fr/f/u24l1s4/p/esup-filemanager.u24l1n67/max/render.uP?pCm=view

IV- CHAPITRE 3

We demonstrated in chapter 2 that microbial communities in RAS in both water and biofilm is significantly shaped by reared species as monocultures of each pikeperch and tench produced distinct microbial signatures. While the polyculture of both species generated unique microbial structure, characterized by similarities with the monoculture highlighting an additive assembly but also generated new emergent communities driven by interspecies interactions. The polyculture of pikeperch and tench showed an important impact reducing the risk of pathogens as the abundance of Clostridiaceae and Mycobacteriaceae families in polyculture in comparison with monocultures, while promoting beneficial families like Rhodobacteraceae. These results suggest that fish biodiversity can potentially improve microbial balance and RAS resilience and overall system sustainability. However, we still don't know which of the specific fish associated microbiota gills, skin or gut is the main vectorization pathway implementing this unique microbial signature in water and biofilm in different rearing modalities in RAS.

So, in this final chapter, we aimed to explore microbial transfers between both fish species and with water and tank's wall biofilm compartments and to identify which of the fish associated microbiota of gills, skin or gut is the main vectorization pathway of microbial inoculation in mono- and polyculture over time.

Investigating the impact of fish microbiota of gut, skin and gills mucous of pikeperch (*Sander lucioperca*) and tench (*Tinca tinca*) in monoculture and biculture on microbial dynamics and inoculation in Recirculating Aquaculture Systems

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Abstract

Recirculating Aquaculture Systems (RAS) are closed-loop systems that filter and reuse water, relying on complex microbial communities to maintain water quality and fish health. However, little is known about how fish species influences microbial dynamics in these systems. Fish-associated microbiomes, including those of the gut and skin and gills mucous, are in continuous contact with their culture system. Their composition, shaped by intrinsic species traits and extrinsic environmental factors, is complex, tissue-specific and essential for host health. However, little is known about how fish microbiota interact and exchange microbial communities with extrinsic matrices (water and biofilms) of RAS microbial communities, particularly in polyculture. This study investigated how the microbiomes of intrinsic matrices (gut, gills, and skin mucus) of pikeperch (*Sander lucioperca*) and tench (*Tinca tinca*) reared in monoculture and polyculture influence the microbial dynamics and inoculation of extrinsic matrices (water and biofilm) in RAS. 16S rRNA metabarcoding revealed distinct species- and tissue-specific microbial compositions and distinct inoculation contributions of intrinsic matrices to water and biofilm microbiomes in different rearing modalities in RAS. Source tracking showed that among fish-associated matrices, microbial colonization of water and biofilms in polyculture, before stabilizing over time, originated mainly from pikeperch skin and gills mucus and tench gut microbiota. At the end of the experiment, polyculture notably increased beneficial taxa such as Rhodobacteraceae and Staphylococcaceae in tench digesta but also elevated Vibrionaceae in skin mucous, which can include pathogenic species. Overall, these results demonstrate that fish species and rearing modality jointly shape host-associated microbiotas and RAS extrinsic microbiomes, providing

new insights for managing microbial communities in polyculture in RAS.

Keywords : Fish microbiota, microbial dynamics, vectorization pathways, monoculture, polyculture, pikeperch, tench, Recirculating Aquaculture Systems (RAS)

1) Introduction

Aquaculture, the world's fastest-growing seafood production sector, now plays a critical role in global food security, having surpassed capture fisheries in 2022 to become the primary source of seafood and compensate for the overexploitation of wild stocks (Bondad-Reantaso et al., 2023; Campanati et al., 2022; FAO, 2024). However, the sustainable expansion of aquaculture is constrained by significant environmental challenges. Aquaculture is highly vulnerable to climate change, which pushes species beyond their thermal limits (Engelhard et al., 2022; Ficke et al., 2007) and degrades water quality through eutrophication, oxygen depletion, and increased disease risk (Engelhard et al., 2022; Luo, 2023). Simultaneously, the sector generates its own pressures, primarily through water pollution from waste that causes algal blooms and eutrophication (Sarker et al., 2024; Streicher et al., 2021), and through the misuse of antibiotics, which fosters antimicrobial resistance (Campanati et al., 2022; Martinez-Porchas and Martinez-Cordova, 2012). Overcoming these interconnected environmental hurdles is critical for the sector's long-term viability and food security role. These issues raise major concerns about the overconsumption of water and space and regarding environmental impacts. To mitigate environmental impacts, policymakers are investing in innovative systems like Recirculating Aquaculture Systems (RAS), closed-loop facilities that minimize interaction with natural ecosystems, seen as a sustainable solution for the future of aquaculture (Roy et al., 2025). The core principle of a Recirculating Aquaculture System (RAS) is to intensively farm fish in a controlled, closed environment while continuously filtering and reusing the water (Bartelme et al., 2017; Bregnballe, 2015; Vielma et al., 2022). The maintenance of optimal water quality and the production of healthy aquaculture products in these systems depend on a complex microbial community, playing an essential role in water purification processes (Schreier et al., 2010). The microbial communities of RAS can be divided into two principal niches: sessile communities attached to surfaces like biofilters, tank’s walls, and associated with aquatic organisms and planktonic organisms suspended in the water column (Blancheton et al., 2013; Crab et al., 2007). Microbes can be autotrophs or heterotrophs. Autotrophic and heterotrophic bacteria in biofilters play roles in the convertion of toxic fish wastes such as ammonia originated from urine, feces, or uneaten feed into nitrite and then less toxic nitrate (Godzieba et al., 2025; Zhang et al., 2023) while heterotrophic bacteria can also degrade organic matter (Blancheton et al., 2013; Gupta et al., et al., 2024; Rurangwa and Verdegem, 2015). The number of

RAS farms has increased steadily especially in developed countries over recent years (Badiola et al., 2018). But their widespread adoption is limited because of the complex and costly system designs, requiring substantial initial investment and sophisticated engineering, and their high-energy consumption and high GHG emissions (Ahmed and Turchini, 2021). RAS also remains vulnerable to disease introduction in rearing tanks via external sources such as infected fish stock or contaminated water (Dahle, 2022; Davidovich et al., 2020; Espinal and Matulić, 2019; Khadka et al., 2025). Poor management may further create favorable conditions for pathogen proliferation, underscoring the need for specialized expertise in microbial and disease control (Aich et al., 2020). For example, *Vibrio, Aeromonas, and Pseudomonas* are identified as opportunistic pathogens in RAS, where they persist in various niches like water column, associated with fish microbiota and biofilms (Gupta et al., 2024; Sergaliev et al., 2021). Bacterial biofilms in RAS can act as reservoir for bacteria including pathogens that can be released through system water (Harrison et al., 2020; Al Dakdouki et al., 2025). Operational challenges in RAS include the need for attention and deeper understanding of microbial compartment to maintain system stability.

To date, RAS are mainly used for intensive production of a single species "monoculture" due to their simplicity (Thomas et al., 2020). However, in monoculture, fish use less efficiently feed and resource, as a single species rarely exploits all water column volume (surface and bottom), leading to accumulation of feed wastes, water quality deterioration and therefore risk of development of opportunistic pathogens (Amoussou et al., 2022; Dawood et al., 2025; Kozłowski et al., 2014). Meanwhile, interest in polyculture practice within recirculating aquaculture systems (RAS) is recently gaining more attention as an alternative to monoculture (Pěnka et al., 2021, 2024; Amoussou et al., 2022; Thomas et al., 2021, 2022).. By cultivating complementary species together, polyculture can potentially enhance both efficiency and sustainability of production systems by taking advantage of noncompetitive species coexistence and interspecific interactions, which can improve RAS functioning (Dawood et al., 2025; Pěnka et al., 2024a; Thomas et al., 2020). In polyculture in RAS, fish exploit water volume and feed resources more efficiently through synergistic interactions, whereby unused nutrients from one species can be utilized by another (Dawood et al., 2025) improving therefore the sustainability and resilience (Medeiros et al., 2017). Also, the presence of one species can enhance the productivity of others by increasing biomass yield such as pikeperch (*Sander lucioperca*) production improved when reared with tench (*Tinca tinca*) in the same RAS (Thomas et al., 2020). However, polyculture is only very rarely applied in RAS due to the limited proofs of polyculture's financial return and to the need to manage the combined complexity of polyculture and RAS (Thomas et al., 2021). For a successful RAS polyculture system, selecting compatible species (based on behavioral responses and zootechnical parameters), to minimize detrimental interactions,

prevent feeding competition, and accommodate behavioral differences in swimming and feeding (Amoussou et al., 2022; Dawood et al., 2025; Pěnka et al., 2024a; Thomas et al., 2020) and appropriate stocking densities and feeding rates are critical (Hossain and Islam, 2006; Silknetter et al., 2020; Thomas et al., 2022). In addition, outbreaks of microbial diseases are also one of the main key drawbacks of the adoption of polyculture in RAS. Introducing multiple species may elevate the risk of disease transmission (Amoussou et al., 2022) which causes concerns about fish welfare and public health risks. It was also reported that some species combinations may trigger spillovers of pathogens (Amoussou et al., 2022; Thomas et al., 2021) causing a serious problem to RAS microbiome. Transfer of bacteria between the fish and the surrounding environment has been reported (Muziasari et al., 2017; Zeng et al., 2021).

Fish are in direct interaction with water and can serve as carriers of microbial communities affecting the microbiome of the system (water and biofilm) through their skin, gills and gut microbiota (De Bruijn et al., 2018; Schreier et al., 2010; Xie et al., 2023; Yan et al., 2019). Diverse studies have shown that the bacterial community composition of the gastrointestinal tract of a fish species was similar to their surroundings water (Chen et al., 2022; Zeng et al., 2020). For instance, a study on tilapia larvae in RAS found a high correlation between the bacterial community in the water and the gut microbiota of the larvae (Giatsis et al., 2015).

Microbial populations inhabiting fish skin, gills, and intestinal tract are specific for each body site (Minich et al., 2022). These fish microbiota exhibits high diversity and is essential for the health of farmed fish (Dai et al., 2021). It is shaped by intrinsic factors (related to host species), such as gastrointestinal tract morphology (Smith et al., 2015), as well as extrinsic factors, including the environmental conditions prior to capture of fish (Dulski et al., 2020; Egerton et al., 2018). Additionally, nutrient inputs from excess feed, organic matter, and fish feces have been shown to influence skin and gut microbial communities of fish in aquaculture ponds (Debnath et al., 2025). Polyculture experiments in RAS have assessed growth performance (Dawood et al., 2025; Thomas et al., 2022, 2020), nutrient management (Dawood et al., 2025; Pěnka et al., 2024a), and fish welfare (Amoussou et al., 2022), without considering microbial community composition. Outside of RAS, microbial communities have been well characterized in polyculture (ponds, lakes), showing that co-cultured fish species can shape distinct microbial assemblages (Bartelme et al., 2017; Bui et al., 2024; Changey et al., 2025; Guan et al., 2020; Yan et al., 2019).

To date, it is known that microbial diversity in RAS is important and impacted significantly by multiple factors: (1) physico-chemical variables of water (pH, temperature, O_2, ammonium and nitrite levels), (2) the density of reared fish and (3) fish species (Changey et al., 2025; Sergaliev et al., 2021). However, characterization of the bacterial communities in fish polyculture within RAS remains

understudied and no study mentions the main fish microbiota pathway (gills, skin or gastrointestinal tract) responsible for microbial inoculation of RAS (water and biofilms) in the context of polyculture. This represents a critical gap, as the interplay between host species and system microbiota may influence both fish health and system microbial stability. Addressing this gap will provide new insights into how multi-species rearing impacts microbial ecology in RAS through their microbiota.

In the present study, the first and main aim was to investigate the effect of polyculture in RAS on microbial communities of the microbiome of the system (sessile and planktonic). This is why a biculture of pikeperch (*Sander lucioperca*), a valuable farmed species in RAS and highly consumed in Europe (Bousseba et al., 2025; Jakubavičiūtė et al., 2024; Pourhosein-Sarameh and Falahatkar, 2024), with tench (*Tinca tinca*) was selected. These two freshwater species have similar rearing abiotic requirements (i.e. pH, temperature, salinity, dissolved oxygen, and light parameters (Baekelandt et al., 2018; Pula et al., 2018). Recent studies showed compatibility in their feeding behaviors as pikeperch feeds in the water column while tench allows the consumption of uneaten feed settling at the bottom. Additionally, pikeperch-tench polyculture showed an increase in the growth (biomass gain) of pikeperch when reared in polyculture with tench in comparison with pikeperch monoculture, without affecting pikeperch's survival (Lecocq et al., 2019; Thomas et al., 2020) but the authors did not investigate the effect of this biculture on microbial communities of the system or on the fish physiological and immune status. In this study, we compare microbial communities' structure in pikeperch–tench biculture with the respective monocultures of each species, by metabarcoding of the rRNA16S gene. The aim is to understand the microbial dynamics in RAS from fish microbiota, both between reared species and among distinct compartments within RAS, with particular emphasis on identifying the primary fish microbial pathway responsible for system inoculation: digestive tract, gills, or skin which is still unknown in RAS. Secondly, we investigated the effect of this biculture (centered on pikeperch) on fish survival, growth and welfare (stress level and immunity) compared to monoculture of each species alone under the same culture conditions (initial densities per tank, food and feed quantity and water parameters).Our findings will allow a better understanding of the effecr of fish species on the overall RAS microbiome.

2) Materials and methods

2.1. Experimental facilities

The study was carried out at the Platform of Experimental Aquaculture (PEA) of the Faculty of Sciences and Technologies (University of Lorraine, Nancy, France) (registration number for animal experimentation D54–547-18). It was conducted from 18 September 2023 to 15 January 2024 and

lasted a total of four months. Twelve independent temperature-controlled rooms, referred to as ecotrons, were used in this experiment. Each ecotron operated as a closed system equipped with its own water recirculation setup. At the core of each system was an experimental unit consisting of a fiber and polyester resin tank (fish tank, 2400 L; dimensions: 2 m × 2 m × 0.8 m), connected to a water recycling circuit with a flow rate of 3,000 L/h. Water from the tank first passed through a mechanical drum filter (mesh size: 63 µm), then flowed sequentially into a 300 L buffer tank (serving as a pumping reservoir), a 55 W ultraviolet sterilization reactor, and finally into a 450 L fluidized biological bed filter filled with media (Helix-type).

2.2. Experimental design and fish

The ecotrons were thoroughly cleaned and disinfected to prevent recontamination by microorganisms from previous use (Al Dakdouki et al., 2025). The cleaning protocol included high-pressure washing of the tanks, chemical disinfection with hydrogen peroxide and chloramine T (two highly effective disinfectants), and physical treatments such as high-temperature exposure and system drying (Arvin and Pedersen, 2015; Bigarré et al., 2017; Linley et al., 2012; Quezada-Rodriguez et al., 2022) and the mix of biofilter media. Furthermore, a solution of nitrifying bacteria was introduced into all ecotrons to prevent ammonium accumulation following fish introduction.

During the cleaning/disinfection phase of the ecotron, fish were acclimated and maintained under optimal rearing conditions specific to each species. Pikeperch (sourced from La Ferme Intégrale, 26730 La Baume d'Hostun, France) were stocked for almost 3 months in two available acclimatation ecotrons at the PEA, at a density of 60 kg per ecotron. Tench (sourced from BAJCSHAL kft., 9062 Kisbacs, Hungary) were stocked in 9 tanks of 700-liter each at 12 kg per tank for almost 4 months.

For the experiment, three rearing modalities were established. Pikeperch weighting 133 ± 75 g and tench of 142 ± 57 g from acclimatation ecotrons were distributed in a total of 12 ecotrons at the beginning of the experiment, with four replicates per modality (n = 4), ensuring a comparable initial biomass of 8 kg of fish per ecotron, as follows: monoculture of pikeperch sander lucioperca (P), monoculture of tench tinca tinca (T), and polyculture of pikeperch with tench (50%-50% in initial biomass) (Poly).

All through the experiment, fish were fed a constant standardized daily ratio of Legouessant Percid Grower Floating No. 4 feed of 1.5% of the mean fish biomass of the total biomass in each ecotron recommended for pikeperch (Baekelandt et al., 2018; Kozlowski et al., 2010). Penka et al. showed that pikeperch fed a ratio of 1.5% showed higher body weight (51.5 ± 16.1 g) and total length (188.2 ± 17.8 mm) than groups that received lower feed ratio (Pěnka et al., 2024b). The quantity of feed (g) is

adjusted once per month based on the mean biomass measured monthly per ecotron. In all ecotron, fish received deliberately the same daily feed quantity, because feed is an intensive input into the water that may cause a contamination directly of microbial communities in the tank or indirectly by modifying the concentrations of nutrients (Abollo et al., 2024; Marmen et al., 2021), thereby avoiding any possible unwanted microbial variability between tanks that might affect the reliability of the results. The food portion per day was partitioned into two equal rations and distributed, by simply throwing the food in the tanks, at fixed times (at 09:00 A.M. and 4:00 P.M.). The photoperiod was set to 16 h of light, starting at 7:00 AM, with simulated dawn and dusk transitions of 30 minutes each, at a light intensity of 20 lx. Water physico-chemical parameters were monitored regularly, with daily measurements of temperature (23.9°C ± 0.1), dissolved oxygen (7.27 mg.L-1 ± 0.06), and pH (7.48 ± 0.01) using a multi-parameter probe, and spectrometric analyses of ammonium (NH_4^+) (0.161 mg.L-1 ± 0.025) and nitrite (NO_2^-) concentrations (0.066 mg.L-1 ± 0.012), and were homogenous between all ecotron during the experiment.

2.3. Biological variables measure on fish

Ethics approval was granted for fish handling through a formal request submitted beforehand reference APAFIS#43927_2023062309077777_v2. All fish used were returned to their original tank after experimentation.

Four growth control were performed monthly to assess fish growth parameters and to adjust the feeding ratio according to the total biomass and monthly blood sampling was also performed to assess fish stress parameters throughout the four months period of the experiment. At the beginning of the experiment high mortality of fish, mostly o pikeperch, was recorded and the pikeperch were thin, underweight and did not eat well in the first month of the experiment. That is why the pikeperch in poor conditions were removed and euthanized and then replaced with new fish from the same initial stock in good conditions that still have been growing in the acclimatation tanks ensuring a comparable initial biomass of 10 kg per tank. We considered initial time for growth control and sampling after this new transfer of fish and it's important to note that slight significant differences were found at this stage between the mean weight of tench in monoculture (150.6 ± 37.9 g**)** and in polyculture (165.5 ± 45.9 g) (P-value = 0.045).

For this, 30 fishper ecotron were collected in monoculture while the totality of the fish per ecotron were collected in polyculture because the number of fish per species was less than 30, and weight (W) and total length (TL) of these were measured. Zootechnical variables of fish, including specific growth rate (SGR), mortality percentage (dead), coefficients of variation for weight (CVw) and total length

(CVtl), weight gain (WG), Fulton's condition factor (FCF), total biomass, biomass gain, and feed conversion ratio (FCR) were then calculated at Tf for each ecotron according to the following formulae:

Dead (%)=100 × (number of dead fish/the total number of stocked fish)

$CV(w)= [\sigma(w)/\mu(w)] \times 100$

$CV(tl)= [\sigma(tl)/(\mu(tl)] \times 100$

$WG= \mu(w)\ final - \mu(w)\ initial$

FCF= (W/TL^3)×100

$SGR= [(\ln(W)final - \ln(W)initial/d] \times 100$

Total biomass (per tank)= total number of fish in the tank/(μ(w)of the fish mesured in the tank

Biomass gain (per tank)= final biomass – initial biomass

FCR= Total food supply (g)/Biomass gain (g)

Standardized feed quantity for all tanks was calculated based on the formulae:

Standardized feed quantity (g)= (μ of total biomass of all tank ×feed ratio)/100

Where:

σ is the standard deviation and μ is the mean.

d is the number of days.

As for biological variables related to stress, blood samples were taken from 3 fish/species/tank at a time were captured from each one of the 12 tanks and anesthetized by immersion in Tricaine Methanesulfonate (MS-222, 150 $mg \cdot L^{-1}$) buffered with sodium bicarbonate (Na_2CO_3, 150 $mg \cdot L^{-1}$). Blood samples were collected early in the morning before any manipulation at T0 and Tf to measure plasma cortisol and glucose concentrations, as well as hematocrit levels, which serve as indicators of stress and allow assessment of the physiological status of the fish. Subsequently, 1 mL of blood was withdrawn from the caudal vein at the caudal peduncle of the fish using a heparinized needle-syringe and collected in 1.5 mL Eppendorf tubes, within a maximum of 5 minutes, to minimize bias associated with capture-induced stress.

For hematocrit (Ht) measurement, 0.1 mL of blood was drawn into microhematocrit capillary tubes (sealed with modeling clay to prevent leakage during centrifugation) and centrifuged in a HAEMATOKRIT 210 centrifuge at 12,000 rpm for 5 minutes. The hematocrit percentage was then determined directly using a calibrated reading plate.

One drop of blood was used for the measurement of glucose concentration with a medical glucometer.

Eppendorf tube containing fish blood were then centrifuged immediately at 12,000 rpm for 5 minutes. The plasma (supernatant) was collected and stored at −80 °C until quantification of cortisol using an ELISA kit (Kit KAPDB270, DiaSource).

2.4. Sampling strategy for microbial analysis

2.4.1. Water and biofilm sampling

Samples of water and biofilms (from the tank walls) were collected in triplicate at different time points: Ti (in acclimatation tanks before the start of the experiment), T0 (2 days after the distribution of fish among the 12 ecotrons), T1, T2, T3 and Tf (with one month between each sampling time). Water samples were taken directly from the upper 30 cm of the tanks' water using 500 mL sterile bottles. Biofilm samples were obtained by scraping the walls of the rearing tanks and transferring the material into sterile 15 mL Falcon tubes.

2.4.2. Fish skin and gills mucous and gut microbiota

Three fish at a time were captured from each one of the 12 tanks and anesthetized by immersion in Tricaine Methanesulfonate (MS-222, 150 $mg \cdot L^{-1}$) buffered with sodium bicarbonate (Na_2CO_3, 150 $mg \cdot L^{-1}$). From each fish, fecal samples were collected first by drying the urogenital area, stripping the fish, and transferring the feces into sterile Eppendorf tubes. Skin mucous was then sampled from the left flank at mid-body, within a delimited area defined by a stencil, by swabbing the surface three times for 10 seconds each (horizontally, vertically, and again horizontally) with a sterile swab; the swab tip was cut and stored in a sterile Eppendorf tube. Gill mucous was finally collected by swabbing each side of the gills for 15 seconds, and the swab tips were stored in the same manner as for skin mucus. Following sampling, fish were returned to their original tanks, and all samples were stored at –20 °C until DNA extraction.

Samples of skin and gills mucous and feces were collected in triplicate at different time points: Ti (in acclimatation tanks before the start of the experiment), T0 (2 days after the distribution of fish among the 12 ecotrons), T1, T2, T3 and Tf (with one month between each sampling time)..

2.5. DNA extraction and amplicon sequencing

100 ml of the collected water were filtered through Whatman® NucleoporeTM Track-Etched membranes of 0.22 μm pore and extraction was performed using DNeasy PowerWater kit (Qiagen,

Hilden, Germany). Microbial DNA from biofilm samples were extracted using the NucleoSpin Soil Kit (Macherey-Nagel) following cell disruption with mechanical force with FastPrep-24™.
Microbial DNA from feces (digesta), gill and mucus were all extracted using the NucleoSpin Soil Kit (Macherey-Nagel) following cell disruption with mechanical force with FastPrep-24™.
All collected DNA were eluted in 50 μL of DNase-free water. Concentration and purity of the DNA extractions was assessed by measuring both 260/280 nm and 260/230 nm ratios with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA). DNA was stored at -20°C until further analysis.
Metabarcoding targeting on the hypervariable V3–V4 region of the bacterial 16S rRNA gene was performed. Amplicon library preparation and sequencing were performed by Novogene Co., Ltd. (Beijing, China). In brief, the V3–V4 region of the 16S rRNA gene was amplified using the primer pair 357F/806R with a dual-barcoding strategy, following the protocol described by Kozich et al. (2013).

2.6. Bioinformatic analysis

Sequencing data of microbial samples were subsequently imported into the FROGS pipeline (Find Rapidly OTU with Galaxy Solution) implemented on a galaxy instance (v.2.3.0) (http:// sigenae-workbench.toulouse.inra.fr/galaxy/) (Escudié et al., 2018). After quality control, 16S paired-end sequences were merged into contigs with VSEARCH (Rognes et al., 2022). Sequences were dereplicated before being clustered using SWARM algorithm (v.2.1.5) (Mahé et al., 2022) with a first denoising step using an aggregation distance equal to 1 with the fastidious swarm option. Chimera were removed using VSEARCH (Rognes et al., 2022). Filters were applied to remove contaminants from phiX database and clusters with an abundance below a 0.005% threshold (Bokulich et al., 2013). The taxonomic assignation of each OTU was performed using the BLAST tools (Camacho et al., 2009) against the database 16S SILVA Pintail 100 138.1. A taxonomy-based filter was applied to retain clusters showing an identity >95% and a coverage >95% with the reference database. Finally, a post-processing step merged clusters that shared a similar taxonomic affiliation and exhibited both an identity >99% and a coverage >99% among them. “Multi-affiliation” indicates that different taxa are assigned to the same OTU, such that a unique taxonomic assignment cannot be determined.

2.7. Statistical analysis

Statistical analyses for zootechnical and physiological parameters were carried out using the R

software version 4.4.0.

The data obtained from bioinformatic analysis were further filtered to remove outlier samples. Thus, samples showing a number of reads under 10 000, a number of ASVs less than 10, or a Shannon index fewer than 0.05 were discarded. Non-microbial clusters (i.e., Mitochondria) were also removed from the dataset. The 'Phyloseq' package (1.52.0) was used to compute alpha diversity indices (Richness, Shannon, Simpson, Berger–Parker). Statistical comparisons were performed using ANOVA followed by Tukey's HSD test with the 'agricolae' package (1.3-7) or using Kruskal–Wallis tests followed by Dunn's post-hoc tests (with BH correction) with the 'rstatix' package (0.7.2). Microbial beta-diversity was explored using the Aitchison distance. Ordination and graphical representation were carried out with the 'phyloseq' package after applying a centered log-ratio (CLR) transformation to the dataset using the 'microbiome' package (1.30.0). Statistically significant differences were assessed by PERMANOVA with the 'pairwiseAdonis' package (0.4.1) and ANOSIM with the 'vegan' package (2.6-10). Composition barplots were generated using the 'ggplot2' package (3.5.2) after clustering taxa at the family level and selecting the 11 most abundant families. Pairwise comparisons among these families were conducted using both DESeq2 (1.48.0) and SIMPER ('vegan' package) analyses to ensure cross-validation of the results.

Clusters shared between fish intrinsic matrices (gills, skin, gut) and extrinsic matrices (water and biofilms) at each sampling time were identified and visualized with Venn diagrams using the 'MicEco' package (0.9.19). Clustering of rearing modalities at Ti and TF based on their OTU profiles was performed with the 'pvclust' package (2.2-0) on the Aitchison distance, using the ward.D2 clustering method and a number of bootstrap replications of 10,000. The values "au" (%) represented on the tree are the approximately unbiased p-value. Values > 95% allow the clusters to be considered strongly supported by the dataset. To identify and quantify microbial community transfers (vectorization pathways) throughout the experiment times, the FEAST source-tracking algorithm (Shenhav et al., 2019) was applied through the 'FEAST' package (0.1.0). Computations were performed individually within each of the four ecotrons for each rearing modality, with between one and three sinks per ecotron, and without predefined source and sink assignment. Results were subsequently averaged.

To assess the fish intrinsic matrices communities, specific to monoculture or polyculture conditions, a LEfSe equivalent method was applied in R using the diff_analysis() function of the 'MicrobiotaProcess' package (1.20.1). Clusters representing less than 0.1% of the total abundance or with less than 20% prevalence were removed. The function was parameterized with a normalization value of 1e6, a Kruskal–Wallis test p-value threshold of 0.05, a Wilcoxon test p-value threshold of 0.01 to eliminate false positives, and a log10(LDA) ≥ 4.

3) Results

3.1. Fish growth and physiology

The zootechnical and physiological parameters measurement and calculation at Tf, 120 days after the beginning of the experiment was summarized in (Tables 2, 3, 4).

For pikeperch, the statistical analysis of zootechnical and physiological parameters revealed no significant differences in the mean of final weight, total length, mortality percentage, CVtl, WG, FCF, and SGR as well as Ht, glucose and cortisol levels between pikeperch reared in monoculture and pikeperch reared in polyculture with tench. Only CVw was found significantly higher in pikeperch monoculture than polyculture (26.1 ± 3.5% > 18.6 ± 3.6% in polyculture; p=0.025) (Table 2).

Conversely, tench in polyculture exhibited significantly a higher mean of final weight (336.8 ± 33.2 g > 281.5 ± 18.3 g; p=0.036) and TL (25.5 ± 0.7 cm >. 24.3 ± 0.5 cm; p=0.034) compared to tench in monoculture. Meanwhile, no significant differences were detected in the mean of WG, mortality percentage, CVtl, FCF, SGR and Ht, glucose and cortisol levels between T and Poly modalities (Table 3).

In addition, no significant differences were found in the mean of total biomass, biomass gain, and feed conversion ratio between all three modalities (Table 4).

Table 2. Zootechnical and physiological parameters (μ ± σ) of pikeperch (*Sander lucioperca*) in RAS (n=4) measured in different rearing modalities: monoculture of pikeperch P and polyculture of pikeperch and tench (*Tinca tinca*) Poly, at Tf 120 days after the beginning of the experiment.

Biological variables	P	Poly	Statistical test and P-value
W (g)	541.5 ± 49.3 a	530.1 ± 31.6 a	Student, P-value = 0.714
SGR (%)	1.292 ± 0.113 a	1.164 ± 0.151 a	Student, P-value = 0.231
TL (cm)	37.2 ± 0.7 a	36.5 ± 0.4 a	Student, P-value = 0.143
CVw (%)	26.1 ± 3.5 a	18.6 ± 3.6 b	Student, P-value = 0.025
CVtl (%)	8.5 ± 1.3 a	6.8 ± 1.2 a	Student, P-value = 0.103
WG (g)	356.5 ± 49 a	327.9 ± 39.8 a	Student, P-value = 0.402
FCF	1.03 ± 0.04 a	1.09 ± 0.04 a	Student P-value = 0.083
Dead (%)	6.25 ± 7.43 a	4.75 ± 6.44 a	Student P-value = 0.754
Ht (%)	30.29 ± 3.25 a	29.67 ± 2.04 a	Student P-value = 0.758
Glucose (mg/dl)	122.25 ± 16.26 a	104.08 ± 6.75 a	Student P-value = 0.108
Cortisol (ng/ml)	144.97 ± 27.50 a	162.26 ± 21.29 a	Student P-value = 0.361

a,b : Different letters in the same row indicate significant differences ($p < 0.05$).

Table 3. Zootechnical and physiological parameters (μ ± σ) of tench (*Tinca tinca*) in RAS (n=4) measured in different rearing modalities: monoculture of pikeperch P and polyculture of pikeperch (*Sander lucioperca*) and tench Poly, at Tf 120 days after the beginning of the experiment.

Biological variables	T	Poly	Statistical test and P-value
W (g)	281.5 ± 18.3 a	336.8 ± 33.2 b	Student, P-value = 0.036
SGR (%)	0.752 ± 0.049 a	0.853 ± 0.136 a	Student, P-value = 0.239
TL (cm)	24.3 ± 0.5 a	25.5 ± 0.7 b	Student, P-value = 0.034
CVw (%)	33.9 ± 4.2 a	38.9 ± 3.8 a	Student, P-value = 0.134
CVtl (%)	9.4 ± 1.3 a	12.3 ± 2.6 a	Student, P-value = 0.109
WG (g)	130.8 ± 14 a	171.3 ± 35 a	Student, P-value = 0.099
FCF	1.91 ± 0.03 a	1.97 ± 0.05 a	Student P-value = 0.078
Dead (%)	4.04 ± 3.23 a	10.25 ± 13.97 a	Student P-value = 0.448
Ht (%)	21.42 ± 4.65 a	23.33 ± 6.45 a	Student P-value = 0.648
Glucose (mg/dl)	65.17 ± 6.81 a	73.17 ± 7.89 a	Student P-value = 0.177
Cortisol (ng/ml)	126.03 ± 58.34 a	88.08 ± 39.50 a	Student P-value = 0.328

a,b : Different letters in the same row indicate significant differences ($p < 0.05$).

Table 4. Zootechnical parameters of pikeperch (*Sander lucioperca*) and tench (*Tinca tinca*) in RAS (n=4) in different rearing modalities: monoculture of pikeperch P, monoculture of tench T and polyculture of pikeperch and tench Poly, at Tf 120 days after the beginning of the experiment (μ ± σ).

Biological variables	P	Poly	T	Statistical test and P-value
Biomass (g)	17673 ± 1733 a	19119 ± 2199 a	17973 ± 1095 a	ANOVA P-value = 0.49
Biomass Gain (g)	7576 ± 1779 a	8694 ± 2020 a	7837 ± 1397 a	ANOVA P-value = 0.653
FCR	2.024 ± 0.475 a	1.758 ± 0.389 a	1.918 ± 0.320 a	ANOVA P-value = 0.651

a,b : Different letters in the same row indicate significant differences ($p < 0.05$).

3.2. Microbial analysis

3.2.1. Initial microbial communities associated with fish

3.2.1.1. Alpha-diversity

Bacterial ASV richness was reported as the total number of amplicon sequence variants (ASVs) per sample of fish-associated intrinsic matrices (gills, mucus, gut) taken at Ti during the acclimatation period (fig. 24). The bacterial richness of pikeperch gut microbiota were found significantly lower than tench, while in contrast, the number of ASV in pikeperch gills were found significantly higher than tench gills. However,for the skin mucous, the difference in microbial richness between pikeperch and tench is considerable but it did not reach statistical significance ($p = 0.051$).

As for ASV diversity (approximated by Shannon index calculation), pikeperch gills showed significantly higher bacterial diversity than tench gills. But no significant differences were recorded between ASV diversity of gut and skin mucous microbiota of either reared fish (fig. 24).

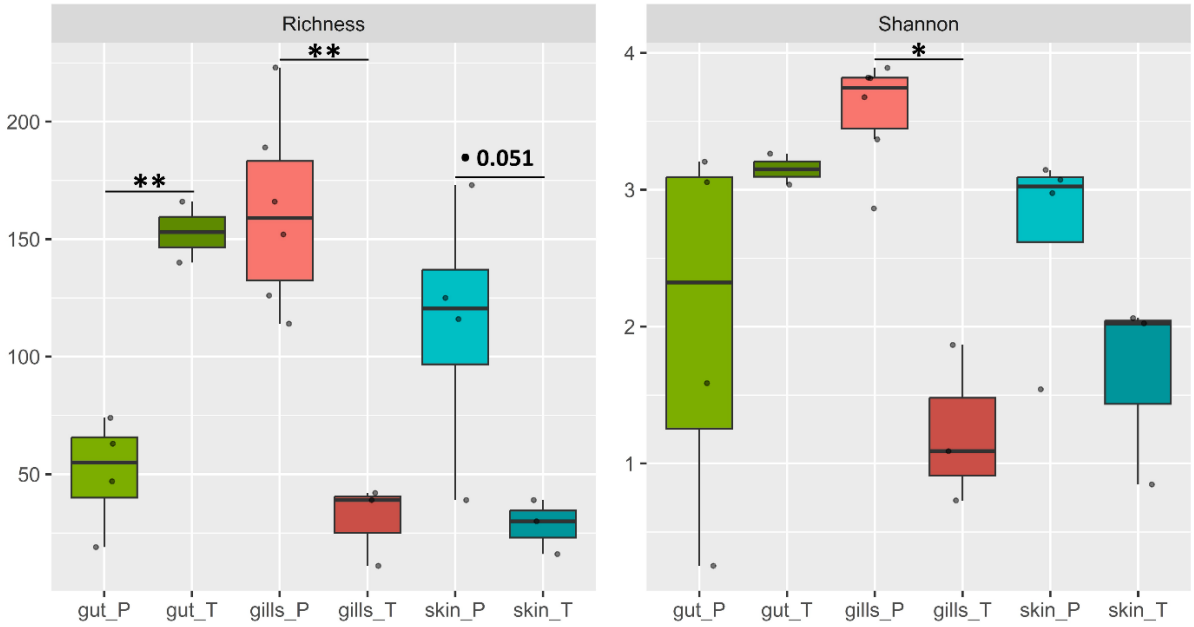

Figure 24. *Microbial alpha-diversity indexes between gut, gills and skin mucous of pikeperch and tench measured at Ti (during acclimatation phase before the start of the experiment). Alpha-diversity is presented as a measure of species richness (number of ASVs) on the left and as a measure of Shannon diversity index (biodiversity index) on the right. Stars above the box-plots indicate significant differences between fish species for each fish matrix; *p < 0.05, **p <0.005 and ***p <0.001.*

3.2.1.2. Composition of initial fish intrinsic microbiotas

The composition of bacterial communities was also examined at Ti at the family level (fig.25A). This figure shows the relative abundance of the 12 majority families found in the gut, gills and skin mucous of stocked pikeperch and tench during acclimatation period. Based only fig. 25A, clear differences are observed between microbial composition profiles of gut, gills and mucous microbiotas between pikeperch and tench.

For digesta samples, statistical analysis (DESeq2) showed significant higher abundance of Bacilliaceae and Peptostreptococcaceae families and lower abundance of JG30-KF-CM45 and Rhodobacteraceae in pikeperch digesta compared to tench. As for the composition of gills, fig. 25A and DESeq2 analysis showed significantly higher relative abundance of Flavobacteriacea, Oxalobacteriaceae, Pseudonocardiaceae and Saprospiroceae but lower abdundance of Corynebacteriaceae, Weeksellaceae and Xanthonomonadaceae in gills of pikeperch in comparison with tench gills. Finally, the composition profile of skin mucous supported by DESeq2 analysis revealed that pikeperch skin exhibit significantly greater abundance of Pseudonocardiaceae, Rubrobacteriaceae and Salinisphaeraceae than tench skin mucous.

In addition, a heatmap was performed showing clusterical analysis based on ASV level to identify differences and similarities between the microbial composition of the different intrinsic matrices of pikeperch and tench (fig. 25B). Regardless of the fish species, the bacterial community composition

showed that fish digesta samples are highly different than skin and gills mucous samples. Meanwhile, the clusterisation data showed that skin and gills composition are grouped by species and not by tissue like gut.

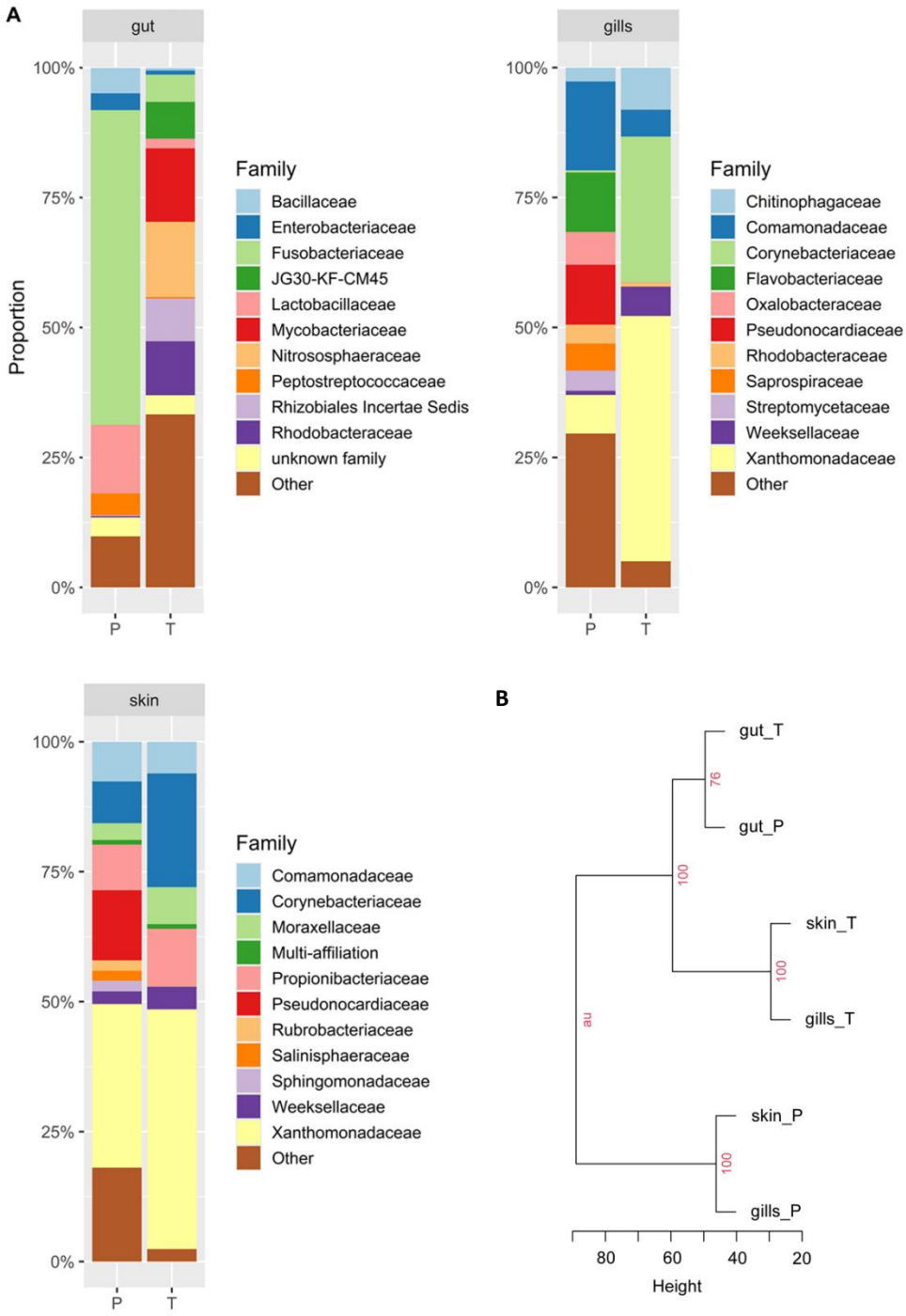

Figure 25. (A) *microbial composition and relative abundances of the 12 most abundant OTU of bacterial families at Ti in gut, gills and skin mucous between fish species P (pikeperch monoculture in acclimatation), and T (tench monoculture in acclimatation) and* ***(B)*** *heatmap showing clusterical analysis based on ASV level illustrating the microbial community composition between intrinsic microbial matrices of P and T at Ti.*

3.2.2. Main intrinsic microbial vectorization pathway inoculating water and biofilms

3.2.2.1. Species effect in monoculture

The Venn diagramm analysis illustrates the temporal dynamics of microbial community sharing between fish-associated intrinsic matrices (gills, mucus, gut) and extrinsic compartments of RAS (water and biofilm) in monocultures of pikeperch P and tench T (fig. 26).

In pikeperch monoculture, gills exhibited the highest proportion of shared microbial communities with both water and biofilm at Ti and during the experiment after T0 reaching 65% of shared communities with biofilm and 77% with water at Tf. Skin mucus also contributed substantially to a considerable proportion of 42% of shared communities with both water and biofilm samples at T0. That sharing of microbial communities between P skin mucous and water and biofilm samples remain considerable at all sampling times. Meanwhile, pikeperch digesta recorded the lowest sharing of microbial communities in either acclimatation tanks and all along the experiment, sharing a proportion of 15% and 14% with biofilm and water respectively at Tf.

As for tench monoculture, gills samples taken at Ti exhibited a low proportion of shared microbial communities with water and biofilm, but this proportion increased greatly at T0 recording, all through the experiment, the highest sharing proportion of microbial communities among intrinsic fish matrices and reaching 68% of communities shared with biofilm and 73% shared with water at Tf. Additionally, from T digesta, 30% and 28% of microbial communities were shared respectively with biofilm and water samples at T0. Considerable proportion of shared taxa between fecal communities and water and biofilm samples remain all through the experiment. Meanwhile, skin mucous of T recorded the lowest sharing of microbial communities among intrinsic matrices of tench. The proportion of shared taxa increased reaching a maximum of 37% of skin communities shared with biofilm and 40% shared with water at T2, but it decreased beyond this time point reaching a shared percentage of only 19% and 20% with biofilm and water respectively at Tf. Interestingly, the sharing profiles between water and biofilm were highly similar regardless of fish species.

Heatmap representation was also performed, assessing temporal “source tracking” of microbial communities in water and biofilm samples in monocultures P and T (fig. 27).

In P modality, the source tracking data showed that the initial colonization phase (Ti–T0) of the biofilm was primarily driven by microbial inoculation from skin (25.48%) and acclimatation water (27.35%) alongside a considerable proportion of unidentified sources “unknown”. Similarly, initial colonization phase (Ti–T0) of water of P was also mainly driven by microbial inoculation from skin (13.89%), acclimatation water (36.11%) and unidentified sources. After the initial phase, the microbial communities of biofilm in P remained relatively stable over time until Tf, with approximately 40%

similarity between successive sampling times. In addition, water compartment continued to participate in biofilm inoculation up to T3 and decreased at Tf while gills contributed to 15.95% of biofilm microbial communities at this time. As for water samples, microbial communities of water exhibited even greater stabilization than biofilms of P over time, recording high similarities between successive sampling times (78.13% at T2) reaching 62.83% at T3-Tf along with a partial contribution of gills to microbial inoculations of water (19.27%).

In monoculture of tench, fecal inputs primarily contributed to 12.29% of microbial communities of biofilm and 36.55% of water communities in the initial colonization phase (Ti–T0) alongside a considerable proportion of unidentified sources “unknown” contributing importantly to 84.80% of microbial communities of biofilm at T0. Beyond initial phase, the biofilm became increasingly stable and robust over time reaching at Tf an important percentage of contribution of 84.27% of microbial communities from T3. In addition, from T0 onward, the water compartment emerged as a recurrent inoculation source up to T3. Similarly, water microbial communities also become highly stabilized through experiment time recording high similarities between successive sampling times notably at (T2–T3) (73.03%) in addition to slight contributions of 10.83% of gut recorded at T2 and of biofilms at Tf.

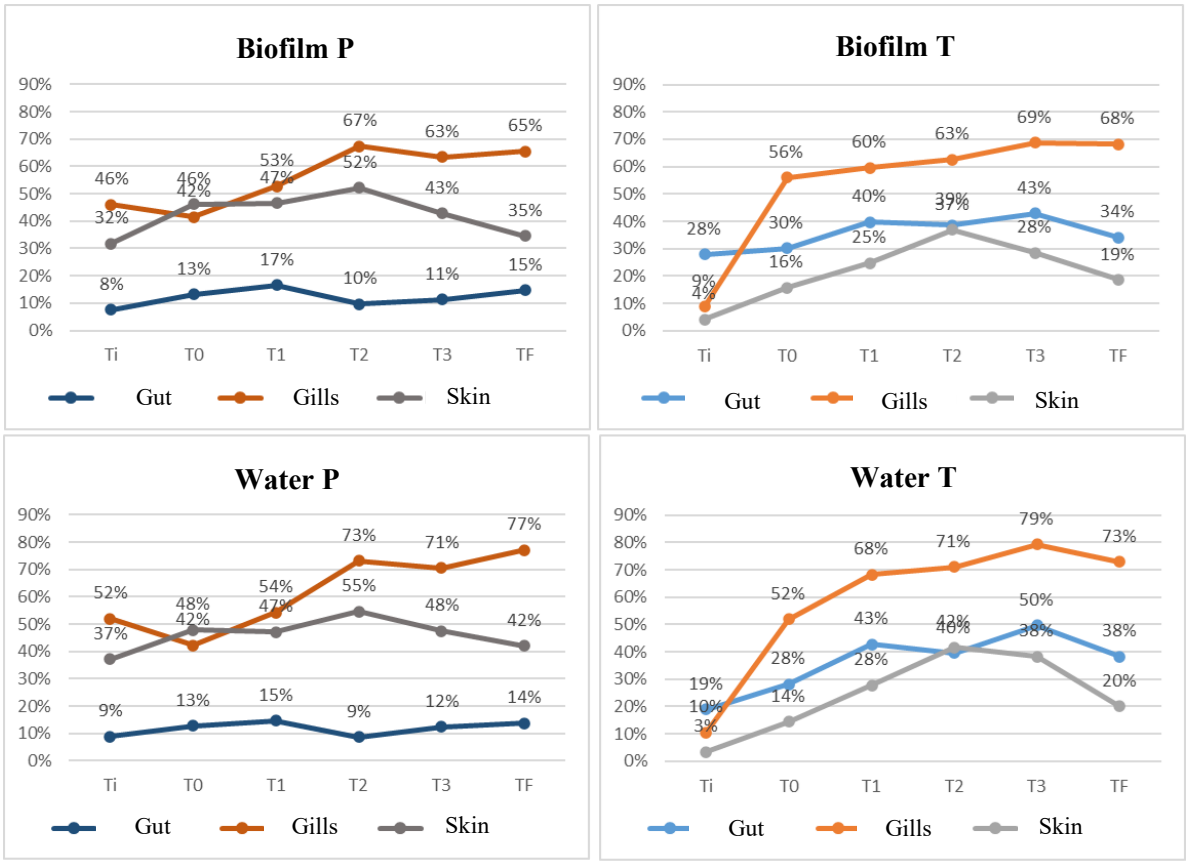

Figure 26. *Curves extract from Venn diargamm result representing the sharing of microbial communities in monoculture of pikeperch (mono_P) on the left and monoculture of tench (mono_T) on the right, between the fish's intrinsic matrices with the water and biofilm communities over time.*

Biofilm P

biofilm	NA	44,27%	49,47%	37,83%	41,69%
feces_P	0,53%	0,08%	0,09%	0,08%	1,08%
gills_P	1,51%	4,89%	0,35%	5,08%	15,95%
skin_P	25,48%	1,01%	0,48%	3,45%	2,39%
water	NA	18,59%	12,25%	12,72%	2,84%
water_accP	27,35%	NA	NA	NA	NA
unknown	45,13%	31,16%	37,35%	40,84%	36,05%
	Ti_T0	T0_T1	T1_T2	T2_T3	T3_TF

Biofilm T

biofilm	NA	37,31%	57,91%	74,33%	84,27%
feces_T	12,29%	0,96%	1,32%	0,50%	5,38%
gills_T	0,61%	2,34%	3,18%	0,84%	2,40%
skin_T	2,01%	0,03%	2,67%	0,03%	0,16%
water	NA	22,19%	11,14%	11,23%	0,91%
water_accT	0,30%	NA	NA	NA	NA
unknown	84,80%	37,17%	23,77%	13,07%	6,89%
	Ti_T0	T0_T1	T1_T2	T2_T3	T3_TF

Water P

biofilm	NA	5,51%	1,64%	3,16%	5,66%
feces_P	1,03%	0,86%	1,48%	5,74%	0,07%
gills_P	5,52%	4,72%	0,66%	6,78%	19,27%
skin_P	13,89%	0,17%	0,01%	5,54%	2,59%
water	NA	45,67%	78,13%	67,05%	62,83%
water_accP	36,11%	NA	NA	NA	NA
unknown	43,45%	43,07%	18,09%	11,73%	9,58%
	Ti_T0	T0_T1	T1_T2	T2_T3	T3_TF

Water T

biofilm	NA	7,97%	0,19%	3,97%	12,08%
feces_T	36,55%	1,16%	10,83%	1,74%	1,89%
gills_T	0,46%	1,33%	6,95%	4,98%	9,30%
skin_T	5,20%	1,44%	0,02%	0,77%	0,02%
water	NA	47,36%	62,51%	73,03%	56,75%
water_accT	3,19%	NA	NA	NA	NA
unknown	54,60%	40,74%	19,50%	15,51%	19,96%
	Ti_T0	T0_T1	T1_T2	T2_T3	T3_TF

Figure 27. *Heatmap representation based on FEAST Algorythm of temporal source tracking of microbial communities in water and biofilm compartments of pikeperch (P) and tench (T) monocultures, showing the relative contributions (%) of fish-associated matrices (gut, skin mucus, and gills) and extrinsic compartments over the rearing period.*

3.2.2.2. Species effect and RAS inoculation in polyculture

The curves extract from Venn diargamm result illustrating the temporal sharing of microbial community between fish-associated intrinsic matrices and water and biofilm in polyculture Poly in fig. 28 showed similar profiles for water and biofilm samples. Overtime, the gills of pikeperch and tench exhibited the highest proportion of shared microbial communities with both water and biofilm. This was followed, in descending order of contribution, by skin mucous of pikeperch, then tench gut, skin mucous of tench, and finally pikeperch gut showing the lowest proportions of shared communities.

The source tracking data represented in heatmaps (fig. 29) revealed that the initial colonization phase (Ti–T0) of the biofilm is primarily driven by the acclimation water of pikeperch (31.57%) and to a lesser extent, by pikeperch skin mucous (11.26%) along with other unknown sources. As for water samples, results showed that initial colonization was primarily driven by the acclimation water of pikeperch (39.2%) with a lower microbial contribution by tench gut (9.96%) and pikeperch skin mucous (8.65%).

For biofilm in polyculture, later contributions from water source (29.3%) and pikeperch gills (15.9%) were considerable at T1, while only 25% of the communities originates from the biofilm in the preceding time point T0. From T2 onward, the biofilm exhibits strong temporal stability, retaining at Tf 86.90% of its previous composition. The contribution of tench-associated sources to the biofilm microbial inoculation remains minimal all through the experiment. As for water in Poly, from T1 onward, water microbial communities increased its stabilization and maintains over half of its composition between successive sampling times recording at Tf a proportion of 64% of shared communities with previous time T3. In addition, later contributions from biofilm (12.77%), pikeperch gut (15.08%) and pikeperch skin mucous (14.81%) were considerable at different time during the experiment.

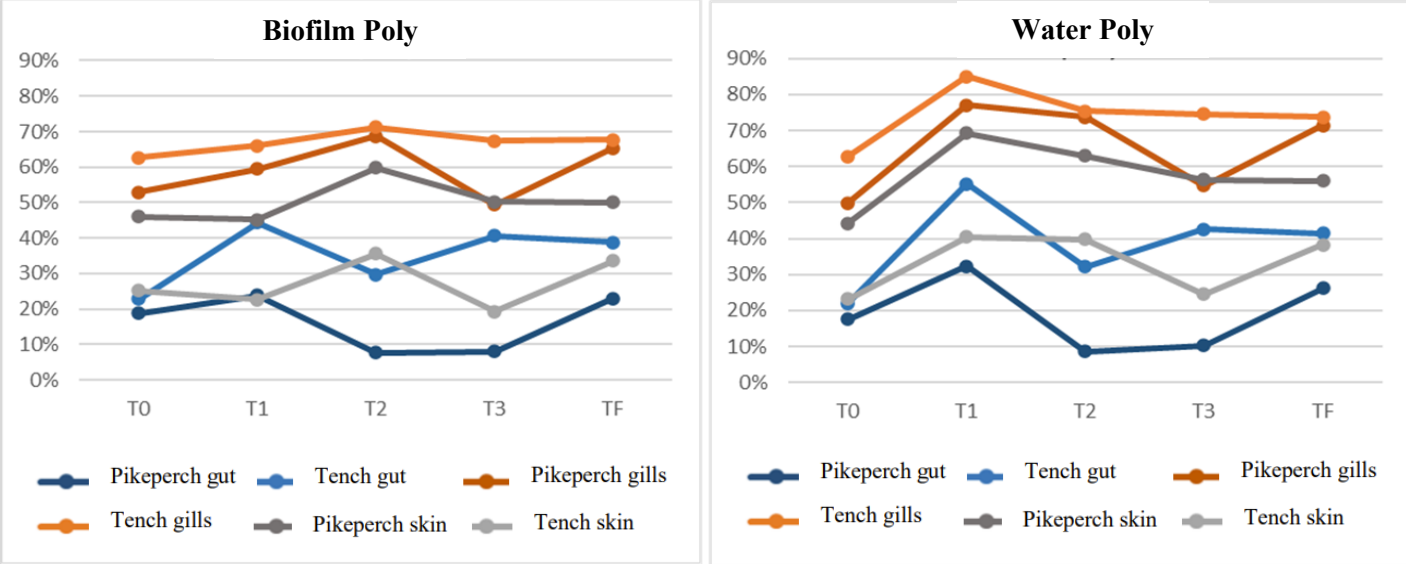

Figure 28. *Curves extract from Venn diargamm result representing representing the sharing of microbial communities in polyculture of pikeperch and tench, between the fish's intrinsic matrices with the water and biofilm communities over time.*

Biofilm Poly

biofilm	NA	25,23%	76,44%	68,15%	86,90%
feces_P	0,09%	0,00%	0,05%	0,01%	0,01%
feces_T	5,99%	0,00%	2,11%	0,11%	0,81%
gills_P	4,25%	15,92%	1,26%	1,81%	0,68%
gills_T	0,04%	2,96%	3,18%	0,35%	2,16%
skin_P	11,26%	0,07%	0,00%	0,00%	0,64%
skin_T	0,15%	0,01%	0,00%	0,34%	0,01%
water	NA	29,26%	3,09%	7,56%	0,53%
water_accP	31,57%	NA	NA	NA	NA
water_accT	0,45%	NA	NA	NA	NA
unknown	46,20%	26,54%	13,88%	21,66%	8,25%
	Ti_T0	T0_T1	T1_T2	T2_T3	T3_TF

Water Poly

biofilm	NA	12,77%	8,77%	0,68%	1,78%
feces_P	3,49%	0,01%	5,21%	15,08%	0,00%
feces_T	9,96%	2,16%	0,20%	2,66%	0,07%
gills_P	5,44%	3,22%	2,59%	0,51%	0,86%
gills_T	0,00%	5,82%	8,23%	4,60%	8,59%
skin_P	8,65%	0,44%	0,69%	1,01%	14,81%
skin_T	0,01%	0,01%	0,09%	1,17%	0,02%
water	NA	38,74%	53,14%	63,17%	64,27%
water_accP	39,20%	NA	NA	NA	NA
water_accT	0,47%	NA	NA	NA	NA
unknown	32,78%	36,84%	21,08%	11,12%	9,60%
	Ti_T0	T0_T1	T1_T2	T2_T3	T3_TF

Figure 29. *Temporal source tracking of microbial communities in water and biofilm compartments of polyculture of pikeperch and tench Poly, showing the relative contributions (%) of fish associated matrices (gut, skin and gills mucous) of pikeperch (_P) and tench(_T) and extrinsic compartments over the rearing period.*

3.2.3. Microbial communities of intrinsic matrices at Tf

Changes in the community structure of intrinsic matrices of fish species between modalities at Tf were analysed with a Principal Coordinate Analysis (PCoA) performed on the metabarcoding results (fig.30). The figure shows a clear separation primarily driven by fish species, with pikeperch and tench samples forming two distinct groups for gut and mucous.

Based on PCoA, the analysis of beta diversity revealed distinct clustering patterns in the gut microbiota of fish between both fish species and rearing modality. The first two axes of the PCoA explain a total of 44.5% of the observed variance. Similarly, for fish gills, the PCoA shows a clear separation of

microbial communities between all modalities for both species. The tench (Poly_T) and pikeperch from polyculture (Poly_P) cluster closely together but far from the monocultures of both species. However, the first two axes of the PCoA explain only a total of 29.2% of the observed variance. Finally, for skin mucous, the PCoA plot showed distinct clustering patterns in the microbial communities of skin between fish species and rearing modality while the first two axes of the PCoA explain a total of 21.9% of the observed variance.

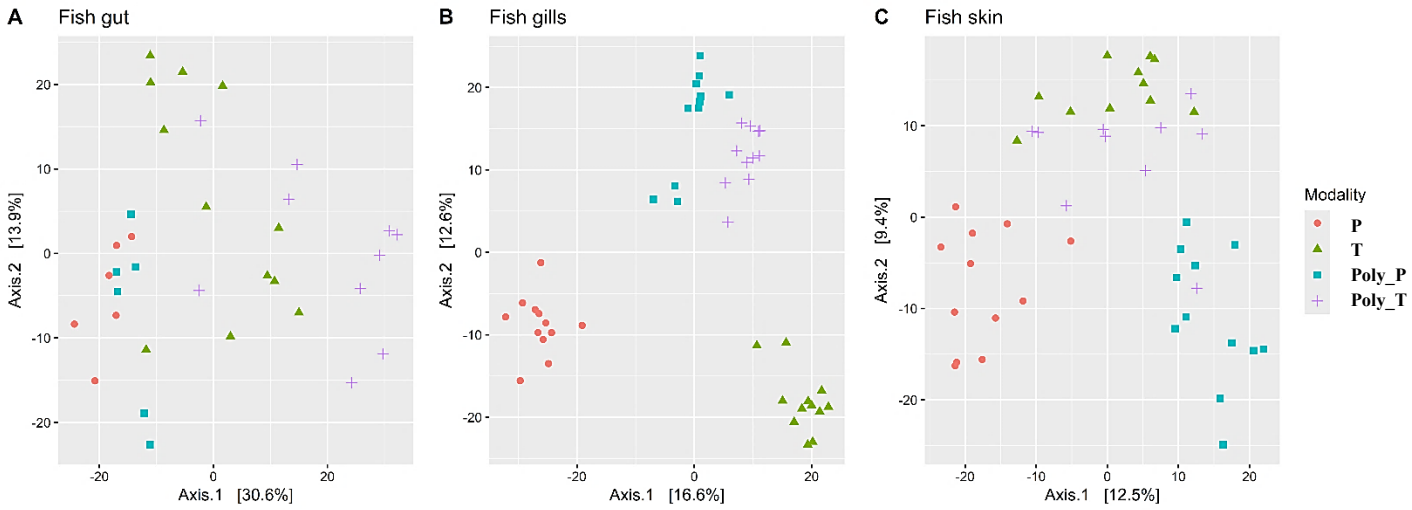

Figure 30. *Principal coordinate analysis (PCoA) of distances between microbial communities of intrinsic microbiota of fish among modalities at Tf. The colors of the symbol are red: P (pikeperch monoculture), green: T (monoculture of tench), blue: Poly_P (pikeperch in polyculture) and violet: Poly_T (tench in polyculture).*

The composition of bacterial communities was also examined at Tf at the family level (fig.31). This figure shows the relative abundance of the 12 majority families found at Tf in the gut, gills and skin mucous of stocked pikeperch and tench 120 days after the start of the experiment.

Based on histograms profiles, the comparison of digesta composition between pikeperch and tench in monoculture and polyculture showed that gut microbiota appears to be highly different and grouped by species more than rearing modality. Moreover, based on the figure and statistical analysis (DESeq2), tench gut microbiota in polyculture and in monoculture showed higher abundance of Staphylococcaceae, Rhodobacteraceae, Neisseriaceae, Rhizobiaceae and Streptococcaceae families compared to P and Poly_P. In addition, based on the figure and statistical analysis (DESeq2), tench digesta in monoculture showed significantly higher abundances of Neisseriaceae and lower abundances of Rhodobacteraceae, Rhizobiaceae, Staphylococcaceae and Rhizobiales Incertae Sedis than tench digesta in polyculture (fig. 31A).

As for gills samples, figure 31B shows a close microbial composition between the gills of species no matter the rearing modality. Based on the figure and DESeq2 analysis, gills of tench in polyculture

showed just significant higher abundance of Weeksellaceae than pikeperch in polyculture. Moreover, gills of P exhibited higher relative abundance of Moraxellaceae but lower Oxalobacteraceae and Caulobacteraceae in comparison with pikeperch in polyculture. Additionally, gills of tench in monoculture showed significantly lower abundance of Flavobacteriaceae, Caulobacteraceae and Oxalobacteraceae than tench in polyculture.

Finally, the figure 31C and DESeq2 analysis revealed that skin of tench in polyculture exhibit significantly higher abundances of Vibrionaceae and Moraxellaceae along with lower relative abundance of Xanthonomonadaceae than mucous of pikeperch in polyculture. Comparing pikeperch in monoculture and polyculture revealed that skin mucous of P harbored significant higher abundance of Moraxellaceae but lower Xanthomonadaceae than in skin of Poly_P. Similarly, tench in monoculture exhibited some differences with Poly_T that showed significantly higher abundance of Vibrionaceae but lower abundance of Xanthomonadaceae in comparison with skin of T based on DESeq2 analysis.

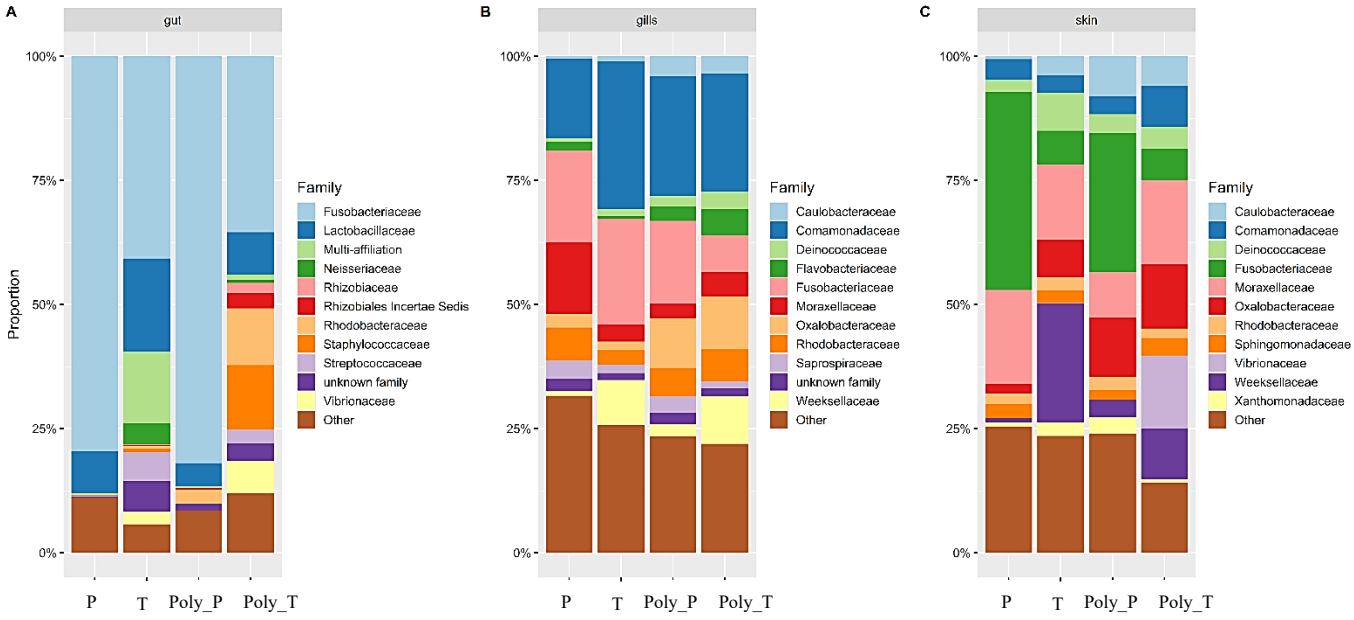

Figure 31. *Microbial composition and relative abundances of the 12 most abundant OTU of bacterial families at Tf in feces (A), gills (B) and skin mucous (C) between pikeperch in monoculture P; tench in monoculture T; pikeperch in polyculture Poly_P and tench in polyculture Poly_T.*

4) Discussion

The main aim of this study was to investigate how the microbiomes of intrinsic matrices (gut, gills, and skin mucus) of pikeperch and tench influence the microbial dynamics and inoculation of extrinsic matrices (water and biofilm) in RAS reared in monoculture versus polyculture. The microbiomes of

fish whether in the gut or in the mucous of the gills and skin surface, important for preserving host health, are complex, diverse and notably specific for each body site (Moschos et al., 2022; Kanika et al., 2025). Microbial diversity of fish microbiota is strongly influenced by intrinsic factors related to species phenotype and traits such as intestinal morphology (Smith et al., 2015; Minich et al., 2022) as well as extrinsic factors such as environmental conditions and nutrient input through feed and waste especially mucosal surfaces that are in direct contact with water (Dulski et al., 2020 ; Egerton et al., 2018; Minich et al., 2020; Deng et al., 2023). In order to focus on the effect of fish species and rearing modalities, fish were maintained in homogenous rearing conditions (such as feed ratio and water quality parameters) in all ecotrons but it was also essential to consider the uncontrollable effect of fish biological traits on their associated microbiotas.

4.1. Physiological and zootechnical traits of fish in monoculture vs polyculture

Physiological and zooetchnical variables were compared per fish species between monoculture and polyculture. Our results showed that mentionnned zooetchnical variables (measured and calculated) of pikeperch were homogenous between monoculture and polyculture except for the coefficient of variation for weight (CVw) that differed between groups (26.1% in P vs 18.6% in Poly, $p = 0.025$). In polyculture, pikeperch weight is notably more uniform (desirable), while higher heterogeneity in pikeperch weight in P can indicate that there could have been more intraspecific competition between pikeperch in monoculture which may be economically disadvantageous and lead to reduced market uniformity (Rapp et al., 2019; Thomas, 2021). In fact, intraspecific competition is a common aspect of pikeperch social behavior and usually results in the formation of hierarchies with dominant individuals especially at high stocking densities resulting in feed loss and reduced growth of some pikeperch due to competition among themselves in monoculture (Thomas, 2021). The overall mean values obtained of zootechnical parameters of pikeperch showed that they reached good commercial sizes (≈530–541 g, ~37 cm) (Nagy et al., 2022; Steinberg et al., 2019) as well as acceptable SGR (1.16–1.29%·day^{-1}), final WG (≈328–356 g) and FCF (1.03–1.09) within predictable range for adult pikeperch of similar weight range and acceptable mortality rate (4.75–6.25%) (M'Hetli et al., 2011; Nikolić et al., 2023; Péter et al., 2023; Rapp et al., 2019). In contrast, tench in polyculture achieved significantly higher final weight and total length (336.8 g, 25.5 cm in Poly vs 281.5 g, 24.3 cm in T; $p<0.05$) which are achievable values for tench (Bezmaternykha and Shcherbina, 2018) whereas SGR, CVw, CVtl, FCF and final WG were not different between the two rearing modalities. In addition, the final biomass, biomass gain, and FCR were similar between the three rearing modalities. When compared to values reported in the literature, the FCR obtained for pikeperch (1.76 in poly and 2.02 in P) appears to be relatively high (Steinberg, 2018) Previous studies have shown that *Sander*

lucioperca receiving a similar daily feeding rate of 1.5% can reach better feed efficiency under RAS conditions, with FCR values ranging from 1.00 to 1.39 (Pěnka et al., 2024a; 2021).

Physiological parameters of both tench and pikeperch including cortisol, glucose and hematocrit, related to the species and the farming conditions (Rożyński et al., 2019), were not affected by the rearing modality. Given that all fish were maintained in the same rearing conditions and handled identically, compared with reported ranges for medium-sized pikeperch (>400 g), hematocrit values of reared pikeperch (530–540 g) obtained in this study around 30% correspond well to documented range for healthy pikeperch (Movahed et al., 2016). Meanwile, plasma glucose concentrations (104–122 mg/dL) were high compared to reported values (60–80 mg/dL) for unstressed pikeperch of similar weight range groups (Ende et al., 2021) indicating a stimulation of gluconeogenetic processes possibly as a stress response (Suljevic et al., 2015). As for cortisol, the primary physiological indicator of stress, our results (145–162 ng/mL), suggest acute stress response compared to reported values for unstressed pikeperch (<100 ng/mL) weighting 337.4–318.7 g (Falahatkar and Poursaeid, 2014) which align with the high glucose levels, as cortisol induces gluconeogenesis (Martín Gallardo et al., 2010). Meanwhile,the physiological parameters observed of tench in this study are consistent with those reported in the literature for adult fish of similar size reared under controlled conditions. For tench, hematocrit values (21–23%) and glucose levels (≈65–73 mg/dL) fall within the normal range of non-fed tench at 22–24 °C (De Pedro et al., 2005; Demaël et al., 1980). As for cortisol, little is reported on cortisol levels for tench of similar conditions of this study. Our results showed cortisol concentrations (≈88–126 ng/mL) which acceptable range for tench and within the range of unstressed Cyprinidea (50–150 ng/mL) such as common carp but this range can be a cause immunosuppression for teleosts (Pottinger et al., 2000). These results indicate good physiological conditions of tench while pikeperch might have experienced some acute stress due to its high sensitivity to the stress probably due to handling, netting, anaesthesia or other stressors (Pourhosein-Sarameh and Falahatkar, 2024).

Thanks to these findings, it can be noted that polyculture of pikeperch and tench could be an interesting option for reducing heterogenic growth and possible intraspecific competition between pikeperch while potentially improving tench growth in comparison with their monocultures highlighting the potential benefits of the implementation of polyculture of pikeperch with tench in RAS compared with its monoculture. More importantly, data of this work showed that pikeperch and tench biological traits were mostly homogenous between rearing modalities. This can allow to minimize the possible variability between the final microbial diversity and composition of fish microbiota associated with the fish characteristics (growth and stress level) between fish in different rearing modalities.

4.2. Microbial communities

Our results at Ti confirm the existence of a species-specific effect of fish on microbial communities of gills and skin mucous and gut and of fish, both in terms of alpha and beta diversity, but also a tissue-specific effect between the different intrinsic matrices, particularly for the fecal inputs. While fecal microbial communities were primarily matrix-dependent, the microbial compositions of skin and gill mucus were largely determined by the fish species (fig.25B). The low microbial richness and diversity in tench skin mucous (fig. 24) may be linked to a more stringent selective pressure, which could result in only specific microbial taxa that are capable of establishing and persisting within this mucosal matrice.These findings align with literature studies demonstrating that fish microbiomes exhibit species-specific microbial signatures as well as matrix-specific compositions within individual species (Minich et al., 2022; Minich et al., 2021; Moschos et al., 2022). The composition representation, at Ti during acclimatation period before the beginning of the experiment (fig. 25A) permitted to highlight that gut microbiota of pikeperch at Ti appears to be associated with a dominant representation of Bacilliaceae and Peptostreptococcaceae families also reported to dominate in gut and intestines of pikeperch in literature (Dulski et al., 2018; Tran et al., 2021). The family Bacillaceae including *Bacillus* species, are used as probiotics. Multiple studies have reported the capacity of Bacillus to produce extracellular enzymes improving fish health and their antimicrobial capacities against fish pathogens via spore forming ability and production of bacteriocin or other related compounds (e.g. *Bacillus amyloliquefaciens)* (Elsadek et al., 2023; Midhun et al., 2017; Santos et al., 2018; Tarnecki et al., 2019). Peptostreptococcaceae family is also a common commensal bacterial family found in higher abundance in healthy fish including species that play important role in maintaining gut homeostasis (Huang et al., 2020). Whereas our result showed that tench fecal inputs exhibit high abundances of Rhodobacteraceae belonging to Proteobacteria the most abundant phylum reported to be found in tench gut microbiome (Dulski et al., 2020). Meanwhile the microbiota of skin and gills mucous exhibited different compositions than gut and differed also between species, as Flavobacteriacea, including *Flavobacterium* and reported in fish skin and gill mucous of Atlantic Salmon (*Salmo salar)* for example (Birlanga et al., 2022) and Pseudonocardiaceae, Saprospiroceae and Oxalobacteraceae appears to be overrepresented in pikeperch gills but not in tench gills dominated by Corynebacteriaceae, also found in the gills of *Labeo Rohita* (Walke et al., 2015), Weeksellaceae and Xanthonomonadaceae. While pikeperch skin mucous exhibited an overrepresentation of Pseudonocardiaceae; found in skin mucous of Gilthead Sea Bream (*Sparus aurata*) and more abundant at high stocking densities (Toxqui-Rodríguez et al., 2024); Rubrobacteriaceae and Salinisphaeraceae, found less abundant in tench skin mucous. Despite the direct contact of gills and skin mucous with the

surrounding microbiome of the system and their importance to fish health, as the first line of pathogen invasion (Guivier et al., 2020; Wang et al., 2010), the microbial composition of these matrices has not been well reported for pikeperch and tench to our knowledge. Overall, these findings show that the overall microbial compositions of our species, based on the 12 majority families found in fish digesta and skin and gills mucous, differ among species and among the three intrinsic matrices.

The microbiomes of these fish intrinsic matrices exist in continuous interaction and exchange microbial communities with the surrounding environment , so the aquatic culture system acts both as a reservoir of microbes and as a selective shaper of microbial communities trought physico-chemicals interactions (Bruno et al., 2023; Muziasari et al., 2017; Zeng et al., 2020). However, deciphering how fish microbiota shape the sessile and planktonic microbial communities of RAS is difficult because of the complex dynamics underlying microbial communities' assembly and stabilization. The temporal dynamics of microbial communities and information on the main intrinsic microbial pathway of fish (skin mucus, gill mucus, gut) inoculating water and biofilms compartments in RAS, particularly in the context of polyculture, has not been investigated.

Our results provide the first clear information about initial inoculation of RAS water and biofilms by fish associated microbiota. First, in monoculture, the source tracking analysis represented in heatmap (fig. 27) showed that initial inoculation of biofilm and water in pikeperch monoculture was mainly driven by P skin mucous and acclimatation water, while in contrast, in tench monoculture, gut were the main driver for initial microbial inoculation. Meanwhile, in polyculture (fig. 29), the initial colonization of the biofilm (T0) appeared to be mainly driven by acclimation water of pikeperch, pikeperch skin mucous microbiotas whith a very low contribution of tench gut microbiota compared with T modality. This aligns with figure 24 and can be explained by the higher microbial richness of pikeperch skin mucous compared to tench and higher richness of tench gut microbiota than pikeperch.The unexpected low contribution of pikeperch gut microbiota compared to tench can be explained by different digestive transit and morphylogy of the gastrointestinal tract (Egerton et al., 2018) but can be suggestively explained by the different quantities of fecal waste per species released in the system since we did not have that information. Beyond the initial inoculation phase, the biofilm and water microbiomes in monocultures stabilized at T1 and maintained similar community structures with minimal changes throughout the experiment.

Notably, biofilm in T (84.27%) showed a considerably higher stabilization of than P modality (41.69%) at Tf suggesting that .A possible explanation is that the species-specific differences in host–microbiome or microbial interactions specific to the tench monoculture favored a more resilient and self-sustaining biofilm community than those in the pikeperch system, suggesting that species-specific differences may influence the degree of biofilm community stabilization in RAS. In addition, source

tracking data of both monoculture P and T whowed that the water microbiome contributed highly to the initial colonization of the biofilm, indicating that water serves as a primary inoculum source for biofilm formation. In contrast, the biofilm community did not considerably contribute to the microbial composition of the water, suggesting a higher microbial transfer from the water to the biofilm rather than reciprocal exchange between these compartments.A This may be related to the water circulation and biofilm forming process, by planktonic bacteria adhesion and release, which are constantly dynamically interconnected (Gonçalves and Gagnon, 2011; King et al., 2004; Rojas-Tirado et al., 2017). In contrast in polyculture, after the initial inoculation, biofilm microbial communities were still influenced by pikeperch gills mucous, due to its high microbial richness and diversity compared to gills' microbiota of tench (fig. 24),and water microbiome at T1 then become highly stable from T2 until Tf. These results highlight a minimal contribution of tench microbiotas to the inoculation of water and biofilm of RAS in comparison with pikeperch microbiotas. Although skin mucous of tench is thicker than pikeperch, its microbial contribution to inoculation of water and biofilm in polyculture is not notable. This result along with low microbial richness may suggest that tench skin mucous is more robust and selective than pikeperch skin microbiota due to its richness in glycoproteins and other compounds with bactericidal activity (Díaz-Puertas et al., 2023). The biofilm stabilization in polyculture appeared to occur at T2 later than in monocultures P and T, suggesting the presence of multiple species introduces complex microbial interactions that extend the initial colonization and delay biofilm stabilization compared to monocultures. In addition, the contribution of water microbiomes to the colonization of biofilm lower than the results obtained in monocultures P and T this can be explained by the high biofilm stability (76.44%) attained since T2 in Poly compared to P and T as the biofilm becomes more robust to colonization by external microbes from water. Water in polyculture also stabilizes after the initial colonization but with a remained considerable contribution of pikeperch gut microbiota, despite their low microbial richness, and skin at T3 and Tf respectively. In poly, the contribution of pikeperch gut microbiota to water inoculation along with the low contribution of tench gut microbiota to biofilm especially in the initial colonization of water compared with T modality suggests that polyculture conditions and microbial interactions allow the persistence or the suppression of certain host-associated microbes compared to fish monocultures. Therefore, we can suggest that fish species-specific microbial communities and the interspecific interactions influence microbial colonization and stabilization, resulting in a unique microbial signature in polyculture. Moreover, it is important to note that in all rearing modalities P, T and Poly, an unidentified fraction "unknown" contributed importantly to the inoculation of the system especially at the beginning of the experiment, at T0 an T1. This inoculation was more pronounced in P biofilm that remained overtime. which correspond to unidentified non sampled microbial sources that can

potentially result from selective pressures altering taxa abundances such as accumulation of organic waste and the apparition of Physa (snails) observed mainly in pikeperch monocultures or inoculation from other external sources, highlighting the complexity of microbial colonization dynamics in RAS Although Venn curves showed that the gills shared the highest number of taxa with both water and biofilm in all rearing modalities all through the different sampling times (fig. 26 and 28), the source tracking data showed a low contribution of gills as microbial vectors influencing the composition water and biofilms in RAS all through the experiment (fig. 27 and 29) which aligns with previous studies showing that fish gills are more highly influenced by environmental microbiome (Clinton et al., 2024; Minich et al., 2020). Finally, the last objective was to assess the influence of rearing modality and species diversification on fish microbiotas compared to monocultures.Concerning microbial communities associated with intrinsic matrices of fish at Tf, the differences were observed at the level of beta diversity representation. PERMANOVA analyses (SI) combined with PCoA representations (fig. 30) showed that the microbiome structure of gills and skin mucous and gut of pikeperch and tench differ between species but also between rearing modalities. Our results showed significant differences between pikeperch and tench microbiotas in monoculture in comparison with their microbiome in polyculture. Particularly, PCoA representation of skin mucous showed that its microbial structure of pikeperch in monoculture clustered separately from pikeperch in polyculture while in contrast tench skin mucous microbiota in T and Poly modalities clustered closely. These results suggest that microbiota of tench mucous is more robust and less influenced by rearing modality than pikeperch's skin microbiota. In contrast, gills microbiota structure of pikeperch and tench in polyculture were clearly found clustered closer than gills of both species in monocultures P and T. While gills microbiota of pikeperch and tench were found more grouped by fish species at Ti (fig. 25B), this observation suggests that shared environmental conditions and extrinsic RAS microbiome drive the microbial communities structure of gill microbiota in both species more strongly than skin mucous and gut microbiotas which was similarly reported in research showing that fish gills are more influenced by environmental microbiota than skin and gastrointestinal compartments (Minich et al., 2020).

To characterize the fish microbiotas at the end of the experiment, DESeq2 analysis and composition representation revealed that gut microbiota appears to be still more grouped by species than rearing modality. However few families differed significantly between tench digesta sampled in monoculture vs polyculture. Tench gut microbiota in Poly were overrepresented by Rhodobacteraceae, Rhizobiaceae Rhizobiales Incertae Sedis and Staphylococcaceae found less abundant in digesta in T modality which exhibited higher abundance of Neisseriaceae. Staphylococcaceae taxa, such as *Staphylococcus edaphicus,* are often detected in fish guts and have been tested as probiotic candidates inhibiting fish pathogens (Istiqomah et al., 2018; Rahayu et al., 2024), meanwhile some species are a

significant concern in aquaculture (Kamala and Sivaperumal, 2023) like *Staphylococcus aureus* which can be commensal but also an important pathogen of fish and humans causing economic losses in farming and responsible for high human mortality rates (Akarsu et al., 2022; Matuszewska et al., 2023). Rhodobacteraceae has also been reported as a probiotic candidate, playing crucial functional roles in maintaining host health including stability of fish gut microbiome, production of antibacterial compounds, biosynthesis of vitamins and other metabolic processes (D'Alvise et al., 2014; Guo et al., 2020; Sha et al., 2025). On the basis of these results, we can suggest that polyculture improve tench health by increasing the abundance of commensal gut associated like Staphylococcaceae and Rhodobacteraceae species. Skin and gills microbiome composition of pikeperch and tench are poorly studied especially in RAS. DESeq2 results showed that gills mucous of pikeperch in P and tench in T exhibited important differences in microbial composition at the family level. Meanwhile, in polyculture only a few families were abundantly different between the gills' microbiome of both species. Moreover, the microbial composition of gills mucous differed between rearing modalities for each species. As pikeperch in monoculture exhibited and overrepresentation of Moraxellaceae but not Oxalobacteraceae and Caulobacteraceae in comparison with Poly, and as for tench, gills microbiome in T modality recorded lower abundance of Flavobacteriaceae, Caulobacteraceae and Oxalobacteraceae than tench in Poly. These findings strengthens the hypothesis that gills' microbiome is highly shaped by rearing modality and undergo continual restructuring by extrinsic matrices of RAS regardless of fish species. For instance, Oxalobacteraceae have been identified in the gut microbiomes of fish (Xia et al., 2014) while Caulobacteraceae were reported in aquatic habitats notably in biofilm forms (Bodenmiller et al., 2004; Entcheva-Dimitrov and Spormann, 2004; Fiebig et al., 2019; Heinrich et al., 2019) but also associated with fish gills (Ghotbi et al., 2022). In addition, comparing pikeperch in monoculture and polyculture revealed that in P modality skin mucous harbored significant higher abundance of Moraxellaceae but lower Xanthomonadaceae than skin microbiome of pikeperch in Poly. Similarly, tench skin microbiome in T and Poly exhibited significant differences as higher abundance of Vibrionaceae were reported in the skin microbiome of tench in Poly in comparison with skin of T exhibiting overrepresentation of Xanthomonadaceae. Vibrionaceae abundanes found in tench skin in Poly was also higher than its abundance in pikeperch skin microbiota. Vibrionaceae (especially *Vibrio* spp.) family is found dominant on skin-mucous surfaces of fish such as wild eel (*Anguilla Anguilla)* (Carda-Diéguez et al., 2017) and as a core taxon in skin microbiota of wild Arctic char (*Salvelinus alpinus*) (Amill et al., 2024). However,many serious opportunistic pathogenic species from this family have been reported, causing health problems for both fish and humans. For instance, *Vibrio* was identified as a biomarker for infected fish (Cámara-Ruiz et al., 2021; Carda-Diéguez et al., 2017).

These findings suggest that fish species and species diversification (rearing modality) exhibit unique

and specific microbial influence on fish microbiotas and on the microbial dynamics between fish and water and biofilm microbiome in RAS. This understanding paves the way for better understanding of the potential effect of polyculture systems on crucial microbial compartments in RAS, and the further possibility to manage species diversification and microbial interactions to improve RAS design and biosecurity.

5) Conclusion

To conclude, our result showed that fish gut microbiota are more tissue-specific while the microbiotas of skin and gills mucous are more species-specific. Among fish tissues, the initial colonization of water and biofilm in P seem to be originated mainly from skin mucus, whereas in T, gut played the predominant role during the initial inoculation phase. But more importantly, in polyculture, pikeperch microbiome (skin and gills) was the major contributor to the inoculation of extrinsic matrices of RAS especially biofilm, while the contribution of tench remained secondary only through gut. While gills microbiomes found as the most connected compartment to water and biofilm in all rearing modalities exhibited a minimal microbial vectorization/inoculation potential and seemed to be more environmentally shaped than mucus or gut microbiotas.

This study highlights that fish species with distinct microbial signatures across skin, gill, and fecal matrices strongly influence microbial dynamics in RAS. In polyculture, pikeperch played a dominant role in biofilm establishment, and tench showed limited impact. Overall, these findings provide the first information about the effect of fish diversification on fish intrinsic matrices and on the colonization and vectorization of RAS extrinsic matrices in polyculture. Our findings suggest that species diversification can affect fish/microbiome interactions, guiding future management of microbial processes in polyculture in RAS for improved system stability and health.

CRediT authorship contribution statement

F. Changey and P. Fontaine conceived and planned the experiments. S. Al Dakdouki, F. Changey and P. Fontaine carried out the experiments and contributed to sample preparation. S. Al Dakdouki carried out DNA extractions. A. Laprie and F. Changey planned and carried out data analysis. F. Changey, L. P. Fontaine was in charge of overall direction and planning. F. Changey and S. Al Dakdouki took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Declaration of Competing Interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript. No approval from research ethics committees was required to accomplish this study as it did not involve any animal experimentation. The datasets generated during the current study are available from the corresponding author upon reasonable request.

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V- General Discussion

1) Optimizing microbial communities' management in RAS

The success of RAS system is directly linked to the structure and composition of microbial communities impacting water quality, system resilience and fish health (Attramadal et al., 2014; Blancheton et al., 2013; Nayak, 2010; Vadstein et al., 2018). Microbial ecology has been suggested as a strategy to manage microbiome in RAS, guiding the microbial communities toward a beneficial state (Bentzon-Tilia et al., 2016; Blancheton et al., 2013; Vadstein et al., 2018). However, considering the results obtained in the three chapters, more studies should investigate the microbial compartment in RAS to sharpen our knowledge about the factors shaping its microbial structure.

1.1. "Memory Effect" of biofilms : The need for microbial standardization as a prerequisite for for comparative studies

The first chapter of this thesis functioned as a methodological pre-experiment to standardize the initial microbial conditions across all ecotrons, thereby enabling a reliable assessment of fish species effects on microbial community dynamics in RAS in next chapters. We demonstrated that biofilms act as a persistent microbial "memory," capable of reseeding the water column after cleaning, consequently maintaining a strong historical imprint that generates tank-specific microbial signatures. This intrinsic resilience poses a major confounding factor for comparative microbial ecology in RAS if tanks are not homogenized prior to experimentation. In fact, in engineered aquatic systems such as RAS, residual biofilms act as persistent and structured microbial recorders of microbial cells, rearing conditions and physico-chemical factors that encoded past selective pressures **(**Bartelme, 2018; Schreier et al., 2010). Microbes in layers of biofilms can communicate via cell-to-cell signaling called quorum sensing, activating their detachment and providing a reservoir that can repopulate water after refilling (Donlan, 2002; Talagrand-Reboul et al., 2017). The β-diversity analysis, in our study, highlighted considerable heterogeneity in initial microbial communities' structure between all tanks after the first refill but also after routine raw cleaning using SP treatment. We provided substantial evidence confirming that persistent microbes and cumulative recording capacity of biofilm of different past microbial signature "tank's history : previous experiments" significantly influence water microbiome after start-up of the systems. These persistent microbes generated from tanks' history can reenter RAS water after refill and can therefore colonize new surfaces. This may modify future microbial compartments in many ways including resource consumption, production of metabolites and microbial interactions and

dynamics such as mutualism, competition influencing the establishment of future colonizers of RAS. When microbial communities differ between tanks, variations in system performance (e.g. nutrient recycling, opportunistic heterotrophs growth, pathogen occurrence) can occur independently of the experimental treatments and objectives. By homogenizing the microbial state, inter-tank variability is minimized, ensuring that all tanks operate under comparable microbial conditions as similar as possible allowing thereby researchers to attribute observed differences in their studies more confidently to experimental factors rather than tank's microbial history. This highlights the need to manage, the biofilms "memory" and "tanks' history" effect representing a critical consideration for RAS management when designing research on microbial communities in RAS. We proved that routine simple physical cleaning, SP treatment, was insufficient to erase this microbial "memory", as residual biofilms rapidly recolonized the system. This can be explained by the strong structural resilience and ecological persistence of biofilms, which allow microorganisms embedded within the EPS matrix to withstand disturbance and persist especially in niches that are inaccessible to cleaning. Moreover, within biofilms, microbes exhibit functional adaptations such as reduced metabolic activity, increased stress tolerance and quorum-sensing, that enhance survival during cleaning stress and promote rapid regrowth. Consequently, the remaining biofilm acts as a microbial reservoir, rapidly dispersing cells into the water column through sloughing and detachment from biofilms once favorable conditions return, accelerating the reinstate of the tank's previous microbial signature. This tank's history effect underscores the difficulty of resetting microbial assemblages in RAS through physical cleaning alone and highlights the need for controlled homogenization strategies to achieve homogenous reproducible starting microbial conditions across systems. We tried combined chemical and thermal treatment whith complete replacement of the biofilter media T2, though effective at decreasing the microbial variability between ecotrons, this treatment reduced microbial richness and decreased significantly several bacterial orders such as *Planctomycetes*, frequently observed in biofilters in RAS while some species can be functional and beneficial for nitrogen cycling, notably through anammox process (Ma et al., 2021; Van Kessel et al., 2011). Such a loss of diversity and functional redundancy could be compromising for nitrification efficiency and overall biofilter stability, suggestively resulting in slower biofilter maturation, delayed establishment of beneficial microbial groups, OM accumulation and increased susceptibility to functional collapse or opportunistic heterotroph blooms during system start-up (Summerfelt and Penne, 2005; Losordo et al., 2009; Michaud et al., 2006; Michaud et al., 2014; Attramadal et al., 2012; Vadstein et al., 2018; Attramadal et al. 2016). This aligns with the findings of Bischoff et al., suggesting that used biofiltration media already colonized can lead to the immediate start nitrification in a culture system stocking low biomass of fish (Bischoff et al., 2015).

This highlights the need for a better protocol that reduce historical microbial effect of tanks while

preserving the core functional microbiome. This is why, our standardized mixed-media disinfection protocol T1, which combined chemical and thermal treatments while seeding the system with a mix of the existing biofilms in all the ecotrons, achieved the dual objective of significantly homogenizing the microbial state among tanks and preserving microbial diversity. This treatment significantly reduced inter-tank heterogeneity, maintained microbial richness and diversity, and provided a controlled yet ecologically realistic microbial baseline. The mixing of biofilter media from all ecotrons redistributed a balanced pool of microbial taxa, ensuring that each tank received the same microbial inoculum. This suggest that this treatment allow the elimination of a proportion of historical tank-specific microorganisms while simultaneously reintroducing representative and functionally beneficial microbial communities shared across all systems. As a result, recolonization after refill followed a comparable trajectory across tanks, driven by a shared and diverse microbial reservoir, suitable for controlled experimental studies on fish-microbiome interactions. This standardized condition was then applied prior to the next experiment assessing sessile and planktonic microbial communities in different rearing modalities (monoculture and polyculture) in RAS, allowing us to attribute observed differences to fish species and species diversification rather than uncontrolled tank history.

1.2. Fish microbiota as a driver of RAS microbial communities: Experimental design for reducing biological and environmental variability

We hypothesize that fish microbiota is a major factor innoculating or /and shaping microbial communities of water and biofilms in RAS. Some studies have reported correlations between fish-associated microbiota and the surrounding water in aquaculture systems, highlighting strong interactions between host and environment (Dulski et al., 2020; Minich et al., 2020; Deng et al., 2023; Kim et al., 2021, Giatsis et al., 2015; Zeng et al., 2020). Little is known about how fish microbiota interact and exchange microbial communities with extrinsic matrices (water and biofilms) of RAS, particularly in polyculture. We hypothezied that the constant interaction between fish intrinsic matrices microbiotas of gut, gills and skin mucous and their surrounding environment promotes the dissemination of host-associated microorganisms into the surrounding water, influencing the structure and dynamics of the microbial communities of water and biofilms of RAS. However, the composition of fish microbiota itself is not static and can fluctuate in response to numerous factors (Egerton et al., 2018; Smith et al., 2015; Minich et al., 2022; Moschos et al., 2022; Kanika et al., 2025). The microbial composition and diversity within RAS water and biofilters are known to be highly influenced by multiple environmental and operational factors, including feed, organic matter accumulation and water physicochemical parameters (e.g., pH, temperature, dissolved oxygen, nitrogenous compounds)

(Godzieba et al., 2025, Minich et al., 2020; Singh et al., 2025; Minich et al., 2021). Because fish-associated microbiota are themselves responsive to environmental conditions, we fixed a uniform feed ratio of 1.5% all through the experiment and carefully monitored water quality parameters to ensure homogenous rearing conditions across ecotrons. In this study, temperature, dissolved oxygen, pH, and nitrogenous compounds (ammonium and nitrite) were measured. The results confirmed that water quality parameters were within optimal ranges for reared pikeperch and tench and remained homogenous between ecotrons and throughout the experiment, as no significant differences were observed among the three rearing modalities. Although a slightly higher mean pH value was recorded in pikeperch monoculture (7.51) compared to the other treatments (7.45–7.47), this minimal variation was not considerable in terms of its potential impact on fish physiology and microbial communities. This slight difference may be related to species-specific differences in metabolic activity and feed utilization and accumulation, or microbial nitrification dynamics which could have influenced CO_2 and ammonia turnover along with bicarbonate addition used to control alkalinity and pH within the initial startup of the system (Montjouridès et al., 2025). The composition of fish microbiota is also influenced by intrinsic biological traits such as species phenotype, feeding behavior, gastrointestinal tract morphology, and stress physiology (Kanika et al., 2025; Minich et al., 2022; Moschos et al., 2022; Smith et al., 2015; Soh et al., 2024; Uren Webster et al., 2020) which are more difficult to control. Because of this, we ensured a uniform initial stocking biomass along with similar and homogenous biological traits of pikeperch and tench found at the beginning of the experiment for each species among the different rearing modalities. An initial mortality event mainly occurred in pikeperch during the first month of the experiment as many underweight pikeperch were initially used to ensure that fish had homogenous weight in all ecotrons. As a consequence, this mortality can be attributed to acute stress during transport and transfer of fish from stocking tanks to the 12 experimental ecotrons or during the acclimation to these ecotrons (Péter et al., 2023; Refaey and Li, 2018) or can be also attributed to early handling at T0 (Pourhosein-Sarameh and Falahatkar, 2024) leading to reduced feed intake or/and immunosuppression and elevated mortality in sensitive pikeperch. These dead and underweight fish were replaced at T1 ensuring uniform starting conditions across all rearing modalities. After that, the mortality rates at Tf (4-10%) were acceptable and homogenous for both species in all three modalities. We also examined the final zootechnical and physiological variables of pikeperch and tench in monoculture and polyculture by characterizing growth and stress-related parameters to further strengthen the assessment of host microbiota–RAS microbiome relationship. First, it should be taken into consideration that the number of measured fish per species in polyculture were slightly lower than in monoculture (30 fish). Pikeperch as been chosen as species of interest, due to its high commercial value (Jakubavičiūtė et al., 2022; Kestemont et al., 2015), exhibited good

growth performance, achieving commercial sizes (~530–541 g) (Nagy et al., 2022; Steinberg et al., 2019) but SGR (1.16–1.29%·day^{-1}) and FCF (1.03–1.09) were not optimal values but still within an acceptable range reported in the literature for pikeperch of similar weight range (Nikolić et al., 2023; M'Hetli et al., 2011; Péter et al., 2023; Rapp et al., 2019). This can be linked with stress effects as studies reported that it can depress growth and feed activity in teleosts (Canosa and Bertucci, 2023) and has shown that high cortisol reduce food intake and mass gain which can translate into lower SGR and FCF. This aligns with physiological results of pikeperch, similar in monoculture and polyculture, showing elevated plasma glucose (104–122 mg/dL) and cortisol (145–162 ng/mL) levels compared to values reported for unstressed individuals, suggesting activation of cortisol-mediated gluconeogenesis and an acute stress response possibly linked to low stress tolerance of pikeperch to stress (Ende et al., 2021; Falahatkar and Poursaeid, 2014; Martín Gallardo et al., 2010; Suljevic et al., 2015). However, all pikeperch biological and physiological variables were similar between monoculture and polyculture except for weight uniformity (CVw) that was significantly improved in polyculture compared to monoculture, suggesting that the presence of tench may have mitigated intraspecific competition among pikeperch and reduced hierarchization common in pikeperch monoculture, leading to more homogeneous growth (Rapp et al., 2019; Thomas, 2021). In contrast, tench displayed enhanced growth performance in polyculture, reaching significantly greater final weight and total length, while maintaining similar final WG, CVw, CVtl, FCF and SGR to monoculture, consistent with literature values for tench of similar weight range (Bezmaternykh and Shcherbina, 2018). While physiological variables of tench, including hematocrit, glucose, and cortisol, were within expected physiological range of unstressed tench and Cyprinidea (Demael et al., 1980; de Pedro et al., 2005; Pottinger et al., 2000; Rożyński et al., 2019) and were similar in monoculture and polyculture. Final biomass, FCR and biomass gain were similar between the three rearing modalities. Overall, the observed homogeneity in most biological traits between modalities minimizes potential confounding effects of fish traits, biomass and stress variability on microbiota composition, allowing a clearer interpretation of host–microbiota–environment relationships.

1.3. Baseline characterization of specific microbial signatures in pikeperch and tench

Under these uniform starting conditions, we aimed to better understand how the microbiomes of intrinsic matrices (gut, gills, and skin mucus) of pikeperch (*Sander lucioperca*) and tench (*Tinca tinca*) reared in monoculture and polyculture influence the microbial dynamics shaping the microbial communities of extrinsic matrices (water and biofilm) in RAS. At the beginning of the experiment, pikeperch and tench microbiota were characterized. Microbial composition of gut microbiota of

pikeperch and tench was unsufficiently reported (Dulski and al., 2018; Wang et al., 2024; Dulski et al., 2020) and the microbial composition of gills and skin microbiota of these species has not been reported. This baseline is essential to disentangle initial state of host-microbiotas driven by origin habitat and environmental factors to better understand the mechanisms underlying microbial dissemination in experimental ecotrons.Recent advances in NGS technology have made it simpler to examine more the microbial compartment of RAS (Boughner and Singh, 2016; Drønen et al., 2021).

During the acclimation period (Ti), we demonstrated that despite the different gastrointestinal morphology of pikeperch and tench, their gut microbiota clustered primarily by matrix (gut) rather than host species, whereas gill and skin microbiotas were more strongly species-specific. This can be likely because gut physicochemical conditions (gut vs. external mucosa) is the dominant driver of microbial community structure, while mucosal surfaces (gill and skin) are more susceptible to environmental factors especially gills as these external mucosal tissues are in direct contact with surrounding water (Guivier et al., 2020; Minich et al., 2020; 2021; Zhang et al., 2019).

Meanwhile, the alpha diversity analysis showed that the bacterial richness of pikeperch gut microbiota was significantly lower than that of tench. This difference may be attributed to feeding behavior or interspecific variations in gastrointestinal tract morphology which may influence microbial diversity through selective nutrient availability and intestinal conditions (Egerton et al., 2018), as tench is a stomachless freshwater teleost (Kaminski et al., 2025; Noaillac-Depeyre and Gas, 1976), whereas pikeperch possesses a well-developed stomach adapted for intermittent feeding digestion (Yanes-Roca et al., 2018). Whereas pikeperch gills harbored significantly higher microbial richness and diversity (approximated by Shannon index), compared to tench gills, possibly linked to differences in gill morphology (Alsafy et al., 2025; Zayed and Mohamed, 2004) or mucous composition (Fekih-Zaghbib et al., 2023). Skin mucous richness showed a trend toward higher values in pikeperch, although this difference did not reach statistical significance ($p = 0.051$). At the taxonomic level, differential abundance analyses revealed distinct microbial signatures for each species and tissue. Pikeperch gut microbiota was found dominated by Bacilliaceae and Peptostreptococcaceae, commensal bacterial families commonly found in higher abundance in healthy fish associated with probiotic functions, gut homeostasis, and antimicrobial activity (Elsadek et al., 2023; Huang et al., 2020; Santos et al., 2018; Tarnecki et al., 2019; Midhun et al., 2017), while tench gut microbiota was enriched in Rhodobacteraceae, consistent with previous reports of Proteobacteria dominance in this species (Dulski et al., 2020). In gills, pikeperch exhibited higher abundances of Flavobacteriaceae, Oxalobacteriaceae, Pseudonocardiaceae, and Saprospiraceae, whereas tench gills were dominated by in Corynebacteriaceae, Xanthomonadaceae and Weeksellaceae, reflecting host-specific microbial colonization patterns. Skin mucous microbiota of pikeperch skin was enriched in Pseudonocardiaceae,

Rubrobacteriaceae, and Salinisphaeraceae, but significantly less abundant in tench skin. In the composition representation (fig. 25A), tench skin and gills microbiota seemed to host lower diversity than pikeperch with a dominance of Corynebacteriaceae and Xanthomonadaceae. This could be due to species-specific differences that influence microbial colonization and persistence on these external mucosal surfaces. This may be reflected by host specific factors such as mucous composition, epithelial structure, and immune activity that differ markedly between cyprinids and percids (Ebran et al., 2000; Gomez et al., 2013; Khan et al., 2025; Reverter et al., 2018). Tench produces mucous rich in glycoprotein and antimicrobial compounds (Díaz-Puertas et al., 2023; Subramanian et al., 2008), which may impose stronger selective pressure on microbial colonization, leading to lower diversity and dominance of a few adapted taxa (Cerezo et al., 2024). In contrast, pikeperch external mucous seemed to be less stringent providing a more permissive surface, supporting greater bacterial richness and diversity. Overall, these findings reinforce our knowledge on the gut and external mucosal microbiotas of pikeperch and tench reared in RAS. We demonstrated that pikeperch and tench host distinct microbial communities across gut, gills, and skin mucous, reflecting both species identity and tissue-specific selective pressures. In addition, we emphasize the need to account for host identity in RAS microbiome studies as the distinct microbial profiles of gills and skin may suggestively serve as critical barriers and modulators of pathogen exposure, with potential consequences for fish health and disease resilience. These insights provide a foundation for future studies on host–microbiota–environment interactions in RAS and for designing management strategies to optimize microbiome-mediated benefits.

1.4. Fish species as biological shapers of extrinsic microbial communities in RAS

1.4.1. Initial colonization of water and tank walls biofilms

Correlations between fish associated microbiota and surrounding environment was reported in RAS systems but only in monoculture (Minich et al., 2020; 2021).

At the initial colonization phase (T0), we observed distinct microbial inoculation patterns between pikeperch and tench monocultures, reflecting species-specific pathways of microbial transfer to the RAS environment. In the pikeperch system, both Venn diagram and source tracking analyses indicated that early establishment of microbial communities in water and biofilm seems to be predominantly driven by skin mucous and acclimation water. In contrast, the tench monoculture exhibited a markedly different pattern, where gut microbial communities contributed most strongly to the initial microbial inoculation of both water and biofilm. This suggests that the early microbial colonization of the RAS

environment in monoculture is shaped by a dominant microbial vectorization pathway specific to each fish species, reflecting differences in host physiology, microbiota composition, and microbial release mechanisms. In pikeperch monoculture, the predominance of skin-derived inoculation implies that mucosal surfaces act as major dispersal interfaces, likely due to constant contact with surrounding water and sloughing of mucous layers that harbor abundant microbial assemblages (Benhamed et al., 2014; Wainwright et al., 2024). Conversely, in tench, the stronger contribution from gut microbial communities highlights the importance of gut-associated microbiota as a key seeding source, potentially linked to higher fecal output or more active microbial shedding from the intestinal tract. These species-specific inoculation routes establish distinct initial microbial fingerprints in the RAS compartments, which may influence subsequent communities' succession, biofilm maturation, and overall microbial stability within the culture system. We hypothezied that gut-associated microbiota will be the primary inoculum source of water and biofilm, but this was not valid in pikeperch monoculture. This allow us to suggest that pikeperch is likely to release fewer viable gut microbes into the system which can be linked to digestive transit, lower fecal output, and differences in gut morphology compared with tench. As tench is stomachless, it may need to be supported by diverse functional bacteria helping in digestion while carnivorous fishes like pikeperch have a stomach and can easily digest and assimilate without the need for diverse functional bacterial assembles (Egerton et al., 2018; Huang et al., 2020; Kaminski et al., 2025; Noaillac-Depeyre and Gas, 1976; Yanes-Roca et al., 2018). As a result, the skin mucous microbiota of pikeperch constantly interacting with the surrounding water becomes the dominant pathway for microbial colonization of RAS compartments in pikeperch monoculture. Collectively, these findings demonstrate that the early microbial colonization of RAS compartments in monocultures is strongly influenced by host species and their different matrices, with pikeperch favoring skin-derived inoculation and tench favoring gut-derived contributions ultimately setting distinct trajectories for subsequent microbial community assembly and stabilization.

In this study, we focus on microbial communities under polyculture conditions in RAS because this practice offers several potential advantages compared to monoculture, including more efficient use of space and feed resources, enhanced nutrient recycling, and improved water quality (Dawood et al., 2025; Thomas et al., 2022). Previous studies on fish polyculture in RAS have primarily focused on zootechnical performance, nutrient partitioning, and welfare (Thomas et al., 2020; Pěnka et al., 2024a). However, these investigations largely overlooked the microbial dimension of RAS, leaving the structure and function of microbial communities under species diversification conditions poorly understood. Beyond RAS, several studies have explored microbial communities' dynamics in polyculture systems, showing that species interactions can profoundly influence microbiome

composition and functionality (Gao et al., 2020; Bartelme et al., 2018; Zhao et al., 2018; Liu et al., 2023). For example, Lalramchhani et al. demonstrated that the prevalence of pathogenic *Vibrio* species was lower in shrimp–milkfish polycultures than in shrimp monocultures in brackish water ponds (Lalramchhani et al., 2019), highlighting the potential of species diversification to promote healthier microbial assemblages. However, the microbial inoculation and influence of fish-associated microbiota on the RAS extrinsic microbiome microbiome remain unexplored in polyculture settings.

To date, no study has characterized the contribution of fish microbiota as vectorization pathways to the structuring of sessile and planktonic microbial communities in polyculture within RAS. Addressing this gap will provide new insights into how multi-species rearing can impact microbial ecology in controlled aquaculture environments. To support the broader adoption of polyculture practices in RAS, our study aimed to investigate how the microbiota of pikeperch and tench of the gills, skin, and gut contribute to shaping the microbial communities of water and biofilms. The present work thus provides the first detailed characterization of microbial community structure and temporal dynamics under species diversification in RAS, offering new insights into how host-specific microbiota interactions can influence microbial ecology and stability in RAS.

At the initial colonization phase (T0) in polyculture of pikeperch and tench, the biofilm and water compartments were predominantly inoculated by pikeperch-associated sources. Source tracking revealed that the acclimation water of pikeperch contributed the largest proportion of microbial communities to both biofilm (31.6%) and water (39.2%), followed by a moderate contribution of pikeperch skin mucous to biofilm (11.3%) and water (8.7%) and tench gut microbiota playing only a minor role in seeding the water (9.96%), indicating that its early contribution to system colonization is less important than pikeperch. This aligns with microbial composition results, as at T0 water communities in polyculture exhibited a relatively high abundance of Rhodobacteraceae also found in tench gut microbiota at Ti, suggesting that Rhodobacteraceae possibly originated from tench gut microbiota were released into the water via feces and contributed to the early colonization of water in polyculture.. Interestingly, a considerable proportion of unidentified (“unknown”) microbial sources participated in the initial inoculation of water and biofilms in all modalities notably in biofilms of tench monoculture which can be explained by the contribution of non-sampled environmental reservoirs or selective pressures such as organic waste accumulation not directly associated with the analyzed fish matrices. This fraction can also include microbial communities originated from the distributed mix of biofiltration media subsequent to the application of standardizing treatment T1, or the presence of other organisms (e.g., Physa snails), emphasizing the complexity of microbial ecology in RAS.

Overall, these results demonstrate that, in polyculture, pikeperch-associated microbiota dominate the

initial microbial inoculation of RAS compartments. This is possibly due to the higher microbial richness and dispersal potential of pikeperch skin and the immediate influence of acclimation water input introduced in the systems during fish transfer. The minimal contribution from tench gut microbiota at T0 in polyculture with pikeperch, compared with the higher contribution observed in tench monoculture, may reflect rearing modality effect on microbial inoculation efficiency, potentially influenced by the competitive dominance of pikeperch-associated microbiota during the early colonization phase. It may suggest the presence of priority effects, where microbes from pikeperch (through skin mucus and acclimation water) establish first and occupy ecological niches in the biofilm and water, thereby limiting the initial colonization of tench gut-derived microbes. Consequently, the difference in early microbial colonization of water and biofilm between polyculture and monocultures may set distinct trajectories and lead to unique microbial assemblages in polyculture compared with either monoculture. These findings highlight the importance of host species phenotype in shaping the early microbiome of shared RAS environments.

1.4.2. Microbial temporal dynamics and stabilization in monoculture vs. polyculture

Beyond the initial colonization phase, microbial communities' dynamics in both biofilm and water compartments exhibited species-specific patterns in monocultures. In pikeperch monoculture, biofilm communities stabilized relatively early but maintained only moderate temporal similarity (~ 40%) between successive sampling times with a considerable contribution of inoculation from water, pikeperch gills (up to ~16% at Tf) and high constant contribution of unknown sources whereas biofilm in tench monoculture showed higher stabilization (~84% similarity at Tf) with only considerable contribution of water. This suggests that biofilm in pikeperch monoculture is permissive to microbial inputs, reflecting a more dynamic and variable microbial assembly and that tench-associated microbiota or microbial interspecies interactions favored the establishment of a more robust and self-sustaining biofilm maintained by water inoculation and less reliant on additional host or unknown sources. In addition, the consistently inoculum of water for biofilm formation (up to T3), whereas the minimal contribution of biofilms to the colonization of water compartment, emphasize a unidirectional microbial transfer likely driven by water circulation and the biofilm formation as the initial stage of biofilm formation begins with the adhesion of planktonic (free-floating) bacteria to surfaces, a process influenced by factors such as bacterial appendages (e.g., flagella and pili), which then proliferate and produce EPS matrix, leading to the development of a mature biofilm with cells potentially detaching and returning to the planktonic state, facilitating the colonization of new surfaces (Zhao et al., 2023; Rather et al., 2021; Berlanga and Guerrero, 2016) such as other zones of tanks walls and fish surfaces.

Meanwhile in polyculture, microbial communities' dynamics in both biofilm and water compartments displayed distinctive patterns compared with monocultures. Following the initial inoculation (T_0), biofilm communities were primarily influenced by the water and pikeperch gill microbiota with notably poor contribution of tench, suggestion that in polyculture of pikeperch and tench, pikeperch-associated microbiota dominate early biofilm colonization in polyculture, while tench-associated microbes contribute minimally, likely due to differences in microbial richness and diversity observed in (fig. 24) or competitive interactions. Interestingly, biofilms in polyculture achieved strong temporal stability (~86.9% retained at Tf), which occurred later (from T2 onward) than in either monoculture (at T1), suggesting that interactions between multiple species microbial communities extend the colonization phase and delay stabilization compared to single-species systems. As for water, the source tracking data reveal distinct differences in water microbial dynamics between polyculture and monoculture P and T. As in polyculture, water microbial communities were drived by contributions from the biofilm and contributions from pikeperch gut (up to 15% at T1–T2) and skin microbiota (14.81%), whereas tench gut microbiota contributed minimally compared to monoculture T. Contributions from tench-associated microbiota, remained minimal throughout the experiment, suggesting that pikeperch play a dominant role in shaping sessile and plankyonic communities in polyculture, while tench have a minimal influence compared to their contribution when reared in monoculture which can be related to competitive interactions that limit the contribution of tench microbiotas. We expected a considerable contribution of tench skin mucous to the system inoculation, because tench exhibit thick skin mucous, which was not observed in either polyculture or monoculture T. This suggest that skin mucous of tench is more selective and robust, potentially due to glycoproteins and other bactericidal compounds limiting microbial colonization (Díaz-Puertas et al., 2023).

These findings highlight how host traits, microbial richness, and interspecific interactions shape water and biofilms microbial dynamics in multi-species RAS. The low contribution of tench suggests that microbial interactions and priority effects in polyculture favor the establishment and persistence of pikeperch-associated communities, potentially suppressing less competitive tench-associated microbes. This suggest that the differences in inoculation pathways, the dominance of pikeperch-associated communities and the delayed stabilization of biofilms in polyculture can lead to a unique microbial signature in polyculture distinct than monoculture the RAS which aligns with the finding in chapter 2. We observed clear and consistent structuring of both planktonic and biofilm communities by fish species, with significant beta-diversity separation and distinct taxonomic assemblages in each modality. Our beta diversity analyses based on Bray–Curtis at Tf, 120 days after the beginning of the experiment, revealed that fish species composition strongly structures microbial communities in both water and biofilm. Significant differences were observed between the three rearing modalities even

two days after fish introduction (T0), indicating a rapid impact of fish presence on microbial communities. At the final time point (Tf), water communities remained clearly separated by modality, with PCoA projections explaining 41.1% of the variability and pairwise comparisons confirming significant differences among all treatments. In contrast, biofilm communities displayed a different pattern: while tench monoculture and polyculture clustered partially together, the structure of microbial communities in pikeperch monoculture clustered farther and more dispersed, suggesting greater inter-tank heterogeneity under pikeperch monoculture. Pairwise analyses further highlighted strong differences involving P (P vs T and P vs Polyculture), whereas T vs Polyculture showed moderate but significant separation. This aligns with the results of source tracking showing higher biofilm stabilization in polyculture similarly to tench monoculture while in contrast biofilm in pikeperch monoculture exhibited lower stabilization at Tf (41.69%) with a high considerable proportion of unknown inoculation (fig. 27). This can be partially explained by the accumulation of the Physa (snails) in pikeperch monoculture while tench in monoculture and polyculture consumed these unexpected organisms as the Venn diagram visualizing the ASV shared between the biofilm samples and snail samples in pikeperch monoculture at Tf showed that the biofilms and the snails share a percentage of 44% of ASVs indicating a direct influence of their specific microbiota. However, 52% of the ASVs were specific to biofilms and not related to the presence of snails suggesting a possible indirect effect via biofilm grazing (Davidson et al., 2019), which could lead to dominance of certain taxa and risk of colonization by opportunistic species, supported by alpha diversity results that showed a decrease in the microbial diversity in pikeperch monoculture P. The 52% fraction specific to biofilms could also explained by intrinsic fish–microbiota interactions and other environmental factors shaping biofilms in pikeperch monoculture. Together, these results indicate that fish species and rearing modality exert differential influences on RAS extrinsic microbiome.

1.4.3. Synergistic effects of species microbiota in polyculture: Reduced pathogens, enhanced beneficial microbiota

To further support these findings, microbial composition and hierarchically clustered (Ward.D2 method) heatmap at Tf showed that water and biofilm in the three rearing modalities were dominated by different bacterial families. Pikeperch monoculture exhibited a decrease in alpha diversity over time favored the overrepresentation of Chromobacteriaceae Clostridiaceae and Chromobacteriaceae in the water column and Rhizobiaceae, Fusobacteriaceae, and Listeriaceae in biofilms, whereas tench monoculture favored the proliferation of Mycobacteriaceae and exhibited increased alpha diversity

over time. Pikeperch-tench polyculture shared some bacterial families with P such as Hyphomicrobiaceae in biofilms and T such as Rhizobiaceae in water and Pseudonocardiaceae in biofilm. However, the overall microbial signature in polyculture seemed different than P and T modalities, as polyculture exhibited lower abundance of Haliangiaceae, Saprospiraceae, Aquaspirillaceae and Comamonadaceae in water compared to P and T. Pikeperch-tench polyculture was also dominated by specific microbial families including Spirosomaceae and Rhodobacteraceae commonly found in tench gut, Fusobacteriaceae found in pikeperch gut, Rubrobacteriaceae in skin mucous of pikeperch, which aligns with source tracking results. Interestingly, polyculture generated a unique microbial signature which is not simply additive microbial communities that combined microbial species of both monocultures but shifted towards high abundancy of groups, notably Rhodobacteraceae and Spirosomaceae (Yanes-Roca et al., 2024; Attramadal et al., 2014), while reducing the relative abundance of Clostridiaceae and Mycobacteriaceae, highly found in P and T respectively, two families which could host opportunistic or pathogenic behavior (Cortés-Sánchez, 2021; Davidovich et al., 2020). Similarly, biofilm communities in polyculture exhibited a higher relative abundance of Rhodobacteraceae and Nitrospiraceae than the other modalities, both include beneficial taxa for water quality in RAS, while showing a reduction in Listeriaceae family found more abundant in the pikeperch monoculture. This bacterial family includes pathogenic species such as *Listeria monocytogenes* ,a causative agent of listeriosis in fish, posing risks to fish and human health (Sasikumar et al., 2024). These findings in biofilm and water highlight possible potential benefits of polyculture in enhancing RAS resilience and improving fish health by lowering microbial families with potential risk of presence of pathogen and thereby disease occurrence.

Overall, our findings highlight the potential vectorization/inoculation role of fish species in modulating microbial ecology within RAS, with potential implications for system stability, biofiltration efficiency, and fish health. The distinct microbial profile observed in polyculture indicates that interactions between pikeperch- and tench-associated microbial populations give rise to an emergent community structure that differs from the mere sum of their monoculture microbiotas. This suggests that species diversification in RAS generates a distinct composite microbial signature emerging from interspecies interactions between host associated microbial populations especially in the water. Overall, these findings emphasize the dominant role of fish species and rearing modality in shaping both planktonic and sessile microbial communities and the complex interactions that arise in these systems (e.g. mutualism, competition) which aligns with the findings of Changey et al., who stated that the most important driver of bacterial communities and ARG distribution pattern in aquaponics is fish species (Changey et al., 2025). This highlights the importance of the choice of fish species in polyculture in terms of the impact of species combination on microbial ecology in RAS.

1.5. Resulting final structure and composition of fish microbiota

Finally, the aim that follows is to assess how the structure and composition of fish species microbiota are influenced in RAS by extrinsic communities in different rearing modalities. To date, no study has characterized the fluctuation of fish gills, gut and skin microbiota in polyculture within indoor RAS. This represents a critical gap, as the interconnection between different species in the same tank and with their RAS environment is still unexplored. In the end of the experiment Tf, the structure and composition of fish-associated microbiota of feces, gills and skin were markedly different between fish species and rearing modality. Gut microbiota showed a closer clusterisation by fish species For gut microbiota, the PcoA representation showed that microbial profiles clustered by species rather than modality, reflecting species-specific microbial assemblages suggesting that host identity remains the principal driver of gut microbial community structure less affected by extrinsic microbial matrices. Nevertheless, tench in polyculture exhibited increased relative abundances of Staphylococcaceae, Rhodobacteraceae, Neisseriaceae, Rhizobiaceae, and Streptococcaceae compared to both pikeperch and tench in monoculture. Suggesting that interspecific interactions in polyculture promote the enrichment of specific bacterial taxa within tench gut microbiota, possibly through indirect microbial exchange, shared environmental exposure, or altered nutrient dynamics within the RAS system. Similarly, PCoA and DESeq2 analyses revealed distinct clusterisation of skin mucous communities between species and rearing modalities, but with closer clusterisation and microbial structure of tench skin microbiota in polyculture and monoculture. This indicates that the external skin microbiota of pikeperch is more responsive to polyculture conditions than that of tench, suggesting that tench skin microbiota is more selective and robust, possibly due to host factors (mucus secretion rate, skin sloughing) or because tench possess a more restrictive skin mucosal composition due to glycoprotein and antimicrobial compounds (Díaz-Puertas et al., 2023; Subramanian et al., 2008) and stronger innate antimicrobial barriers exhibiting thereby species-specific filtering of extrinsic colonizers (Diwan et al., 2023; Ebran et al., 2000; Gomez et al., 2013; Khan et al., 2025; Rakers et al., 2013; Reverter et al., 2018; Thomsson et al., 2022). Tench in polyculture showed higher abundances of Vibrionaceae and Moraxellaceae and lower Xanthomonadaceae compared with pikeperch, while both species exhibited notable shifts between monoculture and polyculture, reflecting differences in host physiology (e.g. mucus composition), and interspecific microbial transfer through contact or shared water. In contrast, gill microbiota of both species displayed a closer clusterisation by modality than species, as tench and pikeperch from polyculture clustered closely together and distinct from their respective monocultures.

This pattern suggests potential microbial exchange or environmental homogenization in shared water systems influencing gill microbiota. Enrichment of Weeksellaceae in tench gills and Moraxellaceae in pikeperch under polyculture while exhibiting lower abundance of Oxalobacteraceae and Caulobacteraceae abundances compared with P, suggest that polyculture fosters unique microbial assemblages that influence significantly gill microbiota of both pikeperch and tench. This aligns with studies reporting that gills is more influenced by environment than skin mucous and gut microbiotas in RAS (Minich et al., 2020).

These results demonstrate that while host species identity remains the dominant determinant of microbial structure of gills, polyculture in RAS systems promotes cross-species microbial interactions and environmental filtering that reshape community composition, especially in gill microbiota potentially influencing host health (Birlanga et al., 2022). Overall, these findings allow us to suggest that the rearing modality can modulate microbial composition within each host compartment.

Overall, our findings highlighted that polyculture impact the dynamics interactions between main RAS microbial compartments : water, biofilms and fish microbiota and that the inoculation of water and biofilms in RAS differs depending on the rearing modality and stocked species. This understanding paves the way for better polyculture management in RAS, integrating species diversity and their microbial interactions.

2) Future directions

Our findings support a potential shift toward polyculture practices in RAS, ultimately yielding high quality of diverse fish species per one rearing unit with careful management of microbia compartment.We suggest that fish assemblages represents a promising practice for developing reproducible, resilient, and health-optimized microbial ecosystems in indoor RAS. However, to deepen our understanding of the complex mechanisms underlying microbial interactions in RAS.

Future investigations should aim to integrate functional metagenomics in order to identify specific beneficial and detrimental microbial taxa while elucidating their functional roles within the system for example, in nutrient cycling processes such as nitrification, denitrification, and organic matter degradation, as well as in the suppression of opportunistic or pathogenic microorganisms. Monitoring the evolution of these microbial interactions over time, in response to a stress factor such as high temperature exposure (Lusiastuti et al., 2024) or antimicrobial exposure (Kampouris et al., 2022), would provide valuable insights into if microbial communities in polyculture can contribute to better overall system stability, resilience, and water quality in responses to stressors. Advanced molecular tools, such as metagenomics or metatranscriptomic (Zhi et al., 2014), could be employed to

characterize not only the microbial composition but also the associated functional activity. Understanding these mechanisms in greater detail would help identify a polyculture model able to improve biofilter performance, enhance fish health, and reduce disease risks. Ultimately, such knowledge would contribute to the development of evidence on the benefits of polyculture in RAS.

The effect of fish species in RAS implies that the choice of reared fish species and fish combinations can create very unique microbial composition that can be beneficial or detrimental. Investigations should further explore other : species combinations, stocking densities, and life stages and how they affect the composite microbiome in polyculture, including both beneficial and potentially pathogenic microorganisms. Metagenomic approaches could provide insights into functional gene expression, and metabolic pathways, revealing how microbial communities in polyculture in RAS impact water quality, biofiltration efficiency, and fish physiological responses.

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Abstract

The production of fish in aquaculture has been experiencing a significant increase to meet the growing demand for food due to the exponential growth of the world population. Recirculating Aquaculture Systems (RAS) have been designed as a more sustainable approach to intensive fish production and are increasingly adopted for their ability to minimize water use and environmental discharge through advanced water treatment, despite higher operational costs. Understanding the dynamic changes and maturation of microbial communities is essential for optimizing RAS performance. A critical, yet underexplored, aspect of RAS is the microbial ecology, including the complex interactions between microbial communities in the water, biofilms, and on the fish (the microbiota). This study investigates the dynamic of colonization of microbial communities in RAS in monoculture of tench (*Tinca tinca*), monoculture of pikeperch (*Sander lucioperca*), and polyculture of both species together. Specifically, we examined the impact of fish species through its specific microbiota of skin, gills and feces on the change in the structure and the diversity of microbial communities in RAS.

To investigate this, we used high-throughput DNA sequencing and the V3-V4 region of the 16S rRNA gene to analyze and compare microbial communities in the water and biofilms but also in the skin, gills and gut of reared fish. Our findings showed different microbial profiles between different modalities suggesting the presence of fish species effect on the structure of planktonic and sessile microbial communities of water and biofilms in RAS with a unique composite microbial composition in polyculture that does not simply correspond to additive microbiome of monocultures, presenting potential advantages in favorizing beneficial taxa over potential pathogens. The inoculation of water and biofilms by fish microbiotas was found only at the beginning of the culture cycle and differed between modalities. As skin mucus was the main fish vectorization pathway in pikeperch monoculture, feces were the main pathway in tench monoculture and in polyculture mainly pikeperch skin mucus followed by a less important inoculation by tench feces. It was also shown that over time, bacterial communities in the biofilters and water undergo stabilization and become importantly less influenced by reared species microbiota. Overall, this work confirms that fish biodiversity is a key driver of microbial structure in RAS, leading to emergent communities with possible potential benefits for system function and pathogen control. It provides a foundational understanding for optimizing sustainable polyculture practices in RAS by integrating species-specific microbial interactions and opens new methodological avenues for future research.

Keywords : fish diversification, polyculture, Recirculating Aquaculture Systems (RAS), microbial communities, microbial ecology, fish microbiota, biofilms.

Résumé

La production de poisson en aquaculture a connu une augmentation significative pour répondre à la demande alimentaire croissante de la population mondiale. Les systèmes d'aquaculture en circuit fermé (RAS) ont été conçus comme une approche plus durable pour la production intensive de poisson et sont de plus en plus adoptés pour leur capacité à limiter l'utilisation de l'eau et les rejets dans l'environnement grâce à un recyclage de l'eau, et ce malgré des coûts opérationnels plus élevés. Comprendre les changements dynamiques et la maturation des communautés microbiennes est essentiel pour optimiser les performances des RAS. Un aspect critique, bien qu'encore peu exploré, est l'écologie microbienne des RAS, incluant les interactions complexes entre les communautés microbiennes présentes dans l'eau, les biofilms et les poissons (le microbiote). Cette étude vise à évaluer la dynamique de colonisation des communautés microbiennes dans les RAS gérés en monoculture de tanche (*Tinca tinca*) ou de sandre (*Sander lucioperca*), ou en polyculture associant ces deux espèces. Plus spécifiquement, nous avons examiné l'impact de l'espèce de poisson, via les microbiotes spécifiques de leur peau, leurs branchies et leurs fèces, sur l'évolution de la structure et de la diversité des communautés microbiennes dans les RAS. Pour étudier cela, nous avons utilisé la technique de séquençage de l'ADN à haut débit (région V3-V4 du gène de l'ARNr 16S) pour analyser et comparer les communautés microbiennes. Nos résultats ont montré des profils microbiens différents selon les modalités d'élevage, suggérant la présence d'un effet espèce de poisson sur la structure des communautés microbiennes planctoniques et sessiles dans les RAS. La polyculture présente une composition microbienne composite unique qui ne correspond pas simplement à l'addition des microbiomes des monocultures, en favorisant des taxons bénéfiques et défavorisant la présence de potentiels pathogènes. L'inoculation de l'eau et des biofilms par les microbiotes des poissons n'a été observée qu'au début du cycle d'élevage et différait selon les modalités. Alors que le mucus de la peau a été la principale voie de vectorisation chez le sandre en monoculture, les fèces ont été la voie principale chez la tanche en monoculture. En polyculture, l'inoculation a été principalement assurée par le mucus de la peau du sandre, suivie par une contribution moindre des fèces de la tanche. Il a également été démontré qu'au fil du temps, les communautés bactériennes des biofiltres et de l'eau se stabilisent et deviennent bien moins influencées par le microbiote des espèces élevées.

Dans leur globalité, ces travaux confirment que la biodiversité piscicole est un facteur clé déterminant la structure microbienne dans les RAS, conduisant à des communautés émergentes présentant de potentiels bénéfices pour le fonctionnement du système et le contrôle des pathogènes. Ils fournissent une base pour une meilleure compréhension et une optimisation des pratiques de polyculture durable en RAS en considérant les interactions microbiennes spécifiques. Cela ouvre de nouvelles perspectives

pour les futures recherches.

Mots-clés : diversité ichtyologique, polyculture, aquaculture en circuit fermé (RAS), communautés microbiennes, écologie microbienne, microbiote des poissons, biofilms.

Résumé détaillé en français

Objectif global :

L'objectif global de ce travail est d'évaluer comment la biodiversité ichtyologique influence la diversité et la dynamique microbienne dans les systèmes aquacoles fonctionnant en circuit fermé (RAS), considérés comme une alternative aux systèmes d'élevage ouverts. Les RAS améliorent la protection de l'environnement et augmentent la résilience des élevages aquacoles (indépendance vis-à-vis des hydrosystèmes naturels). L'étude explore les interactions complexes entre les espèces de poisson via leurs microbiotes d'une part et l'eau et les biofilms d'autre part, en examinant les effets des modalités d'élevage (monoculture ou polyculture) sur la structuration et la stabilité des communautés microbiennes.

Introduction générale :

L'aquaculture consiste à élever des organismes aquatiques, comme des poissons, mollusques, crustacés ou plantes, dans des conditions plus ou moins contrôlées pour augmenter la production. Elle joue un rôle clé dans la sécurité alimentaire et le développement économique des populations humaines. Depuis 1950, sa production a fortement augmenté, dépassant ainsi les captures issues de la pêche traditionnelle en 2022. La demande croissante en produits aquatiques continue de stimuler sa croissance, principalement en Asie, où la Chine domine le secteur.

Cependant, l'aquaculture fait face à des défis environnementaux, sociaux et économiques croissants, liés à la pollution et la surexploitation des ressources et des habitats aquatiques, accentués par le changement climatique. Son développement rapide entraîne une eutrophisation des milieux, une perte de biodiversité et une propagation de pathogènes dans les écosystèmes, tandis que la demande mondiale en produits aquatiques continue d'augmenter. La durabilité du système aquacole repose donc sur l'équilibre entre production, protection de l'environnement, responsabilité sociale et rentabilité économique.

On peut distinguer deux types d'aquaculture : la monoculture et la polyculture. L'intensification des élevages en monoculture (culture d'une seule espèce dans un même système), bien que généralement rentable, est souvent perçue comme une menace pour les écosystèmes. La promotion d'approches plus durables au niveau des pratiques aquacoles est donc essentielle pour répondre à la demande en produits aquatiques tout en limitant ses impacts. Dans ce contexte, le développement de la polyculture est une

piste de progrès à prospecter.

La polyculture associe plusieurs espèces pour une meilleure utilisation des ressources disponibles en profitant de leurs complémentarités. L'intégration de la polyculture aux RAS, pour diminuer encore plus leur impact sur l'environnement, est une piste de progrès originale. Les RAS permettent de limiter la consommation d'eau car elle est recirculée et recyclée à 90-95%. Ces systèmes, bien que coûteux, offrent un contrôle précis des paramètres d'élevage (ex : température, pH, O_2, luminosité) propres à chaque espèce de poisson et une réduction des volumes d'effluents rejetées vers l'environnement. La maîtrise des communautés microbiennes des RAS, où les communautés bactériennes jouent un rôle crucial dans la filtration biologique, la dégradation de la matière organique et donc le maintien de la qualité de l'eau, est un enjeu majeur.

La polyculture en RAS est considérée comme une alternative à la monoculture et représente une voie de développement pour promouvoir des systèmes d'élevage plus résilients en bénéficiant de la coexistence des espèces et de leurs interactions (Thomas et al., 2021). La polyculture doit permettre une meilleure productivité car les poissons utilisent plus efficacement le volume de l'eau et les ressources trophiques (aliment, biofilm). De plus, la polyculture pourrait améliorer le bien-être des poissons via l'intégration d'espèces bénéfiques ou auxiliaires comme la tanche (*Tinca tinca*) qui présente un mucus ayant des propriétés particulières. Cependant, une telle approche nécessite d'associer des espèces compatibles ayant un intérêt économique et les mêmes exigences physicochimiques. De plus, les conséquences d'une association de plusieurs espèces de poisson sur les communautés microbiennes des RAS n'ont pas été étudiées à ce jour. Ce manque de connaissances nécessite d'engager des recherches sur l'écologie microbienne des RAS dans un contexte de polyculture.

L'écologie microbienne étudie la diversité et les interactions des microorganismes entre eux et avec leur environnement. Dans les RAS, les micro-organismes jouent des rôles majeurs surtout dans les biofiltres, notamment dans le recyclage de substances toxiques via la conversion de l'ammoniac toxique en nitrate moins toxique pour les poissons par l'action de bactéries nitrifiantes et le processus de nitrification. De plus, certaines bactéries ont des effets bénéfiques et peuvent prévenir la prolifération de pathogènes grâce à leur potentiel bactéricide et donc réduire le risque de maladies en inhibant la croissance de pathogènes nocifs tels *qu'Aeromonas hydrophila*. L'étude de l'écologie microbienne dans les RAS est cruciale pour la maîtrise d'un environnement optimal favorisant la croissance des poissons. Les interactions entre les bactéries entraînent la formation de biofilms robustes qui peuvent aussi contenir des agents pathogènes ou des pathogènes opportunistes ce qui pose

un défi à la durabilité et la biosécurité des RAS. Ainsi, comprendre l'organisation des biofilms et des communautés microbiennes en RAS offre des perspectives significatives pour améliorer la résilience et l'efficacité de ces systèmes innovants.

Dans les RAS, les communautés microbiennes sont majoritairement présentes dans les biofilms, l'eau et les poissons. Les microorganismes de l'eau et du biofilm contribuent à la décomposition des matières organiques et à la régulation de la qualité de l'eau, mais peuvent aussi inclure des pathogènes. Les microbiotes des poissons, spécifiques à chaque tissu (intestin, peau, branchies), jouent un rôle clé dans la digestion, l'immunité et la protection contre les maladies. Ces communautés sont influencées par des facteurs intrinsèques (morphologie, génétique) et extrinsèques (alimentation, environnement) et sont spécifiques à chaque tissu et à l'hôte. Les avancées dans le séquençage de l'ADN microbien ont permis de mieux comprendre la complexité de ces communautés.

La dynamique microbienne dans les RAS décrit l'établissement, l'évolution et les interactions des communautés microbiennes dans les différents compartiments du système, notamment l'eau, les biofilms et les poissons. Au démarrage du RAS (mise en eau), les microbiotes ne sont pas encore stabilisés et passent par des phases d'inoculation, de croissance et de maturation, influencées par les niveaux de nutriments, les déchets organiques, l'alimentation et les conditions physico-chimiques de l'eau, telles que la température, le pH et l'oxygène dissous. Les biofilms, qui se développent sur les surfaces des filtres, tuyaux et bassins, constituent des compartiments particulièrement dynamiques considérés comme des réservoirs microbiens, servent de niches pour les microorganismes sessiles et planctoniques, et contribuent à la redistribution ces microorganismes vers l'eau et les poissons par détachement ou dispersion en favorisant des échanges bidirectionnels constants entre les compartiments. L'eau elle-même agit comme un vecteur central, hébergeant des microorganismes libres et/ou attachés aux particules et influençant la composition microbienne des poissons par contact direct. Les microbiotes des poissons sont spécifiques à chaque tissu (intestin, peau, branchies).

Enfin, bien que de nombreuses études aient montré les échanges qui existent entre l'eau, les biofilms et les microbiotes des poissons, peu d'informations existent sur l'effet de la diversité des espèces de poisson sur les dynamiques microbiennes en RAS, ce qui limite la compréhension de l'influence des poissons sur l'assemblage microbien et constitue un enjeu majeur pour l'optimisation de la polyculture dans les RAS.

En général, chaque espèce de poisson possède un microbiote spécifique qui pourrait inoculer le système en co-culture, mais la dynamique et les interactions microbiennes restent inconnues en RAS.

Il est donc essentiel de mieux comprendre l'écologie microbienne des RAS dans un contexte de polyculture. Alors que les recherches actuelles se concentrent souvent sur les monocultures en RAS, elles n'examinent pas comment les interactions interspécifiques en polyculture pourraient favoriser des synergies microbiennes bénéfiques ou, au contraire, accroître le risque de propagation de pathogènes. L'objectif principal de l'étude est d'évaluer les communautés microbiennes dans un RAS dans un contexte de polyculture de sandre et de tanche, par rapport aux monocultures respectives de chaque espèce. Les deux espèces sont compatibles et ont des exigences abiotiques similaires : pH, température, salinité, oxygène dissous et lumière. Le premier objectif est d'évaluer comment la présence de ces deux espèces en polyculture influence la diversité microbienne planctonique et sessile dans un RAS via leurs microbiotes en comparaison avec leurs monocultures.

En fait, les poissons peuvent inoculer les RAS via leurs microbiotes cutané, branchial et intestinal, influençant ainsi les communautés microbiennes de l'eau et des biofilms, et pouvant aussi véhiculer des pathogènes. Les mécanismes précis de transfert microbien, leur dynamique et leur stabilité à long terme restent peu connus en RAS, surtout en polyculture. Dans ce contexte, cette étude vise plusieurs objectifs principaux : (1) comprendre les transferts microbiens potentiels entre les espèces de poisson et en direction des compartiments « eau » et « biofilm » en identifiant les principaux vecteurs responsables de l'inoculation du système (branchies, peau, tractus digestif), (2) évaluer la spécificité microbienne des tissus et des espèces dans des élevages conduits en monoculture ou polyculture, et (3) examiner si la diversité microbienne stabilisée et la composition finale du microbiote des RAS en polyculture sont associées aux traits zootechniques (poids, taille) ou physiologiques (niveau de stress) des poissons. Globalement, ce projet de thèse permettra de mieux comprendre les interactions microbiennes liées aux espèces élevées et d'optimiser les pratiques de polyculture en RAS.

Chapitre 1 : Sélectionner un protocole de standardisation de l'état microbiologique initial des RAS afin de réduire l'hétérogénéité initiale des écotrons générée par l'historique des bassins d'élevage

Dans le premier chapitre de cette thèse, on vise à homogénéiser l'état microbien initial des différents systèmes RAS « ecotron » pour limiter la variabilité inter-écotron initiale présente après le premier remplissage des bassins d'élevage. Trois traitements expérimentaux ont été comparés : un protocole standard correspondant à un lavage de routine (SP), un traitement combinant une désinfection chimique (Chloramine T, H_2O_2) et le mélange des médias de biofiltration originaux issus de tous les

ecotrons (T1), et un traitement intégrant la même désinfection chimique (T1) associée au remplacement complet des médias de biofiltration par des médias neufs (T2). L'étude de la diversité microbienne (16S rRNA) a montré qu'il existe bien une grande variabilité initiale entre les ecotrons due à l'historique d'élevage de chaque bassin qui engendre des différences dans la structure des communautés microbiennes entre les écotrons. Le nettoyage de routine s'est avéré inefficace pour pallier ce problème de variabilité. Par opposition, les traitement T2 et T1 ont montré une réduction significative de l'effet de l'historique des bassins avec cependant une richesse microbienne plus élevée pour T1 par rapport à T2. Ces résultats mettent en évidence l'importance de la gestion de l'historique des bassins en RAS et fournissent un protocole de standardisation/homogénéisation des communautés microbiennes initiales des ecotrons, prérequis indispensable avant de démarrer l'étude des effets de la polyculture.

Chapitre 2 : Influence de la biodiversité ichtyologique sur les communautés microbiennes sessiles et planctoniques dans les systèmes d'aquaculture en circuit fermé (RAS)

Maintenant que nous avons défini un protocole pour homogénéiser l'état microbien initial de toutes nos unités d'élevage, le deuxième chapitre explore l'effet de la diversité des espèces de poisson (diversité ichtyologique) sur les communautés microbiennes sessiles (biofilms) et planctoniques (eau) en RAS. Les communautés microbiennes de l'eau et du biofilm dans trois modalités expérimentales ont été comparées : monoculture de sandre (P), monoculture de tanche (T) et polyculture sandre–tanche (P_T), via une approche de métabarcoding du gène rRNA 16S.

Nos résultats montrent l'existence d'un effet spécifique à l'espèce de poisson sur la structure microbienne de l'eau et des biofilms, tant en termes de diversité alpha que de diversité bêta. Nous avons trouvé que la polyculture présente une composition particulière et spécifique des communautés qui ne correspond pas simplement à l'addition des compositions microbiennes des monocultures de chaque espèce. La coexistence des deux espèces a induit la présence d'un effet additif partiel, par lequel des taxons provenant des deux systèmes sources sont conservés en polyculture avec une structuration spécifique des communautés microbiennes de l'eau et du biofilm. Cent vingt jours après le début de l'expérience, la structure microbienne de l'eau était marquée par une favorisation des familles bactériennes bénéfiques dont les *Rhodobacteraceae* et *Spirosomaceae* bien connues pour leur capacité à convertir l'ammonium et à dégrader la matière organique respectivement, et une diminution de l'abondance des familles *Aeromonadaceae* et *Clostridiaceae* au sein desquelles des espèces ont été

décrites comme étant d'importants agents pathogènes des poissons dans les environnements aquacoles, ce qui constitue un facteur de risque sanitaire. Cette complémentarité microbienne pourrait être liée indirectement aux différences comportementales et physiologiques entre les poissons, qui influent sur la nature des excrétions par exemple ou directement à la différence de leurs microbiotes associés à la peau, les branchies et le tractus gastrointestinal. De même, en polyculture, le biofilm a montré une abondance plus importante de familles *Rhodobacteraceae* et *Nitrospiraceae,* connues pour leurs rôles dans la biofiltration, tout en diminuant l'abondance de la famille des *Listeriaceae* trouvée abondante en monoculture du sandre, qui inclut certaines espèces pathogènes. En fait, le biofilm en monoculture de sandre a montré une signature microbienne particulièrement distincte de celles des deux autres modalités d'élevage. Cela s'explique probablement par l'apparition et la persistance de gastéropodes probablement remontés par les canalisations d'évacuation des eaux usées des systèmes d'élevage en monoculture de sandre (rétro-lavage des filtres mécaniques), alors qu'ils n'ont pas persisté dans les bassins contenant des tanches, prédateurs naturels de ces mollusques. L'influence de ces gastéropodes sur la structure du biofilm observée en monoculture de sandre peut être la résultante d'une contribution directe de leur propre microbiote, supportée par les similitudes (44 %) trouvées entre les escargots et le biofilm ou un effet indirect via le broutage du biofilm, pouvant entraîner la disparition de certaines espèces microbiennes et permettre la colonisation par d'autres espèces opportunistes.

Pour conclure, dans ce chapitre nous avons prouvé que la présence de différentes espèces de poisson dans les systèmes RAS influence de manière significative la composition et la structure des communautés microbiennes. Dans l'ensemble, ces résultats montrent que le microbiome de l'eau et du biofilm dans une polyculture de sandre et de tanche se caractérise par des processus de structuration microbienne à la fois additifs et émergents, pilotés par les interactions interspécifiques. Cela souligne l'impact important de la diversité des espèces de poisson sur l'écologie microbienne dans les RAS.

Chapitre 3 : Microbiotes des branchies, de la peau et des fèces des poissons en tant que voies de vectorisation microbienne dans une polyculture sandre-tanche en circuit fermé (RAS)

Les poissons interagissent directement avec l'eau et peuvent transférer leurs communautés microbiennes au microbiome du système d'élevage (eau et biofilm) via leurs microbiotes de la peau, des branchies et du tube digestif. Il est influencé par des facteurs intrinsèques liés à l'espèce et à l'anatomie intestinale, ainsi que par des facteurs extrinsèques comme les conditions environnementales ou l'apport de nutriments via l'alimentation et les déchets.

Dans ce dernier chapitre, le but était de caractériser les dynamiques des communautés microbiennes à plusieurs temps et d'identifier la voie principale de transfert microbien (mucus de peau, mucus des branchies ou fèces) vers l'eau et le biofilm en RAS dans une polyculture sandre-tanche et des monocultures de chaque espèce (metabarcoding du gène rRNA 16S). Ces résultats permettent de mieux comprendre comment les espèces de poisson influencent le microbiome global des RAS.

L'analyse de l'alpha diversité sur les échantillons de microbiote des poissons, prélevés durant la phase d'acclimatation (Ti), a révélé que les fèces des sandres ont moins de richesse que ceux des tanches alors que les branchies des sandres ont montré une richesse et une diversité significativement plus importantes que chez les tanches. De même, nous avons observé des différences de profil des compositions microbiennes entre les microbiotes des matrices des sandres et des tanches surtout pour les fèces et les branchies. Cela confirme ce qui a été rapporté dans la littérature concernant la spécificité microbienne des microbiotes de chaque espèce mais aussi les spécificités entre les différentes matrices de poisson. La clusterisation (Pearson) selon le partage des Operational taxonomic units (OTUs) a également révélé un trait important montrant que la matrice fèces possède une composition propre et différente de celles du mucus de la peau et des branchies.

En outre, l'analyse des communautés microbiennes a montré que les branchies des poissons partagent le plus de taxons avec l'eau et le biofilm, particulièrement chez la tanche. Bien que le mucus de la tanche soit plus riche et abondant que celui du sandre, il s'est avéré que c'est la matrice qui présente le moins de partage avec l'eau et le biofilm alors que chez le sandre, les fèces sont la matrice qui partage le moins de communautés microbiennes vers l'eau et le biofilm. Ces observations suggèrent que les voies de transfert microbien vers l'environnement diffèrent selon l'espèce et le compartiment biologique considéré.

Dans les monocultures de sandre, la colonisation initiale du biofilm tout au début de l'expérience à T0 est principalement due au mucus de la peau et à l'eau. Au-delà de ce temps, la contribution des matrices de poisson a diminué fortement et le biofilm reste relativement stable au cours du temps, l'eau continuant d'inoculer le biofilm. Dans l'eau, la colonisation initiale suit le même schéma (Ti-T0) via la peau et l'eau, puis sa composition reste stable (45-78 %). Aux temps T3 et TF, les branchies et le biofilm contribuent partiellement àl'inoculation microbienne de l'eau.

Pour la monoculture de tanche, la colonisation initiale du biofilm et de l'eau est en grande partie liée aux branchies et à une fraction inconnue, potentiellement due à des pressions sélectives ou à d'autres sources externes comme l'air. Le biofilm devient de plus en plus stable et robuste au fil du temps, et à

partir de T1, le partage est principalement entre l'eau et le biofilm. Par opposition, pour la monoculture de tanche, la colonisation initiale est en grande partie due aux fèces et à une fraction inconnue.

En polyculture, le rôle de la tanche dans la mise en place du biofilm semble très minoritaire. En effet, la colonisation initiale du biofilm en polyculture est majoritairement provoquée par l'eau, dans laquelle les sandres ont été acclimatés avant d'être transférés dans les bassins expérimentaux, ainsi que par le mucus de la peau et les branchies du sandre. Alors que dans l'eau en polyculture, la colonisation initiale (Ti-T0) est majoritairement provoquée par l'eau d'acclimatation des sandres, les fèces des tanches et le mucus des sandres.

Ces résultats montrent que bien que les branchies partagent la proportion la plus importante avec les communautés de l'eau et du biofilm, il apparaît que leur contribution en tant que voie de vectorisation influençant la composition des communautés de ces matrices extrinsèques reste modérée. De plus, la structure microbienne des branchies des espèces sandre et tanche a montré une clusterisation plus proche en polyculture qu'en monoculture. On pourrait donc conclure que ce sont plutôt les communautés extrinsèques des RAS qui influencent le microbiote des branchies chez les deux espèces. Cela correspond avec les données de la littérature qui montrent que les branchies des poissons sont plus influencées par l'environnement que le mucus de la peau et le tractus gastrointestinal.

Finalement, à la fin de l'expérience, l'analyse de la bêta-diversité a montré des différences de composition des différentes matrices de poisson pour chaque espèce entre sa monoculture et sa polyculture. L'analyse des communautés microbiennes a montré également des différences dans les clusters signatures qui dominent dans les différentes matrices pour chaque espèce en monoculture en comparaison avec la polyculture à l'exception des fèces du sandre qui n'ont pas été influencés par la modalité d'élevage.

Cette étude met en évidence le rôle déterminant de l'espèce de poisson dans la structuration et la dynamique des communautés microbiennes au sein des systèmes d'aquaculture en circuit fermé . Les résultats confirment que chaque espèce possède une signature microbienne propre au niveau des matrices internes (fèces, branchies, mucus).

Chez le sandre, la colonisation initiale du biofilm et de l'eau provient principalement du mucus cutané et de l'eau, suivie d'une stabilisation progressive des communautés. En revanche, chez la tanche, les fèces et les branchies jouent un rôle majeur dans la phase initiale d'inoculation. En polyculture, la mise en place du biofilm est largement influencée par le microbiote du sandre, la contribution de la tanche restant secondaire. Par ailleurs, les branchies apparaissent comme le compartiment le plus connecté à

l'eau et au biofilm, bien que leur rôle de vecteur microbien soit faible. Leur microbiote semble davantage façonné par l'environnement, contrairement au mucus et surtout aux fèces qui conservent une spécificité liée à l'espèce.

Enfin, les analyses de bêta-diversité ont révélé des différences significatives entre les monocultures et la polyculture, confirmant que le type d'élevage influence la composition microbienne de l'eau, du biofilm et des microbiotes des poissons sauf pour les fèces du sandre.

Dans l'ensemble, ces résultats soulignent que la polyculture modifie les dynamiques d'interaction entre les microbiotes des poissons et le microbiome du système d'élevage, et mettent en évidence le rôle prépondérant des conditions environnementales dans la structuration du microbiote branchial. Cette compréhension ouvre la voie à une meilleure gestion de la polyculture en RAS, en intégrant la diversité des espèces et leurs interactions microbiennes.

Discussion générale et conclusions

Les biofilms jouent un rôle central dans la stabilité opérationnelle des RAS, agissant comme une mémoire biologique du système, mais peuvent aussi être un facteur de risque contenant des agents pathogènes prêts à se disséminer dans l'eau du système. La diversité microbienne dépend à la fois des conditions physico-chimiques, de la gestion des filtres et du choix des espèces cultivées. La biodiversité des poissons, par la diversité de leurs microbiotes, agit comme un facteur influençant les communautés microbiennes sessiles et planctoniques en RAS et présente un potentiel de renforcement des fonctions de filtration biologique tout en diminuant le risque d'émergence de pathogènes, mais cette approche est encore très peu étudiée et nécessite une attention particulière vu l'importance du compartiment microbien en RAS et les risques liés à la santé du poisson et de l'homme (consommateur).

Ces travaux apportent une contribution majeure à la compréhension de l'écologie microbienne en RAS et des interconnexions entre les différents compartiments microbiens majeurs. On a montré l'influence de l'espèce de poisson sur le microbiome des RAS (eau et biofilm) et notamment qu'elle impacte la structure et les dynamiques microbiennes dans les RAS, avec des signatures microbiennes distinctes au niveau des microbiotes de la peau, des branchies et des fèces propres à chaque espèce. Le mucus épidermique du sandre est suggéré comme étant la voie de vectorisation principale inoculant l'eau et le biofilm, tandis que la tanche contribue majoritairement par ses fèces mais en faible proportion par

rapport à d'autres facteurs. À notre connaissance le mucus cutané du sandre a été peu étudié. En polyculture, le sandre joue un rôle dominant dans l'établissement du biofilm, alors que la tanche présente un impact limité.

La thèse ouvre également vers de nouvelles perspectives en proposant d'étudier d'autres combinaisons d'espèces et/ou de changer les densités d'empoissonnement ou même d'ajouter un facteur de stress comme une antibiothérapie pour découvrir si la polyculture favorise ou s'oppose à la prolifération des bactéries antibiorésistantes et à la dissémination des gènes d'antibiorésistance qui sont de plus en plus rapportés comme facteurs de risque majeurs menaçant la durabilité et la résilience des systèmes aquacoles.