Off-Flavour Producing Bacteria in Aquaculture

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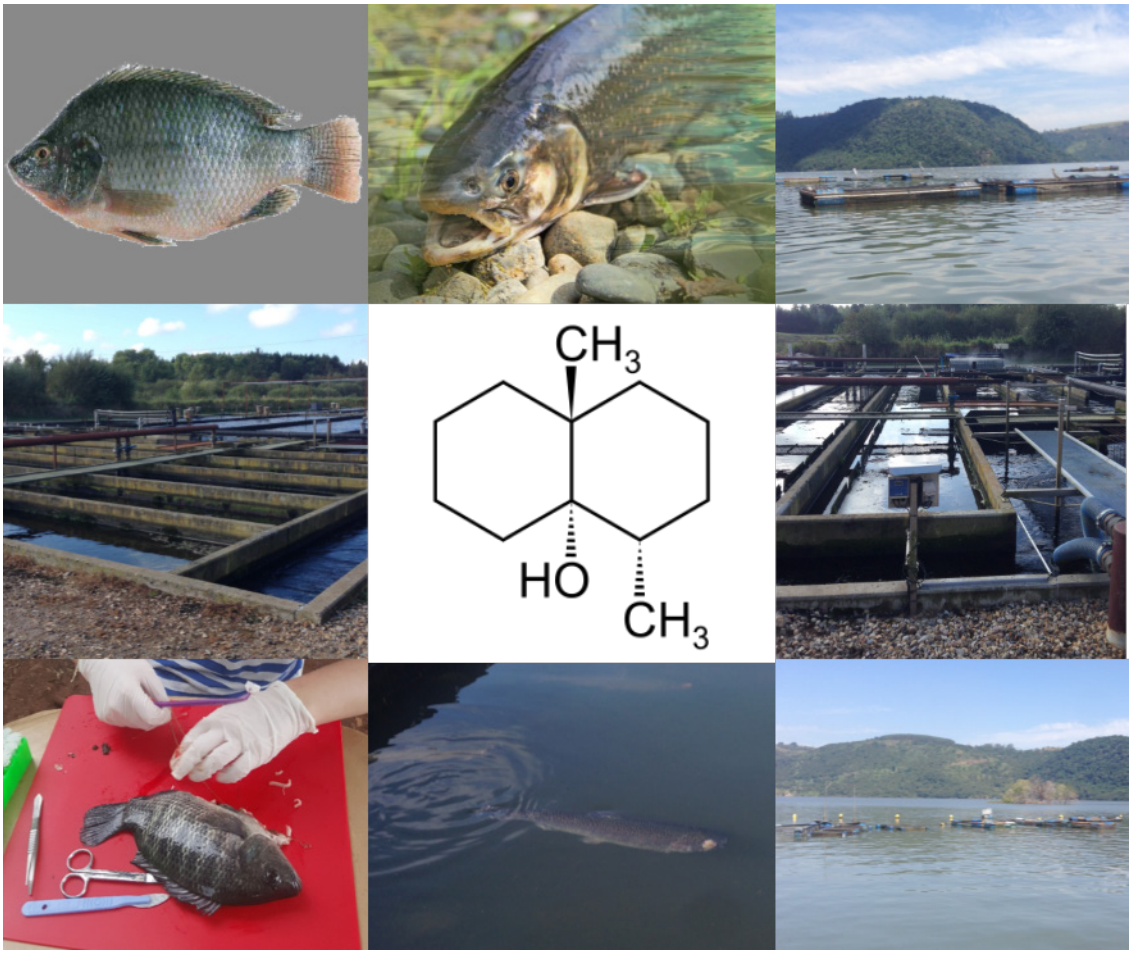

BY
MIE BECH LUKASSEN

DISSERTATION SUBMITTED 2017

AALBORG UNIVERSITET

OFF-FLAVOUR PRODUCING BACTERIA IN AQUACULTURE

by

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Preface

This dissertation is submitted in partial fulfilment of the requirements for obtaining the degree of Doctor of Philosophy (PhD). This dissertation contains the results of research undertaken between May 2014 and July 2017 at the Department of Chemistry and Bioscience of Aalborg University, under the supervision of Professor Jeppe Lund Nielsen. The work presented here was performed as a work package under the EU funded project "Towards off flavour free finfish aquaculture - Spacetaste" together with the project “IMProved quality of cultured fish for human CONsumption” (IMPCON). The dissertation consists of an introduction summarising literature relevant to the area of research and six scientific papers that are included as appendices.

First and foremost, I sincerely would like to thank my supervisor Jeppe Lund Nielsen for his perpetual guidance, optimism and believing in me. I would like to thank him for many interesting discussions we have had, he is very knowledgeable both scientific and non-scientific and I have for sure learned a lot.

Secondly, I would like to thank all of my colleagues, both past and present, at Aalborg University. Thank you to my office roommates for making the office a cheerful place to work. A special thanks to Henrik and Franzi for our many many scientific and non-scientific discussions over the years and to Nadieh for always helping when I was fighting with my computer. I would also like to say thanks to Søren for being my "go-to-guy" for technical MiSeq support. A special thanks to Freya for always helping when I was in trouble and for making each day fun to go to work. Thanks a lot Teis, without you, I would not have been where I am. Thanks to all of my collaborators. Thanks to the technicians Jane, Marianne and Susanne for their help and technical support throughout my project.

Lastly, I would like to thank my friends and family for their support and for keeping me grounded during my project. My deepest thank to my husband Niels and our two kids, Markus and Mynthe, for bearing with me and supporting me throughout the duration of my PhD project. I could not have done it without your patience and comprehension. You are the wind beneath my wings.
Finally, thanks to you, the reader of this thesis.

I hereby declare this is my original work.

Mie Bech Lukassen

Aalborg, July 2017

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English summary

Off-flavours are of great concerns in various system such as drinking water, food production and aquaculture. Geosmin, with its extremely low detection threshold for humans, seems to be the most widespread off-flavour compound in aquaculture, entering through the gills of the fish and accumulates in the adipose tissue. Tainted fish needs to be depurated in clean water for several days and even weeks to ensure geosmin is no longer traceable. This increases the workload and production cost for the aquaculture owner. Geosmin is a secondary metabolite of microbial origin and the known geosmin producers encompass *Actinomycetales*, especially *Streptomyces*, myxobacteria and *Cyanobacteria*.

The objective of this thesis was to reveal the diversity and identity of geosmin-producing bacteria in aquaculture and to provide strategies for elimination of geosmin. Several molecular biological methods were utilized in order to provide knowledge on the identity, prevalence and activity of geosmin-producing bacteria.

In the first paper, the diversity of geosmin-producing bacteria was investigated in six European recirculated aquaculture systems (RAS) and revealed a more diverse population than previously known. Phylogenetic analysis on known geosmin-producing bacteria from a public database and producers found in the investigated RAS using culture-independent methods was performed. Several unidentified groups of *Actinomycetales*, myxobacteria and two closely related groups affiliating with the genus *Sorangium* was found to represent the geosmin-producing bacteria in the systems. This confirmed that the gene, *geoA*, encoding for the geosmin synthase were a suitable molecular marker for investigating the geosmin-producing community. Specific primers targeting four novel putatively geosmin-producing bacteria, a group of *Actinomycetales*, myxobacteria and the two *Sorangium* groups, were designed. A TaqMan quantitative polymerase chain reaction (qPCR) approach was applied to quantify the groups showing geosmin-producing populations to constitute of 0.007-0.9 % of the entire microbial community. Despite this small fraction, they still create severe off-flavour problems in aquaculture.

In the second paper, the geosmin-producing bacterial population were investigated in 26 European RAS. Various environmental parameters were measured in order to find any statistical correlating to geosmin and the geosmin-producing bacteria in aquaculture. Multivariate data analysis revealed that the organic load (chemical oxygen demand (COD)), temperature, phosphate and redox potential were auto inducers for bacteria harbouring the *geoA* gene as well as the production of geosmin. Furthermore, sorangial *geoA* were shown to be one of the main geosmin-producer in the investigated RAS. These results provide knowledge to which factors control the geosmin production and could help designing new RAS with reduced geosmin levels.

In the third paper, the effect of organic load on geosmin levels was studied in laboratory scale RAS. Organic load (biological oxygen demand (BOD)) were manipulated in order to increase their levels. Positive statistical correlations were observed between geosmin, BOD and transcripts of *geoA*. Additionally, total ammonia and pH correlated positively with geosmin. This study confirmed organic load to be a regulator for elevated geosmin levels and this knowledge provides the aquaculture sector an environmental parameter to microbial manage the geosmin-producing bacteria.

In the fourth paper, the microbial community were monitored in a RAS for a period of nine months, revealing a relatively stable community, which reflects the surrounding environment. Monitoring the microbial community provides a possibility to maintain the stability and performance of RAS and observations in this study give a first insight into the community in RAS over a longer period.

In the fifth paper, the habitat and abundance of geosmin-producing bacteria were investigated in six Brazilian aquaculture. The relative number of geosmin-producers were similar in the water, stool, intestine and on the skin, approximately 0.2 % of all bacteria. Putatively geosmin-producing bacteria found in the intestinal mucous layer of the fish suggests that geosmin uptake potential occurs in the intestines and not through the gills as previously believed. This observation could be of high importance in the attempt of developing of geosmin elimination strategies. If the tainting occurs through the uptake of geosmin in a small confined space within the intestines, then instead of focusing on water treatment strategies applying substances able of changing the microbiome e.g. probiotic/prebiotics or adding chelators could be a successful approach.

In the sixth paper, the microbiome of fish feed with a dietary supplementation of 0.1%, 1% and 5% β-glucans were investigated in order to analyse the effect of this treatment. The β-glucan treated fish revealed a statistically significant change in the microbiome compared to a control group not feed with β-glucans. Additionally, the metabolites in the fish blood were measured to investigate the impact to the fish when feed with β-glucans. Several metabolites either increased or decreased when feed with the dietary supplementation of β-glucans compared to the control group. Changing the microbiome and metabolites of fish is applicable with prebiotics and therefore also to modify fish health.

In this thesis, the first study applying a large dataset to reveal environmental parameters affecting geosmin and bacteria harbouring the *geoA* gene were conducted. Furthermore, geosmin-producing bacteria were found for the first time applying molecular tools to exist in the intestinal tract of fish in same quantities as in the water and lastly the microbiome of fish feed with β-glucans together with changed metabolite response were for the first time documented.

Dansk resumé

I systemer såsom drikkevand, fødevareproduktion og akvakultur er afsmag en stor belastning. Geosmin, som har en ekstrem lav detektionsgrænse for mennesker, virker til at være det mest omfattende afsmags stof i akvakulturer. Det absorberes gennem gællerne og akkumuleres i fiskenes fedtvæv. Geosmin indeholdende fisk skal holdes i rent vand i dagevis, endda uger, for at få udskilt lugtstoffet, så det ikke længere er sporbart. Dette øger arbejdsbyrden og produktionsomkostningen for akvakultur ejerne. Geosmin er en sekundær metabolit med oprindelse fra mikrober, hvoraf kendte geosmin producenter omfatter aktinobakterier, specielt streptomyceter, myxobakterier og cyanobakterier.

Formålet med denne afhandling var at afdække diversiteten og identiteten af geosmin producerende bakterier i akvakulturer og at fremsætte eliminations strategier for geosmin. Flere molekylærbiologiske metoder blev anvendt for at fremskaffe viden omkring identitet, prævalens og aktivitet af de geosmin producerende bakterier.

I den første artikel blev diversiteten af geosmin producerende bakterier undersøgt i seks europæiske recirkulerede akvakultur systemer (RAS). Disse viste en større mangfoldig population end tidligere troet. En fylogenetisk analyse af kendte geosmin producerende bakterier blev foretaget med data fra en offentlig database, samt data fundet ved kultiverings uafhængige metoder fra de undersøgte RAS. Flere uidentificerede grupper af aktinobakterier, myxobakterier og en myxobacterial gruppe, kaldet Sorangium, viste sig at repræsentere bakterierne, som producerer geosmin i systemerne. Dette bekræftede, at genet *geoA,* som koder for geosmin syntasen, var en egnet molekylær markør for at undersøge den geosmin producerende populationen. Primere der specifikt rammer tre nye mulige geosmin producerende bakterier, en aktinobakteriel gruppe, myxobakterier og Sorangium, blev designet. En TaqMan qPCR fremgangsmåde blev anvendt for at kvantificere de geosmin producerende bakterier, hvilket viste systemerne indeholdt 0,007-0,9 % af disse bakterier i forhold til hele den bakterielle population tilstede. Til trods for denne lille fraktion geosmin producerende bakterier tilstede udgør de et alvorligt afsmag problem i akvakulturer.

I den anden artikel blev den geosmin producerende population undersøgt i 26 europæiske RAS. Forskellige miljømæssige faktorer blev målt for at detektere statistiske korrelationer til geosmin og geosmin producerende bakterier i akvakultur. En multivariat dataanalyse afslørede at organisk belastning (kemisk iltkrav (COD)), temperatur, fosfat og redox potentiale var inducere af bakterier med *geoA* genet og for produktionen af geosmin. Desuden viste resultatet, at Sorangium var hovedproduceren af geosmin i de undersøgte RAS. Disse resultater fremsætter viden om hvilke faktorer, der kontrollerer geosmin produktionen og kan potentielt assistere i design af nye RAS med reducerede geosmin niveauer.

In den tredje artikel, blev effekten af organisk belastning på geosmin undersøgt i RAS i laboratorium skala. I disse RAS blev organisk belastning (biologisk iltkrav (BOD)) manipuleret med henblik på at forøge BOD niveauerne. Positive korrelationer mellem geosmin, BOD og udtrykt *geoA* blev observeret. Desuden korrelerede geosmin også positivt med ammonium ioner og pH. Dette studie bekræftede, at organisk belastning var en regulator for forhøjede geosmin niveauer og denne viden

muliggør mikrobiel management af geosmin producerende bakterier ved at ændre på den organiske belastning.

I den fjerde artikel blev den mikrobielle population overvåget i et RAS over en periode på ni måneder. Dette viste en relativt stabil population som reflekterede det omgivende miljø. Overvågning af det mikrobielle samfund giver mulighed for at opretholde stabiliteten og ydeevnen i RAS og observationerne i dette studie giver et først indblik i det mikrobielle samfund i RAS over en længere periode.

I den femte artikel blev levestedet og hyppigheden af geosmin-producerende bakterier undersøgt i seks brasilianske akvakulturer. De relative forhold af geosmin producerende bakterier i vandet, fæces, tarmene og på skindet var ensartet, omkring 0,2 % af alle bakterier. Mulige geosmin producerende bakterier fundet i tarmens slimlag i fisken foreslår, at geosmin optagelsen foregår i tarmene og ikke gennem gællerne som tidligere troet. Denne observation kunne være meget vigtig i forsøget på at udvikle elimination strategier for geosmin. Såfremt optagelsen af geosmin foregår over et begrænset område i tarmen, kunne fokus flyttes fra vandbehandlingsstrategier over på at benytte stoffer der kan ændre mikrofloraen i tarmen med for eksempel probiotika/prebiotika eller chelatorer. Dette kunne være en succesfuld metode for elimination af disse bakterier i tarmen.

I den sjette artikel blev mikrobiomet undersøgt i fisk fodret med et supplement af β-glukaner på henholdsvis 0,1, 1,0 og 5,0 % for at analysere effekten af disse behandlinger. Fiskene fodret med β-glukaner viste en statistisk signifikant ændring i deres mikrobiom i forhold til kontrolgruppen, der ikke modtog et supplement af β-glukaner. Desuden blev metabolitterne i fiskens blod målt for at undersøge effekten på fiskene, når de blev fodret med β-glukaner. Flere metabolitter blev enten forøget eller mindsket som følge af kostsupplementet med β-glukanerne i forhold til kontrol gruppen. Det er derfor muligt at modificere fisks mikrobiom og metabolitter med prebiotika og dermed at ændre på fiskenes helbred.

I denne afhandling blev det første studie udført, hvor et stort datasæt blev anvendt for at identificere miljømæssige faktorer, der påvirker geosmin og geosmin producerende bakterier. Endvidere blev geosmin producerende bakterier fundet i tarmen, ved hjælp af molekylære metoder, i samme mængder som i det omgivende vand. Afslutningsvis blev det for første gang dokumenteret, at fisk fodret med β-gukaner ændrer deres mikrobiom og metabolitter i blodet.

List of supporting papers

Papers included in the thesis:

Paper 1

Lukassen, M.B., Saunders, A.M., Sindilariu, P.-D., Nielsen, J.L., 2017. Quantification of novel geosmin-producing bacteria in aquaculture systems. Aquaculture 479, 304-310.

Paper 2

Lukassen, M.B., Schram, E., Nielsen, J.L. Impact of water quality parameters on geosmin levels and geosmin producers in European aquaculture. Submitted.

Paper 3

Lukassen, M.B., Schram, E., Nielsen, J.L. The effect of organic load on the production of geosmin in moving bed biofilter reactors. In preparation.

Paper 4

Lukassen, M.B., Podduturi, R., Jørgensen, N. O. G., Nielsen, J. L. Population dynamics of geosmin-producing bacteria in a Danish full-scale RAS. In preparation.

Paper 5

Lukassen, M.B., Bjerregaard, S. M., Podduturi, R., Jørgensen, N. O. G., Petersen, M. A., David, G. S., da Silva, R. J., Nielsen, J. L. The fish microbiome and its potential impact on uptake of the flavour compound geosmin. Submitted.

Paper 6

Lukassen, M.B.*, Skov, J.*, Nielsen, J. L. Gut microbiome- and immunomodulation by dietary β-glucan in rainbow trout (*Oncorhynchus mykiss*). In preparation.

*Equal contribution

Objectives of the PhD project

The overall objective of this PhD project was to study the microorganisms involved in geosmin production in aquaculture. This was to be achieved by applying different molecular biological methods to provide knowledge to phylogenetic identification, abundance and physiology in order to produce the foundation for potential ways to decrease presence and activity. Furthermore, the objective was to identify the gut microbiome response to a supplementation of prebiotics. The specific objectives of the project were:

- To identify the diversity of geosmin-producing bacteria in recirculated aquaculture systems using a cultivation-independent method.

- Investigation of environmental parameters affecting the levels of geosmin and geosmin-producing bacteria using a large dataset applying a multivariate statistical analysis approach.

- To analyse the habitat of geosmin-producing bacteria in Brazilian aquaculture.

- Identification of the microbial community including the geosmin-producing microbes in a time series of nine month in a Danish indoor recirculating aquaculture plant.

- To identify the gut microbiome in rainbow trout feed with and without a dietary supplementation of β-glucans. Furthermore to analyse the metabolite response in the blood of the fish due to the prebiotic additive.

1 Introduction

1.1 Aquaculture

Modern capture fisheries encounter negative impacts on marine and freshwater ecosystems such as overfishing, killing of undersized fish or non-target species and destruction of coral reef ecosystems (Delgado et al., 2003; Johannes and Ripen, 1996). In fact, in 2013 31.4 % of fish stocks were estimated to be overfished which clearly indicates the requirement of alternative methods. One of the reasons for these problems relates to the rapid increase in world population and with a consumption of more than 20 kg fish annual per capita current capture fisheries cannot fulfil the required quantities. Therefore, fish production in aquaculture has been a popular and necessary solution to this increasing demand. Aquaculture covers the farming of aquatic organisms such as fish, molluscs, crustaceans and aquatic plants and can take place in fresh, brackish or marine water. To distinguish between aquaculture and capture fisheries, FAO utilizes the definition of aquaculture that there needs to encompass ownership of the stock throughout the rearing period to qualify to the term aquaculture (FAO, 2016).

In 2014 aquaculture production of fish amounted to 44.1 % of the total fish industry on a national basis (Figure 1) and in 35 countries the aquaculture production exceeded the conventional capture fisheries (FAO, 2016).

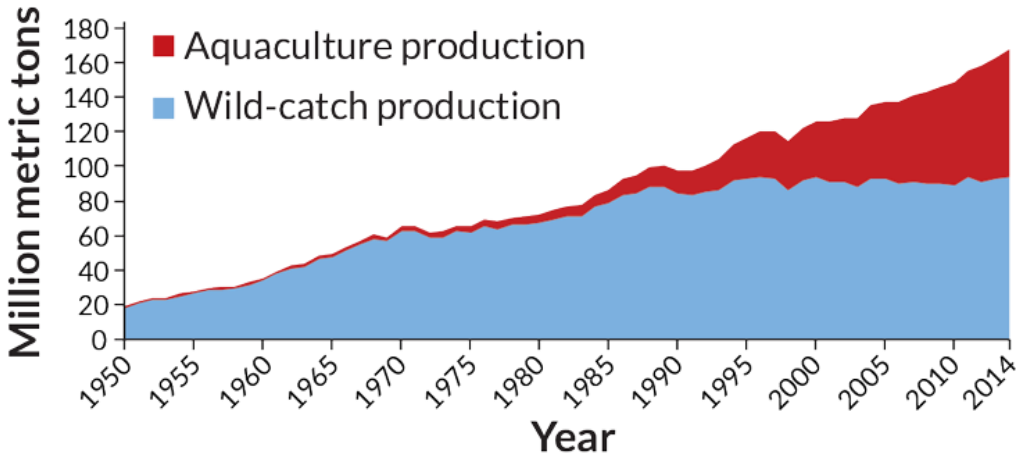

Figure 1: The distribution of aquaculture and capture production during time (Kwok, 2016).

Traditional aquaculture has seriously impacted the nearby environment covering disease outbreak to the surrounding wildlife stocks and altering of the genetic pool of the wild stocks through escaped fish. Furthermore, the effluent from the traditional aquaculture is discharged into the surrounding ponds and rivers inducing potentially eutrophic pollution problems in the nearby environment. Besides nutrients, these compounds also cover various chemicals, antibiotics and undigested feed (Clifford et al., 1998; Delgado et al., 2003; Tendencia and de la Peña, 2001). To ensure long-term sustainability for the aquaculture it is crucial to reduce these negative environmental impacts. As a consequence hereof the Danish government has enacted new stricter environmental regulations in 1987, which specify maximum levels of nitrogen, phosphorus and organic substances in the effluent (Larsen, 2005). A method to decrease the pollution of the environment is to reuse the water in recirculated aquaculture

systems (RAS). With these systems, less effluent is drained off to the surrounding ponds. Furthermore, water is a scarce resource on the planet and aquaculture, and in particular pond aquaculture, are one of the most water consuming methods for rearing of animals. Therefore, with the increasing demand for fish for consumption, aquaculture needs to develop new strategies in order to reduce their water consumption (Boyd and Gross, 2000). RAS has shown to be a cost-effective method to obviate these needs and is gaining utilization around the world.

1.1.1 Recirculating aquaculture systems

RAS can be indoor or outdoor tank-based systems and they can be freshwater or marine water systems, although farming freshwater fish is the most common choice (Burnell and Allan, 2009; Tal et al., 2009). In these semi-closed systems where 80-95 % the water are typically recirculated, the water quality is more easily controlled making it possible to farm fish in higher densities. Successful systems have even been conducted on 100 % recirculated aquaculture systems, so-called zero-discharge recirculating systems. The advantages of RAS are that they need limited water resources, a constant monitoring of the environment parameters in order to improve rearing conditions, fish can be reared at high densities, and with shorter production time due to high food conversion factors (Dalsgaard et al., 2013; Gelfand et al., 2003; Guttman and van Rijn, 2008; Singh et al., 1999).

1.1.1.1 Compartmental structure

The structure of RAS can vary but the following compartments are generally present: a fish production tank, mechanical filtration, biological filtration and oxygenation (Gelfand et al., 2003; Summerfelt et al., 2004; Tal et al., 2009). A schematic overview of the RAS components is outlined in Figure 2.

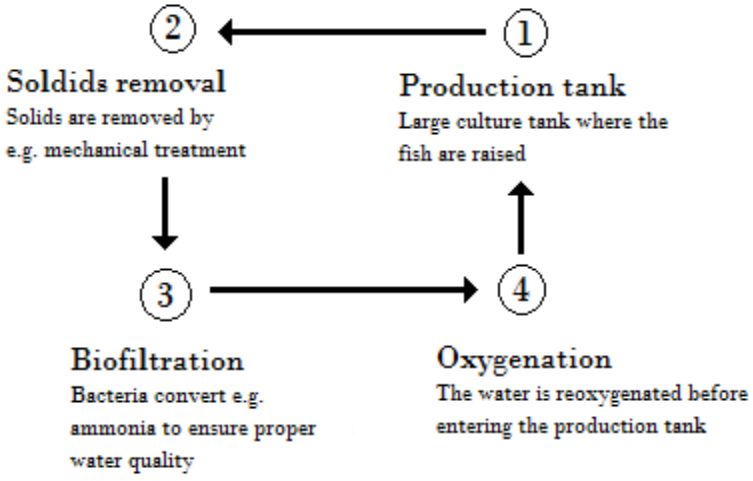

Figure 2: Overview of the different steps in a RAS.

The production tanks can vary in size, shape, material and numbers depending on fish species and accessibility. Outdoor RAS will typically be built into the ground in concrete whereas production tanks in indoor RAS typically will be round compartments standing on the ground made of e.g. plastic. It is Important to design the tank size to fit the capacity of other components in the system especially size of the biological filter compartment. The mechanical filters are often round drum filters with a 40–

80 µm mesh size, removing suspended solids which would otherwise affect the water quality negatively (Dalsgaard et al., 2013; Schrader et al., 2013). Biofilters can consist of moving bed (approximately 1 cm in diameter) or fixed beds (150 and 750 m^2/m^3) (Figure 3A and B).

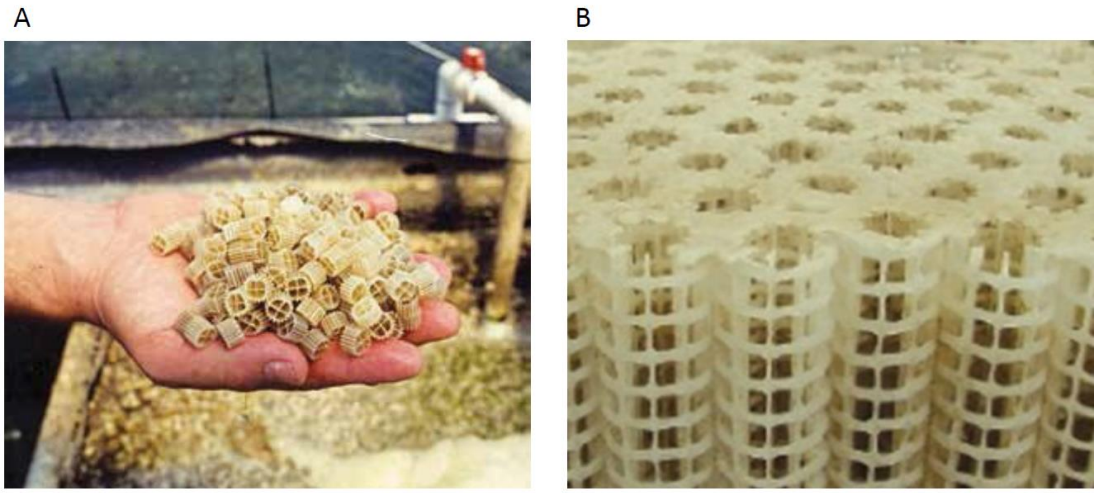

Figure 3: A) Moving bed biofilters. B) Fixed bed biofilters. Modified from (USDA, 2009)

It is within the biofilter compartments that e.g. the fish excretory product ammonia is removed. Exposure to high concentrations of ammonia leads to various diseases in the fish and are therefore toxic to the fish. The removal of ammonia is dependent on nitrifying microorganisms who can convert the compound into non-toxic nitrogen (N_2) (Foesel et al., 2008) and will be described in the next section. Finally, reoxygenation of the water is conducted before entering the fish production tank to ensure optimal conditions for fish respiration (Summerfelt et al., 2000). Optimal water quality ensuring healthy fish depends on effective management of the microbial communities within the system.

1.2 Microbiology in aquaculture

The microbial community in aquaculture is of great importance to ensure effective management of the fish production (Michaud et al., 2009). Microbial consortia can develop from indigenous communities or can be introduced by fish feed, through contact with staff or visitors and by the external (skin) and internal (gut) microbiota of the fish (Sharrer et al., 2005). The role of some of these aquatic microorganisms is to degrade organic matter and recycle harmful nutrients such as ammonia and to keep optimal stable water quality, but also in terms of preventing the proliferation of harmful bacterial species (Attramadal et al., 2012b; Foesel et al., 2008; Sugita et al., 2005). In RAS, two very important groups of bacteria are present: autotrophs, which contribute to nitrification of ammonia mainly in the biofilters and heterotrophs, which degrade organic matter and nitrate into the gaseous nitrogen (N_2) maintaining a proper water quality for the fish to live in (Gao et al., 2012; Michaud et al., 2009; Sugita et al., 2005). The autotrophs cover the two groups of nitrifying ammonia-oxidising bacteria (AOB) and archaea (AOA) and nitrite-oxidising bacteria (NOB). The AOB converts ammonia into nitrite, which is subsequently converted into nitrate by NOB in the two-step reaction: (Dalsgaard et al., 2013; Foesel et al., 2008; Kuhn et al., 2010):

$NH_4^+ + 1.5\ O_2 \Rightarrow NO_2^- + 2H^+ + H_2O$ (AOB)

$NO_2^- + H_2O => NO_3^- + 2H^+ + 2e^-$ (NOB) (Schreier et al., 2010)

Recently, it has been shown that the nitrification of ammonia into nitrate does not need two different organisms but can be carried out by one specialized bacteria denoted commamox who can perform the entire conversion of ammonia into nitrate itself. (Costa et al., 2006; Daims et al., 2015; van Kessel et al., 2015). Similar, another group of microorganisms converts ammonia and nitrite into nitrogen in one reaction designated anammox (Kuenen, 2008). Although, a coexistence is beneficial with autotrophic and heterotrophic bacteria the latter mentioned risk to outgrow the nitrifying bacteria because of a much faster growth rate. The bacteria compete over the present oxygen in the system and an excess of the heterotrophic bacteria risking the nitrification to reduce. High levels of organic matter deriving from digested feed, uneaten feed and debris are impossible to avoid in RAS, but reducing the amount of organic matter in the biofilters, e.g. by application of a drum filter helps controlling the quantity of these bacteria (Blancheton et al., 2013; Gao et al., 2012; Hagopian and Riley, 1998; Leonard et al., 2002; Michaud et al., 2006; Sugita et al., 2005). The microbial composition was shown to differ between aquaculture ponds, RAS, flow-through systems and semi-closed containment systems, but some phyla were the most abundant in all studies: *Proteobacteria, Bacteriodetes, Actinobacteria* and *Planctomycetes* (Attramadal et al., 2012a, 2012b; Martins et al., 2013; Qin et al., 2016; Rud et al., 2016; Smith et al., 2012; Tang et al., 2014; Lukassen et al., submitted (Paper 2)). The phylum *Cyanobacteria* was mainly found in outdoor aquaculture with a (sub)tropical climate (Petersen et al., 2014; Qin et al., 2016; Lukassen et al., submitted (Paper 5)). Furthermore, in RAS the composition of the microorganisms in the water are constantly influenced by external factors such as make-up water dilution and disinfection while the microbial composition in the biofilter is more sheltered, which results in varying organisms in these two RAS compartments (Rud et al., 2016; Lukassen et al., submitted (Paper 2)). High performance with healthy fish in aquaculture, therefore, depends on the microbial community in aquaculture which again are affected by chemical properties such as salinity and pH (Bakke et al., 2016; Liu et al., 2015; Tal et al., 2003). For instance, the abundance of *Actinobacteria* decrease when ammonia and nitrate increase and *Bacteroidetes* correlate positively with ammonia concentration (Jiang et al., 2016; Qin et al., 2016). These environmental changes and therefore microbial alteration may manifest themselves as activators or suppressor of certain metabolic pathways in the aquatic systems (Bentzon-Tilia et al., 2016). Disease outbreaks caused by bacterial pathogens in aquaculture have a tremendous economic impact for the fish farmers risking loses of entire stocks (Moriarty, 1999; Verschuere et al., 2000). Diseases can be caused by the opportunistic pathogens *Vibrio, Aeromonas, Flavobacterium, Yersinia Nocardia*, *Aeromona*s and *Streptococcus* (Gatesoupe, 1999; Schmidt et al., 2000; Schreier et al., 2010; Shi et al., 2012). Elimination of these pathogens were often conducted utilizing antibiotic therapy resulting in antibiotic resistance strains, which forced the sector to consider the use another approach such as pro – and prebiotics (Gatesoupe, 1999; Ringø and Song, 2016; Schmidt et al., 2000). Studies have suggested RAS biofilters to be more sensitive to pathogenic outbreaks while other studies propose RAS to less sensitive due to less interaction with the surroundings and the often applied disinfection units (Blancheton et al., 2013; Sharrer et al., 2005).
Various studies on the identification of the microbial communities in aquaculture have been conducted but still, a detailed characterisation of the aquaculture environment in respect to its residing microbiota, its functions and microbial interactions are at an early stage. With time, microbial management in aquaculture could lead to optimised operational conditions and aquaculture performance.

1.2.1 Gut microbiota of fish

The gut microbiota refers to a complex and dynamic microbial ecosystem, which colonises and resides in the intestines of the host. This microbiome has a high impact on the health of the host as it provides the host protection from colonisation of pathogens, promotes nutrient supply, retention of immunity and homeostasis (Figure 4) (Delzenne and Cani, 2008; Nicholson et al., 2005). The microbial composition in the gut is affected by intake of feed, gender, life cycle and the aquatic environment, which is shaped by e.g. temperature and farming conditions (Huang et al., 2016; Iehata et al., 2015; Kohl and Yahn, 2016; Sugita et al., 1983). In fact, changing the feed has been shown to have a large effect on the microbiome showing microbial manipulation can occur with adjusting the feed composition (Miyake et al., 2015). Furthermore, each fish species has its own microbiome, however, a study revealed that the gut microbiome of different fish species were more similar when growing in the same environment than two identical fish species living in different environments (Dehler et al., 2016; Hennersdorf et al., 2016). During antibiotic treatment or pollution of the aquatic environment with unwanted chemicals, the microbial composition in the gut can be massively altered and result in an elimination of entire bacterial populations (Bakke-McKellep et al., 2007; Navarrete et al., 2008).

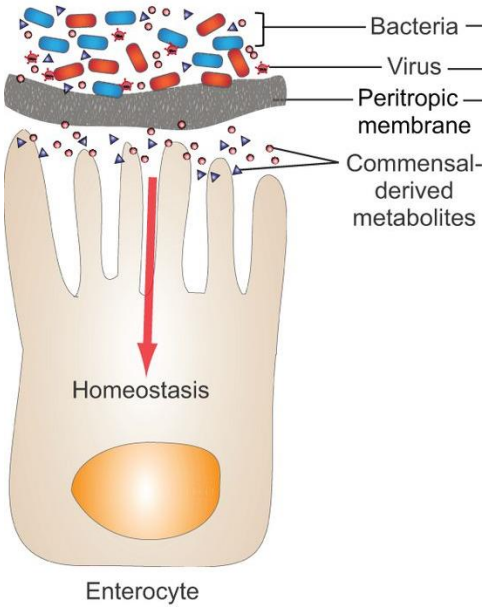

Figure 4: The microbiome within the gut of the fish and their metabolites helps maintain homeostasis (Wong et al., 2016).

Two groups of bacteria are usually present in the fish gut: autochthonous (adherent) and allochthonous (transient) (Kim et al., 2007; Ringø et al., 2003). The first mentioned group is tolerant to the low pH present in the gastric acid helping them to retain their viability so they can attach to the intestinal mucous layer (Onarheim and Raa, 1990; Savage, 1989). The allochthonous group are not able to colonise in this environment and are therefore only present for shorter periods in the gut (Ringø and Birkbeck, 1999). Aerobic as well as anaerobic bacteria represent the bacterial community in the fish gut (Trust et al., 1979). The role of the majority of the bacteria present in the fish microbiota are still unknown but several studies have investigated their function in the search of e.g. health indicators, but more research in this area are needed (Bentzon-Tilia et al., 2016).

As mentioned, a healthy microbiome prevents colonisation of pathogens in the gut (Sugita et al., 1997). Additives to the feed such as probiotics and/or prebiotics (in combination called synbiotics) can help altering the microbiome into a community, which offers the host a health benefit to the host (Figure 5). Altering the microbial community in the gut has shown to affect the metabolism of nutrients, cholesterol and xenobiotic compounds (Cebra, 1999; Falk et al., 1998; Sadzikowski et al., 1977).

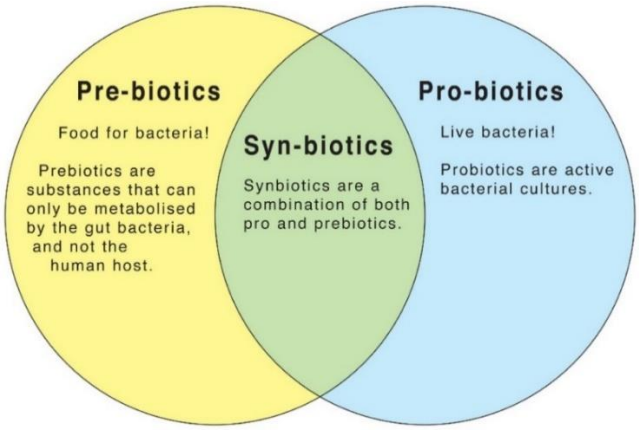

Figure 5: Prebiotics and probiotics enhance the health of the host. Combined they are termed synbiotics. (Fibres, 2015).

Probiotics are living bacteria, which often are administrated in aquaculture as water additive or incorporated in the feed. They have shown to provide increased immunity and survival by competitively exclude pathogens (Figure 6) and by producing compounds as antibiotics and organic acids, which decomposes the pathogens (Austin et al., 1995; Queiroz and Boyd, 1998). Additionally, probiotics can improve growth by enhancing the feed conversion efficiency (El-Dakar et al., 2007; Zhou et al., 2010). Over time the probiotics will lose viability and additional bacteria have to be supplied (Lauzon et al., 2010). The first application of probiotics in aquaculture was conducted 30 years ago and since that it has been widely used especially as an alternative to antibiotic therapy, which has caused the establishment of antibiotic resistance microbes (Gatesoupe, 1999; Kozasa, 1986; Schmidt et al., 2000; Standen et al., 2015; Verschuere et al., 2000). The limitations of probiotic are e.g. that establishment of a healthy microbiome relies on the already present bacteria in the gut. It is implausible to rely on an exogenous addition of probiotic solely can prevent pathogens to colonise the gut and failure can happen then bacterial species, which are not an existing part of the microbiome, are utilized (Verschuere et al., 2000).

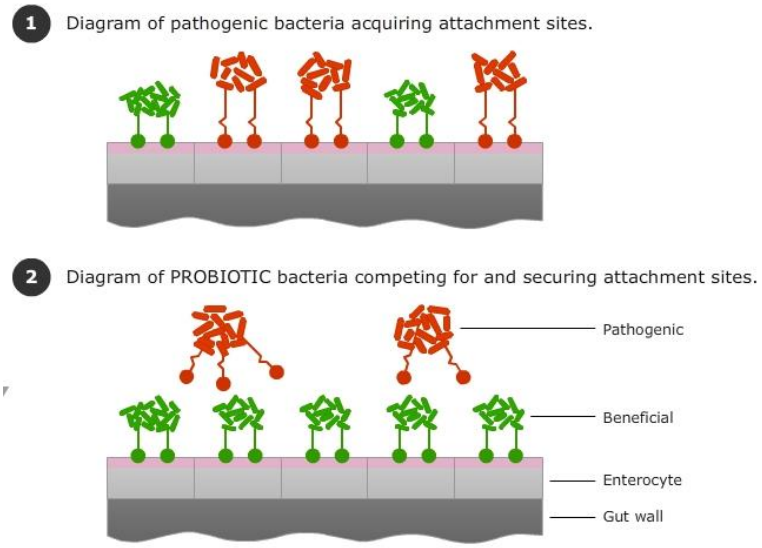

Figure 6: 1) Pathogenic bacteria attach to the peritrophic membrane. 2) The presence of beneficial bacteria limit the adhesion of pathogenic bacteria to the intestine. Modified from (Science Learning Hub, 2011).

Prebiotics are compounds, which acts as a fertiliser for the healthy microbiome already present in the gut. The prebiotics used in aquaculture today is usually deficient-digestible mono-, oligo-, or polysaccharides that can only be metabolised by bacteria and not directly by the host (Cummings et al., 2001; Gibson et al., 2015). In rearing of mammals and fish various selection of prebiotics has been tested: mannan-oligosaccharides, fructo-oligosaccharide, galacto-oligosaccharides, inulin and β-glucans (Macfarlane et al., 2007; Olsen et al., 2001; Skov et al., 2012; White et al., 2002). These additives can elevate specific species such as *Bifidobacter* and *Lactobacillus*, which have a beneficial health effect on the host (Costalos et al., 2008; Tuohy et al., 2005). Furthermore, several health improving reactions have been documented from the use of prebiotics e.g. enhancement of digestive enzymes (protease and amylase), improved growth, immunity and disease resistance (Kihara et al., 1995; Li and Gatlin III, 2004; Staykov et al., 2007; Xu et al., 2009). However, caution should be taken when administrating prebiotics as illustrated by a study on inulin were a high concentration dose of inulin had damaging effect on the host (Olsen et al., 2001). The prebiotics are metabolised in the gut into short-chain fatty acids such as lactic acid, butyrate and acetate, which again are utilized by the host (Roberfroid, 1993; Velazquez et al., 1997). In aquaculture, the use of prebiotics, without the additive of a probiotic, is still in its infancy even though some shows promising applicability as immunostimulants. β-Glucans has shown most promising effects on fish health (Arena et al., 2014; Dalmo and Bøgwald, 2008; Ringø and Song, 2016) and feed containing β-glucans are commercially manufactured (Cook et al., 2001; Jung-Schroers et al., 2016; Kühlwein et al., 2014). The ability for β-glucans to alter the gut microbiome have been proven only by a few studies (Jung-Schroers et al., 2016; Kühlwein et al., 2014; Skjermo et al., 2006; Lukassen et al., in preparation (Paper 6)). Several bacterial species were influenced and their abundance altered due to β-glucan treatment compared to the control. Furthermore, fish feed with β-glucans showed to have a significantly different metabolite profile in the blood indicating that the impact of β-glucans on the host occur through modifying the microbiome (Lukassen et al., in preparation (Paper 6)).

This successful modulation of the fish microbiome indicates the possibility to alter the composition of the gut microbiome by eliminating or generating favourable conditions for different bacterial species and hereby changing the host physiology. Therefore, the potential in manipulation the microbiome

has great potential for obtaining a deeper understanding of the importance of the fish gut microbiome and its function, and how managing the feed provided to the fish could lead to more optimised management and performance of aquaculture.

1.3 Off-flavour in aquaculture

Off-flavour in aquaculture originates mainly from geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneol (MIB) which have an earthy and musty flavour, respectively. Geosmin seems more frequent to be the source of off-flavour problems than MIB (Gerber and Lechevalier, 1965; Houle et al., 2011; Schrader and Summerfelt, 2010). The organisms responsible for geosmin production in aquaculture are bacteria (Auffret et al., 2011; Guttman and van Rijn, 2008; Schrader and Summerfelt, 2010) although eukaryotic fungi and plants also have been identified as capable of producing geosmin (Bacha et al., 2015; Freidig and Goldman, 2014; La Guerche et al., 2005). Since these geosmin-producing fungi have only been detected in soil, juice and wine (La Guerche et al., 2005) and no plants are present in aquaculture, these are not considered a particular problem in this system. Geosmin is an aromatic compound with volatile properties which exists as (+) and (-) enantiomers with the odour causing (-) enantiomer being 10 times more potent than the (+) enantiomer (Bentley and Meganathan, 1981; Gerber and Lechevalier, 1965; Saito et al., 1996; Watson et al., 2007). The off-flavour compound geosmin has hydrophobic properties and due to this characteristic, it accumulates in the fish tissue where fish with a higher fat content tends to accumulate higher levels of geosmin resulting from the hydrophobic feature (Tucker, 2000). Geosmin absorption is relatively fast whereas the excretion is much slower (Rurangwa and Verdegem, 2015). The main uptake route of geosmin is generally assumed to be through the gills, however, uptake of geosmin has also been proposed to occur through skin, intestine and stomach, although the time required to detect the off-flavour in the flesh was 15 to 70-fold longer than through the gills (From and Hørlyck, 1984). Geosmin-producing bacteria were found in the stomach and the intestinal mucous layer of fish suggesting that fish may also feed actively on potential geosmin-producing microorganisms (Gutierrez et al., 2013; Watson et al., 2016; Lukassen et al., submitted (Paper 5)). When geosmin accumulates in the fish, it results in tainted fish with an earthy smell and taste causing the need for depuration of the fish in clean water, which delay the harvest and subsequently sale. In aquaculture, this was estimated in 2003 to increase the cost up to 0.25 US$/kg fish (Hanson, 2003). Geosmin has a human detection threshold down to 4 ng/L making it extremely potent and an extensive problem in the aquaculture industry (Watson et al., 2016).

Aside from aquaculture, geosmin also causes problems in various systems such as drinking water, wine and liquor production (Behr et al., 2013; Du et al., 2011; Ludwig et al., 2007).

1.3.1 Characterisation of geosmin

Terpenes are secondary metabolites, which are highly diverse and exist in various forms with various chemical modifications. When the terpenes are functionally modified in their chemical structure they are referred to as terpenoids and this functional structure are known to be extremely diverse causing highly diverse functional roles (Gershenzon and Dudareva, 2007; Harrewijn et al., 2002). They are produced by plants, liverworts, fungi and bacteria and are well studied due to their many

functionalities (Yamada et al., 2015). Several bacterial terpenes have been identified and the main groups are monoterpenes, sesquiterpenes and diterpenes (Dickschat, 2016). More than 50,000 terpenes have been isolated and characterised and associated with functions such as electron carriers, mediators of polysaccharide assembly, hormones, communication and defence agents, photosynthetic pigments and as off-flavour compounds (Langenheim, 1994; Yamada et al., 2015). A relatively minor fraction of these many terpenes have been identified in bacteria but one frequently found terpenoid in some phyla is the off-flavour compound geosmin, which belongs to the tertiary alcohol group of sesquiterpenes (Figure 7) (Dickschat et al., 2005; Giglio et al., 2008; Schöller et al., 2002; Yamada et al., 2015).

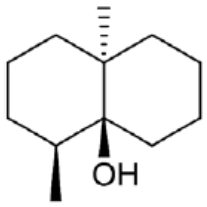

Figure 7: The chemical structure of geosmin.

1.3.2 Geosmin-producing bacteria

The phyla *Actinobacteria* (particularly *Streptomyces*) and *Cyanobacteria* are known to cover geosmin-producing bacteria in aquatic environments. Additionally, a number of myxobacteria have been proposed as potential geosmin-producers (Bacha et al., 2015; Dickschat et al., 2005; Guttman and van Rijn, 2008; Yamada et al., 2015; Lukassen et al., 2017 (Paper 1)). *Myxococcales,* various unidentified species of *Actinomycetales*, and species associated with the genus *Sorangium* were revealed in to be the main geosmin-producing bacteria in European RAS (Lukassen et al., 2017 (Paper 1)). Utilizing a culture-independent method, geosmin-producer were quantified to consist of 0.001-1 % of all bacteria in the systems (Lukassen et al., 2017 (Paper 1)). In these European RAS no *Streptomyces* were found. This finding was supported by observations in Brazilian aquaculture and RAS in which no *Streptomyces* were found (Lukassen et al., submitted (Paper 2 and 5); Lukassen et al., in preparation (Paper 4)). Previously, *Streptomyces* were designated to be one of the most dominant geosmin-producing bacterial group in RAS using culture-dependent methods (Guttman and van Rijn, 2008; Klausen et al., 2005), but this view is challenged by newer culture-independent based investigations (Auffret et al., 2013, 2011; Schrader and Summerfelt, 2010; Lukassen et al., 2017 (Paper 1); Lukassen et al., submitted (Paper 2)). The myxobacterial group *Sorangium* were found with specific quantitative polymerase chain reaction (qPCR) primers to be the most abundant genus in European RAS applicable of producing geosmin (Lukassen et al., submitted (Paper 2); Lukassen et al., in preparation (Paper 4)) supporting the finding that *Sorangium* and *Nannocystis* were detected in an investigated RAS (Auffret et al., 2013).

In Table 1, a list of known geosmin-producers is displayed. All actinobacterial species found in the table affiliate within the order of *Actinomycetales*, as proposed by a recently study (Lukassen et al., 2017 (Paper1)), which is also supported by the observation that all *Actinomycetales* harbour the gene responsible for geosmin synthesis, except for one species (Yamada et al., 2015). The genus *Streptomyces* covers a variety of different species capable of producing geosmin (Du et al., 2013; Klausen et al., 2005). A relatively long list of *Cyanobacteria* are known to be able to produce geosmin

and this phylum is often associated with off-flavour problems in ponds and especially in water reservoirs and aquaculture from the (sub)tropics (Ho et al., 2012; Lukassen et al., submitted (Paper 5)). The known geosmin-producers in the phyla *Proteobacteria* are all in the order of *Myxococcales* (Auffret et al., 2013; Dickschat et al., 2005). Table 1 shows the identified geosmin-producing species, yet recent studies reveal a variety of unknown geosmin-producing bacteria awaiting to be unveiled (Lukassen et al., 2017 (Paper 1)).

Table 1: Known geosmin-producing bacteria.

Phylum	Genus	Reference
Actinobacteria	*Streptomyces*	Guttman and van Rijn, 2008
Actinobacteria	*Kitasotospora*	Auffret et al., 2011
Actinobacteria	*Frankia*	Auffret et al., 2011
Actinobacteria	*Saccharopolyspora*	Watson et al., 2016
Actinobacteria	*Nocardia*	Robin et al., 2006
Actinobacteria	*Actinosynnema*	Auffret et al., 2011
Actinobacteria	*Micromonospora*	Hamlin et al., 2008
Actinobacteria	*Actinomycetales spp*	Lukassen et al., 2017 (Paper 1)
Cyanobacteria	*Oscillatoria*	Izaguirre et al., 1982
Cyanobacteria	*Lyngbya*	Jüttner and Watson, 2007
Cyanobacteria	*Phormidium*	Robin et al., 2006
Cyanobacteria	*Schizothrix*	Jüttner and Watson, 2007
Cyanobacteria	*Anabaena*	Izaguirre et al., 1982
Cyanobacteria	*Microcoleus*	Robin et al., 2006
Cyanobacteria	*Aphanizomenon*	Jüttner and Watson, 2007
Cyanobacteria	*Planktothrix*	Jüttner and Watson, 2007
Cyanobacteria	*Symploca*	Jüttner and Watson, 2007
Cyanobacteria	*Calothrix*	Watson et al., 2016
Cyanobacteria	*Cylindrospermum*	Watson et al., 2016
Cyanobacteria	*Nostoc*	Giglio et al., 2008
Cyanobacteria	*Pseudanabaena*	Houle et al., 2011
Cyanobacteria	*Synechococcus*	Watson et al., 2016
Proteobacteria	*Myxococcus*	Dickschat et al., 2005
Proteobacteria	*Stigmatella*	Dickschat et al., 2005
Proteobacteria	*Nannocystis*	Auffret et al., 2013
Proteobacteria	*Sorangium*	Auffret et al., 2013

1.3.3 Geosmin synthesis

Studies have shown that organisms produce geosmin utilizing the isoprenoid biosynthetic pathways: 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, the mevalonate (MVA) pathway and the Leucine pathway (Figure 8). These pathways lead to the formation of farnesyl diphosphate (FPP), which is a precursor for geosmin production. The genes encoding for the MEP pathway was originally found in the cyanobacterial *Synechocystis* even though members of this genus do not normally produce geosmin. This indicates, however, that the MEP pathway also functions in other cyanobacterial species though this has not been investigated further (Ajikumar et al., 2008; Jüttner and Watson, 2007; Proteau, 1998). Both the MEP and MVA pathways were found in *Streptomyces aeriouvife* and another *Streptomyces* species revealed to utilize the MEP pathway, shown by labelling of 1-deoxy-D-xylulose (Seto et al., 1996; Spiteller et al., 2002). The myxobacteria *Myxococcus xanthus* and *Stigmatella aurantica* produced labelled geosmin when labelled mevalolactone and Leucine was added indicating use of the MVA and Leucine pathway (Dickschat et al., 2005). Studies have proposed the MVA pathway to be used by *Streptomyces* to geosmin production in the stationary growth phase and the MEP pathway as the dominant pathway during active growth (Seto et al., 1998, 1996). It seems that different bacterial groups utilize different biosynthetic pathways for the synthesis of geosmin.

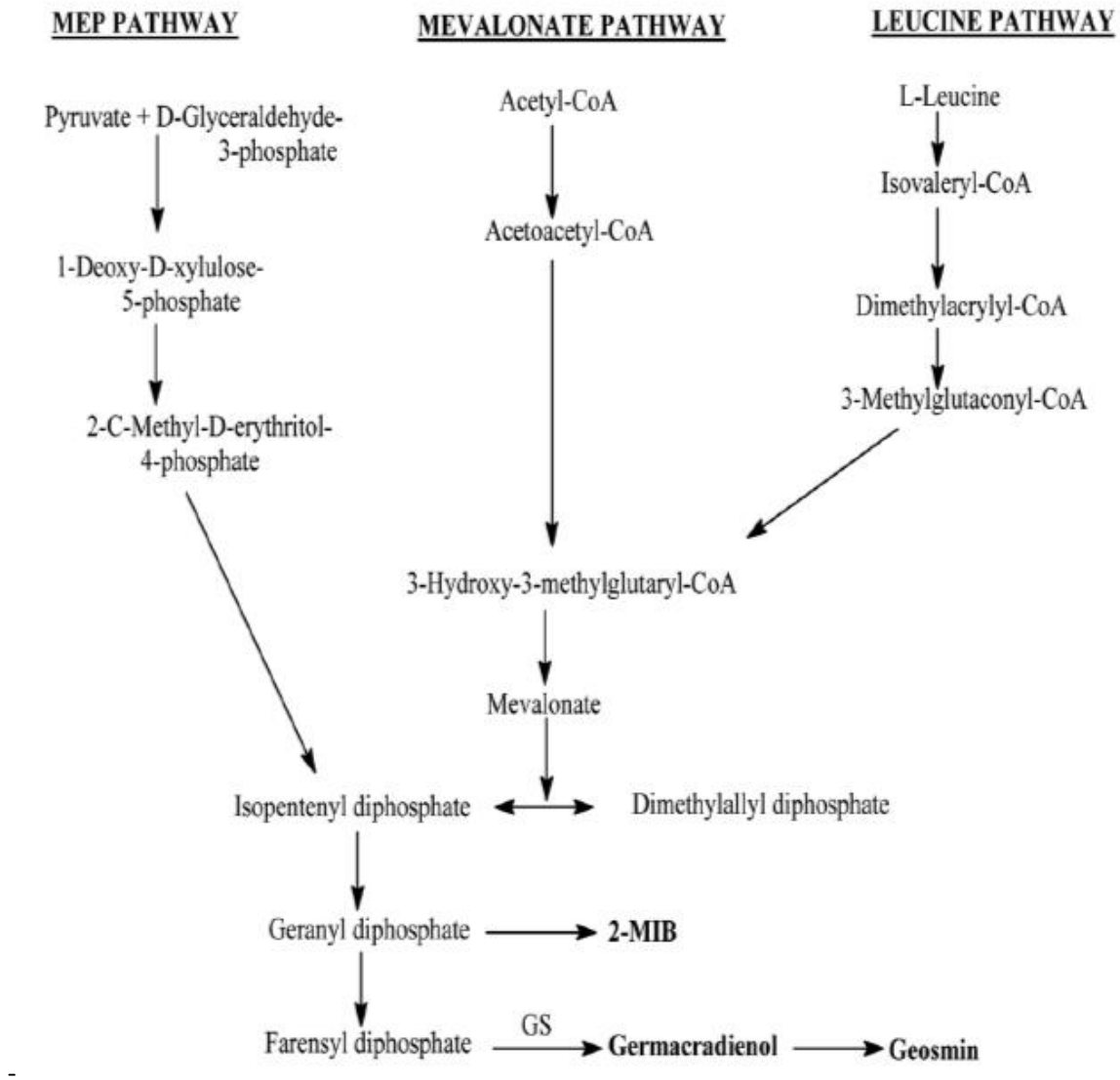

Figure 8: Biosynthesis of geosmin in bacteria (Jüttner and Watson, 2007).

As stated previously, the MEP and MVA pathway both synthesise FPP, which can be converted into germacrene D or geosmin (Figure 9).

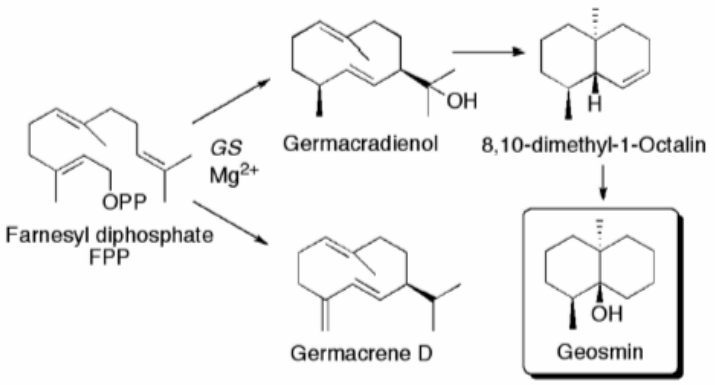

Figure 9: The conversion of FPP to geosmin (Giglio et al., 2008).

The mechanism that allows FPP, the acyclic precursor of all sesquiterpenes, to convert into geosmin is catalysed solely by the enzyme geosmin synthase. The function of this bifunctional enzyme was first discovered in *Streptomyces coelicolor* as a 726 amino acid protein, encoded by the 2181 bp SCO6073 gene (Cane and Watt, 2003; Gust et al., 2003). The geosmin synthase was also found in *S. avermitilis* with a 78 % sequence identity and 85 % similarity to the gene found in *S. coelicolor* (Cane et al., 2006). Additionally, genome sequencing of a variety of *Streptomyces*, *Frankia*, *Saccharopolyspora* and myxobacteria revealed genes with highly conserved regions compared to the gene SCO6073 found in *S. coelicolor* and also *Cyanobacteria* were shown to harbour the gene encoding geosmin synthase (Giglio et al., 2008; Ludwig et al., 2007; Yamada et al., 2015). The synthase consists of an N-terminal and a C-terminal region with a high degree of pairwise homology (Figure 10).

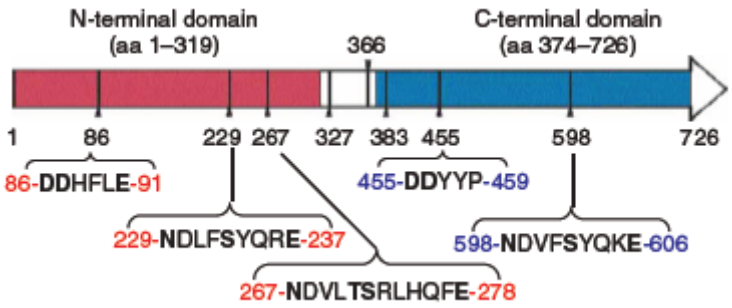

Figure 10: The protein outline of geosmin synthase showing the N-terminal and C-terminal with a high degree of pairwise homology (Jiang et al., 2007).

The N-terminal of the synthase catalyses the Mg^{2+}-dependent cyclization of FPP to a 85:15 ratio mixture of germacradienol and germacrene D whereas the C-terminal was first proposed to be catalytically silent. Later, it was discovered that the C-terminal in contrary catalyses the Mg^{2+}-dependent formation of germacradienol into geosmin (Gust et al., 2003; Jiang et al., 2007, 2006; Jiang and Cane, 2008). These geosmin synthases have conserved Mg^{2+} binding motifs where it for the N-terminal involves a motif with an aspartate-rich DDHLFE sequence (80-100 amino acids from the N-terminus) and the NSE triad ND(L/I)FSY(Q/E)RE motif (approximately 140 amino acids downstream the N-terminus). Additional, the C-terminal displays similar conserved Mg^{2+}-binding motifs: DDYYP and ND(V/I/L)FSYQKE. These motifs are found in all sesquiterpene synthases and are essential for converting FPP into geosmin (Christianson, 2006; Jiang et al., 2006; Yamada et al., 2015). The gene encoding for the geosmin synthase is denoted *geoA*. This gene has shown to be applicable as a functional molecular marker for analysing geosmin-producing bacteria due to the absence of multiple horizontal gene transfer (Giglio et al., 2008; Yamada et al., 2015; Lukassen et al., 2017 (Paper 1)). The microbial diversity harbouring the *geoA* gene was investigated in RAS using a degenerated primer pair (Cyc), targeting the overall putatively geosmin-producing bacterial group, revealing a much more diverse community than previously estimated and investigation in detection the real community is still needed (Ludwig et al., 2007; Lukassen et al., 2017 (Paper 1)). This primer pair, together with newly designed primers targeting specific geosmin-producing groups, provides the possibility to study the source of off-flavour in aquaculture for potential producers (DNA) and for active bacteria expressing the *geoA* gene (RNA) (Lukassen et al., in preparation (Paper 3)).

1.3.4 Factors controlling the presence of geosmin

Various environmental factors influence the level of geosmin in aquaculture by either increasing or decreasing the off-flavour compound. Both up- and downregulating mechanisms are of high relevance in the development of a geosmin elimination strategy. Several studies have shown that high levels of phosphate and organic load (TOC and COD) appear to elevate the geosmin production (Parinet et al., 2010; Sarker et al., 2014; Schrader and Blevins, 2001; Lukassen et al., submitted (Paper 2); Lukassen et at., in preparation (Paper 3)). (Guttman and van Rijn, 2008) and (Dionigi and Ingram, 1994) found that oxygen had a positive effect on geosmin when *Streptomyces* were the main geosmin-producer. Thus to minimise geosmin, these factors should be as low as obtainable without harming the fish. A previous study had shown the reverse impact on geosmin, namely that oxygen had a negative impact on geosmin levels, but this is probably due to a difference in which geosmin-producers are present in the systems (Lukassen et al., submitted (Paper 2)). Furthermore, high levels of temperature and nitrate increase the level of geosmin in *Cyanobacteria* and *Streptomyces in vitro* (Dionigi and Ingram, 1994; Oh et al., 2017; Zhang et al., 2009). Yet, the impact of nitrate is ambiguous as nitrate also has shown to inhibit geosmin production in *Cyanobacteria* and in a full-scale RAS it had no effect (Saadoun et al., 2001; Schrader et al., 2013). Both temperature and nitrate were confirmed to elevate geosmin (Lukassen et al., submitted (Paper 2)). High levels of ammonia and total nitrogen induced the geosmin levels in drinking water systems and in a pure culture study of *Cyanobacteria* (Parinet et al., 2010, 2012; Saadoun et al., 2001) whereas the micronutrients copper, zinc and iron all inhibited the formation of geosmin in pure culture studies (*Cyanobacteria* and *Streptomyces*) (Saadoun et al., 2001; Schrader and Blevins, 2001). Acidifying the water results in hydrolysis of geosmin, however, the optimum pH level of the different fish species limits the possibility to lower this factor below a given level (Hsieh et al., 2012; Kim et al., 2016). Another study proposes to keep the pH above 5 to ensure intracellular geosmin will not be released (Qian et al., 2014). Bacterial interactions also influence the production of geosmin, which was shown in a study employing an *Anabaena* strain and evaluate the inhibiting effects by adding other bacterial strains (Aoyama, 1995). *Streptomyces avermitilis* decreased the production of geosmin *in vitro* when the concentration of salinity increased (Řezanka and Votruba, 1998). Previous studies have also shown that the redox potential plays a role in regulating the geosmin production, however, the geosmin-producing bacteria were not identified in this study (Parinet et al., 2012).

1.3.5 Geosmin analyses

Off-flavour in aquaculture can often be detected simply by smelling to the water (olfactometry) and the fish when the levels are above the human detecting threshold. For this, specially trained sensory panels with an expertise in detecting the presences of different off-flavour compounds are required, however, this method for detection geosmin is challenged by the varying sensitivity among people towards detecting geosmin (Burr et al., 2012; Houle et al., 2011). In many studies, the exact determination and quantification of geosmin in both water and fish fillets are of high value for investigation of off-flavour problems in aquaculture. For these analyses, analytical and molecular methods can be used.

1.3.5.1 Analytical methods

Numerous different analytical methods have been used to detect geosmin. Often extraction of the volatile compound is important to ensure proper determination/quantification and in earlier days this was performed with solvent extraction or steam distillation extraction (SDE). These methods are only sufficient when the analyte is present in high concentration (Conte et al., 1996; Gerber and Lechevalier, 1965; Johnsen and Kuan, 1987; Saadoun et al., 2001). For extraction and concentration of geosmin prior to detecting, methods such as dynamic headspace, static headspace, liquid-liquid microextraction, closed loop-stripping analysis, stir-bar sorptive extraction and solid-phase extraction are conducted (Bauld et al., 2007; Breheret et al., 1999; Cortada et al., 2011; Davidson et al., 2014; Izaguirre et al., 1982; Petersen et al., 2014). One of the most popular methods which are applied in several studies is the solid-phase microextraction (Arthur and Pawliszyn, 1990; Guttman and van Rijn, 2008; Lloyd and Grimm, 1998). As geosmin is a volatile compound gas chromatography (GC) almost always is coupled to a mass spectrometer (MS) as the main choice for detecting/quantifying geosmin. This method has shown limit of detection levels down to 1 ng/L (Auffret et al., 2011; Guttman and van Rijn, 2012; Parinet et al., 2011; Yu et al., 2014). The SPME GC-MS setup is displayed in Figure 11. The GC-olfactometer, where a specialist sniffs the compound separated by the GC, is also a widely used instrumental method together with the electronic nose. The latter technique is mainly used as a screening technique to replace expensive sensory panels and requires additional confirmation (Du et al., 2011; Son et al., 2015).

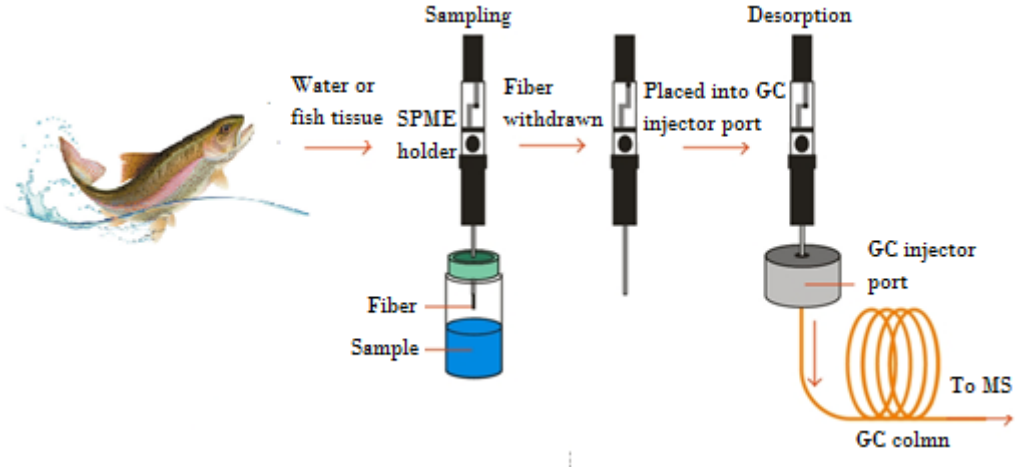

Figure 11: The setup for the SPME GC-MS method. Modified from (Schmidt and Podmore, 2015).

1.3.5.2 Molecular biological methods

Most bacteria have shown to be unable with present approaches to cultivate *in vitro* meaning that they will not be classified by cultivation based approaches (Achtman and Wagner, 2008). Even though molecular methods are applied after cultivation, a selection towards the bacteria cable of cultivating has occurred, which could be avoided sequencing DNA from an environmental sample. Furthermore, quantitative PCR (qPCR) has been applied for detecting of geosmin-producing bacteria to a greater extent in the previous years (Auffret et al., 2013; Su et al., 2013; Lukassen et al., 2017 (Paper 1); Lukassen et al., submitted (Paper 2); Lukassen et al., submitted (Paper 5)). Sequencing and qPCR are two essential methods for understanding the microbial community in aquaculture and both bacterial

DNA and RNA are applicable (Lukassen et al., in preparation (Paper 3)). The usage of DNA provides information on the microbial community composition and possible changes in abundance when e.g. operational changes occur. The limit of DNA studies is the lack of information of the bacterial gene expression *in situ*, which transcriptomic studies can provide (Ludwig et al., 2007; Wang et al., 2014; Lukassen et al., in preparation (Paper 3)). The potential of transcriptomics applied for aquaculture exploring the geosmin-producing population has not been of high focus yet, but have the possibility to provide novel insight into this system. The sequencing and qPCR methods will be presented in the following sections, even though these methods are well established, because of their essential role in the manuscripts prepared for the thesis.

Next generation sequencing

In 1977 Sanger sequencing was developed and has been the method of choice when sequencing clone libraries generated from bacterial DNA for the next 30 years. The urge for less expensive technology and high-throughput sequencing forced the industry to come up with an alternative next-generation sequencing (NGS) approach. The Roches 454 pyrosequencing platform was the first widely used approach, but this has been surpassed by the Illumina sequencing platform (Kozich et al., 2013; Salipante et al., 2014; Schuster, 2007). For sequencing, the 16S rRNA gene has been a standard phylogenetic marker used in many bacterial sequencing studies (Patel and Jain, 2012; Woese and Fox, 1977). Figure 12 depicts the 16S rRNA gene amplicon sequencing approach starting with an environmental sample, extraction of the total bacterial community DNA, amplification of the 16S rRNA genes, sequencing the genes, clustering the sequences into OTUs and finally detecting the taxonomy in the environmental sample, which can be used for phylogeny and community composition analyses. The taxonomy is assigned by comparing the DNA sequence to a 16S rRNA gene databases such as SILVA, Greengenes and MiDAS (McDonald et al., 2012; McIlroy et al., 2015; Quast et al., 2013).

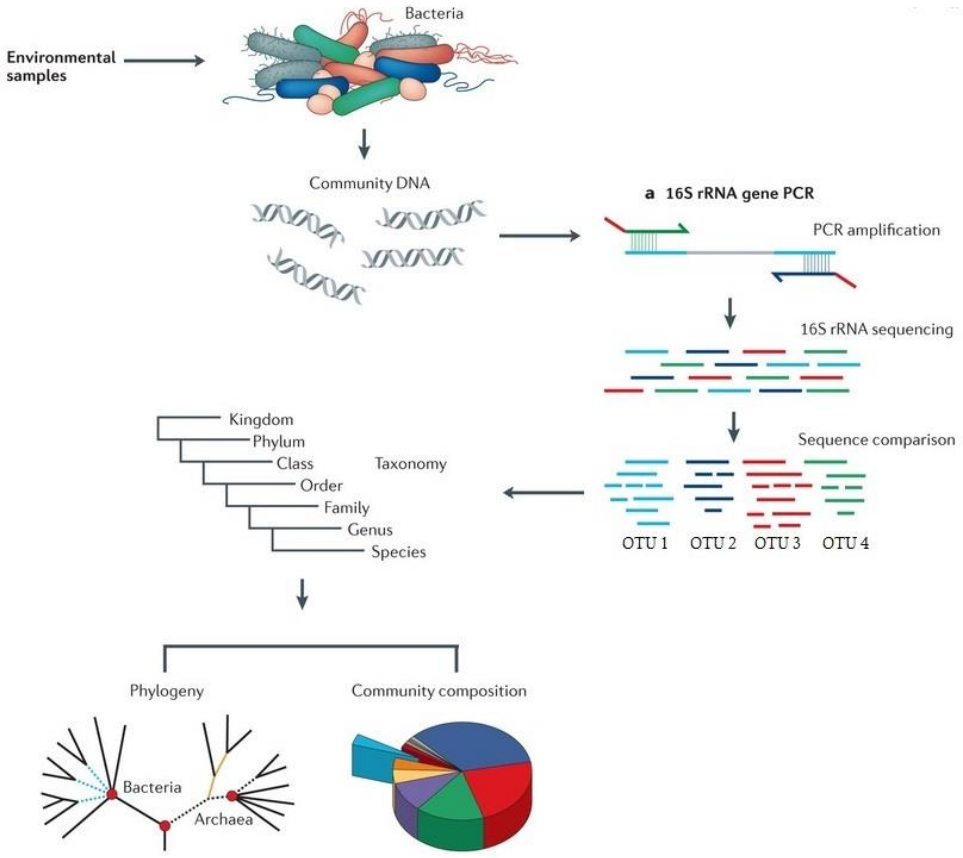

Figure 12: The NGS method for 16S rRNA gene amplicon sequencing. Modified from (Lasken and McLean, 2014)

NGS is not without biases and first of all the extraction method is a subject of bias due to the variations in the cell wall structures between gram positive and gram negative bacteria. Furthermore, it is extremely important to extract the DNA in the exact same manner if sequencing data is to be compared. In addition, differences in 16S rRNA gene copy number from the different microbial species introduce bias, resulting in species with many copy numbers appear more abundant. Also, a bias worth to mention is that the DNA extraction, and thereby NGS, does not distinguish between viable and dead cells. Finally, the primer choice affects the resulting sequencing data (Albertsen et al., 2015; Větrovský et al., 2013). Despite the biases in DNA extraction and NGS, it is a widely used and applicable method. NGS is also usable for other functional genes e.g. the *geoA* gene would be possible to sequence and thereby identify the microbial community in an environmental sample harbouring the gene responsible for the utilization of FPP to geosmin. As more sequence data becomes accessible, information at the DNA level it will provide a better understanding of the responsible microbes for geosmin production and support the development of new diagnostic assays.

Quantification of geosmin-producing bacteria

Early detection of geosmin-producing bacteria in aquaculture are crucial for preventing off-flavour in the fish. qPCR provides a tool making this possible and several studies have been conducted in aquatic environments targeting the *geoA* gene in different species (Auffret et al., 2013; Su et al., 2013).
qPCR enables the quantification of specific genes in environmental samples by detecting the copies of the relevant genes in a PCR amplification procedure. This amplification process is detected by a

fluorescent signal deriving from the produced amplicon, and thus the earlier the gene is amplified the lower the threshold cycle (C_t) will be, meaning the higher amounts of target genes. In contrary, a higher C_t means a lower amount of target sequence (Bustin et al., 2009; Taylor et al., 2010). The qPCR approach is illustrated in Figure 13. To do absolute quantification with qPCR a standard curve with known amount of target DNA is utilised (Nolan et al., 2006).

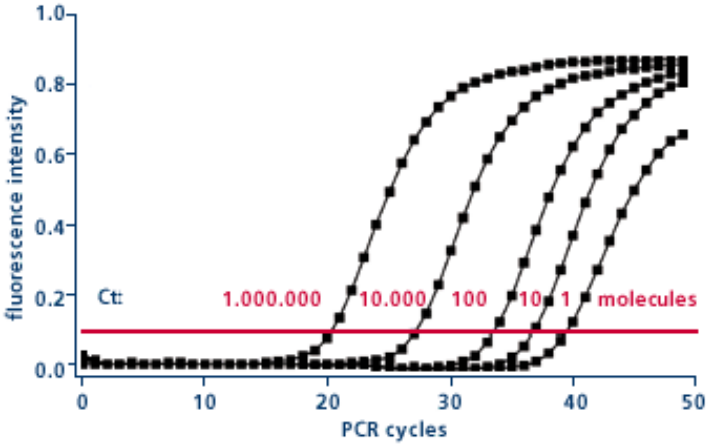

Figure 13: The qPCR approach in quantifying unknown genes.

The fluorescent signal can be detected by non-specific binding (SYBR green) and specific binding (TaqMan). The commercially available SYBR green dye function by binding to the synthesised double-stranded DNA resulting in emission of the fluorescence. At the end of each PCR cycle, the fluorescence amount is measured to monitor the increasing amount of amplified DNA (Figure 14). SYBR green has the advantages of being easy to use and is inexpensive. The disadvantage is that it is a non-specific dye, which risks binding to non-target molecules and primer dimers (Morrison et al., 1998; Noble and Fuhrman, 1998). Taqman is also a commercially available dye, which apply a hydrolysis probe with a fluorophore and a quencher attached to the 5' end and 3' end, respectively. The probe then binds inside the target sequence and during amplification, the polymerase cleaves the probe to separate the fluorophore and quencher resulting in increased fluorescence, which is measured after each PCR cycle (Figure 14). The advantages of this method is the higher level of specificity due to the probe, primer dimer does not result in fluorescence, multiplexing is possible because of differently available fluorophores. Disadvantages are the increased expenses and that a unique probe for each target has to be designed before the analysis (Heid et al., 1996).

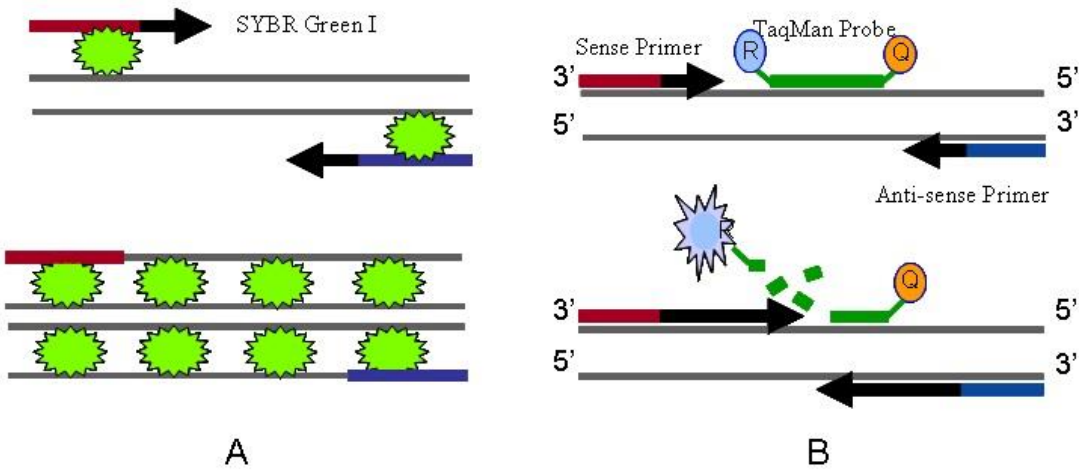

Figure 14: A) The fluorescence dye binds unspecific to the double stranded DNA in the SYBR green approach. B) The probe binds a specific DNA target releasing fluorescence signal only when the target is amplified (Xu et al., 2011).

1.4 Management of geosmin in aquaculture

An aquaculture system not suffering from geosmin can be achieved by a removal or prevention strategy, which can involve both chemical, physical and biological procedures. Various treatments to eliminate geosmin by removal have been studied and tested *in vitro* and in commercial RAS. Ozone, chlorine and UV are often used as disinfection units, but they are not especially effective in degradation of geosmin even though UV/VUV showed to degrade geosmin better than UV (Ho et al., 2002; Kutschera et al., 2009). Furthermore, granular activated carbon (GAC) and powdered activated carbon (PAC) have shown promising results in the adsorption of geosmin, but this adsorption is adversely affected by the presence of natural organic material, causing reduced effectivity (Cook et al., 2001; Cook and Newcombe, 2004; Hung and Lin, 2006). Biological removal is a less expensive approach than installing the chemical/physical treatment units and are, therefore especially interesting. Certain bacterial strains have revealed to be able to degrade geosmin making it possible to apply biological degradation of the compound in aquaculture but more research has to be conducted before it is an applicable method in full-scale aquaculture (Guttman and van Rijn, 2012, 2009; Ho et al., 2007). A relatively easy method to eliminate geosmin in a controlled system as RAS (and to some extend open aquaculture) is by changing the environmental conditions in the system. Obviously, this depends on knowledge on which parameters to change. In this thesis, several environmental factors were found to correlate either positively or negatively with geosmin and/or geosmin-producing bacteria and by avoiding these factors, elevating geosmin production prevention could be achieved (Lukassen et al., submitted (Paper 2)). The main triggers for elevated geosmin levels are high concentration of COD, temperature, redox potential, phosphate and nitrate. As expected due to the finding of redox potential, pH correlates negatively with geosmin meaning that a low pH elevates geosmin concentration in the water. Surprisingly, low concentrations of oxygen also trigger geosmin levels to raise (Lukassen et al., submitted (Paper 2)), however, high levels of oxygen was found to induce geosmin in another study (Lukassen et al., in preparation (Paper 3)) and may reflect the present geosmin producer. These environmental parameters found to affect bacteria harbouring the *geoA* gene and geosmin production (Lukassen et al., submitted (Paper 2); Lukassen et al., in preparation

(Paper 3)), provides the possibility to manage the microbial community and thereby to control the performance of the microorganisms in aquaculture. This shall be performed with care not changing a parameter in too high a degree or altering too many parameters, jeopardising the well-performing bacteria in the system, but just enough to destabilise the geosmin-producing bacteria and allow other microorganisms to outcompete them.

Geosmin-producing bacteria were found in the gut of the fish in Brazilian aquaculture indicating the release of geosmin inside the fish instead of the formerly acknowledged route through the gills (From and Hørlyck, 1984; Howgate, 2004; Lukassen et al., submitted (Paper 5)). If this is the case water treatment will properly not be effective as this does not reach the bacteria in the gut but as diet has shown to modulate the microbiota this could be a feasible method to eliminate the geosmin-producing bacteria inside the gut reducing the absorption of geosmin. Most of these recommendations are manageable in aquaculture and could lead to production systems with less geosmin.

2 Conclusions and perspectives

The objectives of this thesis were to investigate the microbial communities in aquaculture in order to gain a better understanding of the diversity, presence and habitat of geosmin-producing bacteria within these systems. Furthermore, analysing for correlations between geosmin and geosmin-producing bacteria was carried out to provide knowledge on their dependencies for various water quality parameters. The obtained information, therefore, provide importing insight into the geosmin-producing bacteria, which can be used in the creation of an elimination strategy. Lastly, an investigation of the of fish gut microbiome response during β-glucan treatment provided a proof that administrating prebiotics could provide the capability to change the microbial composition inside the gut and influence the health of the host.

The putatively geosmin-producing microbial compositions were investigated in six European recirculated aquaculture systems (RAS) (Lukassen et al., 2017 (Paper 1)). The *geoA* gene was previously suggested as a suitable molecular marker for targeting geosmin-producing bacteria, which were confirmed in this study. A more diverse group of geosmin-producing bacteria than previously assumed was found in the full-scale RAS. Furthermore, no *Streptomyces* were detected in any of the systems but interestingly, several unidentified groups of the order *Actinomycetales*, myxobacteria and two closely related groups affiliating with the genus *Sorangium* showed to be abundant in these systems. A Taqman assay using specific TaqMan qPCR primers were developed to quantify these unidentified *geoA*-containing bacteria and this assay revealed a small but potent fraction of geosmin-producing bacteria in RAS. The proportions of the geosmin causing organisms were only in the range 0.007 - 0.9 % of the total bacterial count but still causing measurable off-flavour in the systems. While constituting such a small fraction of the entire bacteria population it could be hypothesised that an elimination strategy for removal of these organisms is manageable without risking the well-functioning microbes present.

The newly designed specific primers were used to determine the geosmin-producing bacteria in 26 European RAS together with primers targeting the overall geosmin-producing composition (Lukassen et al., submitted (Paper 2)). The *Sorangial* group showed to be most abundant and statistically correlated to geosmin, and thus indicate that this genus is the main contributor to geosmin production in the investigated systems. Furthermore, geosmin revealed to correlate positively to COD, temperature, phosphate and redox potential whereas oxygen and pH correlated negatively to geosmin. These results provide a better understanding of the environmental parameters controlling the production of geosmin and the geosmin-producing population in RAS offering knowledge to the elimination of these microbes.

The statistically based observation that high concentrations of organic matter (COD) correlate with elevated geosmin levels was investigated in a laboratory scale RAS in order to confirm the hypothesis and establish a mode of action (Lukassen et al., in preparation (Paper 3)). Well-controlled systems were manipulated to increase the BOD concentration, and the content of geosmin, BOD and transcripts of *geoA* confirmed that the presence of high organic load concentrations affects geosmin production on the transcriptional level through an upregulation of the *geoA* gene expression.

The microbial community was analysed in a Danish RAS (Lukassen et al., in preparation (Paper 4)). Monitoring the microbiota revealed a relatively stable community for a period of nine months, reflecting the environmental changes. This first insight into the microbial community in a RAS and the factors shaping it could assist in the developing of monitoring tools for the RAS operator in the future.

The presence and abundance of geosmin-producing bacteria were analysed in six Brazilian aquaculture (Lukassen et al., submitted (Paper 5)). Multivariate data analysis and quantification of the *geoA* gene revealed a high content of geosmin-producing microorganisms inside the fish gut. This indicates that geosmin might be taken up together with the feed and released inside the intestinal tract when the cells are lysed. This challenge the general perception that the major route of geosmin uptake occurs through an uptake of dissolved geosmin over the gills. Therefore, manipulation of the gut microbiome by providing probiotics/prebiotics might be a future strategy for eliminating off-flavour caused by geosmin in aquaculture.

The gut microbiome was investigated in rainbow trout in order to determine the bacterial response to a dietary treatment with β-glucans (Lukassen et al., in preparation (Paper 6)). It could be shown that the β-glucan treatment statistically significant alters the microbiome where specific bacterial genus increases/decreases more strongly affected by the higher dose of β-glucans administrated. Furthermore, the metabolic profile revealed also to change for the fish receiving β-glucans. This knowledge indicates the gut microbiome can be manipulated in order to influence the metabolites in the host.

In conclusion, it was possible to investigate the microbial communities in various systems and environments and geosmin-producing bacteria were identified and quantified in all investigated aquaculture. The gut microbiome was proposed to have a higher significance in geosmin accumulation than previously believed and the potential of manipulating the composition in the microbiome was proved.

Microbial management opens up new possibilities to control the performance of aquaculture especially in RAS, which are closed ecosystems in which it is possible to control various parameters. Managing the microbial community can be performed either by changing the environmental parameters (pH, nitrate, organic load) or through bioaugmentation (addition of known microbes in the system). Environmental parameters have a large impact on the geosmin-producing bacteria and other microorganisms within the systems so knowledge on which parameters have an impact on the microbes that are beneficial or harmful for the system could provide the basis for microbial management in future aquaculture.
Identification of the microbial community in aquaculture could provide knowledge of biological indicators for upcoming increased geosmin production, but more research is needed to propose potential candidates. To obtain a higher resolution of the low abundant geosmin-producing bacteria can be achieved by amplicon sequencing the functional *geoA* gene. A challenge at present is that the databases used for identifying the bacteria harbouring the functional genes is incomplete due to the many unsequenced bacterial species. Research data of high coverage libraries might provide increased linkage of such unclassified organisms harbouring *geoA* and serve as databases in depth analysis of *geoA* containing populations in aquaculture.

Probiotics and prebiotics provide the potential to increase or decrease specific bacterial strains in the gut microbiota, making it possible to eliminate pathogens and other unwanted groups e.g. geosmin-producing bacteria. The identification of suited prebiotics require additional research on the effect of new compounds and synergetic effects. Combinations with the inductions of e.g. stress hormones might facilitate to identify suitable compounds.

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