The application of environmental DNA (eDNA) methods for the monitoring and detection of aquatic microorganisms in aquaculture

Inaugural dissertation
of the Faculty of Science,
University of Bern

presented by

Jessica M. Rieder

from the United States of America

Supervisors of the doctoral thesis:
Prof. Dr. Claudia Bank, Institute of Ecology and Evolution
Prof. Dr. Irene Adrian-Kalchhauser, Institute of Fish and Wildlife Health

Institute of Ecology and Evolution

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Prof. Dr. Marco Herwegh

Don't worry about what you don't know

Life's a dance you learn as you go

J. M. Montgomery

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Acknowledgments

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Summary

The use of environmental DNA (eDNA) as a monitoring tool is indispensable in microbial ecology studies and pathogen surveillance programs, particularly in aquaculture. eDNA is generally defined as DNA extracted from environmental samples and has revolutionized the sampling and characterization of microbial communities. However, our understanding of microbial functional composition and interactions within complex ecosystems remains limited, with major knowledge gaps. Advances in molecular technology have shifted microbiological studies from lab-based to natural-population studies. Nonetheless, identifying the environmental drivers that shape natural microbial populations is often challenging, making the detection of key drivers difficult or even impossible.

This dissertation explores and assesses the potential of eDNA as a monitoring and biosecurity tool in recirculating aquaculture systems (RAS). The advantage of studying microbial communities within RAS lies in their semi-closed, compartmentalized, and controlled nature while still maintaining a semi-natural environment. This allows for an examination of the pressures that influence these communities beyond basic lab-based community interactions. Additionally, the simplified microbial community composition within RAS facilitates the identification of interactions and key drivers, making RAS an ideal candidate for studying microbiome-governed systems with direct implications for animal health.

Chapter 1 provides a comprehensive analysis of various molecular parameters, such as primer selection and sequencing methods used to characterize microbial communities in two commercial RAS perch farms. A comparison was made between the performances of different sequencing approaches, including three types of 16S short amplicon sequencing, PacBio long-read amplicon sequencing, and amplification-free shotgun metagenomics. The results revealed that the choice of 16S rRNA primers and the length of amplicons affected certain values, such as diversity measures, the number of assigned taxa, or the differentiation of amplicon sequence variants (ASVs). However, these factors had no significant impact on the spatio-temporal patterns observed between sample types, farms, and time points. This suggests that 16S rRNA sequencing is adequate for community studies. These findings demonstrate that adopting a tiered sequencing approach offers a viable strategy for gathering extensive information about microbial communities, thereby facilitating essential research on community evolution dynamics. Nonetheless, in scenarios where specific target species or applied questions are the focus, employing single-method approaches, such as quantitative real-time PCR, has proven to be more practical and cost-effective. Notably, both methods have the potential to improve farm management practices.

Chapter 2 presents a comprehensive analysis of the spatiotemporal dynamics of bacteria across the six RAS farms, encompassing both freshwater and brackish systems. This investigation yielded valuable insights into the influence of salinity on microbial community structure, highlighting a distinct separation

between salt-adapted and freshwater species. Moreover, discernible patterns emerged between circuits in a farm that reared animals at different stages of life. These findings suggest that environmental factors such as stocking density, nutrient load, and management practices also play a significant role in shaping microbial communities. Additionally, this study identified various pathogens, including several with zoonotic potential, posing risks to both farmed animals and personnel. Consequently, this discovery emphasizes the necessity for rigorous pathogen monitoring and the implementation of safe working procedures. In summary, shotgun metagenomics proved to be a powerful tool for exploring spatio-temporal patterns and detecting pathogens within recirculating aquaculture systems. These findings contribute valuable knowledge to the field and underscore the importance of microbial communities for the sustainability of such systems.

Chapter 3 compares the efficacy of lyophilization and oven-drying as drying methods and assesses the shelf life of dried quantitative polymerase chain reaction (qPCR) reactions targeting two aquatic pathogens, *Aphanomyces astaci*, the causative agent of crayfish plague and *Gyrodactylus salaris*, known as salmon fluke, which affects wild and aquaculture populations. This study demonstrated the feasibility of preparing dried qPCR reactions for the detection of aquatic pathogens, making them suitable for field-based pathogen surveillance programs.

Chapter 4 highlights the various molecular tools available for pathogen surveillance in aquaculture. Given the threats posed by pathogen spread and disease emergence to social, economic, and food security, this chapter raises awareness among aquaculture managers regarding existing molecular solutions.

Chapter 5 provides an explanation of the nuances of DNA extraction for researchers new to eDNA with limited molecular training. This chapter clarifies the purpose and impact of common DNA extraction steps, enabling researchers to combine and optimize protocols according to their specific requirements.

I. Introduction

I.1 What is aquaculture?

Aquaculture, an age-old tradition of cultivating aquatic plants and animals for human consumption, has a rich history dating back to approximately 4,000 years in ancient China. The earliest written texts on koi (*Cyprinus rubrofuscus*) cultivation in pond-based systems originated during this period (Rabanal, 1988). During Roman times, fish farming was prevalent across Europe (Marzano, 2018), and recent research on landforms in the Bolivian Amazon has revealed pre-Hispanic (1595–1635 AD) fish weirs (Erickson, 2000). Throughout these historical periods, aquaculture has remained a subsistence practice with low intensity.

In the late 20th century, aquaculture practices underwent a significant transition from small-scale farming to large-scale commercial industries. Until the 1970s, aquaculture accounted for a relatively small proportion of the total seafood output, producing only a few million tons annually, whereas capture fisheries dominated with an output of nearly 40 million tons. However, in recent decades, the aquaculture sector has experienced remarkable growth (Figure 1A), and in 2020 reached a record high of 20.2 million tons (FAO, 2022). This notable growth can be ascribed to the increasing global demand for fish and other aquatic animals, the stagnation of capture fisheries, and advancements in cultivation technology. As a result of rapid growth, aquaculture has emerged as the fastest-growing animal food-producing sector, surpassing capture fisheries as a primary source of aquatic protein (FAO, 2022).

Within the European Union, the primary emphasis in aquaculture centers on specific categories of aquatic life forms. To illustrate, marine fish production encompasses Atlantic salmon, *Salmo salar* (26.90%), Gilthead seabream, *Sparus aurata* (9.91%), European seabass, *Dicentrarchus labrax* (9.86%), and Atlantic bluefin tuna, *Thunnus thynnus* (6.1%). Conversely, freshwater fish aquaculture is primarily characterized by the breeding of rainbow trout, *Oncorhynchus mykiss* (12.99%), and common carp, *Cyprinus carpio* (3.00%). Concurrently, mollusk-focused aquaculture involves the cultivation of Pacific cupped oyster, *Magallana gigas* (8.90%), Blue mussel, *Mytilus edulis* (4.70%), Mediterranean mussel, *Mytilus galloprovincialis* (3.97%), and the Japanese carpet shell, *Ruditapes pilippinarium* (2.92%) (European Commission, 2021).

Notably, even though they do not feature in the top 10, the rearing of Eurasian perch (*Perca fluvitilis)* and zander (*Sander lucioperca*) are emerging contenders in freshwater aquaculture. For instance, the combined production of these two species reached approximately 1400 metric tons, equivalent to a value of roughly 8.5 million EUR. This marks nearly a threefold increase since the year 2000, when the production stood at approximately 490 metric tons (FAO, 2019), underscoring the promising market

potential for these species. Furthermore, promoting the cultivation of these species will contribute to the diversification of European freshwater aquaculture, all the while catering to the demand for premium aquatic protein.

The success of aquaculture can be attributed to its versatility and adaptability. Aquaculture is conducted in diverse environments, including coastal, marine, and inland settings. It encompasses a wide range of production systems, from low-intensity ponds and cages to sophisticated recirculating and flow-through systems. Moreover, the diversity of cultivated species in aquaculture far exceeds that in livestock farming. In 2017 alone, over 400 species were farmed, including finfish, mollusks, crustaceans, and algae (FAO, 2020; Stentiford et al., 2022). This remarkable range of species demonstrates the dynamic nature of aquaculture and its capacity to cater to diverse consumer preferences and market demand.

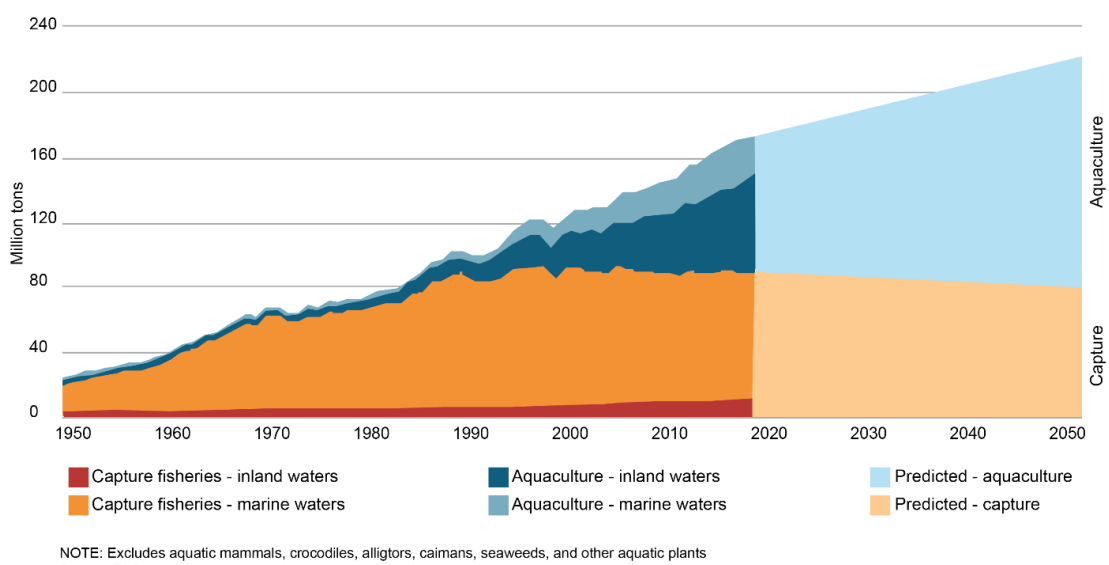

Figure 1: Capture fisheries versus aquaculture production and forecasts. *Based on data presented in the 2018 FAO State of World Fisheries and Aquaculture Production report, a comprehensive analysis was conducted to compare the trends in capture fisheries and aquaculture production, categorized into inland and marine waters, spanning the period from 1950 to 2020. Furthermore, future projections were shown to estimate the anticipated output for capture fisheries and aquaculture from 2020 to 2050, revealing a consistent upward trajectory for aquaculture production.*

The continuous expansion of aquaculture has yielded substantial socioeconomic and ecological benefits. Societally, aquaculture has emerged as a vital contributor to global initiatives aimed at enhancing nutrient and food security by providing affordable aquatic protein sources to regions that previously lacked access. Moreover, its economic growth potential can trigger transformative effects in coastal and rural areas, fostering economic prosperity (Blaalid, 2020). Ecologically, aquaculture is widely regarded as a positive force that offers numerous advantages. These include amplified production

through technological advancements that ensure food security (Midtlyng et al., 2011), reduced dependence on agricultural and wild fishery inputs for feed by cultivating non-feed organisms such as mussels, and improved water quality in areas where extractive organisms are present. Extractive species can be primary producers, such as algae or seaweed, that transform inorganic nutrients into organic biomass, or secondary producers that use organic material from the water column (e.g., shellfish) or seafloor (e.g., sea cucumbers) as food. Such improvements have been associated with enhanced biodiversity at neighboring sites (Gentry et al., 2020; Mehrani et al., 2020). Additionally, aquaculture practices exhibit greater feed conversion efficiency and occupy a smaller spatial footprint than capture fisheries (Jennings et al., 2016; Lester et al., 2018) and land-based agriculture (Froehlich et al., 2018).

Nonetheless, the rapid growth of the aquaculture sector has raised concerns regarding social and environmental issues. Socially, the sector faces considerable challenges, with economic interests often overshadowing its social and cultural considerations. Institutional and sectoral issues have contributed to inequitable outcomes in aquaculture expansion, resulting in an uneven distribution of benefits, loss of livelihoods, and instances of human rights abuses, such as indications of bonded labor (Nakamura et al., 2018) and unacceptable working conditions (Brugere et al., 2023). Furthermore, gender disparities prevail globally, impeding equal participation and benefit-sharing in the aquaculture value chain. Gender biases manifest differently across countries; for instance, certain countries such as Myanmar exhibit biased gender and social norms that confer greater control and use of household assets to men as the primary income providers. Similarly, in Bangladesh, male dominance in decision-making processes pertaining to aquaculture releases valuable inputs from women. African countries also witness limited access to loans and technical skills training for women owing to collateral requirements, impeding their involvement in the aquaculture sector (Adam & Njogu, 2023; Brugere et al., 2023). Finally, the existing constraints on accessing environmentally sustainable aquafeeds extend beyond considerations of nutrition and environmental impact. They also encompass economic and socio-cultural aspects related to the availability of these feeds and the implementation of policies aimed at ensuring quality assurance (D'Abramo, 2021). These examples underscore the slow progress toward achieving equality and fairness within the aquaculture industry.

Aquaculture practices, if not carefully developed and managed, can have significant negative environmental impacts, particularly when prioritizing economic gains over social and environmental considerations. One example is the financially driven shrimp-farming industry in Asia, which has led to extensive mangrove deforestation and degradation. For instance, Sri Lanka experienced a displacement of 36% of its mangroves between 1992 and 2012 because of shrimp farming activities, which were later abandoned (Huxham, 2015). Other ecological consequences include nutrient accumulation, which can deteriorate water quality and potentially trigger algal blooms or create hypoxic to anoxic dead zones (Nichols & Hogan, 2022). The widespread use of wild fish as feed for aquaculture stocks (Naylor et al.,

2000), the potential spillover of pathogens from aquaculture facilities to natural environments (Bouwmeester et al., 2021), and the introduction of non-native species either through farm, escapees or intentional co-introduction with aquaculture stock (Ju et al., 2020) are additional challenges associated with aquaculture practices.

Addressing these challenges is crucial for the sustainable development of aquaculture and its alignment with the United Nations' Sustainable Development Goals (Krause et al., 2020; Troell et al., 2023). One approach to achieving sustainability is the adoption of recirculating aquaculture systems. These semi-closed systems minimize environmental impacts by reducing water requirements and waste production and by preventing spillover into natural environments, thus ensuring greater environmental compatibility (e.g., reduced water usage and waste generation) (Boyd et al., 2020; Stentiford et al., 2020). Implementing recirculating aquaculture practices aligns aquaculture with the principles of sustainable development and contributes to the achievement of UN Sustainable Development Goals (United Nations, 2016).

I.1.1 Recirculating aquaculture

Recirculating aquaculture systems (RAS) are artificial structures used for inland aquaculture, primarily for the cultivation of shrimp and finfish. These semi-closed systems offer numerous advantages over open water systems. They are typically located indoors or in sheltered areas, allowing for control over water parameters and protection from environmental factors. RAS operate on an almost closed circuit (Figure 2), reducing the impact of seasonal variations and enabling stricter implementation of biosecurity measures. The adoption of recirculating aquaculture has witnessed rapid global growth, especially in regions with unsuitable environmental conditions, stringent regulations for wastewater discharge, and limited land and water resources (Martins et al., 2010; Espinal & Matulić, 2019).

The concept of RAS originated in Japan in the 1950s but gained substantial attention in the 1970s. Initially developed for warm freshwater species such as channel catfish, striped bass, and tilapia, RAS underwent a revival in the 1980s. During this period, the standardization of terminology, units of measurement, and water quality reporting was established. This progress has paved the way for technical improvements that enhance the viability of RAS. Currently, RAS systems are highly versatile and can be adapted to cultivate a wide range of aquatic species, including freshwater, brackish, marine, and cold-water species. They are employed for various purposes, such as rearing juvenile marine and salmonid fish, breeding broodstocks, and farming rare exotic species in closed systems to prevent the genetic contamination of local populations (Ahmed & Turchini, 2021).

RAS are technologically intensive and incur higher overhead costs than traditional aquaculture methods. These systems rely on machinery to maintain optimal water parameters, including heaters for temperature control, pumps for water movement and aeration, and drum filters to remove large waste

particles (Figure 2). Some RAS facilities have incorporated automatic feeding systems to ensure precise and timely feeding. The adoption of high-tech machinery incurs substantial initial investments and requires ongoing expenses for energy consumption and maintenance.

Moreover, microbial communities play a crucial role in RAS by contributing to water purification and nutrient cycling. The biofilter, a compartment that provides a large surface area for the growth of beneficial nitrifying microorganisms, is where biological filtration is performed. Microorganisms within the biofilter convert toxic metabolic byproducts such as ammonia and nitrite into less harmful nitrates. Maintaining suitable water quality is paramount for RAS managers as it directly affects the health of cultured organisms. Consequently, a significant number of microbial studies within RAS have focused on investigating biofilter microbial communities (Bagchi et al., 2014; Bartelme et al., 2017, 2019; Hüpeden et al., 2020; Y. Ma et al., 2021).

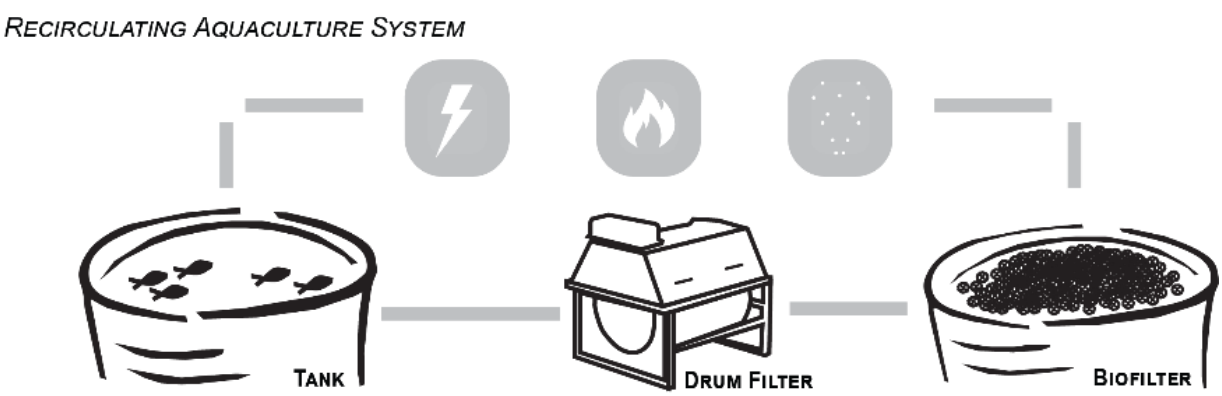

Figure 2: Example of recirculating aquaculture system (RAS) setup. *The design behind an RAS is that water continuously recirculates between the compartments. For example, after leaving the rearing tank, the water is directed to the drum filter, where solid particles, including food remnants and organic waste, are effectively separated and eliminated. The filtered water then proceeds to the biofilter, where specialized microorganisms actively degrade organic substances and facilitate various biochemical cycles, such as nitrification. To ensure optimal conditions for the cultured organisms, the water is subsequently oxygenated, heated to the appropriate temperature, and subjected to ultraviolet (UV) light treatment, effectively sterilizing it. Finally, the treated water is reintroduced into the rearing tank to complete recirculation.*

I.1.1.1 Microorganisms of RAS

Microbial communities within recirculating aquaculture systems (RAS) consist of both beneficial and pathogenic species (Austin & Austin, 2016). These communities comprise a diverse range of microorganisms that can have both positive and negative impacts on system efficiency, metabolic rates, and animal health.

On the one hand, beneficial species such as nitrifying bacteria play a crucial role in maintaining water quality through the completion of the nitrification cycle (Figure 3, Table 1). Biofilters, specifically designed to maximize surface area, harbor high concentrations of nitrifying bacteria. Ammonia-oxidizing bacteria (AOB), such as the freshwater genera *Nitrosomonas* and *Nitrosospira* (Purkhold et al., 2000)

and marine water genus *Nitrosococcus* (Woese et al., 1985), complete the first part of the nitrification cycle by breaking down ammonia. Nitrite-oxidizing bacteria (NOB), represented by members of the phyla *Nitrospinota*, *Nitrospirtota*, *Pseudomonadota*, and *Chloroflexota* (Schreier et al., 2010), complete the second part of the nitrification cycle by breaking down nitrites into nitrates. However, it was recently discovered that complete nitrification could be accomplished by comammox bacteria (Figure 1.3) (Daims et al., 2015; van Kessel et al., 2015), overturning our understanding of nitrification cycling. Comammox bacteria have been identified in various aquatic systems, including marine (Bartelme et al., 2017), brackish RAS (Blancheton et al., 2013; Rurangwa & Verdegem, 2015), freshwater aquaponic systems (Heise et al., 2021), and wastewater treatment plants (Maddela et al., 2022).

Additionally, beneficial heterotrophic bacteria provide a wide range of services, both at the system level and for animal health. They contribute to water quality (Bossier & Ekasari, 2017) by decomposing organic matter and removing nitrogen, thereby mitigating the potential negative impacts of sub-par water qualities. Additionally, they occupy available niches, preventing the establishment and proliferation of pathogenic bacteria (Blancheton et al., 2013). Heterotrophic bacteria play a significant role in digestion, morphological development, disease prevention, and as dietary components for detritivores in aquaculture species such as shrimp, tilapia, and carp (Hagopian & Riley, 1998; Ebeling et al., 2006). Some commonly occurring heterotrophic genera in RAS biofiltration include *Pseudomonas*, *Paracoccus*, and *Comamonas* (Schreier et al., 2010).

On the other hand, a wide range of pathogenic organisms, including bacteria, fungi, fungi-like organisms, and viruses (Table 2), can also be found in RAS. Opportunistic bacteria are the primary causative agents of disease outbreaks in aquaculture (Boutin et al., 2013). The occurrence of such outbreaks is contingent on the fulfillment of three conditions: (1) the presence of a virulent pathogen, (2) a susceptible host that may be experiencing stress or immunosuppression, and (3) unfavorable environmental conditions (Derome et al., 2016). Common bacterial pathogens in fish farming include *Aeromonas salmonicida*, *Vibrio anguillarum*, *Aliivibrio salmonicidia*, and *Yersinia ruckeri*, which are responsible for furunculosis, vibriosis, cold-water vibriosis, and red-mouth disease, respectively (Blancheton et al., 2013). Common fungal or fungal-like pathogens encountered in aquaculture, species belonging to the genera *Saprolegnia* (Pavić et al., 2022) and *Branchiomyces* (Mondal et al., 2023) are commonly observed. These fungi are categorized as secondary tissue invaders and primarily affect hosts that have experienced traumatic injuries or primary infections or are under stress due to suboptimal environmental conditions (Sarkar et al., 2022). Whereas viral hemorrhagic septicemia (VHS) is one of the most fatal viral diseases in the farmed rainbow trout (*Onocrhynchus mykiss*), with an estimated annual loss exceeding 40 million pounds (LaPatra et al., 2016).

Overall, microorganisms are a fundamental aspect of RAS. The presence of beneficial species is vital for water quality, animal health, and operation success. Unfortunately, pathogens are also part of the

microbial communities, and maintaining a completely pathogen-free system is difficult, if not impossible. Therefore, proactive and preventive measures should be implemented to foster stable community and pathogen-reduced communities.

I.1.1.2 Introduction and spatial distribution of microorganisms

The introduction and spatial distribution of microorganisms in RAS play a pivotal role in operational success. These microorganisms can originate from a variety of sources, both natural and anthropogenic, including intake water, air, farmed animals, feed, equipment, and even personnel or visitors, as documented in previous studies (Blancheton et al., 2013).

Once introduced, the spatial distribution of these microbes within the RAS environment is not homogenous. Microbial populations tend to establish themselves throughout the various components of the RAS (Rurangwa & Verdegem, 2015), including the water column, biofilters, and the surfaces of equipment and infrastructure, based on suitable conditions and available resources. For instance, nitrifying bacteria, essential for ammonia conversion in RAS, often find their niche within the biofilters, where they form biofilms to efficiently process ammonia and nitrite (Hüpeden et al., 2020).

Intentional managerial endeavors are also undertaken to regulate and manipulate these microbial communities. For instance, microbial solutions containing predetermined species may be introduced to guide colonization and enhance system performance. For example, biofilter start kits can be applied to aid the establishment of nitrifying species, thus allowing for a faster start-up time.

Understanding and manipulating the introduction and spatial distribution of microbes in RAS is crucial for maintaining water quality, optimizing nutrient cycling, and ensuring animal health (El-Saadony et al., 2021) while also mitigating potential pathogenic risks. Effective management of these microbial communities is central to the sustainable and efficient operation of RAS in aquaculture systems (Rurangwa & Verdegem, 2015; Hüpeden et al., 2020).

I.1.2 Aquaculture conclusion

In summary, aquaculture holds significant potential for meeting the growing demand for seafood, reducing pressure on wild fisheries, and driving economic development. However, it faces substantial challenges related to environmental impact, feed sustainability, and disease management. Addressing these challenges, along with the implementation of effective prevention and control measures (**Chapter 4**), is essential for ensuring responsible and sustainable growth of the aquaculture sector, in alignment with the United Nations Sustainable Development Goals.

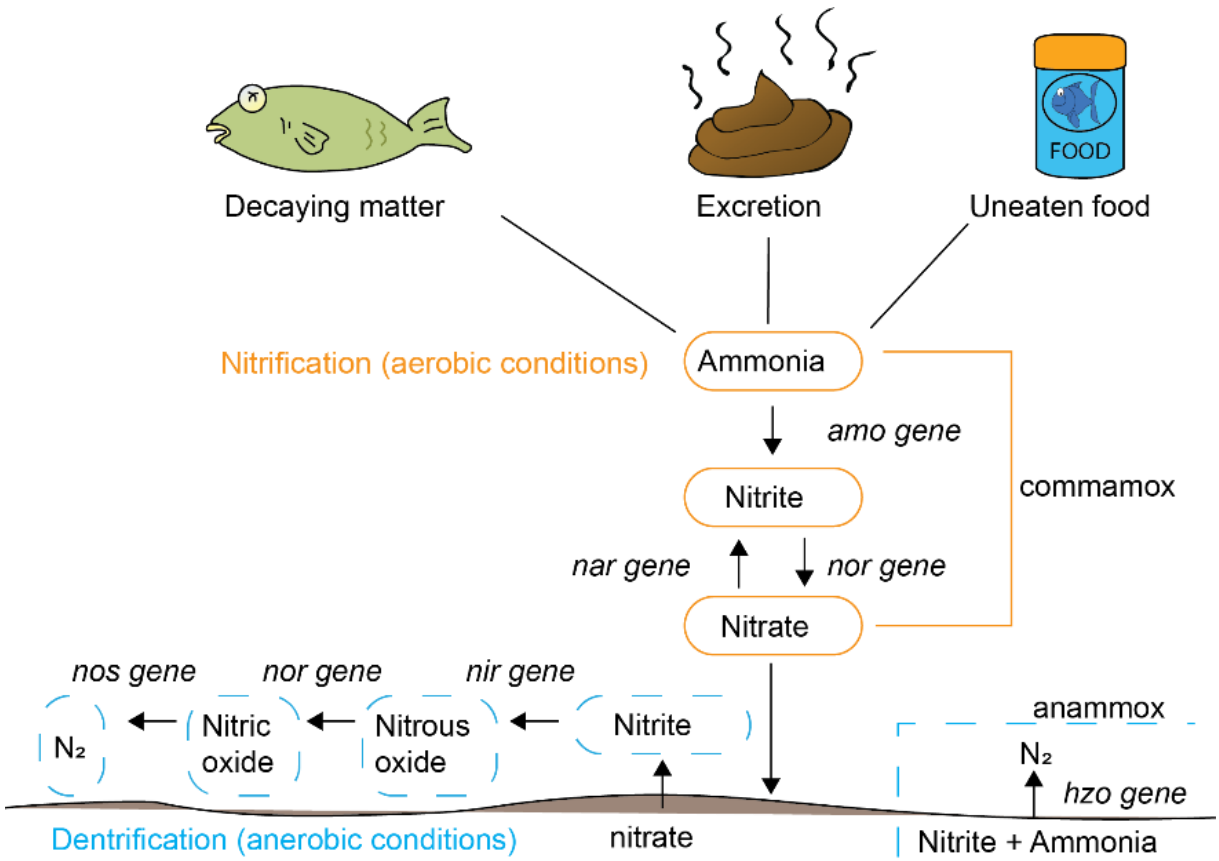

Figure 3: Nitrification and denitrification processes. Nitrification occurs under aerobic conditions and involves the conversion of ammonia (NH_3) to nitrate (NO_3^-), as illustrated in orange. Conversely, denitrification occurs under anaerobic conditions and converts nitrate (NO3 −) into nitrogen gas (N2), as illustrated in blue. The figure also highlights the relevant genes associated with different steps of ammonia conversion.

Process	Sub-process	Organisms involved
Nitrification	bacterial autotrophic ammonia oxidation	*Nitrosomonas europaea, N. eutropha, Nitrosospira multiformis, Nitrosococcus oceanus, N. halophilus, Nitrosolobus sp., Nitrosovibrious sp.*
	bacterial heterotrophic ammonia oxidation	*Alcaligenes faecalis, Pseudomonasputida, Paracoccus denitrificans, Thermus, Azoarcus Bacillus licheniformis*
	arachael ammonia oxidation	*Nitrosopumilus maritimus, N. adriaticus, N. piranensis, N. koreensis, Nitrosotalea devanterra*
	nitrite oxidation	*Nitrobacter winogradskyi, Nitrospira, Nitrococcus mobilus, Nitrospina gracilis*
Comammox	complete ammonia oxidation to nitrate	*Nitrospira sp.*
Denitrification	bacterial heterotrophic nitrite reduction	*Alcaligenes faecalis, Paracoccus denitrificans, P. halodenitrificans, Pseudomonas aeruginosa, P. stutzeri, Thiobacillus denitrifcans, Azospirillum brasilense*
	bacterial heterotrophic nitric oxide reduction	*Alcaligenes faecalis, Pseudomonas stutzeri, Paracoccus halodenitrificans, Paracoccus denitrificans*
	bacterial heterotrophic nitrous oxide reduction	*Alcaligenes sp., Azospirillum sp., Bacillus sp., Pseudomonas sp.,Thiobacillus versutus, Thiosphaera pantotropha*
	bacterial autotrophic denitrification	*Rhodobacter sp., Thiomicrospira sp., Hydrogenophaga sp., Thiothrix sp., Thiobacillus denitrificans, Sulfurimonas denitrificans*
	fungal denitrification	*Aspergillus niger*
	archael dentrification	*Halobacterium denitrificans, Pyrobaculum aerophilum, Haloferax denitrificans*
Anammox	anaerobic ammonia oxidation	*Planctomyces, Gemmata, Isosphaera, Candidatus Brocadia, Candidatus Kuenenia, Candidatus Anammoxoglobus*

Table 1: Overview of nitrification and denitrification processes, subprocesses, and organisms involved. *The table outlines the main sub-processes involved in nitrification, commamox, denitrification, anammox, and the organisms responsible for these processes. Adapted from Preena (2021).*

Genus	Species	Group	Disease or symptoms	Reported hosts	References
Acinetobacter		Bacteria	acinetobacter disease	Atlantic salmon, channel catfish	Austin & Austin, 2016
Aeromonas	*allosaccharophila*	Bacteria	Aeromonas septicemia	shrimp, farmed fish	Austin & Austin, 2016
Aeromonas	*bestiarum*	Bacteria	acute hemorrhagic septicemia or chronic skin ulcers	fish	Austin & Austin, 2016
Aeromonas	*caviae*	Bacteria	Aeromonas septicemia	fish	Austin & Austin, 2016
Aeromonas	*hydrophila*	Bacteria	motile Aeromonas septicemia	freshwater fish	Austin & Austin, 2016 Irshath et al., 2023
Aeromonas	*jandaei*	Bacteria	Aeromonas septicemia	rainbow trout, gizzard shad	Austin & Austin, 2016
Aeromonas	*salmonicida*	Bacteria	furunculosis	farmed fish	Austin & Austin, 2016 Irshath et al., 2023
Aeromonas	*sobria*	Bacteria	Aeromonas septicemia	farmed fish, striped bass, channel catfish, eel	Austin & Austin, 2016
Aeromonas	*veronii*	Bacteria	Aeromonas septicemia	freshwater fish	Austin & Austin, 2016
Aliivibrio	*salmonicida*	Bacteria	cold-water vibriosis	wild and farmed Atlantic salmon	Austin & Austin, 2016
Aliivibrio	*logei*	Bacteria	skin lesions	Atlantic salmon	Austin & Austin, 2016
Aliivibrio	*wodanis*	Bacteria	winter ulcer disease	Atlantic salmon	Austin & Austin, 2016
Bacillus	*cereus*	Bacteria	branchio-necrosis	carp, striped bass	Austin & Austin, 2016
Bacillus	*mycoides*	Bacteria	ulceration	channel catfish	Austin & Austin, 2016
Bacillus	*subtilis*	Bacteria	branchio-necrosis	carp	Austin & Austin, 2016
Candidatus	*arthromitus*	bacteria	disease outbreaks	eels, yellowtail, Atlantic salmon, Chilean salmon	Austin & Austin, 2016
Carnobacterium	*maltaromaticum*	Bacteria	pseudokidney disease, meningoencephalitis	lake whitefish, trout, salmon	Austin & Austin, 2016
Chryseobacerium	*piscicola*	Bacteria	skin and muscle ulceration	Atlantic salmon, rainbow trout	Ilardi et al., 2009
Chryseobacterium	*balustinum*	Bacteria	Flavobacteriosis	marine fish	Austin & Austin, 2016
Clostrid	*botulinum*	Bacteria	botulism, visceral toxicosis	salmonids, channel catfish	Austin & Austin, 2016
Corynebacterium	*aquaticum*	Bacteria	exophthalmia	striped bass	Austin & Austin, 2016
Cytophaga	*rosea*	Bacteria	gill disease	salmonids	Austin & Austin, 2016

Edwardsiella	*tarda*	Bacteria	red pest disease	marine fish, rainbow trout, Japanese yellowtail, grey mullet	Austin & Austin, 2016 Irshath et al., 2023
Eubacterium	*tarantellae*	Bacteria	eubacterial meningitis	striped mullet	Austin & Austin, 2016
Flavobacterium	*branchiophilum*	Bacteria	bacterial gill disease	salmonid, freshwater fish	Austin & Austin, 2016
Flavobacterium	*columnare*	Bacteria	columnaris disease	tilapia, Atlantic salmon, rainbow trout	Austin & Austin, 2016 Irshath et al., 2023
Flavobacterium	*psychrophilum*	Bacteria	bacterial coldwater disease	culture freshwater fish species	Austin & Austin, 2016 Irshath et al., 2023
Flavobacterium	*hydatis*	Bacteria	gill disease	salmonids	Austin & Austin, 2016
Flavobacterium	*johnsoniae*	Bacteria	gill disease, skin disease	carp, rainbow trout, barramundi, longfin eel	Austin & Austin, 2016
Flavobacterium	*oncorhynchi*	Bacteria	bacterial gill disease	salmonids	Austin & Austin, 2016
Flavobacterium	*succinicans*	Bacteria	bacterial gill disease	rainbow trout	Austin & Austin, 2016
Francisella	*philomiragia*	Bacteria	francisellosis	rainbow trout	Austin & Austin, 2016
Francisella	*noatunensis*	Bacteria	francisellosis, visceral granulomatosis	tilapia, striped bass, Atlantic salmon, Atlantic cod, three-line grunt	Austin & Austin, 2016
Hafnia	*alvei*	Bacteria	hemorrhagic septicemia	rainbow trout, cherry salmon	Austin & Austin, 2016
Hahella	*chejuensis*	Bacteria	red egg disease	tilapia	Austin & Austin, 2016
Janthinobacterium	*lividum*	Bacteria	anemia	rainbow trout	Austin & Austin, 2016
Klebsiella	*pneumoniae*	Bacteria	fin and tail disease	rainbow trout	Austin & Austin, 2016
Lactococcus	*garvieae*	Bacteria	vascular endothelium, streptococcicosis	farmed fish, coho salmon, rainbow trout	Shahin et al., 2022 Irshath et al., 2023
Lactococcus	*piscium*	Bacteria	lactobacillosis, pseudokidney disease	rainbow trout	Austin & Austin, 2016
Micrococcus	*leteus*	Bacteria	micrococcosis	rainbow trout	Austin & Austin, 2016

Moritella	*marina*	Bacteria	winter ulcer disease	Scottish farmed salmon	Austin & Austin, 2016
Moritella	*viscosa*	Bacteria	winter ulcer disease	Atlantic salmon	Austin & Austin, 2016 Irshath et al., 2023
Mycobacterium	*marinum*	Bacteria	necrotizing granuloma like tuberculosis	rainbow trout	Austin & Austin, 2016 Irshath et al., 2023
Mycobacterium		Bacteria	fish tuberculosis (mycobacteriosis)	fish	Austin & Austin, 2016
Nocardia		Bacteria	nocardiosis	fish	Austin & Austin, 2016
Pasteurella	*skyensis*	Bacteria	disease outbreaks	rainbow trout	Austin & Austin, 2016
Photobacterium	*damselae*	Bacteria	disease outbreaks	shrimp and farmed fish	Austin & Austin, 2016 Matanza & Osorio, 2020
Piscirickettsia	*salmonis*	Bacteria	coho salmon syndrome, salmonid rickettsial, septicemia	salmon, sea bass	Austin & Austin, 2016 Irshath et al., 2023
Pseudomonas	*anguilliseptica*	Bacteria	red spot, winter disease	rainbow trout, cod, eel, black spot sea bream	Austin & Austin, 2016 Irshath et al., 2023
Pseudomonas	*fluorescens*	Bacteria	generalized septicemia	fish	Austin & Austin, 2016
Pseudomonas	*luteloa*	Bacteria	generalized septicemia	rainbow trout	Austin & Austin, 2016
Pseudomonas	*pseudoalcaligenes*	Bacteria	skin ulceration	rainbow trout	Austin & Austin, 2016
Pseudomonas	*putida*	Bacteria	hemorrhagic ascites, ulceration	rainbow trout	Austin & Austin, 2016
Renibacterium	*salmoninarum*	Bacteria	bacterial kidney disease	salmonid fish	Austin & Austin, 2016
Rickettsia		Bacteria	red mark syndrome, strawberry disease	rainbow trout	Austin & Austin, 2016
Serratia	*liquefaciens*	Bacteria	septicemia	Arctic charr, Atlantic salmon	Austin & Austin, 2016
Sporocytophaga		Bacteria	saltwater columnaris	salmonids	Austin & Austin, 2016
Staphylococcus	*aureus*	Bacteria	eye disease, jaundice	catfish, silver carp	Austin & Austin, 2016
Streptobacillus	*moniliformis*	Bacteria	disease outbreaks	sole, brill, and turbot	Austin & Austin, 2016
Streptococcus	*parauberis*	Bacteria	streptococcosis	fish and shellfish	Austin & Austin, 2016

					Irshath et al., 2023
Streptococcus	*phocae*	Bacteria	haemorrphages	fish	Austin & Austin, 2016
Streptococcus	*dysgalactiae*	Bacteria	streptococcosis	Amur sturgeon, amberjack, Nile tilapia, yellowtail	Austin & Austin, 2016
Streptococcus	*agalactiae*	Bacteria	meningoencephalitis	carp, grouper, rainbow trout, silver pomfret, tilapia	Austin & Austin, 2016
Streptococcus	*ictalurid*	Bacteria	streptococcosis	channel catfish	Austin & Austin, 2016
Streptococcus	*iIniae*	Bacteria	acute septicemia, meningoencephalitis, streptococcicosis	freshwater and marine fish	Austin & Austin, 2016
Streptococcus	*phocae*	Bacteria	Streptococcosis	Atlantic salmon	Austin & Austin, 2016
Tenacibaculum	*soleae*	Bacteria	disease outbreaks	carp, Mexican golden trout	Austin & Austin, 2016
Tenacibaculum	*ovolyticum*	Bacteria	larval and egg mortalities	halibut	Austin & Austin, 2016
Vagococcus	*salmoninrum*	Bacteria	lactobacillosis, pseudokidney disease, peritonitis, septicemia	Atlantic salmon, brown trout, rainbow trout	Austin & Austin, 2016
Vibrio	*alginolyticus*	Bacteria	Zoea syndrome, septic hepatopancreatic necrosis, shell disease	coastal fish, shrimp	Austin & Austin, 2016
Vibrio	*anguillarum*	Bacteria	shell disease, zoonotic pathogen causes septicemia, vibriosis	shrimp, marine fish	Austin & Austin, 2016 Irshath et al., 2023
Vibrio	*campbellii*	Bacteria	Zoea syndrome, septicnecrosis, earth mortality syndrome	shrimp	Austin & Austin, 2016
Vibrio	*cholerae*	Bacteria	zoonotic pathogen causes septicemia, ascites disease	eel, catfish	Austin & Austin, 2016
Vibrio	*cincinnatiensis*	Bacteria	zoonotic pathogen causes	Atlantic salmon	Austin & Austin, 2016

			septicemia		
Vibrio	*fluvialis*	Bacteria	zoonotic pathogen causes septicemia, shell disease	shrimp, marine fish	Austin & Austin, 2016
Vibrio	*furnissii*	Bacteria	zoonotic pathogen causes septicemia	wild and farmed fish, mollusks, and crustaceans, eel	Austin & Austin, 2016
Vibrio	*harveyi*	bacteria	luminescent vibriosis, early mortality syndrome, acute hepatopancreatic necrosis, zoonotic causes septicemia, eye disease (blindness)	shrimp, marine fish	Austin & Austin, 2016
Vibrio	*logei*	Bacteria	luminescent vibriosis	shrimp	Austin & Austin, 2016
Vibrio	*mediterranei*	Bacteria	luminescent vibriosis	shrimp	Austin & Austin, 2016
Vibrio	*metschnikovii*	Bacteria	zoonotic pathogen causes septicemia	olive flounder, farmed fish	Austin & Austin, 2016
Vibrio	*mimicus*	Bacteria	Zoea syndrome, septic necrosis, shell disease	shrimp, grass carp, yellow catfish	Austin & Austin, 2016
Vibrio	*nigripulchritudo*	Bacteria	summer syndrome in grow out	shrimp	Austin & Austin, 2016
Vibrio	*ordalii*	Bacteria	zoonotic pathogen causes septicemia	shrimp and marine fish	Austin & Austin, 2016
Vibrio	*orientalis*	Bacteria	luminescent vibriosis	shrimp	Austin & Austin, 2016
Vibrio	*owensii*	Bacteria	early mortality syndrome	shrimp	Austin & Austin, 2016
Vibrio	*panaeicida*	Bacteria	summer syndrome in grow out	shrimp	Austin & Austin, 2016
Vibrio	*parahaemolyticus*	Bacteria	early mortality syndrome, Zoea syndrome, zoonotic septic necrosis, shell disease	shrimp, kelp grouper	Austin & Austin, 2016
Vibrio	*splendidus*	Bacteria	shell disease, luminescent vibriosis	shrimp	Austin & Austin, 2016
Vibrio	*vulnificus*	Bacteria	zoonotic septic necrosis, shell disease	shrimp and marine fish	Austin & Austin, 2016
Weissella	*ceti*	Bacteria	wieissellosis	rainbow trout	Austin & Austin, 2016

Yersinia	*ruckeri*	Bacteria	enteric redmouth disease, salmonid blood spot	salmonid fish	Austin & Austin, 2016 Irshath et al., 2023
Iridovirus		DNA virus	shrimp hemocyte iridescent virus	white leg shrimp, grouper, farmed fish	Crane & Hyatt, 2011
Ranavirus		DNA virus	lethargic and erratic swimming patterns	fish	Crane & Hyatt, 2011
Lymphocystivirus		DNA virus	tumor-like growth	fish	Crane & Hyatt, 2011
Megalocytivirus		DNA virus	systemic infections	wild and farmed freshwater and marine fish	Crane & Hyatt, 2011
Cyprinivirus		DNA virus	hemorrhagic disease	freshwater eels	Crane & Hyatt, 2011
Aphonomyces	*invadans*	Fungi	epizootic ulcerative syndrome	rainbow trout	Sarkar et al., 2022
Aphonomyces	*euteiches*	Fungi	epizootic ulcerative syndrome	rainbow trout	Sarkar et al., 2022
Aspergillus	*flavus*	Fungi	aspergillosis	spotted snakehead	Sarkar et al., 2022
Aspergillus	*fumigatus*	Fungi	aspergillosis	spotted snakehead	Sarkar et al., 2022
Aspergillus	*niger*	Fungi	aspergillosis	spotted snakehead	Sarkar et al., 2022
Cladosporium	*sphaerospermum*	Fungi	greenish fungal growth with a chalky white mat, kidney damage	red snapper	Sarkar et al., 2022
Exophiala	*angulospora*	Fungi	lesions that lead to a loss of body fluid	Atlantic cod	Gozlan et al., 2014
Fusarium	*oxysporum*	Fungi	mycosis	Nile tilapia, carp, eel, catfish, barb	Sarkar et al., 2022
Fusarium	*solani*	Fungi	mycosis	Nile tilapia, carp, eel, catfish, barb	Sarkar et al., 2022
Mucor	*circinelloides*	Fungi	disoriented swimming, lethargy, imbalance, and nonulcerative dermal masses	yellow catfish	Gozlan et al., 2014
Ochroconis	*humicola*	Fungi	dark pigmented patches	marine fish	Gozlan et al., 2014
Penicillium	*corylophilum*	Fungi	erratic behavior, anemia, skin	red snapper	Gozlan et al., 2014

			patches		
Phoma	*herbarum*	Fungi	abnormal swimming behavior, exophthalmia, multiple rounded areas of muscle softening, protruded hemorrhagic vents, and abdominal swelling	catfish, chinook salmon, Nile tilapia	Gozlan et al., 2014
Saprolegnia	*diclina*	Fungi	saprolegniosis	rainbow trout, freshwater fish, and crustaceans	Sarkar et al., 2022
Saprolegnia	*parasitica*	Fungi	saprolegniosis	rainbow trout, freshwater fish, and crustaceans	Sarkar et al., 2022
Saprolegnia	*salmonis*	Fungi	saprolegniosis	rainbow trout, freshwater fish and crustaceans	Sarkar et al., 2022
Sphareothecum	*destruens*	Fungi	the rosette agent; causes high rates of morbidity and mortality	freshwater fish	Gozlan et al., 2014
Achlya	*bisexualis*	Oomycetes	cotton-like growth of mycelia	flathead grey mullet	Gozlan et al., 2014
Achlya	*klebsiana*	Oomycetes	cotton wool-like lesions causing skin destruction	Nile tilapia, catfish	Gozlan et al., 2014
Achlya	*americana*	Oomycetes	cotton wool-like lesions causing skin destruction	whitefish	Gozlan et al., 2014
Achlya	*oblongata*	Oomycetes	cotton wool-like lesions causing skin destruction	whitefish	Gozlan et al., 2014
Achlya	*ambisexualis*	Oomycetes	cotton wool-like lesions causing skin destruction	rainbow trout	Gozlan et al., 2014
Aphanomyces	*parasiticus*	Oomycetes	ulcerative syndrome	whitefish	Gozlan et al., 2014
Aphanomyces	*frigidophilus*	Oomycetes	ulcerative syndrome	whitefish, brown trout	Gozlan et al., 2014
Aphanomyces	*invadans*	Oomycetes	ulcerative syndrome	various fish species	Gozlan et al., 2014

Alphanodavirus		RNA virus	covert mortality nodavirus	shrimp	Leong, 2008
Alphavirus		RNA virus	sleeping disease	salmonid fish	Bruno et al., 2006
Isavirus	*salmon isavirus*	RNA virus	infectious salmon anaemia virus	Atlantic salmon	Bruno et al., 2006
Novirhabdovirus	*oncorhynchus 2 novirhabdovirus*	RNA virus	viral hemorrhagic septicemia virus	rainbow trout, turbot, flounder	Bruno et al., 2006
Novirhabdovirus	*oncorhynchus 1 novirhabdovirus*	RNA virus	infectious hematopoietic necrosis virus	sockeye salmon, rainbow trout	Bruno et al., 2006
Tilapinevirus	*tilapia tilapinevirus*	RNA virus	tilapia lake virus	tilapia	Bruno et al., 2006
Vesiculovirus		RNA virus	blister-like lesions	various freshwater fish	Bruno et al., 2006

Table 2: Overview of the identified aquatic pathogens based on taxonomic groups. *The table provides a compilation of the reported pathogens found in both wild and aquaculture organisms. Pathogens are grouped based on their respective taxonomic groups. The reported disease or symptoms associated with each pathogen is provided. Please note that this table serves as a general overview and does not include an exhaustive list of all the reported pathogens or potential hosts*

I.2 What is Environmental DNA?

Environmental DNA (eDNA) denotes the genetic material obtained from environmental samples encompassing soil, water, or air. The concept of utilizing genetic material from such samples was initially introduced by Pace et al. (1986), who proposed that DNA extracted from the environment could be used to investigate the natural diversity of microorganisms. Initially, DNA extracted from environmental samples was described using terms such as "DNA isolated from environmental samples" (Somerville et al., 1989) or referred to the specific targeted organism, for example, "bacterial DNA" (Steffan et al., 1988) or "microbial DNA" (Paul & Myers, 1982). It was not until 1987 that Orgam et al. (1987) coined the term "environmental DNA."

The value of environmental DNA has been recognized in various scientific disciplines. Since the late 20th century, eDNA has been widely employed in microbial research, including parasitology (Bass et al., 2015) and microbial restoration ecology (Tessler et al., 2023). More recently, eDNA studies have expanded to encompass macrobial species such as amphibians (Ficetola et al., 2008) and fish (Sagova-Mareckova et al., 2021). However, microbial and macrobial eDNA fields have diverged in their definitions of eDNA. Microbial studies typically collect bulk samples containing intact organisms that are subsequently lysed to extract DNA. In contrast, macrobial studies view eDNA as DNA released or shed by macroorganisms obtained from environmental samples rather than whole organisms. This difference in perspective has led to an ideological shift between the two fields.

To address the ambiguity surrounding the terminology, there was a call to clarify the definition of "eDNA." In response, Taberlet et al. (2012) proposed the following definition: "DNA that can be extracted from environmental samples without first isolating any target organisms." This definition offers the advantage of not assuming the state of the sampled DNA (extracellular or intracellular, tissue fragments, gametes, etc.) and is not restricted to a specific taxonomic group. However, debate on what constitutes eDNA has persisted. In 2020, Pawlowski et al. (2020) suggested that the generic definition of eDNA, as originally formulated, should be employed, encompassing the DNA of all organisms present in environmental samples, including microbial, meiofaunal, and macrobial taxa. However, a two-tier approach for documenting the type of eDNA sample should be used, involving 1) the environmental origin of the sample (e.g., soil and water) and 2) the targeted taxonomic group (e.g., microbial). This metadata documentation is crucial for reader comprehension and future comparative studies.

As of 2023, two definitions of eDNA continue to be used in ecological studies: sensu *lato*, which is often used in global biodiversity surveys to analyze microbial, meiofauna, and macrofauna communities, and sensu *stricto*, which often refers to the extracellular DNA of macrobial organisms for conservation biology to monitor invasive and/or endangered species, as well as in ecology studies to survey biodiversity patterns (Pawlowski et al. (2020) for an in-depth explanation of the two).

In the context of this dissertation, the term eDNA is used according to the overarching definition

put forth by Taberlet et al. (2012), which states that eDNA encompasses all DNA that can be extracted from environmental samples. Furthermore, the two-tier approach recommended by Pawlowski et al. (2020) specifies the types of samples collected, and the targeted taxa were adopted and implemented.

I.2.1 How is eDNA collected and sequenced?

Comprehensive analysis of eDNA samples necessitates the implementation of a diverse set of protocols throughout the workflow, encompassing DNA capture, extraction, PCR amplification, sequencing, and data analysis (Figure 4). The initial step of eDNA analysis involves the collection of an environmental sample of interest. Common methods for aquatic sample capture include filtration, swabbing, and, more recently, passive collection techniques (Kirtane et al., 2020). Subsequently, DNA is extracted and purified to eliminate unwanted cellular components and potential inhibitors that may impede downstream processes. Various methods can be used for DNA extraction, each offering distinct advantages and disadvantages (**Chapter 5**). Following the successful extraction of genomic DNA, the DNA is ready for sequencing. However, the choice of the sequencing approach relies on the specific objectives of the project. If the aim is species-specific identification, DNA barcoding techniques (Hebert et al., 2003) can be employed to target specific genomic regions for classification. In contrast, if the objective is to assess the community composition, then community-based technology can be used, such as metabarcoding or shotgun metagenomics.

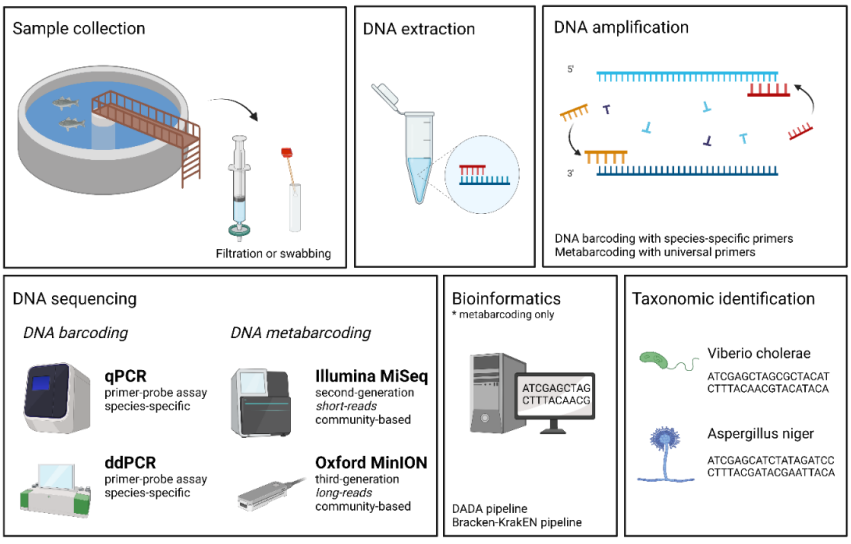

Figure 4: General flow of eDNA processing. *1) Sample collection: eDNA is captured via filtering or swabbing; 2) DNA extraction: DNA is extracted via column-based kits, phenol-chloroform, or silica beads; 3) DNA amplification and sequencing: DNA can be amplified with species-specific primers and sequenced with PCR-based technology or amplified with universal primers and sequenced with next- or third-generation sequencers; 4) Bioinformatics: metabarcoding approaches produce data that must be filtered through a bioinformatics pipeline to clean up the sequencing reads; and 5) taxonomic identification: the classification of present organisms within a sample.*

I.2.2 How is eDNA sequenced?

Environmental DNA (eDNA) analysis can be conducted through the utilization of a diverse array of sequencing methodologies. These encompass barcoding, metabarcoding, and shotgun metagenomics,

each representing distinct sequencing approaches aimed at the taxonomic classification of species. In the barcoding approach, species-specific primers are employed to facilitate the identification of either a single species or, in the case of multiplexing, a select few species when multiple primers are simultaneously employed.

Metabarcoding, on the other hand, extends the concept of barcoding by enabling the concurrent identification of numerous taxa within a single clade from a given environmental sample. This approach allows for a broader taxonomic scope and a comprehensive assessment of biodiversity within a specific group of organisms.

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I.2.2.1 Barcoding

DNA barcoding is a method of specimen identification using short, standardized fragments of DNA. This method utilizes species-specific assays to determine the presence or absence of a particular species within a sample (Hebert et al., 2003) and is commonly employed for bacteria monitoring (Nguyen et al., 2018; Lewin et al., 2020). However, if assays are not properly designed, off-target binding (i.e., primer binding to regions outside the intended target) to closely related species may occur, leading to false positives. Primer design for barcoding can be challenging and time-consuming; however, once designed, these primers can be used in any molecular laboratory to generate and interpret data (Lutz et al., 2020).

DNA barcoding sequencing relies primarily on PCR-based technology, which can be conducted in a conventional molecular laboratory or with field-portable devices (**Chapter 4**). This flexibility makes barcoding methods particularly suitable for biomonitoring programs aimed at monitoring opportunistic bacteria (Rieder et al., 2023), invasive species (Lutz et al., 2020; Bruce et al., 2021), or rare/elusive species (Rees et al., 2014). Traditional lab equipment includes polymerase chain reaction (PCR), quantitative PCR (qPCR), and, more recently, digital droplet PCR (ddPCR), all of which exhibit high sensitivity. However, qPCR and ddPCR methods offer advantages over traditional PCR as they eliminate the need for post-PCR procedures, such as gel analysis, provide quantitative data that are essential for assessing abundance, and can detect genes down to a few copy numbers (Orioles et al., 2022). In recent years, portable qPCR machines have been developed, eliminating the need for expensive infrastructure and equipment (**Chapter 4**). The portable qPCR device Biomeme Franklin has proven to be a valuable tool in monitoring programs for detecting *Flavobacterium psychrophilum* in a mesocolumn study (Nguyen *et al.*, 2018) and *Aphanomyces astaci* in Norwegian waterways (Sundell & Haukaas, 2023).

I.2.2.2 Metabarcoding

Metabarcoding is an amplicon sequencing technology that enables simultaneous sequencing and identification of numerous taxa within a sample (Taberlet, et al., 2012). This approach harnesses the capabilities of "universal" primers and DNA sequencing technologies to amplify sequence-specific DNA regions known as "barcodes." These barcodes are short DNA segments that are unique to a particular clade but possess sufficient variability for taxonomic classification. For instance, the 16S rRNA gene is commonly sequenced for bacterial classification, whereas the internal transcribed spacer (ITS) region is widely used to analyze fungal diversity. The resulting sequencing data generates millions of reads, which are subsequently filtered using a bioinformatics pipeline. Depending on the pipeline employed, this process yields either an operational taxonomic unit (OTUs) table (Blaxter et al., 2005) or an amplicon sequence variant (ASVs) table (Callahan et al., 2016). These tables serve as the basis for subsequent analyses of the community composition and diversity. Metabarcoding has revolutionized biodiversity monitoring and research by providing a powerful tool for assessing and comprehending the genetic diversity of complex ecosystems as well as enabling the classification of non-culturable microbial species.

I.2.2.3 Shotgun metagenomics

Shotgun metagenomics, a primer-free sequencing method, has emerged as a powerful technique for analyzing the genetic material of complex microbial communities. This approach has significantly contributed to our understanding of microbial composition in diverse environments, ranging from soil (Chen et al., 2022) and natural water bodies (Zhao et al., 2023) to artificial water-based systems (i.e., RAS) communities (Rieder et al., 2023) (**Chapter 3**). Unlike targeted approaches, shotgun metagenomics involves sequencing DNA fragments from the entire microbial community, e.g., bacteria, archaea, and viruses. Through computational reconstruction, sequencing reads are assembled to provide a comprehensive overview of the microbial community, including its genetic content, community structure, and diversity. Moreover, functional analysis (Chen et al., 2022) can be performed to identify genes and pathways associated with specific functions such as nutrient cycling, metabolism, and pathogenicity. This type of data provides insight into ecosystem processes, microbial interactions, and disease mechanisms.

Despite the numerous advantages offered by shotgun metagenomic sequencing, several limitations should be acknowledged. One prominent challenge is the substantial volume of data generated during sequencing, which necessitates substantial computational resources for data storage, processing, and analysis. These resources are often institutional or company-based and may not be readily accessible to the broader public. Furthermore, the complex nature of metagenomic data makes it difficult to accurately reconstruct genomes, particularly in scenarios with high microbial diversity, where identifying rare species becomes challenging. Additionally, low signal-to-noise ratios can

interfere with the precise differentiation of genetically similar species (Pust & Tümmler, 2021). Lastly, the presence of host DNA or contaminants in the sample can complicate the analysis and interpretation of results, necessitating the careful implementation of bioinformatics strategies (Liu *et al.*, 2021) to effectively remove unwanted reads and minimize bias in downstream analyses.

I.2.3 eDNA conclusion

In summary, the integration of eDNA and sequencing technologies has resulted in a revolutionary transformation in the field of molecular ecology. This combination has empowered researchers to explore microbial communities with unprecedented depth and scalability, thereby surpassing the limitations of traditional laboratory-based studies. Using these technologies, novel species have been discovered, intricate microbial interactions have been unraveled, and valuable insights into the functional capabilities of microbial communities have been gained. These advancements have been observed in diverse ecosystems spanning natural environments (Chevallereau et al., 2022; Yonathan et al., 2022) and engineered artificial systems (Maddela et al., 2022; Rieder et al., 2023). Furthermore, strategic integration of multiple sequencing methods can optimize the extraction of maximal information while minimizing costs (Rieder et al., 2023). While challenges persist, continuous technological advancements and software development are anticipated to overcome these hurdles. By harnessing the full potential of eDNA and sequencing technologies, our understanding of microbial ecology can be profoundly advanced.

I.3 What are the applications of eDNA in recirculating aquaculture systems?

The application of eDNA methods has significantly advanced our understanding of microbial ecology and the presence of pathogens in recirculating aquaculture systems (RAS). Although the application of eDNA microbial ecology in RAS is still in its early stages, previous investigations have shed light on several crucial aspects essential for sustainable management practices. Many studies have focused on exploring and expanding our understanding of microbial communities in relation to system and animal health (Almeida et al., 2021; Rieder et al., 2023) as well as highlighting the efficacy of eDNA as a powerful tool for the early detection of pathogens (Bastos Gomes et al., 2017; Bohara et al., 2022). The increasing integration of RAS into aquaculture practices further emphasizes the need for continued microbial ecology studies, as they play a vital role in deepening our knowledge of microorganisms within these systems.

The emergence of eDNA and advancements in sequencing techniques have revolutionized microbial ecology. These technological breakthroughs offer a comprehensive and high-resolution perspective of the entire microbial community. In the past, the study of biodiversity and ecological dynamics in recirculating aquaculture systems (RAS) was restricted to culturable organisms, providing an incomplete picture. However, by analyzing eDNA samples, researchers can now obtain a holistic

understanding of the microbial diversity, community structure, and functional potential within aquaculture systems. Previous studies have shown the distinctiveness of various aspects, including (1) the differentiation between water and biofilm communities (Rud et al., 2017; Rieder et al., 2023), (2) the spatial distribution of communities (Bartelme et al., 2019; Rieder et al., 2023), and (3) the provision of functional services, such as nutrient recycling (Hüpeden et al., 2020). By employing eDNA studies, targeted management strategies (Rurangwa & Verdegem, 2015; Bentzon-Tilia et al., 2016) can be developed, leading to optimized system designs that promote the growth of beneficial species while preventing the establishment of pathogenic organisms.

Environmental DNA has emerged as a valuable tool for monitoring and early detection of pathogens, aligning with the 3Rs goals of Replacement, Reduction, and Refinement in animal research. Routine collection and analysis of eDNA data can serve as an effective early warning system, eliminating the need for animal sacrifice in routine monitoring and promoting ethical practices while enhancing profitability. One notable advantage of eDNA is its flexibility in terms of sequencing platforms, each offering different levels of information (Rieder et al., 2023). Currently, barcoding is widely recognized as the gold standard for pathogen surveillance. These methods exhibit high versatility, allowing for both laboratory- and field-based processing (**Chapter 4**). Field-based studies can use lyophilized assays (Rieder et al., 2022) in conjunction with low-tech lateral flow strips, portable devices (Bastos Gomes et al., 2017; Biomeme, 2022), or high-tech automatic samplers for continuous monitoring, making them suitable for farms with varying budgets. Metabarcoding methods provide a community-level perspective (Rieder et al., 2023), enabling the detection of shifts in community structure that can serve as indicators of potential disease outbreaks (Peters et al., 2018), particularly when combined with water quality data (Nguyen et al., 2018). In contrast, metagenomic data offer valuable insights into the interactions between pathogens and their hosts (Amarasiri et al., 2021), elucidating the underlying mechanisms of pathogenicity, host specificity, and immune responses. These insights will contribute to the development of targeted therapies and vaccines, ultimately advancing the prevention and treatment of infectious diseases.

I.3.1 Application of eDNA sampling in RAS conclusion

In conclusion, the utilization of eDNA has significantly advanced our understanding of molecular ecology in aquaculture, offering a non-invasive, comprehensive, and high-resolution approach to studying microbial communities, exploring ecological interactions, and monitoring pathogens. The application of eDNA in research within RAS has already yielded valuable insights and will continue to enhance our understanding of the intricate dynamics within these systems. However, future investigations should prioritize the study of microbial interactions and functional services, particularly in the context of predictive modeling that can inform managers about the responses of these communities to environmental disturbances. By incorporating advanced knowledge and predictive models, scientists and managers can fully leverage eDNA data to develop effective and actionable microbial management strategies.

In conclusion, the utilization of eDNA has significantly advanced our understanding of molecular ecology in aquaculture, offering a non-invasive, comprehensive, and high-resolution approach to studying microbial communities, exploring ecological interactions, and monitoring pathogens. The application of eDNA in research within RAS has already yielded valuable insights and will continue to enhance our understanding of the intricate dynamics within these systems. However, future investigations should prioritize the study of microbial interactions and functional services, particularly in the context of predictive modeling that can inform managers about the responses of these communities to environmental disturbances. By incorporating advanced knowledge and predictive models, scientists and managers can fully leverage eDNA data to develop effective and actionable microbial management strategies.

Environmental Microbiome

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Metagenomics and metabarcoding experimental choices and their impact on microbial community characterization in freshwater recirculating aquaculture systems

Jessica Rieder[1,2,3], Adamandia Kapopoulou[2,3], Claudia Bank[2,3] and Irene Adrian-Kalchhauser[1*]

Abstract

Background Microbial communities in recirculating aquaculture systems (RAS) play a role in system success, nutrient cycling, and water quality. Considering the increasing socio-economic role of fish farming, e.g., regarding food security, an in-depth understanding of aquaculture microbial communities is also relevant from a management perspective, especially regarding the growth, development, and welfare of the farmed animal. However, the current data on the composition of microbial communities within RAS is patchy, which is partly attributable to diverging method choices that render comparative analyses challenging. Therefore, there is a need for accurate, standardized, and user-friendly methods to study microbial communities in aquaculture systems.

Results We compared sequencing approach performances (3 types of 16S short amplicon sequencing, PacBio long-read amplicon sequencing, and amplification-free shotgun metagenomics) in the characterization of microbial communities in two commercial RAS fish farms. Results showed that 16S primer choice and amplicon length affect some values (e.g., diversity measures, number of assigned taxa or distinguishing ASVs) but have no impact on spatio-temporal patterns between sample types, farms and time points. This implies that 16S rRNA approaches are adequate for community studies. The long-read amplicons underperformed regarding the quantitative resolution of spatio-temporal patterns but were suited to identify functional services, e.g., nitrification cycling and the detection of pathogens. Finally, shotgun metagenomics extended the picture to fungi, viruses, and bacteriophages, opening avenues for exploring inter-domain interactions. All sequencing datasets agreed on major prokaryotic players, such as *Actinobacteriota*, *Bacteroidota*, *Nitrospirota*, and *Proteobacteria*.

Conclusion The different sequencing approaches yielded overlapping and highly complementary results, with each contributing unique data not obtainable with the other approaches. We conclude that a tiered approach constitutes a strategy for obtaining the maximum amount of information on aquaculture microbial communities and can inform basic research on community evolution dynamics. For specific and/or applied questions, single-method approaches are more practical and cost-effective and could lead to better farm management practices.

Keywords 16S rRNA gene, Amplicon sequencing, Shotgun metagenomics, DADA2, ASVs, MiSeq, PacBio, Short-reads, Long-reads

*Correspondence:
Irene Adrian-Kalchhauser
irene.adrian-kalchhauser@unibe.ch
Full list of author information is available at the end of the article

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Introduction

Recirculating aquaculture systems (RAS) are a valuable alternative to the limited sustainable capacity of capture fisheries. They are discussed as a long-term sustainable offset for capture fisheries [1] and a means to meet the nutritional demand for high-quality animal protein. RAS cultivate freshwater species such as rainbow trout (*Oncorhynchus mykiss*), pike-perch (*Stizostedion lucioperca*), Arctic char (*Salvelinus alpinus*), and sturgeon (order *Acipenseriformes*) [2] and range from small privately-owned enterprises to industrial-sized corporations. The indoor, closed-circuit design of RAS provides independence from seasonal conditions, allows for biosecurity measures, and reduces the product-to-market distance when situated inland [3].

Microbial communities in RAS play a crucial role in overall system success, nutrient cycling, water quality, and animal health [1, 4–12]. These communities are often actively maintained in the biofilter section of the system, which is designed to maximize the surface area with sand, granulated active carbon, or synthetic carrier material. Biofilter microbial communities perform various services, such as removing toxic metabolic products (e.g., ammonia, nitrite, nitrate, sulfide, and sulfate) and organic waste. Some prominent representatives of the oxidizing ammonia genera found in RAS biofilters are *Nitrosomonas*, *Nitrosospharea*, and *Nitrosospira* [13], as well as ammonia-oxidizing archaea and *Nitrotoga* species [14, 15].

Conversely, pathogenic components of microbial communities in RAS constitute a significant challenge for the fish farm industry. Fish-related disease outbreaks threaten the livelihood of farmers and food security [16] and incur an estimated $6 billion loss yearly [17] due to stock loss. Also, water-associated off-flavoring bacterial groups may adversely impact the quality of the final product [13]. Different management approaches, such as cleaning and disinfection regimes, aim to reduce opportunistic pathogen species such as *Aeromonas* or *Flavobacterium* [18] but could potentially open niches for pathogenic species and promote undifferentiated microbial growth.

Managing microbial communities in RAS is not straightforward and poses complex challenges. It has been proposed that monitoring and targeted manipulation of RAS microbial communities, based on a thorough characterization of interactions and community dynamics, may improve aquaculture management strategies [19–21]. However, RAS microbial research lags behind compared to other microbe-dependent industries, such as wastewater treatment. Furthermore, the interactions between different compartments, management operations, microbial community structure, and how community assemblages differ across facilities are only beginning to be understood [1]. Previous microbial studies have analyzed the biofilter communities in RAS farming lumpfish (*Cyclopterus lumpus* L.) [8], Atlantic salmon (*Salmo salar*), Pacific white shrimp (*Litopenaeus vannamei*), half-smooth tongue sole (*Cynoglossus semilaevis*) and turbot (*Scopthalmus maximus*) [22], but have not investigated other RAS compartments. Furthermore, inter-study comparisons are problematic because non-standardized protocols (e.g., DNA extraction, amplification, or taxonomic assignment) impact the results and conclusion [23–27]. Lastly, global studies are scarce [28], so the characterization of RAS microbial community patterns and keystone taxa remains incomplete.

In recent years, next-generation sequencing technology has led to various methods by which microbiomes can be studied. Three commonly used methods are short- and long-read sequencing, targeting the 16S gene, and shotgun metagenomics, which targets all sequences within a sample. Short-amplicon sequencing requires primers that may target one or multiple variable regions of the genes. The major drawback of short-amplicon sequencing is the lack of resolution required for species identification. Also, primer choice can introduce biases for or against certain taxonomic groups [9, 23, 29]. Long-amplicon sequencing targets all variable regions of the 16S gene, thus increasing resolution for species identification and eliminating primer choice biases. Unfortunately, both short- and long-amplicon 16S sequencing mainly target bacteria and omit other microbes, such as fungi and archaea. Shotgun metagenomics, a primer-free method, targets all sequences within a sample, allowing for the identification of all organisms present at a sufficient frequency. However, low signal-to-noise ratios may interfere with the species-level differentiation of genetically similar species. Recent studies have started combining different sequencing approaches to reduce sequencing costs, increase resolution, and gain broader knowledge than any singular method could provide [30, 31].

This study investigates the effect of sampling and analysis strategies on the inference of microbial community composition in RAS. We collected samples from two freshwater RAS to compare the ability of four primer sets, a primer-free approach, and three sequencing approaches (Fig. 1) to identify key microbial dynamics and improve future sampling and methods decisions. First, we show that primer-specific results at early analysis steps do not lead to distinct biological conclusions. Second, we demonstrate that 16S short-read sequencing is sufficient to detect spatio-temporal developments and dynamics in the context of a RAS system. Finally, we evaluate the ability of the different sequencing approaches to describe the spatio-temporal patterns and identity of microbials in

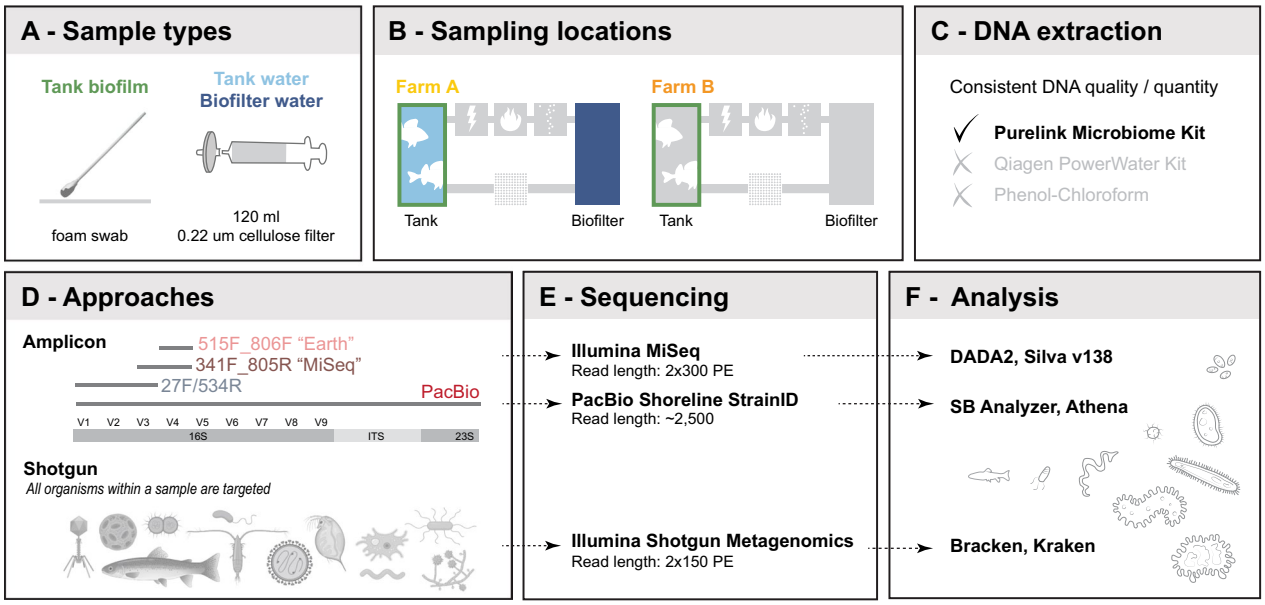

Fig. 1 Study design and experimental steps. **A** Three types of samples were taken. Tank biofilm was collected by rubbing a foam swab against the sidewall of a tank. Tank and biofilter water was collected by filtering 120 ml of water through a 0.22 um cellulose filter. **B** Samples were taken at two farms, A and B. In farm A, all three sample types were collected. In farm B, sampling focused on tank biofilm. **C** Three DNA extraction methods were compared. The Purelink Microbiome Kit outperformed other DNA extraction methods in quality, quantity, and consistent yield. **D** Two amplicon approaches (short and long reads) and an amplification-free shotgun approach were used. **E** Short amplicons were sequenced on an Illumina MiSeq with a v3 2 × 300PE kit. Long amplicons were sequenced with the PacBio Shoreline StrainID kit, producing an average read length of 2500 bp. Shotgun sequencing was performed with a 2 × 150PE Illumina kit. **F** Illumina MiSeq sequencing data were processed with the DADA2 pipeline, and ASVs were blasted against the SILVA v138 database for taxonomic assignment. PacBio long-read data was processed with the SBAnalyzer program and taxonomically assigned using Athena. Shotgun metagenomics sequencing data were processed with an in-house pipeline that uses the Kraken-Bracken method

different compartments of RAS, followed by a discussion of the distinct value of each sequencing approach for different research questions and farm management.

Materials and methods

Sampling sites

The study includes two commercial-size Swiss RAS farms (A and B) with distinct ownership and operational management procedures. Farm A breeds perch (*Perca fluviatilis*), raising offspring from egg to approximately 15 g and features several life-stage-specific circuits with independent filtration systems. Fish are moved to the next circuit when they reach a certain cutoff weight. Fish of approximately 10–15 g are raised in 13.2 m^3 tanks at a stocking density of around 30 kg/m^3. After each batch, a stringent disinfection regimen is applied. First, the biofilter is disconnected from the circuit to protect the microbial community from disinfection solutions. Next, the tanks are emptied, followed by a four-step cleaning regimen, (1) a high-power jet wash with hot water, (2) brushing down the tank walls and floor with soap, (3) a static acid–base treatment of the tanks and pipes with neutralizing steps in between, and (4) spraying the tanks with alcohol. Finally, the tanks are dried entirely before refilling and restocking the next batch of fish. Farm A uses multiple feed brands depending on the life stage of the fish (Bernaqua, Bio-Mar, and Alltech Coppens).

Farm B is situated > 100 km from Farm A in a different catchment. Farm B raises two fish species: perch, obtained from Farm A at around 15 g, and pike-perch (*Sander lucioperca*), obtained at the fingerling stage from another provider. Both species are raised to slaughter weight within a single circuit in concrete tanks (120 m^3). The stocking density varies between 30 and 60 kg/m^3 based on the size of the fish. Cleaning regimens are applied once a tank is emptied. However, there is no strict cleaning disinfection timeline because of grading and moving the fish into new tanks, which might already be occupied. The disinfection protocol consists of (1) washing the empty tank with high-pressure hot water and (2) spraying Virkon S as a disinfection solution, followed by refilling with water and stocking with the next batch of fish. Farm B feeds with Alltech Coppens Supreme pellets of varying sizes according to fish size. Both farms use agitated biofilters with floating plastic biofilter carriers to supply the necessary surface area to

foster microbial communities. Farm identities and locations are confidential.

Sample types

Three sample types were collected: tank biofilm, tank water, and biofilter water (Fig. 1A). First, biofilm samples were collected with a sterile, single-use foam swab (Merck—product was discontinued) by rubbing one side of the swab back and forth approximately ten times across a ~10 × 10 cm area of the tank wall about 6 cm below surface water level and repeating the procedure on the same area with the other side of the swab. After swabbing, the swab was placed into a 2 ml Eppendorf tube, the stick was broken off, and the closed 2 ml tube was stored on ice. Biofilm replicates were taken with an approximately 2 cm gap between them. Next, using a sterile 500 ml plastic beaker, 500 ml of water were collected from the same tank as the tank biofilm sample, followed by on-site filtering of 120 ml of water using a 60 ml sterile, single-use syringe (Faust) and a 0.22 um mixed cellulose filter (Millipore, Merck) contained in a Whatman 47 mm plastic filter holder (Whatman, Merck). Replicates were taken from the same beaker, thoroughly mixing the water before the replicate was sampled. After filtration, the filters were placed in a 2 ml Eppendorf tube and stored on ice. Finally, biofilter water samples were collected, with a new sterile beaker, in the same way and from the same circuit as tank water and biofilm samples. All samples were transported back to the Institute for Fish and Wildlife Health, University Bern, on ice and stored at − 80 °C until further processing.

Sampling scheme

The sampling scheme aimed to maximize insights into differences and similarities between replicates, sample types, time points, analysis methods, within-farm compartments, and farms. In Farm A, two sampling events on different dates occurred in the circuit that houses 12–15 g perch. The first sampling event took place on June 25th, 2020 and consisted of collecting tank wall biofilm (samples 4–6), tank water from the same tank as the biofilm (samples 7–9), and biofilter water from the same circuit (samples 10–12). The sampling took place less than a week after the last tank cleaning. A second sampling took place on November 4th, 2020 and involved the collection of tank wall biofilm (samples 1–3), from a second tank within the same circuit, several weeks after the last cleaning of the tank. In farm B, sampling took place on November 23rd, 2020, that consisted of collecting tank wall biofilm from two tanks (samples 13–15 (tank 1) and 16–18 (tank 2)). Negative control samples were collected for the June 25th, 2020, sampling event but were not sequenced. The negative water control was filtered the same way as the on-site water samples, using distilled water instead of system water. The negative swab sample consisted of unpacking a swab on-site and placing it into a 2 ml tube without swabbing a surface. An overview of all samples is provided in Additional file 1.

DNA extraction

Three DNA extraction methods were tested on pre-trial water and swab samples for optimal and consistent DNA yield and quality (Fig. 1C) because suboptimal lysis conditions can introduce stochastic bias against gram-positive bacteria, which have a thick, difficult-to-lysis outer wall. Tests included (1) the Purelink Microbiome DNA Purification Kit (Thermofisher), which is optimized for microorganism lysis, (2) the DNeasy PowerWater Kit (Qiagen), which is optimized for the isolation of genomic DNA from filtered water samples, and (3) phenol–chloroform extraction, which has been shown to produce high DNA yield from environmental samples [32]. The PowerWater kit produced inconsistent yields (results not shown), whereas the Phenol–Chloroform approach produced higher DNA yield but was contaminated by phenol carry-over, resulting in low DNA purity. The Purelink Microbiome kit consistently produced the highest quality and yield and was subsequently used for the study. Before extraction, frozen filters were crushed in a 2 ml Eppendorf tube with sterile 1000 ml pipette tips, increasing exposure to the lysis buffer. Bead-beating was performed in a TissueLyser set to full speed for 10 min per the manufacturer's instructions.

Sequencing

Short amplicon

The performance of four amplicon-based 16S-targeting approaches was compared regarding amplification, read quality, and taxonomic and biological conclusions (Fig. 1D). Three amplicons designed for short-read Illumina sequencing included 16S variable regions V4 (primers 515F + 806R, hereafter referenced as "Earth"; [33]), V3-4 (primers 341F + 805R, hereafter referenced as "Miseq"; [34], and V1-3 (primers 27F [35] + 534R [36]; hereafter referenced as "27F_534R"; Table 1). One amplicon designed for long-read PacBio sequencing with the Shoreline StrainID kit included 16S, ITS, and 600 bp of the 23S gene [37]; Table 1). The Shoreline Complete StrainID kit uses a patented StrainID primer set.

Optimal amplification conditions suitable for all three short amplicons were determined by gradient PCR and reducing cycle number as much as possible. The PCR included 12.5 µl of KAPA HiFi HotStart Ready Mix (Roche, Switzerland), 5 µl of each primer (0.2 µM stock concentration), and 12.5 ng of DNA plus water to a total volume of 25 ul. PCR cycling

Table 1 Primers used in this study

Targeted region	Short-hand	FW primer	FW primer sequence	FW primer length	FW melting temperatures	REV primer	REV primer sequence	REV primer length	REV melting temperatures	Amplicon length	Primer target species
16S: V3-4	MiSeq	341F	5′-CCT ACG GGN GGC WGC AG-3′	17 bp	58–60 °C	805R	5′-GAC TAC HVG GGT ATC TAA TCC-3′	21 bp	52–60 °C	~ 465 bp	Bacteria
16S: V4	Earth	515F	5′-GTG YCA GCM GCC GCG GTA A-3′	19 bp	64–68 °C	806R	5′-GGA CTA CNV GGG TWT CTA AT-3′	20 bp	54–58 °C	~ 300 bp	Bacteria & Archaea
16S: V1-3	–	27F	5′-AGA GTT TGA TCC TGG CTC AG-3′	20 bp	58 °C	534R	5′-ATT ACC GCG GCT GCT GG-3′	17 bp	56 °C	~ 500 bp	Bacteria
16S-ITS-23S	PacBio	–	Proprietory	–	–	–	Proprietory	–	–	2500 bp	Bacteria

Illumina MiSeq forward and reverse primers carried overhang adapters (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-Forward primer, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-Reverse primer) for compatibility with Illumina index and sequencing adapters

FW Forward primer, *REV* Reverse primer, *bp* Base pairs

numbers (14, 16, 18, 20, 22, and 25) were tested at annealing temperatures between 54 and 58 °C for all three primer pairs. Based on agarose gel electrophoresis evaluations of amplification success, the following protocol was derived: initial denaturation at 95 °C for 3 min, 20 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 20 s), and final elongation at 72 °C for 5 min. In addition to all samples, four positive controls (Zymobiomics microbial community standard (Zymo Research)) were amplified with this protocol. In addition, samples 19–21 were introduced at this step and are technical PCR-level replicates of sample 2 amplified with Earth primers. Notably, sample 19 yielded no sequencing data.

The preparation of 16S rRNA gene amplicons for the Illumina MiSeq System was designed and performed at the Next Generation Sequencing Platform, University of Bern, according to the "16S Metagenomic Sequencing Library Preparation" protocol (Illumina, art #15,044,223 Rev. B). The quantity and quality of the cleaned amplicons were assessed using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32854) and an Agilent Fragment Analyzer (Agilent) with an HS NGS Fragment Kit (Agilent, DNF-474), respectively. Next, the index PCR step was performed as in the protocol except using IDT for Illumina DNA/RNA UD Indexes Set A (Illumina, 20,027,213), MyFi Mix (BIOLINE, BIO-25050) and the inclusion of a no template control (NTC). Then the amplicon libraries were assessed for quantity and quality, as described above, using fluorometry and capillary electrophoresis. The remainder of the protocol was followed, except that the library pool was spiked with 10% PhiX Control v3 (Illumina, FC-110-3001) to compensate for reduced sequence diversity. Finally, the library was sequenced at 2 × 300 bp using a MiSeq Reagent Kit v3, 600 cycles (Illumina, MS-102-3003) on the MiSeq sequencing instrument. The run was assessed using Illumina Sequencing Analysis Viewer 2.4.7. We used Illumina bcl2fastq conversion software v2.20 to demultiplex the library samples and convert generated base call files into FASTQ files. Short-read sequencing, before filtering, resulted in a total of 4,808,910 (27F_534R, samples only), 4,816,559 (Earth, samples only), and 5,149,263 (MiSeq, samples only) reads. Read numbers at all filtering steps are available in Additional file 1.

Raw data from Illumina amplicon sequencing were uploaded to the SRA NBI databank. Project ID and accession codes are documented in the "Availability of data and material" section.

Long amplicon

Long amplicon PacBio sequencing was performed at the Next Generation Sequencing Platform, University of Bern. The quantity and quality of the extracted DNA were assessed using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32854) and an Agilent Femto Pulse system with an Ultra Sensitivity NGS kit (Agilent, FP-1101), respectively. The DNA was then amplified using dual-unique barcoded primers targeting 16S-ITS-23S, using the StrainID kit from Shoreline Biome using strain ID Set Z, Barcodes T1-T16 (Shoreline Biome, STRAIN-Z-SLB). This approach involves a single-step PCR, consisting of primers containing the barcode and target-specific primer, generating amplicons ready for SMRTbell template prep and subsequent sequencing on the PacBio Sequel System. The protocol from input DNA to SMRT sequencing was followed according to the Shoreline Wave for PacBio Technical Manual, following all parameters for the Strain ID workflow. As well as the input DNA of interest, a no template control (NTC), and two community controls (ZymoBIOMICS Microbial Community DNA Standard and ZymoBIOMICS Microbial Community DNA Standard II (Log Distribution) (Zymo Research, D6305 and D6311, respectively) were included. The generated library was SMRT sequenced using a Sequel binding plate 3.0 and a sequel sequencing plate 3.0 with a 10 h movie time on a PacBio Sequel system on their own SMRT cell 1 M v3. The library was loaded at 9 pM and generated 15 Gb and 284,296 HiFi reads.

Raw data from PacBio amplicon sequencing were uploaded to the SRA NBI databank.

Shotgun metagenomics

Illumina shotgun metagenomics sequencing was performed at the Next Generation Sequencing Platform, University of Bern. The extracted DNA was assessed for quantity, purity, and length using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32854), a DeNovix DS-11 FX spectrophotometer, and an Agilent FEMTO Pulse System with a Genomic DNA 165 kb Kit (Agilent, FP-1002-0275), respectively. Sequencing libraries were made using an Illumina DNA Prep Library Kit (Illumina, 20,018,705) in combination with IDT for Illumina DNA/RNA UD Indexes Set B, Tagmentation (Illumina, 20,027,214) according to the Illumina DNA Prep Reference Guide (Illumina, 10,000,000,254 16v09). Six PCR cycles were employed to amplify 30 ng of tagemented DNA. Pooled DNA libraries were sequenced paired-end on a NovaSeq 6000 SP Reagent Kit v1.5 (300 cycles; Illumina, 20,028,400) on an Illumina NovaSeq

6000 instrument. The run produced, on average, 159 million reads/sample. The quality of the sequencing run was assessed using Illumina Sequencing Analysis Viewer (Illumina version 2.4.7) and all base call files were demultiplexed and converted into FASTQ files using Illumina bcl2fastq conversion software v2.20.

Raw data from Illumina shotgun metagenomics sequencing were uploaded to the SRA NBI databank.

Read processing

Short amplicon

Illumina short-reads were processed with the DADA2 v. 1.14.1 (Divisive Amplicon Denoising Algorithm 2) [38] pipeline. The DADA2 pipeline includes the inspection of read quality, quality filtering and trimming of reads, dereplication and error rate learning, sample inference for the determination of true sequence variants, merging of reads, construction of sequence table, removal of chimeric reads, and taxonomic assignment. Each primer dataset (Earth, MiSeq, 27F_534R) was first run independently through the DADA2 pipeline, then the Earth and MiSeq fastq files were combined into one file, which was processed with DADA2 ("Combined dataset"). Primers were removed with the DADA2 *trimLeft* function: *trimLeft* = *c*(19, 20) for Earth primers, *trimLeft* = *c*(17, 21) for MiSeq primers, and *trimLeft* = *c*(20, 17) for primer pair 27F_534R. Base pairs with a quality score below 30 at the end of the read were removed using the DADA2 *trimRight* function: *trimRight* = *c*(10, 90) for Earth and MiSeq primers and *trimRight* = *c*(30, 100) for primer pair 27F_534R, based on visual inspection of the quality plots (Additional file 1). All other *filterAndTrim* parameters were set at the default values. The DADA2 function *mergePairs* was applied in the individual and combined datasets to align the denoised forward reads with the reverse complement of the corresponding denoised reverse reads, producing a merged "contig" sequence. By DADA2 defaults, merged sequenced are only output if the forward and reverse reads overlap by at least 12 bp and are identical in the overlapped region. Unfortunately, for primer pair 27F_534R, after the removal of bp with a quality score less than 30 merging the noised forward reads and the reverse complement of the corresponding denoised reverse read was not possible as too many base-pairs were removed. For the remove bimera denova step, the *minfoldParentOverAbundance* parameter was set to 5 for individual datasets and 8 for the combined dataset. The naïve Bayesian classifier method was used for all datasets, with the default *minboot* = 50 (bootstrap confidence values: Additional file 2).

After DADA2 filtering, the datasets retained the following amount of reads 3,195,326 (66.4% average) for the 27F_534R dataset, 4,044,627 (average 83.8%) for the Earth dataset, 4,212,750 (81.7%) for the MiSeq dataset, and 8,344,294 (81.5%) (per primer: Earth: 4,128,007 and MiSeq: 4,216,287) for the Combined dataset (Additional file 1). Reads from the technical samples (20 and 21) and mock communities were removed from the total amount of reads reported above.

Individual datasets were used to quantify individual primer pair read quality, while the Combined dataset was used to quantify alpha and beta diversity, technical replication reproducibility, MDS analysis, and enriched ASVs. Sequencing quality was analyzed using the percentage of reads with a Phred score equal to or larger than 30 for each sample type and primer. Microbial taxonomic alpha-diversity (intra-sample) was calculated using Richness and Shannon indices as implemented in the R package phylsoseq [39]. Species beta-diversity (inter-sample) was estimated using the Bray–Curtis dissimilarity metric, while the dissimilarity between groups was visually assessed with multidimensional scaling (MDS) plots.

Long amplicon

PacBio Shoreline long reads were demultiplexed without primer trimming, palindromes were removed, and reads with lengths smaller than 200 base pairs were filtered out using the SBAnalyzer software (Shoreline Biome).

Shotgun metagenomics

Illumina shotgun metagenomics reads were high quality, requiring no filtering.

Taxonomic assignment

Short amplicon

Short-read data was assigned to taxonomic units with the SILVA v.138 gene reference database. After DADA2 processing, the Earth dataset contained 10,941 ASVs, with 196 ASVs assigned to the mock community sample and 3 ASVs assigned to both the mock community and samples. Of the 10,742 ASVs found within the samples, 10,501 were assigned to Bacteria, 14 to Archaea, 57 to Eukaryota, and 170 could not be assigned. For the MiSeq dataset, 6102 ASVs remained, with 20 ASVs assigned to the mock community and 2 ASVs assigned to both the mock community and samples. Of the 6080 ASVs found within samples, 6095 were assigned Bacteria, 2 to Archaea, 2 to Eukaryota, and 3 could not be assigned. For the combined dataset, 18,072 ASVs remained, with 236 ASVs assigned to the mock community and 3 ASVs assigned to both the mock community sample and samples. Of the 17,833 ASVs found within the sample, 17,822 were assigned to Bacteria, 16 to Archaea, 61 to Eukaryota, and 173 could not be assigned (Additional file 2).

Sample data were managed using the R package *phyloseq* (v1.30.0) (McMurdie and Holmes, 2013), and plots were generated using the R package *ggplot2* (v.2.2.1) [40].

Long amplicon

Long read data were taxonomically assigned with the Athena database v2.2, resulting in 99.3% of reads successfully classified (196,749 reads). An abundance table, a taxonomic classification list for each species, and a list of samples assigned to each read were created (Additional file 3). The initial goal was to compare output after running short- and long-reads through the DADA2 pipeline. However, the low read depths of the samples due to the mock community sample vastly outnumbering the samples during sequencing made this approach no longer possible. Therefore, the abundance table was analyzed manually for the spatial distribution of species.

Shotgun metagenomics

The raw reads of the metagenomics samples were classified according to their taxonomy using kraken2 [41]. This software classifies reads according to their best matching location in the taxonomic tree. Bracken was used to estimate the species abundance [42], using the taxonomy labels assigned by kraken2 to estimate the number of reads originating from each species present in the sample.

Data analysis

Short amplicon

Read quality was assessed based on the percentage of reads with a Phred score greater than 30 for each primer.

Microbial taxonomic alpha-diversity (intra-sample) was evaluated with the Richness and Shannon indices implemented in the *microbiome* R package [43]. Species beta-diversity (inter-sample) was estimated with Bray–Curtis distances, using the ordinate function in the *phyloseq* package, to understand similarities and differences in community composition independent of primer choice, within-farm compartments, farm identity, and time point in the production cycle. The dissimilarity between samples was assessed by multidimensional scaling (MDS).

Community composition was analyzed between primers, replicates, sample types, and farms by comparing the relative abundance of the top 9 phyla, all other phyla (Other), and not assigned (NA).

ASV enrichments were analyzed with a PERMANOVA non-parametric multivariate test using the adonis function in the R package *vegan* (v.2.5.7) [44] to determine which ASVs were significantly enriched between tank samples of farm A and between farms. The top 20 enriched ASVs coefficients were plotted.

All analyses were completed in RStudio 1.4.1717 [45].

Long amplicon

The ten most abundant species were identified for each sample type per farm based on the total number of reads after both replicate reads were summed together. Abundance was compiled and plotted for these species to understand spatial and abundance distribution across sample types and farms. Markedly, some replicates have less than ten dots because the top species was only detected in one replicate.

Shotgun metagenomics

Phyla with at least 0.5% or more of the total reads were retained to analyze the overall community composition. A Sankey plot using the R *network3D* v.0.4 package [46] was plotted to compare the community composition across the domains. In addition, relative abundance bar graphs were plotted to quantify community composition variance at the replicate, sample type, and within-farm compartments.

All figures were prepared for publication using Adobe Illustrator 2021.

Results

We used a tiered sequencing approach to analyze RAS microbial communities. Therefore, the results obtained from each sequencing dataset cannot be compared directly but complimentarily. Combining the datasets offers a more profound knowledge of the RAS than any one sequence approach could accomplish.

Short amplicon

Read quality

The overall read quality was satisfactory, with Earth, MiSeq, and 27F_534R primers producing Phred scores ≥ 30 for 89.3%, 86.6%, and 78.5% of reads, respectively (Fig. 2A). However, the lower read quality and the longer amplicon length of primer pair 27F_534R led to difficulties merging the forward and reverse reads using the merge function. Therefore, we decided to remove this primer from downstream analyses as it could not be processed in the same fashion as the other two primers.

Taxonomic assignment

Regarding taxonomic assignment, Earth and MiSeq amplicons performed similarly at a higher-level classification (e.g., phylum, order) but diverged at a lower-level classification (e.g., ASV). The Earth dataset identified 37 phyla, whereas the MiSeq dataset identified 34 phyla. However, the MiSeq dataset assigned 99 more genera at the genus level than the Earth dataset (470 vs. 371, respectively) (Additional file 2). Although the MiSeq

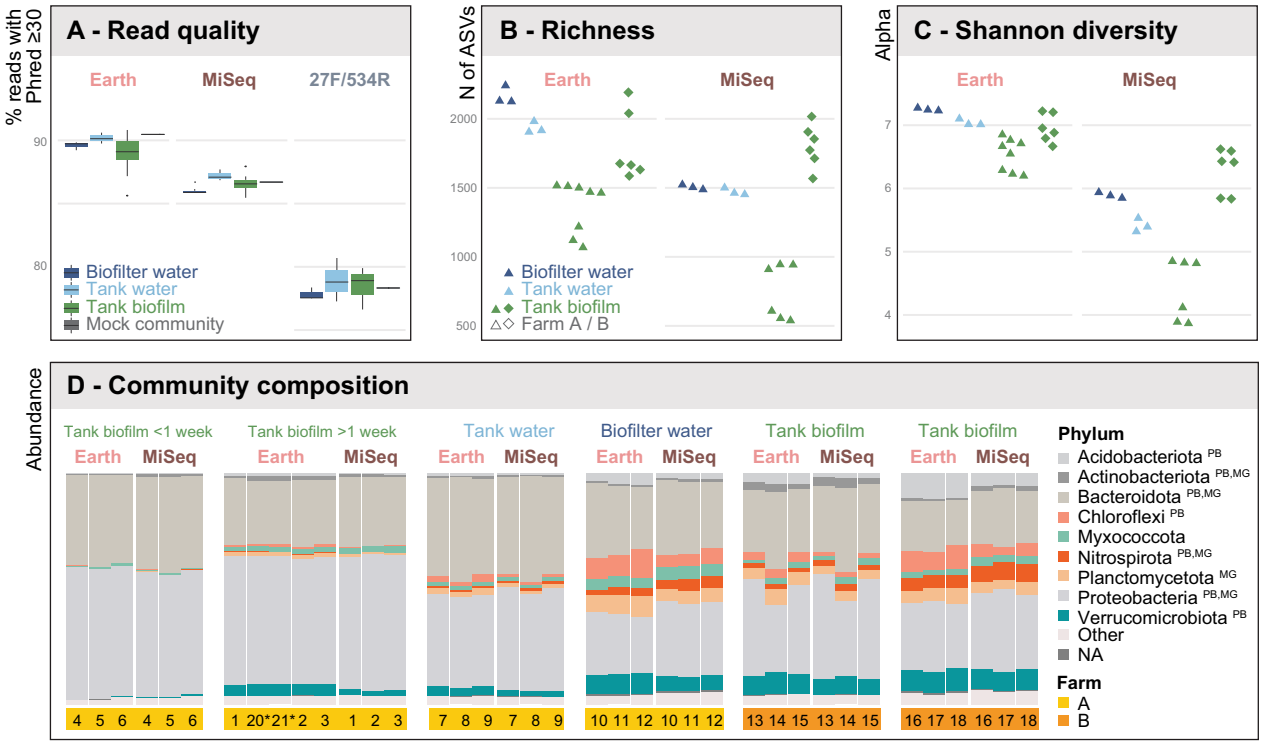

Fig. 2 16S primer choice affects sequencing quality and diversity measures but not community-level results. **A** Read quality. Earth and MiSeq primers yielded reads with high sequencing quality, whereas primer set 27F/534R yielded lower read quality and was excluded from downstream taxonomic analyses. Between sample types, read quality was comparable for each primer set. **B** Richness. Earth primers yielded higher alpha richness based on ASVs across all sample types. In farm A, biofilter water featured the most ASVs, followed by tank water and, finally, tank biofilm. Richness in farm B's biofilm was as high (Earth) or higher (MiSeq) than farm A's biofilter water. **C** Diversity. Samples amplified with Earth primers displayed higher alpha diversity (Shannon index) than samples amplified with MiSeq primers. Patterns are overall similar to richness (panel B). The similar diversity patterns suggest that it would be possible to compare community studies using different primers at the relative scale. **D** Community composition. The biological and technical replicates (samples 2, 20–21) were highly similar in composition, suggesting that primer selection does not impact spatio-temporal findings at the phyla level and indicates that reproducible results can be obtained with short amplicon sequencing. Indications for succession can be seen in the tank biofilm samples, with increasing complexity from young to older biofilm. Finally, Farm B's tank biofilm samples resembled Farm A's biofilter water samples, suggesting that microbial communities with RAS become similar in complexity over time, potentially reaching a stable, mature state. Abbreviations after the phylum name indicate that the phylum was detected in other platform datasets; PB = PacBio, MG = Metagenomics

primers could identify more taxa, Earth primers resulted in higher alpha diversity, both for richness (Earth: ranged: 1070–2240 compared to MiSeq: ranged 441–1962) and Shannon diversity (Earth: ranged: 6.12–7.32 compared to MiSeq: ranged 3.83–6.18) (Fig. 2C; Additional file 3). Within farm A, alpha richness was highest in biofilter water (Earth average: 2166 and MiSeq average: 1504), followed by tank water (Earth average: 1934 and MiSeq average: 1477) and tank biofilm, which was influenced by the age of the biofilm (Earth average: young 1135 vs. mature 1497 and MiSeq average: young 465 vs. mature 829). Within farm B, the tank biofilm average richness was similar between the two tanks (Earth: tank1 1793 vs. tank2 1805, MiSeq: tank1 1665 vs. tank2 1815). The Shannon diversity between sample types within farm A mirrored the pattern of richness, with biofilter water having the highest average diversity (Earth: 7.28, MiSeq: 5.88), followed by tank water (Earth: 7.07, MiSeq: 5.42), and the different aged biofilm samples (Earth: young 6.21 vs. mature 6.72, MiSeq: young 3.98 vs. mature 4.83). Farm B's tank biofilm samples had similar average Shannon diversity values (Earth: tank1 6.91 vs. tank2 7.10, MiSeq: tank1 5.59 vs. tank2 6.08) (Additional file 3).

Community patterns

Amplicon choice did not affect the composition of the microbial community at higher taxonomic levels. Community composition for distinct sample types, replicates, and the derived spatio-temporal patterns were very similar between the two amplicons (Figs. 2D and 3A, B). Subtle biases for/against specific phyla (e.g., *Chloroflexi*, favored by Earth; *Myxococcota* and *Plantomycetota*, favored by MiSeq; Fig. 2D) did not affect the inferred overall community structure, which was virtually

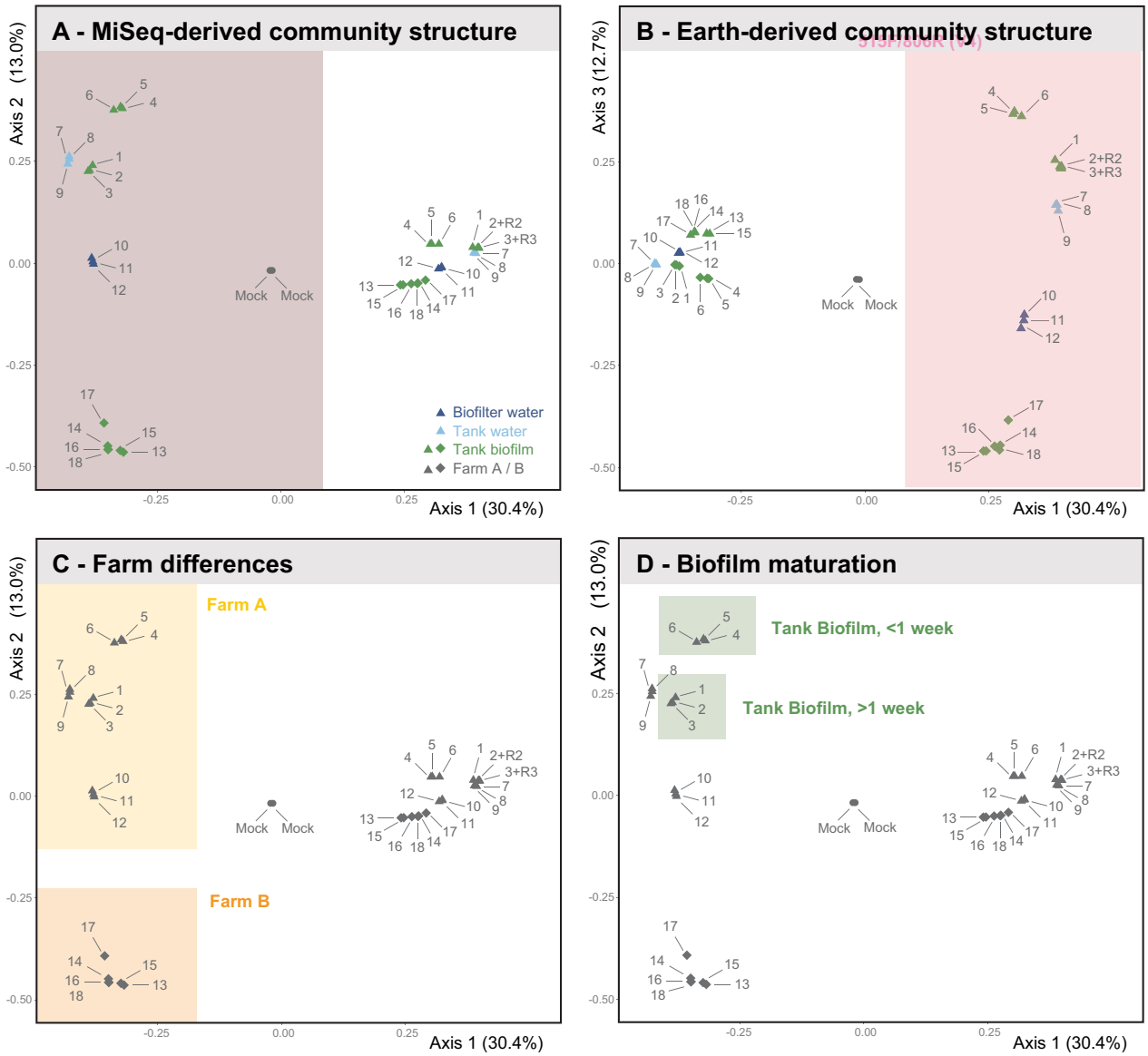

Fig. 3 Impact of primers, sample types, farms, and production cycles on community patterns derived from 16S sequencing. Multidimensional scaling analysis using the Bray–Curtis distance matrix is visualized with MDS plots at the phylum level. **A** MiSeq primers. Axes 1 and 2 achieve a clear separation of all samples. Replicates cluster together closely to the point of overlapping, whereas sample types, compartments, and farms form separate clusters. **B** Earth primers. Earth primers achieve an identical overall pattern but in a distinct area of the morphospace. The mock communities cluster in the exact location independently of primer choice, further stressing the equivalence of primers at this level of analysis. **C** Farms. Farms separate along axis 2. Panel C visualizes this for MiSeq primers, but the pattern holds with Earth primers (Panel B). **D** Tank and Time. Both primers can distinguish the biofilm samples collected in farm A from two tanks of different operational times but within the same circuit, emphasizing that a short-read approach is sufficient to achieve fine-scale resolution at the community level

identical for both amplicons according to MDS analyses (Fig. 3A and B).

As revealed by the MDS analysis, multiple factors influence the community patterns, with environmental farm conditions being the primary driver (Fig. 3C), followed by sample type (Fig. 3A and B). Sample types featured distinct community compositions, but the same sample types did not necessarily cluster together (e.g., tank and biofilter water vs. biofilm). For example, farm B's tank biofilm was more similar to farm A's biofilter water than farm A's tank biofilm samples. Biofilm age also drove differences between community richness and dominating genera (Figs. 2D and 3D), with the young vs. mature biofilm consisting of 108 vs. 152 genera (Earth) or 126 vs.

190 genera (MiSeq), respectively. Upon further inspection, farm B's tank biofilm included 288 and 356 genera, whereas farm A's tank biofilm included 166 and 462 genera for Earth and MiSeq, respectively (Additional file 4).

Enriched ASVs

The differential enrichment of specific ASVs further drove the differences between communities and amplicons. Both primers agreed on differential enrichment of *Chryseobacterium* and *Hydrogenophaga* in Farm A tank water, but they disagreed regarding the biofilm samples, with differential enrichment of *Rhizobiaceae* and *Ideonella* (MiSeq) vs. *Comamonadaceae* and *Sphaerotilus (Earth)* (Fig. 4). Considering the close clustering of these samples in morphospace (Fig. 3) these results could explain the taxa driving this separation. When comparing biofilm from farm A and farm B, ASVs differentially enriched in farm A were affiliated with *Rhizobiaceae* and *Ideonella* (MiSeq) and *Rhizobiales* and *Sphaerotilus* (Earth), while ASVs affiliated with members of *Aeromonas* and *Flectobacillus* (MiSeq and Earth) were differentially enriched in farm B (Fig. 4). Notably, Earth and MiSeq datasets agreed about the presence of taxonomic groups harboring pathogens, e.g., *Chryseobacterium*, *Flavobacterium*, and *Aeromonas*. These results show that at the level of ASVs, biases are introduced by primer choice.

Long amplicon

The low number of reads obtained from the long-read amplicon approach (a consequence of harsh lysis conditions and over-sequencing of the mock community standard) prohibited overall community statistics approaches. Nevertheless, taxonomic conclusions of biological interest could be derived from the 10,041 reads obtained, which resulted in the identification of 204 species (Additional file 5).

Similar to the short-read data, species-level data obtained with long-reads emphasize the unique features of farms and, to a lesser extent, compartments (Fig. 5). Seventeen of the top enriched species were affiliated with biofilm samples. However, only five were shared between farms, including *Sphaerotilus natans*, a bacterium responsible for bulking, *Streptococcus thermophiles*, a commonly used probiotic bacterium, and *Nitrospira defluvii*, a bacterium that aids nitrification. Twelve species were exclusively detected in farm A, and four were specific to farm B. Within farm A, many of the species were detected in at least two compartments, except *Thermomonas sp.* SY21 and *Haliscomenobacter hydrosissis* that were detected in all compartments. However, *Lysobacter tolerans* and *Paracoccus aminovorans* were found explicitly in farm A's biofilm. The two water-type samples (biofilter and tank) from the same circuit featured similarities and differences when inspecting the top enriched species, with *Flavobacterium aquatile*, *Propionibacterium freudenreichii*, and *Limnohabitans sp.* 63ED37-2 detected in tank water, and *Corynebacterium casei*, C. *variable*, *Nitrospira defluvii*, and *Brevibacterium yomogidense* detected in biofilter water. Finally, in farm B's biofilm, *Aeromonas hydrophila*, a common secondary invader known to cause a broad spectrum of infections, was also differentially enriched.

Shotgun metagenomics

The shotgun metagenomics data corroborated amplicon findings and extended the picture beyond prokaryotes (75.55%) and included eukaryotes (23.97%), archaea (0.24%), and viruses (0.24%) (Additional file 6). Focusing on phyla with at least 0.5% or more of the total reads, a dataset comprising 96.34% of all reads identified ten phyla. Three-fourths (75.26%) of these reads were assigned to prokaryotic phyla, indicating that competition with eukaryotic reads was not an issue (Fig. 6A). Shotgun sequencing agreed with the patterns detected by amplicon sequencing. The top phyla were *Proteobacteria* (54.72% of total reads), *Actinobacteria* (9.47% of total reads), and *Bacteroidetes* (8.05% of total reads) (Fig. 6A) for all samples (Fig. 6B). The eukaryote phyla comprised *Arthropoda* (10.29%), with fish food and spider colonies as the most likely source; Chordata (8.04%), with the farmed European perch (*Perca flavescens*) as the source; and *Ascomycota* (sac fungi); and *Streptophyta* (green algae and plants) (Additional file 6).

Among lower abundance phyla (0.50–0.08% of reads), 16 additional taxa, from a virus group to eukaryotic groups, were detected. The virus group was *Uroviricota,*

(See figure on next page.)

Fig. 4 Taxonomic units unique for specific primers and/or compartmens according to 16S sequencing. Permanova coefficients indicate which ASVs are most characteristic for (but not necessarily most abundant in) a particular compartment. Uppercase captions indicate the lowest classified order (O = Order, F = Family, G = Genus). Taxonomic units containing aquaculture pathogens are marked with an asterisk. Primer pair differences (different emerge at the ASV level. We found that water and biofilm samples from the same farm and circuit differ in differentially enriched ASVs, which is vital for understanding taxa diversity and functional services within different sample types. Notably, both primers could identify pathogenic groups within the farms, e.g., Chryseobacterium, Flavobacterium, and Aeromonas

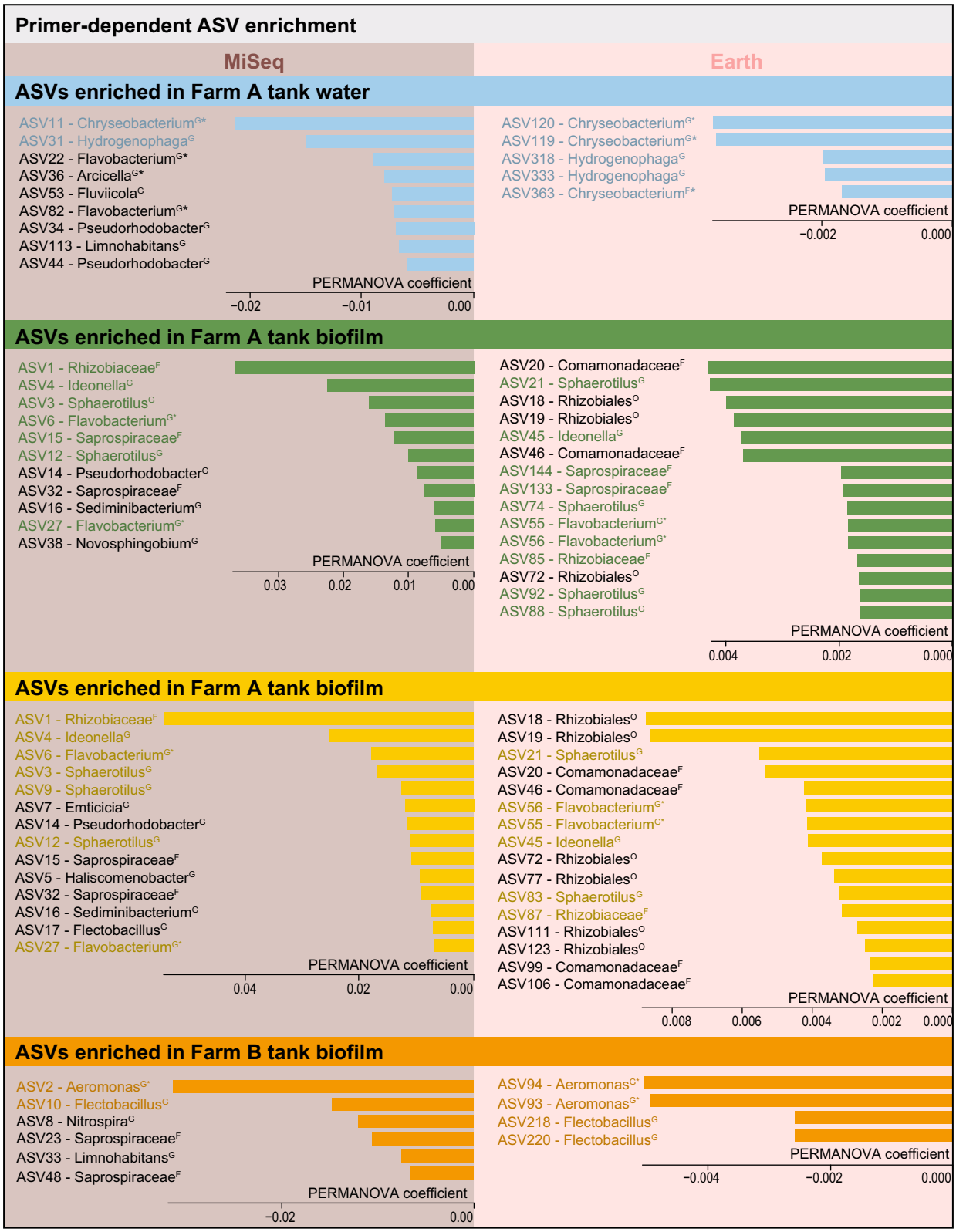

Fig. 4 (See legend on previous page.)

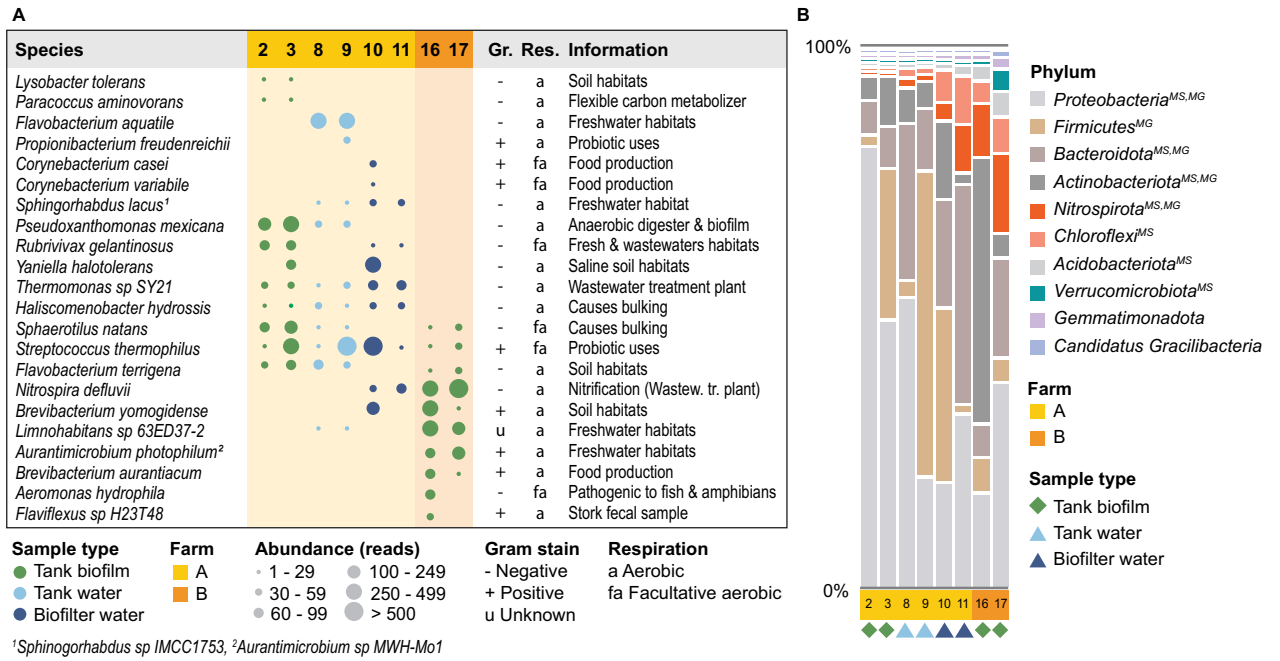

Fig. 5 PacBio sequencing results (10 most abundant species identified in each compartment). The top ten species were identified by adding the reads from both replicates and then identifying the ten most abundant. The farms (yellow vs. orange) feature distinct communities, with distinct compartments within farm A sharing more species biofilm samples between farms. For example, *Haliscomenobacter hydrossis*, a species known to cause bulking, is ubiquitous and unique to farm A. Only three species, *Sphaerotilus natans*, *Streptococcus thermophilus*, and *Flavobacterium terrigena*, featured among the most abundant species in both farms. *Aeromonas hydrophila* was obtained from a tank in farm B that had an active Aeromonas spp. outbreak. The table further includes gram stain, respiration method, and additional information about the particular species. Updated species names are denoted as footnotes. Green diamonds represent tank biofilm, light blue triangles represent tank water, and dark blue triangles represent biofilter water. Uppercase captions indicate that the phylum was also detected in the other datasets; *MS* MiSeq, *MG* Metagenomics

a dsDNA-tailed bacteriophages virus. Four out of the six low-abundance bacteria phyla were also detected in other platform datasets. For instance, *Verrucomicrobiota, Acidobacteriota,* and *Chloroflexi* were detected in the MiSeq and PacBio datasets, and *Gemmatimonadota* was detected only in the PacBio dataset (Additional File 6). In addition, *Euryarchaeota*, a methane-producing archaean, was detected. The eukaryotes included invertebrates such as *Mollusca* (mollusks), *Echinodermata (starfish, sea cucumber and urchins,* etc.*)*, *Cnidaria (jellyfish, sea anemones,* etc.*)*, *Nematoda* (roundworms), and *Platyhelminthes* (flatworms). Additionally, *Basidiomycota* (fungus), *Chlorophyta* (green algae), and *Apicomplexa* (protozoan) were detected (Additional file 6).

As expected, pathogenic species were detected at even lower read abundance levels. The ten most abundant pathogenic bacteria included *Flavobacterium psychrophilum* (0.071%), *Aeromonas veronii* (0.031%), *A. hydrophila* (0.029%), *F. branchiophilum* (0.026%), *F. columnare* (0.015%), *A. caviae* (0.014%), *A. salmonicida* (0.005%), *Vibrio vulnificus* (0.004%), *V. parahaemolyticus* (0.004%), and *A. jandaei* (0.003%) (Additional file 6). The PacBio data for farm A's tank water samples also identified *A. hydrophila*, *A. salmonicida*, and *A. veronii*.

Discussion

Microbial communities are the drivers and determinants of a successful RAS, but their composition, interactions, and spatio-temporal dynamics are often unknown. Targeted research in RAS is required to shed light on how these communities form, interact and provide services. On the one hand, such knowledge will lead to better management, innovative RAS design, and procedures to manipulate communities. On the other hand, such research will extend our understanding of the rules governing community ecology and evolution beyond controlled lab systems. In this paper, we compare the distinct layers and types of information obtained by distinct methodological approaches from short-read to shotgun metagenomics. We demonstrate that each method can present a cost-effective technique to monitor particular aspects of microbial communities within RAS.

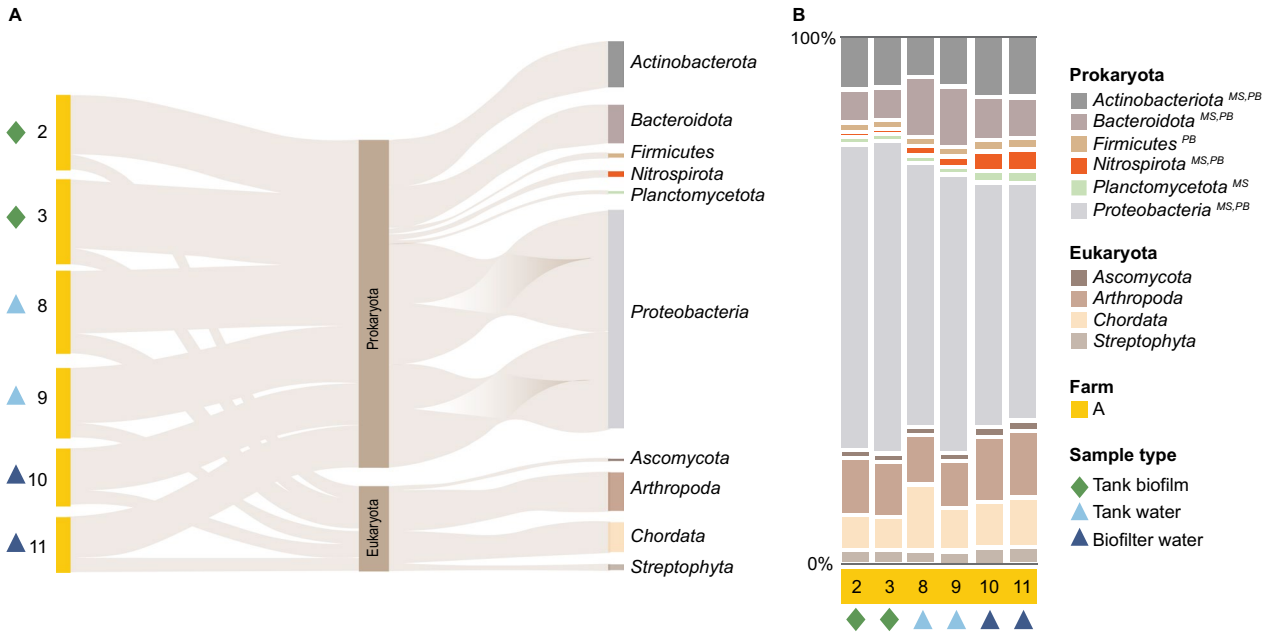

Fig. 6 Shotgun metagenomics data for phyla with at least 0.5% or more of the total reads. **A** Sankey diagram displaying each sample's prokaryotic and eukaryotic phyla. The prokaryotic phyla are the dominant group in each sample, with Proteobacteria as the most abundant group. Eukaryotic phyla consist of (1) Arthropods likely introduced via the feed, (2) Chordata represented by the farm-raised European perch (Perca fluviatilis), (3) Streptophyta, which consists of green algae and land plants and (4) Ascomycota, sac fungi, representing the largest phylum of fungi. **B** Relative abundance plots illustrate the community composition similarities and differences between replicates, sample types, and within-farm compartments. Notably, Proteobacteria are less abundant in biofilter water than in tank biofilm. Uppercase captions indicate that the phylum was detected in the other datasets; *MS* MiSeq, *PB* PacBio. Green diamonds represent tank biofilm samples, light blue triangles represent tank water, and dark blue triangles represent biofilter water

Primers, pipelines, and platforms

Variations in protocols concerning primers and amplification, sequencing platforms, quality filtering, and clustering parameters affect conclusions in microbial ecology. For example, primer bias will occur in any study that includes an amplification step. Understanding how these biases affect biological conclusions is essential, especially in a dynamic field such as aquaculture, where no consensus has been reached concerning methods. However, aquaculture microbiome research widely employs 16S rRNA sequencing as a cost-effective method for surveying microbial communities [1, 7, 10, 47, 48]. Primer selection for short-read sequencing is potentially the most influential step during aquaculture microbial community analysis, as primers directly select for or against specific groups based on the targeted 16S v-region [23, 27, 29, 49, 50].

In our study, primer pair 27F_534R underperformed, an unexpected result as this primer pair was successfully used with active sludge collected from a wastewater treatment plant [23]. We attribute this to our approach of co-sequencing all amplicons. Shorter fragments sequence more efficiently, and 27F_534R amplicons were likely out-competed by the shorter MiSeq and Earth amplicons [51]. This would explain the decrease in both read numbers and read quality with increasing amplicon size (Earth > MiSeq > 27F_534R; Fig. 2A). Therefore, the 27F_534R amplicon, which in theory would offer higher taxonomic resolution due to its increased length [9, 52], could still be adequate for future RAS samples, but should not be combined with shorter fragments during sequencing.

Minor differences in ASV richness between Earth and MiSeq primers did not impact the spatio-temporal patterns and biological conclusion, even though primer bias was detectable at higher taxonomic resolution (Fig. 4). This implies that community studies can potentially be compared at higher taxonomic levels even when different 16S rRNA primers were used. However, the significance of biases at high taxonomic resolution is somewhat uncertain, particularly since previous findings with the same primers differ at the family level. For example, Earth primers have been reported to underestimate the abundance of *Chloroflexi* and *Actinobacteria* in active sludge [23], while in our study, *Chloroflexi* appeared to be overrepresented with the Earth primers, while *Actinobacteria* was similarly represented by both primers (Fig. 2D).

In summary, our results suggest that short-read sequencing is adequate for exploring the spatio-temporal dynamics and community composition at higher taxonomic levels. Because of its low cost, ease of implementation and the availability of well-validated pipelines, 16S rRNA sequencing remains a powerful approach. It has the potential as a monitoring tool in larger-scale RAS farms that incorporate research and design projects into their annual budgets.

Long-read sequencing approaches are recommended to improve taxonomic resolution [53–56] and are desirable in a context where species-specific pathogen identification is relevant. A current drawback is that long-read methods require a large amount of high-quality starting material, thus making them unsuitable for environmental studies that often have low DNA yield [57] and high levels of amplification inhibitors. Also, including a mock community, as recommended for normalization [25], can compromise sequencing depth. The methodological requirements associated with environmental samples containing gram-positive bacteria, i.e., harsh lysis conditions, compromised our long-read approach that was further impaired when paired with high-quality community standards during sequencing. When aiming for high-quality long DNA fragments for long-read sequencing, lysis methods and the inclusion of mock standards require thorough optimization. We conclude that the taxonomic resolution of the PacBio approach is beneficial in exploring functional services and species identification, especially pathogenic ones. However, the approach might not be optimal for a large-scale spatio-temporal study that requires quantitative results and may suffer from challenges in DNA quantity or quality.

In contrast to the aforementioned short- and long-read approaches, amplification-free shotgun metagenomics are not impeded by primer bias. In addition, genome-wide information, read count and genome size can be used to calculate biogenomic mass—a proxy for biomass [58]. Species-independent functional profiling based on the presence or absence of genes is another benefit of metagenome data. Finally, shotgun metagenomics sequences all genetic information rather than just one taxon. RAS microbial ecosystems also harbor archaea [18], fungi [59, 60], and viruses [61], which all interact, compete for resources, and aid or deleteriously impact the system. Therefore, shotgun metagenomics represents the most thorough approach for characterizing RAS microbial communities.

In our study, most reads obtained by shotgun metagenomics were of microbial identity, but additional relevant taxa (especially viruses, archaea, and fungi) were detected (Additional file 6), confirming the effectiveness of the approach to provide a wholistic picture. Importantly, the metagenomics data mirrored the amplicon data, confirming the validity of the three sequencing approaches to reach relevant biological conclusions at higher taxonomic levels. The similarity in community patterns also supports our previous conclusion that the impact of primer bias in amplicon approaches is negligible at higher taxonomic levels of analysis. Shotgun approaches are, therefore, highly promising and could be further functionalized by stepping toward an RNA-focused metatranscriptomic approach [62, 63].

The selection of a suited bioinformatics pipeline for analyzing sequencing data is a critical step in microbial studies. Currently, six bioinformatics pipelines are commonly used for 16S rRNA gene amplicon data analysis [64], and all have the potential to introduce bias through sequencing errors [65]. DADA2 is an increasingly used pipeline that shows high sensitivity, can differentiate sequences at single-base resolution, and clusters sequences into ASVs [64]. ASVs are advantageous over OTUs because they represent true sample sequence variants, unlike OTUs that are derived from traditional clustering, which can be prone to sequencing errors and biases based on the algorithm used or the fixed identity threshold value. A large body of literature on aquaculture microbiomes works with operational taxonomic units (OTUs). However, aquaculture studies using ASVs are on the rise, including studies on host-microbiome interactions [66], microbial dynamics in RAS [10], and microbial dysbiosis during a *Tenacibaculosis* outbreak [67] that could provide relevant data for meta-analysis studies.

Our results support several conclusions on method choice with transfer potential to other studies. First, primer bias does not compromise higher-level spatio-temporal conclusions of 16S approaches as long as a sufficient number of high-quality reads are obtained. Importantly, relative differences in community composition between data obtained with different primers can safely be compared, whereas we recommend avoiding comparing absolute statistics of microbial communities analyzed with different primers or lower taxonomic levels. Second, the requirements and challenges of long-read approaches complicate quantitative spatio-temporal community analyses but have value in species-level identification. Lastly, our results agree with other studies on the benefits of hybrid sequencing approaches [68–71]. The combination of three different sequencing methods yielded an in-depth overview of spatio-temporal dynamics and species-level information that would otherwise have been difficult to obtain.

Community composition

Combining three different sequencing approaches allows for an in-depth assessment of microbial communities,

including potential functional aspects. The dominating phyla in both the short-read amplicon (Fig. 2D) and the shotgun approach (Fig. 6) were *Bacteroidetes* and *Proteobacteria,* which agrees with previous short-read RAS studies (marine RAS: [7, 10, 12, 72]; freshwater RAS: [47]). *Bacteroidetes* contain species that are specialized in the degradation of complex polymers and the cycling of carbon and protein-rich substance [73, 74] and tend to be attached to particles or surfaces [7]. For example, *Flavobacteria*, a class in *Bacteroidetes*, were recently discovered to play a major role in nitrous oxidation–reduction, the final step of denitrification [75]. *Proteobacteria* are a diverse phylum containing nitrifying and denitrifying genera [18], which play a major role in nutrient recycling and remineralization of organic matter [76–78], essential steps for the operation of RAS.

A key finding of this study is the strong impact of the sample site and sample type on results and conclusions, as seen across the different datasets. Differences between biofilm and water samples have been reported before, e.g., for a sole RAS [10], a flow-through lumpfish farm [8], and an Atlantic salmon RAS [6], albeit only at higher taxonomic resolution. We show that overall community composition and species presence/absence differ not only between biofilm and water, but also between different compartments of the same circuit and between biofilm successional stages. Within the MiSeq data, differentially enriched ASVs were detected between the tank water and biofilm. The tank water differentially enriched ASVs belong to the genera *Chryseobacterium*, *Flavobacterium*, and *Hydrogenophaga*. *Chryseobacterium* [79] and *Flavobacterium* [81, 82] include opportunistic pathogens that impact fish health, resulting in devastating losses in wild and farmed fish stock worldwide. Furthermore, *Chryseobacterium* species are suspected of playing a role in spoilage [82] and being multidrug-resistant [83], which is a danger to both animals and humans. Differentially enriched ASVs in tank biofilm were *Rhizobiales*, *Ideonella*, *Comamonada*, and *Sphaerotilus*, which are involved in nutrient recycling processes or water quality. Notably is *Ideonella*, a small genus group composed of four species, with one species, *Ideonella sakaiensis*, capable of degrading PET, a polymer widely used in food containers, bottles, and synthetic fibers [84]. Since plastics are used in RAS for biofilter media (e.g., biofilter carriers), the presence of a potentially plastic-degrading species has implications for replacement and repair costs. The PacBio data showed that certain species were compartment-specific. For example, *Lysobacter tolerans* only occurred in the tank biofilm samples. They are capable of producing peptides that can damage the cell walls or membranes of other microbes and are regarded as an untapped source for producing novel antibiotics [85]. Species only found in the tank water included *Flavobacterium aquatile,* a species typically found in waters containing a high percentage of calcium carbonate—a characteristic of many Swiss waterways [86]—and *Propionibacterium freudenreichii*, an essential bacteria in the production of Emmental cheese, a Swiss cheese [87]. This type of information is essential for managers when choosing the type of sample to take for monitoring and diagnostic purposes and at the same time is promising regarding the use of RAS as models for spatiotemporal community dynamics.

Another key result is the major impact of community maturation state on biofilm community results. The biofilm succession process entails a non-random process controlled by attachment events, movement, and cellular interactions that induce the non-random spatial organization of biofilms [88]. As biofilms develop, they increase in volume and surface area, creating gradients of conditions that open niches, e.g., for anaerobic species [89]. This additional habitat complexity increases species richness and functional services, such as degrading organic compounds, cycling of nutrients, or preventing the establishment of pathogenic species through niche exclusion. At the same time, biofilms may act as a pathogen haven and/or reservoir [90]. For example, *Aeromonas hydrophila* (found in farm B, Additional files 5 and 6) can form thick layers that allow them to evade disinfection or antibiotic treatments [82, 91] while enabling the spread of antimicrobial resistance genes [92]—an area we are excited to explore with future shotgun metagenomics data.

In aquaculture management, biofilms are regularly removed during cleaning procedures, leaving them in a continuous state of recolonization. The impact of the removal and the resulting successional processes on ecological functions and animal health in RAS is unknown, but frequent disruption may potentially open up niches to pathogenic species while preventing the establishment of beneficial slow colonizers. A study by Rampadarath et al. [93] showed that within the first 24 h of biofilm formation, *Proteobacteria* microbials were the most dominant, followed by *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, and *Verrucomicrobia*. Some of the most prominent bacterial fish pathogens are distributed across the phyla *Proteobacteria* and *Bacteroidetes*, which are early colonizers. In our data, we find the beneficial *Nitrospira defluvii* only in mature samples (farm A: biofilter water and farm B: tank biofilm), suggesting that these species are late colonizers and that frequent biofilm removal could prevent their establishment and negatively impact denitrification.

We look forward to further disentangling the impact of frequent disruption and recolonization processes on biofilm communities and identifying factors promoting the establishment of healthy communities after a disruption. Identifying key steps towards colonization with beneficial communities could reduce start-up and operation costs [1], prevent the establishment of pathogens [94], and lead to healthier stock [95].

Finally, community patterns between farms are suggestive of an "island-biogeography" effect, where distinct communities develop in largely isolated habitats. Other aquaculture facilities studies have reported such effects [1, 96]. The long-read data clearly distinguishes farm communities (Fig. 5), with *Haliscomenobacter hydrossis* (i.e., causes bulking) [97] and *Streptococcus thermophiles* only being present in farm A. Furthermore, the between-farm biofilm communities only had three species in common: *Sphaerotilus natans*, another bulking species [98], *Streptococcus thermophilus*, and *Flavobacterium terrigena*. In the present case, the conclusion is that farm conditions such as design, management styles, source water, environmental parameters (e.g., temperature, salinity [99], pH) in addition to farmed species, fish feed, and nutrient concentrations [10, 103], combined with stochastic assembly processes of dispersal and colonization [100], supersede the continued exchange of microbial communities through the regular delivery of juveniles from farm A to farm B.

Disease and health

Understanding the potential pathogenic risks within a RAS is vital from the perspective of economic success but also to preserve animal health and wellbeing. The emergence and spread of pathogens accompany the current growth and rapid progress of aquaculture. Aquaculture disease outbreaks can be catastrophic to the industry, causing an estimated worldwide loss of more than US$6.0 billion per annum [101].

The shotgun metagenomics approach detected various pathogenic species in farm A that pose a risk to fish health and can ultimately result in disease outbreaks (Additional files 5 and 6). *Flavobacterium psychrophilum* (0.09% of total reads), the causative agent for bacterial coldwater disease, and *Aeromonas veronii* (0.04% of total reads), causing freshwater fish sepsis and ulcer syndrome, were the most abundant pathogens detected. Interestingly, these species are not typically associated with perch but with freshwater salmonid fish, such as rainbow trout (*Oncorhynchus mykiss*). However, a potential risk in animal farming is the emergence of spillovers and strains with altered host specificities. In addition, ubiquitous pathogens known to infect a wide range of freshwater fish, including perch, were detected across both systems at lower abundances and predominantly in tank water, including *Flavobacterium branchiophilum,* the causative agent of bacterial gill disease; *Aeromonas hydrophila,* the causative agent of motile aeromonas septicaemia; and *Flavobacterium columnare,* the causative agent of columnaris disease.

The development of nonpharmaceutical controls for pathogens in animal farming is vital for animal and public health. Antibiotic resistance poses one of the greatest human health and sustainability challenges of the 21st century [102]. Antibiotics have fostered the emergence of resistance genes and the promotion of horizontal gene transfer and mutagenesis in aquatic bacteria [103]. One proposed alternative method is bacteriophage therapy, which uses naturally-occurring bacteriophages to target specific bacteria species or strains of bacteria, such as *Ackermannviridiae* sp. or *Myoviridae* sp. Both phage groups were present in the studied farms (Additional file 6). However, phage therapy is still in its infancy, with only a handful of successful phage therapies for the 150 different bacterial pathogens of farmed and wild fish (e.g., *A. hydrophila* in loaches, *F. columnare* in catfish, and *F. psychrophilum* in rainbow trout) [104]. Our results show the potential of shotgun genomics to support the development of additional innovative phage therapies or other pathway-based disruptive measures.

Conclusion

Our results show that microbial communities in RAS are highly dynamic and site-specific despite the permanent circulation of water throughout the system. Additionally, management routines create a state of continuous succession and recolonization, especially for biofilm communities. Finally, commonly used 16S primers can detect spatio-temporal development and dynamics across RAS compartments, sample types, and farms, but cannot provide the resolution required for species or strain identification, which is critical knowledge for RAS managers.

The results presented here contribute to quantifying the microbial community and dynamic and complex interactions in RAS. Further research of microbial communities in aquaculture is necessary to harvest the full power of these micro- —but mighty—organisms during farm management (e.g., during biofilter start-up or disease prevention), to extract basic biological principles (e.g., the link between environmental stressors and microbiome dysbiosis), and to clarify medically relevant interactions (e.g., between host-microbiome-environment interaction and disease development).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-023-00459-z.

Additional file 1. Information regarding samples, primers used for amplification, and reads per samples.

Additional file 2. Information regarding ASVs for each dataset and the assigned taxa.

Additional file 3. Information regarding the alpha values for each dataset.

Additional file 4. Information regarding taxa identified during the different maturation stages of biofilm.

Additional file 5. Information regarding taxa identified with the PacBio sequencing data.

Additional file 6. Information regarding taxa identified with the Illumina shotgun metagenomics data.

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Author contributions
All authors conceived and designed the analysis, CB and IAK acquired funding with the help of JR and AK, JR collected the data, JR and AK performed the data analysis, all authors performed data interpretation, JR wrote the manuscript draft, and all authors edited and reviewed the manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are available in the SRA NBI databank: Illumina short-amplicon: project ID: PRJNA757614; accession codes between SRR18029926–SRR18029932 and SRR18029942–SRR18029955). PacBio long-amplicon: project ID: PRJNA757614; accession codes between SRR18029933-SRR18029941; and Illumina Metagenomics: project ID: PRJNA757614; accession codes between SRR20005812–SRR20005817.

Code availability
Code used to analyze data is available at 10.5281/zenodo.7553703. The abundance table output of the Shoreline PacBio sequencing is included within the article and its supplemental files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, Institute for Fish and Wildlife Health, University of Bern, Länggasstrasse 122, 3001 Bern, Switzerland. [2]Division of Theoretical Ecology and Evolution, Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, 3012 Bern, Switzerland. [3]Swiss Institute of Bioinformatics, Quartier Sorge - Batiment Amphipole, 1015 Lausanne, Switzerland.

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I. CHAPTER 2:

The characterization of fresh and brackish water microbial communities within six recirculating aquaculture systems (RAS) across Switzerland using a shotgun metagenomics approach

Jessica Rieder, Adamandia Kapopoulou, Claudia Bank & Irene Adrian-Kalchhauser

Keywords: shotgun metagenomics, recirculating aquaculture, freshwater, brackish water, perch, zander, shrimp, animal health

I.1 Abstract

Recirculating aquaculture systems have emerged as a promising solution for sustainable animal protein production, characterized by reduced water and carbon footprint. The success of RAS depends on the vital roles of the microbial organisms. However, our understanding of large-scale patterns of microbial dynamics and their impact on system efficiency and animal health remains limited. To address this knowledge gap, we conducted a comprehensive three-month experiment involving six RAS farms in Switzerland specializing in perch, zander, and shrimp production.

The objective of our study was to investigate microbial community diversity and dynamics, pathogen occurrence, and metabolic functions within RAS. Within each farm, sampling was conducted at various locations, including tanks, biofilters, and drum filters, as well as within different circuits of each farm when feasible. This sampling scheme, which encompassed different salinities, life stages, production events, and management styles, allowed for the capture of the impacts of environmental conditions, recolonization phases, and disease outbreaks.

We sequenced 496 environmental samples across different matrices (biofilm, biofilter carriers, and water) using a shotgun metagenomic sequencing approach. The resulting dataset comprised an average of 43 million reads per sample. Our findings underscore the significant influence of environmental factors, such as salinity, farm conditions, cultivated animal species, and sample types, on shaping bacterial communities within the RAS. Salinity has emerged as a primary driving factor, evident from the distinct separation between brackish and freshwater species. This finding highlights the evolutionary adaptation of bacterial species to environments with different salinities. Moreover, our results reveal distinct spatiotemporal patterns observed among farms, circuits, and compartments, indicating the stochastic nature of community establishment within the RAS. These patterns reflect the dynamic and complex nature of microbial communities within RAS, which are influenced by various factors that shape their composition and structure. However, the stochastic nature of these patterns makes it challenging to identify predictive measures based on observed trends.

Our findings provide crucial insights for aquaculture managers by offering valuable information on optimal RAS management practices during homeostasis and disease events. These findings contribute to our understanding of the microbial ecology in RAS and have practical implications for enhancing system efficiency, promoting animal health, and supporting sustainable aquaculture practices.

I.2 Introduction

Aquaculture is a rapidly expanding sector in the global food industry, contributing over 52% of the total harvest weight of aquatic animals for human consumption (FAO, 2022). This sector exhibits high diversity in terms of farmed species, with more than 400 species of animals cultivated (Stentiford et al., 2020) across various production systems, water environments, and geographic locations. Commonly cultivated species include freshwater species such as tilapia, catfishes, and marine species, including salmonids (e.g., Atlantic salmon, coho salmon, and chinook salmon) and shrimp.

Recirculating aquaculture is gaining prominence as a sustainable alternative to capture fisheries and is predicted to become the dominant method for future aquaculture production (Ahmed & Turchini, 2021). This approach offers several advantages, including a semi-closed loop design, known as a recirculating aquaculture system (RAS), which enhances biosecurity measures and reduces the water footprint due to efficient water reuse through mechanical filtration, biological filtration, and disinfection measures, year-round production independent of seasonal conditions, and the ability to be situated inland, thereby minimizing product-to-market distance (Martins et al., 2010; Ahmed & Turchini, 2021). RAS are also versatile systems engineered to mimic the ecology of natural systems for rearing various farmed organisms such as finfish, shrimp, shellfish, and algae.

Microbial communities within the RAS play critical roles in maintaining system health and stability, including nutrient cycling, water quality maintenance, and animal health (Rud et al., 2017; Roalkvam et al., 2019; Infante-Villamil et al., 2021; Rieder et al., 2023). These communities are often actively maintained in the biofilter, a compartment engineered to maximize the surface area for the establishment and growth of nitrifying species. Throughout the rest of the farm, resident species that naturally establish and are often distinguished by sample matrices, such as biofilms vs. water communities (Bakke et al., 2017; Almeida et al., 2021; Rieder et al., 2023). Variation between the community composition of biofilms and water results in the provision of different functional services.

However, the presence of pathogenic species within the microbial communities of RAS presents significant challenges to the aquaculture sector. Disease outbreaks can negatively impact the sustainability and growth of the industry, leading to substantial economic losses estimated at USD 6 billion annually (Stentiford et al., 2012). These organisms are primarily opportunistic pathogens that are widely distributed. However, some species also have zoonotic potential, posing a serious risk to aquaculture personnel. Among the commonly detected bacterial pathogens in freshwater RAS are *Aeromonas* spp., *Flavobacteria* spp., *Photobacterium damsela*, *Plesiomonas shigelloides*, and *Shewanella putrefaciens*. Additionally, while *Vibrio* spp., the causative agent of vibriosis, can occur in freshwater RAS, they are more commonly found in marine RAS (Austin & Austin, 2007). Regarding the zoonotic species, *Bacillus cereus, Shigella* spp., *Vibrio* spp., and *Photobacterium damsela* pose significant risks to humans. Disinfection and treatment measures are inherently risky, including the

unintended removal of healthy bacteria (Rieder et al., 2023), which may create niches for other pathogenic species or promote uncontrolled microbial growth and the emergence of resistant strains.

Aquaculture managers recognize the importance of maintaining a healthy microbiome in aquaculture systems and acknowledge the potential risks associated with imbalanced microbial communities, such as elevated ammonia levels or disease outbreaks (Smith et al., 2012). However, effective management of microbial communities within the RAS is challenging owing to the influence of external and internal factors, including water quality and complex microbial interactions. Adding to this complexity, most microbial ecology studies in this context are primarily descriptive (Prosser, 2020), lacking direct applicability to practical management approaches. Finally, many microbial studies in RAS predominantly rely on 16S rRNA sequencing, which restricts the detection of microorganisms and limits a comprehensive understanding of the entire microbial community. Therefore, a more holistic sequencing approach is required to unravel the complexities of microbial communities and facilitate informed decision-making in aquaculture management.

Although not routinely used, shotgun metagenomics is a promising method for analyzing complex genomes present in environmental samples. This approach offers several advantages over 16S metabarcoding sequencing, including the simultaneous sequencing of organisms across all major domains, exploration of metabolic functional pathways, and the potential for the discovery of new species/strains. However, the application of metagenomics in aquaculture is still limited, partly because of challenges such as high cost and data storage and processing issues (Martínez-Porchas & Vargas-Albores, 2017; Rieder et al., 2023). Future research should consider a tier-sequencing approach (Rieder et al., 2023) that combines metabarcoding and metagenomic data, as it offers a cost-effective solution while maximizing the information obtained, which could result in improved management decisions, more effective treatment plans, and potential detection of new species or strains.

This study investigates the spatiotemporal dynamics of microbial communities in six Swiss RAS farms using a shotgun metagenomics methodology. The sampling strategy includes two freshwater RAS farms dedicated to perch cultivation, one freshwater RAS farm cultivating perch and zander, two freshwater RAS farms exclusively cultivating zander, and one brackish-water RAS farm focused on shrimp production. We explore the influence of various environmental factors, management practices, and spatial and temporal variables on the composition and dynamics of microbial communities within these RAS farms. First, we show that salinity parameters strongly determine community composition, with distinct communities being detected between brackish and freshwater farms. We also showed that these communities are further influenced by management styles, different life stages of farmed animals, and cleaning/disinfection procedures. Second, we demonstrate that shotgun metagenomic sequencing is sufficient to detect spatiotemporal developments and dynamics in RAS, highlighting the heterogeneous distribution of species in RAS. We also highlight the detected pathogens and discuss

their implications on both animal and farm personnel health.

I.3 Material and Methods

I.3.1 Sampling sites

This study encompasses six commercial-scale RAS farms in Switzerland, denoted as farms A–F. These farms differ in terms of their locations and operational management practices. Specifically, farm A raises both perch (*Perca fluviatilis*) and zander (*Sander lucioperca*); farms B and C focus exclusively on the cultivation of perch, while two other farms, E and F, exclusively cultivate zander in freshwater RAS. Finally, Farm D specializes in shrimp cultivation, specifically whiteleg shrimp (*Penaeus vannamei*), using a brackish water system. All farms use agitated biofilters containing floating plastic biofilter carriers, which provide adequate surface area to support and promote the growth of beneficial microbial communities.

I.3.1.1 Farm A

Farm A is an individual-circuit RAS located in the Canton Bern region of Switzerland, which obtains water from the Lötschbert-Basistunnel. This farm cultivates two fish species: perch (obtained from Farm B) and zander (acquired at the fingerling stage from another supplier). Upon arrival, the imported 15 g perch are initially placed in 20 cm^3 tanks, with a stocking density ranging from 42-72 kg/m^3 (Figure 1A), for approximately 1.5 months. In the larger, growing-out tanks (120 m^3), perch and zander are raised at a stocking density of 30-60 kg/m^3, until they reach the desired slaughter weight (Figure 1A).

Farm A does not follow a strict timeline for cleaning and disinfection, as fish are often moved into already occupied tanks. However, when the tanks are empty, they undergo the following disinfection protocol: first, tanks are disconnected from the circuit to protect the microbial community in the biofilter; second, they are washed with high-pressure hot water and subsequently sprayed with Virkon S, a disinfection solution, over the tank walls and bottom; and finally, they are washed with freshwater to remove Virkon S. After disinfection steps are completed, the tanks are stocked with a new batch of fish.

Farm A feeds with various fish feeds for the different species and sized fish, such as Alltech Coppens Start Premium 1.5 mm, Star Alevin 2.0 mm), and Perca 3-4.5 mm. The farm uses an automatic feeding system for the feeding process.

I.3.1.2 Farm B

Farm B is a freshwater multi-circuit RAS located in the Canton Vaud region of Switzerland. The farm sources its water from the municipal drinking system and specializes in breeding perch from eggs to approximately 15 g. The farm transfers fish between circuits based on set cut-off weights. The first two circuits accommodate perch ranging from 0.5 to 5 g, housed in 4.64 m^3 tanks with a stocking density ranging from 0.5 to 30 kg/m^3 (Figure 1B). The third circuit houses perch weighing 10 and 15 g in 13.2

m^3 tanks, with a stocking density of approximately 30 kg/m^3.

Farm B follows a rigorous disinfection regimen applied after each stocking batch. Tanks are disconnected from the circuit to protect the microbial community in the biofilter. The tanks are emptied and subjected to a four-step cleaning process, which involves a high-power jet wash using hot water and brushing the tank walls and floor with soap. Subsequently, the farm performs a static acid-base treatment on the tanks and pipes, with neutralizing steps in between. Finally, the tanks are sprayed with alcohol and left to air-dry for several days before the next batch of fish is stocked.

Farm B feeds with multiple feed brands, determined by the age and size of the fish. These brands include Bernaqua, BioMar, and Alltech Coppens.

I.3.1.3 Farm C

Farm C is a freshwater multi-circuit RAS located in the Canton Valais region of Switzerland. The farm sources its water from the Lötschberg catchment and focuses on raising imported 15 g of perch obtained from farm B to slaughter weight. Fish are transferred through independent circuits of varying tank sizes based on their weight.

Circuits 1 and 2 are reserved for perch between 15 and 35 g, where they are raised in 25 m^3 tanks with a stocking density ranging from 38 to 87 kg/m^3 (Figure 1C). In circuit 4, fish weighing between 130 and 200 g are grown in 130 m^3 tanks, with a stocking density ranging from 51 to 78 kg/m^3.

Farm C follows a strict disinfection protocol. Tanks are disconnected from the circuit and then treated with Steinfels Foam C disinfectant for 15 minutes, rinsed with high-pressure cold water, and air-dried – time permitting - before the next batch is stocked.

Farm C feeds with multiple grain sizes of Alltech Star Alevin Star Supreme food, selecting the appropriate size based on the age of the fish, ranging from 2.0 mm to 4.5 mm.

I.3.1.4 Farm D

Farm D is a single-circuit brackish water RAS located in the Canton St. Gallen region of Switzerland. This farm cultivates vannamei shrimp (*Penaeus vannamei*). The farm sources its water from Lake Constance and adds salt to a salinity level of 25-28 ppt (parts per thousand) to create an appropriate brackish-water environment.

During the sampling period, shrimp were imported from the United States 24 days post-hatching and reared until they reached slaughter weight (180 days post-hatching) at an approximate stocking density of 1 kg/m^3 (Figure 1D). The farm raises shrimp in one tank for the entire growing-out process.

Farm D implements weekly proactive disinfection measures by adding Wofasteril to the water at a ratio of 3.2 L per 150 m^3 as a preventative measure.

The daily feeding regime includes Copen Deluxe 2.0 as their primary food source. In addition, the farm introduced probiotics into the system. This includes using rice bran at a rate of 6 L per week for 150 m^3 of water and yellow cap and green cap probiotics, both provided at a rate of 15 g per week for

150 m^3 of water.

I.3.1.5 Farm E

Farm E is a freshwater multi-circuit RAS situated in the Canton Valais region of Switzerland. The farm sources water from the Schreendbach spring. Established in August 2020, it farms zander, starting from fingerling size (approximately 10 g) and raising them until they reach the desired slaughter weight, for example, 800 g, which takes approximately 1.5 years. The farm receives the fingerlings from the Lyss breeding facility.

The zander fish at farm E are housed in a series of interconnected tanks with a volume of 140 m^3 at a stocking density of 51-59 kg/m^3 (Figure 1E). The water flow follows a linear pattern, with clean water entering tank 1 and exiting tank 4, where it returns to the filtration system for conditioning.

Farm E does not have a specific daily or weekly cleaning routine in place as it employs a “low disturbance” policy.

Farm E feeds Aller Aqua

I.3.1.6 Farm F

Farm F is a freshwater farm operating as a single-circuit system in the Canton Basel region of Switzerland. The farm sources water from the Baselbieter Quellwasser. Zander fry (20 - 50 g) are obtained from FTN AquaArt AG, an indoor aquaculture and stock fish farming company in Switzerland. The fries are reared until they reach the desired slaughter weight of approximately 800 g.

The zander are raised in tanks with a depth of 0.8 m and maintained at a stocking density of 45 kg/m^3 (Figure 1F). Periodically, the fish undergo grading based on their length and, if necessary, are moved to a new tank.

Preventive measures to maintain water quality and health include adding 6 kg of salt daily to the water, resulting in salinity levels ranging from 0.1 to 0.2 ppt. Furthermore, a 1,200 W UV light was installed between the first and second sampling events for additional disinfection measures.

Farm F feeds Le Goussant Turbot.

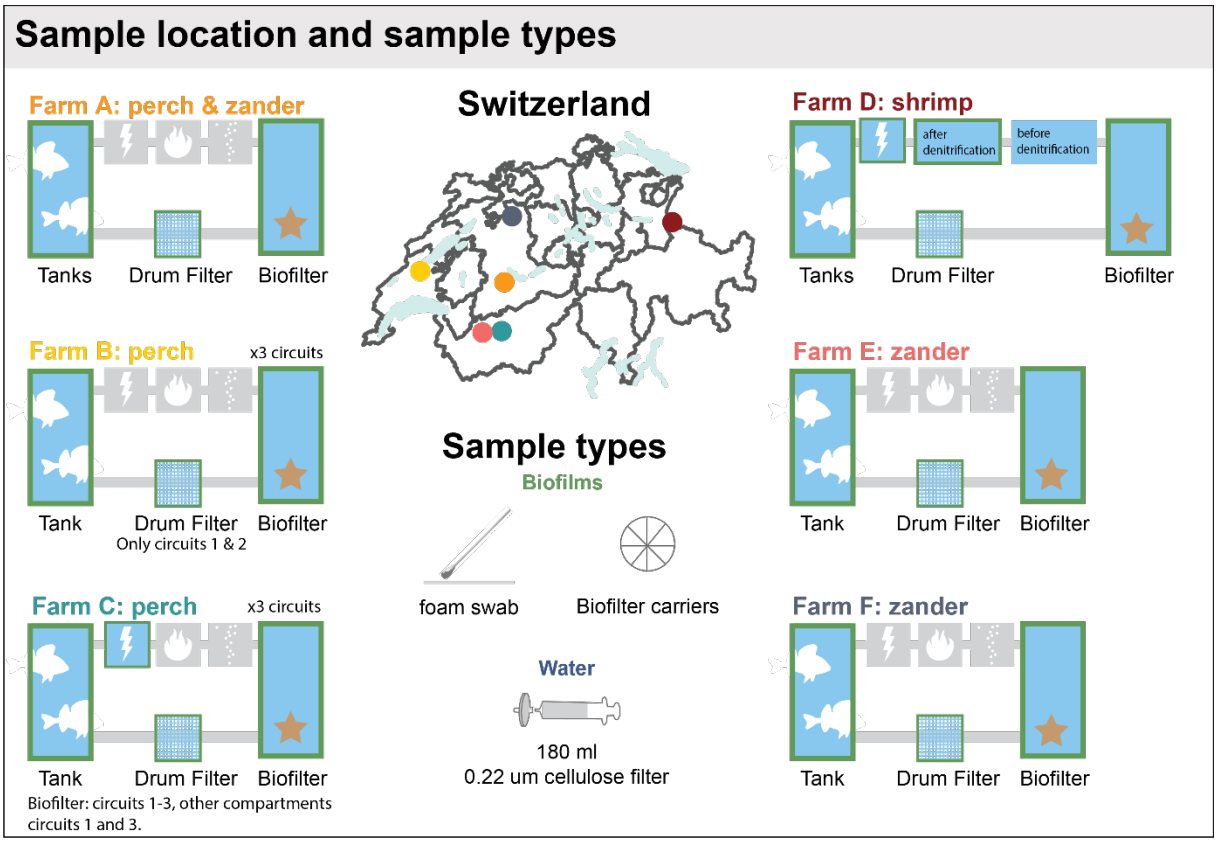


Figure 1: Diagram illustrating the sampling sites and types of samples collected from each farm. *The compartments filled in blue represent water sampling, the compartments outlined in green represent biofilm sampling, and the brown star within the biofilter compartment indicate the collection of biofilter carriers. (**A**) Single-circuit perch and zander RAS. The sampling scheme consisted of collecting biofilm and water samples from tanks, drum filters, biofilters, and biofilter carriers. (**B**) Multi-circuit perch RAS. The sampling scheme consisted of biofilm and water samples from tanks, drum filters (only circuits 1 and 2), biofilters, and biofilter carriers across the circuits unless denoted differently. (**C**) Multi-circuit perch RAS. The sampling scheme consisted of biofilm and water samples from the tanks (circuits 1 and 3), drum filters (circuits 1 and 3), biofilters (all three circuits), and biofilter carriers (all three circuits). Additionally, within circuit 1, biofilm and water samples were collected from the UV compartment. (**D**) Single-circuit shrimp RAS. Sampling consisted of a distinct scheme, which involved the collection of biofilm and water from tanks, UV, biofilter, and before denitrification, as well as after denitrification of water and biofilm samples from the tank outflow pipe. (**E** and **F**) Single-circuit zander RAS. The sampling scheme consisted of collecting biofilm and water samples from tanks, drum filters, biofilters, and biofilter carriers. Please note that the schematic representation is not drawn to scale and serves as a simplified illustration to protect the confidentiality of farms.*

I.3.2 Sample types

Various sample matrices were collected to analyze the variation in community composition among biofilms, biofilter carriers, and water. In addition, biofilm and water samples were collected from various compartments within the farm. The following samples were collected from the different compartments: tank biofilm and water, drum filter biofilm and water, biofilter biofilm, water and carriers, UV biofilm and water, tank outflow pipe biofilm, before denitrification biofilm and water, and after denitrification water (Figure 2).

We used sterile, single-use foam swabs (Whatman FTA) to collect the biofilm samples. The swab was rubbed back and forth approximately ten times on each side across a designated area of

approximately 10x10 cm, situated approximately 6 cm below the surface water level. An additional biofilm sample was obtained near the bottom of the tank wall. Following swabbing, we placed the swab into a 2 ml Eppendorf tube; the stick was detached, and the sealed tube was stored on ice. Three biofilm replicates were collected, with an approximate 2 cm gap between each replicate. Subsequent sampling was conducted from different areas of the tanks or compartments to ensure no overlap of the sampling area.

We collected water samples using sterile 250 ml polypropylene bottles (Thermo Scientific, Nalgene). We filtered 180 ml of water from each bottle through a 0.22 um mixed cellulose filter (Millipore, Merck) housed in a Whatman 47 mm plastic filter holder (Whatman, Merck). Subsequently, we placed the filters in a 2 ml Eppendorf tube and stored them on ice.

Furthermore, the biofilter carriers were collected, placed in individual sterile zip-lock bags, and stored on ice.

All samples were transported to the Institute for Fish and Wildlife Health, University of Bern, and maintained at low temperatures using ice. They were stored at -20°C until further processing.

I.3.3 Sampling scheme

We developed a sampling design to capture comprehensive insights into temporal variations, differences, and similarities within and between farms and their compartments (Figure 1).

We conducted six biweekly sampling events commencing in October 2021 in farms A, B, and C. In farm A, we collected the following RAS compartments and sample types during sampling days 1, 29, and 57 (sampling events 1, 3, and 5), biofilm (surface and bottom), and water samples from four tanks (one zander tank, three perch tanks), a drum filter, and a biofilter, along with biofilter carriers. During sampling days 16 and 74 (sampling events 2 and 6), all previously mentioned samples were collected, except for tanks that house the imported fries, which were not operational at those times.

In farm B, samples were collected from three independent circuits. During sampling days 18, 46, 60, and 88 (sampling events 1, 3, 4, and 6), we collected the following samples from circuit 1: biofilm (surface) and water from two tanks, the drum filter, and the biofilter; in circuit 2, biofilm (surface and

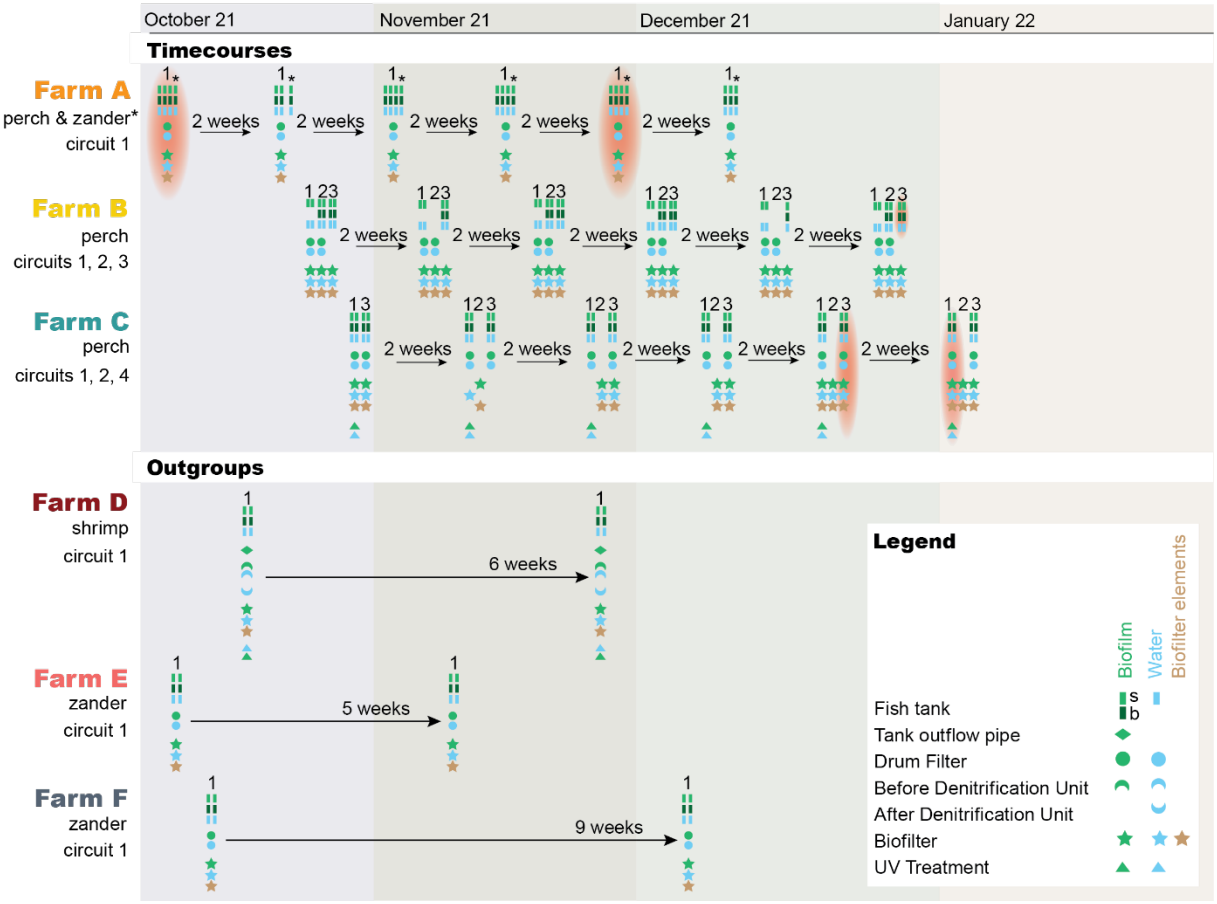


Figure 2: **Overview of sampling events, samples collected, and disease outbreaks**. *We implemented a sampling scheme across multiple farms, compartments, and time points to investigate the spatiotemporal dynamics of the microbial communities. Farms A-C were sampled bi-weekly over six weeks to examine the microbial communities within these semi-interconnected farms. At the same time, farms D-F were sampled twice. The numbers above each column indicate the circuits sampled within each farm. In farm A, the asterisk denotes the tank that specifically cultivated the zander. The red ovals indicate the occurrence of a disease outbreak.*

bottom) from two tanks, surface biofilm from the drum filter and biofilter, and water from all sampled locations. In circuit 3, the sampling was similar to that of circuit 2, except that we collected no drum-filter samples. In addition, we collected biofilter carriers from all three circuits. We followed a similar sampling scheme on sampling days 32 (sampling event 2) and 75 (sampling event 5), except that we collected no tank samples from circuit 2 because it was inactive during the sampling period. Furthermore, on sampling day 75, we sampled only one tank from circuit 3, as it was undergoing a system-wide shut down for maintenance.

In farm C, we sampled three independent circuits. During sampling day 25 (sampling event 1), within circuit 1, biofilm (surface and bottom) and water samples were collected from two tanks, the drum filter, UV compartment, and biofilter, along with biofilter carriers. In circuit 3, the same samples were collected except for the UV samples, as UV treatment was not applied in this compartment. On sampling day 39 (sampling event 2), the tank, UV, and drum filter sampling remained the same as on sampling day 25. Biofilter water was collected from circuits 1 and 3, whereas biofilter biofilms and carriers were collected from circuits 2 and 3. For sampling days 53 and 71 (sampling events 3 and 4),

the only change occurred in the biofilter sampling, with biofilm and water samples collected from circuits 2 and 3. Finally, sampling days 80 and 95 (sampling events 5 and 6) encompassed all compartments in all three circuits, and all types of samples were collected.

We completed two sampling events at farms D, E, and F. In farm D, for both sampling events, biofilm samples were collected from the tank walls (surface and bottom) tank outflow pipe before denitrification, biofilter, and after UV treatment. Water samples were collected from the tanks after denitrification, biofiltration, and UV treatment. We also collected biofilter carriers. In farms E and F, for both sampling events, biofilm (surface and bottom) and water samples were collected from two tanks, the drum filter and biofilter. Additionally, we collected biofilter carriers.

I.3.4 DNA extraction

DNA extraction was performed using the Purelink Microbiome DNA Purification Kit (Thermo Fisher), which is specifically designed to efficiently lyse microorganisms. The manufacturer's protocol was modified to optimize the lysis step. Initially, the silica beads supplied with the kit were transferred to a 5 ml Eppendorf tube, allowing a larger sample surface area to be exposed during bead beating. Subsequently, we transferred the samples to a 5 ml tube containing the beads. To facilitate lysis, 1,500 µL of lysis buffer and 100 µL of lysis enhancer were added to the samples, which were incubated for 3 h at 65°C and 600 rpm using a thermoshaker (Hettich). Bead beating was performed for 15 min at the maximum speed on a Genie 2 vortex mixer equipped with a horizontal tube adaptor. Following bead-beating, the samples were centrifuged at 3,900 × g for 5 min, and 500 µL of the supernatant was carefully transferred to a clean 1.5 µl Eppendorf tube. The subsequent extraction steps were performed according to the manufacturer's instructions.

I.3.5 Sequencing

We conducted shotgun metagenomic sequencing at the Next Generation Sequencing Platform, University of Bern. Before sequencing, the quantity, purity, and length of the extracted DNA were evaluated. Quantification was performed using a fluorometer (Thermo Fisher Scientific Qubit 4.0) and the Qubit dsDNA HS Assay Kit. We performed purity assessment using a DeNovix DS-11 FX spectrophotometer. At the same time, we determined the length of the DNA fragments using the Agilent FEMTO Pulse System with the Genomic DNA 165 kb Kit from Agilent.

Sequencing libraries were prepared using the Illumina DNA Prep, Tagmentation Library Kit M in combination with IDT for Illumina DNA/RNA UD Indexes Sets A-D, following the guidelines outlined in the Illumina DNA Prep Reference Guide. A notable modification to the protocol involves the use of a tiered PCR cycle approach for targeted DNA amplification. This approach allows for a minimum number of PCR cycles, reducing the potential for amplification bias. The number of PCR cycles used varied based on the DNA yield of each sample, ranging from 5 to 17 cycles, to have a sufficient quantity to amplify

30ng of tagemented DNA.

The resulting DNA libraries were assessed for quality using a Thermo Fisher Scientific Qubit 4.0 fluorometer and an Agilent Fragment Analyzer with an HS NGS Fragment Kit. A total of 496 DNA libraries were pooled in an equimolar fashion, resulting in two pools, one with 252 libraries and the other with 244 libraries. Paired-end sequencing was performed using two iSeq 100 i1 Reagent v2 300-cycle kits on an Illumina iSeq 100 instrument. Following the iSeq 100 sequencing runs, the library pools were reassessed, and rebalancing was performed as necessary. The libraries were then subjected to paired-end sequencing using two NovaSeq 6000 S4 Reagent Kits v1.5 300-cycle kits on an Illumina NovaSeq 6000 instrument. On average, each sample yielded approximately 43 million reads. The quality of the sequencing run was assessed using Illumina Sequencing Analysis Viewer (version 2.4.7), and the resulting base call files were demultiplexed and converted into FASTQ files using Illumina bcl2fastq conversion software (version 2.20).

I.3.6 Read processing

We used a reference-based pipeline to analyze the shotgun metagenomic data. The raw read quality using FastQC software (v0.11.7). Notably, the raw reads exhibited high quality across the board, thus necessitating no trimming or filtering procedure.

I.3.7 Taxonomic assignment

MetaPhlAN, a computational software that uses marker genes, was used to profile the composition of microbial communities, specifically bacteria, at the species level. The advantage of this software is that it allows for unambiguous taxonomic assignments and accurately estimates organismal relative abundances (Blanco-Míguez et al., 2023).

I.3.8 Data analysis

Community composition was analyzed across farms, compartments, sample types, and time points by comparing species with more than 10% relative abundance within a sample. For example, if present ≥ 10% within a sample, it was retained; otherwise, it was assigned to the “Other” category.

Using the no-cutoff dataset, we assessed alpha diversity using the Richness and Shannon diversity indices. Alpha diversity was analyzed by comparing different sample types from each compartment within a farm. Richness refers to the direct count of all species present, whereas the Shannon diversity index is the sum of the proportion of each species relative to the total number of species in the community; therefore, it accounts for both abundance and evenness.

We assessed beta diversity to investigate similarities and dissimilarities in community composition among all farms, within-farm compartments, sample types, and time points (Tuomisto, 2010). This study quantified beta diversity using the Bray-Curtis dissimilarity metric, which provides a non-phylogenetic approach. After computing the distances or dissimilarities between samples based on

their bacterial community compositions, a non-metric multidimensional scaling (NMDS) approach was employed to ordinate the data. The NMDS analysis allows for the visualization of complex relationships and patterns in the data.

Alpha and beta diversity quantifications and computations were performed using the metaMDS function in the vegan package in R (Oksanen et al., 2020).

All figures were plotted in R and prepared for publication using the Adobe Illustrator 2021 software.

I.4 Results

We used an in-depth metagenomic sequencing approach to analyze RAS microbial communities. We directly compared samples to explore the spatiotemporal patterns of microbial communities across different RAS farms that vary in environmental conditions such as salinity, management styles, and farmed animals.

I.4.1 Taxonomic assignment

Taxonomic assignment analysis led to the identification of 693 species in the 496 samples. We classified these species without applying any cut-off or removing the negative controls. However, when a threshold of 1% was applied, only 256 unique species were classified. By examining different sample types with the same 1% cut-off, the number of uniquely detected species was as follows: species in biofilm samples ($N_{samples}$ = 264), 46 species in chip samples ($N_{samples}$ = 42), and 151 species in water samples ($N_{samples}$ = 186). Considering all sample types, we detected 416 unique species across the 492 samples. Notably, 17 species across the four negative control samples overlapped with the species detected in the samples.

I.4.2 Alpha diversity

We assessed two alpha diversity indices, namely the observed species and the Shannon index. Richness analysis revealed variability among sample types collected from different compartments within a farm. Generally, water samples exhibited higher alpha richness than the tank biofilms and biofilter carriers. Drum filter water demonstrated the highest richness in farms A, B, E, and F (62.0, 59.5, 60.5, and 63.0, respectively). However, this was not the case in farm C, where tank water had the highest richness (41.5), or in farm D, where the pre-denitrification compartment yielded the highest richness, 72.5. However, the biofilter carriers consistently yielded the lowest richness across all the farms. We observed a notable observation in the drum filter of farm B within circuit 2, where richness was often reduced when the tanks were shut down between rearing stocks (Figure 2C).

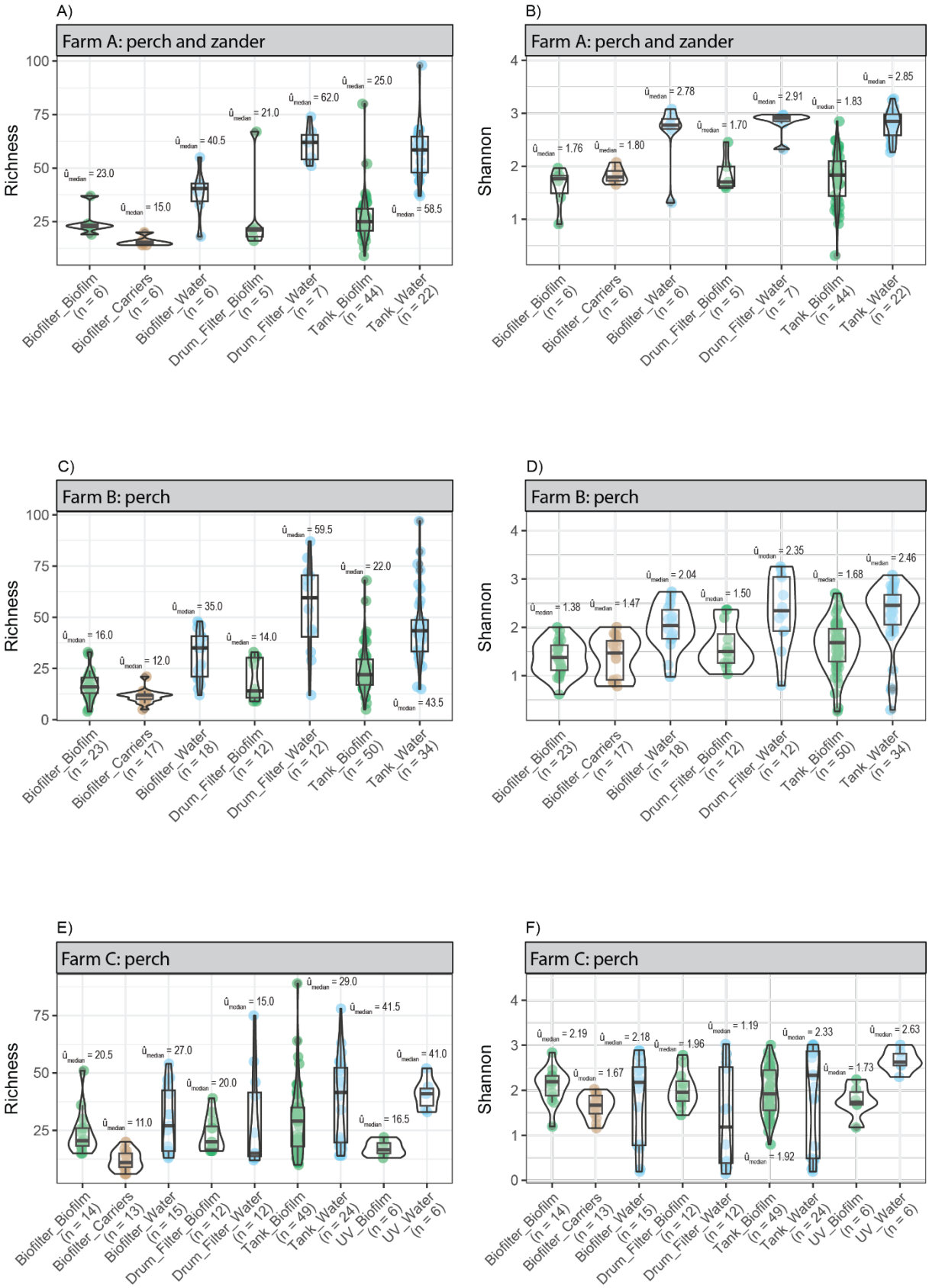
The Shannon diversity results mirror those of richness in that we detected distinct variations among sample types collected from different compartments within a farm. The highest diversity was observed in drum filter water samples, with farms A and E exhibiting median values of 2.19 and 2.23, respectively.

In farm B, tank water displayed the highest diversity, with a median value of 2.46. Similarly, the highest diversity was observed in farm F in the biofilter water samples, with a median value of 2.26. Finally, farm D exhibited the highest diversity in the denitrification biofilm samples, with a median value of 2.46.

I.4.3 Community composition patterns across farms

Community composition displayed substantial spatiotemporal variations across farms, compartments, salinity levels, and sample types. Notably, salinity concentration emerged as the primary factor along axis 1 of the NMDS plot, differentiating brackish water communities from freshwater communities (Figure 4A), indicating a robust adaptation to varying salinity concentrations. Moreover, despite the similar water quality and farmed animals, each freshwater farm exhibited a distinct composition, as seen along axis 2 (Figure 4B), underscoring the influence of general farm conditions on community structure. Additionally, sample type played a crucial role in shaping community composition, as water and biofilm communities exhibited distinct compositions (Figures 4C – 4H), with only a limited number of species detected in water and biofilms. Finally, the impact of management decisions on community composition is evident in Figure 4F, which illustrates the shift in water communities following the installation of a UV light during the two sampling events.

Across freshwater farms, certain species were always present despite different water sources and low salinity shifts, such as in farm E, highlighting their remarkable adaptability to diverse water conditions (Figures 5 – 7, 9, 10). The species detected across all freshwater farms included *Gemmatimonas aurantiaca*, a bacterium involved in phosphorus cycling, and *Nitrosomonas ureae*, a bacterium involved in nitrogen cycling. However, across the three core farms of the study (A, B, and C), *Chlorobi* bacterium OLB7, which contributes to nitrogen cycling; *Sphaerotilus natans*, known to cause sludge bulking; and *Cytophagaceae* bacterium BCCC1, *Acidovorax* sp. GW101 3H11 and *Flectobacillus* sp. BAB 3569 was consistently identified in the present study. Nitrospira defluvii, a nitrogen-cycling bacterium, was consistently detected across farms B, C, E, and F. In contrast, *Devosia* sp 66 22, a bacterium with bioremediation potential, was found in the microbial communities of farms A, B, C, and F.


A)
Farm A: perch and zander
Richness
Biofilter_Biofilm (n = 6)
Biofilter_Carriers (n = 6)
Biofilter_Water (n = 6)
Drum_Filter_Biofilm (n = 5)
Drum_Filter_Water (n = 7)
Tank_Biofilm (n = 44)
Tank_Water (n = 22)
B)
Farm A: perch and zander
Shannon
C)
Farm B: perch
Richness
Biofilter_Biofilm (n = 23)
Biofilter_Carriers (n = 17)
Biofilter_Water (n = 18)
Drum_Filter_Biofilm (n = 12)
Drum_Filter_Water (n = 12)
Tank_Biofilm (n = 50)
Tank_Water (n = 34)
D)
Farm B: perch
Shannon
E)
Farm C: perch
Richness
Biofilter_Biofilm (n = 14)
Biofilter_Carriers (n = 13)
Biofilter_Water (n = 15)
Drum_Filter_Biofilm (n = 12)
Drum_Filter_Water (n = 12)
Tank_Biofilm (n = 49)
Tank_Water (n = 24)
UV_Biofilm (n = 6)
UV_Water (n = 6)
F)
Farm C: perch
Shannon

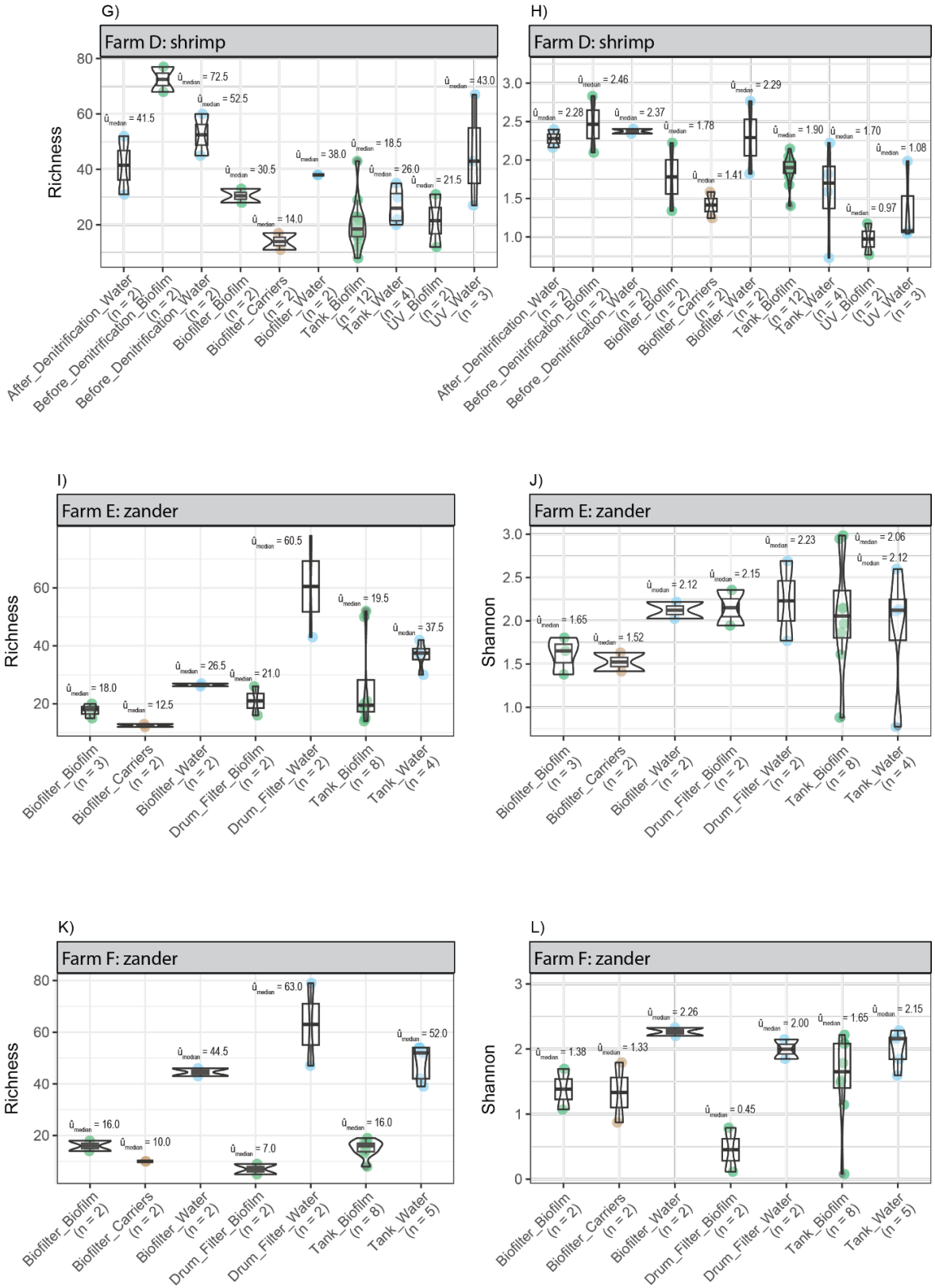


Figure 3: **Alpha diversity was assessed using the Richness and Shannon indices to compare sample types across compartments within a farm**. *Richness is shown in the left columns (panels: **A, C, E, G, I,** and **K**). In farms **A, B, D, E**, and **F**, the water samples featured the highest richness, followed by biofilm and biofilter carriers. In farm C (panel **E**), the drum filter biofilm displayed the highest richness, followed by water. Diversity is shown in the right columns (panels: **B, D, F, H, J**, and **L**). Overall, the patterns were similar to those of richness.*

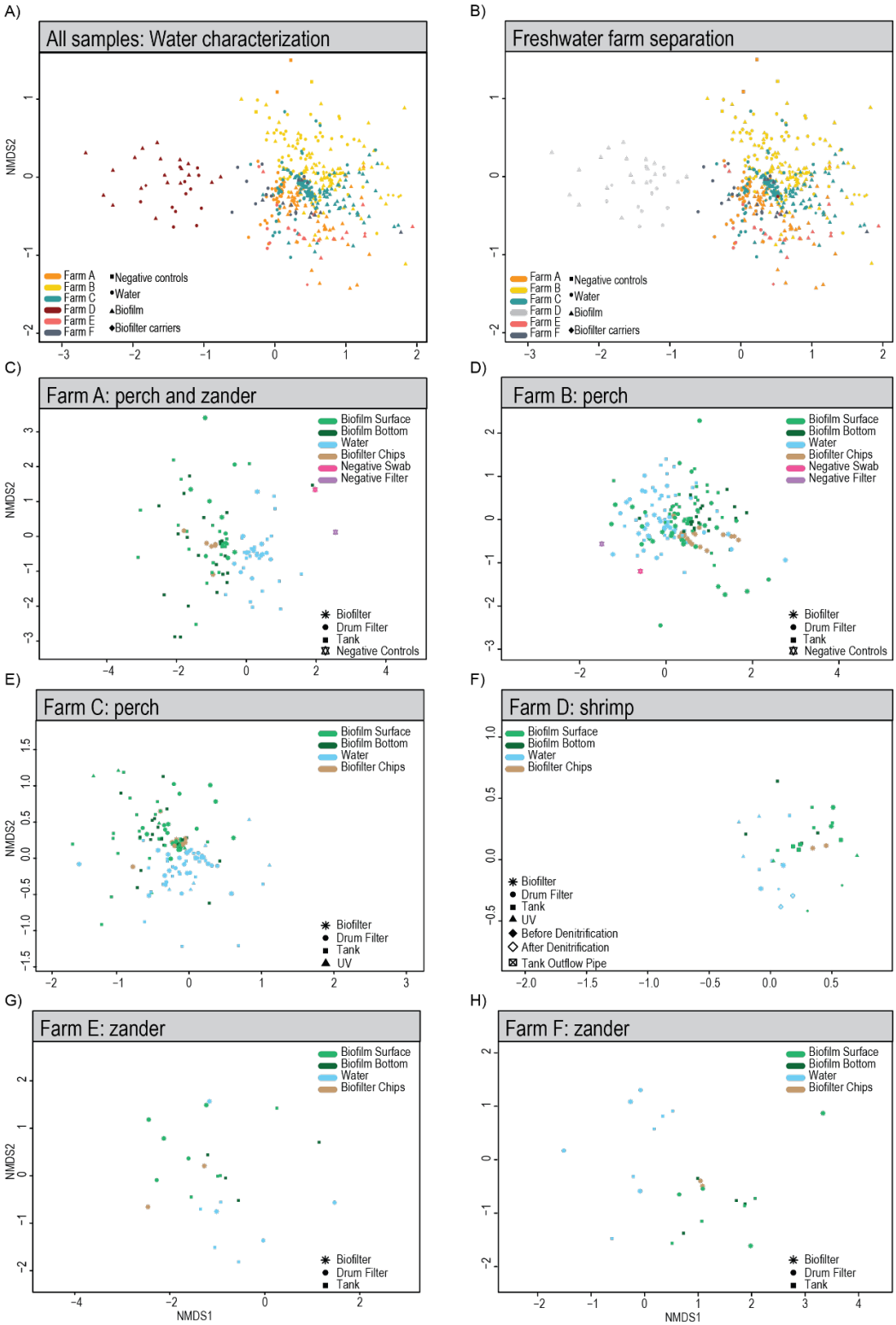


Figure 4: NMDS plots illustrating the influence of salinity, farm, and sample type on community patterns, as determined by shotgun metagenomic sequencing. *The analysis employed non-metric multidimensional scaling (NMDS) using the Bray-Curtis distance matrix, visualized with NMDS plots at the species level. In the analysis of 496 samples, axis 1 (**A**) clearly distinguishes brackish water communities from freshwater species, while axis 2 (**B**) indicates differentiation among freshwater farms. Across all farms, there was a noticeable separation between water and biofilm communities, with this distinction varying along axes 1 (**C–D**) and axes 2 (**E–H**). Notably, in farm F (**H**), introducing a UV light between the two sampling periods led to a pronounced shift in the community composition of the water samples, as seen along axis 2.*

I.4.4 Community composition and distribution within farms

We observed a consistent community composition across all compartments in farm A despite the different life stages and species in the tanks. This finding suggests a certain degree of homogeneity in the microbial communities throughout the farm. *Gemmatimonas aurantiaca*, a bacterium involved in phosphorus cycling, was the dominant species within biofilms (Figures 5A and B) but was not detected in water communities. The high abundance of *G. aurantiaca* indicates nutrient-rich water, potentially leading to eutrophication and reduced dissolved oxygen. In contrast, *Photobacterium damselae*, a pathogenic species that can cause vibriosis in a variety of marine animals, including fish, and an opportunistic pathogen in humans that can cause fatal necrotizing fasciitis in humans, is abundant in water communities, especially in zander tanks, posing a severe risk to fish and workers (Figure 5C).

In farm B, the microbial community composition displayed dynamic changes across different sample matrices and circuits. The biofilm community composition exhibited significant variation across compartments in circuits 1 and 2, likely due to the frequent shutdowns of these circuits and subsequent recolonization of biofilms, a process influenced by stochastic factors (Figure 6A). However, it is worth noting that after a recent stocking event on sampling day 88, a substantial abundance of *Sphaerotilus natans* was detected in the drum filter (Figure 6A), which is typically detected in high-nutrient water, indicating the impact of increased nutrient load on the system following a stocking event. In circuit 3, both the tank and biofilter biofilm communities underwent substantial compositional shifts on sampling day 75, which coincided with the start of a full system shutdown (Figure 6A) and a significant reduction in nutrient load. The tank community was dominated by *Brevundimonas diminuta*, a slow-growing bacterium. In contrast, Cutibacterium acnes, an opportunistic pathogen, dominated the biofilter community instead of the expected *Nitrospira japonica*, a nitrifying bacterium that dominated other sampling events. The biofilter carriers exhibited a relatively more stable community composition within the circuit but displayed variations between circuits (Figure 6B). The biofilms in circuits 1 and 2 were dominated by *Gemmatimonas aurantiaca* and *Devosia* sp 66 22, both of which are involved in phosphorus cycling. In contrast, *Nitrospira japonica*, a slow-growing nitrifying bacterium, was exclusively detected in circuit 3. The water communities exhibited a similar composition across sampling events, circuits, and compartments, which was expected to a certain extent because the same water source is used across all circuits (Figure 6C).

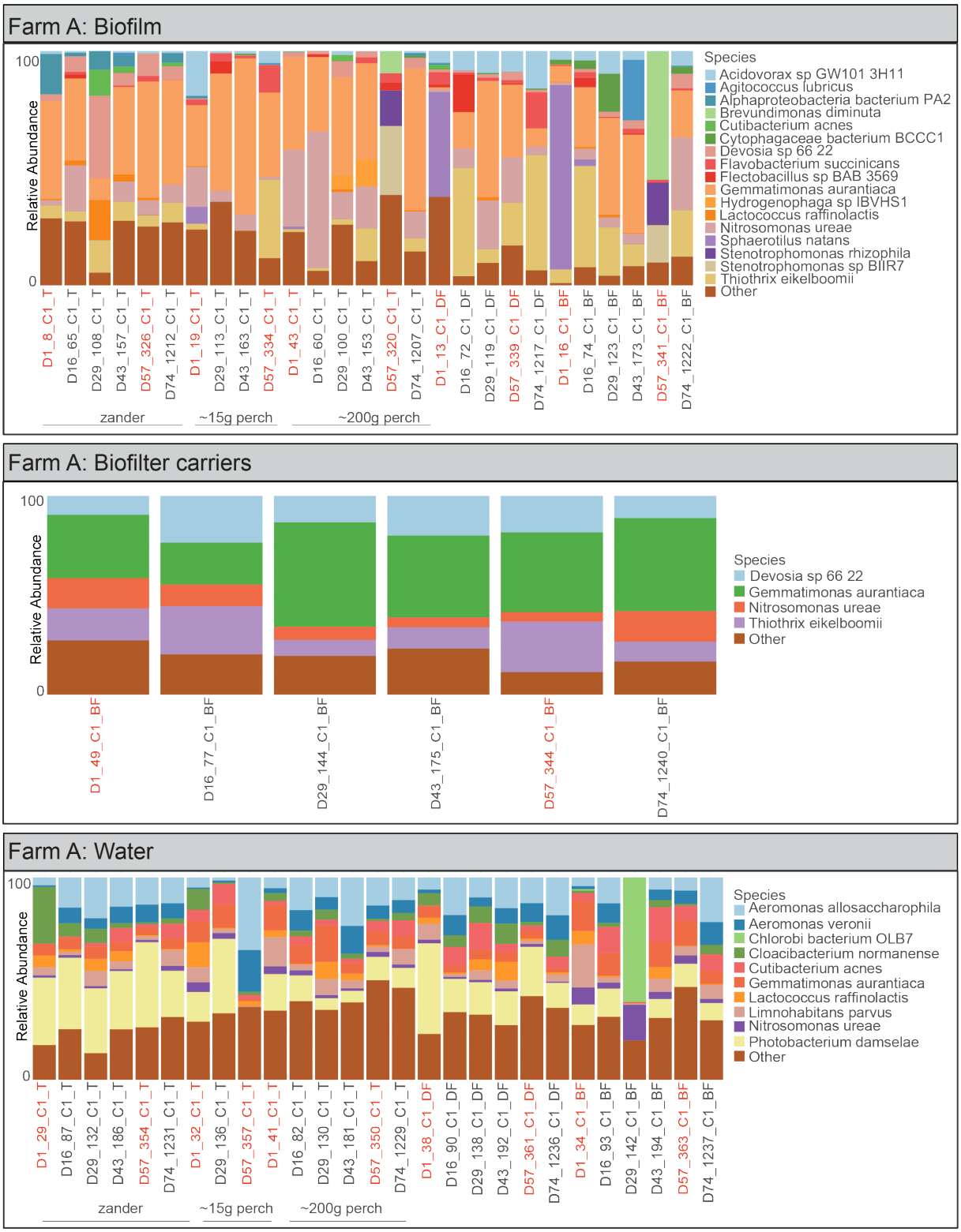


Figure 5: **Community composition across sample types within farm A**. *A consistent community structure was observed across all circuits, encompassing biofilm and water samples, even though different species were being farmed together. This finding suggests that the homogenizing effect of a single circuit plays a more significant role in shaping community composition than the host microbiota. The sample names are structured as follows: the first position represents the sample day, the second represents the sample number, the third represents the circuit, and the last represents the compartment (T = tank, DF = drum filter, and BF = biofilter). The sample names highlighted in red denote the disease outbreaks.*

In farm C, similarities and distinctions were observed between the biofilm and water communities

of the two circuits, which housed fish at different life stages. Similar species, commonly associated with freshwater water communities, were detected in both circuits. For example, the biofilm communities consistently included *the Chlorobi* bacterium OLB7, a bacterium involved in nitrogen cycling. Except on sampling days 25 and 53, this species was not detected in the biofilter biofilms of circuit 1 (Figures 7A and B). Another species consistently detected across circuits, compartments, and sample types was the freshwater bacterium *Sediminibacterium sp. FEMGT703* (Figure 7). Notable differences in community composition were also observed between biofilms and water communities. When comparing the tank biofilm communities between the two circuits, it was found that the tank biofilm from circuit 1 consistently included *Acidovorax* sp GW101 3H11, which was largely absent and sometimes completely absent from the tank biofilms of circuit 3. A similar pattern was observed in drum filter biofilms, with *Nitrospira defluvii* being noticeably present in circuit 3 but absent in circuit 1 (Figure 7A). Distinct community compositions were also revealed between the water communities of different circuits. In circuit 1, where powdered feed and a higher feeding rate occurred, *Sphaerotilus natans*, a species associated with nutrient-rich water, was consistently present (Figure 7C). This species exhibited an exceptionally high abundance in the UV water compartment, where competition might be reduced because of UV treatment, which may have a greater effect on sensitive community species. However, *S. natans* was absent in circuit 3, which houses larger perch that are fed a more solid feed pellet. Another distinctive community composition was observed within circuit 3, which undergoes frequent oxygenation events, unlike circuit 1. In this circuit, *Aurantimicrobium* sp MWH Mo1 dominated the microbial community, an aerobic freshwater microorganism that thrives in well-oxygenated environments (Figure 7B).

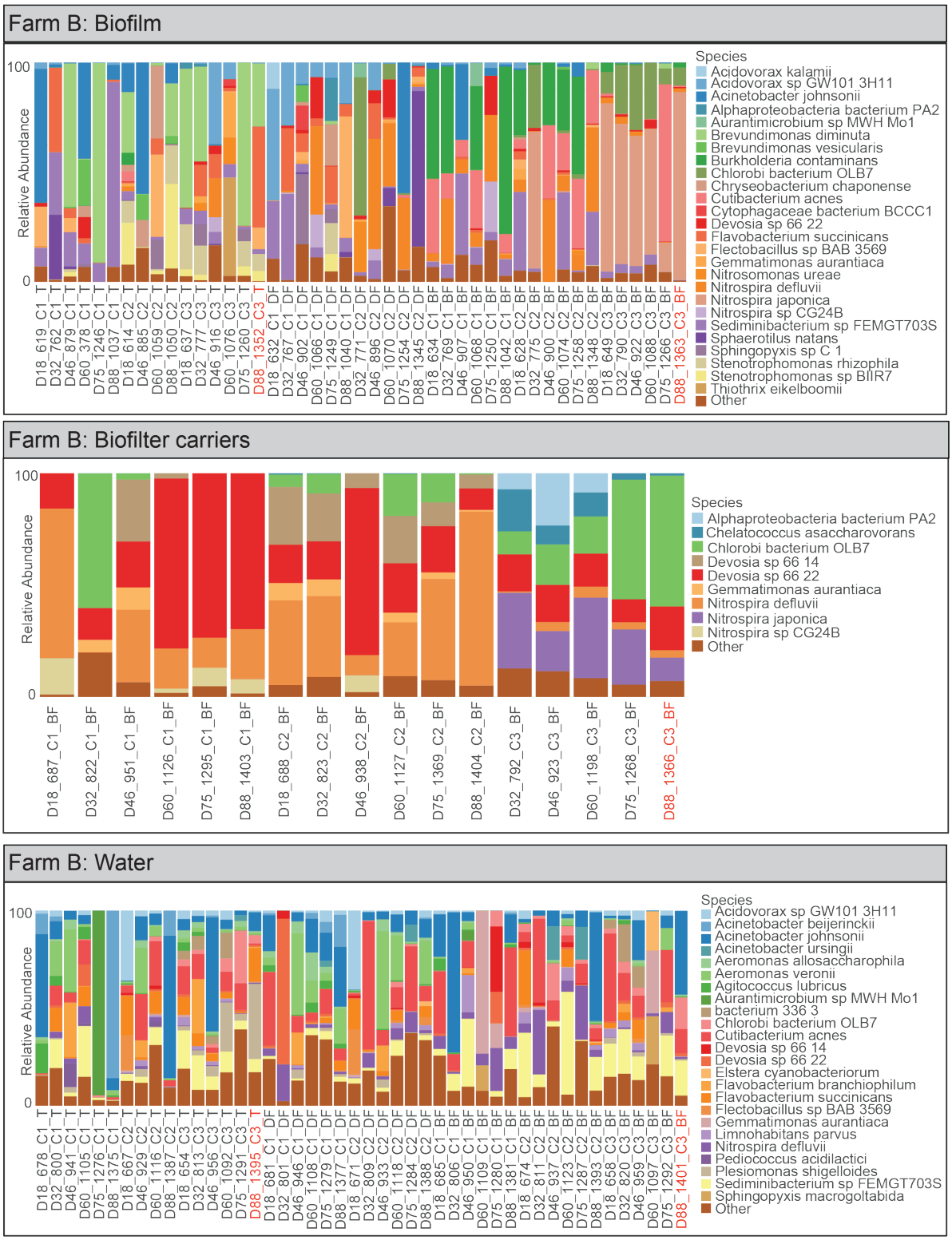


Figure 6: **Community composition across sample types within farm B**. *The community composition revealed distinctions between circuits 1 and 2, which accommodate ~ 1 g of fries following the same feeding, density, and management plan. In contrast, circuit 3 houses ~ 15 g of fries and adheres to a different feeding, density, and management approach. These findings suggest that life stage, nutrient loads, and management differences between circuits 1, 2, and 3 influence the community composition. The sample names are structured as follows: the first position represents the sample day, the second represents the sample number, the third represents the circuits, and the last represents the compartment (T = tank, DF = drum filter, and BF = biofilter). The sample names highlighted in red denote the disease outbreaks.*

Farm D, which specializes in shrimp farming in brackish water, exhibited a distinct community

composition compared with freshwater farms. The biofilm communities within the tank and biofilter compartments displayed greater dynamicity in their composition than those in the UV compartment during the two sampling events (Figure 8A). A notable difference was observed in the abundance of *Ruegeria pomeroyi*, a marine generalist, in the UV biofilm communities. In contrast, the biofilter carrier biofilm communities remained almost identical between the two sampling events, with a notable prevalence of *Defluviimonas denitrificans*, which is a species involved in nitrogen cycling. This finding suggests a stable and well-established biofilter community that aligns with the desired goals of the farm managers (Figure 8B). Similar to the biofilm communities, the water communities also exhibited distinct compositions between the tank and biofilter compartments compared to the UV compartment. *Phaeobacter italicus* was dominant in the UV water communities compared with the other compartments (Figure 8C).

In farm E, the microbial community demonstrated a relatively consistent structure across its compartments, which aligns with the non-disruptive approach taken by the management team of this farm. The farm follows a low-disturbance policy, refraining from routine cleaning and allowing the microbial communities to develop and reach a stable state naturally. The biofilm communities and biofilter carriers consistently exhibited stable community compositions (Figures 9A and B). However, greater variations in community composition were observed within the water communities (Figure 9C), suggesting a potentially more dynamic nature of water communities.

Farm F exhibited a distinct community composition between the biofilm and water communities. The tank biofilm communities displayed notable differences in composition between the two sampling events, with *Chryseobacterium chaponense*, a pathogenic species that predominantly dominated the community. The drum filter biofilm is primarily dominated by *Nitrospira defluvii*, a species involved in nitrogen cycling, which would not be expected to have a dominant presence in the drum filter but in the biofilter. Although *Nitrospira defluvii* was also detected in the biofilter, it had a much lower abundance (sampling day 6) and was almost absent on sampling day 67. The presence of *Thiothrix eikelboomii*, a sludge-bulking organism, in the tank and biofilter biofilm on sampling day 6 suggests the presence of nutrient-rich water (Figure 10A). In contrast, biofilter-carrier biofilm communities exhibit greater stability in their composition. However, similar to the biofilm communities, *Nitrospira defluvii* was minimally present or absent in the biofilter carrier biofilm communities (Figure 10B). The water communities consistently maintained a stable composition across all compartments and sampling days. However, they were dominated by *Photobacterium damselae*, an opportunistic pathogenic species that poses risks to farmed animals and personnel (Figure 10C).

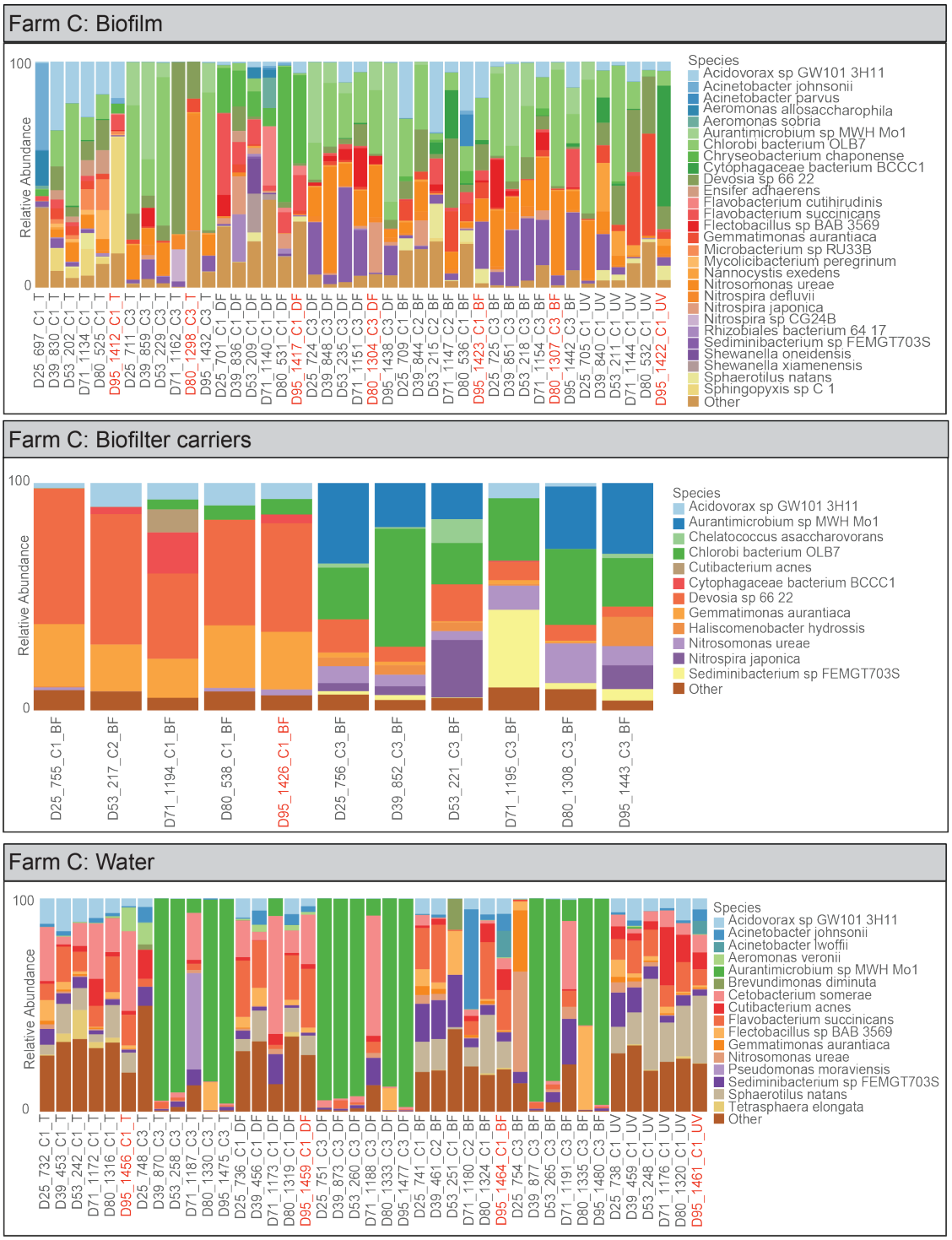


Figure 7: **Community composition across sample types within farm C**. *The community composition clearly distinguishes between the two circuits at the compartment and sample matrix levels. Circuit 1 accommodates approximately 15g fries, which are raised at a different density and fed according to a distinct schedule compared to circuit 3, where perch weighing around 200g are raised. These findings strongly imply that the life stage of farmed organisms, nutrient loads, and management variations between circuits can influence community structure. The sample names are structured as follows: the first position represents the sample day, the second position represents the sample number, the third position represents the circuit, and the last position represents the compartment (T = tank, DF = drum filter, BF = biofilter, UV treatment). The sample names highlighted in red denote the disease outbreaks.*

I.4.5 Spatial distribution of functional services

The diversity of nutrient-cycling species was relatively constrained in farm A, which was often challenged by disease outbreaks and underwent frequent treatments. Across all sample types, *Gemmatimonas aurantiaca*, a bacterium involved in phosphorus cycling, and *Nitrosomonas ureae*, a bacterium contributing to nitrification, were observed (Figure 5). In contrast, the *Chlorobi* bacterium OLB7, a nitrogen-fixing bacterium, was exclusively detected in water samples (Figure 5C).

Within farms B and C, the presence of the same nutrient-cycling species suggests that these species play a fundamental role in the nutrient-cycling processes within freshwater systems. However, the abundance and spatial distribution of these species varied across circuits, compartments, and sample types within each farm (Figures 6 and 7), suggesting that additional drivers within farms shape the presence of these species. *Chlorobi* bacterium OLB7, responsible for nitrogen fixation, was detected in all sample types from farm B, with a dominant presence in circuit 3 (Figure 6). However, in farm C, similar abundances were detected across all compartments but only in the water communities (Figure 7C). *Gemmatimonas aurantiaca*, which is involved in phosphorus cycling, was detected across all sample types in both farms. In farm B, it was present in the tanks, drum filters, and biofilters of circuits 2 and 3. In farm C, it was detected across all circuits and compartments, with the highest abundance in the biofilm communities of the UV compartment and biofilter carriers in circuit 1 and the biofilter water of circuit 2 (Figure 7). Four species involved in the nitrification process were detected within the farms; however, their detection and abundance varied between circuits and compartments, affecting nitrogen cycling rates. *Nitrospira defluvii* was detected in all sample types but showed contrasting patterns between the two farms. It was most abundant in circuits 1 and 2 of farm B, which houses 1 g perch fry and undergoes frequent shutdowns. In contrast, it was most abundant in circuit 3 of farm C, which housed ~ 200 g perch and experienced fewer shutdowns. *Nitrosomonas ureae* was detected only within the biofilm communities of circuits 2 and 3 in farm B (Figure 6A) but in all sample types and compartments within farm C (Figure 7). *Nitrospira japonica* was detected in the tank biofilms of circuits 2 and 3 and the biofilter carriers of circuit 3 in farm B, and across both circuits and all compartments, except the drum filter of circuit 1 in farm C (Figures 6A and B). *Nitrospira* sp. CG234B was detected in the biofilm communities of the biofilter (circuit 1), drum filter, biofilter carriers (circuits 1 and 2), and tanks (circuit 3). In farm C, it was only detected in the tank biofilm of circuit 3 (Figure 7A). However, *Nitrospira japonica* and *Nitrospira* sp CG234B were absent in the water communities (Figure 7C).

In farm D, several of the detected species were involved in various forms of nutrient cycling or degradation. *Methyloceanibacter marginalis* and *Ruegeria pomeroyi* are associated with carbon cycling processes. *Ahrensia marina* is involved in the enzymatic cleavage of dimethylsulfoniopropionate to dimethylsulfide, whereas *Alteromonas macleodii* is responsible for the degradation of sugars and amino acids. Finally, *Donghicola tyrosinivorans* is involved in tyrosine degradation.

Farm E contained beneficial species involved in nutrient cycling and organic matter degradation.

Acinetobacter johnsonii, known for its role in organic matter degradation, was identified in drum filter and biofilter water communities (Figure 9C). *Gemmatimonas aurantiaca*, which is responsible for phosphorus cycling, was detected across all sample types, with a dominant presence in the biofilter carriers (Figure 9B). Additionally, four species involved in nitrogen cycling were identified across sample types, including *Candidatus Nitrospira nitrificans*, *Elstera cyanobacteriorum*, *Nitrosomonas urea*, and *Nitrospira defluvii*, with the first two species unique to this farm. *Candidatus Nitrospira nitrificans* were exclusively detected in the drum filter (Figure 9A), designed to remove large waste particles, whereas *Elstera cyanobacteriorum* was only detected in the water samples (Figure 9C).

Farm F identified nutrient-cycling species similar to other freshwater farms. *Gemmatimonas aurantiaca* was detected in the biofilter biofilms (Figure 10A). *Nitrosomonas ureae* was present in the tank, drum filter, biofilter biofilms, and biofilter carriers (Figures 10A and C). Finally, *Nitrospira defluvii* was detected in both biofilms and biofilter carriers, with a high abundance in drum filter biofilms (Figure 10A).

I.4.6 Spatial distribution of pathogenic species

Several opportunistic bacterial pathogens have been consistently detected in RAS, presenting potential risks to the health of farm animals and employees. These pathogens can cause diseases or symptoms, resulting in stock mortality or illnesses. This study confirmed the presence of similar opportunistic bacterial species, as reported in previous studies. Interestingly, the detection of these pathogens varies, with some being exclusively detected in water or biofilm samples, whereas others are detected across different sample types.

The number and specific species of pathogens varied among the three core farms. Farm B showed the highest count of detected pathogen species (*N* = 11), followed by farm C (*N* = 8) and farm A (*N* = 6), which experienced the highest incidence of disease outbreaks (Figures 5–7). Within these three farms, *Aeromonas veronii*, known to affect poikilothermic animals such as fish and cause hemorrhagic septicemia, was exclusively identified in the water communities (Figure 5C). Conversely, *Brevundimonas diminuta* (Figures 5A and 6A), *Aeromonas allosaccharophila* (Figure 7A), *Chryseobacterium chaponense* (Figures 6A and 7A), and *Brevundimonas vesicularis* (Figure 6B) were detected only in the biofilm samples. Furthermore, certain species were found in both the biofilm and water communities across the farms, including *Flavobacterium succinicans* (Figures 6A and C, 7A and C) and *Cutibacterium acnes* (Figures 5A and C, 6A and C, and 7A and C). *Brevundimonas diminuta* was also present in both the water and biofilm samples but never in both sample types within a single farm. For example, in biofilm communities, it was detected in farms A and B (Figures 5A and 6A), whereas in farm C, it was detected in water communities (Figure 7C). Notably, *B. diminuta* exhibited a significant increase in relative abundance within the biofilter on sampling day 57, coinciding with a reported disease outbreak from management (Figure 5A).

In farm D, the presence of opportunistic bacterial pathogens was limited, with only two species detected. *Vibrio parahaemolyticus* was found in biofilm and water communities (Figures 8A and C), while *Vibrio vulnificus* was exclusively detected in water communities (Figure 8C). Both species are known causative agents of vibriosis.

In farm E, three opportunistic pathogens were identified across sample types and compartments. *Chryseobacterium chaponense*, known to cause disease in rainbow trout, was detected in drum filter biofilms (Figure 9A), indicating its ability to thrive in high-nutrient environments. In contrast, *Cutibacterium acnes*, which can potentially cause intestinal granulomas, and *Plesiomonas shigelloides*, which are known to induce subcutaneous hemorrhagic ulcers, were found in water communities across all compartments (Figure 9C).

In farm F, two opportunistic bacterial pathogens were identified in the biofilm and water communities. *Chryseobacterium chaponense* was the predominant species detected in the tank biofilm samples collected on sampling day 67 (Figure 10A), raising concerns about a potential disease outbreak. *Photobacterium damselae*, known to cause hemorrhaging and ulcerative skin lesions, poses a risk to workers as it can lead to necrotizing fasciitis and is the dominant species found in all water communities (Figure 10C).

Figure 8: **Community composition across sample types within farm D**. *The results demonstrated community variation among biofilm and water samples, while the biofilter carrier communities exhibited remarkable consistency across sampling events, suggesting a relatively stable environment within this specific compartment. Additionally, the UV compartment shows a distinct composition compared to the other compartments, affecting both biofilm and water communities, indicating a significant impact of UV treatment on community composition. The sample names are structured as follows: the first position represents the sample day, the second position represents the sample number, the third position represents the circuit, and the last position represents the compartment (T = tank, DF = drum filter, BF = biofilter, and UV = UV treatment).*

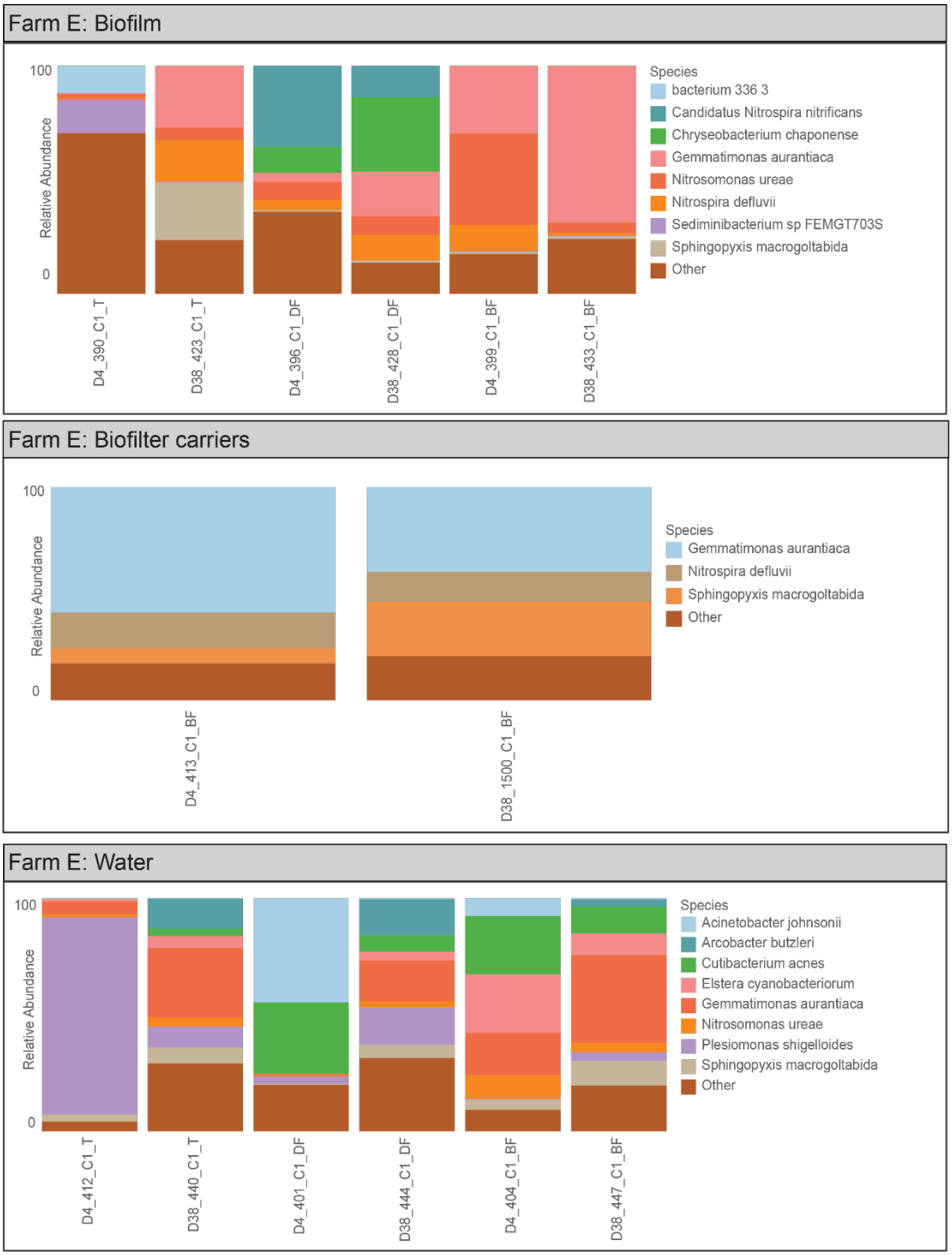


Figure 9: **Community composition across sample types within farm E**. *The findings indicate a high variance in biofilm and water samples, while the biofilter biofilm communities remain remarkably consistent across sampling events, implying a relatively stable environment within this particular compartment. In addition, nutrient-cycling species were detected in higher abundance in the biofilter biofilms and biofilter carriers, for example, Gemmatimonas aurantiaca, Nitrospira defluvii, and Nitrosomonas ureae (biofilms only). The sample names are structured as follows: the first position represents the sample day, the second represents the sample number, the third represents the circuit, and the last represents the compartment (T = tank, DF = drum filter, and BF = biofilter).*

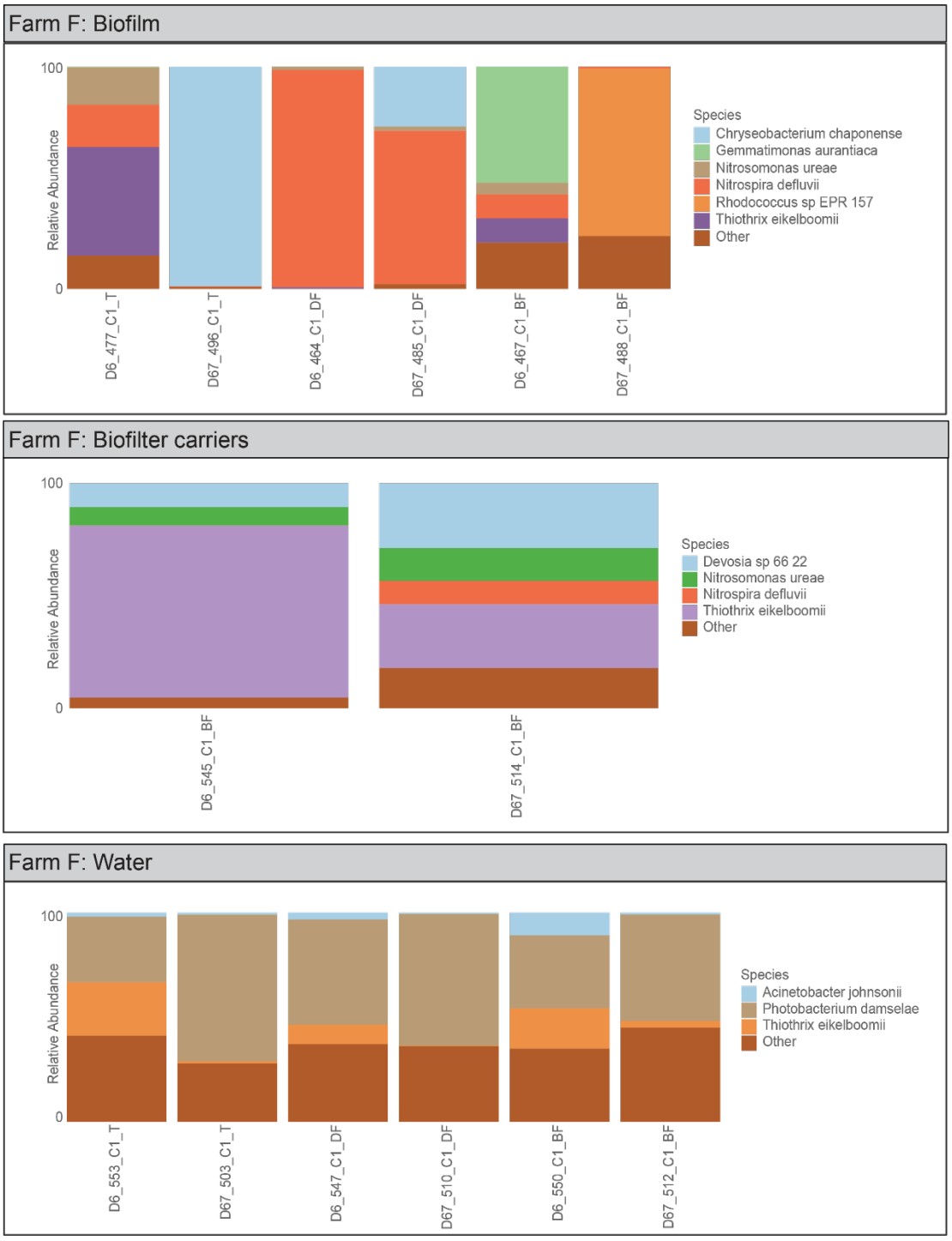


Figure 10: **Community composition across sample types within farm F**. *Notable variability is observed among the different compartments in the biofilm communities. This implies potential variations in biofilm developmental stages during natural succession or due to distinct environmental pressures in these compartments, leading to contrasting structures. Notably, a pronounced shift is evident in the tank biofilms during the second sampling event, where Chryseobacterium chaponense, an emerging pathogen, dominates the community, potentially serving as an indicator for a disease outbreak. Conversely, the biofilter biofilms and water communities demonstrate compositional stability over time. Another noteworthy finding in water communities is the prevalence of Photobacterium damselae, a zoonotic pathogen posing health risks to both animals and personnel. The sample names are structured as follows: the first position denotes the sample day, the second position represents the sample number, the third position indicates the circuit and the last position designates the compartment (T = tank, DF = drum filter, and BF = biofilter).*

I.5 Discussion

Microbial communities play a critical role in the functioning of RAS, as they are responsible for essential processes, such as nutrient cycling and maintaining animal health. Understanding the dynamics of these microbial communities is vital for ensuring the success of RAS. However, current knowledge in this field is limited, particularly concerning large-scale spatio-temporal patterns and the identification of core microbial species required for stable communities. Revealing key driving forces that shape these communities, such as environmental parameters, including salinity, pH, temperature, intra- and inter-species dynamics, including competition for available resources or niches, and ecological processes, such as dispersion, colonization, and succession, are required for predictive measures that can then be directly applied in real-world situations. Gaining more profound knowledge of community composition and ecological drivers could be used to develop targeted interventions and management strategies for the maintenance of desired microbial community compositions and functions, innovative technologies that optimize nutrient cycling, minimize disease risks, and identify key microbial species that contribute to system stability and performance.

This study conducted a large-scale spatio-temporal analysis using shotgun metagenomics data to analyze the microbial community composition, distribution, and patterns across six semi-closed RAS systems comprising fresh and brackish water-farmed species. A key finding of this study was the notable influence of salinity on community composition (Figure 4A) but not on species richness (Figure 3). These findings are consistent with those of a previous study conducted by Dong *et al*. (2022), which demonstrated that salinity had no significant impact on bacterial richness but played a crucial role in shaping community shifts and reducing microbial activity. The reduction in microbial activity with increasing salinity is further discussed in a review by Liang *et al.* (2023). Similarly, Bakke et al. (2017) investigated the impact of different salinity concentrations (12, 22, and 32 ppt) on community composition in post-smolt salmon RAS, revealing distinct microbial communities among the different systems and emphasizing the prominent role of salinity as a driver of the community structure. Overall, this finding adds to the extensive evidence of the influence of salinity on community composition, highlighting its crucial role as a fundamental driver, particularly in aquatic environments (Herlemann et al., 2011).

Another key finding was the strong effect of the sample site and matrix on the results and conclusions. Differences between biofilm and water communities have been reported previously in an Atlantic salmon RAS (Bakke et al., 2017), a flow-through fish farm for lumpfish (Roalkvam et al., 2019), a post-smolt salmon RAS (Almeida et al., 2021), and a perch RAS (Rieder et al., 2023). However, these findings showed that biofilm communities had a higher richness than water samples, which directly contradicts our findings in this study, which revealed the highest richness in water samples (Figure 4A, C, E, G). This discrepancy may be attributed to technical issues related to the biofilm collection method,

such as inadequate swab material for aquatic sampling, potentially leading to ineffective capture of biofilm communities, resulting in lower diversity. At the same time, the DNA extraction method may not have been sufficiently harsh to remove biofilms from the carriers, resulting in lower richness. Future optimization should be conducted for the extraction of DNA from biofilter carriers.

Furthermore, our findings indicate that community composition shows greater dynamics in circuits that experience frequent disturbances such as tank cleaning or shutdown/startups. Communities within these circuits exhibited a highly stochastic pattern (Figures 5-10A and C), unlike compartments with fewer disturbances, such as biofilter carrier communities, which displayed a more consistent community pattern across sampling events (Figures 5-10B). Notably, the tank biofilm communities, which were regularly removed during routine cleaning procedures, showed continuous recolonization and high stochasticity, supporting the findings of Rieder *et al.* (2023). This observation aligns with a study conducted by Aguilar *et al*. (2020), which demonstrated that homogenizing dispersal, a stochastic process, is the primary assembly mechanism at a short-term scale (e.g., daily or weekly), whereas homogeneous selection, a deterministic process, becomes the primary assembly mechanism at a larger scale (e.g., yearly). The impact of biofilm removal and subsequent successional processes on ecological function and animal health warrants further investigation.

Another notable finding from this study was the consistent distinction between UV-treated water communities and water communities of other compartments in farm D (Figures 8A and C). However, this distinction was not observed in farm C (Figures 7A and C). One potential explanation for this difference could be the variance in water turbidity between the two farms, with farm C potentially having more turbid waters. It is known that high turbidity can reduce the effectiveness of UV treatment (Liltved et al., 1995), which may result in a lesser impact on community structure and a more homogenous composition compared to other compartments. Although UV treatment is a common procedure for reducing pathogen load, limited research has been conducted on its impact on the microbial community, especially in freshwater systems. However, recent studies on marine RAS have shown significant regrowth of bacteria, including fast-growing and possibly opportunistic species, following UV treatment. Regrowth of bacteria after UV treatment has been linked to altered microbial community composition, resulting in negative effects on marine larval health and survival (Attramadal et al., 2021; Dahle et al., 2023). To gain a deeper understanding of these dynamics in freshwater systems, future studies should investigate the relationships between UV treatment, specifically concerning ecological functions, and the impact on animal health.

I.5.1 Disease and health

Understanding the potential disease risks associated with RAS is of utmost importance, as it has profound implications for the economic viability and sustainability of the aquaculture industry and the health and well-being of farmed animals. Opportunistic pathogenic bacteria pose a significant

challenge, severely restricting the successful cultivation of aquatic species globally and resulting in substantial economic loss. Recent estimates indicate that the annual global economic loss due to infectious diseases in aquaculture exceeds USD 6 billion (Stentiford et al., 2012, 2020).

Compared to other aquaculture systems, such as large ponds or flow-through systems, RAS experiences greater water quality fluctuations, making the system more susceptible to instability. Changes in water quality, such as temporary increases in ammonia or nitrite levels, can lead to stress among animals, thereby increasing the risk of disease and potential losses. Opportunistic bacterial populations can become concentrated within RAS, with certain bacteria, including *Aeromonas* spp., *Vibrio* spp., *Streptococcus* spp., and *Flavobacterium* spp. thriving in recirculating systems (Howell, 2022). The effective control of pathogens in RAS requires a comprehensive understanding of their presence, transmission routes, and strategies to mitigate their abundance (Wright et al., 2023).

Our study revealed various opportunistic bacterial species across farms and different sample types, highlighting the challenges in maintaining a pathogen-free environment. Within the three semi-interconnected freshwater farms (A, B, and C), we consistently detected *Aeromonas veronii*, *A. allosaccharophila*, *Brevundimonas diminuta*, and *Flavobacterium succinicans*, illustrating their wide dispersal across different water sources and their ability to colonize diverse habitats. *Aeromonas* species can cause diseases in various farmed aquatic organisms, including fish and shellfish, affecting different parts of the animal, such as the skin, fins, gills, and internal organs. The severity of these diseases can range from mild symptoms to acute infections, leading to high mortality rates in the affected population (Huang et al., 2020; Huang et al., 2022). *Flavobacterium* species are particularly relevant in fish farming, where they cause a range of diseases collectively known as flavobacteriosis. Despite over a century of scientific research, controlling and preventing flavobacteriosis has proven challenging, resulting in significant global stock loss (Loch & Faisal, 2015).

Certain pathogens exhibit specific preferences for particular sample matrices. For instance, in freshwater RAS, *Aeromonas sobria, Brevundimonas vesicularis,* and *Chryseobacterium chaponense* are exclusively detected in biofilms. Biofilms can act as reservoirs for recurrent infections (Normington et al., 2021) and can protect against water-based treatments. *Aeromonas* sobria has been implicated in various diseases in different fish species, including perch (*Perca fluviatile*; (Wahli et al., 2005) and red garra (*Garra rufa*; (Majtán et al., 2012). *Chryseobacterium chaponense* and *Brevundimonas vesicularis* are emerging opportunistic pathogens with limited information regarding their interactions and contributions to animal health, underscoring the need for further research to elucidate their ecological roles and potential impacts on animal health (Kämpfer et al., 2011; Ryan & Pembroke, 2018).

In contrast, *Acinetobacter lwoffii*, *Aeromonas veronii*, *Flavobacterium branchiophilum*, *Photobacterium damselae*, and *Plesiomonas shigelloides* were exclusively detected in water samples. *Acinetobacter lwoffii* has been reported as an emerging pathogen for fish, causing pathological lesions,

hemorrhage, degeneration, necrosis, and inflammation. It is also a significant human pathogen associated with nosocomial infections such as bacteremia, pneumonia, and meningitis (Cao et al., 2018). *Aeromonas veronii* is a widely distributed pathogen that can infect various species, including freshwater fish, amphibians, birds, and red meat animals, resulting in serious losses to the aquaculture industry and threatening food safety. Additionally, *Aeromonas veronii* can cause infections in humans, particularly in individuals with weakened immune systems, resulting in sepsis, gastroenteritis, and other diseases (T. Li et al., 2020). *Flavobacterium branchiophilum* is the primary causative agent of bacterial gill disease, a severe condition affecting various cultured freshwater fish species, particularly salmonids (Touchon et al., 2011). *Photobacterium damselae*, found exclusively in water communities, is a zoonotic agent capable of causing a range of diseases in fish, including skin lesions, ulcerations, hemorrhage, and septicemia. Severe cases can result in high mortality rates. In humans, especially those with compromised immune systems, they can cause soft tissue infections and necrotizing fasciitis (Matanza & Osorio, 2020). *Plesiomonas shigelloides* was detected in diseased silver carp (*Hypophthalmichthys molitrix*), a widely cultivated fish, and was associated with mortality (Behera et al., 2018).

Furthermore, we observed that specific pathogens adapted to salinity levels. *Vibrio parahaemolyticus* and *Vibrio vulnificus*, the causative agents of vibriosis, were exclusively detected in the shrimp farm, indicating their salt-adapted nature. Both pathogens pose significant challenges to the shrimp industry and sometimes lead to farm closures (Jayasree et al., 2006; Letchumanan et al., 2015).

Given the zoonotic properties of many of these opportunistic bacteria, implementing effective biosecurity measures and proper hygiene practices in RAS settings is crucial to minimize transmission and mitigate the impact of these pathogens on fish and human health. By identifying and addressing potential disease risks, aquaculture operators can enhance production efficiency, reduce economic losses, and ensure the health and welfare of farmed animals and personnel, thereby ensuring the long-term sustainability and viability of the aquaculture sector.

I.6 Future outlooks and conclusion

The preliminary results in this chapter demonstrate that microbial communities within the RAS exhibit high levels of dynamics and site specificity despite continuous water circulation throughout the system. This study adds to the already abundant data on the effects of salinity and environmental factors, such as water parameters or disturbances, on microbial community structure.

Future endeavors for the manuscript will extend beyond this initial exploration. The plans include conducting a more comprehensive community composition analysis encompassing all microorganisms present. This analysis aims to untangle the influence of frequent disruption and recolonization processes on biofilm and water communities and investigate the temporal patterns in greater detail

across farms, circuits, and compartments. Additionally, functional genes will be explored to determine the functional services provided within these systems and to understand whether community composition fluctuations truly affect the provision of these functions.

Collaborative efforts with other research groups will also be undertaken to delve into in-depth bacteriophage analysis and to analyze microbial evolutionary changes, enabling the development of more targeted treatment plans. These future projects aim to provide a more comprehensive understanding of the intricate dynamics and evolutionary aspects of microbial communities within the studied systems.

Future research on RAS microbial communities is essential to fully harness their potential. This includes utilizing their capabilities during biofilter start-up or disease prevention, uncovering fundamental biological principles related to the relationship between environmental stressors and microbiome dysbiosis, and elucidating medically relevant interactions between the host-microbiome-environment and disease development. This knowledge will pave the way for sustainable and effective management strategies for the aquaculture industry.

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Note

Detecting aquatic pathogens with field-compatible dried qPCR assays

Jessica Rieder [a,1], Pedro M. Martin-Sanchez [b,c,1], Omneya A. Osman [d], Irene Adrian-Kalchhauser [a], Alexander Eiler [b,d,*]

[a] Institute for Fish and Wildlife Health, University of Bern, Länggassstrasse 122, 3012 Bern, Switzerland
[b] Section for Aquatic Biology and Toxicology, Department of Biosciences, University of Oslo, P.O. Box 1066, Blindern 0316, Oslo, Norway
[c] Instituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, Avenida Reina Mercedes 10, 41012 Seville, Spain
[d] eDNA Solutions AB, Bjorkasgatan 16, SE-43131 Molndal, Gothenburg, Sweden

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ABSTRACT

Field-ready qPCR assays with extended shelf-life support monitoring programs for emerging aquatic pathogens and enable quick conservation and management decisions. Here, we developed, validated, and tested the shelf-life of qPCR assays targeting *Gyrodactylus salaris* and *Aphanomyces astaci* with lyophilization and air-drying.

Pathogenic microorganisms are a major threat to aquatic and terrestrial ecosystems. Globalization (international trade, transportation, and urbanization) and anthropogenic global changes have fostered the spread of pathogens (McIntyre et al., 2017; Guenard, 2021), resulting in biodiversity decline and economic losses. Three relevant aquatic pathogens with negative economic and ecological implications are: (i) the monogenean salmon parasite *Gyrodactylus salaris* (*Gs*) that colonizes the skin, gills, and fins of salmon and has caused widespread losses in both wild and farmed Atlantic salmon (Bakke et al., 1992; Rusch et al., 2018), (ii) the oomycete crayfish pathogen *Aphanomyces astaci* (*Aa*) that elicits crayfish plague in native European, Asian, and Australian crayfish species and causes massive population die-off events (Martín-Torrijos et al., 2021), and (iii) the amphibian-targeting panzootic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which originated in Asia, spread globally because of amphibian trade, and has decimated >500 amphibian species over the past half-century (Fisher and Garner, 2007, 2020; Scheele et al., 2019).

The analysis of environmental DNA (eDNA) is an emerging tool for quick and relatively inexpensive monitoring and detection of aquatic pathogenic organisms (Amarasiri et al., 2021). As a result, scientists, governmental agencies, and companies are increasingly incorporating eDNA methods into (semi)-automatic sampling machines coupled to portable real-time quantitative PCR (qPCR) thermocyclers for continuous on-site pathogen monitoring of waterways (Thomas et al., 2020; Sepulveda et al., 2019, 2020). However, a remaining challenge is the requirement of cold storage for key reagents, which prohibits their use in field-operating machinery. Reagents that can be dried and stable at room temperature (RT) are commercially available. However, they have not been independently evaluated for their applicability and true shelf-life regarding eDNA monitoring of pathogens.

This study describes field-ready storable dried qPCR assays for three aquatic pathogens, *Gs, Aa,* and *Bd*, all based on previously published and optimized primers and probes (Table 1). For *Gs* and *Aa* assays, we compared two different drying methods, lyophilization and air-drying, respectively, and the amplification efficiency of dried assays across a time series (Table 1). The dried *Bd* assay was not evaluated for shelf-life, so results are not shown, but it was tested at several time pointsafter drying and showed promising results.

All three assays targeted the ribosomal DNA internal transcribed spacer 1 (ITS1) region and were evaluated for reproducibility and sensitivity in a wet, freshly-made state. The standard curves were generated using serial dilutions of synthetic double-stranded DNA fragments (gBlocks, Integrated DNA Technologies, Inc., Leuven, Belgium) encompassing the primer/probe target regions of the three

* Corresponding author at: Section for Aquatic Biology and Toxicology, Department of Biosciences, University of Oslo, P.O. Box 1066, Blindern 0316, Oslo, Norway.
E-mail addresses: jessica.rieder@unibe.ch (J. Rieder), p.m.martin-sanchez@ibv.uio.no, pmartin@irnase.csic.es (P.M. Martin-Sanchez), omneya@ednasolutions.se (O.A. Osman), irene.adrian-kalchhauser@unibe.ch (I. Adrian-Kalchhauser), alexander.eiler@ibv.uio.no (A. Eiler).
[1] These authors contributed equally to this work.

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Table 1
qPCR assays evaluated in this study.

Target[a] (Reference)	Forward primers (conc.)	Reverse primers (conc.)	TaqMan probe (conc.)	IPC[b]	gBlocks / reference sequences (Acc. No)[c]	qPCR program	Drying method	Shelf-life tested
Gs (Rusch et al., 2018)	Gsal-208F (0.75 µM)	Gsal-149R (0.75 µM)	Gsal-188P-MGB2 (0.25 µM)	Yes	Gs_124–289 (DQ898302)	2 min 95 °C; 45 cycles (10 s 95 °C, 1 min 60 °C)	Lyophilization	Yes
Aa (Vrålstad et al., 2009)	AphAstITS-39F (1.2 µM)	AphAstITS-97R (1.2 µM)	AphAstITS-60 T (0.3 µM)	No	Aa_1–152 (AM947023)	2 min 95 °C; 45 cycles (5 s 95 °C, 20 s 60 °C)	Air drying	Yes
Bd (Boyle et al., 2004)	ITS1–3 Chytr (0.9 µM)	5.8S-Chytr (0.9 µM)	Chytr-MGB2 (0.25 µM)	Yes	Bd_26–271 (AY598034)	2 min 95 °C; 50 cycles (10 s 95 °C, 1 min 60 °C)	Lyophilization	No

[a] *Gs*: *Gyrodactylus salaris*; *Aa*: *Aphanomyces astaci*; *Bd*: *Batrachochytrium dendrobatidis*.
[b] IPC: Internal Positive Control including a template DNA and its complementary TaqMan probe and primers.
[c] gBlocks names refer to the selected positions in the corresponding reference sequences, whose GenBank Accession numbers are detailed.

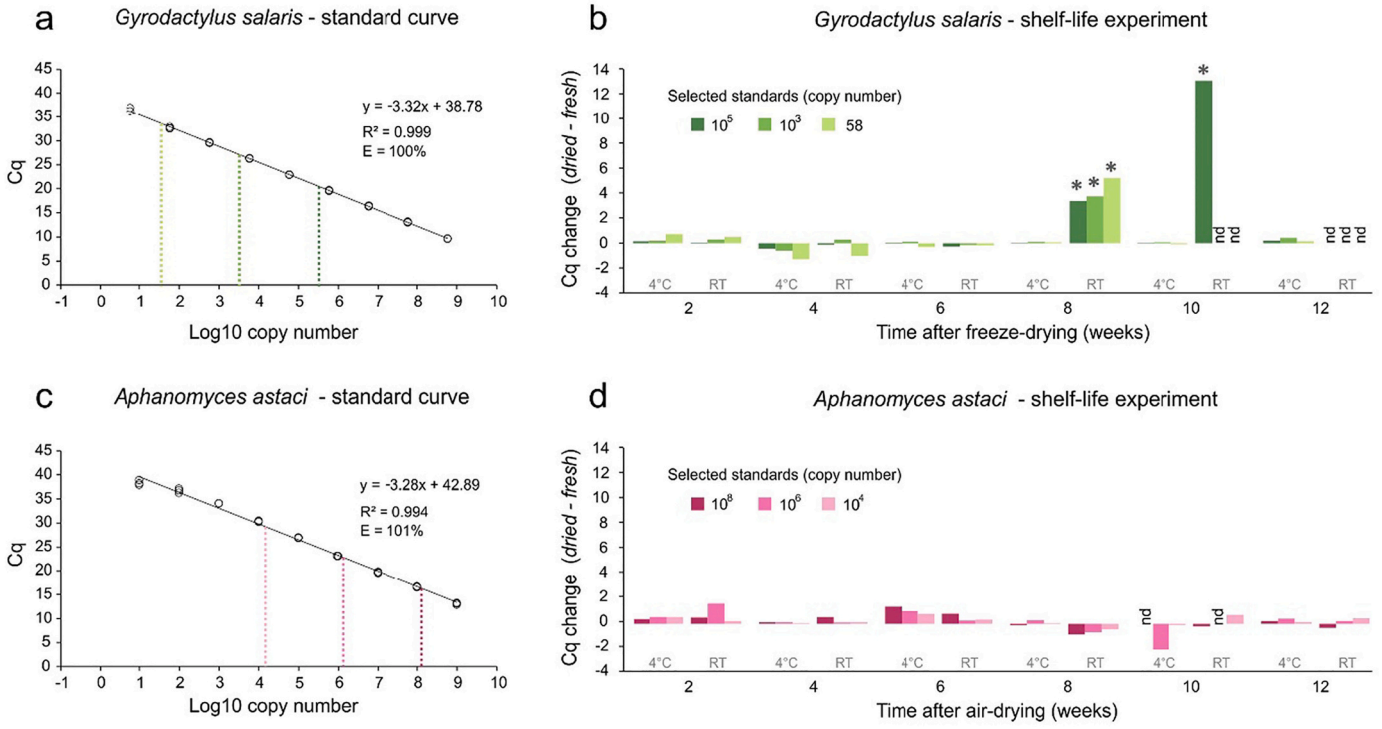

Fig. 1. Validation and stability results for the *Gyrodactylus salaris* (*Gs*) and *Aphanomyces astaci* (*Aa*) dried assays. (a + c) Standard curves of TaqMan-based qPCR amplification of *Gs* (a) and *Aa* (c) using fresh assays and gBlocks fragments. Standard curves were plotted using all three replicates for each serial dilution. The dotted lines represent the three concentrations used in each shelf-life experiment. (b + d) Shelf-life experiment results for *Gs* (b) and *Aa* (d) over 12 weeks, testing three concentrations and two different storage temperatures (4 °C and room temperature - RT). These results are shown as changes in Cq values compared to fresh assay controls (y-axis = Cq dried – Cq fresh); where a positive Cq indicates that the sample amplified less well than the control, and a negative Cq indicates that the sample amplified better than the control; a perfect match in Cq values of the fresh and dried assays is indicated by a zero. Concentrations for each assay were selected within the linear quantification range of the standard curve. Asterisks indicate Cq changes associated with the degradation of the assays (see details in Fig. 2). nd: non-detected qPCR signals.

assays (Table 1; Fig. 1a,c; Supp. Material Fig. S1).

After generating baseline data for the wet assays (Fig. 1a,c), the efficiency and shelf-life of dried assays for *Gs* and *Aa* were evaluated with a 12-week time-series experiment (Fig. 1b,d). The *Gs* assays were prepared using SensiFAST Lyo-Ready Mix (Meridian Biosciences, Bioline Assays Ltd., London, UK) with an exogenous internal positive control (IPC; Applied Biosystems, Waltham, MA, USA), which allows for the assessment of both the overall integrity of assays and the potential false negatives (PCR inhibition) in future environmental analyses. The IPC kit includes a synthetic template DNA with its corresponding primers and TaqMan probe (VIC-labeled probe, in contrast to the FAM-labeled probes used for the three target assays). *Gs* assays (final drying concentrations: qPCR Mix: 1×; forward and reverse primer: 0.75 µM; probe: 0.25 µM. Total volume in molecular-grade water: 18 µl) were frozen at −80 °C for 24 h and then lyophilized at −50 °C and < 0.1 mbar for 4 h with a FreeZone 2.5 Liter Benchtop (Labconco, Kansas City, MO, USA). *Aa* assays were prepared with Air-Dryable qPCR Mix (Meridian Biosciences, Bioline Assays Ltd) (qPCR Mix: 1×; forward and reverse primer: 1.2 µM; probe: 0.3 µM. Total: 15 µl) and air-dried at 60 °C for 60 min using a drying oven (Memmert UE 200–800; Memmert Gmbh, Schwabach, Germany) with a fan speed of 100% (drying time and temperature optimization, not shown); no IPC was used (Table 1). Both assays were vacuum-sealed in bags with silica beads, placed in darkness, and stored at either 4 °C or RT (21 °C ± 1 °C). qPCR analyses comparing dried vs. fresh assays were conducted every two weeks post-drying. The dried *Gs* assays were reconstituted with 18 µl of molecular-grade water and 2 µl of gBlocks, while the dried *Aa* assays were reconstituted with 15 µl of molecular-grade water and 5 µl of gBlocks. Three different concentrations of the gBlocks fragments were used as standards for *Gs* (5.8×10^5, 5.8×10^3 and 58 copies of Gs_124–289) and *Aa* (1.9×10^8, 1.9×10^6 and 1.9×10^4 copies of Aa_1–152) (Fig. 1).

We find that in three of four conditions (i.e., *Gs*: 4 °C, *Aa*: 4 °C, RT),

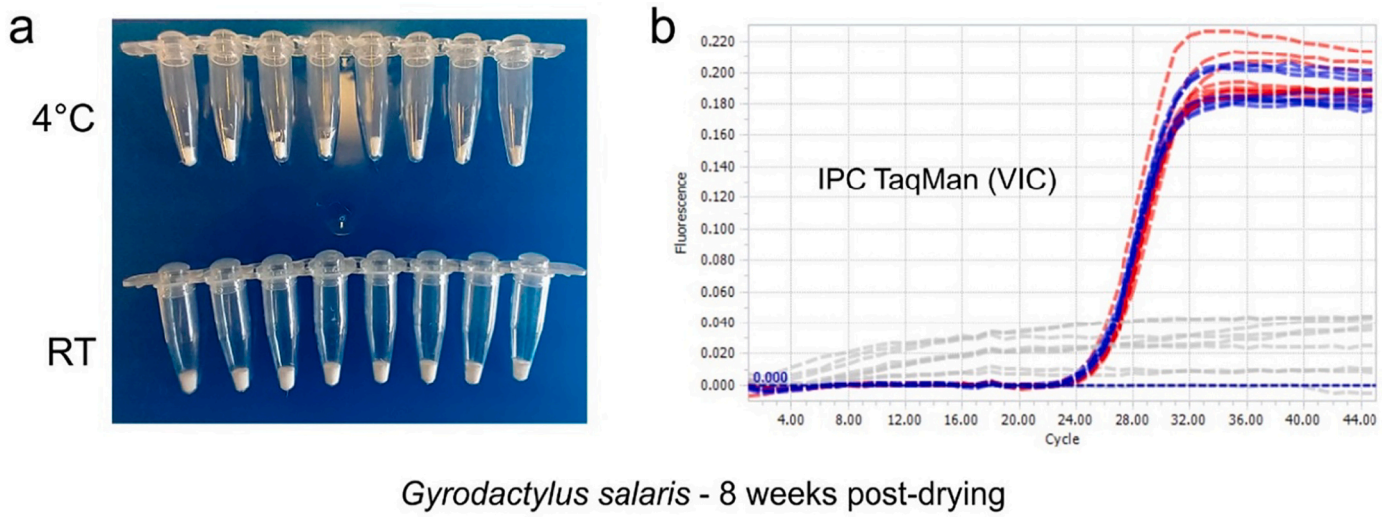

Fig. 2. Partial degradation of the freeze-dried *Gyrodactylus salaris* qPCR assays. (a) Representative of dried qPCR reagents. (b) Amplification curves for the internal positive controls (IPC; VIC signals). Note the poor performance of the dried assays stored at room temperature (grey IPC curves) compared to those stored at 4 °C (red) and fresh controls (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dried assays perform equally well as fresh assays even after 12 weeks (3 months) of storage. However, the *Gs* assays stored at RT declined in performance at week 8, with increased Cq values compared to the control and anomalous IPC signals (Fig. 1b, indicated by the asterisks; Fig. 2). In optimum conditions, with stable reagents and lack of PCR inhibitors (often present in environmental DNA samples), the Cq values for IPC (VIC fluorescence) should be 25 ± 2, as shown in Fig. 2b for the assays stored at 4 °C and fresh controls. At week 10, only the highest concentration could be detected, and by week 12, all concentrations were undetectable (Fig. 1b). Since the aim was to develop *Gs* assays stable at RT, further optimization is required to make this assay stable at RT beyond week 8. However, the assay may be suitable for settings that allow for a combination of storage conditions, e.g., long-time storage at 4 °C and short-term exposure to RT during field-based studies. Air-dried *Aa* assays were stable until the end of the experiment at all concentrations and in both storage conditions. An anomaly occurred in week 10 when the highest concentration of the 4 °C stored group and the medium concentration of the RT stored group were not detected. Since results at the following timepoint, in week 12, were on par with the control group, we assume that this anomaly was likely a result of the drying position in the oven, possibly because of unequal airflow across all samples, which can affect drying efficiency (communication with the company); a future issue that would need to be addressed.

The development of field-ready diagnostic assays is vital for detecting and controlling emerging diseases quickly on-site. Here, we provide proof-of-concept data for field-ready qPCR assays that could be further coupled with portable field-use qPCR machines to detect and monitor aquatic pathogens. Additional steps include further optimization to increase shelf-life and to enable transferability to (semi)-automatic microfluidic devices. A possible method for the latter is proposed by Xu et al. (2021), where the addition of liquid nitrogen to the master mix formed a transferable ball.

We demonstrate the feasibility of preparing dried, long-term stable qPCR reactions that can be reconstituted with water and a DNA template. All assays would be suitable for field-based conservation monitoring programs.

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CRediT authorship contribution statement

Jessica Rieder: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Pedro M. Martin-Sanchez:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Omneya A. Osman:** Methodology, Writing – review & editing. **Irene Adrian-Kalchhauser:** Funding acquisition, Supervision, Writing – review & editing. **Alexander Eiler:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106594.

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I. CHAPTER 4:

The future of pathogen detection in aquaculture: Mobile labs, eDNA, CRISPR and metatranscriptomics

Jessica Rieder, Anastasiia Berezenko, Alexandra Meziti, Irene Adrian-Kalchhauser

Keywords: pathogen surveillance, detection and monitoring, molecular tools, CRISPR-CAS, metagenomics and metatranscriptomics

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I.1 Abstract

Inland recirculating aquaculture is a rapidly growing food industry that provides sustainable, locally sourced, high-quality protein. However, this growth is associated with emerging challenges related to pathogen spread and disease emergence. Detecting and managing diseases in aquaculture is in its infancy compared to other animal farming sectors because of the immense diversity of species, the use of wild animals, and limited knowledge regarding pathogens, host responses, and disease mechanisms. Also, recirculating aquaculture intimately depends on beneficial bacterial communities for the clearance of waste products and the maintenance of water quality, but opportunistic bacteria are also a natural part of these communities. To fulfill its potential as a sustainable protein source of the future, the aquaculture sector requires improved tools to detect and monitor pathogens as well as the state of the entire microbial system.

In this perspective, we explain how current molecular approaches and technological advancements offer promising solutions for pathogen detection and system monitoring in aquaculture. Firstly, we describe how molecular diagnostics are moving towards point-of-care and on-site detection through miniaturized laboratory equipment, more robust workflows that are independent of cool chains, and monitoring the environment instead of fish health. Secondly, we discuss how methodologies from other scientific disciplines are spilling over to aquaculture, including Crispr-CAS protocols for pathogen detection and “omics” approaches for in-depth characterization of microbial states.

We anticipate that these methods hold significant potential for disease surveillance and management in the dynamic field of aquaculture. Together, these methods will empower the next generation of aquaculture managers to implement timely and targeted interventions and improve their disease management strategies.

I.2 Introduction

Aquaculture is a highly dynamic sector with significant annual growth rates. Currently, global inland aquaculture produces 50 million tons of food annually (FAO, 2022). Aquaculture growth has been fueled by advancements in rearing technology, global declines in natural fisheries increased per capita incomes across societies, and increased demand for fisheries-independent aquatic animal protein. Also, the growth of the sector depends heavily on continued technological and methodological development.

Pathogen spread, and disease emergence are a growing global concern for aquaculture managers. Disease outbreaks in aquaculture cause an estimated global annual loss of USD 6 billion (World Bank, 2014). Similar to any animal farming industry, these outbreaks are related to a multitude of factors, such as operational practices, rearing densities (Saraiva et al., 2022), biosecurity measures, environmental parameters (e.g., water quality), and stock properties, such as limited genetic diversity (Wright et al., 2023). However, the prediction, detection, and treatment of diseases in aquaculture are more challenging than in other animal farming industries. First, aquaculture uses over 400 evolutionarily and behaviorally unique species (Stentiford et al., 2022) that often react to similar conditions in opposing manners. For example, Atlantic salmon *Salmo salar* reared at high densities shows increased stress and disease susceptibility (Ellison et al., 2020), while the inverse is true for territorial Nile tilapia *Oreochromis niloticus* (Ellison et al., 2018). Second, the aquatic setting and a more limited set of behaviors associated with stress, pain, and disease in fish complicate visual and behavioral inspections. Therefore, disease detection often occurs late, the window of opportunity for rapid treatment is narrow (Rupp et al., 2019), and outbreaks can result in complete stock loss. In addition, most aquatic pathogens lack specific and consumer-safe treatments. Therefore, prevention and early detection methods constitute the first line of defense in aquaculture, and tools that support early detection are key to the continued and sustainable growth of the sector.

Fortunately, several technological and methodological developments promise to facilitate prevention and early detection through rapid and specific pathogen detection and identification. Point-of-care molecular-based tools have already been developed for microorganisms such as *Flavobacteria* spp. (Nguyen et al., 2018; Mabrok et al., 2021) and *Aphanonmyces astaci* (Strand et al., 2020). In the near future, more on-site, user-friendly detection methods for rapid pathogen testing that can be implemented in routine disease management programs will alleviate some of the challenges associated with the management and prevention of disease. This review aims to summarize current infrastructural and methodical developments that are expected to impact pathogen detection and management processes in the aquaculture sector. This includes miniaturization of laboratory equipment, the development of more robust assays, the rise of environmental DNA methods, and the spillover of methods such as CrispR-CAS and metagenomics to the aquaculture sector.

I.2.1 Molecular techniques for field-based diagnosis

The three current obstacles to early disease detection and rapid response in aquaculture contexts include (1) the requirement for access to and sample exchange with a diagnostic lab with trained personnel (most often located off-site, which necessitates transport of delicate samples), (2) the requirement for controlled conditions and infrastructure for diagnostics (expensive and large machinery, cold chains, and temperature-sensitive reagents are commonly required), and (3) the necessity to catch and handle fish to obtain samples, which is time-intensive and runs counter to animal welfare considerations. These three obstacles particularly impact disease detection in contexts where diagnostic labs are scarce, and/or time is of the essence, and/or resources are limited.

Below, we describe how (1) point-of-care technology allows for the detection of microorganisms on-site, thus "bringing the lab to the farm," (2) field-compatible molecular methods eliminate the necessity for tightly controlled environmental parameters, thus achieving "independence from the lab infrastructure," and (3) environmental DNA/RNA analysis techniques detect pathogens from swabs and water samples rather than from the fish themselves, thus "moving away from fish handling." Together, these three advances promise to reduce the effort and time required for detection and allow for more rapid responses and successful aquaculture treatments.

I.2.2 Bringing the lab to the farm

Mobile lab technologies include miniaturized and portable qPCR machines, DNA sequencing devices, and microfluidic devices for DNA isolation or amplification. They can be kept at a facility or brought to the facility, e.g., by a veterinarian, and reduce the need for sample transport to a central laboratory. They thus may reduce turnaround times, mitigate the risk of sample loss or damage during transport, and enable diagnostics in areas without centralized lab infrastructure.

Portable qPCR machine models are a key technology for the specific and quantitative detection of individual pathogens on farms. Quantitative PCR is widely used in diagnostic testing because of its specificity, sensitivity, quantifiability, and high-throughput potential. However, standard qPCR machines are often large, heavy, expensive, and must be serviced. Portable qPCR machines such as the Biomeme Franklin or the Bio Molecular Systems Mic are smaller and lighter (footprints:101.3 mm x 182 mm x 89.8 mm and 130 mm x 150 mm x 150 mm, and weights 1.20 kg and 2 kg, respectively). They also offer faster turnaround times (< 60 min per run). Depending on the model, regular calibration services or access to electricity (except for batteries) may not be required. Generally, portable qPCR devices rely on the same reagents as other qPCR devices. The parameter sacrificed for miniaturization is sample throughput. Compared to the traditional 96-well format, portable machines process fewer reactions at the same time, for example, 48 (Bio Molecular Systems Mic) or 9 (Biomeme Franklin). However, throughput is usually not a concern for point-of-care testing.

Portable sequencing devices also enable pathogen identification in real-time and on-site. Sequencing methods are used in diagnostic testing when species-specific tests are not available or when symptoms cannot be associated with testable candidate pathogens. Sequencing approaches are less quantitative than qPCR. In return, they can detect novel or unknown pathogens. Standard sequencing machines are even bulkier, heavier, more expensive, and less accessible than qPCR machines. Therefore, handheld portable devices, such as Nanopore MinION, promise new avenues for real-time molecular monitoring. For example, portable sequencing devices have been used for infectious disease surveillance and pathogen genotyping (Delamare-Debouteville, 2021). Gallagher et al. (2018) demonstrated the rapid and accurate sequencing of salmonid alphavirus and infectious salmon anemia virus, two viruses that affect global salmonid aquaculture. Handheld devices are also affordable: a Nanopore Flongle flow cell run is suitable for virus and bacteria sequencing and currently runs at USD 90.

Finally, microfluidic technology can reduce workflows that usually require large, heavy, and expensive laboratory equipment and space to a few square centimeters. These miniaturized platforms enable precise manipulation and analysis of small volumes of fluids and can sequentially combine several steps of molecular protocols, such as sample preparation, nucleic acid amplification, and detection, within a single chip or cartridge (see reviews by Gorgannezhad *et al.* (2019) and Kulkarni and Goel (2020) for a more in-depth discussion of designs and options). The integration of several steps into one lab-on-a-chip also reduces the risk of contamination. Microfluidic devices have been successfully used to detect viruses, including decapod iridescent virus 1 (DIV1), white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus, infectious spleen kidney necrosis virus, koi herpesvirus, Iridovirus, the *Enterocytozoon hepatopenaei* parasite, and various bacteria *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio harveyi*, *V. alginolyticus*, *V. anguillarum*, *V. parahaemolyticus*, *V. vulnificus,* and *Pseudomonas aeruginosa* (Chang et al., 2013; Zhou et al., 2021; Hu et al., 2023; Guptha Yedire et al., 2023).

In summary, various portable devices are available for bringing the lab to the farm. Their small size, affordability, and operability under field conditions may enable pathogen monitoring on farms with limited or no access to central laboratories or diagnostic facilities. Consequently, farm managers can implement measures faster and in a pathogen-targeted manner, thus reducing animal loss and the need for treatment.

I.2.3 Independence from controlled environments

Generally, molecular assays are quite unforgiving with regard to environmental parameters. They require stringent cooling chains for sustained activity or depend on tightly controlled temperature sequences, e.g., for successful DNA amplification. Recently matured approaches such as the lyophilization of assays or the isothermal amplification of nucleic acids create independence of

conditions that are difficult to maintain in field settings. These developments have been fueled by the need for on-site pathogen surveillance in human health programs in remote areas (Song et al., 2022) but are easily transferable to aquaculture settings.

Lyophilization (freeze-drying) can promote the independence of temperature-sensitive enzymes from the cooling chain. Traditional PCR and qPCR reagents and enzymes quickly lose activity when exposed to temperatures above -20°C. The lyophilization of pre-prepared assays, including all reagents and enzymes, produces formulations that can be transported, stored at room temperature, and reconstituted with water on-site (Rieder et al., 2022). An additional benefit of lyophilization is the ability to prepare and quality control batches, thus reducing the variability associated with the individual preparation of assays. Also, lyophilized assays can be stored for prolonged time periods. Lyophilization is, therefore, likely most beneficial in resource-limited or remote settings, where access to continuous refrigeration and/or uninterrupted electricity is challenging.

Isothermal protocols create independence from the precise temperature cycling required by traditional PCR and qPCR detection methods. Isothermal enzymes amplify DNA at constant and low temperatures, which can be achieved using incubators or water baths. Two isothermal protocols are currently available: loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). RPA runs at 37°C (M. A. Williams et al., 2022), whereas LAMP runs at 65°C (Notomi et al., 2000). Both are time-efficient (< 60 min) (Notomi et al., 2000; Sullivan et al., 2019) and sensitive, detecting low copy numbers of DNA (e.g., ~1.06 copies for white spot syndrome virus) (Sullivan et al., 2019) and can be combined with microfluidic devices (Giuffrida & Spoto, 2017; Kant et al., 2018). LAMP has been used to detect *Vibrio parahaemolyticus* (Anupama et al., 2020) and *V. vulnificus* (Tian et al., 2022), two known fish pathogens (Novoslavskij et al., 2016). RPA has been used to detect viruses such as *Penaeus stylirostris* denso virus (Jaroenram & Owens, 2014), white spot syndrome virus (Sullivan et al., 2019), infectious hypodermal and hematopoietic necrosis virus (X. Xia et al., 2015), Cyprinid Herpes virus-3 (Prescott et al., 2016), abalone herpes-like virus, red-spotted grouper nervous necrosis virus (Gao et al., 2018), and bacteria including *Flavobacterium columnare* (Mabrok et al., 2021), *Edwardsiella ictaluri* (H. Li et al., 2022), *V. parahaemolyticus* (Geng et al., 2019), *Tetracapsuloides bryosalmonae* (Soliman et al., 2018), and *Francisella noatunensis* subspecies o*rientalis* (Shahin et al., 2018).

Colorimetric readouts enable machine-independence regarding the readout of detection assays. Lateral flow strips, for example, are low-budget and change color in case a target is detected. This human-perceptible readout eliminates the need for expensive and sensitive equipment such as spectrophotometers or fluorometers that detect non-human-perceptible readouts of more standard lab assays. In liquid colorimetric assays, the reaction changes color upon detection.

Combining these three approaches could have transformative power for pathogen detection in

aquaculture. Colorimetric readouts have already been coupled to lyophilized assays and isothermal amplification methods (Faye et al., 2015; Mabrok et al., 2021; Sukonta et al., 2022; Zingg et al., 2023). The release from cold-chain requirements, temperature-controlled equipment, and machine-dependent readout methods provides ultimate independence from classical laboratory infrastructure.

I.2.4 Moving away from fish handling

The recent rise of environmental DNA (eDNA) and RNA (eRNA) methods has been instrumental in modernizing and transforming species and pathogen surveillance. Both rely on the detection of pathogens from environmental samples, such as water, soil, or air, instead of samples obtained from diseased or carrier organisms. In comparison to organismic samples, eDNA/RNA methods are non-invasive, equally sensitive, and align with the UN Sustainability goals (United Nations, 2016) and the 3Rs objectives (Replace, Reduce, and Refine) concerning better animal welfare. These and other characteristics of eDNA/RNA approaches predispose them to adaptation to aquaculture management.

Early detection is critical for rapid mitigation responses, and environmental sampling can potentially detect the presence of pathogens in low abundance at or even before the start of an outbreak. Furthermore, eDNA analyses can improve aquaculture biosecurity measures if applied to potential contamination sources (e.g., transport water) and thus can reduce the spread of pathogens between farms. So far, eDNA methods have, for example, been used to detect the ectoparasitic flukes *Gyrodactylus salaris* (Fossøy et al., 2020), *Tetracapsuloides bryosalmonae* (Sieber et al., 2020), *and Flavobacterium psychrophilum* (Nguyen et al., 2018).

Environmental DNA detects pathogen presence, but given the opportunistic nature of many fish pathogens, other tools are required to distinguish active outbreaks from low levels of pathogen presence. Here, the analysis of the less stable environmental RNA is useful to discriminate between the two. eRNA is unstable in the environment (reported to last for 13-24 hours (Marshall et al., 2021; S. A. Wood et al., 2020)), and detection, therefore, means that molecules were recently produced and shed. eRNA-based detection, therefore, requires frequent sampling (Miyata et al., 2021) and is generally less well-established than eDNA. A successful application was the detection of *Bonamia ostreae*, a protozoan parasite that induces significant mortality in flat oyster *Ostrea edulis* populations (Mérou et al., 2020).

Environmental DNA and RNA have great potential in routine monitoring programs, even though many questions remain unanswered (see review by Bass (2023)). Conceptually, managers using eDNA approaches need to understand the requirements for case- and site-specific validation and optimization and must be familiar and comfortable with concepts of probability, false negatives, limits of detection, and false positive rates.

Various molecular developments are available today that can create independence from laboratory infrastructure, laboratory conditions, and animal samples when monitoring fish health in aquaculture.

While most of them do require specific expertise, they also have the potential to empower aquaculture managers to independently monitor parameters that matter to them, from specific pathogen concentrations (portable qPCRs or microfluidics) to the entire microbiome state (eDNA and handheld sequencers). They can also empower veterinarians in remote areas to conduct certain tests during occasional farm visits or to perform virtual consultations from a central location based on results obtained and shared by the manager. Therefore, an essential skill for future aquaculture specialists is deciding which methods are helpful in particular infrastructural and geographical contexts.

I.3 Method spillovers to aquaculture

In addition to the above modifications to methods that are already applied in pathogen monitoring in human and veterinary medicine, methods have recently started to spill over from other fields of science into aquaculture applications. These approaches were originally developed for basic research purposes but have more recently been adapted to "diagnose" the state of aquaculture. Crispr-Cas applications have moved from genome modification to highly specific pathogen detection, and metagenomics has moved from evolutionary biology to monitoring tools of microbiomes in all kinds of environments.

I.3.1 CRISPR-Cas

A genome-editing tool that has recently been adopted for diagnostic assays is "clustered regularly interspaced short palindromic repeats" (CRISPR). CRISPR-based diagnostics present a highly specific and sensitive detection alternative to PCR and qPCR (Phelps, 2019) that can be coupled with isothermal amplification (Table 1) and low-tech lateral flow strips. The targeted pathogen is recognized through an oligonucleotide bound to a CAS protein (both provided by the experimenter). The CAS proteins scan the genetic material within a sample for matches with the oligonucleotide. If a match occurs, the CAS proteins bind to the target DNA and, similar to genome modification protocols, initiates a cleavage reaction that, in this case, is designed to release a detectable signal. Currently, three distinct approaches have been used for aquaculture: SHERLOCK, DETECTR, and RAA.

The Cas13-based SHERLOCK (Sensitive High Efficiency Reporter unLOCKing) method was initially developed in 2017 to rapidly and sensitively detect Zika and Dengue viruses (Gootenberg et al., 2017). SHERLOCK combines isothermal amplification by recombinase polymerase amplification (RPA) with highly specific Cas13-based detection of transcribed amplicons followed by fluorescence reporting (Kellner et al., 2019) and requires standard laboratory settings and equipment. Its successor, SHERLOCKv2 or STOP (SHERLOCK Testing in One Pot), adopted a loop-mediated isothermal amplification (LAMP) with a heat-tolerant Cas12b and was successfully used for Covid detection (Joung et al., 2020). Major *et al.* (2023) adopted STOP for shrimp aquaculture diagnostics and detected viral targets (e.g., white spot syndrome virus and Taura syndrome virus) in shrimp tissue samples in 30

minutes without the need for thermocycling.

METHOD	PROTEINS	AMPLIFICATION	DETECTION	TARGET	SENSITIVITY	TIME	REF
SHERLOCKV1	Cas13a	RPA	Fluorescence, Colorimetry	DNA/ RNA	1.06 copies (10 copies /colorimetry)	60 mins	Sullivan, 2019
SHERLOCKV2	Cas12b	LAMP	Fluorescence	DNA/ RNA	100 copies	30-60 mins	Major, 2023
DETECTR	Cas12a	RPA	Fluorescence, Colorimetry	DNA	40 copies (200 copies/ colorimetry)		Li, 2022
RAA-CRISPR/ CAS12A	Cas12a	RAA	Fluorescence	DNA	2 copies	40 mins	Xiao, 2021

Table 1: **Overview of CRISPR-based technologies describing the benefits and flexibility of different methods and the proteins involved.**

DETECTR (DNA endonuclease targeted CRISPR trans-reporter) also combines RPA with the Cas12a enzyme. Using a fluorescent readout, DETECTR has been used to confirm Scale Drop Disease (Sukonta et al., 2022), Hepatopancreatic Microsporidiosis (Kanitchinda et al., 2020), and Acute Hepatopancreatic Necrosis Disease (C. Li et al., 2022). More recently, DETECTR has been adapted for colorimetric lateral flow strips (< 10 minutes for results) (C. Li et al., 2022). To reduce the risk of carryover contamination, a one-step procedure was developed for detecting Acute Hepatopancreatic Necrosis Disease (P. Wang et al., 2023). A noteworthy aspect of DETECTR is the ability to differentiate between closely related species (M.-A. Williams et al., 2019), which suggests a potential use with biosensor devices. Such one-device, no-manipulation approaches are key to realizing the full potential of eDNA-based live monitoring systems (Phelps, 2019).

Finally, RAA-CRISPR/Cas12a (recombinase-assisted amplification CRISPR-CAS approach) represents an adaptation of DETECTR for detecting *Vibrio vulnificus* (Xiao et al., 2021). RAA takes approximately 40 minutes, and the results can be detected with a UV lamp. Such simplicity and reported sensitivity make it a promising method for early on-site detection of vibriosis.

In summary, CRISPR-based diagnostics represent a highly effective, specific, robust, and potentially low-resource approach and carry real potential for the aquaculture industry. Future developments will likely include quantitative approaches to determine the abundance of the target pathogen in addition to its presence. Also, associated technical innovations for detection, such as a smartphone-based fluorescence reader for the CRISPR Diagnostic of SARS-CoV-2 (Samacoits et al., 2021), could be adopted for aquaculture applications.

I.3.2 Metabarcoding, metagenomics, and metatranscriptomics

Metabarcoding, metagenomics, and metatranscriptomics are powerful -omics approaches to gain insights into the composition and functional activities of microbial communities. Metabarcoding focuses on specific genetic markers from specific groups of interest (e.g., bacteria; 16S sequencing represents a barcoding approach), while metagenomics analyzes genomic material from the entire microbial community (allowing for simultaneous identification of bacteria, archaea, viruses, and bacteriophages). Metatranscriptomics focuses on RNA molecules and provides information on species presence, but also on function and current activity state.

Metabarcoding allows managers to identify specific groups of organisms within the system by sequencing regions of a targeted gene. This approach provides valuable insights into the structure, dynamics, and interactions of complex communities. It is especially valuable in studies involving large-scale surveys as it allows for efficient and cost-effective screening of multiple samples, providing a rapid assessment of spatial-temporal patterns (Rieder et al., 2023). However, it is important to note that a limitation of metabarcoding data is the inability to achieve species-level identification. Consequently, this hinders the detection of pathogens and the identification of key functional roles performed by the microbial communities within the system. Nonetheless, metabarcoding is a versatile tool for obtaining snapshots of community dynamics and patterns.

Metagenomics allows managers to identify all organisms present within the system, including pathogens or potentially novel species, from all kingdoms of life – bacteria, fungi, parasites, viruses, and bacteriophages. This information can shed light on the microbial community structure, their roles in nutrient cycling, disease dynamics, and the overall ecological dynamics within a system (Martínez-Porchas & Vargas-Albores, 2017; Nogueira & Botelho, 2021b). Therefore, metagenomics enables the prediction of the functional potential of the microbial community. For a side-by-side comparison of metabarcoding and metagenomics approaches, see (Rieder et al. (2023)). Both metabarcoding and metagenomics are useful to characterize the steady state of a system, to compare compartments or farms, or to monitor long-term changes in the community (e.g., across seasons or during the setup of a new biofilter).

Metatranscriptomics provides insights into the active functional processes of microbial communities. By exploring which genes are actively transcribed and expressed, managers gain insight into the molecular machinery and metabolic activities occurring within the system. Metatranscriptomics unravels critical processes such as nutrient uptake and metabolism, stress responses, host-microbe interactions, and the expression of antimicrobial resistance genes. Recent studies have used metatranscriptomics to examine shifts in microbial community activity through the seasons (Sutherland et al., 2022) and to investigate pathogen-host dynamics, particularly concerning pathogen load (Rey-Campos et al., 2022). Metatranscriptomics data can serve as a surveillance tool by monitoring the activity of processes involved in nutrient cycling (Hook et al., 2021; Marshall et al., 2021)

or for tracking the activity of antimicrobial-resistant genes (Wang et al., 2020). Overall, metatranscriptomics is more sensitive than metabarcoding or metagenomics when it comes to monitoring short-term changes in the system, for example, upon treatments or operational changes.

Two main challenges need to be tackled to enable wide-scale application of -omics approaches for achieving better and sustainable aquaculture practices. First, the financial aspect remains a significant hurdle. While the costs of high-throughput sequencing are steadily decreasing, they remain high when studying the large number of samples required for industrial-scale studies or monitoring. Second, skilled lab workers and trained bioinformaticians are indispensable for ensuring accurate and consistent results when working with -omics data. Both challenges can be met through collaborations between the aquaculture industry and the academic sector, with funding from the aquaculture industry supporting applied scientific research and science generating new knowledge and insights beneficial to aquaculture managers.

In summary, integrating -omics approaches allow for a comprehensive understanding of microbial communities and their functional activities. By combining microbial composition and gene expression information, researchers can link specific microbial taxa to their functional roles and identify key processes driving system performance or animal health. This integrated approach can lead to the development of targeted interventions, improved management strategies, and the discovery of novel beneficial microorganisms for sustainable and efficient aquaculture practices.

I.4 Conclusion

In conclusion, pathogen surveillance in aquaculture (and other fields) currently benefits tremendously from advances made in molecular biology, engineering, medicine, and basic science. The field currently witnesses a revolution regarding point-of-care diagnostics and on-site pathogen surveillance, with various innovations facilitating lab-free, fish-free, and refrigeration-free detection, identification, and characterization of pathogens. In addition, community sequencing approaches allow unprecedented insights into the effects of management interventions, cleaning procedures, stocking cycles, and other operational practices on the overall microbiome of tanks and biofilters. Together, these methods will empower the next generation of aquaculture managers to implement timely and targeted interventions and improve their disease management strategies.

II. CHAPTER 5:

A guide to DNA extraction protocols for ecologists, conservation managers, and eDNA researchers
Jessica Rieder, Elaine Jemmi, Irene Adrian-Kalchhauser

Keywords: molecular methods, environmental DNA, DNA isolation

II.1 Abstract

Ecologists, conservation biologists, and environmental managers are increasingly dependent on environmental DNA (eDNA) data for research and decision-making. Decisions in DNA extraction methodologies can introduce significant biases into eDNA datasets; coupled with the inherent complexity of eDNA samples, the optimization and troubleshooting of DNA extraction protocols are pivotal for the successful execution of eDNA projects. Knowledge about the basic steps and principles of DNA extraction is, therefore, an essential skill in eDNA work. However, traditional education in ecology, conservation, and environmental management typically does not include in-depth training in molecular methods. While DNA extraction box kits are extremely valuable in this context, they may fail in delivering desired results with eDNA samples, necessitating protocol adaptations or an educated selection of alternative approaches.

The primary objective of this paper is to enable scientists with an ecological background who utilize DNA extraction protocols to understand the four key steps of DNA extraction and to use this expertise to their advantage. Furthermore, we describe the purpose of commonly used reagents and chemicals, point out alternatives for each key step, explain the impact of certain choices on DNA integrity and purity, and advocate for an adaptable “mix and match” protocol when applicable.

We anticipate that the paper will enable field ecologists to develop a deeper understanding of the mechanisms and chemistry behind DNA extraction. Thus, allowing them to make educated decisions regarding the best DNA extraction method for their research. Our intention is not to furnish comprehensive, step-by-step protocols but rather to offer guiding principles and highlight alternative solutions, thereby stimulating focused exploration into specific methodological aspects. Finally, we hope this paper acts as a useful resource to support knowledge transfer and teaching.

II.2 Introduction

Environmental DNA has become a central analyte in ecological research, biodiversity assessment strategies, and conservation practices. eDNA is used to determine the presence/absence of individual species (Nguyen et al., 2018; Lutz et al., 2020) as well as to describe the composition of entire communities (Rieder et al., 2023). Species-specific approaches are commonly used for pathogens (Bastos Gomes et al., 2017; C. K. Howell et al., 2019) or invasive species (Nevers et al., 2018; Lutz et al., 2020; Rusch et al., 2020), while barcoding, metabarcoding or metagenomics approaches are used to assess biodiversity (Creer et al., 2016; Deiner et al., 2017; Taberlet et al., 2018) and taxonomic richness (Deiner et al., 2017; Rieder et al., 2023). Today, eDNA is widely accepted as a powerful alternative to conventional capture- or culture-based methods in species and ecosystem monitoring.

Consequently, a substantial number of originally field-trained biologists and taxonomists are embracing approaches that rely on the isolation of DNA. As pointed out by numerous publications (Adamowicz et al., 2014; Albertsen et al., 2015; Felczykowska et al., 2015; Corcoll et al., 2017), choices made at this step can have serious impacts on detection probabilities and experimental outcomes. For community composition studies, inefficient DNA extraction may lead to over- or underrepresentation of species and biases (Rieder et al., 2023). Inadequate extraction may result in poor DNA quality and quantity, lead to false results and conclusions, or cause a project to fail. However, choosing or developing a DNA isolation protocol tailored to the specifics of the sample and the requirements of the downstream steps requires a certain understanding of the DNA extraction process and the purpose of the chemicals used (Table 1). Many field-trained biologists, therefore, find this task daunting.

This paper is tailored to readers who are not lab-trained molecular biologists but nonetheless need to decide on a sample- and purpose-appropriate DNA extraction approach for precious samples or need to improve, simplify, or scale up a DNA extraction protocol. We would like to enable researchers to see through the jungle of protocol voodoo that either magically produces DNA or fails inexplicably and realize that DNA extraction protocols are actually surprisingly robust to change and adaptation and can often be tweaked to greatly improve efficiency or facilitate handling. Our objective is to enable researchers to understand what a particular section of their protocol does, modify existing protocols, confidently mix and match kits with non-kit protocols, and make educated choices when selecting a DNA extraction method according to their experimental needs. We do not aim to provide step-by-step instructions – these are better obtained elsewhere - but aim to synthesize overarching principles and the purposes of certain protocol steps and point out aspects that are particularly important or particularly malleable. To this end, we list various approaches to achieve the same outcome, describe the role of key reagents, explain the impact of various approaches on DNA integrity and purity, and address common troubleshooting issues and solutions.

II.3 The four steps

Four basic steps are required to move from a heterogeneous sample to pure, concentrated DNA. These include 1) disruption ("lysis") of the material, 2) separation of DNA from other cellular material, 3) removal of salts, and 4) concentration and recovery of DNA (Figure 1). Steps three and four can also occur in reverse order, particularly in extractions not relying on a DNA-binding matrix. Of relevance, "dirty" protocols can skip some of these steps. For example, a rough disruption step without any cleanup may suffice to perform a non-quantitative PCR on an abundant target. Understanding which part of a specific protocol achieves which of these steps is the start of any protocol optimization, adaptation, and customization. For example, upstream steps from one protocol or kit can often be combined with downstream steps from other protocols. Also, protocols can often be paused between, sometimes for weeks, given appropriate storage conditions.

Category	Role and mechanism of action	Commonly used in DNA extraction	Abbreviation	Comments
Detergents/surfactants	Detergents insert their long hydrophobic tails in between the hydrophobic/uncharged regions of proteins and membranes, thus helping to break them up. They also prevent non-specific associations between plastics and cellular components.	Cetyl Triethyl Ammonium Bromide Sodium Dodecyl Sulfate Triton X-100 Tween 20	CTAB SDS	Cationic detergent. Anionic surfactant. Nonionic surfactant. Nonionic surfactant. Reduces the adsorption of DNA to plastic tubes.
Chaotrophic salt*	Chaotropic salts disrupt the hydrophilic / charged interactions of proteins. They thus complement the action of detergents.	Sodium Iodide Guanidine Thiocyanate Guanidine Hydrochloride	NaI Guanidine HCL	Aids in membrane lysis and DNA binding to the silica resin; disrupt the association of nucleic acids with water, thus providing optimal conditions for their transfer to silica.
Degrading enzymes	Enzymes ending in "ase" work on whatever substrate is mentioned in the first part of the enzyme name.	Proteinase K Rnase		Digests proteins complementary to detergents and chaotropic salts Digests RNA. It is very specifically used if RNA is a problem for downstream applications and is not generally required for DNA isolation.
Organic solvents*	Attracts charged water molecules away from the DNA and thus reduces the solubility of the DNA in water.	Phenol-Chloroform-Isoamylalcohol		
Salt		Sodium Chloride Sodium Acetate	NaCl NaOAc	
Alcohol	Interferes in the interaction of water molecules with DNA, and thus, together with the salt, reduces the solubility of the DNA in water.	Isopropanol Ethanol	2-Propanol EtOH	
Chelating agent	Chelators sequester ions from the solution. DNase and RNase enzymes require calcium and magnesium ions for functioning. Adding chelators, therefore, inactivates these enzymes and reduces the degradation of nucleic acids. Chelators also, however, impact DNA polymerases, which require magnesium and therefore need to be removed before, e.g., PCR.	Ethylene Diamine Tetraacetic Acid	EDTA	Binds divalent metal ions such as calcium and magnesium.
Buffering agent	DNA is, chemically, an acid and, therefore, degrades in basic pH conditions. Similarly, enzymes used during DNA extraction (e.g., Proteinase K) need appropriate pH conditions to work. Buffering agents help maintain a stable pH during reactions or storage.	Tris(hydroxymethyl)aminomethane Tris(hydroxymethyl)aminomethane Hydrochloride Tris-EDTA buffer	Tris Tris-HCL TE buffer	EDTA is good for stability, as is inhibits/degrades Dnases, but interferes with enzymatic applications such as PCR.

Table 1: Chemicals commonly used in DNA extractions. DNA extraction protocols of all kinds (from self-prepared solutions to ready-to-use kits) use similar categories of chemicals. Detergents, chaotropic salts, or degrading enzymes help to break up materials. Chelating agents and buffering agents protect DNA. Salts and alcohols promote a separation of DNA from water. Importantly, compounds serving similar purposes can often substitute for each other. *[Hazard]

II.3.1 Step 1: Cell lysis

The lysis step disrupts cellular membranes and structures, releases DNA from the nucleus, and removes any proteins that are bound to it. Lysis can be achieved through three approaches: 1) mechanical disruption, 2) chemical digest, and 3) enzymatic digest, which can be used as stand-alone, in sequence, or in combination (Figure 1A). DNA extraction kits often combine mechanical disruption by shaking with chemical disruption by a buffer containing salt and detergents and enzymatic digest by an enzyme such as Proteinase K. The sample lysis step is crucial with regard to the amount and integrity of recovered DNA and is a key step with regard to optimization and mix-and-match approaches. For example, a lysis step from a kit can be followed up by centrifugation and precipitation, or a home-made lysis protocol can be combined with column-based separation and purification.

Two aspects of the lysis step have a particular impact on the recovered DNA. First, cell lysis exposes the DNA to degrading enzymes such as DNases. Therefore, lysis conditions are designed to prevent DNA degradation through fast processing at cold temperatures or the use of high denaturing temperatures or chemicals such as high salt, detergents, or organic solvents that inactivate DNases. Second, complex samples commonly contain both easy- and hard-to-lyse components. Harsh lysis methods that also disrupt the latter, such a high temperatures, sonication, and mechanical beating, can negatively impact DNA integrity, and DNA may have become too fragmented for downstream sequencing, e.g., long-read sequencing. Gentle lysis, such as freeze/thaw cycling or manual grinding, may be less destructive for DNA but fail to recover all DNA from hard-to-lyse components. As a result, specific taxonomic groups maybe misrepresented in the final sample. Therefore, sample composition and experimental aim both need to inform the choice of lysis method.

II.3.1.1 Mechanical disruption

Mechanical methods release DNA by disrupting the surrounding biological materials through physical force. Common approaches are grinding in liquid nitrogen, dounce homogenization, boiling, freeze-thaw, bead beating, and sonication (see cell lysis review by Islam et al. (2017) for additional processes). Grinding, dounce, bead beating, and sonication apply shearing forces, create heat by friction, and are therefore carried out on ice or in cold conditions. Boiling disrupts cell membranes and proteins through entropic destabilization and protein denaturation, while freeze-thaw cycles induce the formation of ice crystals, which disrupt cell walls.

Considerations when using mechanical disruptions are mostly related to sample composition, handling, and DNA integrity. Combining multiple mechanical methods ensures that more uniform lysis occurs, especially regarding hard-to-lysis species (Z. Y. Ma et al., 2020) (Figure 1B). The more a method depends on equipment (e.g., sonication or bead beating), the better it can be standardized (e.g., as opposed to manual grinding in liquid nitrogen). Some methods, high temperatures, sonication, and mechanical beating, have a higher chance of fragmenting DNA than others - frew/thaw cycling or manual grinding where some methods can be achieved with kitchenware, e.g., manual grinding or

heating, while others require expensive machinery e.g., sonication and bead beating, and some methods can be scaled to high sample numbers e.g., freeze/thaw cycling and heating while others cannot e.g., mechanical beating and sonication. Generally speaking, more time-consuming processes with individual sample handling result in less DNA fragmentation but are harder to standardize.

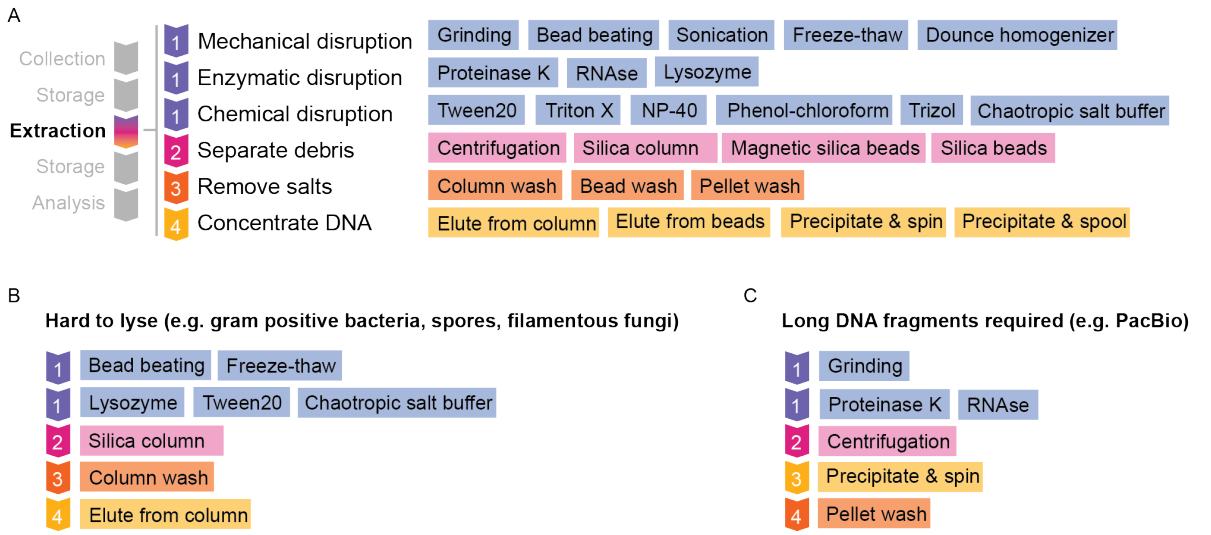

Figure 1: Mix-and-match of four steps and various methods. *A) The four main steps in DNA extraction are (1) cellular lysis, (2) cellular component separation and binding of DNA (3), removal of salts (4), and concentration of DNA (4). Each step can be achieved through a variety of approaches with distinct properties. For example, mechanical disruption can be accomplished through grinding, bead beating, sonication, etc. B) Example of an approach that can be taken for hard-to-lysis organisms, such as gram-positive bacteria or spores. A variety of strong lysis methods are combined with a matrix-based separation protocol. C) Example of an approach that can be taken to recover long DNA fragments. Gentle, DNA-preserving DNA extraction methods are combined with a non-fragmenting recovery protocol to ensure that long DNA fragments are obtained for third-generation sequencing (e.g., PacBio).*

II.3.1.2 Chemical digest

Chemical methods release DNA by disintegrating membranes and proteins with detergents, salts, pH changes, and/or organic solvents, or a combination thereof. Detergents, usually in a 1% or 2% (w/v) concentration, destabilize lipid bilayers. Anionic (e.g., SDS) and cationic detergents (e.g., CTAB) can alter the structure of the proteins and have the most potent effect. Non-ionic detergents (e.g., Triton X-100, Tween 20) are non-denaturating and thus less harsh. Zwitterionic detergents (e.g., CHAPS) share characteristics with ionic and non-ionic detergents, and the extent to which proteins are affected is intermediate (Donnell et al., 2017). For a comparative review of different detergents, see Johnson (M. Johnson, 2022). Chaotrophic salts such as urea guanidine and EDTA destabilize polar structures, mostly proteins. Here, a common issue is that EDTA precipitates at low temperatures and will not be effective in cooled samples. Organic solvents such as phenol or chloroform destabilize lipid bilayers and also denature proteins (Saini et al., 2021). Here, an issue is that organic solvents pose relevant health and environmental hazards.

Chemical lysis does not negatively affect DNA integrity and is suited (if performed without shaking,

beating, or shearing) for long-fragment isolation. It also inactivates DNA-degrading enzymes. For the same reason, however, the chemicals used may interfere with downstream enzymatic methods and need to be removed after lysis. Finally, since the chemicals used are able to dissolve and disrupt organic material, they may pose a health hazard upon direct contact or inhalation.

II.3.1.3 Enzymatic digest

Enzymatic methods release DNA by enzymatically disintegrating cellular components through enzymes such as lysozymes, nucleases, and proteases. Many extraction kits rely on proteinase K, which non-specifically cleaves proteins in cell lysates (e.g., tissue, cell culture, etc.). Other enzymes have more specific effects. For example, lysozymes, labiases, or achromopeptidase can support extractions from gram-positive bacteria that feature a complex protective peptidoglycan layer (see Andrews et al. (Andrews & Asenjo, 1987) and Salazar et al. (Salazar & Asenjo, 2007) for more thorough reviews of enzymatic lysis of microbial cells). For gram-negative bacteria, lysozyme can be combined with detergents (e.g., EDTA) to break the cell wall and membrane (Geciova et al., 2002; Shehadul Islam et al., 2017). Chitinase may be used for the lysis of yeast cells, whereas pectinases can be used for plant cell lysis. Less specific enzymes include mutanolysin and lysostaphin, which are used to extract nucleic acids from susceptible bacteria (Cho et al., 2021); zymolyase, lyticase, and glusulase are commonly used for yeast cell wall degradation (Burden, 2023).

Enzymatic lysis is a very gentle method (Shehadul Islam et al., 2017) and preserves the integrity of DNA, making it a suitable choice for third-generation sequencing projects (Figure 1C). However, even though enzymatic lysis methods may yield higher amounts of DNA, they could impact the DNA quality if not correctly used or result in inconsistent taxonomic composition (Yang et al., 2023). Also, chemical and enzymatic lysis approaches are more scalable to large sample numbers than most machine-dependent physical disruption methods.

II.3.2 Step 2: Cellular component separation and binding of DNA

After lysis, DNA is present as part of a heterogeneous mixture of nucleic acids, proteins, lipids, and other cellular components, as well as salts, enzymes, and detergents from the lysis reagents. These interfere with the efficiency and precision of downstream applications (e.g., PCR, DNA sequencing, and gene expression analysis) and are, therefore, usually followed by separation and cleanup steps. Importantly, some downstream applications – such as robust PCR protocols – might be able to detect abundant targets. A centrifugation to collect cellular debris at the bottom of the tube and dilution of the recovered supernatant to titrate away, e.g., inhibiting salts in the PCR, may be sufficient for non-quantitative detection. In most cases, however, cleanup is required.

Two separation approaches can be taken after lysis. DNA can be separated from the other components by a matrix-free approach (e.g., detergent or organic solvent-based) or in a matrix-

dependent manner (e.g., silica or cellulose). The choice of method usually depends on the sample type and experimental goal, the needs regarding purity, integrity, and amount, and the financial resources.

II.3.2.1 Matrix-dependent

During matrix-dependent methods, the lysis material is brought in contact with a solid phase (matrix) that binds and retains DNA but no other cellular components. Commercial kits typically use such matrix-dependent approaches. Most commonly, the matrix consists of silica or cellulose, though other substrates have been used. The matrix is either packed in a column, through which the sample is forced either by centrifugation or the application of vacuum, or coated on the surface of small, often magnetic beads, which can be separated from the solution by centrifugation or exposure to magnetic tube racks.

The binding of DNA to the matrix is facilitated by particular salt conditions, which is why a binding buffer sometimes needs to be added to the sample after lysis. Chaotropic salts such as guanidine hydrochloride, guanidinium thiocyanate, or hydrochloride (Table 1) are common components of lysis buffers and also promote binding of the nucleic acids to the silica (Berensmeier, 2006). Sodium iodine or sodium perchlorate may also be used but are not as common. In addition, ethanol or other organic solvents may be part of lysis or binding buffers to promote binding. Once the DNA is safely bound to the matrix, the remaining lysis material is removed by centrifugation or vacuum (columns) or pipetting away of the supernatant (beads), leaving matrix-bound DNA behind.

Matrix-dependent kits are user-friendly, support standardization, and allow fast processing of many samples with centrifuge/vacuum capacity as a limiting factor. Magnetic bead-based approaches can be automated and multiplexed with instruments like the Thermo ScientificTM KingFisherTM system. Quality control of the manufacturers minimizes batch-to-batch variability. While kits were originally designed for patient samples such as blood or tissue, variations optimized for water, soil, microbes, or plants are now commercially available. The optimization mostly refers to the treatment of starting materials and lysis conditions.

At the same time, however, kits can result in low DNA yield (Abdel-Latif & Osman, 2017) since DNA loss during binding, washing, or due to incomplete recovery can be substantial (Menchhoff et al., 2020). Also, most kits produce low-molecular-weight DNA (i.e., short fragments, which may or may not matter, depending on downstream applications). Furthermore, the chaotropic salts used to support the binding of DNA to the matrix may inhibit downstream enzymatic reactions such as DNA polymerase amplification (Vandeventer et al., 2012) if not removed properly. Finally, commercially available kits are costly.

II.3.2.2 Matrix-free

Matrix-free DNA isolation protocols exploit the physical and chemical properties of DNA (e.g., hydrophilic and polar/charged) to separate it from other, more lipophilic, and less polar/charged

components and cellular components.

For phenol-chloroform-isoamyl alcohol extraction, a commercially available mixture of these organic solvents is brought into intense contact with the lysis material through vortexing or vigorous shaking. This results in protein denaturation and the dissolution of cellular lipids in the organic solvents. Subsequently, the organic solvents are separated from the aqueous lysis buffer based on their different density during centrifugation. Lipids and highly hydrophobic cellular components end up in the bottom solvent layer, hydrophilic components such as salts and DNA remain in the top watery layer, and protein fragments – which are partly polar and partly lipophilic – collect at the interphase between the layers (sometimes visible as white material). The aqueous upper phase and, thus, the DNA can then be transferred to a new tube, and the process is repeated at least once (more often for very fatty or protein-rich samples, which is usually not a concern for eDNA). To remove phenol, which can seriously interfere with downstream processes, the process is then repeated with chloroform as an organic solvent. In the context of eDNA projects, phenol-chloroform extraction can mitigate issues with PCR inhibitors (e.g., humic acids), which are removed by organic solvents. Also, in combination with ethanol precipitation (see below), this extraction can potentially recover more DNA / and is less affected by DNA loss during the protocol compared to matrix-dependent methods.

An alternative, “quick and dirty” option for separation is to pellet larger debris from the lysis material by high-speed centrifugation, recover the DNA-containing supernatant to a new tube, and follow up with column-based or precipitation-based cleanup. The recovery of DNA is much less quantitative, and long fragments tangled and intermixed with other cellular components will end up in the pellet and be lost, but for certain applications or under certain infrastructural constraints, the approach may yield satisfactory results.

Matrix-free protocols are interesting for low-budget projects and/or situations where personnel/time costs are not a major factor. The quantity, purity, and integrity of DNA obtained after phenol-chloroform extraction is often higher compared to kit approaches (Deiner et al., 2015), which matters for, e.g., long-read sequencing with PacBio or MinION. However, matrix-free approaches don't scale as easily to hundreds of samples as kit approaches. Inexperienced/inadequate pipetting skills could result in the carry-over of organic solvents, particularly phenol, which can be detrimental to downstream enzymatic reactions. Lastly, the chemicals used for matrix-free approaches are harmful to the user and the environment (e.g., phenol) and thus require protective equipment (e.g., chemical hood) and appropriate disposal options.

II.3.3 Steps 3 and 4: Removal of salts and DNA collection/concentration

After the separation step, the DNA is devoid of other cellular components but not yet in a state suited for downstream applications because it is either diluted in a substantial volume of an aqueous solution (e.g., after phenol-chloroform extraction) or bound to a solid phase (when using a matrix-

based approach). In both cases, the DNA must be recovered in a concentrated state, and salts must be removed before the DNA is ready to use in downstream applications. Salts can compromise the buffering capacity of the buffer used for long-term storage or inhibit enzymes used in downstream applications, such as polymerases or restriction enzymes, and are therefore removed by "washing steps" with low salt buffers and at least a 70% ethanol concentration. The low-to-no salt conditions attract small, charged molecules or ions into the wash buffer, while the ethanol keeps the DNA precipitated or matrix-bound. If ethanol concentrations of the wash buffer are lower than 70% (for example, in old buffers due to evaporation), DNA loss will occur during washing. Salt removal is a step to consider for tweaking when DNA concentrations are too low, or DNA quality does not meet expectations. On the one hand, washing steps can be repeated to improve purity. On the other hand, each washing round is associated with DNA loss, and some downstream applications may be quite robust, even in the presence of salts.

The order of recovery and salt removal depends on the approach. In matrix-free methods, where DNA is present in a dissolved aqueous state, DNA recovery precedes salt removal, whereas, for DNA bound to a matrix, salts are removed before recovering the DNA.

II.3.3.1 Matrix substrates: salt removal first, recovery second

Salt is removed from matrix-bound DNA by forcing a wash buffer (low salt, 70% EtOH) through the column by centrifugation. Afterward, residual ethanol still contained in the column is usually removed with an additional spin step, sometimes referred to as a dry spin. Removing residual ethanol before subsequent steps is essential for downstream applications to function properly, and instructions referring to this step should be taken seriously.

For recovery of DNA from the column (a step called "elution"), a low salt buffer is forced through the column. This disrupts the ionic interactions between the DNA and the matrix; the DNA enters the solution and can be recovered from the matrix by centrifugation (column) or pipetting (beads). Elution can be achieved with nuclease-free water, but more commonly, TE buffer is used because it promotes stability during long-term storage (see the section on Storage Considerations below; (Panda et al., 2019)). Elution conditions can impact DNA yield, and various measures can improve recovery from reducing the elution volume to recover more concentrated DNA, pre-warming the elution buffer, incubating with elution buffer for a couple of minutes, or passing the eluted DNA sample through the filter a second time. These are often detailed in the kit protocols.

II.3.3.2 Matrix free: recovery first, salt removal second

To recover DNA from aqueous solutions, DNA is precipitated (a process to separate a solid from a solution) and then collected, e.g., through centrifugation. DNA is a charged molecule and highly soluble in water because the charges on DNA interact with the bipolar water molecules. To disrupt the interaction between DNA and water and allow for their separation, salts and alcohols are used in

combination. Ethanol (Y. Xia et al., 2019) or isopropanol (Green & Sambrook, 2017) can both be used, but isopropanol is often the better choice for eDNA samples for logistical reasons: ethanol requires the addition of 2-3x sample volumes of alcohol and, thus, quite large vessels, whereas isopropanol requires 0.6-0.7 volumes of alcohol (Green & Sambrook, 2017). The most commonly used salt is sodium acetate (Y. Li et al., 2020), but other salts releasing positive ions can be used, too. Factors impacting the efficiency and recovery are a) incubation time – over-night incubations can yield more material (Y. Li et al., 2020), but for high concentration samples, 5 minutes may be just fine) incubation temperature – cold temperatures down to -80°C can improve recovery (in particular of small fragments), but again, for high concentration samples, room temperature may be fine, and c) addition of a carrier material – low amounts of DNA are more easily recovered in the presence of glycogen or unrelated DNA, but again, for high concentration samples, this is not necessary. The main takeaway here is that if recovery is poor, precipitation at -20° overnight and in the presence of a carrier could be attempted. A second takeaway is that the precipitation step can function as a convenient break in the protocol over lunch, during a meeting, or overnight. After precipitation, a high-speed centrifugation step (usually in a cooled centrifuge) collects the precipitated DNA at the bottom of the tube as a clear or sometimes whiteish pellet. Again, time and temperature can improve recovery, and prolonging a centrifugation step usually does no harm. The ethanol-salt supernatant is afterwards removed by pouring or pipetting, and any remaining salts are removed from the pellet by a wash step.

Salt removal occurs after the DNA has been precipitated and pelleted, a step which is usually referred to as “washing.” 70% ethanol in water is added gently to the pellet and incubated with the pellet (usually during cold, high-speed centrifugation with the tube in the same orientation in the centrifuge as before to keep the pellet in place). 70% EtOH is a low-ionic solution, which is attractive for salts but prevents the DNA from going back into the solution. After the centrifugation (which can, again, be extended if logistically helpful), the supernatant is carefully removed without disturbing the (sometimes invisible) pellet. The pellet is then air-dried at room temperature with an open tube lid. Here, the main goal is to remove residual ethanol, which may interfere with downstream steps. Drying can take a long time if the supernatant is not removed completely. Finally, the pellet is resuspended in the long-term storage buffer, most often TE or low-TE (also see section on Storage Considerations).

II.4 Post-extraction remedies

In the eDNA field, samples are usually precious. Repeating a failed experiment or re-extracting from additional samples is very often not an option. Fortunately, some issues of DNA samples can be patched post-extraction, at least to a certain degree. For example, samples with low DNA concentrations can be subjected to vacuum centrifugation to re-precipitation and elution in a smaller volume or to concentration and re-elution on a DNA cleanup column. This may entail some DNA loss in terms of total amount but may yield a concentration of DNA per volume sufficient for, e.g., sequencing library

preparation. Similarly, samples with salt concentrations inappropriate for downstream applications may be diluted, re-precipitated, or subjected to cleanup kits, desalting columns, or drop dialysis on dialysis membranes. Finally, inhibitors are a particular concern for eDNA samples. Inhibitors can prevent amplification from well-concentrated, nicely buffered samples. Again, a simple solution is sample dilution. Also, adding anti-inhibitory compounds, such as BSA or commercially available anti-inhibitors to, e.g., PCR reactions can alleviate some levels of inhibition. Sometimes, a harsh spin to collect whatever inhibitors are still around at the bottom and then carefully taking the volume for PCR from the surface of the sample can also mitigate inhibition.

II.5 Storage considerations

Work with environmental samples often requires prolonged storage of samples, for example, to cover multiple years or seasons or to compare before- and after-scenarios of interventions. A continuous concern for storage is **stability and degradation**, and a perfect extraction can be ruined by suboptimal storage conditions. Generally speaking, DNA is – if isolated and stored correctly – more stable in purified form than in environmental samples. Recommended long-term storage conditions include freezing at -20°C (for prolonged storage, deep freezing at -80°C) in a buffer (e.g., TE buffer) (Panda et al., 2019). At the same time, repeated thawing and freezing of DNA samples promotes fragmentation and degradation. If a sample is going to be used multiple times, it may, therefore, be desirable to aliquot it before freezing. If a sample is going to be used within a week or so, storage at 4°C after extraction and freezing the sample only afterward for long-term storage may, therefore, be beneficial. Also, DNA is more stable at higher concentrations. Eluting or resuspending the DNA in small volumes can be helpful in this regard, as well as adding unrelated DNA to ramp up concentrations (e.g., commercially available high molecular weight mouse DNA). Finally, DNA tends to adhere to plastics, which reduces the concentration available in the sample, particularly for low-concentration samples. Steps to reduce adsorption include adding surfactants to the sample (TET buffer (TE buffer pH 7.4, 0.05% Tween 20), and/or using low-bind tubes. Another concern for storage is **the needs of downstream applications**. These may require minimum concentrations or be sensitive to storage buffer composition. For example, low-EDTA TE buffers should be used if downstream methods are sensitive to chelators such as EDTA (for example, NGS). In summary, planning the storage ahead of time and before embarking on a particular DNA extraction protocol is highly recommended.

II.6 Conclusions

Choosing, redesigning, or optimizing a DNA extraction protocol is always driven by the needs of the project in terms of amount, bias, integrity, and purity (Box 1). These needs are defined by the requirements of downstream applications and data collection. Once the needs are clear, this paper should equip field ecologists with the necessary confidence to mix and match DNA extraction protocols

and kits to suit these needs. The first step towards this goal is to identify which parts of the candidate protocol belong to steps 1, 2, 3, or 4. We also encourage field ecologists to ask molecular colleagues questions about protocols and make use of the community's protocol pillars, e.g., forums like Science Learning Hub, online protocol collections such as Cold Spring Harbour Protocols, or print resources such as the Molecular Cloning book series, to expand their understanding further. Finally, we'd like to point out that small tweaks – from extending a 2h-step to overnight incubation or inserting an additional freeze-thaw cycle to doubling the lysis volume – can greatly facilitate handling or improve protocol efficiency. Knowing which steps can or cannot be changed, omitted, or swapped is key for transitioning from morphological to molecular taxonomy and ecology.

Box 1. Defining project needs

I. **Amount.** How much DNA is required for the downstream application? Does the aim of the project tolerate the loss of DNA, incomplete recovery, or hard-to quantify low-concentration DNA samples? For example, species-specific detection by PCR that aims for a yes/no answer is more tolerant to low DNA concentrations than semi-quantitative community metagenomics.

II. **Bias.** How sensitive is the detection method to bias, and does bias matter at all? Does the aim tolerate unequal extraction of distinct organisms, or will this impact the result? For example, the detection of easy-to-lyse gram-negative prokaryotes is probably insensitive to bias, while a relative quantification of a chitinous fungus with respect to the entire community may be affected by incomplete lysis.

III. **Integrity.** How intact should the DNA be – does the detection process tolerate fragmentation well, or do downstream applications require long contiguous DNA fragments? For example, 16S metabarcoding with short amplicons is pretty robust to fragmentation, while any application that aims for species-level resolution through long-fragment sequencing approaches will require more intact DNA.

IV. **Purity.** How clean should the DNA be? How sensitive is the downstream detection method to contamination with salts, organic solvents, or other inhibitors? For example, next generation sequencing approaches are less tolerant towards contaminations than yes/no detections through ordinary PCR.

III. General discussion and conclusion

This dissertation presents a comprehensive framework that integrates environmental DNA (eDNA) methods and various molecular tools to explore, detect, and classify microbial communities, including pathogens. This research was divided into three main research projects to achieve these objectives.

The first part involved establishing an eDNA pipeline for the analysis of microbial communities in recirculating aquaculture systems (RAS) (**Chapter 1**). This pipeline encompasses the entire process, from sample collection to data analysis, to evaluate the efficacy of eDNA in monitoring microbial communities and the depth of information obtained from different sequencing approaches.

In the second part, using the established eDNA pipeline, a large-scale spatiotemporal project was conducted across six commercial-sized RAS farms, encompassing both freshwater and brackish water systems (**Chapter 2**). Here, I explored the extensive patterns and dynamics of microbial communities within these aquaculture systems to provide valuable insights to aid managers in their decision-making processes.

Finally, a field-compatible qPCR assay was developed for the detection of *Aphonomyces astac*i, the causative agent of crayfish plague (**Chapter 3)**. This assay holds potential as a point-of-care testing tool, possibly with lateral flow strips or (semi)-automatic sampling devices, for rapid and on-site pathogen detection.

In addition to the three research projects, this dissertation includes two chapters discussing the future of molecular applications in aquaculture (**Chapter 4**) and provides a comprehensive understanding of DNA extraction for newcomers in the field of eDNA (**Chapter 5**).

The following sections discuss the questions that my research aimed to answer, followed by a discussion regarding the future potential of recirculating aquaculture.

III.1 Are eDNA methods an effective tool for monitoring microbial communities in RAS?

As evidenced in this dissertation, eDNA-based molecular methodologies demonstrate remarkable effectiveness in monitoring the spatiotemporal patterns of microbial communities (**Chapters 1 and 2**). A considerable advantage of eDNA-based monitoring is its ability to detect and identify a diverse array of microorganisms without the need for direct sampling or culturing of individual organisms. This breakthrough technology has enabled researchers to explore entire microbial communities and gain insights into their compositions, interactions with other species, and responses to environmental disturbances.

To date, few studies have investigated the spatio-temporal patterns and dynamics of microbial communities in RAS. Most research studies have focused on microbial communities within biofilters (Bagchi et al., 2014; Huang et al., 2016; Hüpeden et al., 2020), which are essential for exploring

nitrifying organisms and understanding nitrogen cycling rates but fail to provide a system-wide overview of community composition. The few studies that have explored spatio-temporal patterns across RAS have consistently revealed distinct differences between biofilm and water communities over time (Bakke et al., 2017; Almeida et al., 2021; Dahle et al., 2023; Rieder et al., 2023). These findings are consistent with the results presented in **Chapters 1 and 2** of this dissertation, and can be attributed to the maturation of biofilms, leading to a more complex matrix that supports a diverse community structure of aerobic and anaerobic species (Suarez et al., 2019). Additionally, these studies have revealed the spatial dynamics of communities across different compartments, indicating that microbial communities are not homogenously distributed across the systems, which could impact the metabolic rates offered within a compartment. These findings imply that microbial communities within RAS are highly dynamic, and a system-wide sampling scheme is required to fully understand the microbial composition of a system.

III.1.1 Challenges and solutions

Despite the remarkable progress made in molecular ecology in the aquaculture sector, several challenges still hinder the complete implementation of these advancements. Scientists currently face the following challenges:

III.1.1.1 Challenge and solution 1: Lack of standardization

The lack of standardization presents a significant challenge for achieving consistent and comparable results across various studies and research settings. The absence of widely accepted protocols and methodologies for eDNA sampling, extraction, and analysis introduces variations in data quality, impeding meaningful comparisons between studies (Albertsen et al., 2015; Kumar et al., 2022; Rieder et al., 2023). Moreover, the lack of standardized sampling and processing techniques introduces further variability in data quality and reliability. Biases may be introduced during DNA extraction (**Chapter 5**), PCR amplification (Kelly et al., 2019), and data analysis, hindering the accurate interpretation of the results (Albertsen et al., 2015; Deiner et al., 2015; J. S. Johnson et al., 2019). Additionally, the absence of standardized protocols creates challenges in data management and sharing, limiting the potential for conducting meta-analyses that could offer valuable insights into broader ecological patterns and trends (Petit-Marty et al., 2023).

Further investigations, similar to the study conducted by Rieder et al. (2023), aiming to establish a definitive best practice protocol for monitoring microbial communities in RAS, should be pursued. These future studies have the potential to lead to the development of semi-standardized protocols for eDNA sampling within RAS, providing essential information on the optimal swab material, filter size, filtering amount, and required replicates to be collected. The standardization efforts are of utmost importance as they facilitate meta-analysis studies that could lead to the detection of large-scale

patterns, offering valuable insights that can guide managers in making informed operational decisions and formulating effective microbial management plans.

III.1.1.2 Challenge and solution 2: Incomplete databases

The presence of incomplete databases poses a significant challenge when attempting to accurately identify and classify microbial species from eDNA data. Insufficient reference databases encompassing all relevant microbial taxa hinder the precision of species identification and taxonomic assignment, potentially leading to misinterpretations of microbial community composition. The absence of pertinent sequencing data in the database can result in erroneous or ambiguous taxonomic assignments, further contributing to the misidentification and inaccurate classification of microbial taxa, thereby undermining our comprehension of community composition and diversity (Bagchi et al., 2014; Huang et al., 2016; Hüpeden et al., 2020). Furthermore, incomplete databases impede the precise annotation of functional genes and pathways, limiting our capacity to investigate the ecological roles and metabolic functions of microbial communities. Additionally, these databases frequently lack information on rare and novel microorganisms, which are vital for comprehensively understanding microbial diversity and ecosystem functioning.

Addressing the specific challenges associated with reference databases presents a challenging task, and achieving complete resolution may remain elusive. Nevertheless, there are measures that can be adopted to enhance the databases. A perspective paper by Keck *et al*. (2022) reviewed the seven challenges of reference databases and presented potential solutions for each challenge. Overall, they concluded that the completion of the reference databases concerns the entire scientific community, thus making it the collective responsibility of all scientists to publicly share the genetic data they generate. That metadata conforming to FAIR principles (Rimet et al., 2021) should also be shared and linked to the sequencing data. Second, they pointed out the importance of supporting consortia and working groups that maintain these databases, as their unwavering efforts are indispensable in sustaining these databases that we scientists depend on. Finally, they pointed out there is a need to raise awareness among end-users about the specific challenges faced by reference databases. This wider distribution of knowledge will enable the implementation of tools for improved quality control and curation of workflows tailored to reference databases.

III.1.1.3 Challenge 3 and solution: Descriptive only studies

The majority of microbial studies predominantly exhibit a descriptive nature, which hampers the practical application of their findings and the formulation of predictive measures. These descriptive studies involve the observation and measurement of microbial communities but often lack explanatory insights into the underlying causation of these observations, thereby limiting their overall understanding and practical relevance. For instance, Bakke et al. (2017) demonstrated the similarity of water communities among different compartments within RAS at the same time point, the structural

changes in biofilms and water communities over time, and the significant role of salinity in shaping these communities. However, the direct translation of this information into actionable management practices remains elusive. Similarly, Almeida et al. (2021) identified the dominant phyla across RAS compartments and highlighted the role of water parameters, such as salinity and pH, in shaping these communities. Nevertheless, the precise effects of these associations have not yet been fully elucidated. This pattern is always followed in **Chapter 2** of this dissertation, where my findings mirror the findings of Bakke and Almeida, but identifying key drivers that managers can change to manipulate the microbial communities in a more favorable way remains unstated.

The primary constraint of these studies lies in their lack of being guided by scientific questions or theories, which consequently hinders the advancement of forward knowledge in the field of microbial ecology. While such studies may unveil new species or patterns, they fall short in contributing to a profound comprehension of ecology or ecosystem functioning, necessitating a shift towards a more hypothesis-driven system design. Adopting this approach involves evaluating the necessary parameters, such as the number of species and specific environmental characteristics, to be measured, thereby enabling comprehensive insights into microbial ecology (Prosser, 2020). Therefore, future studies should 1) formulate hypothesis-driven questions, allowing for cause and correlations to be determined, and 2) go beyond short-read data and incorporate a tier-sequencing approach to gain a more holistic understanding of the community composition and functional services (Rieder et al., 2023). These types of study designs will enable a more profound comprehension of the role played by microbial communities in RAS, finally providing information that is valuable to managers.

III.1.2 Conclusion

In summary, eDNA studies present practical and comprehensive approaches to assess microbial diversity, abundance, and community structure in RAS. Environmental DNA methods provide valuable insights into spatiotemporal patterns that can impact system efficiency and offer information on the influence of management practices on microbial communities. Additional research on microbial communities in aquaculture is essential to fully harness the potential of microorganisms for effective farm management, such as optimizing biofilter start-ups or enhancing disease-prevention strategies. Further investigations are required to uncover fundamental biological principles, such as understanding the connections between environmental stressors and microbiome dysbiosis. Lastly, microbial research is needed to reveal medically relevant interactions, particularly those involving host-microbiome-environment interactions, and their role in disease development. Such in-depth studies will provide valuable insights into advancing sustainable aquaculture practices and promoting the health and well-being of farmed species.

III.2 Are eDNA methods an effective tool for monitoring and detecting pathogens

in RAS?

This dissertation provides compelling evidence of the remarkable effectiveness of eDNA-based molecular methodologies in detecting aquatic pathogens (**Chapters 1 and 2**). Environmental DNA-based monitoring tools are increasingly supplanting traditional techniques for early pathogen detection and surveillance (Amarasiri et al., 2021; Farrell et al., 2021; Bass et al., 2023) due to their numerous advantages over conventional methods. One key advantage of eDNA sampling is its non-invasive nature. Unlike traditional sampling methods that necessitate direct organismal sampling, eDNA sampling requires only the collection of water or biofilm samples for detecting aquatic pathogens. This non-invasiveness is especially beneficial as it reduces stress in farmed animals, mitigating the risk of disease susceptibility associated with increased animal stress (Webster et al., 2021).

Furthermore, eDNA exhibits great flexibility in its compatibility with modern molecular technologies, ranging from single-species detection methods like PCR-based applications (Nguyen et al., 2018; Williams et al., 2021; Rieder et al., 2022) to community-wide methods such as amplicon or shotgun metagenomics (Bass et al., 2023; Rieder et al., 2023). The use of shotgun metagenomic data enables the detection of novel or emerging pathogens (Taylor-Brown et al., 2018), significantly enhancing our ability to monitor and respond to emerging diseases. These advancements contribute to increased specificity, sensitivity, and accuracy of analyses (Taberlet et al., 2012), allowing researchers to identify pathogens even at low abundance (MacAulay et al., 2022) and facilitating early intervention measures to prevent spread and mitigate disease outbreaks.

III.2.1 Challenges and solutions

Despite the numerous advantages of eDNA-based surveillance, several challenges currently impede its integration into routine monitoring programs (Sepulveda et al., 2020). Scientists currently face the following challenges:

III.2.1.1 Challenge and solution 1: Sampling design

Designing a robust sampling plan that maximizes the capture of the targeted pathogen can present challenges (Wilcox et al., 2016). The sampling scheme must carefully consider both biological factors, such as the interplay between the pathogen, host, and environmental elements, and technical considerations. For instance, the application of UV light for pathogen control in RAS (Attramadal et al., 2021) may lead to a reduction of pathogen load to undetectable levels (Klymus et al., 2020), potentially resulting in false negative results. Moreover, the distribution of pathogen abundance may not be uniform across farms (Rieder et al., 2023), necessitating meticulous selection of sampling locations for optimal capture and detection.

To ensure adequate detection, multiple replicates or frequent sampling may be necessary, as pathogens could be more prevalent in localized areas rather than uniformly distributed in the water

column. Carrying out a pilot study that focuses on factors such as sample location, collection volume, required replicates, sample type, and site characteristics (Goldberg et al., 2016; Sieber et al., 2020) can help address some of these uncertainties and contribute to a more targeted and effective sampling design.

III.2.1.2 Challenge and solution 2: Transportation and storage logistics

When on-site sequencing is not feasible, several challenges emerge concerning sample storage, transportation logistics, and associated costs. While the expenses of sampling and analysis are typically considered during planning, the costs and logistics involved in transporting samples to and from the sampling site are often overlooked despite their equal importance (MacAulay et al., 2022). Inadequately planned storage and transportation can lead to sample degradation, jeopardizing the quality and validity of the results (Lear et al., 2018). In such instances, it may become necessary to repeat the sampling process, resulting in increased time and costs and causing delays in taking appropriate actions to address disease outbreaks. Therefore, careful consideration of resource availability, particularly concerning transportation and cold storage options, should be an integral part of the planning phase (MacAulay et al., 2022).

III.2.1.3 Challenge and solution 3: Sample processing

eDNA sampling itself is a relatively straightforward process, but the molecular methods involved in sample processing, encompassing DNA extraction to sequencing, introduce increased complexity. While farm managers or personnel can receive training to follow specific protocols, a deeper understanding of these techniques is indispensable for effective methodological decision-making, optimization, and troubleshooting. For instance, grasping the appropriate DNA extraction method, particularly regarding the lysis step (as detailed in **Chapter 5**), and making informed primer choices (Ghyselinck et al., 2013; Albertsen et al., 2015; Darwish et al., 2021; Rieder et al., 2023), can pose challenges for untrained molecular scientists. However, these two steps are of utmost importance to ensure reliable results. Inadequate lysis might hinder the detection of hard-to-lyse species, such as gram-positive bacteria or fungal spores, while erroneous primer selection could lead to insufficient PCR binding, ultimately yielding misleading conclusions.

Therefore, it is advisable to foster cross-field collaboration between scientists and farm managers. This collaborative approach enables managers to access the necessary information for making well-informed decisions while scientists can explore fundamental microbial ecology objectives, thereby advancing the field with fresh insights and knowledge.

III.2.1.4 Challenge and solution 4: Data interpretation

PCR-based sequencing approaches, such as PCR or qPCR, are currently the golden standard for single-species detection. However, interpreting single-species data accurately can be a challenging task. The main obstacle lies in the need for cautious consideration of result accuracy due to the occurrence

of false negatives and false positives (Kelly et al., 2019). On the one hand, false negatives (type II errors) are particularly concerning for managers as they imply the absence of the targeted pathogen, potentially leading to misguided decisions. Factors contributing to false negatives include inhibition or the use of expired reagents that impede amplification, pathogen abundance below the detection threshold, and inadequate sampling schemes, such as insufficient replicates, low sampling volume, or incorrect sampling locations (Evans et al., 2017). To mitigate false negatives, addressing these factors and optimizing the sampling and sequencing methods are crucial steps (Sieber et al., 2020).

On the other hand, false positives (type I errors), indicating pathogen presence when none exists (Evans et al., 2017), should be further confirmed through sequencing or validation by a trained veterinarian. Managers must be aware that a true-positive result does not necessarily indicate an ongoing disease outbreak. Some pathogens may be ubiquitous opportunistic species that cause disease outbreaks only under specific conditions, including the presence of the pathogen, suboptimal environmental conditions, and a stressed host. Therefore, complementary approaches, such as assessing host health and clinical signs, should be employed to better understand the disease status (Rupp et al., 2019).

III.2.1.5 Challenge and solution 5: Limitations in determining active disease outbreaks with eDNA

The inability to ascertain disease outbreaks using eDNA data represents a critical challenge. While eDNA methods offer valuable insights into pathogen presence and diversity, they do not provide the necessary information to determine if there is an ongoing outbreak. However, there is a promising solution on the horizon. eDNA sampling can be complemented with environmental RNA (eRNA), an emerging, non-invasive technology capable of identifying active outbreaks. By targeting active gene transcription, eRNA can provide crucial information about the presence of an active disease outbreak. Although eRNA studies are still in their early stages, they hold tremendous potential (Mérou et al., 2020).

III.2.2 Conclusion

In summary, the implementation of eDNA-based pathogen surveillance programs in RAS offers significant potential for efficient pathogen monitoring and identification. The ability for early detection facilitates proactive management strategies, swift responses, and mitigation of the potential consequences of severe disease outbreaks. Furthermore, the ongoing progress in novel molecular-based technologies for pathogen surveillance presents opportunities for cost reduction and increased options, especially by providing cost-effective pathogen tools for lower-budgeted farms.

III.3 What is the future of recirculating aquaculture?

The future of recirculating aquaculture holds great promise for its sustainable growth and development. To ensure its long-term viability, comprehensive knowledge of the microbial

communities within RAS is required, which will drive system engineering, increase animal health, and lead to effective microbial management practices that support sustainable operations. Additionally, collaborations with other sectors will ensure that the sector aligns with sustainable practices that reduce their energy and water footprint.

The utilization of molecular technologies in RAS represents a valuable approach for gaining profound insights into the complex dynamics and functional capacity of microbial communities. Previous investigations employing next-generation sequencing have demonstrated the potential to uncover spatiotemporal patterns, shifts in community composition during disease outbreaks, and distinctions among communities in different sample matrices (Almeida et al., 2021; El-Sayed et al., 2021; Dahle et al., 2023; Rieder et al., 2023). However, future research will need to move beyond short-read sequencing and incorporate other sequencing technologies that offer deeper insights into functional potential. For example, metagenomics and metatranscriptomics data (**Chapter 4**) could lead to the discovery of novel species with important roles in degradation and nutrient cycling or with the potential to serve as probiotics or natural defenses against pathogenic species. However, by combining the powers of different sequencing approaches (Rieder et al., 2023), comprehensive knowledge could lead to the development of effective management strategies and open up avenues for exploring nature's solutions to environmental challenges (e.g., phage therapy) (R. Liu et al., 2022), offering the aquaculture sector opportunities for sustainable and eco-friendly practices.

Presently, RAS operate with high energy consumption, posing a challenge in achieving sustainability goals, particularly in Europe, where the EU's Sustainable Development Goal 7.3 emphasizes the reduction of energy usage. To effectively meet these objectives and enhance the sustainability of RAS operations, future system designs and operations must be reevaluated. An essential step in this direction involves integrating RAS with other industries, such as agriculture and energy production, to create synergistic systems that optimize resource utilization. For instance, nutrient-rich wastewater generated in RAS can be repurposed as fertilizer for hydroponics or aquaponics, fostering a closed-loop nutrient cycle and minimizing waste. Furthermore, the utilization of waste heat from industrial processes or power plants can be harnessed to maintain optimal temperatures in RAS, thereby improving energy efficiency and reducing environmental impact.

Moreover, a fundamental transformation from traditional single-species RAS to integrated multitrophic aquaculture (IMTA) systems is needed. These integrated systems involve cultivating species at different trophic levels within a circular economic framework, in which the waste produced by one species becomes a valuable resource for others. IMTA systems offer several advantages, including minimization of energy losses and environmental degradation. Additionally, these systems foster sustainable practices while providing a diverse range of edible products. Embracing the principles of circularity and resource optimization, IMTA represents a key pathway for achieving greater

sustainability in recirculating aquaculture and aligning with global sustainable development targets.

In conclusion, the future of the aquaculture sector hinges on collaborative efforts, technological advancements in molecular methods, and commitment to social, economic, and environmental sustainability. By fostering partnerships across disciplines, harnessing the potential of molecular technologies, and implementing sustainable practices, the aquaculture industry can address these challenges, enhance productivity and profitability, achieve UN sustainability objectives, and ensure long-term viability and resilience. Embracing a holistic management approach will contribute to food security, job creation, economic growth, and biodiversity conservation, thus benefiting both human well-being and the environment.

This dissertation makes a considerable contribution to eDNA-based microbial community analysis and pathogen detection in aquaculture. Through a comprehensive framework and innovative research, this study advances the use of molecular tools for improved monitoring and management practices in the aquaculture industry.

Microorganisms will give you anything you want if you know how to ask them

-Kinichiro Sakaguchi

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Declaration of consent

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Registration Number: 11–116-936

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