Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Anders Gjendemsjø

Bacterial colonization of zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*) in gnotobiotic model systems

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
Co-supervisor: Eirik Degré Lorentsen, Lisa Zoé Auclert
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Acknowledgments

This work was conducted in the research group "Analysis and Control of Microbial Systems (ACMS)" at the Department of Biotechnology and Food Science of NTNU Trondheim. Experimental work was also performed with professor Nicolas Derome's research group at Institut de Biologie Intégrative et des Systèmes (IBIS) of Université Laval in Québec, Canada. This collaboration was facilitated by the by International Partnership for Advancing Microbiome-Informed Aquaculture (IPAMA), a research network funded by the Research Council of Norway.

First of all, I would like to thank my supervisor Ingrid Bakke. The time and effort you have devoted to me, my questions, and my experiments, despite your already busy schedule, has gone above and beyond what I could ever expect. Thank you for helping me see the positive sides of failed experiments, and for always greeting me with a smile and an onwards-and-upwards attitude when I come knocking at your office door. I could not have asked for a better supervisor. I would also like to extend my gratitude to Eirik Degré Lorentsen and Amalie Johanne Horn Mathisen for all the help I have received at the ACMS lab, and for always answering my questions with great knowledge and devotion. I could not have done it without your guidance.

I would further like to thank Lisa Zoé Auclert, Pierre-Luc Mercier, and prof. Nicolas Derome of the Derome lab at IBIS. Thank you for your advice and assistance, for providing me with the opportunity to participate in an international research collaboration, and for letting me experience the great city of Québec. To Lisa, thank you for making me feel so welcome at IBIS with your kind demeanour. Your assistance and knowledge during my long nights at the lab was invaluable, and is greatly appreciated.

Lastly, i would like to extend my heartfelt gratitude to all the people who have cheered me on from the sideline. I thank my family and friends for all the support I have received throughout my five years here in Trondheim. It has helped me push through when times have been hard. And to my dearest "Ferns": Thank you for all the endless laughs, and for being my main source of energy and spirit for the last five years. I love you all.

Anders Gjendemsjø
Trondheim, June 2024

Abstract

In the yolk sac-fry stage, farmed Atlantic salmon *(Salmon salar)* are especially susceptible to unfavorable microbial rearing conditions, and high mortalities are frequently reported from the aquaculture industry in this life-stage. This can to some extent be attributed to harmful host-microbe interactions. Increasing our knowledge about these interactions could therefore contribute to the reduction of mortality-related losses in the Norwegian aquaculture industry. A novel approach could potentially be to use established model organisms, such as zebrafish *(Danio rerio)*, as a tool to better predict the effects of host-microbiota interactions in economically important fish species. For this to be applicable, the comparability of microbiota-induced host responses should be investigated.

This study aimed to characterize the initial microbiota of newly hatched zebrafish and Atlantic salmon under gnotobiotic and re-conventionalized conditions, and to assess whether host responses to various bacterial conditions were comparable between the two host organisms on the gene expression level. Unfortunately, due to an unforeseen delay in sample analysis at a third party, gene expression results were not returned in time for them to be included in this work.

In addition, the study set out to determine the growth characteristics of nine bacterial strains previously isolated from the gut of Atlantic salmon at temperatures relevant to the rearing of zebrafish larvae and salmon yolk-sac fry. To achieve this, growth characteristics were first determined for the nine bacterial strains in a general growth medium. Further, gnotobiotic experiments were performed with each host organism, using the same nine bacterial strains. Host organisms were exposed to the bacterial strains both as mono-associations, and as a microbial consortium (SynCom). The community composition of re-conventionalized control conditions kept in both gnotobiotic experiments was also investigated. Bacterial community compositions were analyzed by Illumina sequencing of 16S rRNA gene amplicons.

In the gnotobiotic experiment with salmon yolk-sac fry, gnotobiotic conditions were successfully maintained. In the zebrafish gnotobiotic experiment, indications of bacterial contaminations were observed. Further, there appeared to be a positive correlation between the SynCom strain's maximum growth rates and their colonization success in rearing water and host-associated microbiotas. Although the rearing water and fish tissue microbiotas were distinct, results suggested that the fish tissue microbiota was influenced by the water microbiota, but that host-provided selection factors made them distinguishable. The main finding of this study was that the experimental system design seemed to adversely affect the microbial community composition, as it was observed that rapid-growing heterotrophs dominated the fish tissue and end water microbiotas. This could suggest that the batch-like system design provides a selection regime in which rapid-growing heterotrophs out-compete slower-growing bacteria.

Sammendrag

I plommesekkstadiet er oppdrettslaks *(Salmo salar)* spesielt sårbar for ugunstige mikrobielle betingelser, og høy dødelighet rapporteres ofte fra oppdrettsnæringen i dette livsstadiet. Dette kan til en viss grad tilskrives skadelige vert-mikrobe interaksjoner. Økt kunnskap rundt disse interaksjonene kan derfor potensielt bidra til å redusere dødelighetsrelaterte tap i den norske oppdrettsnæringen. En ny tilnærming kan muligens være å bruke etablerte modellorganismer, slik som sebrafisk *(Danio rerio)*, som et verktøy for bedre å forutsi effektene av vert-mikrobiota interaksjoner i økonomisk viktige fiskearter. For at dette skal være anvendelig, må sammenlignbarheten av mikrobiota-induserte vertsresponser undersøkes.

Denne studien hadde som mål å karakterisere den initielle mikrobiotaen til nyklekt sebrafisk og laks under gnotobiotiske og re-konvensjonaliserte betingelser, og å undersøke om vertsresponsene til de ulike bakterielle betingelsene var sammenlignbare mellom de to vertsorganismene på genuttrykksnivå. På grunn av en uforutsett forsinkelse i prøveanalysen hos en tredjepart, ble dessverre ikke genuttrykksresultatene returnert i tide til å inkluderes i denne studien.

Et annet mål med studien var å karakterisere vekstegenskapene til ni bakteriestammer, som tidligere hadde blitt isolert fra laksetarm, ved temperaturer relevante for oppdrett av sebrafisklarver og plommesekkyngel av laks. Vekstegenskapene til bakteriestammene ble derfor først bestemt i generelt vekstmedium. Videre ble de samme ni bakteriestammene brukt i gnotobiotiske forsøk utført med hver vertsorganisme. Vertsorganismene ble eksponert for bakteriestammene både som mono-assosiasjoner og som et mikrobielt konsortium (SynCom). Den bakterielle samfunnssammensetningen av re-konvensjonaliserte kontrollbetingelser i begge gnotobiotiske eksperimenter ble også undersøkt. De bakterielle samfunnssammensetningene ble analysert ved hjelp av Illumina-sekvensering av 16S rRNA gen-amplikoner.

I det gnotobiotiske forsøket med plommesekkyngel av laks ble de gnotobiotiske betingelsene opprettholdt. I forsøket med sebrafisk ble indikasjoner på en bakteriell kontaminasjon observert. Det så ut til at det var en positiv sammenheng mellom SynCom-stammenes maksimale vekstrater og deres koloniseringssuksess i de vann- og vertsassosierte mikrobiotaene. Selv om mikrobiotaen i vann og fiskevev framsto som forskjellige, antydet resultatene at vertsmikrobiota ble påvirket av vannmikrobiota, men at seleksjonsfaktorer i verten gjorde dem forskjellige. Hovedfunnet i denne studien var at utformingen av det gnotobiotiske eksperimentelle systemet så ut til å påvirke sammensetningen av mikrobielle samfunn negativt, da det ble observert at hurtigvoksende, heterotrofe bakterier dominerte mikrobiotaene assosiert med fiskeverten og vannet på slutten av forsøket. Dette kan tyde på at det batch-lignende eksperimentelle systemet bidrar med et seleksjonsregime der hurtigvoksende, heterotrofe bakterier dominerer.

Abbreviations

AB-SGM Antibiotic Salmon Gnotobiotic Medium

AB-ZGM Antibiotic Zebrafish Gnotobiotic Medium

ASV amplicon sequencing variant

CFU colony forming unit

dpf days post-fertilization

dph days post-hatching

FTS flow-through system

IBIS Institut de Biologie Intégrative et des Systèmes

LARSEM Laboratoire Aquatique de Recherche en Sciences Environnementales et Médicales

NAP Nucleic Acid Preservation buffer

NSC Norwegian Sequencing Centre

OD_{600} optical density at 600 nm

OTU Operational Taxonomic Unit

PCoA Principal Coordinate Analysis

PCR polymerase chain reaction

PVP-I poly(vinylpyrrolidone)–iodine

RAS recirculating aquaculture system

SGM Salmon Gnotobiotic Medium

SIMPER Similarity Percentage Analysis

SynCom Synthetic Community

TSA Tryptic Soy Agar

TSB Tryptic Soy Broth

ZGM Zebrafish Gnotobiotic Medium

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1 Introduction

1.1 Motivation

As the global population grows larger and wealthier, the demand for high-quality, animal-based protein sources is following suit [1]. Compared to other animal protein sources for human consumption, such as beef, pork, and poultry, aquaculture-based sources can still be considered underutilized, as fish only accounts for 7% of all protein produced for human consumption [1, 2]. Due to increased worries about harmful ecological impacts, production volumes from capture fisheries have remained relatively stable over the last thirty years, characterized by a shift towards ecologically sustainable fish stocks and more stringent stock management [1, 3]. The contribution of aquaculture to global seafood production, however, has increased exponentially since the 1960s, driving a total increase in seafood production volumes (Figure 1.1). In 2013, aquaculture surpassed capture fisheries in production volumes globally, and its contribution to total seafood production is anticipated to grow further [1, 3].

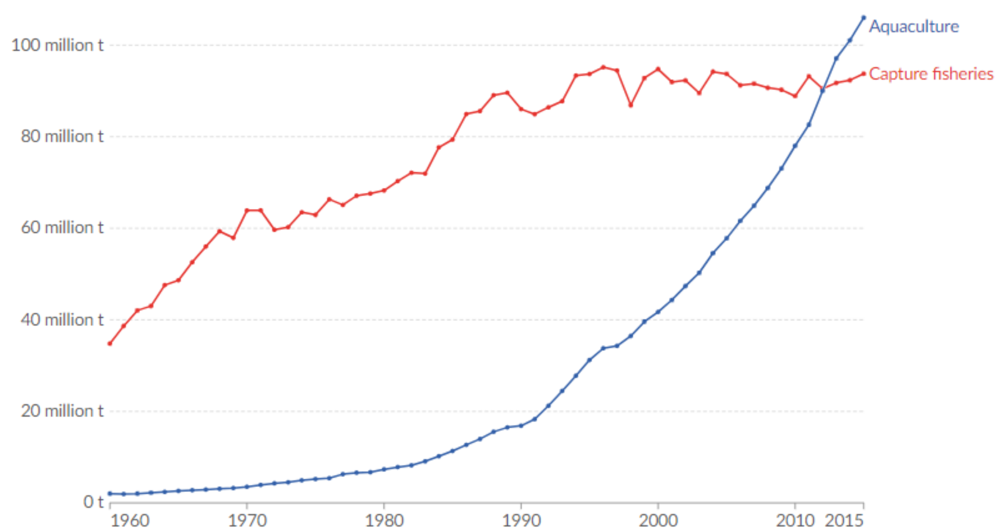


Figure 1.1: The development of global seafood production in tons from 1960 - 2015 from aquaculture (blue) and capture fisheries (red). (Food and Agriculture Organization of the United Nations (via World Bank) – processed by Our World in Data. CC BY [3, 4])

In Norway, aquaculture is already the second-largest export industry, beaten only by the petroleum industry [5]. Atlantic salmon (*Salmo salar*) is by far the most important species in Norwegian aquaculture, with over 1.2 million tons exported in 2023 at an estimated value of approximately 122 billion NOK [6]. This makes Norway the biggest producer of Atlantic salmon globally [5]. Given the forecasted importance of aquaculture as a provider of sustainably sourced, high-quality protein, it is evident that the Norwegian aquaculture industry will be a key contributor in feeding an increasingly larger and wealthier global population.

In an effort to run aquaculture facilities with better animal welfare and lower mortalities, the last decades have seen an increased interest in factors affecting fish biology and health both from academia and the industry itself. Focus has been on a broad but often correlated range of parameters such as water quality [7], rearing facility design [8], feed composition [9], and, most pertinent for this thesis, the effects of the fish- and rearing water microbiota [10].

The current production trend in Norway for rearing Atlantic salmon can be divided into two stages: The Atlantic salmon starts its life in onshore rearing facilities, either in a flow-through system (FTS) where fresh water is continuously moved through the system, or in a more contemporary recirculating aquaculture system (RAS) where water is treated biologically and physiochemically and recirculated. Once smoltification is induced, the salmon is transferred to sea cages for the final grow-out stage until slaughter weight is achieved [11]. RAS typically have a water recirculating degree of > 90%, demanding increased attention to biological and physiochemical water quality parameters, some of which depend on bacterial populations in the RAS itself [10, 11]. The high degree of water recirculation allows for the accumulation of bacterial populations to a larger extent compared to traditional flow-through systems [11]. Understanding the effect of these environmental bacteria, not just on water quality but also on fish health, have therefore become more important with the emergence of RAS as the industry standard for rearing juvenile fish [7, 11].

In a survey of 190 Norwegian onshore salmon smolt production facilities, 44% of all reported mortalities occurred in just one of seven weight groups, when individuals weighed less than 3 grams [12]. This corresponds to the first life stage post-hatching, the yolk-sac fry stage. This stage is characterized by rapid development of adult physiological and immunological functions, making yolk-sac fry especially vulnerable to unfavorable microbial and physiochemical conditions [10]. The high mortalities seen in this life stage can, to some extent, be attributed to harmful host-microbiota interactions [13]. A better understanding of these interactions in the earliest life stages will therefore be imperative to further develop the Norwegian aquaculture industry both economically and ethically.

1.2 Host-microbiota interactions and the ontogenesis of the teleost microbiota

A microbiota can be defined as the collection of all living microorganisms in a specific environment at a specific time, and the term is often used about host-provided environments such as skin, gut, and oral microbiota [14]. Culture-independent methods based on high-throughput sequencing and multi-omics approaches have allowed for a deeper insight into microbial community compositions, diversities, and functions in recent decades. This has led to remarkable progress in our understanding of the intricate commensal relationship between host and microbiota, but many questions remain unanswered [15, 16].

The most studied host-related microbiota is that of the mammalian gut, both in humans and in rodent model organisms. These studies have shown that the gut microbiota is shaped by environmental and geographical factors [17], diet [18], host immune system [19], and development [20]. Reversely, studies have shown that the microbiota influences various host health and development traits. The vast genomic potential present in a diverse microbiota, for example that of the mammalian gut, opens the door for a wide range of functional properties that can benefit the host, including pathogen protection, nutrient provision, and maintaining the structural integrity of the mucosal layer [21, 22]. In addition, it has been shown that the commensal microbiota contributes to and maintains proper immune function through various mechanisms [19, 23, 24]. Although less is known about the detailed roles of fish microbiota compared to mammalian hosts, evidence suggests that it plays a role in similar processes to those observed for mammals, such as the proper development of the gut and immune system [25, 26, 27], protection against pathogen infections [28], nutrient production and uptake, and growth [29].

One major difference between mammals and teleosts is that, in the lack of a stratum corneum, teleosts have an additional mucosal tissue – the skin mucus. It provides a biochemical and physical barrier against potential pathogens and the external environment, and presents itself as a microbial habitat in close spatial proximity to the surrounding water [30]. Although the skin mucosal microbiota is typically reported to be distinct from it, studies show that the environmental microbiota together with the physiochemical selection regime are the main determinants affecting the community composition of the skin mucosal microbiota, in addition to host-provided selection factors [30, 31, 32, 33, 34]. However, the exact functions of the skin microbiota, and the mechanisms involved in its assembly are still not properly understood [33].

The current consensus for how the teleost microbiota establishes in host mucosal surfaces is summarized by Llewellyn et al. (2014). It is thought that whilst still in the developing egg, the embryo remains sterile as it is protected from environmental bacteria by the egg chorion. Bacteria will however colonize the outer surface of the chorion. Once hatched, all available mucosal surfaces of the larvae are colonized by the environmental bacteria and possibly also by those present on the chorion. As soon as the gastrointestinal tract opens, bacteria will colonize the developing gastrointestinal mucosal tissues. When larvae commence active feeding, the microbiota of the feed will have a strong impact on the composition of the gastrointestinal microbiota. As the teleost matures, so will the microbiota, becoming more diverse and increasingly stable [32]. The adult microbiota is the most diverse and stable due to host-provided selection factors in the different host niches [32].

1.3 Studies on the effects of microbiota in salmonids and zebrafish

A prerequisite for studying the effects of microbiota on its host is to establish gnotobiotic model systems. Gnotobiology is the study of model organisms with known, pre-determined bacterial conditions which is decided by the researcher [35]. To obtain gnotobiotic model organisms, they first need to be made germ-free. Once this is achieved, re-inoculation with the desired bacterial conditions can be done. Germ-free derivation procedures have been developed both for zebrafish [25] and, more recently, for Atlantic salmon yolk-sac fry [36].

Few studies have been performed on the microbiota-induced effects on its host in Atlantic salmon, or in salmonids in general, but there are still some examples in literature that shed light on potential interactions. Gómez de la Torre Canny et al. (2023) observed that the presence of commensal bacteria in newly hatched salmon yolk-sac fry gave a greater functional thickness of the skin mucus compared to fry reared in germ-free conditions, as well as an effect on adipose tissue accumulation [36]. Other studies have found that the commensal microbiota of the salmonid species rainbow trout (*Oncorhynchus mykiss*) and brook charr (*Salvelinus fontinalis*) can contribute with protection against bacterial pathogens and antifungal properties [28, 37, 38, 39]. Specifically, Pérez-Pascual et al. (2021) were able to show that whilst germ-free rainbow trout were highly susceptible to infection by the common freshwater fish pathogen *Flavobacterium columnare*, re-conventionalization with eleven culturable species from the trout microbiota gave resistance towards the infection. Through bacterial mono-associations they were able to show that the resistance was conferred by a commensal *Flavobacterium* strain [28].

Compared to the salmonids, studies of microbiota-induced effects on its host in zebrafish can be said to have reached a higher level of sophistication, and there are several examples of such studies in literature. A gnotobiotic zebrafish derivation protocol was established by Rawls et al. in 2004 [25]. Preceding this, the zebrafish was already a well-established vertebrate model organism in many different biological disciplines, such as developmental biology and biomedical research [40]. Advantages of the zebrafish model system includes a fully sequenced genome, high fecundity, rapid oviparous embryonic development, short generation times, and transparent embryos. Standardized molecular methods, assays, imaging techniques, and transgenic lineages were already used routinely before the model system was used to investigate host-microbiota relations. These tools allow for detailed investigations into intricate cellular and systemic responses to different experimental conditions [40]. Compared to rodent models, using zebrafish also allows for experiments with a high number of individuals, which is useful in studies of host-related microbial ecology. The door thus stood ajar for investigating microbiota-induced effects in zebrafish, and was opened by Rawls et al. with the establishment of the gnotobiotic derivation procedure [25].

In the same study, the intestinal microbiota was found to trigger host responses involved in epithelial proliferation, nutrient metabolism, and innate immune system signaling, which were conserved between mice and zebrafish, two seemingly distant vertebrate relatives [25]. This was an important finding, as it showed that host responses to microbiota are evolutionarily conserved, which further implies that findings related

to microbiota-induced effects in model species such as zebrafish might be transferable to other host systems. Later studies have since revealed some of the molecular mechanisms underlying the communication between the zebrafish commensal microbiota and the innate immune system. Some examples are the findings that transcriptional activity of the nuclear transcriptional factor NF-κB, which is important in immune response regulation, is regulated by bacterial factors present in a commensal microbiota [26], that the presence of a commensal microbiota alters neutrophil homeostasis and increases their recruitment to injury [41], or that MyD88 signaling mediates the effects of colonization on the mucosal immune status of the host [42]. Studies in gnotobiotic zebrafish have also revealed some of the details regarding the role of the microbiota in host nutrient metabolism. One study found that a commensal microbiota promotes fatty acid uptake and lipid droplet formation, and their subsequent metabolism in the liver [43].

The well-established set of tools that exists for zebrafish does not exist in the same way for Atlantic salmon, or any salmonids for that matter. Still so, the gnotobiotic derivation protocol developed by Gómez de la Torre Canny et al. has opened for new insights into host-microbiota relations in Atlantic salmon [36]. It is especially suited for studying bacterial community dynamics, but to reach the level of detail that is possible in zebrafish models, there is still a way to go in terms of developing standardized methodologies and molecular tools comparable to those already existing for zebrafish. One advantage which the salmon model system is the physical size of eggs and fry. Salmon are significantly larger and more robust towards physical damage than zebrafish, which eases handling both during the experiment and in subsequent analytical work. Salmon also have a longer yolk-sac stage compared to zebrafish [44, 45]. This allows for studying the host associated microbiota over a longer time period without the need for external feeding.

Although all the details regarding the effects on host health of the teleost fry microbiota are not fully known, the fact that it has been demonstrated that it can interfere with pathogen colonization and affect mucus and immune system development could potentially have implications for current practices in Norwegian aquaculture, where strict sterilization of embryos to avoid pathogen infections is standard. Considering that the microbiota might be especially important in pathogen protection in the earliest life stages when the immune system is not yet fully developed, filling these knowledge gaps and increasing our understanding of the host-microbiota relationship in economically important species such as Atlantic salmon could be important for reducing early-life mortality numbers in the aquaculture industry [46, 47, 48].

1.4 Investigating microbial community compositions

Before the invention of the PCR and the discovery that microbial phylogeny can be derived from nucleic acid sequences, studying microbial communities relied mainly on culture-dependent methods [49]. Considering that we now know that only a small subset of known bacterial species are actually culturable, such methods obviously had their limitations when it came to capturing the actual microbial diversity in a sample [50]. In addition, many bacteria are indistinguishable morphologically and functionally, so separating different taxa based on phenotype is often not possible. [51].

It was Woese and Fox who first suggested the 16S rRNA gene as a marker for phylogenetic classification. Combined with the invention of Sanger sequencing, the 16S rRNA gene sequence quickly became, and still is, the gold standard for studying microbial phylogeny, diversity, and taxonomy [52]. The 16S rRNA gene encodes the prokaryotic small subunit ribosomal RNA, which functions as a major structural component of the 30S ribosomal subunit in bacteria. It also has functional roles, such as providing a ribosomal binding site for the Shine-Dalgarno sequence of the incoming mRNA [53, 54].

The 16S rRNA gene possesses several features which make it the preferred marker for classifying bacteria. Firstly, it is an essential housekeeping gene, with functionally conserved homologs universally distributed. Secondly, the gene's primary structure consists of nine hypervariable regions (v1 – v9) interspaced by highly conserved regions. By using universal primers which bind to the conserved regions one can perform targeted PCR amplification of all bacterial 16S rRNA genes in a sample. The variable regions can then be used for taxonomic diversity studies. By using curated databases which links taxonomy to 16S rRNA gene sequences one can determine the taxonomic classification of the queried source bacteria [55]. The diversity of the sampled microbial community is reflected in the sequence diversity of the sampled 16S rRNA genes. In addition, horizontal gene transfer of the gene is limited [49]

The combination of targeted 16S rRNA gene PCR amplification and subsequent massive parallel sequencing is what is called 16S amplicon sequencing [55]. In Figure 1.2, an overview of a typical workflow for 16S amplicon sequencing is shown. Usually, only a subset of the total 16S rRNA gene which contains one or more hypervariable regions, such as the v3-v4 region, is sequenced. The possibility 16s amplicon sequencing provides for deep characterization of microbial communities, combined with state-of-the art -omics technologies, has revolutionized the scientific fields which involves microbial communities, and they are now central methods in fields such as microbial ecology, environmental microbiology, and environmental biotechnology.

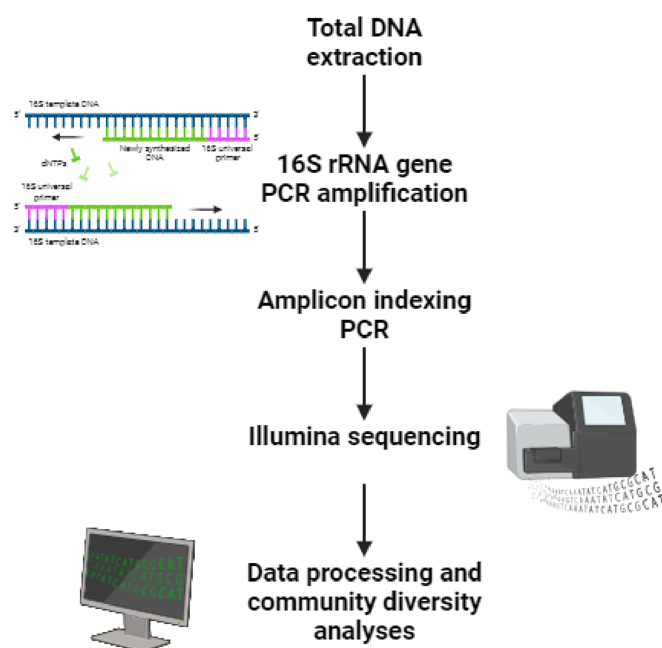


Figure 1.2: A generalized overview of the typical 16S amplicon sequencing workflow. Created with Biorender.com

The composition of bacterial communities are can be analyzed in regards to which bacterial species are present, and at what levels. Depending on the homology to previously classified 16S rRNA gene sequences present in databases, a 16S sequence can result in classification at different taxonomic levels based on a set similarity threshold [49]. Previously, sequencing reads were clustered into "Operational Taxonomic Unit (OTU)" based on sequence similarity. The current trend is to work with amplicon sequencing variant (ASV), as it offers better sensitivity, resolution, and reproducibility compared to OTU-based methods [56]. ASVs are all sequence variants present in a sequencing run after strict quality processing, which are then assumed to represent biologically significant sequences. Assessment of sample diversities involves big sequencing data sets, and requires the use of relevant pipelines and multivariate statistics. Diversity depends both on how many species that are observed, the richness, and to which degree these species are present relevant to one another, the evenness. One can investigate both the diversity within single samples, the α-diversity, or between samples, the β-diversity [57]. Different diversity indices have been developed to quantify the diversity, such as the Shannon diversity index for α-diversity and the Bray-Curtis index for β-diversity [58, 59].

1.5 Aims and objectives

In previous work in the ACMS research group, yolk sac fry of Atlantic salmon has proven to be a sturdy organism with regards to physiological effects of different bacterial associations, showing little to no effects on growth and survival. However, it has been shown that the initial bacterial colonization of a developing teleost host can induce various responses which might not affect growth and survival directly [25]. How, and to what degree these responses are triggered, will depend both on the species of bacteria associated with the host, the host itself, and the colonization success of the bacteria.

The overall aim of this study was to examine if zebrafish could be used as a model to predict host responses in Atlantic salmon to specific bacterial strains. It also set out to investigate colonization of the two different host organisms by a gnotobiotic bacterial community (termed SynCom).

The master project's specific objectives were to:

- Determine growth characteristics of nine previously classified bacterial strains isolated from the gut of Atlantic salmon at temperatures relevant for rearing zebrafish and Atlantic salmon yolk-sac fry

- Prepare axenic zebrafish larvae and Atlantic salmon yolk-sac fry, and perform gnotobiotic experiments in these host models with the bacterial isolates, both as mono-associations and combined in a Synthetic Community (SynCom).

- Quantify the development of bacterial densities in these conditions by means of flow cytometry analyses

- Characterize the resulting fish microbiota and community compositions in the SynCom and re-conventionalized conditions through and 16S amplicon sequencing

- Assess whether host responses on the gene expression level to the initial bacterial colonization are comparable between the two host systems, and evaluate

if zebrafish could be a useful model system for host responses to early bacterial colonization in Atlantic salmon

2 Materials and Methods

2.1 Selection of bacterial strains isolated from salmon

As part of a previous project in the ACMS group, 77 different bacterial strains were isolated from skin and gut samples of Atlantic salmon reared in Salmar's Follafoss facilities, a commercial RAS for smolt production. The bacterial strains were acquired in 2017 by plating them on different media and picking colonies with unique morphologies. They were then taxonomically characterized to the genus level using the RDP Classifier tool based on 16S rRNA gene amplicon sequence data. They have since been stored at -80 ℃. The nine bacterial strains used throughout this thesis were chosen from this strain collection. Representatives from three different phyla (*Actinobacteria*, *Proteobacteria*, and *Firmicutes*) were selected to capture the diversity of the collection. Initially, twenty strains were chosen, but this number was reduced to nine after initial trials on growth characteristics experiments. An overview of the nine strains used throughout this thesis is given in Table 2.1.

2.2 Determining growth characteristics of bacterial strains

To determine basic growth characteristics for the 9 selected bacterial strains, OD_{600}-based growth curves were generated at two temperatures, and a CFU count using the drop plate method was performed. The results of these experiments were also used to determine which concentrations to use of the single-strain cultures in the gnotobiotic zebrafish and salmon yolk-sac fry experiments (Section 2.3 and Section 2.4)

Bacterial growth curves were generated for the nine selected strains (Table 2.1) in the general growth medium Tryptic Soy Broth (TSB) (Appendix A) by measuring the development of the optical density at 600 nm (OD_{600}) in single-strain cultures over time at two different temperatures relevant for rearing zebrafish (22 ℃) and salmon yolk-sac fry (10 ℃) , using either a Tecan Spark® 20M Microplate Reader or a BioLector®Pro micro-bioreactor system (m2p Labs), respectively.

Isolates were retrieved from glycerol stocks stored at -80 ℃ by plating them on Tryptic Soy Agar (TSA) plates (Appendix A) and incubated at room temperature (approximately 22 ℃) for three days until visible growth was obtained for all strains. Colonies were then picked and re-plated on new TSA plates and incubated for an additional three days, giving growth of single colonies. Subsequently, 3 mL of TSB was inoculated with a single colony for each bacterial isolate and incubated for one day (22 ℃, 250 rpm shaking). These liquid cultures where then used to inoculate new 1% cultures which were used for the OD_{600} measurements.

Table 2.1: Overview of bacterial strains used throughout this thesis, their taxonomy as determined by analysis of 16S rRNA gene sequences using the RDP Classifier tool, and weight of host individual from which they originally were isolated. All isolates were obtained from gut samples of Atlantic salmon reared in a commercial RAS facility.

Strain ID	RDP classification	Source host weight (g)
3.28	Actinobacteridae[100%];Actinomycetales[100%];Micrococcineae[100%];Microbacteriaceae[100%]; Microbacterium[100%]	70
3.67	Actinobacteridae[100%];Actinomycetales[100%];Micrococcineae[100%];Micrococcaceae[100%]; Arthrobacter[100%]	21.8
3.24	Actinobacteridae[100%];Actinomycetales[100%];Micrococcineae[100%];Micrococcaceae[100%]; Micrococcus[100%]	70
3.11	Gammaproteobacteria[100%];Enterobacteriales[100%];Enterobacteriaceae[100%]; Hafnia[99%]	21.8
3.78	Gammaproteobacteria[100%];Pseudomonadales[100%]; Moraxellaceae[100%]; Psychrobacter[100%]	70
B40	Bacilli[100%]; Bacillales[100%];Staphylococcaceae[100%];Staphylococcus[100%]	60
B49	Bacilli[100%];Lactobacillales[100%];Streptococcaceae[100%];Lactococcus[100%]	60
3.18	Bacilli[100%];Lactobacillales[100%];Carnobacteriaceae[100%];Carnobacterium[100%]	21.8
3.3	Alphaproteobacteria[100%];Sphingomonadales[100%];Sphingomonadaceae[100%]; Sphingomonas[90%]	4.5

For the experiment at the 22 °C, 150 µL of each 1% culture were aliquoted into three replicate wells on a 96-well plate. The outermost wells of the plate were not used due to the increased risk of evaporation; instead, they were filled with sterile TSB to serve as a negative control. Cultivation conditions for the experiment were shaking at 250rpm at 22 °C, with OD_{600} measurements every hour for 48 hours.

For the experiment at 10 °C, the BioLector®Pro high-throughput micro-bioreactor system (m2p Labs) was used. 800 µL of each 1% culture were aliquoted into their separate wells on a m2p Labs 48-well flower plate, with three replicates of each culture. In addition, three replicate negative controls consisting of sterile TSB were made. Cultivation conditions for the experiment were set to 10 °C, shaking at 1300 rpm, with biomass gain measurements every hour for 190 hours.

The maximum growth rate (μ_{max}) for each individual strain at the two different temperatures was determined from measurements made in the exponential growth phase, using the logarithmic form of the exponential growth equation (Equation 2.1):

$$\ln X_t = \mu t + \ln X_0 \tag{2.1}$$

Here, X_t is the is the biomass concentration at time t, µ is the specific growth rate, and X_0 the biomass concentration at time 0 [60]. Specifically, Microsoft Excel was used to fit a linear regression model to the natural logarithms of the cell density measurements observed in the exponential growth phase. Data points were chosen to give the highest rate of change per time. This was done for all three replicates for each strain. According to Equation 2.2 the average slope of the three replicate regression models is the average μ_{max}.

Minimum doubling time was also determined based on the relationship

$$t_d = \frac{\ln 2}{\mu} \tag{2.2}$$

where µ is the specific growth rate, and t_d the doubling time [60]. Minimum doubling time was found by using the determined μ_{max} in place of µ in Equation 2.2. The end of lag phase was defined as the time point were growth had increased with more than 5% of the highest registered cell density for that specific strain.

2.2.1 Determining relationship between OD600 and CFU/mL using the drop plate method

To determine the relationship between OD_{600} and bacterial concentration, a colony forming unit (CFU) count was performed for all nine strains using the drop plate method [61]. This method allows for parallel CFU counts to be performed on a single petri dish, thus reducing both the workload and the use of laboratory consumables [61].

First, the bacterial isolates were revived on TSA dishes and re-plated to single-colony cultures in the same way as described for the OD_{600} measurements (Section 2.2). For each bacterial isolate, 25 mL of TSB in a 250 mL erlenmeyer flask was then inoculated with a single colony incubated at 22 °C with shaking at 250 rpm overnight or until visible growth was achieved. These cultures were then used to inoculate a new 25mL of

TSB (1%), which were incubated until exponential growth phase was established, determined by the growth curves from the previous experiment (22 °C, 250 rpm shaking). Then, whilst still in the exponential growth phase, three replicate samples of this culture were diluted to an OD_{600} between 0.1 and 0.3. These samples were then further diluted in series to 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}-dilutions. For each of these dilutions, five 10 µL drops were deposited on a TSA plate divided into four, one quadrant for each dilution (Figure 2.1). All dilutions were done with TSB. The plate was then inoculated at ambient temperature (approximately 22 °C) until single, countable colonies appeared. Thus, for each bacterial strain, this resulted in three Petri dishes representing three different OD's, each containing five drops of all four dilutions.

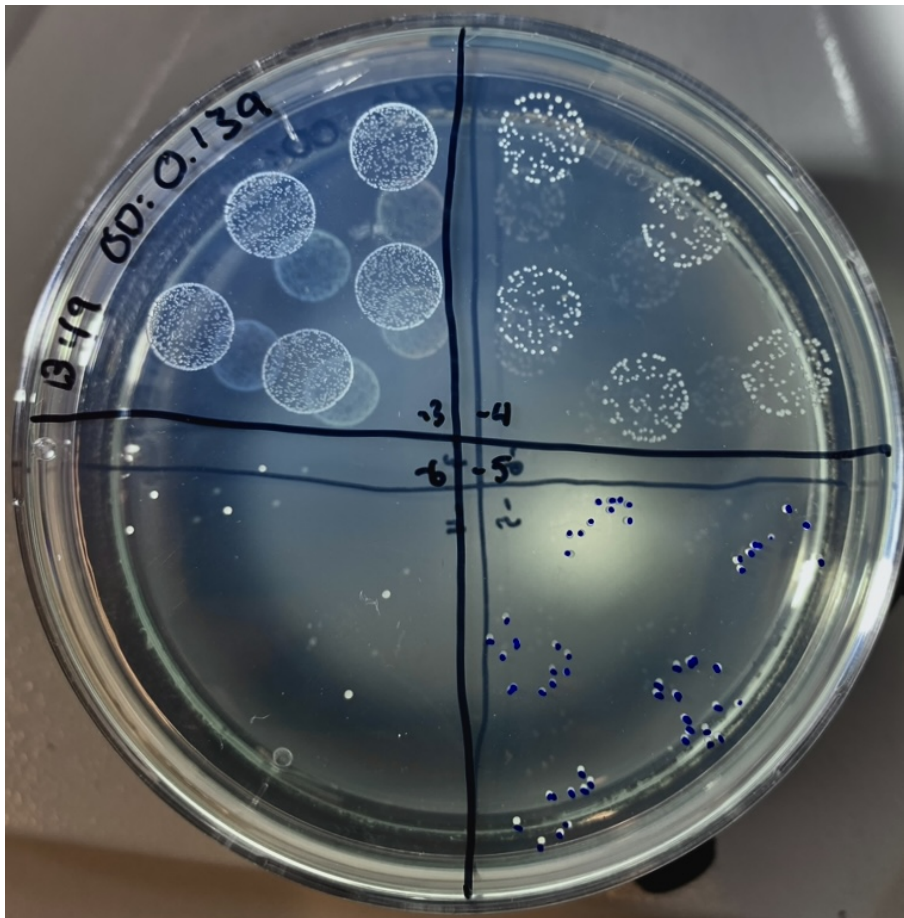


Figure 2.1: The replicate plate for strain B49 (*Lactococcus sp.*) at OD_{600} 0.139 as an example of the drop plate method, showing the setup of each plate with five drops of each of the four dilutions made for that specific OD_{600} replicate. This was done in three different OD_{600} replicates for all nine strains.

The CFU/mL was calculated for each replicate OD_{600} using the dilution quadrant which gave a CFU count of 3 - 35 per drop. Each of the five drops of the given dilution was considered an individual data point for that specific OD_{600} replicate. All three replicate plates were then used to generate a linear regression model for the relationship between OD_{600} and CFU/mL within the given OD_{600} range. These relationships were used in the bacterial association experiments described in Section 2.3 and Section 2.4 to add the correct volume of bacterial culture to the fish wells to achieve the target starting bacterial concentrations. For three of the bacterial isolates (3.78, B40, and B49) which initially gave inconsistent CFU counts between replicates, the experiment was

repeated with five replicate OD's instead of the original three. The regression curves for the relationship between OD_{600} and CFU/mL are given in Supplementary Figure S.1.

2.3 Gnotobiotic experiment in zebrafish (*Danio rerio*)

This experiment was performed at Institut de Biologie Intégrative et des Systèmes (IBIS) of Université Laval in Québec, Canada, in collaboration with professor Nicolas Derome and PhD candidate Lisa Zoé Auclert. Zebrafish embryos and fish lab facilities were kindly provided by Laboratoire Aquatique de Recherche en Sciences Environnementales et Médicales (LARSEM), also located at Université Laval. Training and supervision in gnotobiotic handling and germ-free derivation of zebrafish eggs was provided by Lisa Zoé Auclert.

2.3.1 Overview of experimental design

For this experiment, a total of 39 gnotobiotic cohorts, each consisting of 10-15 wild-type AB zebrafish, were established in 6-well plates to investigate the effects of host association with both mono-associations and a combination (SynCom) of bacterial strains originally isolated from salmon fry on host gene expression and the colonization success of these strains when presented with a novel host system.

An overview of the experimental design is given in Figure 2.2. Twelve different bacterial conditions were investigated: A mono-association for each of the nine bacterial strains, a SynCom condition consisting of equal amounts of all nine strains, a germ-free control condition, and a re-conventionalized control condition using rearing water from the zebrafish hatchery at LARSEM. Three replicate wells were established for each condition. Wells containing only the bacterial inoculum (for SynCom and re-conventionalized conditions) or sterile Zebrafish Gnotobiotic Medium (ZGM) (for the germ-free condition), but no fish, were made to represent the starting bacterial communities of these conditions (proxy wells).

Fertilized zebrafish eggs acquired through natural breeding were provided by LARSEM on the day of fertilization and immediately sterilized as described by Pham et al. [62], before being divided into cohorts and associated with the respective bacterial conditions (detailed descriptions are given in Section 2.3.2 and Section 2.3.3). The experiment was ended at 5 days post-fertilization (dpf). Mortality and hatching progression were monitored daily. Sampling for microbial community characterization and flow cytometry analysis were done at the start and end of the experiment. At the end of the experiment, fish were also sampled for the same purposes, as well as for host RNA extraction and first-strand cDNA synthesis to investigate host responses to the SynCom, germ-free, and re-conventionalized conditions.

2.3.2 Derivation of germ-free zebrafish embryos

The derivation of germ-free zebrafish eggs and the following bacterial association was based on the protocol of Pham et al. with some minor modifications [62], and was supervised and assisted by Lisa Zoé Auclert.

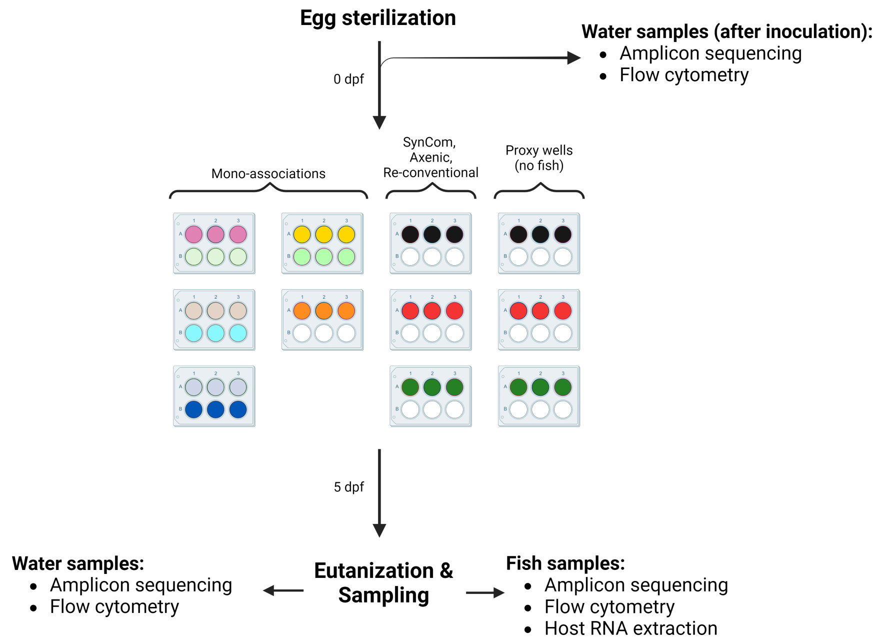


Figure 2.2: Overview of the experimental design for the zebrafish bacterial association experiment. Each well color represents a different bacterial condition. White wells are empty. Except for proxy wells, wells contained 10 - 15 sterilized zebrafish eggs. Created with Biorender.com.

Approximately 450 fertilized eggs were delivered from LARSEM on the day of the germ-free derivation. They were immediately rinsed twice in 50 mL Antibiotic Zebrafish Gnotobiotic Medium (AB-ZGM) (Appendix A) before being incubated at 26 °C in AB-ZGM in individual Petri dishes as described in Pham et al [62]. Following the antibiotic treatment, the eggs were rinsed twice in 50 mL ZGM (Appendix A) before being incubated in 0.1% poly(vinylpyrrolidone)–iodine (PVP-I) solution for $\leq$ 2 minutes. After this incubation, excess PVP-I solution was rinsed off with ZGM, before the eggs were transferred to a 0.003% bleach solution for $\leq$ 20 minutes. After the bleach incubation, the eggs were rinsed in large volumes of ZGM (>400 mL) to remove any excess bleach before being divided into cohorts of 10-15 eggs per well in six-well plates. The eggs were transferred using disposable sterile transfer pipettes, as these secure gentle handling and limit death due to shear forces [63]. Finally, the media volume was adjusted to 5 mL ZGM in each well. Sterility tests were performed by plating 100 µL of water from wells on TSA plates.

2.3.3 Bacterial association of zebrafish embryos

For the bacterial associations, bacterial cultures were prepared in TSB cultures as described in Section 2.2.1. Bacteria were added to the wells from a liquid culture in exponential phase. The target bacterial concentration in each well was 5 $\times 10^5$ CFU/mL. The OD_{600} was determined for each culture, and the relationships between OD_{600} and CFU/mL found in Section 2.2.1 were used to determine the volume of bacterial culture needed to reach the target bacterial concentrations in the wells. The resultant aliquots of the bacterial cultures were placed in eppendorf tubes and diluted to a final volume of 1 mL with ZGM. This dilute culture was then centrifuged at 8000×g for 7 minutes.

The supernatant was discarded to remove TSB media and the pellet resuspended in 1 mL ZGM. 1 mL of media was aspirated from each well and replaced with the bacteria-containing ZGM. To prepare the SynCom aliquote, one-ninth of the volume used for the mono-associations was added in the same eppendorf tube for all 9 strains. For the wells with the re-conventionalized condition, 1 mL of well media was replaced with 1 mL of parent rearing water.

2.3.4 Sample collection

Water samples for flow cytometry analysis were taken at the start and end of the experiment to quantify the bacterial densities of the different bacterial conditions. Samples of whole fish were also taken at the end of the experiment to quantify bacterial densities in skin mucus. For water samples, 100 µL were sampled in three replicates from each bacterial condition and fixated in 900 µL glutaraldehyde solution to a final glutaraldehyde concentration of 0.5%. These samples were then snap-frozen and stored at -80 ℃. Fish samples, also three replicates consisting of one individual from all conditions, were placed in 1 mL 0.5% glutaraldehyde solution, snap-frozen, and stored at -80 ℃.

For analyses of community compositions in the SynCom, re-conventionalized, and germ-free control conditions by 16S rRNA gene amplicon sequencing, water samples were taken at the start of the experiment, as well as water and fish samples at the end of the experiment. The proxy wells were sampled to represent the starting bacterial communities of these conditions. To collect the bacteria in the water, each well's whole volume (5 mL) was sampled in 1 mL batches. These were then centrifuged sequentially at 20000 × g for 10 minutes in the same tube, and the supernatant was removed after each run. The resulting pellet was then snap-frozen in 50 µL of Nucleic Acid Preservation buffer (NAP) prepared according to Camacho-Sanchez et al. [64], and stored at -80 ℃ . At the end of the experiment, four replicate samples, each consisting of three individual fish from replicate wells, were taken from the Syncom, re-conventionalized, and germ-free conditions. These were placed in 1 mL NAP buffer, snap-frozen, and stored at -80 ℃. The remaining water of these wells were also sampled as described for the proxy wells.

Fish samples were taken for RNA extraction and subsequent gene expression analysis at the end of the experiment to investigate host responses to the bacterial colonization. Four replicate samples consisting of three individuals were taken from the SynCom, germ-free, and re-conventionalized conditions, placed in 1 mL TRIzol™ (Invitrogen) and kept at 4 ℃ for one day before further processing as described in Section 2.8. An overview of the samples collected from the zebrafish gnotobiotic experiment is given in Table 2.2.

Table 2.2: Overview of water and fish samples collected in the zebrafish gnotobiotic experiment. The number of replicates of each sample type is given. For the mono-associations, the number of replicates given is for each of the nine conditions. **FC start/end** = Samples for flow cytometry analyses collected at the start or end of the experiment. **16S start/end** = Samples for 16S rRNA gene amplicon analyses collected at the start or end of the experiment. **RNA extraction** = Samples collected for RNA extraction and subsequent gene expression analyses

	FC start		**FC end**		**16S start**		**16S end**		**RNA extraction**	
	Water	Fish	Water	Fish	Water	Fish	Water	Fish	Water	Fish
X9 Mono-associations	3	-	3	3	-	-	-	-	-	-
SynCom	3	-	3	3	4	-	3	4	-	4
Germ-free control	3	-	3	3	4	-	3	4	-	4
Re-conventionalized	3	-	3	3	4	-	3	4	-	4

2.4 Gnotobiotic experiment in Atlantic salmon yolk-sac fry (*Salmo salar*)

The bacterial association experiment with Atlantic salmon yolk-sac fry was performed at ACMS' facilities at NTNU Trondheim, with salmon embryos acquired from Aquagen. Assistance and training in germ-free derivation of Atlantic salmon eggs was kindly provided by Eirik Degré Lorentsen and Amalie Johanne Horn Mathisen of ACMS.

2.4.1 Overview of experimental design

An overview of the experimental design is given in Figure 2.3. The same twelve bacterial conditions that were investigated for zebrafish were also used for the association experiment with the salmon yolk-sac fry host. For the re-conventionalized control condition, rearing water from SINTEF SeaLab's salmon rearing facilities was used. After deriving the eggs germ-free as described in Section 2.4.2, and subsequent hatching in cell culture flasks, individuals were transferred to 6-well plates. 60% media exchanges were performed every second day in the flasks containing the hatching eggs. Six replicate wells were established for each mono-association condition and twelve for the remaining SynCom, germ-free, and re-conventionalized conditions. Each replicate well contained one individual host.

The experiment was ended at 13 days post-hatching (dph). Sampling for microbial community characterization and flow cytometry analysis were done at the start and end of the experiment. At the end of the experiment, fish were also sampled for the same purposes, as well as for host RNA extraction and first-strand cDNA synthesis to investigate host responses to the SynCom, germ-free, and re-conventionalized conditions. All work with salmon yolk-sac fry was done in a dark-room cold lab set at 8 °C.

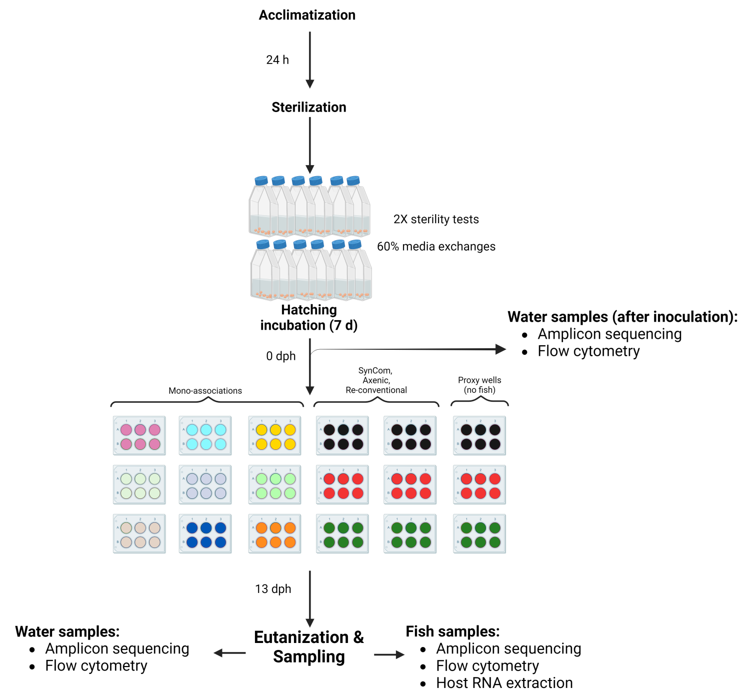


Figure 2.3: Overview of the experimental design for the salmon yolk-sac fry bacterial association experiment. Each well color represents a different bacterial condition. White wells are empty. Except for proxy wells, wells contained one yolk-sac fry individual. Created with Biorender.com.

2.4.2 Germ-free derivation of Atlantic salmon eggs

The germ-free derivation of Atlantic salmon eggs and the following bacterial associations were based on the protocol developed at ACMS by Gómez de la Torre Canny and Fiedler (Appendix B). Fertilized eggs received from Aquagen were kept in a dark-room at all times. They were immediately transferred to a petri dish (13.5 cm Ø) upon arrival and covered in sterile Salmon Gnotobiotic Medium (SGM) for acclimatization (24 hours) (Appendix A). Following this, media was exchanged with Antibiotic Salmon Gnotobiotic Medium (AB-SGM) for antibiotic incubation (24 hours) (Appendix A). On day 3 after arrival, eggs were transferred to 50 mL conic vials in groups of eleven. These were filled with Buffodine solution containing 50 mg/L available iodine for a thirty-minute incubation. Buffodine was then decanted, and eggs were rinsed four times in 50 mL SGM, before being transferred to250 mL cell culture flasks with vented caps (eleven eggs per flask). The described steps were all performed by Eirik Degré Lorentsen and Amalie Johanne Horn Mathisen. Flasks were filled with 100 mL SGM, and 60% media exchanges were performed every second day until hatching.

Sterility tests were performed before and after hatching for all flasks, and included inoculation of brain-heart infusion media, Saboraud dextrose broth, nutrient broth, glucose yeast medium, and two TSA plates with 100 µL SGM hatching media. Any flasks showing growth in any sterility control media were removed from the experiment. 3 dph, salmon yolk-sac fry were transferred to 6-well plates containing 10 mL SGM per well using stringent sterile technique. Each well contained one individual.

2.4.3 Bacterial association of salmon yolk-sac fry

The bacterial associations for salmon yolk-sac fry were performed as described previously for zebrafish in Section 2.3.3.

2.4.4 Sample collection

Samples for flow cytometry and RNA extraction/cDNA-synthesis were collected as described previously for zebrafish in Section 2.3.4, except that only one individual was used per replicate fish sample. Samples for 16S rRNA gene amplicon sequencing however, were sampled in accordance with established routines at ACMS. Four replicate water samples were taken from each of the SynCom, germ-free, and re-conventionalized conditions at the start and end of the experiment, as well as four replicates of fish for each condition after the experiment. For the water samples, the total 10 mL of rearing media (SGM) in a well were aspirated with a syringe and filtered through a Whatman® Nuclepore™ 0.2 µm Track-Etched membrane. The filtrate was discarded, and the filter was stored at - 20 °C for subsequent DNA extraction, described in Section 2.6.1. Fish samples for amplicon sequencing were collected in the same way for salmon yolk-sac fry as for zebrafish, previously described in Section 2.3.4. An overview of the samples collected from the salmon yolk-sac fry gnotobiotic experiment is given in Table 2.3.

Table 2.3: Overview of water and fish samples collected in the salmon yolk-sac fry gnotobiotic experiment. The number of replicates of each sample type is given. For the mono-associations, the number of replicates given is for each of the nine conditions. **FC start/end** = Samples for flow cytometry analyses collected at the start or end of the experiment. **16S start/end** = Samples for 16S rRNA gene amplicon analyses collected at the start or end of the experiment. **RNA extraction** = Samples collected for RNA extraction and subsequent gene expression analyses

	FC start		FC end		16S start		16S end		RNA extraction	
	Water	Fish	Water	Fish	Water	Fish	Water	Fish	Water	Fish
X9 Mono-associations	3	-	3	3	-	-	-	-	-	-
SynCom	3	-	3	3	4	-	4	4	-	4
Germ-free control	3	-	3	3	4	-	4	4	-	4
Re-conventionalized	3	-	3	3	4	-	4	4	-	4

2.5 Optimizing DNA extraction for zebrafish and salmon yolk-sac fry tissue samples

As part of another master project being conducted at ACMS concurrently with the work of this thesis, protocols for extracting bacterial DNA from adult Atlantic salmon skin tissue samples were being optimized, specifically focusing on the first lysis step [65]. The main finding in this work was that vortexing of the tissue sample in lysis buffer followed by an incubation period before removing tissue and proceeding with lysis without host tissue present gave the best results. The lysis buffer provided in the ZymoBIOMICS™ kit was used. To test if this also applied for zebrafish and salmon yolk-sac fry samples, one replicate of each of the bacterial conditions (SynCom, germ-free, reconventionalized) for both host species (six samples in total) were subjected to the following lysis treatment:

- Tissue samples were transferred to lysis tubes containing lysis buffer (600 µL).
- The NAP buffer which tissue samples had been stored in was centrifuged at 10 000 × *g* for five minutes. The supernatant was removed, and the pellet resuspended in 150 µL lysis buffer, which was added back to the lysis tube containing the corrresponding fish tissue sample.
- Samples were vortexed for two minutes, followed by a thirty minute incubation and another two minute vortexing.
- The lysis buffer was then split in equal parts between two lysis tubes, keeping the host tissue in one of them. Fresh lysis buffer was added in both tubes to a total volume of 750 µL.
- Approximately 50 µL 0.1 mm glass beads (Bertin Technologies) were added to all samples. Lysis was performed by bead beating in a Precellys® tissue homogenizer at 5500 rpm in two 30-second cycles.

Lysates were then further processed in the KingFisher™ Flex Purification System as described in Section 2.6.1. The resulting DNA extracts were used as templates in a 16S rRNA gene targeted PCR as described in Section 2.6.2 both in the original concentration, and in 1:10 dilutions. The obtained PCR products were evaluated by gel electrophoresis.

2.6 Library preparation for 16S rRNA gene amplicon sequencing

To investigate the development of the bacterial community compositions in the SynCom, germ-free and re-conventionalized conditions, a DNA library with the isolated 16S rRNA gene target sequences was prepared. This was then sent to the Norwegian Sequencing Centre (NSC) for Illumina sequencing. In the following subsections, the steps in preparing the DNA library are described.

2.6.1 DNA extraction

DNA extraction was done for all water and fish samples prepared for 16S rRNA gene amplicon sequencing analysis (see Table 2.2 and Table 2.3) with the ZymoBIOMICS ™ 96 MagBead DNA kit (Appendix C). The supplier's kit protocol was followed with some deviations. All samples were lysed using Precellys® tissue Homogenizer with approximately 50 µL 0.1 mm glass beads (Bertin Technologies) at 5500 rpm in two 30-second cycles using the lysis buffer (750 µL) provided in the ZymoBIOMICS™ kit.

For the zebrafish water samples, the entire sample volume (50 µL) was added to the lysis buffer before lysing in the Precellys® tissue Homogenizer. The zebrafish fish tissue samples were thawed, and individuals were transferred from their storage NAP buffer to the lysis buffer, where lysis was performed with host tissue, as this lysis method was evaluated as the most optimal for zebrafish tissue samples in Section 2.5. The salmon water samples consisted of a Whatman ® Nuclepore ™ Track-Etched membrane filter. These were transferred directly to the lysis buffer, and homogenization was executed with the membrane filter present. For the salmon fish tissue samples, tissue was transferred to the lysis buffer and vortexed for two minutes, incubated, and then removed, as this lysis method was evaluated as the most optimal for salmon yolk-sac fry tissue samples in Section 2.5. After lysis homogenization, all samples were centrifuged at 10 000 $\times$ g for 1 minute, and 200 µL of the supernatant was used for DNA extraction using the KingFisher™ Flex Purification System. 50 µL of ZymoBIOMICS ™ MagBinding Beads were used for the binding step. The remaining steps were performed as described in the kit protocol (Appendix C), giving a 50 µL eluate of nuclease-free water containing the extracted DNA.

2.6.2 Targeted PCR

The DNA extracts from Section 2.6.1 containing the extracted DNA were used as templates for the targeted PCR amplifications of the v3+v4 region of the bacterial 16S rRNA gene, using broad coverage primers Ill-341F-KI and Ill805R, which consists of a gene targeting sequence and an Illumina adapter sequence (Table 2.4). 1 µL of DNA template, 1X Phusion buffer HF, 200 µM of each dNTP, 0.02 units/µL Phusion Hot-Start DNA Polymerase (all from Thermo Scientific™), and 0.16 µM of each primer (Sigma-Aldrich) made up each PCR in a total volume of 25 µL. PCR cycling conditions are given in Table 2.5. A negative control from the DNA extraction step, as well as a non-template and positive PCR control, was included. The resulting PCR products were checked for quality and size by gel electrophoresis before continuing with normalization.

Table 2.4: Sequences of the Ill-341-KI and Ill-805R broad coverage primers used for amplifying the v3+v4 region of the 16S rRNA gene in the targeted PCR. Gene targeting sequences are marked in bold.

Primer	Sequence	Target region
Ill-341-KI	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN **CCT ACG GGN GGC WGC AG**-3'	v3
Ill-805R	5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN **GAC TAC NVG GGT ATC TAA KCC**-3'	v4

Table 2.5: Cycling conditions for the 16S rRNA gene PCR amplification

PCR step	Time	Temperature [°C]	Cycles
Denaturation	2 min	98	1X
Denaturation Annealing Elongation	15 sec 20 sec 20 sec	98 56 72	38X
Elongation	5 min	72	1X
Cooling	∞	4	1X

2.6.3 Normalization of targeted PCR products

PCR products from the targeted PCR were normalized and purified using the Sequal-Prep ™ Normalization Plate kit from Invitrogen™ according to the kit protocol (Appendix D). This process step ensures that the input concentration of the amplified DNA from the targeted PCR is similar across all samples before the indexing PCR (1 – 2 ng/µL).

2.6.4 Indexing PCR

Before sending the library for sequencing, an indexing PCR was performed on the normalized PCR products. This is done so that samples are distinguishable after being pooled and sequenced. Each sample's normalized PCR product is tagged with a unique combination of forward and reverse sequence indexes, which are added to the PCR products in this step. The Nextera XT DNA Library Preparation Kit A (Illumina™), which consists of a set of indexing primers that allow for up to 96 unique index sequence combinations, was used. For all samples, a PCR consisting of 2.5 µL of normalized template, 1X Phusion buffer HF, 200 µM of each dNTP, 0.02 units/µL Phusion Hot-Start DNA Polymerase (all from Thermo Scientific™), and 2.5 µL of each indexing primer was performed in a total volume of 25 µL, using a unique combination of primers for each sample. PCR cycling conditions are given in Table 2.6. The same controls that were used for the targeted PCR (Section 2.6.2) were included, and the resulting indexed PCR products were again checked for size and quality by gel electrophoresis before continuing with the final normalization.

Table 2.6: Cycling conditions for the Indexing PCR amplification

PCR step	Time	Temperature [°C]	Cycles
Denaturation	2 min	98	1X
Denaturation	15 sec	98	
Annealing	20 sec	55	10X
Elongation	20 sec	72	
Elongation	5 min	72	1X
Cooling	∞	4	1X

2.6.5 Second normalization and pooling of samples

The indexed PCR products were normalized using the SequalPrep ™ Normalization Plate kit as described in Section 2.6.3). The normalized samples were subsequently pooled in a single tube and concentrated with the Amicon® Ultra Centrifugal Filter (Millipore™) according to the accompanying protocol (Appendix E). In addition, a washing step with 500 µL TE-buffer was performed twice after concentration by centrifuging the sample with buffer at 14 000 x g for 10 minutes in the centrifugal filter column. DNA concentration of the final sample was measured in a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific™), and the sample was assessed for purity by gel electrophoresis. The final pooled library was sent to NSC for sequencing by the Illumina MiSeq v3 plattform.

2.7 Data processing and statistical analysis of amplicon sequencing data

2.7.1 Data processing

The amplicon sequencing data was processed using the pipeline **USEARCH v.11** [66]. Due to time limitations, the amplicon sequencing data processing was performed by Ingrid Bakke. The command “merge_pairs” was used to merge forward and reverse reads, trim off primer sequences, and discard merged sequences shorter than 380 bp (base pairs). The merged sequences were quality filtered using the command “fastq_filter” with an expected error threshold of 1. The “unoise3” command was applied to generate ASVs [67]. This step included chimera removal. As recommended in the documentation for “unoise3”, all sequence variants with an abundance less than 8 reads in the total data set were discharged. The “sintax” command [68], together with the RDP trainset reference dataset (v18), was applied to assign taxonomy to the ASVs. Finally, an ASV table was made using the command “otutab”.

The resulting ASV table was inspected manually in Microsoft Excel. All ASVs (9 in total) found in negative controls for DNA extraction and PCR were removed from the ASV table, with the exception of a few ASVs that had very low abundances (maximum 10 reads) in negative controls, but were abundant in the samples. Most of the 9 ASVs removed represented taxa typically associated with human skin (Cutibacterium, Corynebacterium) or water systems (e.g. Acidovorax). In addition, four low-abundant ASVs representing chloroplasts, and one representing the eukaryotic genus *Zea*, were

removed. Next, the ASV table was rarified to 4750 reads per sample by using the command “otutab_rare”. As the sequencing depth varied greatly between samples (7 to 274608 reads), this led to the exclusion of 8 samples which had less than 4750 reads. This applied to 3 water samples obtained from assumed germ-free rearing conditions in the zebrafish experiment, as well as a water sample from the re-conventionalized condition. From the salmon yolk-sac fry experiment, two water samples and one fish tissue sample from the assumed germ-free condition were excluded, as well as a fish tissue sample from the re-conventionalized condition (see Supplementary Table S.1).

For samples collected from the SynCom condition in the salmon experiment, a large number of highly similar ASVs representing the genera that had been included in the synthetic community were identified. The sequencing data for these samples were therefore re-processed using the command “cluster_otus” to perform OTU clustering at a 97% pairwise sequence identity level. An OTU table was made using the “otutab” command, and taxonomy was assigned to the representative sequences for the ASVs using the “sintax” command together with the RDP trainset v18. The OTU table was rarefied to 18300 reads per sample using the command “otutab_rare”.

Finally, the command “sintax_summary” was applied to both the rarified ASV and the SynCom OTU table to generate tables showing the community composition at different taxonomic levels (phylum, class, order, family, and genus). These tables were used for further analyses.

2.7.2 Statistical analysis

β-diversity analyses were performed on the salmon yolk-sac fry SynCom samples, based on the OTU table obtained for these samples from processing of the amplicon sequencing data. Bray-Curtis similarities were calculated using PAST (v4.10) [69] and a Principal Coordinate Analysis (PCoA) ordination plot of two dimensions was generated based on Bray-Curtis similarities for visualization of β-diversity in the SynCom sample groups. PCoA is an analysis method where each sample is fitted in a multidimensional space with as many coordinates as there are samples, and distances between the sample points in the multidimensional space reflects the similarity between samples. The further apart two samples are, the less similar. Then, the two coordinates which contributes most to the variation between samples, the principal coordinates, is used, and the sample point’s positions are projected on these two coordinates, resulting in a two-dimensional plot which can reveal information about the relationship of the sampled bacterial communities within and between different sample groups.

To see whether the community compositions of the three sample groups from the SynCom condition in the salmon yolk-sac fry experiment were significantly different, the one-way PERMANOVA (permutational multivariate analysis of variance) tool in PAST was used, based on Bray-Curtis similarities.

Based the rarefied ASV table obtained after processing of the amplicon sequencing data, the four α-diversity indices Chao-1, observed ASV richness, Shannon’s diversity index, and evenness were calculated for all remaining samples from the re-conventionalized conditions in both gnotobiotic experiments using PAST. The observed ASV richness is the total number of ASVs detected in a sample. Chao-1 is a theoretical estimate of what the total ASV richness in a sample would be if sequencing depth was infinite. The estimate is based on the prevalence of singleton and doubleton reads in the queried sample [70]. Evenness is a measure of the ASVs equitability in a sample.

Shannon's diversity index *(H)* considers both the richness and evenness of a sample. Due to the nonlinearity of the Shannon diversity index, the exponential Shannon diversity index *(e^H)* was used as recommended by Lucas et al. [71].

To assess if the observed α-diversities in the re-conventionalized sample groups were significantly different, an F-test (significance level = 0.05) to assess differences in variability was performed. Based on the result of the F-test, pairwise t-tests (significance level = 0.05) assuming either equal or different variance was performed both between sample groups from within a specific host species experiment (start water vs. end water, start water vs. fish, end water vs. fish) and for the same sample group obtained from the two different different host species experiments (start water zebrafish vs. start water salmon yolk sac-fry, etc.).
β-diversity in the re-conventionalized conditions were also investigated as described for the salmon yolk-sac fry SynCom condition. To determine the ASVs specific contribution to the observed differences between sample groups, the Similarity Percentage Analysis (SIMPER) tool in PAST was used, based on Bray-Curtis similarities.

2.8 Host RNA extraction and cDNA synthesis

RNA extraction from host tissues was done for four replicate samples for each of the SynCom, re-conventionalized, and germ-free conditions using TRIzol™ and subsequent phenol-chloroform RNA extraction as described in the TRIzol™ reagent user guide (Appendix F). This was done for both zebrafish and salmon yolk-sac fry samples. A replicate consisted of three individuals for zebrafish samples and one individual for salmon yolk-sac fry samples. Nucleic acid concentration was determined for each sample using a NanoDrop UV-Vis spectrophotometer before first-strand cDNA synthesis was performed using qScript® cDNA SuperMix in accordance with the supplied manual (Appendix G).

2.9 Flow cytometry data processing

Water samples obtained from both gnotobiotic experiments (see Table 2.2 and Table 2.3) were shipped to **KYTOS**, who performed the flow cytometry analysis. Samples were analyzed on an Attune NxT flow cytometer equipped with a blue (488 nm, 50 mW) and violet (405 nm, 50 mW) laser. Water samples were diluted in Instant Ocean and stained with SybrGreen according to KYTOS protocols for analysis of bacterial densities in water samples.

Raw data received from KYTOS was further processed at NTNU in AttuneTM Cytometric Software (v5.1.1) by Amalie Johanne Horn Mathisen. The quality of the single files was inspected by plotting Counts versus Time and Green Fluorescent (BL1-H, 530/30 nm) versus Time. A gate on Counts versus Time was set to filter out unstable measurements. Red Fluorescent (BL3-H, 695/40 nm) versus BL1-H was plotted to distinguish the background noise from the bacterial cells. Lastly, the bacterial populations were identified by plotting BL1-H versus Side Scatter (SSC-H). The obtained data was then further processed in Microsoft Excel to estimate the bacterial density of the rearing water and fish tissue samples.

3 Results

3.1 Growth characteristics of bacterial strains

Nine bacterial strains which had previously been isolated from the gut of Atlantic salmon fry was selected for the gnotobiotic experiments in this thesis. The goal of this experiment was to determine basic growth characteristics for the strains at temperatures relevant for rearing zebrafish and Atlantic salmon yolk-sac fry, before applying them in the gnotobiotic host models. Growth measurements at 22 ℃ were done in a Tecan Spark® 20M Microplate Reader. For the growth measurements that was to be performed at 10 ℃ a BioLector®Pro micro-bioreactor system (m2p Labs) was used. However, datalogs revealed that although the equipment was set to 10 ℃, temperature unexpectedly increased throughout the experiment. Because of this, the actual average temperature of the experiment in the time-frame at which measurements for determining the μ_{max} was made, was 14.4 ℃. The resultant growth curves are given in the Supplementary Materials.

The mean maximum growth rate (μ_{max}) of three replicates of each individual strain at the two different temperatures was determined from measurements made in the exponential growth phase, as described in Section 2.2. The mean μ_{max} values are given in Table 3.1, together with the mean minimum doubling time and the time spent in lag phase for each bacterial strain. Strain 3.11 *(Hafnia sp.)* had the highest μ_{max} at both temperatures, and strain 3.3 *(Sphingomonas sp.)* the lowest. The reduced culturing temperature affected the strains differently, although all nine strains showed a significant decrease in μ_{max}. The strain with the greatest decrease in μ_{max} was strain B40 *(Staphylococcus sp.)* (90.0%). Strain 3.28 *(Microbacterium sp.)* was least affected by the reduced temperature in terms of μ_{max} , with an average decrease of 54.3 % (Figure 3.1).

Table 3.1: Mean μ_{max} and minimum doubling time with standard deviations, and time spent in lag phase for the nine bacterial strains at 22 and 14.4 ℃.

	22 ℃			14.4 ℃		
	μ_{max} (h^{-1})	Doubling time (h)	Lag phase (h)	μ_{max} (h^{-1})	Doubling time (h)	Lag phase (h)
3.28 *(Microbacterium sp.)*	0.201 ± 0.003	3.5 ± 0.04	9	0.092 ± 0.010	7.6 ± 0.76	16
3.67 *(Arthrobacter sp.)*	0.204 ± 0.009	3.4 ± 0.15	8	0.094 ± 0.003	7.4 ± 0.20	12
3.24 *(Micrococcus sp.)*	0.081 ± 0.010	8.7 ± 0.96	14	0.036 ± 0.004	19.5 ± 2.14	42
3.11 *(Hafnia sp.)*	0.326 ± 0.005	2.1 ± 0.03	3	0.114 ± 0.008	6.1 ± 0.39	8
3.78 *(Psychrobacter sp.)*	0.217 ± 0.001	3.2 ± 0.02	7	0.080 ± 0.003	8.7 ± 0.38	14
B40 *(Staphylococcus sp.)*	0.230 ± 0.012	3.0 ± 0.16	8	0.023 ± 0.010	38.2 ± 19.9	43
B49 *(Lactococcus sp.)*	0.214 ± 0.006	3.2 ± 0.09	4	0.032 ± 0.001	21.5 ± 0.93	11
3.18 *(Carnobacterium sp.)*	0.245 ± 0.001	2.8 ± 0.01	4	0.059 ± 0.007	11.9 ± 1.41	10
3.3 *(Sphingomonas sp.)*	0.067 ± 0.008	10.4 ± 1.26	17	0.009 ± 0.001	78.1 ± 4.62	29

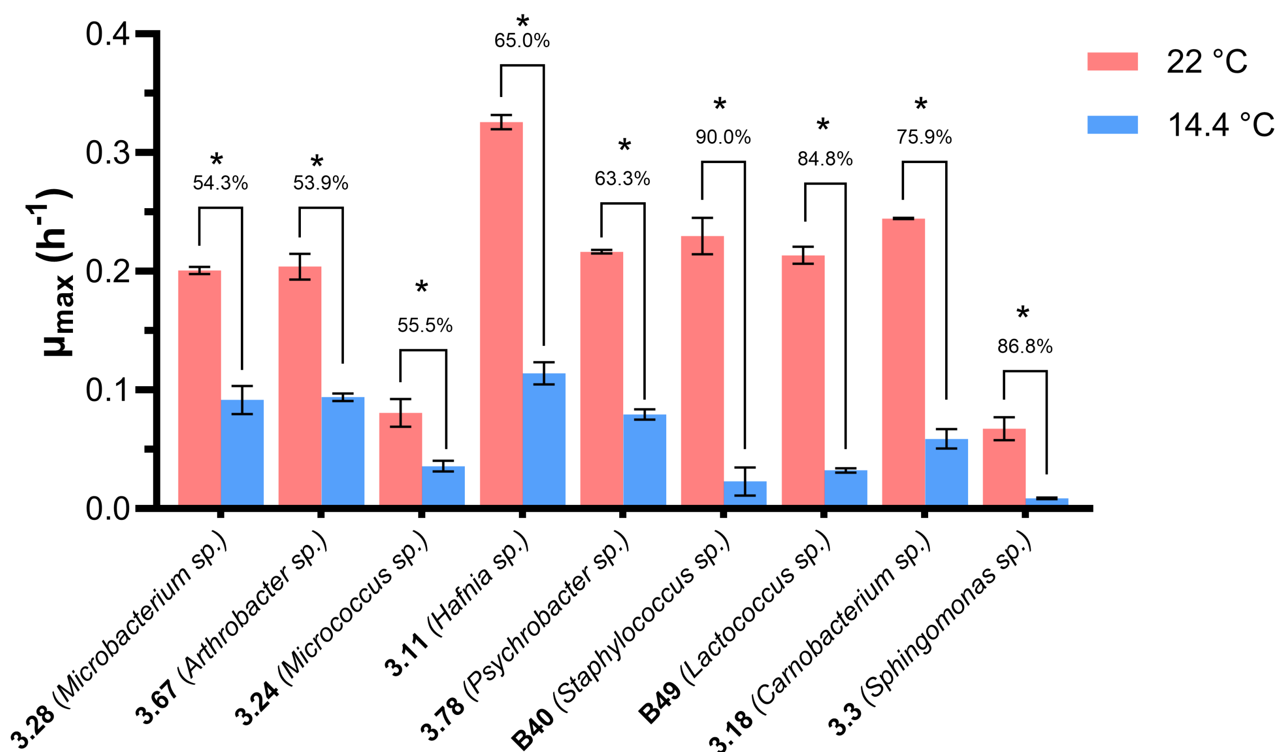


Figure 3.1: Comparison of the mean μ_{max} determined for each strain at 22 and 14.4 ℃. Means were calculated from three replicate cultures for each strain at both temperatures. Error bars indicate the standard deviation of the calculated means. The percent decrease for each strain is shown. Asterisks indicate a significant difference in μ_{max} between the two experiments (two-way t-test, $p = 0.05$).

3.2 Optimization of DNA extraction protocols for zebrafish and salmon yolk-sac fry tissue samples

Achieving consistent PCR amplification of bacterial 16S rRNA genes from salmon yolk-sac fry tissue samples had been a challenge in previous projects in the ACMS group, probably due to low amounts of bacterial DNA compared to the amount of host DNA, and presence of PCR inhibitors in DNA extracts from such samples. Because of this, optimization trials were performed to investigate if the presence of salmon yolk-sac fry tissue in the lysis steps prior to DNA extraction had an effect on PCR outcome. Since there was little experience within the group with zebrafish tissue samples, fish tissue samples from the gnotobiotic experiment in zebrafish was also included in the trials.

Two main conditions were tested for both host samples; homogenization with or without host tissue present in the bead beating lysis step. The obtained DNA extracts were used as templates in a 16S rRNA gene targeted PCR either in their original concentrations, or in 1:10 dilutions. For zebrafish, the best PCR outcome was obtained with the undiluted DNA extracts from samples where host tissue had been included in the homogenization step (Figure 3.2). For salmon yolk-sac fry, undiluted DNA extracts of samples where host tissue had been excluded from the homogenization step gave the best PCR outcome (Figure 3.2). Salmon samples which were homogenized with the host tissue included, resulted in amplification of unspecific PCR products. Thus, salmon yolk-sac fry samples was homogenized without host tissue, and zebrafish samples with

host tissue, for the preparation of the 16S rRNA gene amplicon library.

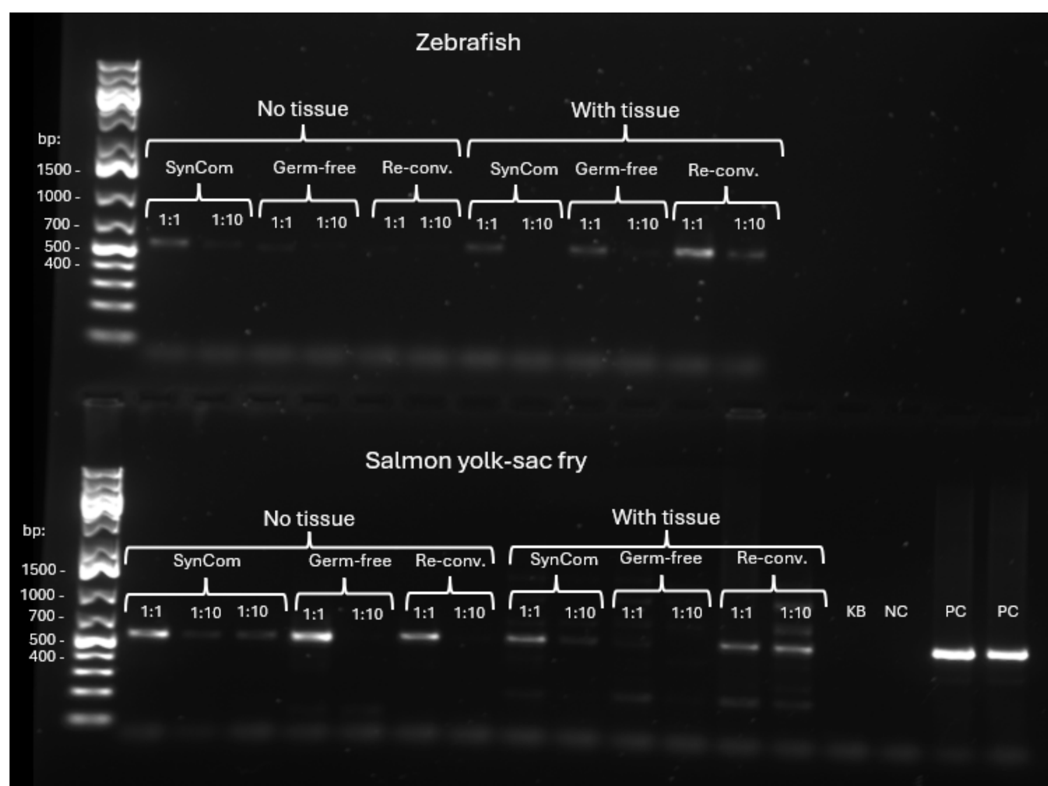


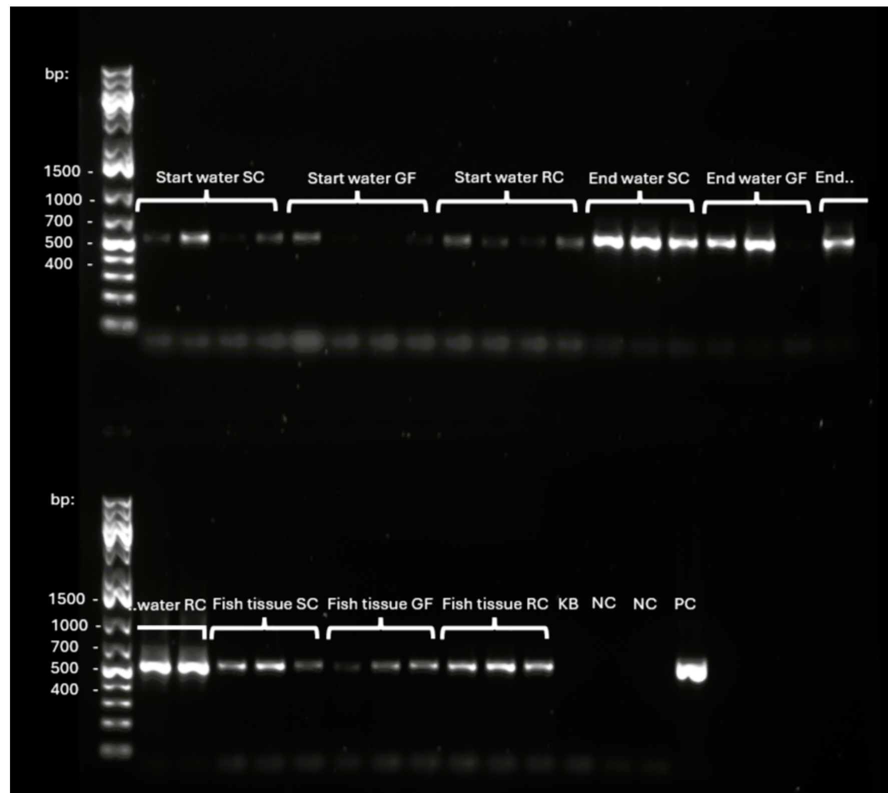
Figure 3.2: Agarose gel showing PCR products of the v3+v4 region of the 16S rRNA gene obtained from the DNA extraction optimization trials on zebrafish and salmon yolk-sac fry tissue samples. For each of the two host species, one fish tissue sample obtained from each of the SynCom, germ-free, and re-conventionalized conditions were used. Bead beating was performed either with or without host tissue, and DNA extracts were used as templates in a targeted PCR amplification reaction in either the original concentration (1:1) or in a 1:10 dilution. PCR reaction conditions are detailed in Section 2.6.2.

3.3 Preparation of 16S rRNA gene amplicon library

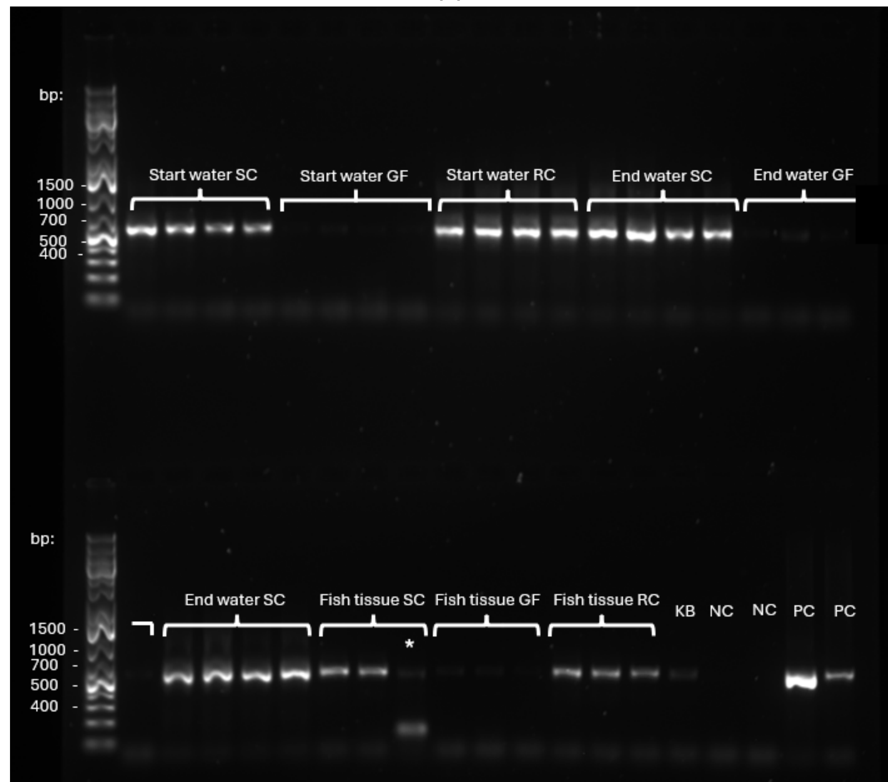
To characterize the water and fish microbiota in the SynCom, germ-free, and re-conventionalized conditions in the two gnotobiotic experiments, the v3+v4 region of the bacterial 16S rRNA gene was amplified and sequenced. DNA extracts from water samples taken at the start and end of the experiments, as well as from fish tissue samples, were used as template in the first targeted PCR (Figure 3.3). PCR products of the expected size of approximately 540 bp were obtained for all samples. For one salmon yolk-sac fry fish tissue sample obtained from the SynCom condition, prominent amplification of unspecific products were observed (Figure 3.3b). PCR products were also observed for several germ-free zebrafish samples, both from water and fish tissue (Figure 3.3a). This indicated that a bacterial contamination potentially was present in these samples.PCR products were not observed for the negative controls for DNA extraction or for non-template PCR controls.

After normalization and purification of the 16S rRNA gene amplicons, a second PCR was performed to add sample-specific indexes to the amplicons. Examination of the resultant PCR products by gel electrophoresis revealed that amplification was less successful than expected for some samples, and also indicated amplification of unspecific products and/or primer-dimers (Supplementary Figure S.2). Therefore, the indexing

PCR was repeated with twelve PCR cycles. This gave more PCR product of the expected size, but also an increased amplification of the undesired products (gel image not shown). Therefore, the first indexed amplicons from the ten-cycles indexing PCR were used to generate the amplicon library which was sent for sequencing. The final DNA concentration obtained for the pooled amplicon library as measured on a NanoDrop UV-Vis spectrophotometer was 31.7 ng/µL (A260/A280: 2.08) (Figure 3.4).



(a)



(b)

Figure 3.3: Agarose gels showing PCR products of the v3+v4 region of the 16S rRNA gene obtained from the targeted PCR in the amplicon library preparation. (a) shows zebrafish and (b) salmon yolk-sac fry samples. DNA extracts from water samples taken at the start and end of the experiments, as well as fish tissue samples were used as template in the PCR. PCR reaction conditions are detailed in Section 2.6.2. The sample indicated with an asterisk in b) showed prominent amplification of unspecific products. **SC** = SynCom, **GF** = germ-free, **RC** = re-conventionalized, **KB** = DNA extraction kit blank control, **NC** = non-template PCR control, **PC** = Positive PCR control.

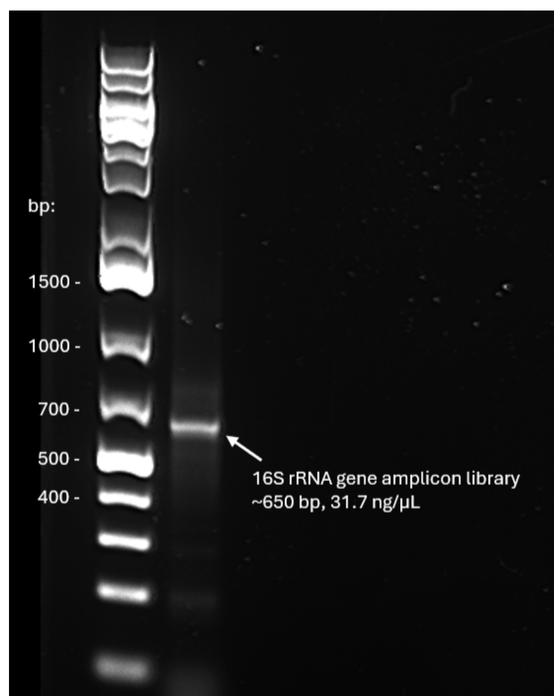


Figure 3.4: Agarose gel electrophoresis of the pooled, indexed 16S rRNA gene amplicon library which was sent to NSC for sequencing.

3.4 Zebrafish gnotobiotic experiment

To investigate the effects of bacterial associations on the zebrafish host at the gene expression level, and the colonization success of the SynCom strains when presented with a novel host system, a gnotobiotic experiment was performed with zebrafish. After the derivation of germ-free eggs, sterility tests were performed on TSA plates, which indicated that the derivation had been successful. Zebrafish were inoculated with mono-associations of the individual bacterial strains, a SynCom consisting of all nine strains, and a re-conventionalized condition using rearing water from the LARSEM zebrafish rearing facilities. In addition, a germ-free control condition was kept. Directly after the germ-free derivation, a mortality of 11.8% was registered for the eggs. Distinguishing between non-viable, unfertilized eggs and eggs where the derivation procedure itself induced mortality proved to be difficult. No further mortalities were registered after inoculation with the different bacterial conditions. Hatching progression proceeded similarly for all conditions, and after 3 dpf, all eggs were hathced.

RNA was successfully extracted for gene expression analysis from fish samples of the SynCom, re-conventionalized, and germ-free control conditions. Yield and purity of the extracted RNA varied between sample, but was generally of acceptable quality, with an average concentration of 26.3 ± 10.5 ng/µL (Supplementary Table S.2). The RNA extracts were then used to perform first-strand cDNA synthesis, which was to be analysed by a cDNA microarray. The cDNA was sent to the Environmental Genomics Facility at University of Windsor (Canada), a cooperation facilitated by IBIS. Unfortunately, the gene expression data were not returned in time for it to be included in this work.

Bacterial densities were determined for three replicate wells of each bacterial condition by flow cytometry (Table 3.2). At the start of the experiment, bacterial densities varied from between $1.09 \pm 0.816 \times 10^4$ bacteria/mL for the germ-free condition to $38.3 \pm$

3.38×10^4 bacteria/mL for the re-conventionalized condition. The bacterial condition with the lowest bacterial density at the end of the experiment was the mono-association with strain 3.28 *(Microbacterium sp.)* with $4.88 \pm 0.910 \times 10^4$ bacteria/mL, whilst 3.18 *(Carnobacterium sp.)* had the highest ($8757.9 \pm 1565.2 \times 10^4$ bacteria/mL. The end/start ratios of bacterial densities in the bacterial conditions indicated that the bacterial density increased in all conditions except for the B49 *(Lactococcus)* mono-association.

Table 3.2: Bacterial densities in water at the start and end of the zebrafish gnotobiotic experiment, and the end/start ratio of bacterial densities for each of the bacterial conditions in the experiment as determined by flow cytometry. The table shows the mean of samples taken from three replicate wells plus-minus the standard deviation. End/start ratio indicates the ratio between the determined end and start densities. Samples were diluted 1:10 and fixated in 0.5% glutaraldehyde. Before flow cytometry analysis, samples were diluted once more 1:2, giving a final dilution of 1:20. Bacterial concentrations were determined based on gated FL1 vs. FSC-A plots obtained from processing of raw data files acquired from KYTOS in the BD Accuri™ software.

Bacterial condition	Start concentration (10^4 bacteria/mL)	End concentration (10^4 bacteria/mL)	End/start ratio
3.28 *(Microbacterium sp.)*	3.57 ± 0.398	4.88 ± 0.910	1.37 ± 0.26
3.67 *(Arthrobacter sp.)*	2.11 ± 0.268	11.6 ± 2.59	5.44 ± 0.80
3.24 *(Micrococcus sp.)*	4.85 ± 1.89	51.9 ± 11.7	13.02 ± 6.78
3.11 *(Hafnia sp.)*	1.10 ± 0.165	96.4 ± 61.6	88.21 ± 53.42
3.78 *(Psychrobacter sp.)*	12.6 ± 1.92	265.9 ± 42.7	21.06 ± 0.84
B40 *(Staphylococcus sp.)*	2.53 ± 0.429	6.73 ± 2.26	2.59 ± 0.43
B49 *(Lactococcus sp.)*	13.0 ± 0.974	9.78 ± 2.31	0.74 ± 0.12
3.18 *(Carnobacterium sp.)*	14.1 ± 2.89	8757.9 ± 1565.2	627.06 ± 53.07
3.3 *(Sphingomonas sp.)*	2.02 ± 0.184	112.2 ± 51.7	58.06 ± 29.51
SynCom	5.29 ± 0.855	1209.6 ± 593.7	217.47 ± 69.29
Germ-free	1.09 ± 0.816	8.21 ± 0.881	11.50 ± 5.28
Re-conventionalized	38.3 ± 3.38	315.7 ± 87.5	8.15 ± 1.77

3.4.1 Characterization of the zebrafish microbiotas

16S rRNA gene amplicon sequencing was performed to characterize the microbiota associated with rearing water and fish from the SynCom and re-conventionalized conditions. Germ-free control samples were also included in the amplicon library, since the targeted PCR amplification performed during library preparation indicated that there potentially was a bacterial contamination present in these samples. Characterization of the water and fish microbiota from the re-conventionalized condition is presented in Section 3.6 together with the corresponding results for the salmon yolk-sac fry experiment.

For the germ-free samples which were kept after rarefaction (see Supplementary Table S.1), the 16S amplicon sequencing data reinforced the suspicion that a bacterial contamination was present. Two ASVs, ASV4 and ASV20, which were both assigned to the family *Oxalobacteraceae*, were abundant in both water samples taken at the end of the experiment, as well as in three of the four fish tissue samples, and accounted for 44.3 – 99.6% of reads in the relevant samples. ASV4 was also present in the fourth fish tissue sample, but in lower abundance compared to the aforementioned samples (0.01% of total reads). In addition, several, less abundant ASVs assigned to the genus *Corynebacterium*, a genus known to be highly abundant in human skin, was observed in several samples [72]. An overview of the ten most common ASVs observed in the zebrafish germ-free samples are given in Supplementary Table S.3. Remaining ASVs observed in these samples occured sporadically across samples and probably represented noise.

For the SynCom condition, ASV11, which was assigned to the genus *Comamonas*, unexpectedly dominated all four fish tissue samples, accounting for 91.5 – 98.6% of all reads, indicating that fish were contaminated by a *Comamonas* strain. The same ASV was also highly abundant in one of the four water sample taken at the end of the experiment (accounting for 66.6% of all reads). ASVs which represented the actual SynCom strains were detected only sporadically and generally in low abundances, varying from a single read in a start water sample, to 62.5% of all reads in another replicate of the same sample type (see Supplementary Table S.5). Generally, SynCom water samples seemed to be dominated by ASVs not showing any particular pattern between samples, probably representing noise. This could indicate that the water sampling method used in the zebrafish experiment perhaps did not work as intended, and that it failed to capture the full microbial diversity in samples. Due to the lack of filters for collecting the water microbiota, water had been sampled by sequential centrifugation as described in Section 2.3.4.

3.5 Salmon yolk-sac fry gnotobiotic experiment

A gnotobiotic experiment was also performed in well plates with salmon yolk-sac fry as the host model. This experiment was performed in the fish lab facilities of ACMS. Sterility tests of the SGM hatching media were performed twice for all flasks containing fish in various liquid and agar culture media (see Section 2.4.2). Microbial contamination was detected for two flasks between the germ-free derivation and association with bacterial strains. These were therefore excluded from the experiment. At 3 dph, wells with salmon yolk-sac fry were inoculated with mono-associations of the individual bacterial strains, a SynCom consisting of all nine strains, and a re-conventionalized condition using rearing water from SINTEF SeaLab's salmon RAS facilities. A germ-free control condition was also kept. All eggs were hatched 13 days after receiving the fertilized eggs. Mortality was generally low throughout the experiment; one egg never hatched, and two hatched individuals died during handling.

RNA extraction and subsequent cDNA synthesis for gene expression analysis was successfully performed also in this experiment. RNA was extracted from fish tissue samples obtained from the SynCom, germ-free, and re-conventionalized conditions. In this experiment, cDNA concentration was measured instead of RNA. Both yields and A260/A280-ratios of the obtained cDNA was higher than for the RNA measured in the zebrafish experiment. The average cDNA concentration obtained was 157.8 ± 32.8 ng/µL (see Supplementary table S.4).

Bacterial densities were determined for three replicate wells of each bacterial condition by flow cytometry (Table 3.3). Bacterial densities varied from between $1.41 \pm 0.226 \times 10^4$ bacteria/mL in the germ-free condition to $140.81 \pm 96.41 \times 10^4$ bacteria/mL in the B40 *(Staphylococcus sp.)* mono-association at the start of the experiment. At the end of the experiment, the lowest bacterial density was found in the mono-association with strain B49 *(Lactococcus sp.)* with $5.68 \pm 1.00 \times 10^4$ bacteria/mL, whilst the 3.28 *(Microbacterium sp.)* mono-association had the highest ($7828.04 \pm 1515.53 \times 10^4$ bacteria/mL). The end/start ratios of bacterial densities in the bacterial conditions indicated that the bacterial density increased in all conditions except for the B40 (*(Staphylococcus*, B49 *(Lactococcus)*, and 3.3 *(Sphingomonas)* mono-associations.

Table 3.3: Bacterial densities at the start and end of the salmon yolk-sac fry gnotobiotic experiment, and the end/start ratio of bacterial densities for each of the bacterial conditions in the experiment. The table shows the mean of samples taken from three replicate wells plus-minus the standard deviation. Samples were diluted 1:10 and fixated in 0.5% glutaraldehyde. Before flow cytometry analysis, samples were diluted further 1:2. Bacterial concentrations were determined based on gated FL1 vs. FSC-A plots obtained from processing of raw data files acquired by KYTOS in the BD Accuri™ software.

Bacterial condition	Start concentration (10^4 bacteria/mL)	End concentration (10^4 bacteria/mL)	End/start ratio
3.28 (Microbacterium sp.)	2.71 ± 0.474	7828.04 ± 1515.53	2915.06 ± 503.33
3.67 (Arthrobacter sp.)	4.04 ± 0.282	104.23 ± 470.03	25.83 ± 11.20
3.24 (Micrococcus sp.)	72.02 ± 96.33	105.13 ± 102.97	23.33 ± 28.66
3.11 (Hafnia sp.)	4.09 ± 0.519	38.73 ± 9.85	9.36 ± 1.45
3.78 (Psychrobacter sp.)	6.20 ± 0.447	8.039 ± 1.20	1.32 ± 0.28
B40 (Staphylococcus sp.)	140.81 ± 96.41	6.12 ± 0.78	0.043 ± 0.021
B49 (Lactococcus sp.)	9.00 ± 3.62	5.68 ± 1.00	0.77 ± 0.38
3.18 (Carnobacterium sp.)	12.24 ± 8.97	382.58 ± 39.81	50.88 ± 26.80
3.3 (Sphingomonas sp.)	12.54 ± 0.58	9.25 ± 1.27	0.74 ± 0.08
SynCom	25.86 ± 5.58	1308.20 ± 652.69	54.53 ± 31.31
Germ-free	1.41 ± 0.226	8.59 ± 0.944	6.40 ± 1.88
Re-conventionalized	17.50 ± 1.46	308.30 ± 87.31	17.66 ± 5.13

3.5.1 Characterization of the salmon yolk-sac fry microbiotas

Water and fish tissue samples from the SynCom and re-conventionalized conditions in the salmon yolk-sac fry gnotobiotic experiment were subjected to 16S rRNA gene amplicon sequencing for characterization of their associated microbiotas. Germ-free samples were also included in the amplicon library, as some of these samples gave low amounts of PCR product. Results for the samples from the re-conventionalized condition is presented in Section 3.6 together with the corresponding results for the zebrafish experiment.

Community profiles for PCR products representing germ-free fish and water samples showed that ASVs were sporadically dispersed among samples and did not show any particular pattern, indicating that they most likely represented DNA contaminations and noise rather than actual bacterial contaminations. The lack of observable PCR products for these samples in the targeted PCR performed during library preparation supports this (Figure 3.3b).

Amplicon sequencing data for the SynCom samples contained several highly similar ASVs representing the genera that had been included in the SynCom. Therefore, an OTU clustering at 97% pairwise sequence identity, and subsequent determination of the bacterial community compositions at the OTU level, was performed (Figure 3.5). OTUs representing the SynCom genera accounted for 92.4 ± 7.7% of the total reads, indicating that the gnotobiotic condition had been successfully maintained. The remaining reads were accounted for by OTUs dispersed sporadically between samples in low abundances, probably representing noise. However, no OTUs representing *Sphingomonas sp.* or *Staphylococcus sp.* were detected. Instead, an OTU representing an *Oceanobacillus sp.* (OTU24) was the fourth most common OTU on average in all samples. Similarly, a *Pseudomonas* OTU was abundant in several samples. This could imply that it was these strains, rather than the *Sphingomonas sp.* and *Staphylococcus sp.* strains which were used for the SynCom condition. Two distinct *Microbacterium* OTUs (OTU8 and OTU175) was observed in most SynCom samples, with OTU8 being the dominant of the two. This could indicate that the *Microbacterium* isolate which was used contained more than one strain, or the two OTUs could represent two gene variants of the 16S rRNA gene from the same strain.

Community profiles also showed that SynCom OTUs had varying relative abundances in start water samples, even though the aim was to inoculate wells with equal amounts of each strain (Figure 3.5). OTU1 *(Psychrobacter sp.)* was the most dominant OTU in start water samples, but had reduced abundance in end water samples, where OTU6 *(Hafnia sp.)* and OTU8 *(Microbacterium sp.)* dominated. OTU8 especially had a great increase in relative abundance in water from the start to the end of the experiment. The *Oceanobacillus sp.* OTU (OTU24) was almost exclusively present in start water samples. Fish tissue samples appeared relatively similar to end water samples, except that they had higher abundances of OTU1 *(Psychrobacter ssp.)* and lower abundances of OTU8 *(Microbacterium sp.)* and OTU21 *(Carnobacterium sp.)*.

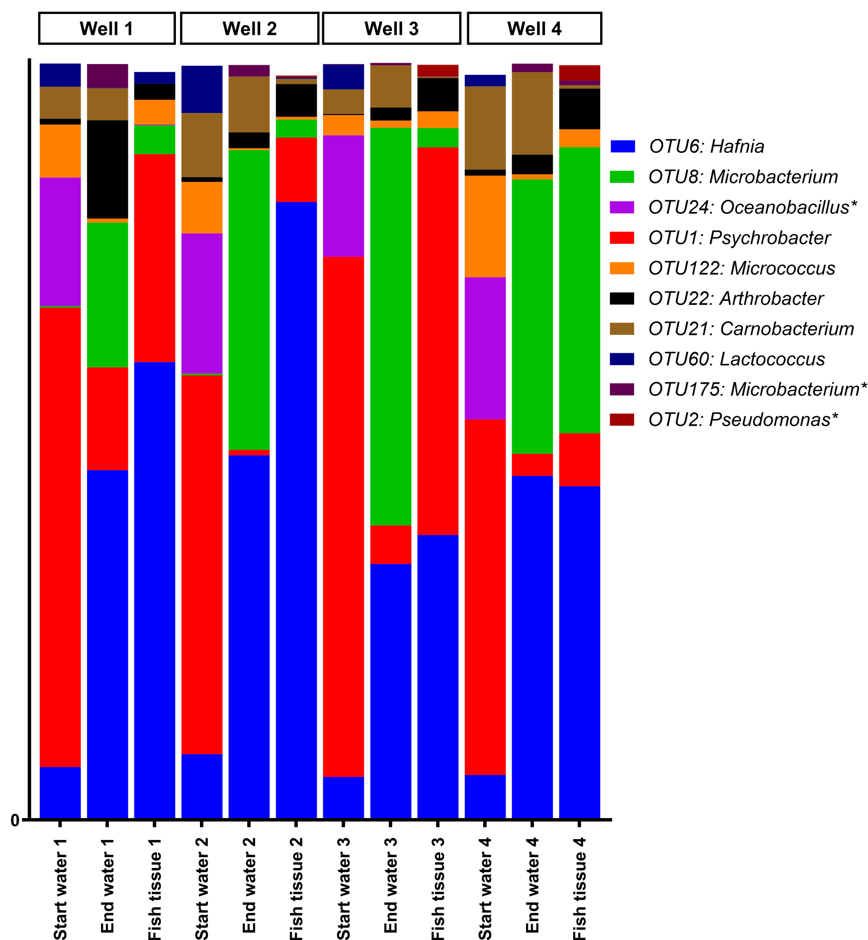


Figure 3.5: Community composition at the OTU level for the SynCom samples. Only the most abundant OTUs, representing nine genera presumably derived from the SynCom strains are shown. OTUs assigned with an asterisk are the additional *Oceanobacillus*, *Pseudomonas*, and *Microbacterium* OTUs which were suspected to also be part of the SynCom inoculum.

To compare microbiota associated with water and fry samples from the SynCom condition, as PCoA ordination plot based on Bray-Curtis similarities was generated (Figure 3.6). The PCoA indicated that the start water communities differed from those of the end water and fish tissue samples, which seemed to be more similar. Furthermore, the PCoA plot also indicated that community profiles varied to a greater extent in the end water and fish tissue samples, compared to the start water samples. This indicates that SynCom communities diverged from the starting conditions throughout the course of the experiment.

A one-way PERMANOVA analysis confirmed that the bacterial community compositions were significantly different both when comparing start water samples to end water ($p = 0.0235$) and fish tissue samples ($p = 0.0241$). This indicated that community profiles had diverged from the starting conditions both in water and in fish tissue samples. Comparing end water samples and fish tissue samples resulted in a p-value of 0.0519. The PCoA ordination plot revealed that the fish tissue sample from replicate well 4 appeared more similar to end water samples than the other three fish tissue replicates (Figure 3.6).

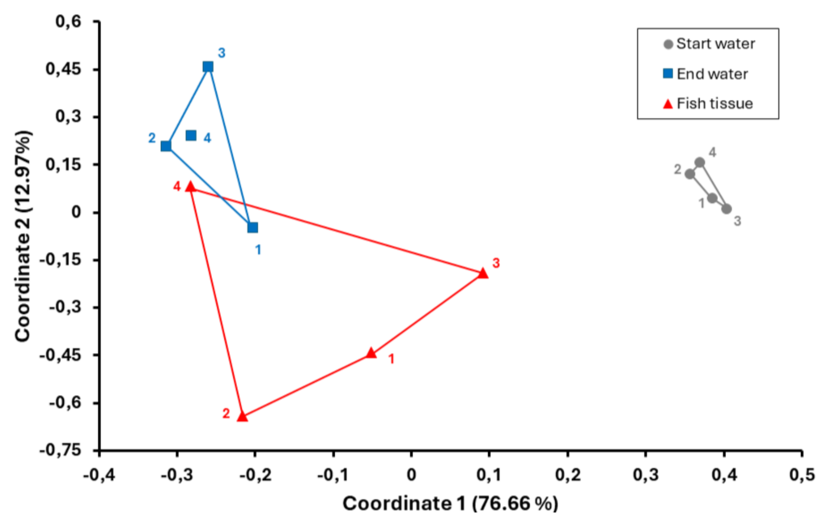


Figure 3.6: PCoA ordination plot of the salmon SynCom samples based on Bray-Curtis similarities, grouped into start water, end water, and fish tissue samples. Individual samples are from four replicate wells (indicated 1 - 4), which contained one salmon yolk-sac fry exposed to the SynCom.

3.6 Bacterial rearing conditions in the re-conventionalized conditions of the zebrafish and salmon yolk-sac fry gnotobiotic experiments

In both the gnotobiotic zebrafish and salmon yolk-sac fry experiments, a re-conventionalized condition was included to represent the bacterial rearing conditions the two host organisms normally would be exposed to in their respective rearing habitats. To characterize the water and fish microbiota, 16S rRNA gene amplicon sequencing was performed on water samples from before and after the experiments, as well as on fish tissue samples.

3.6.1 Alpha-diversity in the re-conventionalized conditions

In the ASV table that had been rarefied to 4750 reads per sample, 236 ASVs were observed in all re-conventionalized samples from the zebrafish experiment. In the salmon yolk-sac fry experiment, a total of 517 ASVs were observed. Combined in the two sample sets, a total of 712 unique ASVs were found, meaning that 41 ASVs were present in both experiments. The average sequence coverage of the observed ASV richness compared to the estimated richness (Chao-1) at the relevant sequencing depth of 4750 reads per sample was $85.7 \pm 5.3\%$ for zebrafish samples, and $81.6 \pm 3.8\%$ for salmon yolk-sac fry samples ($\pm$ SD).

Overall, α-diversity indices showed that water samples from the salmon yolk-sac fry experiment had the highest α-diversity (Figure 3.7). Notably, salmon water samples taken both at the start and end of the experiment had a significantly higher observed and estimated (Chao-1) ASV richness than all other sample types (one-tailed T-tests, $p < 0.05$), including the salmon yolk-sac fry fish tissue samples. This indicated that rearing water microbiota had a higher α-diversity than fish microbiota in this experiment. The significantly higher observed ASV richness in salmon water samples compared to zebrafish water samples in one-tailed t-tests ($p = 2.56 \times 10^{-6}$ for start water samples, $p = 0.023$ for end water samples) is another indication that the sampling method applied for zebrafish water samples might not have worked as intended. The exponential Shannon diversities of zebrafish and salmon fish tissue samples were not significantly different (one-tailed t-test, $p = 0.25$).

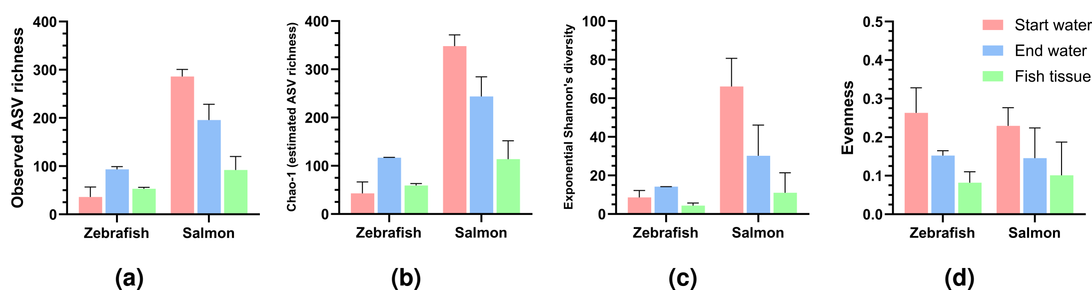


Figure 3.7: α-diversity indices for water and fish samples from the re-conventionalized conditions in the zebrafish and salmon yolk-sac fry gnotobiotic experiments obtained from PAST. **a)** shows the observed ASV richness, **b)** the estimated ASV richness (Chao-1), **c)** the exponential Shannon's diversity index, and **d)** the evenness. Mean values for four replicate samples for a given sample type are shown, except for the zebrafish end water samples (two replicates) and salmon fish tissue samples (three replicates). Error bars indicate the standard deviation between replicate samples of the same type. Diversity indices were calculated based on the ASV table rarefied to 4750 reads per sample.

The evenness of corresponding sample types from the two experiments was observed to be similar (Figure 3.7d). Within each experiment however, the evenness decreased in both water and fish tissue communities compared to the start water community. One-tailed t-tests revealed that this decrease was significant both from start water to end water ($p = 0.0425$) and from end water to fish tissue samples ($p = 0.0164$) in the zebrafish experiment. In the salmon yolk-sac fry experiment only comparison of the start water and fish tissue samples showed a significant decrease in evenness ($p = 0.025$).

3.6.2 Bacterial community compositions in the re-conventionalized conditions

The bacterial community composition was determined on the family level for all samples from the re-conventionalized condition in both the zebrafish and salmon yolk-sac fry gnotobiotic experiments (Figure 3.8). In the water microbiota at the start of the zebrafish experiment, the families with the highest average relative abundances were *Caulobacteraceae* (44.3%), *Comamonadaceae* (average 7.6%), *Sphingomonadaceae* (average 7.3%), and *Pseudomonadaceae* (average 5.2%) (Figure 3.8a). ASVs which were unassigned at the family level made up 8.9% of the communities in these samples on average. Relative abundances in water microbiota varied greatly between replicates already at the start of the experiment. ASVs assigned to the *Weeksellaceae* family for example, had a relative abundance of 19.1% in one replicate, whilst in the remaining three replicates its relative abundance was between 0 - 0.821%. Another example is

ASVs assigned to the *Hyphomicrobiaceae* family, which were not detected in any replicates but one, where it made up 13.7 % of all reads.

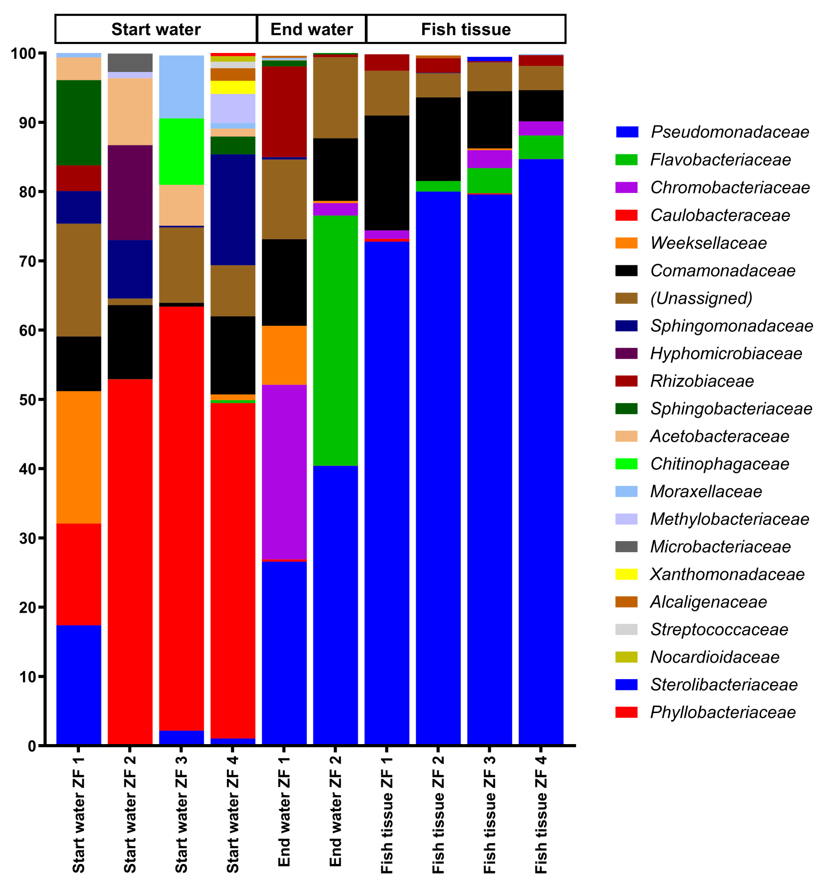
In the start water microbiota of the salmon yolk-sac fry experiment, *Comamonadaceae* (average 22.0%), *Flavobacteriaceae* (average 18.2%), *Pseudomonadaceae* (average 17.6%), *Rhodobacteraceae* (average 15.2%) and *Sphingomonadaceae* (average 6.6%) were the most abundant families (Figure 3.8b). ASVs which were unassigned at the family level made up 14.6% of the communities in these samples on average. Variation between replicate samples were markedly lower compared to the zebrafish experiment, both in terms of which taxa were present, and the relative abundances of these taxa.

The rarified ASV table included only two replicates of the water samples taken at the end of the zebrafish experiment. In these samples, families that were abundant in both replicates were *Pseudomonadaceae* (relative abundances: 26.6% and 40.4%) and *Comamonadaceae* (relative abundances: 12.5% and 9.1%) (Figure 3.8a). ASVs which were unassigned at the family level had relative abundance of 11.5% and 11.7% in "end water 1" and "end water 2", respectively. Some variation between the two replicate samples were observed for this sample type as well. For example, *Flavobacteriaceae* was exclusive to "end water 2" (36.1% relative abundance), whilst *Chromobacteriaceae* and *Rhizobiaceae* were almost only present in the other (relative abundances: 13.1% vs. 0.379% and 8.53% vs. 0.316%, respectively).

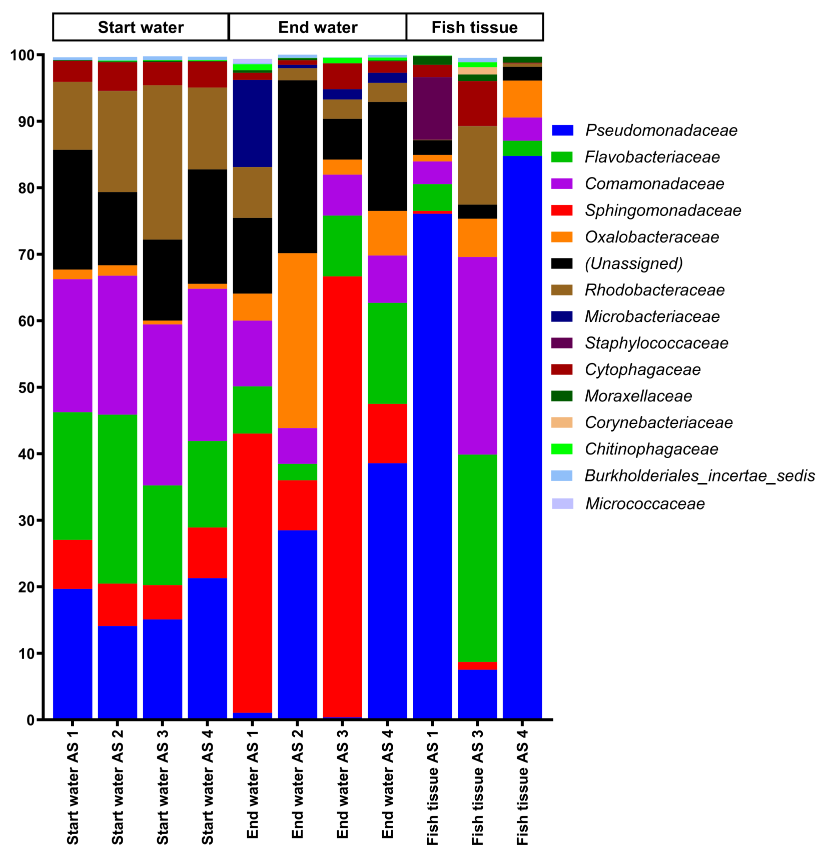
The families with the highest average relative abundances in water samples taken at the end of the salmon yolk-sac fry experiment were *Sphingomonadaceae* (average 31.2%), *Pseudomonadaceae* (average 17.1%), *Oxalobacteraceae* (average 9.8%), *Flavobacteriaceae* (average 8.5%), and *Comamonadaceae* (average 7.1%) (Figure 3.8b). ASVs which were unassigned at the family level had an average relative abundance of 15.0%. In these samples, it was observed that replicates were either dominated by *Sphingomonadaceae* or *Pseudomonadaceae*, but never by both simultaneously.

Microbiota in the zebrafish tissue samples were dominated by *Pseudomonadaceae*, which had an average relative abundance of 79.3% (Figure 3.8a). Another abundant family were *Comamonadaceae* (average relative 10.4%). The variation in the community compositions between replicate samples were markedly lower for fish tissue samples compared to the water samples in the zebrafish experiment.

In two of three salmon yolk-sac fry tissue samples which remained after rarefaction, the bacterial communities were dominated by *Pseudomonadaceae*, with relative abundances of 76.1 and 84.8% (Figure 3.8b). In the third sample, *Pseudomonadaceae* had a relative abundance 7.6%. This sample was instead dominated by *Flavobacteriaceae* and *Comamonadaceae*, with relative abundances of 31.2, and 29.7%, respectively. This indicated a greater variation in microbiota compositions for fish tissue samples compared to water samples in the salmon yolk-sac fry experiment.



(a)



(b)

Figure 3.8: Community compositions at the family level in re-conventionalized samples from a) the zebrafish experiment and b) the salmon yolk-sac fry experiment. All families with relative abundance $\geq$ 0.5% in at least one sample are included. Numbers in sample names indicate the replicate well from which the samples were obtained.

3.6.3 Beta-diversity in the re-conventionalized conditions

The β-diversity of samples from the re-conventionalized conditions was also assessed. A PCoA ordination plot based on Bray-Curtis similarities revealed that zebrafish and salmon yolk-sac fry samples formed distinct clusters, and that salmon yolk-sac fry samples appeared more similar to each other compared to zebrafish samples (Figure 3.9a). A one-way PERMANOVA analysis comparing all zebrafish re-conventionalized samples to all salmon yolk-sac fry re-conventionalized samples confirmed that the bacterial community compositions of the two experiments were, as expected, significantly different (p = 0.0001). SIMPER analysis revealed that the ASVs which contributed most to the difference between the two re-conventionalized conditions were ASV3 (*Pseudomonadaceae*), ASV2 (*Pseudomonadaceae*), ASV14 (*Caulobacteraceae*), and ASV12 (*Sphingomonadaceae*) (Table 3.4). Together, these four ASVs contributed to 38.08 % of the difference between the two groups.

Table 3.4: A summary of the ten ASVs that contributed the most to the differences between the re-conventionalized communities of the zebrafish and salmon yolk-sac fry experiment. Difference contributions were identified by SIMPER analysis based on Bray-Curtis dissimilarities. The analysis was performed in PAST. The ASVs individual contribution, together with the cumulative contribution from top to bottom is given. The mean relative abundance of each ASV in re-conventionalized samples from both the zebrafish and the salmon yolk-sac fry experiment is also shown.

ASV ID	Taxonomy (family level)	Contribution (%)	Cumulative contribution (%)	Mean rel. abundance zebrafish (%)	Mean rel. abundance salmon (%)
ASV3	*Pseudomonadaceae*	14.5	14.5	29.05	0.00
ASV2	*Pseudomonadaceae*	10.35	24.85	0.00	20.65
ASV14	*Caulobacteraceae*	6.992	31.84	13.96	0.00
ASV12	*Sphingomonadaceae*	6.235	38.08	0.00	12.44
ASV17	*Rhodobacteraceae*	2.891	40.97	0.00	5.77
ASV21	*Pseudomonadaceae*	2.735	43.7	5.45	0.00
ASV25	*Comamonadaceae*	2.702	46.4	0.00	5.39
ASV9	*Comamonadaceae*	2.257	48.66	4.51	0.00
ASV29	*Pseudomonadaceae*	1.763	50.42	3.52	0.00
ASV46	*Chromobacteriaceae*	1.646	52.07	3.28	0.00

The PCoA ordination plot for zebrafish samples revealed that the microbiota of the start water, end water, and fish tissue samples differed (Figure 3.9b). The re-conventionalized condition in this experiment, start water samples appeared to be the sample group with the highest variation in community compositions. This is another indication that the sampling method applied for water samples might not have worked as intended. The same PCoA plot also indicated that the rearing water community composition was more similar to fish tissue at the end of the experiment than at the start (Figure 3.9b). One-way PERMANOVA analysis of the zebrafish end water and fish tissue samples showed that these two sample groups were significantly different (p = 0.0478). A similar comparison of the zebrafish start water and fish tissue samples gave a p-value of 0.008.

In the salmon yolk-sac fry experiment, a PCoA showed that start water samples appeared to have more similar community compositions compared to end water and fish tissue samples (Figure 3.9c). As was observed in Section 3.6.2, two of the three fish tissue samples (Fish tissue AS 1 and 3 in Figure 3.8b) appeared to have similar community compositions. The PCoA plot revealed that the remaining sample were more similar to water samples (Figure 3.9c). However, a one-way PERMANOVA comparison showed that start water and fish tissue community compositions were significantly different (p = 0.0285). When comparing the community composition of the rearing water

at the start and end of the experiment, these were also shown to differ significantly (p = 0.0280). When comparing the end water and fish tissue compositions these were not statistically different (p = 0.109). Together, this indicates that the water microbiota at the end of the experiment was more similar to the fish tissue microbiota than to water microbiota at the start of the experiment.

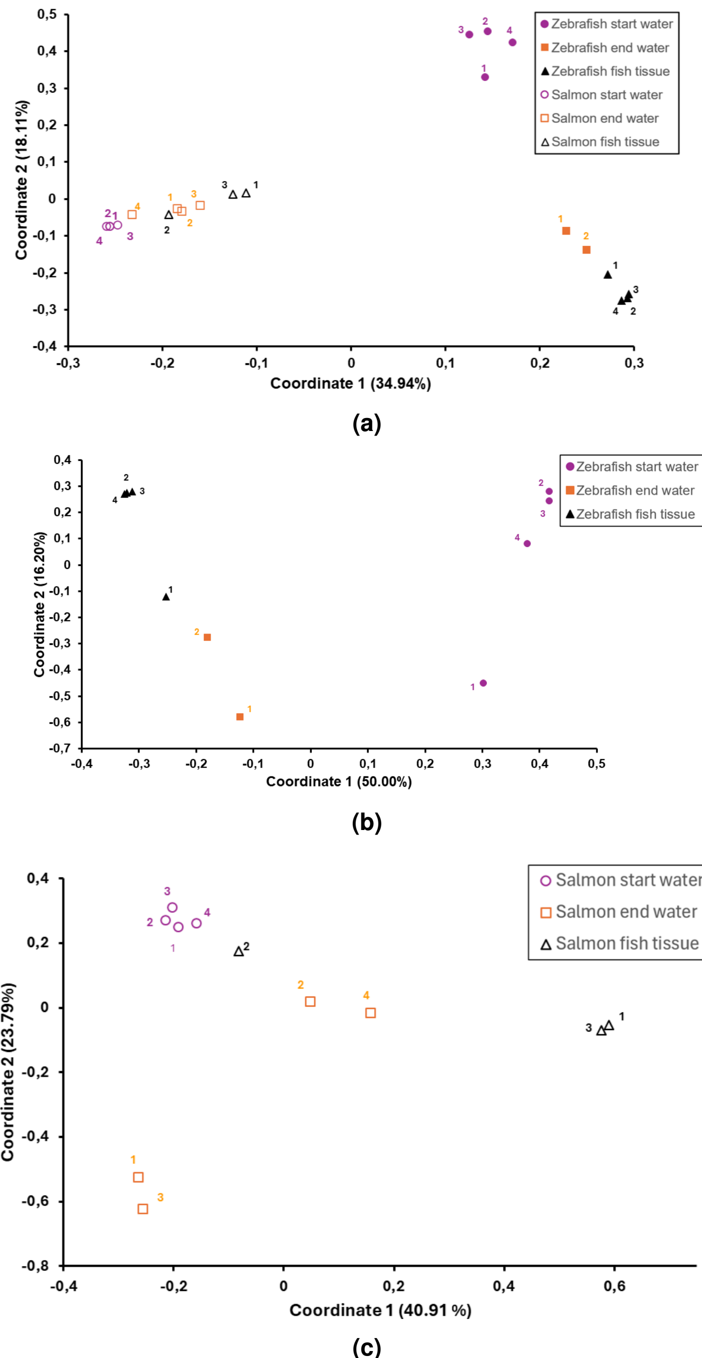


Figure 3.9: PCoA ordination plots based on Bray-Curtis similarities of the re-conventionalized samples from both gnotobiotic experiments. In **a)** all re-conventionalized samples from both experiments are compared. Comparisons of the start water, end water, and fish tissue samples are shown for **b)** the zebrafish and **c)** the salmon yolk-sac fry experiments individually.Numbers indicate the replicate well from which the samples were obtained.

4 Discussion

4.1 Evaluation of experimental work

4.1.1 Quality of amplicon library and amplicon sequencing data

Amplification of unspecific products were observed for some samples in the indexing PCR, particularly in one SynCom and one germ-free salmon yolk-sac fry tissue replicate (see Supplementary Figure S.2). In addition, the amount of PCR products of the expected size after the indexing PCR appeared to be low for most samples. An attempt was made to increase the number of PCR cycles from ten to twelve. This seemed to increase the amount of the product of the desired size, but also gave what appeared to be an even greater increase in the amplification of the shorter unspecific products.

To avoid interference of the unspecific products with the Illumina sequencing, it was decided to continue with the library indexed at ten PCR cycles. The pooled amplicon library was purified by eluation from an agarose gel to remove unspecific PCR products. The resulting sequencing data from this library was of poor quality, with a very low amount of forward and reverse reads which could be merged in the USearch processing. The library was therefore re-sequenced. This resulted in sequencing data which were of high enough quality to be processed into ASVs. Still, for some samples a relatively high proportion of forward and reverse reads could not be merged. This probably contributed to the great variation in sequencing depth that was observed. One of the reasons forward and reversed reads are unable to merge, is that the obtained reads from sequencing are longer than expected. This is a known issue in amplification of bacterial 16S rRNA genes from host-associated microbiotas, due to unspecific amplification of host DNA ([73, 74]. Specifically, the loss of reads was greater for zebrafish samples than for salmon yolk-sac fry samples. For salmon yolk-sac fry samples, PCR protocols are well-established, and co-amplification of the host 18S and 12S rRNA genes are generally not a problem (Ingrid Bakke, verbal communication).

4.1.2 Maintenance of gnotobiotic conditions in the zebrafish and salmon yolk-sac fry experiments

PCR of samples from the germ-free condition in the zebrafish experiment unexpectedly resulted in amplification of bacterial 16S rRNA genes, indicating that a bacterial contamination was present in these samples. The amplicon sequencing data further revealed that two *Oxalobacteraceae* ASVs (ASV4 and ASV20) accounted for most of the reads in all but one germ-free fish tissue samples and in water samples taken at the end of the experiment from the germ-free condition. In the fish tissue sample which had few reads of the *Oxalobacteraceae* ASV, several ASVs assigned to *Corynebacterium* were dominant. The ASVs representing *Oxalobacteraceae* were not classified above the confidence threshold of 0.80 at the genus level, but the data indicated that

they both belonged to the *Undibacterium* genus (confidence tresholds of 0.3758 and 0.5742 for ASV4 and ASV20, respectively). This genus is typically found in various fresh water environments, and have been isolated from zebrafish previously [75, 76]. *Corynebacterium* are common in human skin microbiota and are frequently observed contaminations in amplicon sequencing-based research [72, 77]. It is possible that the *Corynebacterium* ASVs observed in the one fish tissue sample represented a DNA contamination. In the zebrafish SynCom condition, a *Comamonas* ASV which was not part of the SynCom dominated the fish tissue samples. The natural habitats of *Comamonas* include fresh water ponds and rivers, and members of the genus have been isolated from zebrafish microbiota previously [78, 79]. As the SynCom condition was expected to show an increase in bacterial density, flow cytometry measurements are not suitable for detecting a contamination in these samples. If a bacterial contamination was present in the germ-free condition, one would expect that the flow cytometry measurements showed a higher-than-expected bacterial density in these samples. However, these results were less clear on whether a bacterial contamination had occured in the relevant samples. Although they indicated an increased bacterial density in the germ-free condition throughout the experiment, this increase was small compared to some of the other bacterial conditions. This could perhaps indicate that a potential bacterial contamination occurred in the latter stages of the experiment. Still, considering the weaknesses of the flow cytometry data discussed in Section 4.2, and the observations made in the targeted PCR and the amplicon sequencing data, it seems likely that a bacterial contamination was present in the germ-free and SynCom conditions in the zebrafish experiment.

Additionally, ASVs representing the SynCom strains where only sporadically detected in water samples from the zebrafish SynCom condition, contrary to the case for the corresponding samples from the salmon yolk-sac fry experiment. Here, ASVs representing the SynCom genera were the most abundant, and remaining ASVs were accredited to noise. As the same method for establishing the SynCom condition was applied in both experiments, but vastly different results were obtained from the subsequent amplicon sequencing, this indicates, along with other results discussed in Section 4.5 that the water sampling method used in the zebrafish experiment did not work as intended. Details on the execution of the water sampling are discussed in Section 4.1.3.

The completion of the salmon yolk-sac fry gnotobiotic experiment appeared to have been generally successful, as no obvious indications of contamination were observed in the sterility controls of the germ-free conditions, the targeted PCR, nor the amplicon sequencing data for the germ-free and SynCom conditions. Furthermore, the SynCom condition looked to have been successfully established, as the amplicon sequencing data showed that SynCom samples were dominated mainly by OTUs representing the strains used in the condition, with some notable exceptions (for discussion of detailed results based on amplicon sequencing data, see Section 4.4).

There are several possible explanations as to why gnotobiotic conditions were compromised in the zebrafish experiment. One possibility is that the maintenance of sterile conditions when handling well plates throughout the experiment was unsuccessful. The zebrafish and salmon yolk-sac fry experiments differed in this aspect. In the salmon yolk-sac fry experiment, well plates were not manipulated for the monitoring of survival. In the zebrafish experiment, however, well plates had to be moved daily, as hatching progression and mortality had to be monitored in a stereo microscope due to the small size of zebrafish eggs and larvae. With increased handling comes an increased risk

of contaminations, and although handling was kept to a minimum and stringent sterile technique was applied, it is still a possible source of contamination.

Another possible explanation could be that the initial germ-free derivation of the zebrafish eggs failed, and that some bacteria survived the treatment. Protocols for germ-free derivation of fish exists for several species, and both the use of chemical disinfectants and antibiotics differ between protocols [80]. Thus, there is not one general, foolproof method for generating germ-free fish eggs, as species-specific considerations must be taken to optimize sterility without negatively affecting host survival and development. The main difference between the derivation protocols used in this experiment was the use of bleach in the zebrafish experiment, and the range of antibiotics used. In the zebrafish experiment, a mixture of two antibiotics, ampicillin and kanamycin, and one antifungal chemical, amphotericin B, was used (Appendix A). In the salmon yolk-sac fry experiment, an additional four antibiotics were used in addition to those of the zebrafish experiment (Appendix A). In a previous master project at ACMS, it was shown that a *Flavobacterium sp.* was able to survive the germ-free derivation process [81]. Only an increased concentration of oxolinic acid was able to inhibit its growth. This shows that both the composition of antibiotics, as well as the working concentrations of these, is important for obtaining germ-free conditions. The narrower range of antibiotics used in the zebrafish experiment will have increased the risk of bacteria surviving the germ-free derivation of this experiment.

A final possibility is that bacteria were present in eggs intrachorionically. This would lead to bacteria evading both the chemical disinfectants and antibiotics, as neither penetrate the chorion of the eggs, but only sterilize their surface. Although the internal fish egg is generally regarded to be sterile [32], studies have shown that some bacteria, amongst them an *Undibacterium* species, are able to gain access to intrachorionic sites in fish eggs [82]. Interestingly, the two most common ASVs in zebrafish tissue samples from the germ-free condition were assigned to the *Undibacterium* family, although not above the 0.8 confidence treshold. Still, there is a possibility that these ASVs perhaps came from bacteria which resided on the inside of the fish egg chorions.

The routines for performing sterility tests differed between IBIS and ACMS. In the zebrafish experiment performed at IBIS, tests were done on TSA plates. This did not reveal that the germ-free wells were contaminated. At ACMS where the salmon yolk-sac fry experiment was performed, sterility tests were done in four different liquid culture media in addition to TSA plates (see Section 2.4.2). This resulted in the detection of contaminations in two flasks after the derivation of germ-free eggs. These flasks were therefore left out from the subsequent bacterial associations. For both flasks, the contaminations were detected in only one of the five media that were used. It is widely known that most bacteria are unculturable in common growth media, and that when comparing viable counts on growth medium to microscopic counts, there is a great discrepancy in the estimated number of bacteria [50]. A wider range of growth media can counteract this discrepancy to some degree, but ideally, culture-independent methods such as flow cytometry should also be applied to properly control sterility in gnotobiotic experiments [80]. The lack of such a method could be considered a weakness in the experimental protocols followed in this thesis. Particularly, the use of only one growth media to control sterility in the zebrafish experiment should be revised. The same range of control media used for the salmon yolk-sac fry experiment should also have been applied for the zebrafish experiment. In addition, a culture-independent method such as flow cytometry should be included to reveal unculturable potential contaminants. The

fact that a likely bacterial contamination was overlooked in the zebrafish experiment underlines the importance of a diverse range of control media, and preferably also a culture-independent method, when performing sterility tests in gnotobiotic experiments.

4.1.3 Methods for sampling water microbiota

In both the zebrafish and salmon yolk-sac fry experiments, comprehensive sampling was performed both of water and fish. Samples intended for flow cytometry analysis, amplicon sequencing, and host gene expression analysis were collected in both experiments (see Table 2.2 and Table 2.3).

Sampling of water for characterization of the water microbiota was performed differently in the two gnotobiotic experiments. There is no apparent reason as to why water from the zebrafish rearing facilities of LARSEM should have less than half the number of observed ASVs to that of the Atlantic salmon rearing facilities of SINTEF SeaLab. The difference in observed ASVs in fish tissue samples was not significant between the two host species. This, together with the results discussed Section 4.1.2 and Section 4.5 , indicates that the water samples from the zebrafish experiment probably did not represent the full diversity of the water microbiotas. Normally, Millipore® Sterivex™ 0.22 0.22 µm filters are used both by both ACMS and IBIS to sample water microbiota. However, this method was deemed unsuited for the experimental design utilized in this thesis, as these filter units required a higher volume than what was available in each replicate well. In the salmon yolk-sac fry experiment, water was sampled for amplicon sequencing using Whatman® Nuclepore™ 0.2 µm Track-Etched membrane filter. This method is routinely applied in the ACMS group for sampling water microbiota from low volume sources. In the zebrafish experiment, a method of sequential centrifugation was utilized (described in Section 2.3.4) , as Nuclepore™ filters were not available at IBIS. When the sampling was performed, no visible pellet was observed after the last centrifugation, and it is likely that a considerable amount of bacteria were lost in the discarded supernatant, from what was already relatively low bacterial concentrations. These findings imply that the filtering method used in the salmon yolk-sac fry experiment was superior to the sequential centrifugation method applied in the zebrafish experiment in capturing the diversity of the sampled microbiotas.

4.2 Flow cytometry data

The bacterial densities in water from all the applied bacterial conditions, including the mono-associations, were analyzed by flow cytometry of samples taken at the start and end of both gnotobiotic fish experiments. These results indicated that the development of bacterial densities varied greatly between conditions. A decrease in bacterial density was observed in the B49 *(Lactococcus sp.)* mono-association in the zebrafish experiment, as well as in the salmon yolk-sac fry experiment. In addition, two more mono-associations also showed decreased bacterial densities in this experiment (B40 *(Staphylococcus sp.)* and 3.3 *(Sphingomonas sp.)*). Coincidentally, these three strains were also those with the lowest determined μ_{max} in the growth experiment at the lower temperature. This suggests that these strains were not well adapted for colonizing the rearing water, and that in the salmon yolk-sac fry experiment in particular, this might be due to poor growth ability at the relevant rearing temperature. Furthermore, bacterial densities in samples taken at the start of the experiments indicated that for most of

the bacterial conditions, the target concentration of 5×10^5bacteria/mL was not met and was consistently lower. A possible explanation for this is the uncertainties in the determined relationships between OD_{600} measurements and CFU counts, which is discussed in detail in Section 4.4.

Some bacterial strains showed changes in densities which, considering the experimental conditions from which they where obtained, appeared unrealistic. For example, the 3.28 *(Microbacterium sp.)* had a final determined concentration of $7828.04 \pm 1515.53\ 10^4$ bacteria/mL in the salmon yolk-sac fry experiment. This represented an almost 3000-fold increase in bacterial concentration from what was found at the start of the experiment. In the zebrafish experiment, one could expect an even greater increase in bacterial concentration due to the higher rearing temperature, but here the same strain showed an increased concentration of just $1.37 \pm 0.27\ 10^4$ bacteria/mL. This probably indicates that at least some of the flow cytometry measurements of bacterial densities were erroneous. Due to the flow cytometer at ACMS being unavailable because of a technical issue, samples were analyzed by **KYTOS**. As a result of this, samples were collected according to what is the standard protocol at ACMS, which differs from the method of KYTOS, who have developed their own sampling system (KYTOvial). In processing of the raw data retrieved from KYTOS after analysis, some samples appeared over-diluted, as the event counts were especially low (Amalie Johanne Horn Mathisen, verbal communication). This introduces uncertainty into the measurements, apparent by the relatively high standard deviations observed for some bacterial conditions (see Table 3.2 and Table 3.3). The over-dilution made it challenging to distinguish between actual bacterial counts and background noise. Careful consideration should be taken when interpreting results from samples with low bacterial densities, such as those from the germ-free conditions of both gnotobiotic experiments.

4.3 Optimal DNA extraction methods differed for zebrafish and salmon yolk-sac fry tissue samples

In previous projects in the ACMS group, there had been challenges with obtaining consistent PCR amplification of bacterial 16S rRNA genes from samples of yolk-sac fry of salmon, probably due to low amounts of bacterial DNA relative to host DNA, and the presence of PCR inhibitors in the DNA extracts. Trials were therefore performed on a selection of samples from both the salmon yolk-sac fry and zebrafish gnotobiotic experiments, to investigate what were the optimal DNA extraction conditions for these samples, specifically focusing on the first lysis step.

Differing methodologies were shown to give best PCR results for zebrafish and salmon yolk sac fry. For salmon yolk-sac fry samples, the best PCR results were obtained when host tissue was removed before homogenization in Precellys homogenizer. This was consistent with what was found for Atlantic salmon skin tissue samples in a master project being performed concurrently to this work [65]. A plausible explanation for this could be that the presence of PCR inhibitors and large amounts of host DNA when including the yolk-sac fry in the homogenization step interferes with the subsequent PCR reaction. For zebrafish samples, the best PCR results was obtained from samples where homogenization was performed with host tissue present. The effects of host DNA and PCR inhibitors might not have come in to play to the same degree with zebrafish samples as they did with samples of yolk-sac fry of salmon, as the amount of host tissue was considerably smaller in samples. The average weight of zebrafish larvae at 6 dpf have been determined to be less than 2 mg, whilst newly-hatched yolk

sac-fry of salmon typically are > 200 mg [83, 84]. In the DNA extraction, there was one individual per replicate sample for salmon yolk-sac fry, and three for zebrafish. Due to the lower total amount of host tissue in zebrafish samples, it is also probable that the amount of bacterial DNA present in these samples was considerably lower than in salmon yolk-sac fry samples. This could explain why homogenization without zebrafish tissue resulted in less PCR products than what was observed for the salmon yolk-sac fry samples subjected to the corresponding homogenization protocol. The fact that undiluted samples were found to give the best PCR also implies that the amount of bacterial DNA generally was low in samples.

4.4 Bacterial community compositions in SynCom water and fish of the salmon yolk-sac fry experiment

Amplicon sequencing data of samples from the SynCom condition in the salmon yolk-sac fry experiment contained several highly similar ASVs which represented the different SynCom genera. It has been shown that polymerase errors become more common in later cycles of PCR [85]. This could lead to the presence of erroneous sequences in low abundances in the subsequent ASV assignment. In the 16S rRNA gene amplification performed in this work, a fairly high amount of PCR cycles (X38) were performed. Thus, a likely explanation to the observed diversity of ASVs representing SynCom genera could be that they in fact represented the same sequences, but errors introduced in the PCR amplification caused them to be assigned to different ASVs. For this reason, an OTU clustering was performed. In retrospect, an increased annealing temperature could have been considered, as this is known to increase stringency in PCR reactions [86]. It is important to note however, that the PCR reaction parameters used for 16S rRNA gene amplification in this work has been optimized throughout many amplicon sequencing-based projects at ACMS, and that they generally have resulted in high-quality sequencing data.

The resultant OTU table from OTU clustering revealed that nine OTUs accounted for 92.4 $\pm$ 7.7% of total reads in SynCom samples, and that all SynCom strains except for the *Sphingomonas sp.* and *Staphylococcus sp.* were represented within these nine OTUs. The remaining reads were accounted for by OTUs dispersed sporadically between samples in low abundances, probably representing noise derived from background levels of contaminating DNA from the experiment and the different steps performed in library preparation. Instead of *Sphingomonas sp.* and *Staphylococcus sp.*, OTUs assigned to *Oceanobacillus* and *Pseudomonas* were present among the nine most common OTUs. It is therefore probable that these strains were added in place of the *Sphingomonas* and *Staphylococcus* strains as intended. Both an *Oceanobacillus* and a *Pseudomonas* strain were part of the strain collection from which strains were obtained for this thesis, so there is a possibility that a labelling mistake from when the collection was first established led to the unintended substitution of strains. Sequencing of the selected strains 16S rRNA gene was not performed prior to experiments to confirm the strain identities. If this had been done, it could have revealed an accidental substition of strains.

The community profiles of the water collected at the start of the experiment revealed that although the intention was to add all strains in equal concentrations, relative abundances varied both within and between replicate samples. OTU1 and OTU24, representing *Psychrobacter* and *Oceanobacillus*, were particularly abundant. The relation-

ships between OD_{600} measurements and CFU counts were used to determine the amount of each bacterial strain that was added to the SynCom wells. Uncertainties in these relationships relationships could have led to differing strain concentrations used for establishing the SynCom condition. Differences in growth morphologies is a common source of error in CFU counts, as single colonies can arise from multicellular aggregates, giving an underestimation of the actual cellular density [87]. Similarly, cellular aggregates in liquid cultures will lead to inaccurate measurements of turbidity, as the assumed 1:1-correspondence between optical density and cell numbers are broken [87].

Another possibility could be that strains were actually added in more equal amounts than what the OTU-based community compositions suggests, but that PCR biases or differences in gene copy number of the 16S rRNA gene between strains skewered the relative abundances of OTUs [88]. Bacteria can have several copies of rRNA operons, varying from 1 – 15 between different taxa, and this is a common source of uncertainty in microbial community analyses based on 16S rRNA gene sequences [89]. The rrnDB is a database which contains annotated information on 16S rRNA gene copy numbers in prokaryotes [90]. The *Psychrobacter* species present in the rrnDB database had between three to six copies of the 16S rRNA gene, with a mean of 4.4. For *Oceanobacillus* species, only two species were present in the database. These had 16S rRNA gene copy numbers of seven and ten. However, according to the rrnDB database, several of the other SynCom strains which had lower relative abundances in the start water communities, also contained species with similarily high gene copy numbers. *Hafnia sp.* were for example registered with either seven or eight gene copies of the 16S rRNA gene in the rrnDB database, but the *Hafnia* OTU was still significantly less abundant in start water communities compared to the *Psychrobacter* and *Oceanobacillus* OTUs. Therefore, copy number was probably not the sole cause of the different relative abundances observed in these communities. PCR biases, including differences in priming efficacy, as well as the effect of template GC-content on amplification efficacy, are known sources of error when PCR approaches are used for inferring abundances of taxa in amplicon sequencing [88]. It is therefore plausible that such biases could have affected the observed abundances of OTUs in the SynCom samples. However, these biases would probably be conserved for the different strains in the different sample types, except perhaps for the fish tissue samples, where the presence of host DNA also could have an effect on PCR reaction conditions and outcome.

An additional OTU classified as *Microbacterium*, OTU175, also appeared in most SynCom samples. Manual inspection of the OTU table revealed that its pattern of relative abundance among the samples followed that of OTU8, the more abundant *Microbacterium* OTU, with highest relative abundances in end water samples. It is therefore likely that OTU175 represented a second gene copy variant of the 16S rRNA gene derived from the same *Microbacterium* strain. In the rrnDB database, genera from the *Microbacterium* family had 16S rRNA gene copy numbers ranging from one to three with an average of 2.0 [90], so it is possible that the *Microbacterium* strain used in this thesis could have at least two copies of the 16S rRNA gene, giving rise to the two *Microbacterium* OTUs observed.

In the end water samples, OTU6 *(Hafnia sp.)* was the most dominant OTU, and its average relative abundance had increased significantly compared to the start water samples. In the growth characterization experiment, the *Hafnia* strain had the highest μ_{max} out of all strains in the growth experiment at both temperatures, indicating that this strain

was better adapted to growth at temperatures relevant to rearing of salmon yolk-sac fry. *Hafnia sp.* are facultively anaerobic heterotrophs belonging to the Enterobacterales. Representatives of the genus are considered common members of aquatic microbiotas and the gut microbiotas of animals, including fish, and they are known psychrotrophs [91, 92]. Thus, it appears that the *Hafnia* strain was able to establish itself as the dominant strain in the water microbiota through the course of the experiment, enabled by its high μ_{max} at low temperatures. Similarly, OTU8 representing *Microbacterium* had a marked increase in relative abundance when comparing end water to start water. This was also one of the strains with the highest determined μ_{max} in the growth experiment at the lower temperature, only beaten by the *Hafnia* and *Arthrobacter* strains. The OTUs which initially dominated the water microbiota, OTU1 *(Psychrobacter)* and OTU24 *(Oceanobacillus)*, had significantly decreased relative abundances in the end water samples. Together, these results imply that during the course of the experiment, the *Hafnia* and *Microbacterium* strains were able to outcompete the initially dominant *Psychrobacter* and *Oceanobacillus* strains in the water microbiota, aided by their high μ_{max} and ability to grow on the nutrients provided by the presence of the yolk-sac fry host. Furthermore, it seems that a high growth rate in general growth media correlates with a strain's effectiveness in colonizing the salmon yolk-sac fry rearing water, potentially providing a competitive advantage in the investigated system.

The PCoA based on Bray-Curtis similarities revealed that the water microbiotas' composition in the SynCom condition had changed significantly during the course of the experiment (Figure 3.6). When wells are inoculated with the bacterial strains, this could be considered a batch system in which a system-specific selection regime containing a set of niches ready to be exploited by the bacteria is established [93]. The salmon yolk-sac fry supplies organic matter, nutrients, and, together with the rearing water, growth surfaces and environments, for which the bacterial strains will compete. Such a system has the potential to alter the bacterial community composition within the wells, as strains better adapted to the present niches will out-compete other, less well-adapted strains [93]. In other words, the observed alteration in community composition is probably driven by host-provided selection factors promoting competition for resources between the added bacterial strains.

Furthermore, it was also found that end water and fish tissue microbiotas were not significantly different, although their community compositions indicated that clear distinctions could be made between the two sample groups (Figure 3.6). This implies that the end water microbiota was more similar to the microbiota of fish tissue than to that of start water, and that the environmental microbiota had a strong influence on the microbiota of fish tissues. The importance of the environmental microbiota in shaping fish host associated microbiotas has also been noted in previous studies. For example, Fiedler et al. (2023) showed in a gnotobiotic experiment with salmon yolk-sac fry that whether fish was reared in the presence of egg-derived or lake-derived microbiota had a profound effect on the microbiota of both the host skin and gut, and that the effect was more pronounced in skin microbiota [33]. Similarily, Bakke et al. (2013) showed that rearing water microbiota had a greater effect than live feed microbiota on the community composition of host-associated microbiota in cod larvae *(Gadus morhua)* [94].

Fiedler et al. (2023) also reported that variation in community compositions was observed between individual salmon yolk-sac fry from replicate flasks with the same initial bacterial conditions, and that replicate rearing vessels developed distinct system-dependent microbiotas [33]. Similar variation in community structures was also ob-

served between replicate salmon yolk-sac fry of the SynCom condition. One replicate in particular had a microbiota more similar to end water microbiota. A revised one-way PERMANOVA analysis excluding this sample revealed that the remaining fish tissue samples had significantly different microbiota compositions compared to those of the end water samples ($p = 0.0321$). Together, this indicates that although the microbiota of water at the end of the experiment affected the fish microbiota compositions, clear distinctions between the two sample groups were still observable.

This is consistent with what was reported by Vestrum et al. (2020) in a study where the effects of ecological processes on bacterial community assembly in cod larvae were investigated [34]. Here, they found that environmental bacterial communities were dissimilar to those associated with the cod larvae, but that different environmental communities strongly affected the fish communities. Similarly, Bugten et al. found that changes in rearing water microbiota induced by membrane ultrafiltration in a pilot-scale RAS for rearing of Atlantic salmon parr directly influenced the parr hindgut microbiota, but that the communities remained distinct from one another [95]. These distinctions could be a result of host-provided selection factors, as different adaptations are required for growth on fish tissue compared to those needed to grow in the rearing water. Comparison of the community compositions at the family level supports this, as the relative abundance profiles of the two sample groups appear distinct from one another, apart from the one fish tissue sample which appeared to have a more similar microbiota composition to end water samples than to the remaining fish tissue samples (Figure 3.5). Vestrum et al. (2020) showed that stochastic processes such as ecological drift is important for creating variation in the initial community assembly of communities related to teleost hosts [34]. It is likely that such processes contributed to the observed variation in microbiota compositions of fish tissue samples also in this experiment.

4.5 Bacterial community compositions in the re-conventionalized conditions

In the re-conventionalized conditions, 236 ASVs were detected in the zebrafish experiment, and 517 ASVs in the salmon yolk-sac fry experiment. *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were the dominant phyla in the re-conventionalized conditions from both experiments. These phyla have also previously been found to be abundant in salmon yolk-sac fry, both in a small scale experiment were fry were reared in culture flasks in the presence of egg-derived microbiota [33], and in fry from a commercial hatchery facility [96]. In zebrafish, *Proteobacteria* and *Actinobacteria* have previously been suggested as members of a core microbiota [97]. *Bacteroidetes* have also been observed, although it is considered less common than the *Proteobacteria* and *Actinobacteria* [98, 97]. The re-conventionalized water microbiota composition at the start of the zebrafish experiment was found to vary more than what was expected between replicates. In the salmon yolk-sac fry experiment, the corresponding samples had the most similar community compositions between replicates (see Figure 3.8 and Figure 3.9). This fact, along with the great difference in ASV richness between the zebrafish and salmon yolk-sac fry re-conventionalized conditions, are indications that the water sampling method used in the zebrafish experiment did not work as intended (see Section 4.1.3). Fish tissue and end water microbiotas from the re-conventionalized conditions were more similar than the start and end water microbiotas, particularly in the salmon yolk-sac fry experiment. This supports what was found in the SynCom condition, and that has also been observed in previous studies, that the environmental microbiota strongly affects the composition of the host-associated microbiota [33, 94].

Although the overall α-diversity appeared to be higher in the salmon yolk-sac fry experiment, probably as a results of improper water sampling in the zebrafish experiment as discussed in Section 4.1.3, the evenness appeared relatively similar between corresponding sample types from the two experiments (Figure 3.7d). The microbial communities in both experiments, particularly the zebrafish experiment, started out with higher evenness, but ended up with one or a few families dominating communities in the end water and fish tissue microbiotas. In the zebrafish experiment, these families were *Pseudomonadaceae*, *Flavobacteriaceae*, and *Comamonadaceae*. However, some caution should be taken when interpreting these observations due to the suspected shortcomings of the water sampling method discussed previously. In addition, only two replicate end water samples remained after rarefaction. In the salmon yolk-sac fry experiment, end water and fish tissue microbiotas were also highly abundant in *Pseudomonadaceae* and *Flavobacteriaceae*, as well as *Comamonadaceae* and *Sphingomonadaceae*. *Sphingomonadaceae* specifically was only abundant in the end water, and not the fish microbiotas. Some consideration should also be taken for these results, as one replicate fish tissue sample was left out after rarefaction, and out of the three remaining replicates, one differed significantly in community composition from the other two (Figure 3.8b).

The predominant families observed in both experiments are all commonly observed in aquatic environments and fish microbiota, and typically represent rapid-growing aerobic heterotrophs, particularly the *Pseudomonadaceae* and *Comamonadaceae* [87, 99]. From the SIMPER analysis, it was revealed that on the ASV level, single ASVs assigned to *Pseudomonadaceae* and *Comamonadaceae* were dominant in each experiment (see Table 3.4). Manual inspection of the ASV table revealed that the two ASVs representing the *Pseudomonadaceae* in the two experiments had both been determined on the genus level above the confidence threshold level of 0.80 as *Pseudomonas*, showing that two distinct *Pseudomonas* strains were dominant in each experiment. Bates et al. have previously observed that representatives of *Pseudomonas* were dominant in the microbiota of zebrafish embryos and larvae [100]. Similarly to what was observed for the *Hafnia sp.* of the SynCom condition, it appears that the batch-like experimental system preferentially selects for fast-growing heterotrophs, which are able to rapidly utilize the nutrients provided by the presence of host organisms, leading to microbiotas dominated by such species. The observed increase in bacterial density measured by flow cytometry in both re-conventionalized conditions further supports this. For salmon yolk-sac fry fish tissue samples, two of the three replicates had communities which were dominated mainly by *Pseudomonadaceae*, whilst the last replicate's community was dominated by *Flavobacteriaceae* and *Comamonadaceae*, and had a comparatively low abundance of *Pseudomonadaceae*. Variation between replicate samples exposed to the same bacterial conditions has also previously been observed by Fiedler et al. (2023), and similar observations were also made in the SynCom condition, as discussed in Section 4.4 [33].

4.6 The adequacy of the gnotobiotic experimental systems

As already mentioned in the preceding sections, several observations were made in the SynCom condition of the salmon yolk-sac fry experiment, as well as in both of the re-conventionalized conditions, which suggested that the experimental conditions of the gnotobiotic experiments provided a selection regime in which rapid-growing het-

erotrophs ended up dominating the water and fish tissue microbiotas. In Vadstein et al. (1993) the ecological theory concepts of r/K-selection is used to describe how carrying capacity effects microbial community compositions in fish rearing systems, and how these concepts can be used as a tool to steer towards more favorable microbial conditions in such systems [101]. Here, the carrying capacity of a system is defined as the maximum microbial biomass that can be sustained, determined by the system's physical and nutrient-related restrictions [101]. Fast-growing, opportunistic heterotrophs are considered r-strategists. Due to their high growth rates and rapid nutrient turnover, r-strategists will quickly dominate systems were the bacterial load is below the carrying capacity and competition for resources is low. K-strategists are slower-growing, specialized bacteria who are well-adapted to highly competitive systems near or at carrying capacity, due to their higher substrate affinities compared to r-strategists [101]. Attramadal et al. (2014) states that K-selection is advantageous for the survival of reared fish, as it provides a more stable microbial condition that disfavours opportunistic (r-selected) bacteria that could cause detrimental host-microbe interactions [102]. Even though these bacteria might not be specific pathogens, it has been shown high abundances of r-strategists still could have detrimental effects on fish. For example, Vestrum et al. (2018) found that genes associated with infections were up-regulated in cod larvae were *Arcobacter* were abundant in the water and fish-associated microbiotas [103].

Typically, a re-conventionalized control condition is included in gnotobiotic experiments to represent the stable, microbial rearing conditions to which host organisms ideally would be subjected too in healthy rearing conditions or their natural environments [63]. However, when the bacteria of the re-conventionalized conditions were first introduced to the germ-free host organisms in the batch-like conditions of this experiment, this probably represented a sudden increase in the carrying capacity experienced by the bacteria, as they were introduced to a system containing empty niches. Such a change in carrying capacity could potentially open up for proliferation of rapid-growing opportunists, and the system could therefore be said to actually represent an r-selection regime. The fact that end water and fish tissue microbiotas in the re-conventionalized conditions of both experiments were dominated by only a few ASVs representing families typically associated with r-strategist bacteria, such as *Pseudomonadaceae*, supports this. In addition, the *Hafnia sp.* was dominant in end water and fish tissue microbiotas in the SynCom condition. This further indicates that the system is better described as an r-selection regime, as this strain had the highest determined μ_{max} in the growth characterization experiment. This could perhaps represent a more general issue with re-conventionalized controls used in short-term fish gnotobiotic experiments, as most fish gnotobiotic research systems where water exchange does not occur have a similar batch-like design to the systems used in this thesis, although at varying size scales [80]. It would mean that the applied condition in such experiments does not properly represent the natural, healthy microbial conditions as intended, but rather an r-selected, potentially detrimental, condition.

4.7 Future work and perspectives

In this thesis, microbial community dynamics in host-associated microbiotas have been investigated and compared in two gnotobiotic experiments of similar design using two different host organisms; zebrafish and salmon yolk-sac fry. Amongst other findings, it was shown that the experimental system design will have an impact on the investigated microbial community compositions. As a consequence of this, re-conventionalized con-

trol conditions might not actually represent a stable, healthy bacterial condition as is usually the intention. This demonstrates that there still are gaps in the current knowledge on the early bacterial colonization of newly hatched teleost larvae/fry, and the systems used to study such interactions. Considering the substantial loss to mortality in commercial hatchery facilities, often assigned to negative host-microbe interactions, continued efforts should be directed towards better understanding the effects of rearing selection regimes on host-associated microbiotas in the earliest life-stages of economically important teleost species.

Unfortunately, the planned assessment of host responses to the initial microbial colonization of the two host models were excluded from this thesis, due to an unforeseen delay in sample analysis at a third party. In addition, due to the contaminations observed in the zebrafish experiment, this experiment would have had to be repeated in order to perform comparisons betwen the two host organism's responses, had the gene expression analysis data been available. Still, the prospect of utilizing already established model organisms, such as zebrafish, to model host-microbiota interactions in economically important teleost species remains exciting. It has the potential to provide new insights into the intricate relationship between the teleost host and its microbiota. This could further contribute to improved fish health and knowledge-based decision making in the aquaculture industry.

To bring this prospect further, the comparability of host responses between the model organisms needs to be further investigated. Often, findings from different teleost host model systems are extrapolated to aquaculture species. To assume that results are directly comparable might not necessarily be straightforward, considering the enormous diversity within the teleost clade. However, it has already been shown that certain host responses to the presence of a microbiota is conserved on the gene expression level between zebrafish and mice [25]. As zebrafish and Atlantic salmon are more closely related species, it is thus likely that even more host responses are conserved between the two. Still, differences can be expected, and considerations such as the two species differing life cycles, or the fact that their gastrointestinal system has markedly different morphologies and functional capabilities, should be made. Instead of assuming that findings are true across the teleost division when proven in one specific species, a more systematic approach should be taken, investigating the comparability of responses of special interest.

In this context, there is further a potential for investigating the effects of specific bacterial taxa, which could potentially contribute to determining novel probiotic strains, and unveiling the mechanisms behind positive host-microbe interactions on a new level of detail. Currently, probiotic feeds and supplements for aquaculture species with documented effects are commercially available. However, such feeds are used in later life-stages, and must be provided continuously, as the effects are not retained over a prolonged period of time. As host-microbiota effects in the early life-stages of teleosts are linked to the development of the innate immune system, and since teleosts are considered sterile before hatching, it could be possible that the host microbiota is more easily modulated in this life stage, and that more long-term effects could be achieved with the development of probiotic treatments specifically for the earliest life-stages.

5 Conclusions

This study aimed to characterize the initial microbiota of newly hatched zebrafish *(Danio rerio)* and Atlantic salmon *(Salmon salar)* under gnotobiotic and re-conventionalized conditions, and to assess whether host-responses to various bacterial conditions were comparable between the two host organisms on the gene expression level. Unfortunately, due to an unforeseen delay in the analysis of samples prepared for gene expression analysis, these results were not returned in time for them to be included in this work. Still, the prospect of using an already established model organism to model microbiota-induced host responses in economically important fish species remains exciting.

Growth characteristics of nine bacterial strains previously isolated from the gut of commercially reared Atlantic salmon were determined at temperatures relevant for rearing of zebrafish larvae and yolk-sac fry of Atlantic salmon. A *Hafnia* strain was found to have the highest maximum growth rate at both of the investigated temperatures. This experiment showed that although strains were isolated from the same selection regime, their growth rates were variable, and the effect of temperature on maximum growth rate varied between strains.

Two gnotobiotic experiments were performed with the nine bacterial strains in zebrafish and salmon yolk-sac fry host model systems. The established host- and water-associated microbiotas were analyzed by 16S rRNA gene amplicon sequencing. Indications that bacterial contaminations were present in the germ-free and SynCom conditions of the zebrafish experiment was observed, as ASVs assigned to *Oxalobacteraceae* and *Comamonas*, respectively, were abundant in samples from these conditions. Furthermore, the water sampling method applied in the same experiment appeared to be unsuitedfor sampling microbiota.

In the Syncom condition of the salmon yolk-sac fry experiment, ASVs assigned to *Hafnia* and *Microbacterium* dominated end water and fish tissue microbiotas. These strains also had high maximum growth rates at the relevant temperature. In addition, the amplicon sequencing data indicated that the environmental microbiota had an influence on the host-associated microbiota, but clear distinctions between the two microbiotas could still be made, probably as a result of host-provided selection factors.

In the re-conventionalized conditions of the two gnotobiotic experiments, the evenness of the microbiota significantly decreased. This reduction was due to the dominance of one or a few ASVs representing rapid-growing, heterotrophic bacteria, such as *Pseudomonas*, in the final water and host-associated microbiotas. These results suggests that in short-term gnotobiotic experiments with batch-like system characteristics, such systems are better characterized as an r-selection regime, providing a competitive advantage to rapid-growing opportunistic bacteria.

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Supplementary Materials

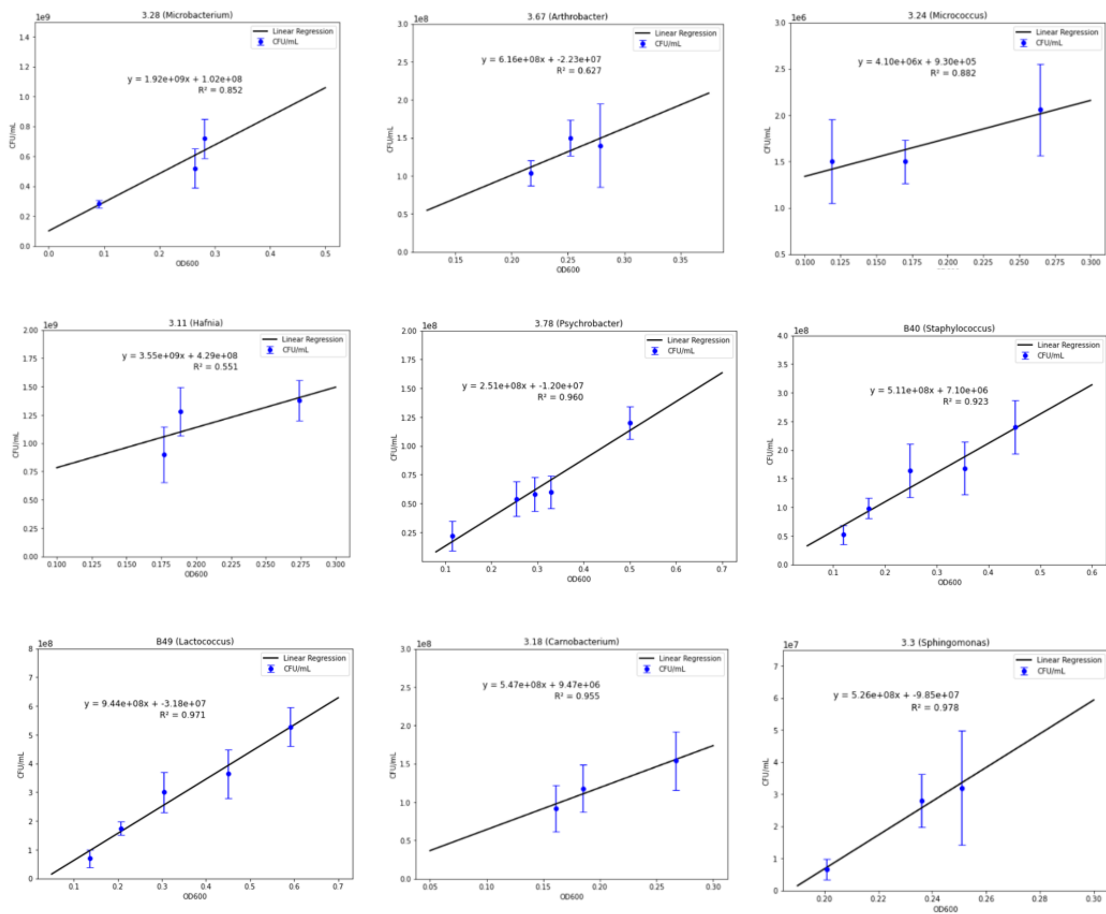
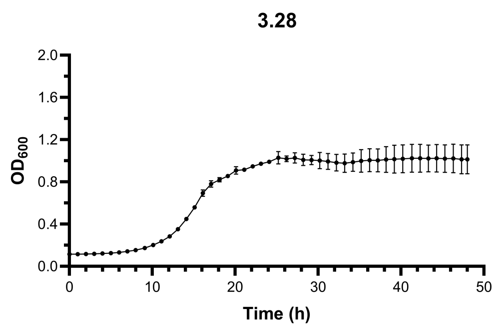


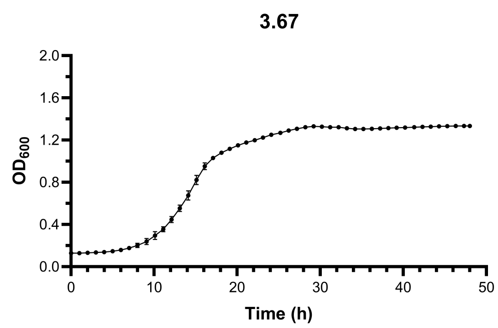
Table S.1: Standard curves for growth characteristics based on OD_{600}-measurements and CFU counts for the nine bacterial strains used in this thesis.

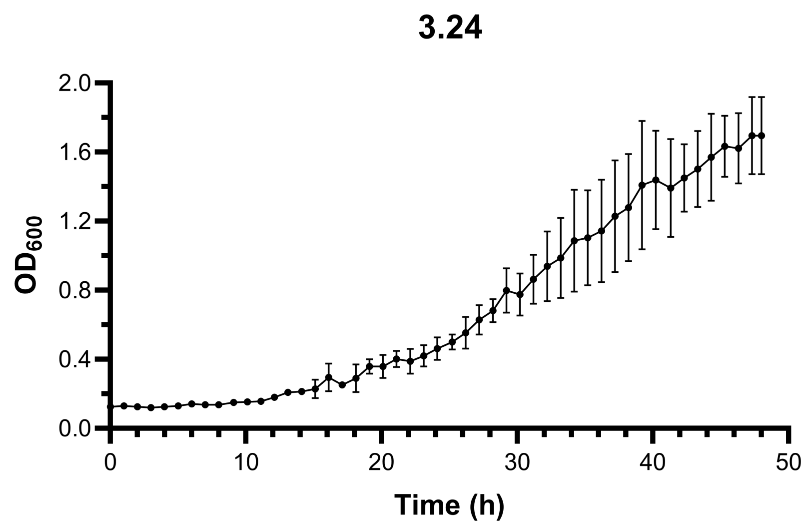
Table S.1: Samples that were discarded after rarefaction of ASV table to 4750 reads per sample. With the exception of one re-conventionalized water sample from the zebrafish experiment, and one re-conventionalized fish tissue sample from the salmon yolk-sac fry experiment, all discarded samples were germ-free control samples.

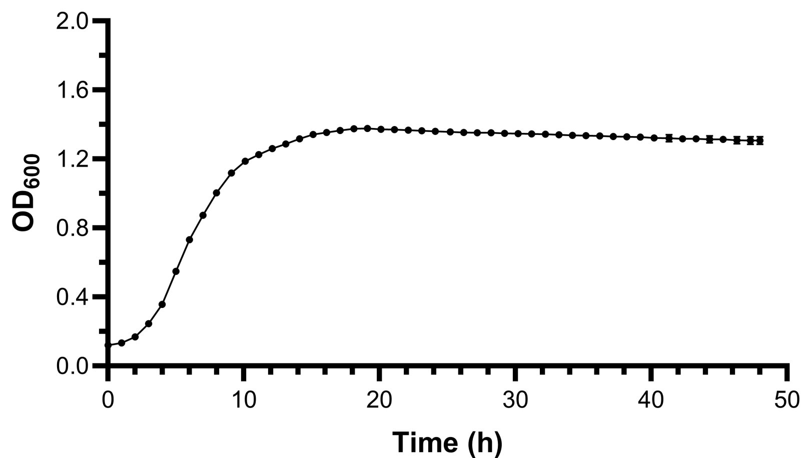
Zebrafish		Salmon yolk-sac fry	
Sample	**Number of reads**	**Sample**	**Number of reads**
Start water germ-free 2	14	Start water germ-free 3	948
Start water germ-free 3	1271	End water germ-free 4	1280
End water germ-free 3	209	Fish tissue germ-free 4	111
End water re-conventionalized 3	1146	Fish tissue re-conventionalized 2	7

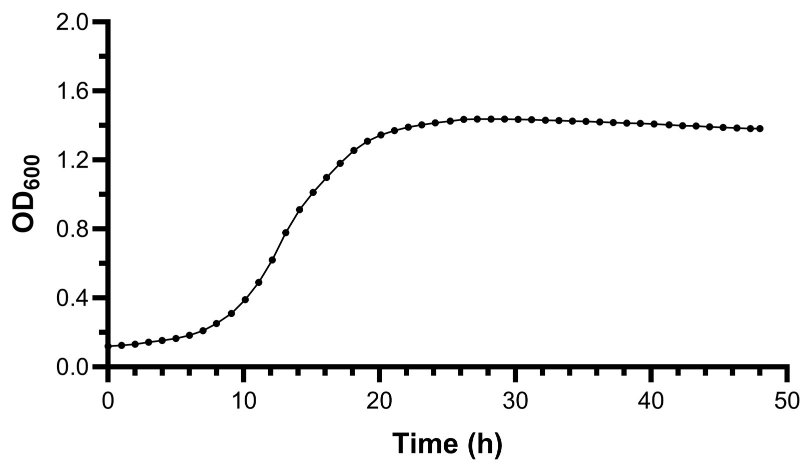
Growth curves based on OD_{600}-measurements at 22 °C for the nine bacterial strains used in this thesis.

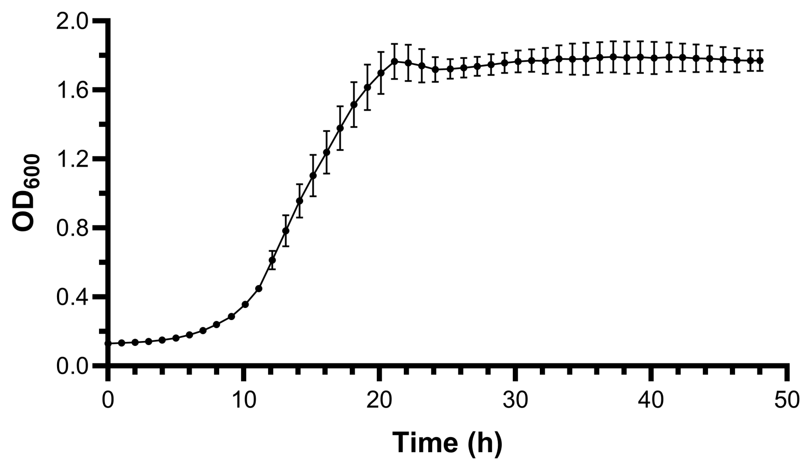


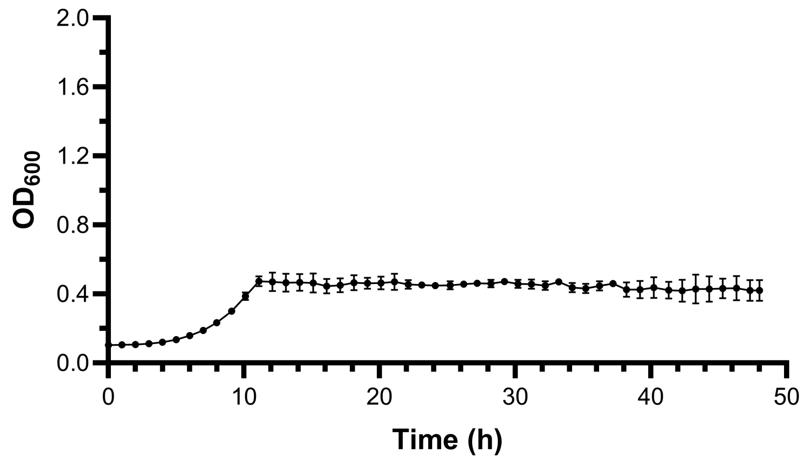


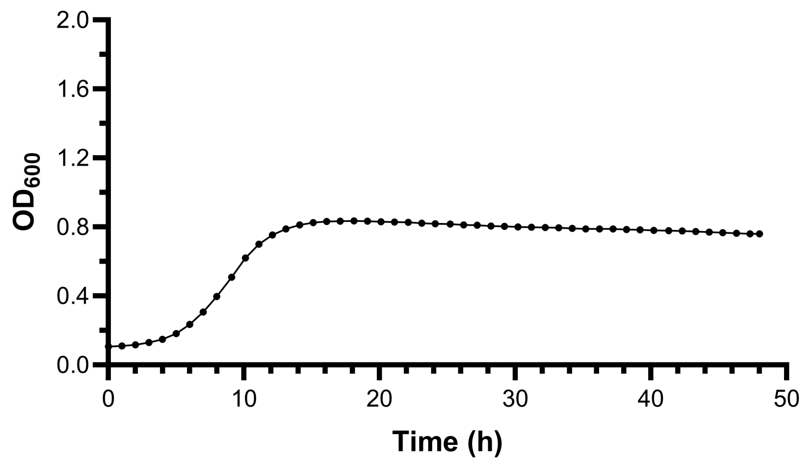


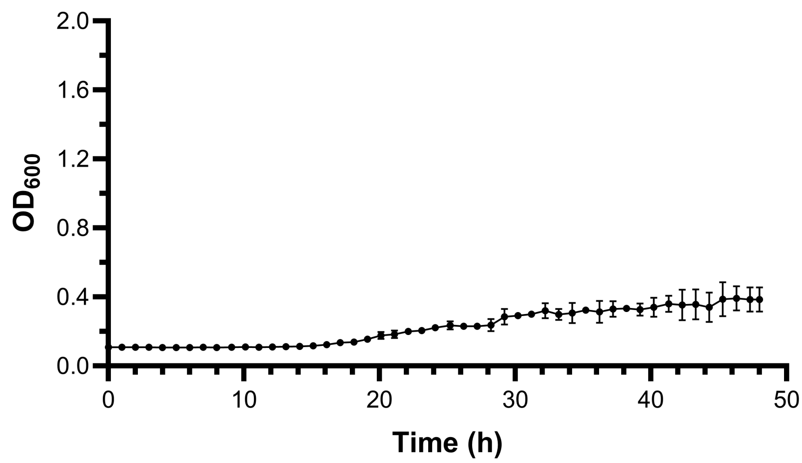

3.11
2.0
1.6
1.2
0.8
0.4
0.0
OD600
0
10
20
30
40
50
Time (h)


3.78
2.0
1.6
1.2
0.8
0.4
0.0
OD600
0
10
20
30
40
50
Time (h)

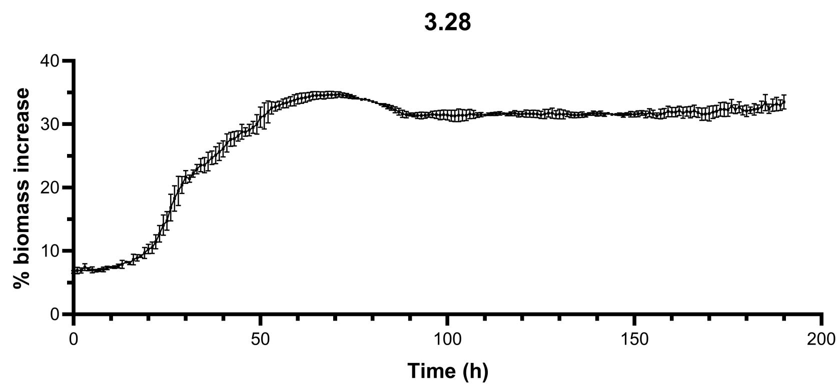

B40
2.0
1.6
1.2
0.8
0.4
0.0
OD600
0
10
20
30
40
50
Time (h)

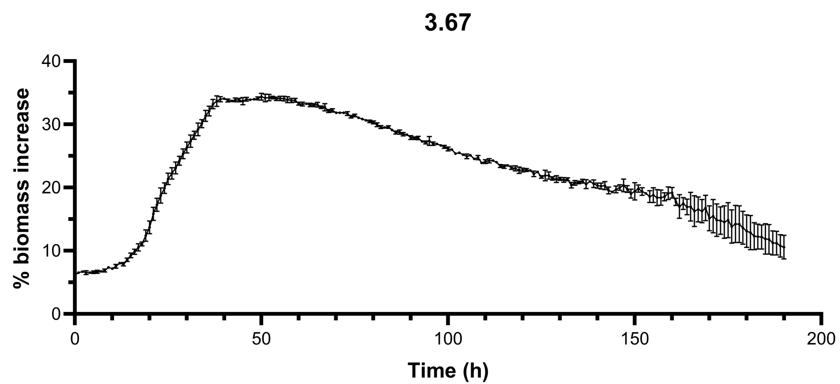

B49
2.0
1.6
1.2
0.8
0.4
0.0
OD600
0
10
20
30
40
50
Time (h)

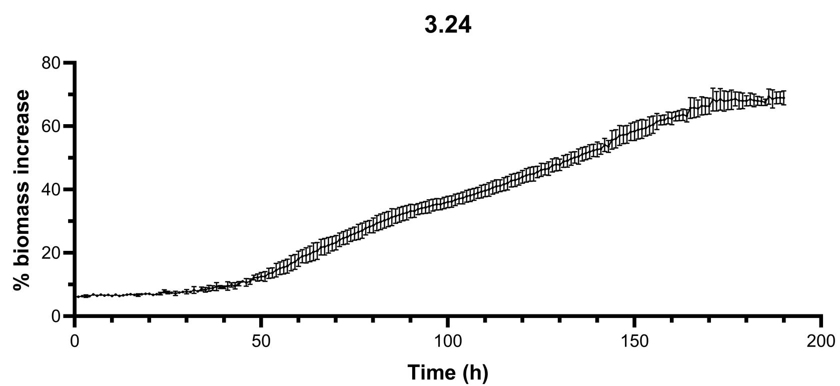

3.18
2.0
1.6
1.2
0.8
0.4
0.0
OD600
0
10
20
30
40
50
Time (h)

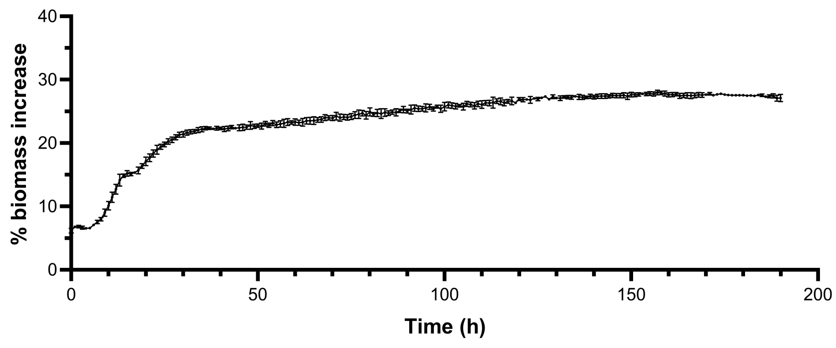

3.3
2.0
1.6
1.2
0.8
0.4
0.0
OD600
0
10
20
30
40
50
Time (h)

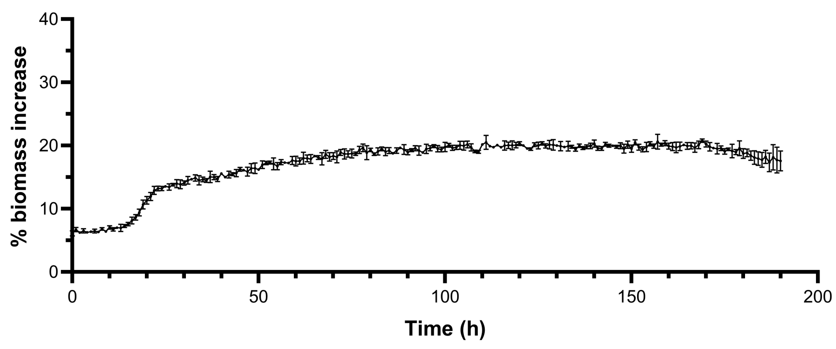
Growth curves based on biomass gain measurements at 14.4 ℃ for the nine bacterial strains used in this thesis.

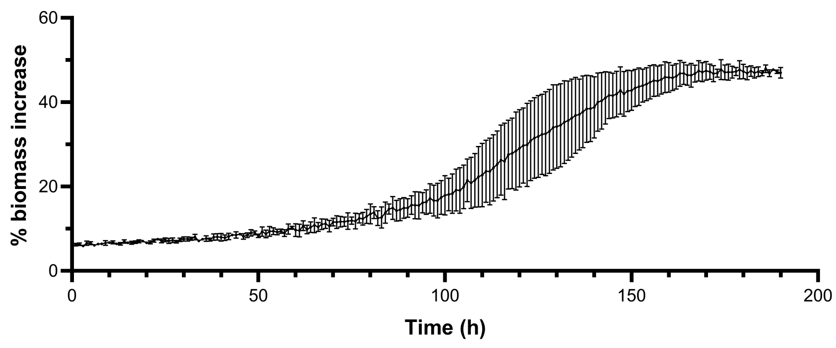


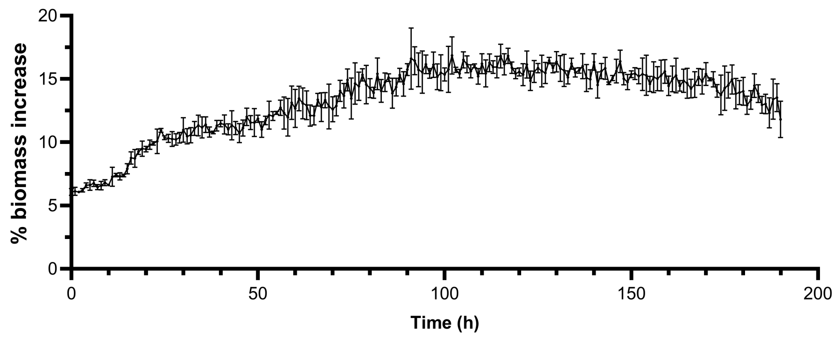


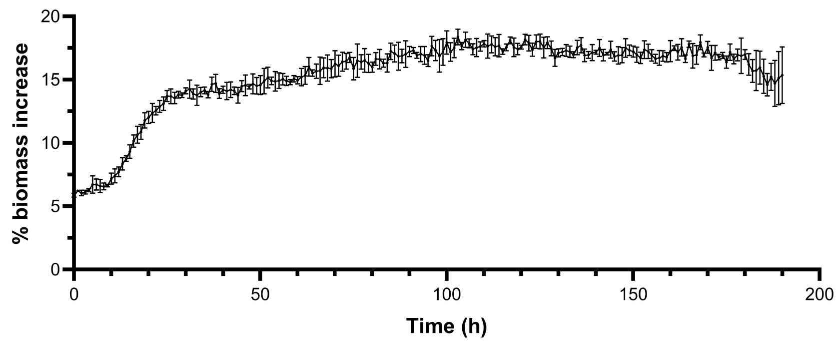


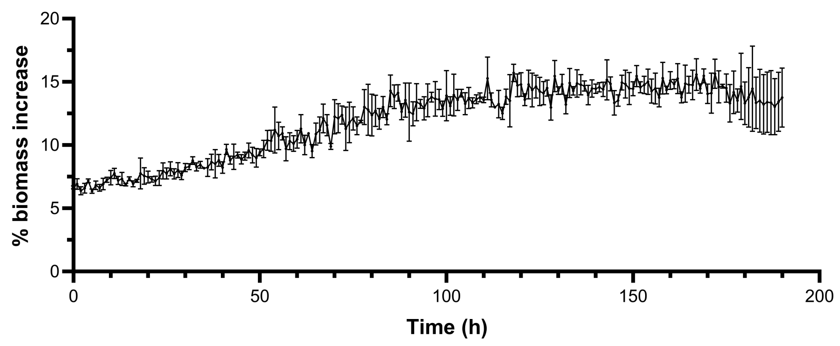

3.11
% biomass increase
Time (h)
0
10
20
30
40
50
100
150
200


3.78
% biomass increase
Time (h)
0
10
20
30
40
50
100
150
200


B40
% biomass increase
Time (h)
0
20
40
60
50
100
150
200


B49
% biomass increase
Time (h)
0
5
10
15
20
50
100
150
200


3.18
% biomass increase
20
15
10
5
0
0
50
100
150
200
Time (h)


3.3
% biomass increase
20
15
10
5
0
0
50
100
150
200
Time (h)

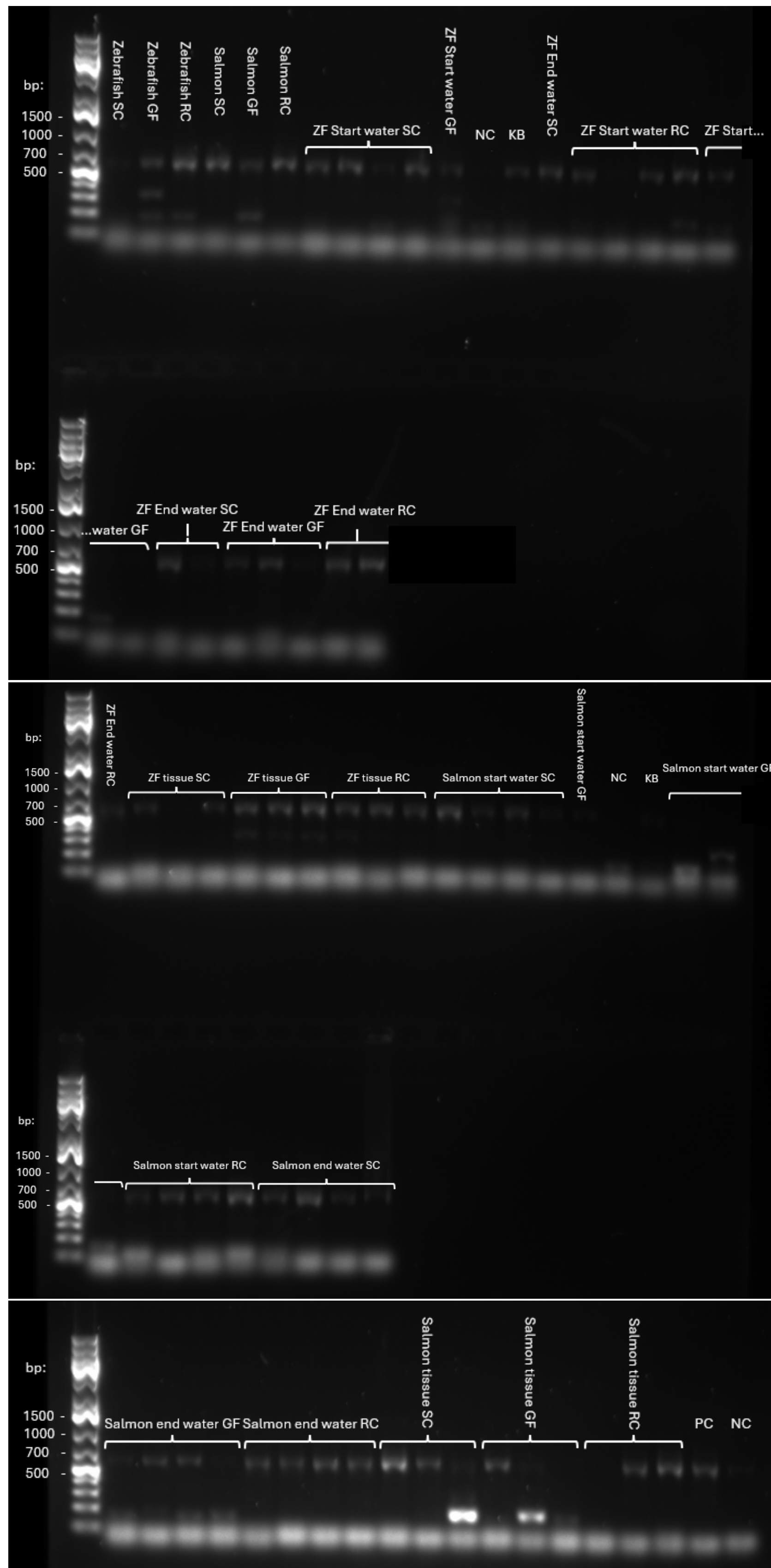


Figure S.2: Gel electrophoresis of PCR products of indexed 16S rRNA genes after indexing PCR at X10 cycles with Nextera XT indexing primers (Illumina™).

Table S.2:RNA concentrations and the A260/A280-ratio obtained after RNA extraction from fish tissue samples for the SynCom, germ-free, and re-conventionalized conditions obtained after the gnotobiotic experiment in zebrafish.

Sample	RNA concentration (ng/µL)	A260/A280
SynCom 1	15.4	1.73
SynCom 2	26.4	1.70
SynCom 3	18.1	1.74
SynCom 4	38.7	1.66
Germ-free 1	20.4	1.70
Germ-free 2	22.4	1.72
Germ-free 3	42.1	1.73
Germ-free 4	29.7	1.76
Re-conventionalized 1	48.3	1.60
Re-conventionalized 2	26.7	1.82
Re-conventionalized 3	13.9	1.73
Re-conventionalized 4	14.6	1.75

Table S.3: The taxonomic classification of the ten ASV's with the highest average presence in germ-free zebrafish samples. Eight germ-free zebrafish samples remained after normalization.

Taxonomic classification	**Average number of reads in zebrafish germ-free samples**	**Number of samples with ASV present (out of eight)**
f:*Oxalobacteraceae*	2510.75	Six
f:*Oxalobacteraceae*	545.25	Five
g:*Corynebacterium*	445.5	One
g:*Corynebacterium*	212	One
f:*Comamonadaceae*	202.5	Three
g:*Corynebacterium*	112.25	One
g:*Rhizobium*	78.75	Three
g:*Corynebacterium*	77.25	One
g:*Cutibacterium*	72.5	Two
g:*Flavobacterium*	65.5	Two

Table S.4: cDNA concentrations and the A260/A280-ratio obtained from cDNA synthesis from RNA extracts obtained from fish tissue samples for the SynCom, germ-free, and re-conventionalized conditions in the salmon yolk-sac fry gnotobiotic experiment. cDNA synthesis products were diluted 1:10 with nuclease-free water before concentration and purity measurements.

Sample	RNA concentration (ng/μL)	A260/A280
SynCom 1	194.5	1.99
SynCom 2	139.9	1.88
SynCom 3	181.5	1.92
SynCom 4	175.4	1.91
Germ-free 1	183.0	1.92
Germ-free 2	185.5	1.91
Germ-free 3	192.5	1.94
Germ-free 4	160.4	1.91
Re-conventionalized 1	158.3	1.90
Re-conventionalized 2	99.8	1.79
Re-conventionalized 3	104.8	1.78
Re-conventionalized 4	117.8	1.84

Table S.5: ASV table showing the 25 ASVs with the highest average abundance in samples from the zebrafish SynCom condition. ASVs representing genera which where part of the SynCom are highlighted in bold.

OTU ID	Start water	Start water	Start water	Start water	End water	End water	End water	Fish tissue	Fish tissue	Fish tissue	Fish tissue	Taxonomy (0.8)
Zotu11	0	0	0	0	0	0	3165	4383	4683	4456	4346	d:Bacteria,p:Proteobacteria,c:Betaproteobacteria,o:Burkholderiales,f:Comamonadaceae,g:Comamonas
Zotu9	0	0	0	0	3974	4079	5	0	1	73	0	d:Bacteria,p:Proteobacteria,c:Betaproteobacteria,o:Burkholderiales,f:Comamonadaceae
Zotu43	0	1867	1449	348	0	2	4	0	0	0	0	d:Bacteria,p:Firmicutes,c:Bacilli,o:Bacillales,f:Bacillaceae 1,g:Peribacillus
Zotu1	**0**	**199**	**1019**	**1296**	**158**	**50**	**327**	**1**	**2**	**0**	**1**	**d:Bacteria,p:Proteobacteria,c:Gammaproteobacteria,o:Pseudomonadales,f:Moraxellaceae,g:Psychrobacter**
Zotu32	2230	0	0	0	0	0	0	0	0	0	0	d:Bacteria,p:Proteobacteria,c:Gammaproteobacteria,o:Gammaproteobacteria incertae sedis,g:Marinicella
Zotu54	0	1848	0	0	0	0	0	0	0	0	0	d:Bacteria,p:Bacteroidetes,c:Flavobacteriia,o:Flavobacteriales,f:Flavobacteriaceae,g:Flavobacterium
Zotu30	**1**	**0**	**1445**	**158**	**1**	**1**	**9**	**0**	**0**	**0**	**0**	**d:Bacteria,p:Firmicutes,c:Bacilli,o:Lactobacillales,f:Carnobacteriaceae,g:Carnobacterium**
Zotu39	1591	0	0	0	0	0	0	0	0	0	0	d:Bacteria,p:Proteobacteria,c:Gammaproteobacteria,o:Thiotrichales,f:Thiotrichaceae,g:Leucothrix
Zotu61	895	0	0	0	0	0	0	0	0	0	0	d:Bacteria,p:Proteobacteria,c:Gammaproteobacteria,o:Thiotrichales,f:Thiotrichaceae,g:Leucothrix
Zotu10	**0**	**0**	**0**	**25**	**12**	**41**	**580**	**0**	**0**	**0**	**42**	**d:Bacteria,p:Actinobacteria,c:Actinobacteria,o:Micrococcales,f:Microbacteriaceae,g:Microbacterium**
Zotu70	0	0	0	669	0	1	0	0	0	0	0	d:Bacteria,p:Actinobacteria,c:Actinobacteria,o:Propionibacteriales,f:Propionibacteriaceae,g:Cutibacterium
Zotu31	**0**	**497**	**0**	**40**	**0**	**2**	**8**	**0**	**0**	**0**	**0**	**d:Bacteria,p:Actinobacteria,c:Actinobacteria,o:Micrococcales,f:Micrococcaceae,g:Arthrobacter**
Zotu33	0	158	81	272	0	0	2	0	0	0	0	d:Bacteria,p:Firmicutes,c:Bacilli,o:Bacillales,f:Bacillaceae 2,g:Oceanobacillus
Zotu154	**0**	**0**	**0**	**357**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**d:Bacteria,p:Firmicutes,c:Bacilli,o:Bacillales,f:Staphylococcaceae,g:Staphylococcus**
Zotu48	**0**	**0**	**0**	**262**	**8**	**8**	**51**	**0**	**0**	**0**	**0**	**d:Bacteria,p:Actinobacteria,c:Actinobacteria,o:Micrococcales,f:Micrococcaceae,g:Micrococcus**
Zotu13	0	0	0	268	4	15	13	0	0	2	0	d:Bacteria,p:Proteobacteria,c:Gammaproteobacteria,o:Enterobacterales,f:Hafniaceae
Zotu7	0	118	0	41	25	26	45	1	0	1	0	d:Bacteria,p:Proteobacteria,c:Gammaproteobacteria,o:Enterobacterales,f:Hafniaceae
Zotu197	0	0	0	225	0	0	0	0	0	0	0	d:Bacteria,p:Actinobacteria,c:Actinobacteria,o:Micrococcales,f:Dermacoccaceae,g:Dermacoccus
Zotu305	0	0	210	0	0	0	0	0	0	0	0	d:Bacteria,p:Proteobacteria,c:Alphaproteobacteria,o:Caulobacterales,f:Caulobacteraceae,g:Brevundimonas
Zotu361	0	0	0	0	0	0	0	208	0	0	0	d:Bacteria,p:Proteobacteria,c:Gammaproteobacteria,o:Pseudomonadales,f:Moraxellaceae,g:Acinetobacter
Zotu151	0	0	190	0	0	0	0	0	0	8	0	d:Bacteria,p:Proteobacteria,c:Alphaproteobacteria,o:Caulobacterales,f:Caulobacteraceae,g:Brevundimonas
Zotu168	**0**	**0**	**137**	**15**	**0**	**2**	**3**	**0**	**0**	**0**	**0**	**d:Bacteria,p:Firmicutes,c:Bacilli,o:Lactobacillales,f:Streptococcaceae,g:Lactococcus**
Zotu81	0	0	0	123	0	5	15	0	0	0	0	d:Bacteria,p:Firmicutes,c:Bacilli,o:Bacillales,f:Bacillaceae 1,g:Bacillus
Zotu82	**0**	**0**	**0**	**119**	**3**	**8**	**4**	**0**	**0**	**0**	**0**	**d:Bacteria,p:Firmicutes,c:Bacilli,o:Lactobacillales,f:Streptococcaceae,g:Lactococcus**
Zotu628	0	0	0	0	0	0	0	0	0	129	0	d:Bacteria,p:Actinobacteria,c:Actinobacteria,o:Micrococcales,f:Microbacteriaceae

Appendices

A - Media solutions

A.1 Tryptic soy broth (TSB)

Composition of tryptic soy broth (TSB) media used for plating bacteria in various experimental sections.

Component	Amount
Tryptic Soy Broth powder	22.5 g
Milli-Q® water	750 mL

A.2 Tryptic soy agar (TSA)

Composition of tryptic soy agar (TSA) media used for plating bacteria in various experimental sections.

Component	Amount
Tryptic Soy Broth powder	22.5 g
Agar powder	11.25 g
Milli-Q® water	750 mL

A.3 Zebrafish Gnotobiotic medium with and without antibiotics (ZGM and AB-ZGM)

Hank's salt stock solutions were already prepared in advance in the Derome lab [104]. The media was prepared as described by Pham et al [62]. For filter sterilization, Nalgene™ RapidFlow™ 0.2 µm filter units were used. For the AB-ZGM, antibiotic stock solutions as described by Pham et al. were also already prepared in the Derome lab [62]. For these, Filtropur 0.2 µm syringe filters (Sarstedt®) were used.

Composition of 1 L Zebrafish Gnotobiotic Medium (ZGM) with and without antibiotics used for rearing of zebrafish.

Component	**Amount**
NaCl	0.8 g/L
KCl	0.04 g/L
Na_2HPO_4	0.0358 g/L
KH_2PO_4	0.06 g/L
$CaCl_2$	0.1872 g/L
$MgSO_4$-$7H_2O$	0.246 g/L
$NaHCO_3$	0.35 g/L
Milli-Q® water	950 mL
- - - - - - - - - - - -	
→ Filter sterilize	1L total volume
Antibiotics for AB-ZGM	**Amount**
Ampicillin stock	50 µL
Kanamycin stock	5 µL
Amphotericin B stock	100 µL
ZGM	49.6 mL
- - - - - - - - - - - -	
→ Filter sterilize	

A.4 Salmon Gnotobiotic Medium (SGM)

Sol Gómez de la Torre Canny, modified from on US EPA/600/4-90/027F artificial water recipe

Salt Stocks

All salt stocks are prepared in fish-only 1L glass bottles pre-autoclaved with a stir bar inside. Also, use fish-only graduated cylinders.

$MgSO_4 \cdot 7H_2O$ 100X
Dissolve 12.3 g in 1 l. Autoclave.

KCl 100X
Dissolve 0.4 g in 1 l. Autoclave.

$NaHCO_3$ 100X
Dissolve 9.6 g in 1 l. Filter sterilize.

$CaSO_4 \cdot 2H_2O$ 5X
Dissolve 0.3 g in 1 L..
This salt takes a few hours to go into solution. I usually prepare the solution and leave it ON in stirring. Once dissolved, autoclave

SGM prep

$MgSO_4 \cdot 7H_2O$ 100X	10 ml
KCl 100X	10 ml
$NaHCO_3$ 100X	10 ml
$CaSO_4 \cdot 2H_2O$ 5X	200 ml
Miiq H_2O	770 ml

	1000 ml

Prepare in pre-autoclaved 1 L fish-only glass bottles and use for measuring the water and the $CaSO_4 \cdot 2H_2O$ 5X fish-only graduated cylinders.
Autoclave prepared SGM and cool down to RT before storing in the fish room.
NOTE: Sometimes the salts go out of solution, after autoclaving and because of the low temperature. Just shake the bottle before the water change. Flasks containing fish have a slightly lower pH so the salt will not precipitate after the water change.

A.5 Antibiotic Salmon Gnotobiotic Medium (AB-SGM)

Antibiotics (ABX) Stock Preparation

Rifampicin (Rif) x

(557303-1, VWR)
Powder stored in locked chemical cabinet
Stock: 50 mg/ml in DMSO
Dissolve 1000mg of powder in 20 ml of DMSO.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.
NOTE—To facilitate dissolving, I recommend splitting powder from original vial, shake at room temperature, and make sure to diffuse any clumps of powder at the bottom of the vial before adding DMSO. Shaking at RT for about an hour helped getting powder into solution.

Kanamycin (Kan) x

(420311-5, VWR)
Powder stored in locked chemical cabinet
Stock: 50 mg/ml in H_2O
Dissolve 1000mg of powder in 20 ml of filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

PenicillinG (PenG) x

(TCIAP1772-25G, VWR)
Powder stored in locked chemical cabinet
Stock: 100 mg/ml in H_2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Ampicillin (Amp)

Powder stored in locked fridge
(171254-5, VWR)
Stock: 100 mg/ml in H_2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Oxolinic acid (Ox) x

(J66637.06, VWR)

Powder stored in the chemical fridge
Stock: 12,5 mg/ml in 0,05N NaOH
Dissolve 1000mg of powder in 80ml of 0,05 N NaOH.
NOTE—0,05 N NaOH was prepared by diluting filter-sterilized 1N NaOH with filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Amphotericin B (Fun)

Stock: 250 µg/ml
Note Alexander Fiedler:
In Spring 2021 I ordered it as powder (P4030-250MG, VWR)
Powder stored in locked fridge
Dissolve x in DMSO
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Erythromycin (Ery) x

(329815-5, VWR)
Powder stored in locked fridge
Stock: 50 mg/ml in 90% EtOH
Dissolve 1000 mg of 20 ml of 96% OH.
NOTE—96% EtOH was prepared by diluting absolute EtOH in filtered/autoclaved mqH_2O (19,2 ml of EtOH + qs 20 ml mqH_2O=
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Antibiotic cocktail preparation

Antibiotic	Working concentration	Volume of stock to add to 1 L SGM
Rifampicin	10 mg/l	200 µl
Erythromicin	10 mg/l	200 µl
Kanamycin	10 mg/l	200 µl
Ampicillin	100 mg/	1000 µl
Amphotericin B	250 ug/l	1000 µl
Penicillin	150 mg/l	1500 µl
Oxolinic acid	22.5 mg/l	1800 µl

NOTE: All these operations should be performed at room temperature. Fish room temperature is too cold for one of the antibiotic stocks to remain liquid.
Eirik: We added 3 times oxolinic acid concentration (1800 µl), however flavobacterium seemed to survive. Thus the next time we used 5 times oxolinic acid (3000µl 37.5 mg/l) which seemed to work fine.

Materials (e.g. for ~20 ABX cocktails)

- 1 SGM bottle.
- 20x 50 ml conical vials.

- 50 ml conical rack.
- 10 ml serological pipette.
- 1000 µl tips and pipette.
- Vaccumm filter unit

1. Thaw the ABX stocks in advance.
2. Prepare laminar flow cabinet by inserting all materials needed in the hood after surface sterilizing them with 70% ethanol. Once the materials are in the hood, expose them to UV light for 30 minutes.

NOTE: Do not irradiate Abx with UV light! Just surface sterilize the Eppendorff tubes or conical vials with Ethanol before inserting in the hood

3. Add the ABX stocks following the table to a sterile SGM bottle inside of the laminar flow cabinet.

4. Filter sterilize the solution Abx cocktail and aliquot 45 ml in sterile 50 ml conical vials.
5. Freeze the aliquots or freshly made Abx work well for derivations.

NOTE: Upon thawing, there will be a white precipitate in the ABx. This does not affect the ability of this solution to inhibit microbial growth.

B - Salmon embryo derivation protocol

02.01.19; Sol Gómez de la Torre Canny; Modified by Alexander Fiedler on the 20.01.21

Material checklist

Some material needs to be prepared and/or ordered in advanced:

- 50 ml serological pipettes (sterile, individually wrapped).
- 10 ml serological pipette (sterile, individually wrapped).
- 50 ml conical vials (sterile).
- Salmon Gnotobiotic Media (SGM, Annex 1) enough at least for the derivation process and for the first few days of water exchanges.
- Antibiotic cocktail in salmon gnotobiotic media (AB-SGM, Annex 2) enough for the embryo dishes received (2 x 40 ml aliquots per 100 embryos).
- 140 mm Petri dishes.
- 250 ml tissue culture flasks.
- Buffodine.
- p1000, p100, and p20 tips and pipette.
- Autoclaved 500 ml glass bottles for embryo rinsing.
- A few sleeves of TSA plates.
- 96-well plates.
- Plating beads.

Day -3 to 0—Material prep

1. Make sure you have enough TSA plates for the cfu counts of hatchery input community, as well as for the cfu counts of the conventionalization water (CVZ-H_2O)
   Note AWF: We stopped counting the hatchery input and the CVZ-H_2O in 2019 since the counts were always very low
2. For the pre-derivation SGM: 4 TSA plates per 100 embryos upon arrival; 4 TSA plates per 1 L bottle for the CVZ-H_2O; 2 TSA plates per flask and 2 TSA plates per 1 L bottle of autoclaved CVZ-H_2O

Day 0—Material preparation

3. Thaw the antibiotic cocktail aliquot (ABX) at room temperature (RT) first thing in the morning.

4. When thawed, transfer to fish room to equilibrate at FRT 6-8° C O/N.

Day 1—SGM acclimation

Atlantic salmon eggs should be received ~1 week before the expected hatching date. Perform all egg manipulations in the "dirty" counter of the fish room after wiping its surface with ethanol.

Material list for dirty bench

- 1 large Petri dishes per 100 embryos.
- 1 set of single use sterile plastic forceps.

1. According to the experimental design, determine how many dishes will be derived and which will remain as "conventionally raised" (CVR).
2. Upon arrival, transfer the salmon embryos to "giant" Petri dishes (140 mm diameter). Transfer ~100 embryos per dish per dish.
3. Add ~75 ml of sterile SGM, which is enough volume to cover the embryos completely without overflowing the Petri dish. Gently swirl embryos to mix them with the media.

Day 2— Antibiotic (ABx) pulse and colony forming unit (cfu) counts of hatchery bacterial input.

The antibiotic pulse should be performed as early as possible to allow the full 24 hours of incubation prior to the derivation on Day 3.
Also, On Day 2 we start with the preparation of the hood for day 3.

Material list for hood
50 ml rack.
50 ml conical vials x (N +1 Petri dishes).
50 ml serological pipets x (N + 1 Petri dishes).
1 bottle of sterile SGM for the CVR water change.
1 x 96 well plate.
1 conical vial with sterile SGM for dilutions.
4 TSA plates x Petri dish with 100 embryos.
2 tube of sterile glass beads
20 µl and 100 µl tips and pipette.
Waste bucket.
2 x 40 ml AB-SGM aliquot per 100 embryos
Note AWF: The following steps can also be performed on the fish bench outside of the hood (except for the CFU determination).

4. Prepare laminar flow cabinet by inserting all materials needed in the hood after surface sterilizing them with 70% ethanol. Once the materials are in the hood, expose them to UV light for 30 minutes.
5. Collect 50 ml of sterile SGM into a sterile conical vial for preparing dilutions. (**Note AWF**: Only needed if you determine the input CFU numbers)
6. For the "giant" Petri dishes that correspond to the GF treatment: Transfer 50 ml of the media in which the embryos were suspended to a sterile conical vial for CFU determination and remove the remaining media with the same pipette.
7. Add the ABx cocktail aliquot (2 x 40 ml; refer to Annex 2)
8. Incubate for 24 h at 6 – 7° C in fish room.
9. For plates that correspond to the CVR treatment: Transfer 50 ml of the media in which the embryos were suspended to a sterile conical vial for CFU determination and remove the remaining media with the same pipette. Fill up the plate with SGM
   NOTE: This may reduce the bacterial load but we need to normalize the water exchanges.
10. Label the TSA plates, and add a small amount of sterile glass beads. Organize them according to the sample and the dilution.
11. Prepare the dilutions by adding 100 µl of undiluted sample (well 1), and 90 ul of sterile SGM (Well 2 to 12). Do a serial dilution of 10 µl starting at the undiluted sample
12. Plate 80 µl of each dilution.
    Note AWF: Step 10-12 are only needed when the hatchery CFU input is determined

NOTE: For the hatchery INPUT, I usually prepare 3 dilutions (D0= undiluted; D1 = 1:10 dilution; D2= 1:100 dilution; and D3=1:1000 dilution)

Preparation of hood for Day 3

13. After starting the antibiotic pulse and performing the cfu count of the input, clean the hood properly, emptying it with exception of the pipettes and the Drummond pipette. Surface sterilize the surface and expose to UV light for 30 minutes.
14. Insert materials in the hood, in as many rounds of UV exposure as necessary:

Material list for the first session (see below):

- 2x 3l autoclaved plastic beakers (to be filled with serological pipettes after being exposure to UV light individually)
- 5 l big waste plastic beaker
- 1000 µl pipette tips (1 box)

NOTE:
The 3l plastic beakers can fit up to 70 x 10 ml serological and 30 x 50 ml serological pipettes. N is the number of GF flasks in the experiments

- 50 ml serological pipettes x N/3 +2 → use one 50 ml pipette for preparing three buffodine conicals

- N GF fish flask
- 2 racks for 50 ml conicals.
- N sterile conical vials
- SGM bottles (1 bottle is roughly enough for 6 GF flasks)

Day 3— Derivation

Because of the large amount of materials that need to go in the hood, I split the work in 2 sessions of UV sterilization. In the first session, I dispense the SGM in the tissue culture flasks where the fish will be housed; as well as in the conical vials where the disinfection solution will be prepared. On the second session I proceed with the derivation itself

1st session

1. Prepare laminar flow cabinet by inserting all materials needed in the hood after surface sterilizing them with 70% ethanol. Once the materials are in the hood, expose them to UV light for 30 minutes. Because of the size of the hood, I recommend to work with 20 flasks at a time.

1. Aliquot 50 ml of SGM in each conical vial

NOTE: Measure carefully here, since this will contain the Buffodine solution.

2. Remove 250 µl of SGM.

NOTE: Buffodine has 1.11% w/w Iodine (~11 g/l). The manufacturer suggests a 1:100 dilution of the buffodine (110 mg/l final concentration). We prepare a 1:200 dilution (0.25 ml Buffodine in 50 ml final volume). We therefore get a Buffodine solution of 50 mg/l of available iodine. Sol tested the concentrations and 50 mg/l should be fine

3. Fill flasks with ~100 ml SGM by carefully pouring from the bottle, and label flasks according to your experimental design.

2nd session

1. Prepare laminar flow cabinet by inserting all materials needed in the hood after surface sterilizing them with 70% ethanol. Once the materials are in the hood, expose them to UV light for 30 minutes.

Materials (derivation of N flasks)

- 2 autoclaved glass bottles of 500 ml (for collecting disinfection solutions during rinses)

- N sterile conical vials (for aliquot the embryos from big Petri dishes)
- N/3 +2 forceps → use one forceps for not more than three flasks; the +2 are backup
- N/4 bottles of SGM (~200 ml SGM needed per flask → one SGM bottle is enough for around 4.5 flasks)
- N/3 +2 forceps → use one forceps for not more than three flasks
- N + 2 10 ml pipettes

2. After the UV, insert the 140 mm petri dishes containing the embryos with AB-GSM and an aliquot of Buffodine into the hood after surface sterilize with 70% Ethanol (insert only one petri dish at a time into the hood to avoid crowding)

3. Finish preparing the disinfection solution by adding 250 µl of Buffodine to prepare a solution of 50 mg/l available Iodine.

4. Transfer 15 (+2 to account for lysed/abnormal embryos) embryos using a pair of single use sterile plastic forceps to an empty conical vial.

5. Add the disinfecting solution and incubate for 30 minutes, occasionally gently rolling the conical vials, and maintaining the conical vials flat to prevent crushing.

NOTE SOL:
To prevent incubating embryos in Buffodine for longer than required, you can stagger the flasks leaving 2 minutes in between each disinfectant solution addition (3-4 min in the begging until you get the practice after a while).
I recommend disinfecting a triplicate at a time. If you have many triplicates to disinfect, you can add disinfection solution maybe within 15 minutes of this incubation count another triplicate of flasks, and add the PVP-I solution to this new triplicate right before you finish the incubation of the last triplicate. This way you can disinfect 6 flasks per hour.

Note AWF: I take three minutes between each flask. The disinfection solution should be in the flasks for 30 min. So every three minutes I add the solution to a new conical with eggs until I am at the 10th conical after 27 minutes. Then I do the rinsing for all the 10 conicals that I prepared in the 30 minutes, so that after 1 h I have disinfected 10 flasks. I recommend working in packs of 20, so to set 20 flasks under the hood, prepare all of them and then add new material under the hood for 20 more flasks.
Pay attention to the order at which you added the disinfection solution.

1. Do a quick rinse by decanting the disinfecting solution into a PVP-I 500 ml autoclaved bottle and pipet out any remnant of the Buffodine solution using a 10 ml pipette. Add one whole volume of sterile SGM. This should happen as fast as possible

2. Do three additional rinses by decanting the SGM and removing the media with a 10 ml serological pipette (four rinses with a total of 200 ml SGM as recommended by the manufacturer).

NOTE SOL:
To do this, I just use one 10ml serological pipette per triplicate. It is important to remove as much Buffodine solution as well as the rinses. Take care to not suck in liquid into the electrical pipette. Use filtered pipettes!

3. Transfer the surface disinfected embryos to the flask where they will be housed for the remaining of the experiments by decanting the conical vial after the last rinse.

Day 7 and onward — Water exchanges and flask maintenance

When the fertilized eggs are received from Aquagen or any other provider, usually a predicted hatching day can be calculated. We usually received them between 8 – 12 days before the hatching.

Materials (per 15)

- *One 50 ml serological pipettes per fish flask*
- *X bottles of SGM, each flask will get 60 ml of SGM. One bottle is enough for 15-16 flasks*
- *Waste bucket.*

1. Prepare laminar flow cabinet by inserting all materials needed in the hood after surface sterilizing them with 70% ethanol. Once the materials are in the hood, expose them to UV light for 30 minutes.
2. Remove 60 ml of the SGM in the flask using a serological pipette.
3. Pour 60 ml from the bottle to the flask

NOTE:
If you are maintaining a concentration constant, you can instead of pouring the sterile SGM from the bottle use a 50 ml serological pipette.

Remember to:

1. Perform 60% SGM changes on Monday, Wednesday, and Friday. Water changes can start on the first Monday after derivation (depending on the day of the

procedure), unless that means that there will be more than 2 days between the derivation and the water change.
2. Avoid popping the yolks of the eggs. The first couple of weeks after hatching the embryos are particularly vulnerable to damage.
3. Remove dead embryos as soon as possible (look for paleness, absence of heartbeat, haemorrhage).
4. To remove embryos, you can use a 10 ml serological pipette and apply gentle pressure to suck the end of the yolk sack.
5. Recount the number or embryos after derivation. Remember, it is easier to count balls than to count swimming larvae. You need this number to calculate the percentage of hatch embryos to determine the experimental hatching day.
6. Once embryos start to hatch (near the predicted hatching date) it may be necessary to record the number of hatched embryos during the weekend, or between water changes. You can record the number of hatched and dead embryos on the bottle itself. Remember to use ethanol resistant marker!!!
7. Fish room temperature should be recorded at the water change. Ideally, the temperature should remain between 6-7°C.
8. Fish room should be kept in the dark, except for the time that the laminar flow cabinet is on.
9. When performing the water changes, always start with the germ-free flasks. To prevent cross contamination, SGM bottles used for the exchange of conventionally raised flasks, should not be used for germ free flasks.

Day 8 to 12, depending on the developmental stage—Post-derivation sterility

When the fertilized eggs are received from Aquagen or any other provider, usually a predicted hatching day can be calculated. Hatching of farmed embryos is quite synchronous, which is not the case for wild embryos. I have adopted as a convention to mark the “experimental hatching day” as the day in which all the flasks have reached 60% hatching. I perform the first sterility test on this day to verify the sterility of the GF flasks before continuing to manipulate the microbial conditions in the flasks as early as 1wpH.

Materials per flask

- 1 pre-autoclaved borosilicate tube with 3 ml of brain heart infusion (BHI, blue caps)
- 1 pre-autoclaved borosilicate tube with 3 ml of Saboraud dextrose broth (SabDex, red caps)
- 1 pre-autoclaved borosilicate tube with 3 ml of nutrient broth (NB, green caps)
- 1 pre-autoclaved borosilicate tube with 3 ml of glucose yeast medium (GY, yellow caps)
- 2 TSA plates.

In addition to the material per flask, we need one **positive control (**any CVR flask) one **negative control** (salmon gnotobiotic media, SGM) and one **mock control** (un-innoculated). These means three sets of media/plates, in addition to the sets for the experimental flasks.

- Tips for 200 µl pipette
- Rack for 16 mm tubes
- SGM bottle for the negative control

1. Prepare laminar flow cabinet by inserting all materials needed in the hood after surface sterilizing them with 70% ethanol. Once the materials are in the hood, expose them to UV light for 30 minutes.
2. Surface sterilize the flasks and the SGM bottle and insert them in the hood.
3. Sample 100 µl from each flask and inoculate the tubes and the plates.
4. Incubate the tubes with liquid media and one of the TSA plate at RT. Incubate the second TSA plate at fish room temperature (FRT) for 1 month. Inspect for growth at 1 week post inoculation (wpi) and 4 wpi for slow growers.

Note AWF: For the last sterility check (before the infection for me), I incubated one set of liquid media at RT and one set at FRT. In addition you can use the flow cytometer to determine whether the flasks are GF.

WRITE DOWN HERE HOW TO DO THE FLOW!!

C - ZymoBIOMICS™ 96 MagBead DNA kit protocol

Protocol

Sample Lysis

For all mixing steps: pipette mix or shake at max speed.
Note: *Shaking speed will depend on sample volume and plate well depth. Use of shaker plates will require user optimization.*

1. Add sample to the **BashingBead™ Lysis Module** using the table below:
   a. If using **ZymoBIOMICS™ BashingBead™ Lysis Rack (0.1 & 0.5 mm, D6002-96-7)**, add 550 µl **ZymoBIOMICS™ Lysis Solution**.
   b. If using **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)**, add 750 µl **ZymoBIOMICS™ Lysis Solution**.

Note: DNA/RNA Shield *has been provided to optionally replace* ***ZymoBIOMICS Lysis Solution*** *to improve DNA integrity.*

Sample Type	Maximum Input
Feces	100 mg
Soil	100 mg
Liquid Samples[1] and Swab Collections[2]	250 µl
Cells (Suspended in PBS)	5-20 mg (wet weight) (2 x 10^8 bacterial and 2 x 10^7 yeast cells)
Samples in DNA/RNA Shield™,[3]	≤ 800 µl

Note*: For samples stored and lysed in* ***DNA/RNA Shield™ Lysis Tubes****, do not add ZymoBIOMICS™ Lysis Solution and proceed to Step 2.*

2. When using the ZymoBIOMICS™ BashingBead™ Lysis Rack (0.1 & 0.5 mm, S6002-96-7), it is recommended to seal the rack with the provided sealing foils. Seal the foils to the rack using a heat sealing device set at 180°C for 4 seconds. Refer to Appendix E for more detailed guidance.

3. Secure in a bead beater fitted with the appropriate holder assembly for your bead beating module and process using optimized bead beating conditions (speed and time) for your device (Appendix D).[4]

   Optional Stopping Point: *Following Step 3 is the best stopping point if breaking up the work is needed. Samples post lysis can be stored for several hours at room temperature or can be stored at – 80 °C for long term storage.*

4. Centrifuge the **BashingBead™ Lysis Module**:
   a. If using **ZymoBIOMICS™ BashingBead™ Lysis Rack (0.1 & 0.5 mm)**, centrifuge at ≥ 4,000 x *g* for 5 minutes.
   b. If using **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)**, centrifuge at ≥ 10,000 x *g* for 1 minute.

5. Transfer up to 200 µl of the deep-well block (not provided). Add 600 µl **ZymoBIOMICS™ MagBinding Buffer.**

   Note: *For samples with excessive amounts of solid particulate, centrifuge at 4,000 x g for 5 minutes to reduce clogging.*

6. Dispense 25 µl of **ZymoBIOMICS™ MagBinding Beads** to each well. Mix well by pipette or shaker plate for 10 minutes.

   Note: *ZymoBIOMICS MagBinding Beads settle quickly, ensure that beads are kept in suspension while dispensing.*

7. Transfer the 96-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.

8. Dispense 500 µl of **ZymoBIOMICS™ MagBinding Buffer** and mix well by pipette or shaker plate for 1 minute.

9. Transfer the 96-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.

10. Dispense 500 µl of **ZymoBIOMICS™ MagWash 1** and mix well by pipette or shaker plate for 1 minute.

11. Transfer the 96-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.

12. Dispense 900 µl **ZymoBIOMICS™ MagWash 2** and mix well by pipette or shaker plate for 1 minute.

    Note: *If high speed shaker plates are used, dispense 500 µl ZymoBIOMICS™ MagWash 2.*

13. Transfer the deep-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.

14. Repeat the wash (Steps 12-13).

15. Transfer the 96-Well Block onto a heating element (55°C) until beads dry (approximately 10 minutes). If no heating element is available, air dry for approximately 20-30 minutes.

16. Dispense 50 µl of **ZymoBIOMICS™ DNase/RNase Free Water** to each well and re-suspend beads. Mix the beads well for 10 minutes and then transfer the plate onto the magnetic stand for 2-3 minutes until the beads pellet[5].

17. Transfer the supernatant (containing the eluted DNA) to a clean elution plate or tube[6].

    The eluted DNA can be used immediately for molecular based applications or stored ≤ -20°C for future use.

D - SequalPrep™ Normalization Plate (96) kit protocol

SequalPrep™ Normalization Plate (96) Kit

Catalog no: A10510-01 Store at room temperature (15–30°C)

Contents and Storage

The components included with the SequalPrep™ Normalization Plate (96) Kit are listed in the table below. Sufficient reagents are included to perform 10 × 96 purification/normalization reactions. Upon receipt, **store all components at room temperature (15–30°C)**. Store plates for up to 6 months.

Components	Quantity
SequalPrep™ Normalization Plate (96)	2 bags of 5 plates each
SequalPrep™ Normalization Binding Buffer	40 ml
SequalPrep™ Normalization Wash Buffer	50 ml
SequalPrep™ Normalization Elution Buffer (10 mM Tris-HCl, pH 8.5)	40 ml

Description

The SequalPrep™ Normalization Plate Kit allows simple, one-step, high-throughput amplicon purification and normalization of PCR product concentration (2–3 fold range) via a limited binding capacity solid phase. Each well of the SequalPrep™ Normalization Plate can bind and elute ~25 ng of PCR amplicon. Eluted PCR amplicon can be subsequently pooled and subjected to a variety of massively parallel sequencing analyses. The SequalPrep™ Normalization Plate is compatible with any automated liquid handling workstations without the need for shakers, magnets, or vacuum. The SequalPrep™ Normalization Plate Kit when used with SequalPrep™ Long PCR Kit provides a complete PCR enrichment and amplicon normalization system that is designed to complement amplicon sequencing workflows such as next-generation sequencing.

The conventional next generation sequencing workflows require laborious sample prep methods consisting of amplicon purification, quantitation, and manual normalization to adjust amplicon concentration. The SequalPrep™ Normalization Plate Kit eliminates the tedious amplicon quantitation and manual normalization steps.

SequalPrep™ Normalization Plate Kits utilize ChargeSwitch® Technology that provides a switchable surface charge depending on the pH of the surrounding buffer to facilitate nucleic acid purification. Under low pH conditions, the positive surface charge of the ChargeSwitch® coating binds the negatively charged nucleic acid backbone. Proteins and other contaminants (such as short oligonucleotide primers) are not bound and are simply washed away.

System Overview

The SequalPrep™ Normalization Plate Kit is a solid phase, high-throughput amplicon purification and normalization system in a 96-well plate format. PCR products (5–25 µl) are added to a SequalPrep™ Normalization Plate well and mixed with the Binding Buffer. DNA binding to the plate is performed at room temperature for 1 hour. The wells are washed with Wash Buffer to efficiently remove contaminants. Purified PCR products are eluted using 20 µl Elution Buffer at normalized concentrations.

System Specifications

Starting Material:	At least 250 ng PCR product (amplicon) per well
DNA Fragment Size:	100 bp to 20 kb
Elution Volume:	20 µl
DNA Yield:	Up to 25 ng per well
Normalization Range:	2–3-fold
Plate Dimensions:	Standard SBS (Society for Biomolecular Screening) footprint, semi-skirted 96-well plate
Plate Capacity:	0.2 ml

Accessory Products

The following products may be used with the SequalPrep™ Normalization Plate Kit. For details, visit www.invitrogen.com.

Product	Quantity	Catalog no.
SequalPrep™ Normalization Wash Buffer	4 × 50 ml	A10510-03
SequalPrep™ Long PCR Kit with dNTPs	1,000 units	A10498
Platinum® PCR Supermix	100 reactions	11306-016
Platinum® PCR Supermix High Fidelity	100 reactions	12532-016
Quant-iT™ PicoGreen® dsDNA Assay Kit	1 kit	P7589
PureLink™ Foil Tape	50 tapes	12261-012
E-Gel® 96 gels 1% (or 2%)	8 gels	G7008-01 (G7008-02)

Part no: 100003531 Rev. date: 5 May 2008

General Guidelines

- Wear a laboratory coat, disposable gloves, and eye protection when handling reagents and plate.
- Always use proper aseptic techniques when working with DNA and use only sterile, DNase-free tips to prevent DNase contamination.
- If you are using only part of the plate for DNA purification, cover unused wells with the Plate Seal and leave them attached while purifying DNA in the other wells. The plates can be stored at room temperature for up to 6 months.
- The SequalPrep™ Normalization Plates are compatible for use with automated liquid handling workstation; the workstation must be capable of handling and manipulating 96-well plates.
- If you are using automated liquid handling workstations for purification, you may need additional Wash Buffer depending on your type of workstation. See previous page for Wash Buffer ordering information.

Generating PCR Amplicon

You can generate the PCR amplicon using a method of choice. General recommendations for generating PCR amplicons are listed below:

- To obtain the best results, we recommend using the SequalPrep™ Long PCR Kit with dNTPs (page 1) which provides a robust system for long-range, high-fidelity PCR for use in next-generation sequencing applications.
- Other commercially available PCR supermixes and enzymes such as Platinum® PCR Supermix (page 1), Platinum® PCR Supermix High Fidelity (page 1), or equivalent are suitable for use.
- Perform PCR in a separate plate. **Do not** use the SequalPrep™ Normalization Plate to perform PCR.
- You need at least 250 ng amplicon per well to use with the SequalPrep™ Normalization Plate (see below).

Sample Amount

To achieve robust normalization, we recommend adding at least 250 ng/well of amplicon. This input amount is easily achieved using only a fraction of most PCR amplification reactions. An average efficiency PCR (20 µl reaction volume) produces product in the range of 25–100 ng/µl, allowing you to purify 5–10 µl using the SequalPrep™ system.

Elution Options

Depending on the nature of the downstream application and target nucleic acid concentrations desired, the SequalPrep™ kit offers the flexibility to elute purified DNA in a variety of options.

The **standard elution** method described in the protocol below is designed to elute purified DNA from each well using 20 µl elution volume to obtain each amplicon at a concentration of 1–2 ng/µl.

The **optional sequential elution** method is designed to sequentially elute multiple rows or columns using the same 20 µl of elution buffer to obtain higher amplicon concentrations. The amplicon concentrations will be additive as sequential wells are eluted. For example, dispense 20 µl of elution buffer into the first column (A1–H1), mix well, and incubate for 5 minutes at room temperature. Then, simply move this column of elution buffer to the next column (A2–H2), and again incubate for 5 minutes. Continue this step to obtain your specific elution needs for the downstream application of choice.

Materials Needed

- PCR reactions containing amplicons of the desired length (see **Generating PCR Amplicon**, above)
- DNase-free, aerosol barrier pipette tips
- *Optional:* automated liquid handling workstation capable of handling and manipulating 96-well plates
- *Optional:* PureLink™ Foil Tape (see previous page)

Binding Step

1. Transfer the desired volume of PCR product (5–25 µl PCR reaction mix, at least 250 ng amplicon/well) from the PCR plate into the wells of the SequalPrep™ Normalization plate.
2. Add an equivalent volume of SequalPrep™ Normalization Binding Buffer.

   For example: To purify 10 µl of PCR product, add 10 µl SequalPrep™ Normalization Binding Buffer.
3. Mix completely by pipetting up and down, or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate.
4. Incubate the plate for 1 hour at room temperature to allow binding of DNA to the plate surface. Mixing is not necessary at this stage.

   Note: Incubations longer than 60 minutes do not improve results. However, depending on your workflow you may perform overnight incubation at room temperature for the binding step.
5. **Optional:** If >25 ng DNA/well yield is desired, transfer the amplicon/Binding Buffer mixture from Step 4 to another, fresh well/plate to sequentially bind more DNA. Perform DNA binding at room temperature for 1 hour.

   Note: After binding is complete, you can remove the amplicon/Binding Buffer mixture from the well and store at –20°C for up to 30 days to perform additional purifications at a later time.
6. Proceed to **Washing Step**, next page.

Washing Step

1. Aspirate the liquid from wells. Be sure not to scrape the well sides during aspiration.

   Note: If you wish to store the amplicon/Binding Buffer mixture for additional purifications at a later time, aspirate the liquid from wells into another plate and store at –20°C for up to 30 days.

2. Add 50 µl SequalPrep™ Normalization Wash Buffer to the wells. Mix by pipetting up and down twice to improve removal of contaminants.
3. Completely aspirate the buffer from wells and discard.

   To ensure complete removal of wash buffer and maximize elution efficiency, you may need to invert and tap the plate on paper towels depending on the pipetting technique or instrument used. A small amount of residual Wash Buffer (1–3 µl) is typical and does not affect the subsequent elution or downstream applications.

4. Proceed to **Elution Step**, below.

Elution Step

Review **Elution Options** (previous page).

1. Add 20 µl SequalPrep™ Normalization Elution Buffer to each well of the plate.

   Note: Do not use water for elution. If you need to elute in any other buffer, be sure to use a buffer of pH 8.5–9.0. If the pH of the buffer is <8.5, the DNA will not elute efficiently.

2. Mix by pipetting up and down 5 times or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate. Ensure that the buffer contacts the entire plate coating (up to 20 µl level).
3. Incubate at room temperature for 5 minutes.
4. Transfer and pool the purified DNA as desired or store the eluted DNA at 4°C (short-term storage) or –20°C (long-term storage) until further use.

Expected Yield and Concentration

The expected DNA concentration is 1–2 ng/µl when using 20 µl elution volume. The expected DNA yield is ~25 ng/well normalized.

Optional: DNA Quantitation

The SequalPrep™ Normalization Plate Kit is designed to eliminate the quantitation and manual dilution steps typically performed for normalization in next-generation sequencing workflows. You can pool the eluted amplicon and use the pooled amplicons directly for your downstream applications without DNA quantitation.

However, if your downstream application requires DNA quantitation, you may determine the yield of the eluted amplicon using Quant-iT™ PicoGreen® dsDNA Assay Kit (page 1). We **do not** recommend using UV spectrophotometric measurements (A_{260}/A_{280} nm), as this method is inaccurate for low DNA concentrations.

Downstream Applications

The SequalPrep™ Normalization Plate Kit is designed to produce purified PCR products with normalized concentrations and substantially free of salts and contaminating primers. PCR amplicons purified from this system can be used individually or pooled in any downstream application for which normalization is an important sample preparation criterion such as next generation sequencing applications.

Pooled amplicons purified using the SequalPrep™ Normalization Plate Kit have produced successful data from massively parallel sequencing-by-synthesis on the Illumina/Solexa Genome Analyzer indicating that the amplicon purity is suitable for other next-generation sequencing platforms (Roche/454 FLX, Applied Biosystems SOLiD™ system). For detailed sample preparation guidelines, refer to the instrument manufacturer's recommendations.

Continued on next page

Troubleshooting

Problem	Cause	Solution
Low DNA yield	Insufficient starting material	Be sure to input at least 250 ng amplicon per well for best results.
	PCR conditions not optimal	Check amplicon on gel to verify the PCR product prior to purification. Use SequalPrep™ Long Polymerase (page 2) for best results.
	Incorrect binding conditions	Be sure to add an equivalent volume of SequalPrep™ Normalization Binding Buffer, mix completely, and incubate for 1 hour during the Binding Step.
	Incorrect elution conditions	Use 20 µl SequalPrep™ Normalization Elution Buffer for elution and ensure that the buffer contacts the entire plate coating (up to 20 µl level). **Do not** use any water for elution.
DNA degraded	DNA contaminated with DNase	Follow the guidelines on page 2 to prevent DNase contamination.
Poor normalization	Insufficient starting material	Be sure to input at least 250 ng amplicon per well for best results.
	Inconsistent pipetting or handling	Avoid introducing bubbles while pipetting and do not scratch the plate surface while pipetting. To avoid pipetting inconsistencies, we recommend using automated liquid handling workstations.
	Incorrect binding conditions	Be sure to add an equivalent volume of SequalPrep™ Normalization Binding Buffer, mix completely, and incubate for 1 hour during the Binding Step.
	Too much (>3 µl) wash buffer remaining	Completely remove wash buffer and if needed, invert and tap the plate on paper towels to remove any remaining wash buffer.

Quality Control

The Certificate of Analysis provides quality control information for this product, and is available by product lot number at www.invitrogen.com/cofa. Note that the lot number is printed on the kit box.

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E - Amicon®Ultra Centrifugal Filter protocol

Required Equipment

Centrifuge with fixed angle rotor that can accommodate 1.5 mL microcentrifuge tubes

CAUTION: To avoid damage to the device during centrifugation, check clearance before spinning.

Suitability

Preliminary recovery and retention studies are suggested to ensure suitability for intended use. See the "How to Quantify Recoveries" section.

Device Storage

Store at room temperature.

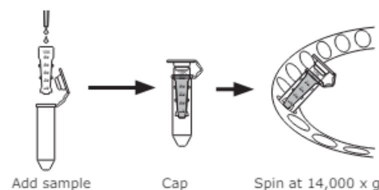
Prerinsing

The ultrafiltration membranes in Amicon® Ultra-0.5 devices contain trace amounts of glycerine. If this material interferes with analysis, pre-rinse the device with buffer or Milli-Q® water. If interference continues, rinse with 0.1 N NaOH followed by a second spin of buffer or Milli-Q® water.

CAUTION: Do not allow the membrane in Amicon® Ultra filter devices to dry out once wet. If you are not using the device immediately after pre-rinsing, leave fluid on the membrane until the device is used.

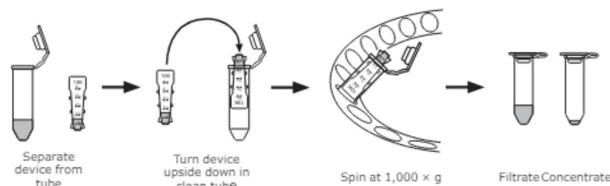
How to Use Amicon® Ultra-0.5 Centrifugal Filter Devices

1. Orient membrane panel facing up.
2. Insert the Amicon® Ultra-0.5 device into one of the provided microcentrifuge tubes.
3. Add up to 500 µL of sample to the Amicon® Ultra filter device and cap it.
4. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.
5. Spin the device at 14,000 × g for approximately 10–30 minutes depending on the MWCO of the device used. Refer to Figure 1 and Table 2 for typical spin times.



6. Remove the assembled device from the centrifuge and separate the Amicon® Ultra filter device from the microcentrifuge tube.
7. To recover the concentrated solute, place the Amicon® Ultra filter device upside down in a clean microcentrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.

   NOTE: For optimal recovery, perform the reverse spin immediately.



F - Trizol™reagent user guide

invitrogen USER GUIDE

TRIzol™ Reagent

Catalog Numbers 15596026 and 15596018

Doc. Part No. 15596026.PPS Pub. No. MAN0001271 Rev. C.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

Invitrogen™ TRIzol™ Reagent is a ready-to-use reagent, designed to isolate high quality total RNA (as well as DNA and proteins) from cell and tissue samples of human, animal, plant, yeast, or bacterial origin, within one hour. TRIzol™ Reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size. TRIzol™ Reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. TRIzol™ Reagent allows for simultaneous processing of a large number of samples, and is an improvement to the single-step RNA isolation method.

TRIzol™ Reagent allows users to perform sequential precipitation of RNA, DNA, and proteins from a single sample. After homogenizing the sample with TRIzol™ Reagent, chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). RNA is precipitated from the aqueous layer with isopropanol. DNA is precipitated from the interphase/organic layer with ethanol. Protein is precipitated from the phenol-ethanol supernatant by isopropanol precipitation. The precipitated RNA, DNA, or protein is washed to remove impurities, and then resuspended for use in downstream applications.

- Isolated RNA can be used in RT-PCR, Northern Blot analysis, Dot Blot hybridization, poly(A)+ selection, in vitro translation, RNase protection assay, and molecular cloning.
- Isolated DNA can be used in PCR, Restriction Enzyme digestion, and Southern Blots.
- Isolated protein can be used for Western Blots, recovery of some enzymatic activity, and some immunoprecipitation.

For DNA isolation, see the *TRIzol™ Reagent (DNA isolation) User Guide* (Pub. No. MAN0016385).

TRIzol™ Reagent can also be used with Phasemaker™ Tubes (Cat. No. A33248) to isolate RNA. Phasemaker™ Tubes creates a solid barrier between the organic and aqueous phases of the TRIzol™ Reagent following sample homogenization which makes separation of phases easier. See the *TRIzol™ Reagent and Phasemaker™ Tubes Complete System User Guide* (Pub. No. MAN0016163) for the full protocol.

TRIzol™ Reagent can also be used with the PureLink™ RNA Mini Kit (Cat. No. 12183018A) which uses spin columns instead of ethanol precipitation to purify the RNA. For additional information, see the *PureLink™ RNA Mini Kit User Guide* (Pub. No. MAN0000406).

Contents and storage

Contents	Cat. No. 15596026 (100 reactions)	Cat. No. 15596018 (200 reactions)	Storage
TRIzol™ Reagent	100 mL	200 mL	2–25°C

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. "MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Table 1 Materials required for all isolations

Item	Source
Equipment	
Centrifuge and rotor capable of reaching 12,000 × *g* and 4°C	MLS
Tubes	
Polypropylene microcentrifuge tubes	MLS
Reagents	
Chloroform	MLS

Table 2 Materials required for RNA isolation

Item	Source
Equipment	
Water bath or heat block at 55–60°C	MLS
Reagents	
Isopropanol	MLS
Ethanol, 75%	MLS
RNase-free water or 0.5% SDS	MLS
(Optional) RNase-free glycogen or 0.1 mM EDTA	MLS

Table 3 Materials required for protein isolation

Item	Source
Equipment	
(Optional) Dialysis membranes	MLS
Reagents	
Isopropanol	MLS
Ethanol, 100%	MLS
0.3 M Guanidine hydrochloride in 95% ethanol	MLS
1% SDS	MLS

Input sample requirements

IMPORTANT! Perform RNA isolation immediately after sample collection or quick-freeze samples immediately after collection and store at –80°C or in liquid nitrogen until RNA isolation.

Sample type	Starting material per 1 ml of TRIzol reagent
Tissues[1]	50–100 mg of tissue
Cells grown in monolayer	1×10^5–1×10^7 cells grown in monolayer in a 3.5-cm culture dish (10 cm^2)
Cells grown in suspension	5–10 × 10^6 cells from animal, plant, or yeast origin or 1 × 10^7 cells of bacterial origin

[1] Fresh tissues or tissues stored in RNA*later*™ Stabilization Solution (Cat. No. AM7020).

Procedural guidelines

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- Use cold TRIzol™ Reagent if the starting material contains high levels of RNase, such as spleen or pancreas samples.
- Use disposable, individually wrapped, sterile plasticware and sterile, disposable RNA-free pipettes, pipette tips, and tubes.
- Wear disposable gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin; change gloves frequently, particularly as the protocol progresses from crude extracts to more purified materials.
- Always use proper microbiological aseptic techniques when working with RNA.
- Use RNase*Zap*™ RNase Decontamination Solution (Cat. no. AM9780) to remove RNase contamination from work surfaces and non-disposable items such as centrifuges and pipettes used during purification.
- Ensure that all materials that come into contact with TRIzol™ Reagent are compatible with phenol, guanidine isothiocyanate, and chloroform.

Lyse samples and separate phases

1. Lyse and homogenize samples in TRIzol™ Reagent according to your starting material.
   - **Tissues:**
     Add 1 mL of TRIzol™ Reagent per 50–100 mg of tissue to the sample and homogenize using a homogenizer.
   - **Cell grown in monolayer:**
     a. Remove growth media.
     b. Add 1 mL of TRIzol™ Reagent per $1 \times 10^5 - 1 \times 10^7$ cells directly to the 3.5-cm culture dish to lyse the cells.
     c. Pipet the lysate up and down several times to homogenize.
   - **Cells grown in suspension:**
     a. Collect the cells by centrifugation and discard the supernatant.
     b. Add 1 mL of TRIzol™ Reagent per 0.25 mL of sample (5–10×10^6 cells from animal, plant, or yeast origin or 1×10^7 cells of bacterial origin) to the pellet.
        Note: Do not wash cells before addition of TRIzol™ Reagent to avoid mRNA degradation.
     c. Pipet the lysate up and down several times to homogenize.

   Note: The sample volume should not exceed 10% of the volume of TRIzol™ Reagent used for lysis.

   STOPPING POINT Samples can be stored at 4°C overnight or at –20°C for up to a year.
2. *(Optional)* If samples have a high fat content, centrifuge the lysate for 5 minutes at 12,000 × *g* at 4–10°C, then transfer the clear supernatant to a new tube.
3. Incubate for 5 minutes to allow complete dissociation of the nucleoproteins complex.
4. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, securely cap the tube, then thoroughly mix by shaking.
5. Incubate for 2–3 minutes.
6. Centrifuge the sample for 15 minutes at 12,000 × *g* at 4°C.
   The mixture separates into a lower phenol-chloroform, an interphase, and a colorless upper aqueous phase.
7. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out.

   IMPORTANT! Avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase.

Proceed directly to "Isolate RNA" on page 2.

To isolate DNA or protein, save the interphase and organic phase. See the *TRIzol™ Reagent (DNA isolation) User Guide* (Pub. No. MAN0016385) or see "Isolate proteins" on page 3 for detailed procedures. The organic phase can be stored at 4°C overnight.

Isolate RNA

1	Precipitate the RNA	**1.1.** *(Optional)* If the starting sample is small (<10^6 cells or <10 mg of tissue), add 5–10 µg of RNase-free glycogen as a carrier to the aqueous phase. **Note:** The glycogen is co-precipitated with the RNA, but does not interfere with subsequent applications. **1.2.** Add 0.5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol™ Reagent used for lysis. **1.3.** Incubate for 10 minutes at 4°C. **1.4.** Centrifuge for 10 minutes at 12,000 × *g* at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. **1.5.** Discard the supernatant with a micropipettor.
2	Wash the RNA	**2.1.** Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis. **Note:** The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C. **2.2.** Vortex the sample briefly, then centrifuge for 5 minutes at 7500 × *g* at 4°C. **2.3.** Discard the supernatant with a micropipettor. **2.4.** Vacuum or air dry the RNA pellet for 5–10 minutes. **IMPORTANT!** Do not dry the pellet by vacuum centrifuge. Do not let the RNA pellet dry, to ensure total solubilization of the RNA. Partially dissolved RNA samples have an $A_{230/280}$ ratio <1.6.
3	Solubilize the RNA	**3.1.** Resuspend the pellet in 20–50 µL of RNase-free water, 0.1 mM EDTA, or 0.5% SDS solution by pipetting up and down. **IMPORTANT!** Do not dissolve the RNA in 0.5% SDS if the RNA is to be used in subsequent enzymatic reactions. **3.2.** Incubate in a water bath or heat block set at 55–60°C for 10–15 minutes.

Proceed to downstream applications, or store the RNA at –70°C.

G - Quantabio qScript cDNA SuperMix protocol

qScript® cDNA SuperMix

Cat No. 95048-025 Size: 25 x 20-µL reactions(1 x 100 µL) **Store at -25ºC to -15°C**
95048-100 100 x 20-µL reactions (1 x 400 µL)
95048-500 500 x 20-µL reactions (2 x 1 mL)

Description
qScript cDNA SuperMix provides a sensitive and easy-to-use solution for two-step RT-PCR. This 5X concentrated master mix provides all necessary components (except RNA template) for first-strand synthesis including: buffer, dNTPs, $MgCl_2$, primers, RNase inhibitor protein, qScript reverse transcriptase and stabilizers. qScript is a RNase H(+) derivative of MMLV reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The unique blend of oligo (dT) and random primers in the qScript cDNA SuperMix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets < 1kb in length. qScript cDNA SuperMix produces excellent results in both real-time and conventional RT-PCR.

Components
qScript cDNA SuperMix — 5X reaction buffer containing optimized concentrations of $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer and stabilizers.

Storage and Stability
Store components in a constant temperature freezer at -25°C to -15°C upon receipt.
Repeated freezing and thawing does not affect functional performance.
For lot specific expiry date, refer to package label, Certificate of Analysis or Product Specification Form.

Reaction Assembly
Place components on ice. Mix, and then briefly centrifuge to collect contents to the bottom of the tube before using.

Component	Volume for 20-µL rxn.	Final Concentration
qScript cDNA SuperMix (5X)	4 µL	1X
RNA template	variable	(1 µg to 10 pg total RNA)
RNase/DNase-free water	variable	
Total Volume (µL)	20 µL	

Note: for smaller reaction volumes (i.e. 10-µL reactions), scale components proportionally.

Reaction Protocol

- Combine reagents in 0.2-mL micro-tubes or 96-well plate sitting on ice.
- After sealing each reaction, vortex gently to mix contents. Centrifuge briefly to collect components at the bottom of the reaction tube.
- Incubate:
  - 5 minutes at 25°C
  - 30 minutes at 42°C
  - 5 minutes at 85°C
  - Hold at 4°C
- After completion of cDNA synthesis, use 1/5th to 1/10th of the first-strand reaction (2-4 µL) for PCR amplification. If desired, cDNA product can be diluted with 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and stored at -20ºC.

Norwegian University of
Science and Technology