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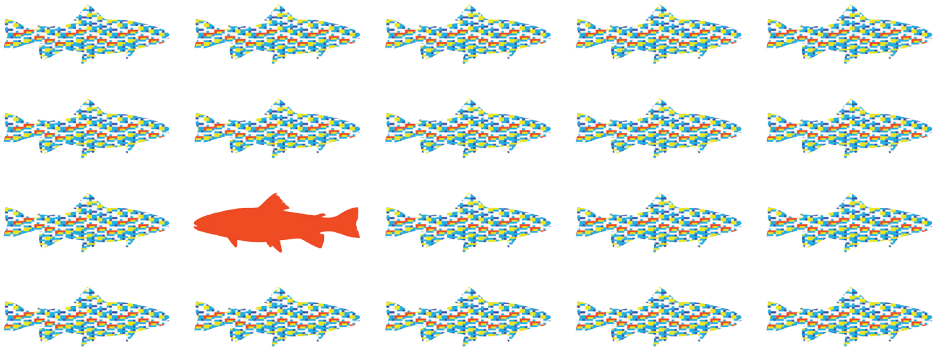
Impact of Fish Farming on Antibiotic Resistome and Mobile Elements in Baltic Sea Sediment



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IMPACT OF FISH FARMING ON ANTIBIOTIC RESISTOME AND MOBILE ELEMENTS IN BALTIC SEA SEDIMENT

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List of Original Publications

This thesis is based on the following articles and manuscript, which are referred to by their Roman numerals in the text:

I. **Muziasari WI**, Pärnänen K, Johnson TA, Lyra C, Karkman A, Stedleft R, Tamminen M, Tiedje JM, Virta M. (2016). Aquaculture changes the profile of antibiotic resistance and mobile genetic element associated genes in Baltic Sea sediments. *FEMS Microbiol. Ecol.* 92(4): fiw052. doi: 10.1093/femsec/fiw052.*****

II. **Muziasari WI**, Managaki S, Pärnänen K, Karkman A, Lyra C, Tamminen M, Suzuki S, Virta M. (2014). Sulfonamide and Trimethoprim resistance genes persist in sediments at Baltic Sea aquaculture farms but are not detected in the surrounding environment. *PLoS ONE* 9(3):e92702.

III. Tamminen M, Karkman A, Lõhmus A, **Muziasari WI**, Takasu H, Wada S, Suzuki S, Virta M. (2011). Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* 45: 386–391.******

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Author Contributions

I. Windi I. Muziasari took part in planning of the sampling and collecting sediment samples. She designed the experimental setup and participated in DNA isolation and practical laboratory works, with exception of the qPCR array analyses. She interpreted the qPCR array results and performed the statistical analyses. She had the main responsibility of the writing of the manuscript.

II. Windi I. Muziasari took part in planning of the sampling and collecting sediment samples. She developed qPCR methods for the resistance gene quantification. She designed the experimental setup and participated in performing DNA isolation, the qPCR analyses and practical laboratory works, with exception of the antibiotic compound quantification. She interpreted the results and performed the statistical analyses. She had the main responsibility of the writing the manuscript and is the corresponding author.

III. Windi I. Muziasari performed qPCR analyses for two tetracycline resistance genes and participated in interpreting the results.

IV. Windi I. Muziasari planned the sampling and collected the fish samples. She designed the experimental setup, modified the DNA isolation method for different types of samples and participated in performing the practical laboratory works, with exception of the qPCR array analyses. She interpreted the results and performed the statistical analyses. She had the main responsibility of the writing the manuscript.

Abbreviations and Definitions

Antibiotic	a medicine or compound that can kill bacteria or limit their growth
Antibiotic resistance	the ability of bacteria to grow in the presence of an antibiotic
ARG(s)	antibiotic resistance gene(s), any gene (or genes) which enables a bacterium to tolerate or resist antibiotics at concentrations which would kill or inhibit the growth of other bacteria
Antibiotic resistome	consists of all the existing ARGs that are capable of conferring resistance towards antibiotics
CARD	comprehensive antibiotic resistance database; an up to date bioinformatic database for antibiotic resistance and is actively being maintained.
16S rRNA gene	ribosome component of the prokaryotic 16S small subunit
DNA	deoxyribonucleic acid, the molecule that carries genetic information in all living systems
Primer Set	a set of oligonucleotides which are hybridized to a target gene; is used in PCR and qPCR reactions
PCR	polymerase chain reaction, a method to detect a target gene
qPCR	quantitative polymerase chain reaction, a method to detect and quantify a target gene
qPCR array	quantitative polymerase chain reaction array, a highly parallel method of detection and quantification which allows hundreds of primer sets in one reaction

C_T value	a value of threshold cycle, the cycle number which is used to calculate the limit of detection in qPCR
DeltaC_T (ΔC_T)	ΔC_T = (C_T target gene - C_T 16S rRNA gene); can be used for relative quantification of a target gene's abundance in qPCR
HGT	horizontal gene transfer, the transfer of genes between organisms
MGE(s)	mobile genetic element(s), a genetic element which is able to move within genomes or between cells
Transposon	a mobile genetic element
Gene cassette	a small mobile genetic element that contains a gene and a recombination site; usually links to integrons
Integron	a genetic element which is able to capture and incorporate gene cassettes
Class 1 integron	the integron of class 1; known to capture ARG cassettes
Juvenile fish	a small fish; when grown to a sufficient size, enters the fish cages at a coastal fish farm
Baltic Sea	a semi-enclosed inland sea of the Atlantic Ocean located in Northern Europe; is bordered by Sweden, Finland, Russia, Estonia, Latvia, Lithuania, Poland, northeastern Germany and Denmark; is one of the earth's largest bodies of brackish water.

Abstract

Antibiotic resistance has become a serious threat to the efficacy of antibiotics used in human and veterinary medicine. Understanding the abundance and prevalence of antibiotic resistance genes (ARGs) in the environmental resistome is important for maintaining the efficacy of antibiotics and predicting a risk of the ARGs spreading in the environment and moving into previously non-resistant bacteria, including human pathogens. Fish farms are an environmental reservoir of ARGs due to the treatment of fish with antibiotics that also are important for human medicine.

The two main topics of this thesis are (1) determining the abundance and diversity of ARGs and mobile elements in sediments impacted by fish farming and (2) investigating the major source of ARGs in the farm sediments in the Northern Baltic Sea. In addition, correlations between ARGs and mobile elements were examined to estimate the potential risk of ARG mobilization in the environment. This study employed a high-throughput qPCR array, which permits quantifying hundreds of ARGs and genes associated with mobile elements in the environmental resistome in a single experiment.

Fish farming impacts the composition of ARGs in sediments below fish farms in the Northern Baltic Sea. However, the impact is local and mostly limited to enrichment of ARGs associated with antibiotics used at the farms. In the current conditions, the risk of ARG spread from the farm sediments to the surrounding sediments is low in the Northern Baltic Sea. However, the enriched ARGs persist in the farm sediments during the 6-year observations even when the selection pressure of the antibiotics is negligible. Moreover, significant correlations between mobile elements and ARGs may imply the persistence of certain ARGs in the fish farming environments and their potential for mobilizing the ARGs to other bacteria including pathogens. The persistence of ARGs at the farm facilities is a threat to the efficacy of the antibiotics against fish diseases, potentially leading to fish production losses. We provide indirect evidence suggesting that certain ARGs are being constantly introduced by feces of the farmed fish into the sediments below the fish farms. Further studies could focus on investigating the development of ARGs in juvenile fish before they are introduced into the Baltic Sea open-cage farms. We conclude that a high throughput qPCR array is a powerful tool that provides unprecedented insights into the ARG composition in the environmental resistome associated with fish farming.

Tiivistelmä

Bakteerien vastustuskyky antibiooteille eli antibioottiresistenssi uhkaa heikentää ihmisten ja eläinten lääkinnässä käytettävien antibioottien tehoa. Bakteerien vastustuskyky aiheutuu antibioottiresistenssigeeneistä, joita bakteerit kantavat perimässään. Ympäristössä tavattavien antibioottiresistenssigeenien määrien ja leviämisen seuraaminen on keskeistä antibioottien tehon säilyttämiseksi sekä riskien ymmärtämiseksi. Erityisen riskin aiheuttavat uudet vastustuskykyiset bakteerikannat, mukaanlukien ihmisille sairauksia aiheuttavat kannat. Kalankasvattamoilla käytetään antibiootteja kalojen sairauksien hoitamiseksi. Kasvatuksen seurauksena kasvattamoiden ympäristössä havaitaan resistenssigeenejä keskimääräistä enemmän.

Tämän väitöskirjan kaksi aihetta ovat (1) antibioottiresistenssigeenien sekä liikkuvien geneettisten elementtien määrien ja monimuotoisuuden selvittäminen Itämerellä sijaitsevien kalankasvatuslaitosten pohjasedimenteissä sekä (2) resistenssigeenien alkuperän selvittäminen. Lisäksi tutkittiin epäsuorasti resistenssigeenien kykyä liikkua uusiin bakteerilajeihin. Tutkimuksissa käytettiin uudenlaista qPCR-pohjaista analyysimenetelmää, joka mahdollistaa satojen resistenssigeenien ja liikkuvien geneettisten elementtien samanaikaisen mittaamisen näytteistä.

Kalankasvatus vaikuttaa paikallisesti antibioottiresistenssigeenien määriin ja monimuotoisuuteen kalankasvattamoiden pohjasedimenteissä Itämerellä. Vastustuskykyä havaitaan erityisesti niitä antibiootteja vastaan, jotka ovat olleet yleisessä käytössä kasvattamoilla. Tulostemme perusteella resistenssigeenien leviäminen kasvattamoiden lähiympäristöön on melko vähäistä. Toisaalta resistenssigeenit säilyvät sedimenteissä kuuden vuoden seurantajakson ajan, vaikka antibioottien aiheuttama valintapaine on ollut heikko. Merkittävä yhteys liikkuvien geneettisten elementtien sekä resistenssigeenien määrien välillä saattaa tarkoittaa uusien vastustuskykyisten kantojen muodostumisen riskiä. Lisäksi resistenssigeenien pysyvyys kalankasvattamoilla on uhka kalankasvatuksessa käytettävien antibioottien teholle, ja saattaa johtaa kasvaneisiin kustannuksiin tuotannon laskun vuoksi. Tutkimus antaa epäsuoraa näyttöä siitä, että tietyt resistenssigeenit saapuvat kasvattamoille kasvatettavan kalan ulosteen mukana. Jatkotutkimuksissa olisi syytä mitata nuorten kalojen kantamia antibioottiresistenssigeenejä ennen kalojen saapumista kasvattamoille. Toteamme lisäksi, että tutkimuksessa käytetty uudenlainen qPCR-pohjainen analyysimenetelmä soveltuu hyvin antibioottiresistenssigeenien tutkimiseen ympäristössä.

Ringkasan

Antibiotik biasanya digunakan untuk pengobatan penyakit infeksi pada manusia atau hewan karena mempunyai efek menekan atau menghentikan metabolisme bakteri. Penggunaan antibiotik di bidang budi daya ikan telah menyebabkan peternakan ikan menjadi salah satu sumber resistensi antibiotik di lingkungan. Resistensi antibiotik adalah kemampuan bakteri menjadi kebal terhadap efek kerja antibiotik. Resistensi antibiotik dikodekan oleh gen-gen resisten yang dapat ditransfer dari satu bakteri ke bakteri lainnya melalui unsur genetik bergerak (*mobile genetic elements*) sehingga gen-gen resisten tersebut dapat menyebar secara luas di berbagai lingkungan. Keseluruhan gen resisten di habitat lingkungan tertentu didefinisikan sebagai resistome antibiotik.

Keberadaan bakteri resisten di peternakan ikan akan menyebabkan terapi antibiotik untuk pengobatan infeksi pada ikan menjadi tidak efektif, yang kemudian akan menyebabkan produksi ikan menurun. Oleh karena itu, dengan memahami komposisi dari resistome antibiotik di lingkungan budi daya ikan, kita dapat memprediksi potensi munculnya gen-gen resisten di peternakan ikan dan mencegah penyebaran gen-gen tersebut ke lingkungan sekitarnya. Hal ini sangat penting terutama pada kemungkinan terjadinya penyebaran gen resisten dari bakteri yang hidup di lingkungan alami ke bakteri patogen yang dapat menyebabkan penyakit. Karena keterbatasan metode untuk menganalisa resistome antibiotik di lingkungan, sampai saat ini studi dampak dari kegiatan budi daya ikan pada resistome antibiotik di lingkungan belum ada.

Dalam disertasi ini, dengan subyek penelitian di dua peternakan ikan di Laut Baltik Utara di Finlandia, dampak dari kegiatan budi daya ikan pada resistome antibiotik di lingkungan sedimen laut di bawah jaring apung ikan dianalisa dengan menggunakan metode genetika molekuler terbaru. Metode tersebut adalah qPCR array komprehensif yang secara menyeluruh dan serentak dapat mendeteksi dan menghitung keberadaan ratusan gen di lingkungan. Tujuan utama disertasi ini adalah untuk 1) mengamati kelimpahan dan keragaman gen-gen resisten dan unsur genetik bergerak (transposon dan integron kelas 1) di sedimen Laut Baltik Utara yang dipengaruhi oleh kegiatan budi daya ikan, 2) menyelidiki sumber utama gen-gen tersebut di sedimen Laut Baltik Utara, dan 3) untuk meprediksi potensi penyebaran gen resistensi di lingkungan budi daya ikan dengan menganalisa korelasi antara kelimpahan gen-gen resistensi dan unsur genetik bergerak.

Hasil penelitian dari disertasi ini berupa tiga artikel yang telah dipublikasikan di *peer-review* jurnal dan satu manuskrip. Penelitian ini menghasilkan empat kesimpulan utama sebagai berikut:

- Kegiatan budi daya ikan mempunyai dampak yang signifikan pada komposisi resistome antibiotik di sedimen laut di bawah jaring apung ikan di Laut Baltik Utara (Artikel **I**). Dampak tersebut bersifat lokal dan hanya terbatas pada pengayaan gen-gen resisten terhadap sulfonamida, trimetoprim, dan oksitetrasiklin (jenis antibiotik yang telah atau sedang digunakan di daerah peternakan ikan). Namun, keberadaan transposon atau unsur genetik bergerak dapat menyebabkan prevalensi gen resisten tertentu di sedimen peternakan ikan dan potensi transfer gen resisten ke bakteri lain termasuk bakteri patogen. Selain itu, penelitian ini juga menunjukkan kecenderungan adanya resistome antibiotik alami di sedimen Laut Baltik Utara yang sebagian besar terdiri dari gen-gen resisten yang memiliki mekanisme pompa efluks.

- Berdasarkan hasil pengamatan selama 6 tahun dari tahun 2006 - 2012, gen-gen resisten terhadap sulfonamida, trimetoprim (Artikel **II**) dan oksitetrasiklin (Artikel **III**) tetap melimpah di sedimen peternakan ikan walaupun tekanan seleksi dari antibiotik-antibiotik tersebut tidak terdeteksi di sedimen. Akan tetapi, karena gen-gen resisten tersebut tidak terdeteksi di kontrol sedimen di luar peternakan ikan, resiko penyebaran gen-gen tersebut dari sedimen peternakan ikan di Laut Baltik Utara ke sedimen sekitarnya cukup kecil. Hal ini menunjukkan bahwa berlimpahnya gen-gen resistensi di peternakan ikan berpotensi besar menjadi masalah bagi industri budi daya ikan, tetapi kurang berdampak pada lingkungan sekitarnya.

- Berdasarkan hasil analisa komposisi resistome antibiotik di saluran cerna ikan-ikan dari peternakan ikan, secara tidak langsung dapat disimpulkan bahwa kotoran dari ikan ternak tersebut adalah sumber utama unsur genetik bergerak dan gen-gen resisten tertentu di sedimen peternakan ikan di Laut Baltik Utara, Finlandia (Manuskrip **IV**).

- Terdapat korelasi yang signifikan antara kelimpahan integron kelas 1 dengan salah satu gen resisten sulfonamida (Artikel **II**) dan antara transposon dengan gen resisten tetrasiklin (Manuskrip **IV**). Pengamatan ini

menunjukkan bahwa integron kelas 1 dan transposon dari unsur genetik bergerak mungkin memainkan peranan besar dalam prevalensi gen-gen resisten tertentu di lingkungan budi daya ikan di Laut Baltik Utara, Finlandia.

Berdasarkan hasil penelitian ini, dapat disimpulkan bahwa qPCR array adalah metode yang ampuh yang dapat memberikan wawasan baru secara komprehensif dalam memahami komposisi resistome antibiotik di lingkungan yang terkait dengan budi daya ikan. Hasil dari penelitian ini diharapkan dapat meningkatkan kualitas manajemen budi daya ikan dan memberikan kontribusi ilmiah untuk mewujudkan lingkungan perairan yang sehat.

1.

INTRODUCTION

1. Introduction

Fish farming is the main form of aquaculture and involves raising fish in pens or cages (FAO, 2007). The fish are usually farmed for food production providing an important protein and nutrient source for the human population. Due to the use of antibiotics, fish farms have been suggested as one of antibiotic resistance reservoirs in the environment (Cabello *et al.*, 2013). The emergence of antibiotic resistance in fish farming environments may lead to inefficient success of the antibiotic treatment at the fish farms and thus results in production losses in the fish farming industry.

On the other hand, antibiotic resistance itself is a natural phenomenon (D'Costa, 2011). Bacteria have been evolving to resist the naturally occurring antibiotics in the environment over the history of life. Antibiotic resistance is encoded by antibiotic resistance genes (ARGs) and the collection of all the existing ARGs is defined as antibiotic resistome (D'Costa, 2006, Wright, 2007). The ARGs can be transferred between bacteria via mobile genetic elements (MGEs) through horizontal gene transfer (HGT) systems which increase the dissemination of ARGs in the environment (Wright, 2010). Therefore, insight into the antibiotic resistome profiles in the fish farming environments is valuable to predict potential ARGs emerging in fish farming and a risk of the ARGs spreading to other environments, including the human pathogens.

However, due to the limitations of methods to study antibiotic resistome in the environment, currently no data exists to explain the impact of fish farming on the environmental resistome. In the following chapters, using fish farms in the Northern Baltic Sea, Finland as research subjects, I explore the impact of fish farming on the resistome composition and mobile elements in the environment. I also investigate the plausible source of ARGs in the fish farming environments. I focus on using culture-independent method, in particular, a high throughput method for detection and quantification of ARGs to observe the composition and quantify the environmental resistome.

1.1. Baltic Sea fish farming

The Baltic Sea is a unique brackish water marine environment. Since 1970s, fish farming has been practiced in the Baltic Sea (Honkanen & Helminen, 2000). Baltic Sea fish farms use an open-cage system in which fish are kept inside a net that allows natural water interchange (FAO, 2007). Because the Baltic Sea has no tides and slow water circulations, the waste of Baltic Sea fish

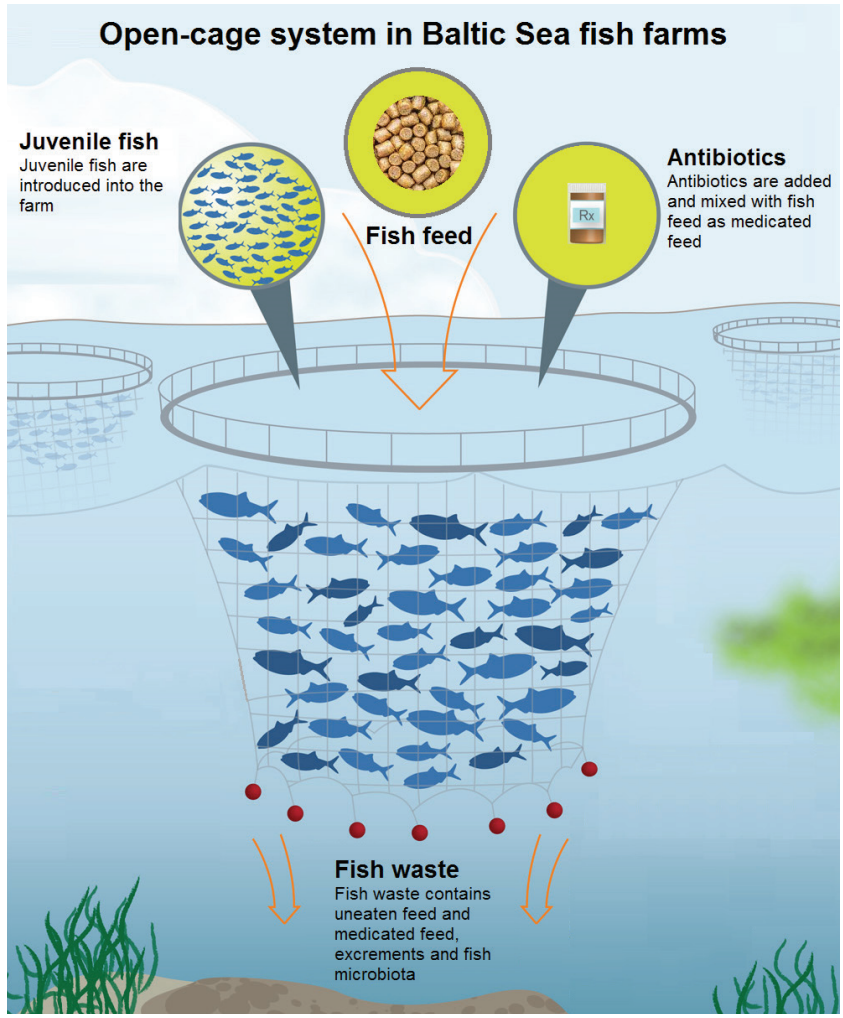


Figure 1. Baltic Sea fish farms use an open-cage system which allows free transfer of water from the farms to the surrounding water and eventually to the sediments (adapted and modified from Ocean Conservancy: Aquaculture).

farms may impact directly the sediments below the farms (Figure 1).

The biggest producers of farmed fish in the Baltic Sea countries are operating in Finland. Food fish production in Finland was approximately 13.6 kilotons with a production value of 56 million EUR in 2013 (Finnish Game and Fisheries Research Institute, 2014). The fish species farmed for the food consumption mainly are rainbow trout (*Onchorhynchus mykiss*) and common whitefish (*Coreganus lavaretus*). In Finland, juvenile fish from the average weight of 5 - 15 g (fry) until the average weight reaches 100 – 500 g (fingerlings) are farmed in freshwater in artificial ponds. The fingerlings are subsequently transferred into net cages in the Baltic Sea brackish water for one or two growing periods (June-November) (Varjopuro *et al.*, 2000).

1.2. Usage of antibiotics in fish farming

Antibiotics are used at fish farms to treat fish infectious diseases caused by bacterial pathogens. The antibiotics generally act in the parts of fish where the rate of blood circulation is high such as in the fish intestine and gills. The administration of antibiotics in fish farming is dependent on the type of the culture system. Water medication and medicated feed are the most common routes of administration (Heuer *et al.*, 2007, Park *et al.*, 2012). Water medication is commonly used in a closed-cage fish farming or ponds where the biomass is small such as in juvenile and larvae farming. The dissolved antibiotics are absorbed by the juvenile fish through the gills, skin and mucosa. Medicated feeds, which are commonly used in the open-cage fish farming, are prepared by adding and mixing the antibiotics into feed, or sprayed, or top-coated onto the fish feed. However, usually the healthier fish eat more than the infected fish. Therefore, usually the usage of medicated feed is prophylactic rather than therapeutic (Park *et al.*, 2012). Uneaten medicated feed along with fish excrements contain undigested antibiotics that may end up in the sediments below the fish cages.

1.2.1. Common antibiotics in fish farming

Table 1 shows typical classes of antibiotics globally used in fish farming: aminoglycosides, (chlor)/(flor)/(am)fenicol, beta-lactams, (fluoro)quinolones, macrolides, nitrofurans, sulfonamides, tetracycline, and trimethoprim. Table 1 also shows the mechanism of antibiotic actions in bacterial cells and the administration route of antibiotics at fish farms. Oxytetracycline, chloramphenicol and oxolinic acid are the most commonly used antibiotics in fish farming worldwide (Sapkota *et al.*, 2008). The antibiotics used in fish farming are also important for human medicines (Sapkota *et al.*, 2008, Heuer *et al.*, 2009, Park *et al.*, 2012).

Table 1. Antibiotics and their mechanisms of action, route of administration at fish farms and importance in human medicine.

Antibiotics	**Classification**	**Mechanism of action***	**Route of administration**	**Importance in human medicine***
Amoxicillin	Beta-lactam	Inhibition of cell wall synthesis	Feed	Critically important
Ampicillin	Beta-lactam	Inhibition of cell wall synthesis	Feed	Critically important
Chloroamfenicol	Amfenicol	Inhibition of protein synthesis	Feed / Water/ Injection	Important
Florfenicol	Amfenicol	Inhibition of protein synthesis	Feed	Important
Erythromycin	Macrolide	Inhibition of protein synthesis	Feed / Water/ Injection	Critically important
Streptomycin	Aminoglycoside	Inhibition of protein synthesis	Feed	Critically important
Furazolidone	Nitrofuran	Disruption bacterial DNA	Feed / Water	Important
Nitrofurantoin	Nitrofuran	Disrupting bacterial DNA	Feed	Important
Oxolinic acid	Quinolone	Inhibition of DNA synthesis	Feed	Critically important
Enrofloxacin	Fluoroquinolone	Inhibition of DNA synthesis	Feed, Water	Critically important
Flumequine	Fluoroquinolone	Inhibition of DNA synthesis	Feed	Critically important
Oxytetracycline, chlortetracycline, tetracycline	Tetracycline	Inhibition of protein synthesis	Feed / Water/ Injection	Critically important
Sulfodiazine, sulfamethoxazole	Sulfonamide	Inhibition of protein synthesis	Feed	Important
Trimethoprim	Trimethoprim	Inhibition of protein synthesis	Feed	Important

*Sources: Sapkota *et al.*, 2008, Heuer *et al.*, 2009, Park *et al.*, 2012, Davies & Davies, 2010.

1.2.2. Use of antibiotics in Baltic Sea fish farms in Finland

In most countries in Europe and North America, the use of antibiotics in fish farming is strictly regulated and requires a prescription from a veterinary professional (Heuer *et al.*, 2007). In Finland, the use is regulated by the Finnish Food Safety Authority (EVIRA). The antibiotics authorized for use in Finnish fish farms are oxytetracycline, a combination of sulfonamide-trimethoprim with the fixed ratio of 5:1 and florfenicol (EVIRA, 2015a, b). The combination of sulfonamide and trimethoprim is used in fish farming (FAO, 2006) since the action of these two compounds is synergistic (Bushby & Hitching, 1968). At Finnish fish farms, the antibiotics are used to contain furunculosis-causing *Aeromonas salmonicida, Flavobacterium psychrophilum* and *Flavobacterium columnare* which cause flavobacteriosis, and pathogens which occur only in sea farms, such as vibriosis-causing *Vibrio anguillarum*, enteric red mouth disease (ERM)-causing *Yersinia ruckeri*, red-spot disease (RSD)-causing *Pseudomonas anguilliseptica*, and edwardsiolosis-causing *Pseudomonas edwardsielloosi* (Viljamaa-Dirks, 2016). In Finland, the antibiotics are mainly used at coastal farms and at a low level when compared to the global standards (EVIRA, 2007). Between 2001 and 2014, approximately 2.3 metric tons of sulfonamide, 0.6 metric ton of trimethoprim, 1.2 metric tons of tetracycline and 0.04 metric ton of florfenicol were used in fish farming in Finland (EVIRA, 2015a, b). However record of antibiotics usage at individual farms, e.g. at the study Baltic Sea fish farms is not available.

1.3. Occurrence of antibiotic resistance genes in fish farming

The main concern of using antibiotics in fish farming is the development of a reservoir of ARGs that may eventually spread to clinically relevant bacteria (FAO, 2006, Heuer *et al.*, 2009). Certain ARGs have in fact first been detected in fish farming environments from which they have spread to clinical settings (Sørum, 1998, Sapkota *et al.*, 2008). An increasing number of studies has documented the occurrence of ARGs in cultured bacteria isolated from fish farming environments worldwide (Table 2). The ARGs found in bacteria isolated from farmed fish, water and sediment samples mostly encoding resistance to tetracycline, sulfonamides, trimethoprim and fluoroquinolone which are common antibiotics used in fish farming (FAO, 2006).

Table 2. ARGs detected in cultured bacteria isolated from fish farming environment

Detected ARGs	Sample	Type of farm	Location	Reference*
tetA, tetD, tetE, sul1, dfrA1, dfrA2, catB2	Fish (skin and gills)	open-cages, freshwater farms	Denmark	Schimdt *et al.*, 2001a
tetB, tetC, tetD, tetG, tetY	Fish (intestine)	open-cages, marine farms	Japan	Furushita *et al.*, 2003
tetM	Sediments	open-cages, marine farms	Japan	Nonaka *et al.*, 2007
dfrA1, tetA, tetB, tetD, tetE, tetH, pse1, oxa2a, ant(3')Ia, aac(6')-Ia	Fish	freshwater, marine farms	South Africa	Jacobs and Chenia, 2007
tet(39), sulII	Sediments	integrated, freshwater farms	Thailand	Agerso & Peterson, 2007
qnr, aac(6')-Ib-cr, floR	Water	brackish-water farms	Egypt	Ishida *et al.*, 2010
sul1, sul2, sul3	Water	integrated, freshwater farms	Vietnam	Hoa *et al.*, 2010
tetA, tetD, tetM, tetE, aadA, mexB, cadA	Fish (intestine, skin, gills and meat) Water,	freshwater and marine farms	Australia	Akinbowale *et al.*, 2007; Ndi & Barton, 2012
tetA, tetG, dfrA1, dfrA5, dfrA7, dfrA12, dfrA15, blaTEM, strA-B, cat-1, mefA	Fish, Water, Sediments	integrated, freshwater farms	Tanzania and Pakistan	Shah *et al.*, 2012

sul1, sul2	Water, Sediments	integrated, freshwater farms	China	Gao *et al.*, 2012
qnrA, qnrB, qnrS, oqxA, aac(6)-Ib-cr, tetA, tetB, tetG tetK, tetM, dfrA1, dfrA5, dfrA12, sul1, sul2, floR, blaTEM, strA-B	Sediments	open-cages, marine farms	Chile	Buschmann *et al.*, 2012; Shah *et al.*, 2014
tetC, tet33, tetK, tet41, tetB, tetL, tet35, tet32, tetB/P, tetL, strA-B, mefE, mel, fexA, mphB	Sediments	open-cages, marine farms	China	Yang *et al.*, 2013
tetA, tetB, tetC, tetD, tetE, tetK, tetL, tetM, tetO, tetQ, tetS, tetQ, tetX	Water	ponds, freshwater farms	Poland	Harnisz *et al.*, 2015

*References are from after year 2000

Studies shown in Table 2 used culture-dependent methods to observe ARG present in fish farming environments. However, the presence of ARGs in cultivable bacteria may underestimate the presence of ARGs in the environment due to the fact that the majority of environmental bacteria are non-cultivable (Amann *et al.*, 1995, Buschmann *et al.*, 2013). The study of ARGs in the environment can be determined with less bias using culture-independent methods (Perry & Wright, 2013). Studies on ARGs using culture-independent methods in fish farming environments have been reported but have only examined 15 or fewer ARGs (Table 3). Based on the comprehensive antibiotic resistance database (CARD; http://arpcard.mcmaster.ca), there are up to 1600 known ARGs (McArthur *et al.*, 2013). Therefore, an in depth investigation of the resistome composition in fish farming environments is presented in this study.

Table 3. ARGs detected in fish farming using culture-independent methods

Detected ARG	Method of detection	Sample	Type of farm and location	Reference
tetA, tetB, tetD, tetE, tetG, tetM, tetO, tetQ , tetS, tetW	PCR	Water and Sediments	ponds, freshwater farms in Wisconsin, USA	Seyfried *et al.*, 2010
sul1, sul2 ,tetB, tetM, tetO, tetQ , tetT, tetW (sul1, sul2, tetM, tetO, tetT, tetW)	PCR, qPCR	Sediments	integrated, freshwater farms in China	Gao *et al.*, 2012
tetM, tetL	Multiplex PCR	Sediments	open-cages, marine farms in Italy	Di Cisare *et al.*, 2013
tetA, tetB, tetC, tetD, tetE, tetL, tetM, tetO, tetQ , tetS, tetX (tetA, tetC, tetL, tetO)	PCR, qPCR	Water	ponds, freshwater farms in Poland	Harnisz *et al.*, 2015
sul1, sul2, sul3, tetM, tetO, tetW, tetS, tetQ , tetX, tetB/P, qepA, oqxA, oqxB, aac(6)-Ib, qnrS	qPCR	Water and Sediments	ponds, freshwater farms in China	Xiong *et al.*, 2015

1.4. Antibiotic resistome in the environment

The antibiotic resistome consists of all bacterial ARGs, including cryptic ARGs which are not necessarily expressed (Wright, 2007). Studies have shown that the ARGs have been present in the environment long before antibiotics have been clinically used by humans (D'Costa *et al.*, 2011, Bhullar *et al.*, 2012). This is consistent with the concept that antibiotic-producing bacteria must produce the resistance for self-protection (D'Costa *et al.*, 2006, Wright, 2007). In the natural environment, ARGs also have non-resistance-related functions such as providing increased fitness and participating in intracellular signaling (Groh *et al.*, 2007, Martinez, 2008).

Because most of clinically relevant ARGs originate from antibiotic-producers in the environment, e.g. from soil bacteria Actinomycetes, several studies have explored the antibiotic resistome from soil bacteria (Riesenfeld *et al.*, 2004, D'Costa *et al.*, 2006). Since then, our understanding of ARG reservoirs in the environment has continuously expanded (Allen *et al.*, 2010, Perry *et al.*, 2014). Monitoring the presence and prevalence of ARGs within the antibiotic resistome of different environments is essential to maintain the efficiency of antibiotics for human and veterinary medicines, especially for ARGs carried by mobile elements (Martinez *et al.*, 2014). However, little is known regarding the environmental resistome in the fish farming environments and its association with mobile elements.

1.4.1. Acquisition of antibiotic resistance genes

Bacteria can be intrinsically resistant e.g. by carrying efflux-pump resistance genes (Perry *et al.*, 2014). The intrinsic ARGs are usually present in bacterial chromosome and are vertically transferred (Davies & Davies, 2010). Bacteria can also acquire the ARGs via mutations in chromosomal genes which occur usually under antibiotic selective pressure or can be acquired from other bacteria through horizontal gene transfer (HGT) (Blair *et al.*, 2015). These acquired ARGs are usually horizontally transferred via (1) transformation by which cells take up naked DNA from the environment, (2) transduction by which DNA is transferred with the help of bacteriophages and (3) conjugation by which DNA is transferred using mobile elements such as plasmid, transposon, Insertion Sequence (IS) and other integrative and conjugative elements.

1.4.2. Mobile elements and horizontal gene transfer

Mobile genetic elements (MGEs) can move the ARGs between genomic locations intracellularly but also between bacterial cells (Frost *et al.*, 2005). Therefore, the MGEs are responsible for horizontal ARG transfer, and can eventually transfer the ARGs to human and animal microbiome (Stokes & Gillings, 2011). The horizontal transfer of ARGs in the environment can be facilitated by a variety of mobile genetic elements such as transposons and integrons (Aminov, 2011). Transposons are grouped into three types: (1) Tn3 family transposon which contains a transposase (TnpA) and a resolvase (TnpR) to transpose intracellularly; (2) composite transposon which mobilizes due to the flanked-IS; and (3) conjugative transposon which has the ability to transpose between bacteria (Hegstad *et al.*, 2010). It has been shown that the abundance of transposase of transposon is correlated with the ARG abundance in the environment (Zhu *et al.*, 2012). Transposons carrying ARGs are found in transferable plasmids from fish farm bacteria (Furushita *et al.*, 2011).

Integrons are genetic elements which capture and incorporate small mobile gene cassettes facilitating the spread of genes located in these gene cassettes (Hall & Collis, 1995, Stokes *et al.*, 2006). The integrons can be carried by larger mobile elements such as plasmids and transposons which promote their wide distribution within bacterial communities (Stadler *et al.*, 2012). Class 1 integrons are known to carry gene cassettes which encode ARGs. Class 1 integrons have been suggested to indicate ARG pollution caused by human activities (Gaze *et al.*, 2011, Gillings *et al.*, 2015) and are also found in fish farming-associated bacteria worldwide (Schmidt *et al.*, 2001b, L'Abee-Lund & Sørum, 2004, Ndi & Barton, 2011).

1.4.3. Mechanism of antibiotic resistance genes

Antibiotics affect bacterial cells through several mechanisms of action by inhibiting cell wall, protein, and nucleic acid synthesis and disrupting bacterial DNA (Davies & Davies, 2012, Park *et al.*, 2012). The bacterial resistance mechanisms include (1) removal of antibiotics from the cell by efflux-pumps, (2) protection of bacterial cell by modification of antibiotic targets and inhibition of antibiotics uptake and (3) deactivation of antibiotics by chemical modification (Wright, 2010). For instance, tetracycline resistance genes include the three mechanisms of resistance (Roberts, 2005, Roberts, 2012) but only efflux-pumps and cellular protection are commonly found in fish farm-associated environments (Table 2 and Table 3). Most of sulfonamide resistance genes encode genes for cellular protection while trimethoprim resistance genes

confer resistance by antibiotic deactivation (Eliopoulos & Huovinen, 2001). Figure 2 shows the bacterial resistance mechanisms to antibiotics.

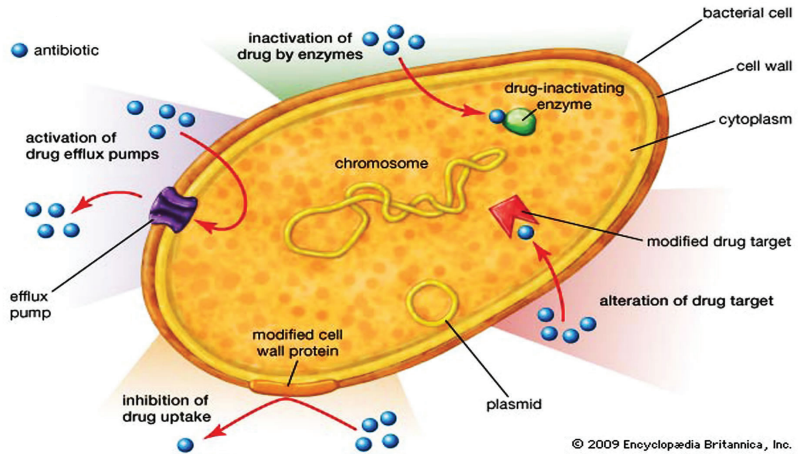


Figure 2. Bacterial antibiotic resistance mechanisms: (1) efflux-pumps, (2) cellular protection (antibiotic target modification or antibiotic uptake inhibition) and (3) deactivation of the antibiotics. The picture is reprinted from Encyclopedia Britannica Inc. (Morier, 2016). Web. 05 Feb. 2016.

1.5. Study of environmental resistome using culture-independent methods

The environmental resistome is often under selective pressure since antibiotics are extensively used in human activities such as animal and fish farming (Allen *et al.*, 2010). This may influence the local resistome composition and promote the transfer of ARGs within bacterial communities. The approaches to study the antibiotic resistome in the environment can be broadly divided into (1) culture-dependent methods and (2) culture-independent methods. Culture-dependent methods involve culturing antibiotic resistant-bacteria and characterizing their genetic properties which are associated with the antibiotic resistance (D'Costa *et al.*, 2006, Bhullar *et al.*, 2012, Walsh & Duffy, 2013, Yang et al., 2013, Shah *et al.*, 2014). Culture-dependent methods only detect those bacteria that can be cultured in laboratory conditions, and unavoidably exclude any information from uncultivable bacteria. Therefore, culture-independent methods are needed as a complementary, unbiased way to study the environmental resistome.

Culture-independent methods are performed by extraction of total bacterial DNA from the environmental samples. ARG presence in total environmental DNA can be detected by polymerase chain reaction (PCR) using a set of forward and reverse primers designed specifically to target partial or complete

ARGs (Aminov *et al.*, 2001, Allen *et al.*, 2010). However, standard PCR does not give information on the amount of gene in the environment. To estimate the amount of ARGs in the environment, quantitative PCR (qPCR) approach is now widely used (Pei, *et al.*, 2006, Luo *et al.*, 2011, Pruden *et al.*, 2012, Czekalski *et al.*, 2014). Very recently, the throughput of qPCR has been increased by orders of magnitude by performing the reactions in a highly parallel qPCR array (Looft *et al.*, 2012, Zhu *et al.*, 2013, Segawa *et al.*, 2013, Wang, *et al.*, 2014, Su *et al.*, 2015, Karkman *et al.*, 2016). Metagenomics, the study of the environmental DNA sequences using next-generation sequencing has also been used to detect ARGs in different environments (Kristiansson *et al.*, 2011, Chen *et al.*, 2013, Bengtsson-Palme *et al.*, 2015, Rowe *et al.*, 2015). Some technical aspect related to the qPCR, qPCR array and metagenomics are described in table 4. In this thesis, qPCR (**II** & **III**) and qPCR array (**I** & **IV**) have been employed to study the composition and abundance of ARGs in fish farm environments.

Table 4. Culture-independent methods to study the environmental resistome.

	qPCR	**qPCR array**	**Metagenomics**
Amount DNA per sample	0.05 µg	2 µg	2 µg
Limit of detection	ca. 1-10 copies of a target gene	ca. 10^2-10^4 copies of a target gene	semi-quantitative*
Throughput	one or few ARGs	hundreds of ARGs	the whole resistome*
Cost per sample	ca. 50 €	ca. 200 €	ca. 500 € - 50000 €**
Infrastructural requirements	qPCR machine; common.	qPCR array; uncommon.	Next-generation DNA sequencing facility; common.
Expert requirements	laboratory	laboratory	bioinformatics
Advantage	Straightforward and inexpensive.	Straightforward, inexpensive and high-throughput.	Very high throughput and potentially detects all resistance genes in a sample.
Disadvantage	Limited throughput and only targets known ARGs	Limited to known ARGs.	Semi-quantitative and requires extensive data analysis.

* Assuming a sufficient sequencing coverage that is practically difficult to achieve.
** The cost is proportional to the required sequencing depth.

1.5.1. Challenges in extracting bacterial DNA from environmental samples

Culture-independent methods involve direct isolation of total bacterial DNA, which covers DNA from both cultivable and non-cultivable bacteria present in the environmental samples. The challenges in extracting and purifying the whole DNA from environmental samples can be due to the presence of inhibitory factors in the environmental samples e.g. organic matter that interferes with the extraction process (Miller, 2001). Also, the challenges can be due to the presence of bacteria with difficult-to-lyse cell walls in particular bacterial communities (Jalava & Jalava, 2002). Several commercial DNA extraction and purification kits are available, however, finding the most suitable DNA extraction kit to give enough good quality DNA from different environmental samples can also be a challenge. Modification or pre-treatment is often added along with the manufactures instruction of the DNA extraction kits (Tournier *et al.*, 2015).

1.5.2. Detection and quantification of antibiotic resistance genes

Both detection and quantification of ARGs can be done using qPCR. The qPCR method combines end-point detection of standard PCR with a corresponding increase of fluorescent reporter that indicates amplicon accumulation during every cycle (Smith & Osborn, 2008). The C_T (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (exceeds background level). C_T values are inversely proportional to the amount of target PCR product in the sample (the lower the C_T value the greater the amount of target gene in the sample). SYBR green, a common fluorescent reporter used in qPCR, binds to all double-stranded DNA and therefore it is essential to use a highly specific primer set to avoid overestimate quantification of targeted ARG in the samples.

Quantification of the unknown targeted ARG numbers in qPCR measurements is determined by comparing the C_T values of target PCR product against a constructed standard curve of known target ARG amount, rather than as an absolute measurement of the amount of ARG number in the environmental DNA samples. For instance, the PCR amplicons of ARG can be cloned into vector plasmids and the resulting ARG plasmids or plasmids isolated directly from resistant bacteria used as standards in qPCR measurements. Another recent method to obtain the qPCR standard, which is also used in this study, is pre-synthesized vector with DNA sequences of the target ARG (**II** & **III**).

1.5.3. High throughput method using qPCR array

The comprehensive antibiotic resistance database, CARD currently lists up to 1600 known ARGs (McArthur *et al.*, 2013). To study the environmental resistome by measuring each of the known ARGs from many samples is a tremendous amount of work and time. Therefore, a high throughput method of detection and quantification is required. This method used in this study, named qPCR array, applies hundreds of primer sets to measure the presence and abundance of hundreds of different ARGs in one DNA sample in one PCR reaction (Looft *et al.*, 2012). Each primer set has to be designed to have similar annealing temperature and to target the conserved sequence areas within ARGs to assess the environmental resistome. The primer design for antibiotic resistance genes has been validated as a part of previous studies (Stedtfeld *et al.*, 2008, Looft *et al.*, 2012, Zhu *et al.*, 2013). The qPCR array requires good quality and high amount of DNA (at least 2 µg) which may be challenging to obtain from a direct environmental DNA extraction.

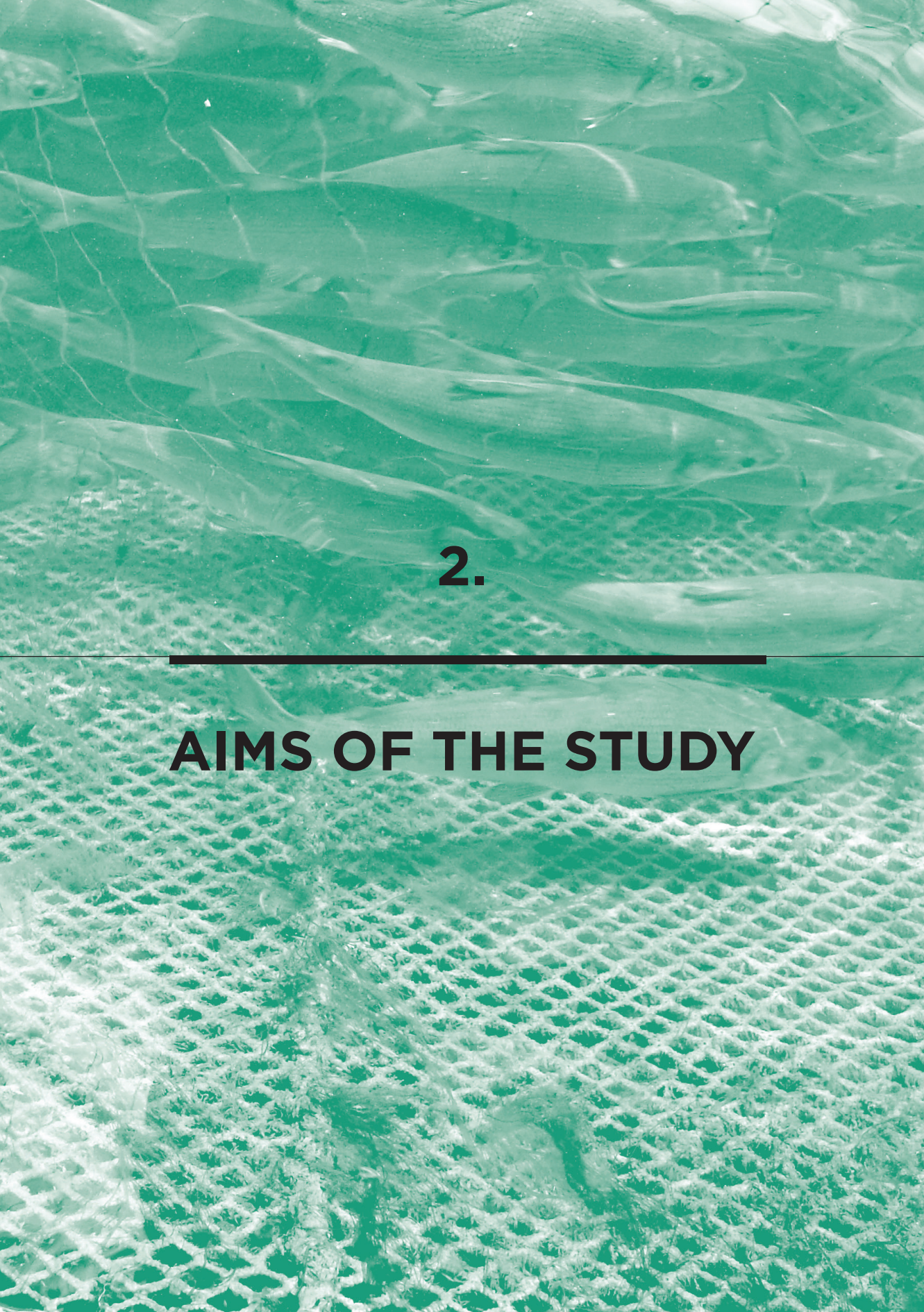
The performance of qPCR array is the same as the standard qPCR reactions except that the qPCR array allows parallel PCR reactions in one run of reaction. There are several well formats in qPCR array including 384, 1536 and 5184-well format. Data analysis in the qPCR array is mainly based on relative quantification using the $\Delta\Delta C_T$ or ΔC_T methods (Schmittgen & Livak, 2008) with normalization of the raw data to a housekeeping gene for example, the 16S ribosomal RNA gene (**I** & **IV**). The qPCR array has recently been used to analyze the ARG profiles from swine feces in a controlled feeding study (Looft *et al.*, 2012), from swine manure and soil compost (Zhu *et al.*, 2013), from glaciers (Segawa *et al.*, 2013), from soil impacted by reclaimed irrigation water (Wang *et al.*, 2014), from sewage sludge compost (Su *et al.*, 2015) and wastewater treatment plants (Karkman *et al.*, 2016), but has not been previously used to study fish farms.

1.5.4. Statistical analysis of antibiotic resistance genes

The qPCR array produces multidimensional data including the presence/absence of a target ARG in the samples and relative quantities for the detected ARGs. Therefore, a multivariate data analysis such as ordination is needed to explore the qPCR array data. In the ordination analysis, samples with similar composition are considered closer to each other, and farther from each other if the composition is different (Zeleny, 2014). To analyze the antibiotic resistome composition in different environmental samples, unconstrained ordination methods are used to simplify the multidimensional-qPCR array data into two

dimensions for visualization. Unconstrained ordination methods use matrix of distances between the samples, for example principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) (Legendre & Legendre, 2012). There are several ecologically relevant methods to calculate the distance matrix such as Gower, Jaccard and Bray-Curtis dissimilarity indices (Faith *et al.*, 1987). To analyze significant differences between the distances of the samples, statistical analysis like permutational multivariate analysis of variance can be used (Oksanen *et al.*, 2016).

To make use of quantitative data from the qPCR and qPCR array experiments, statistical analysis can be used to see whether the abundances of different genes in one sample or between samples are significantly different. For this common statistical analysis like student t-test which calculate the means of abundances can be used. Box-plots can be used to visualize the abundances with five values (the highest and lowest values, the upper and lower quartiles and the median) and notched box-plots can be used to visualize the 95 % confidence intervals between the medians (McGill *et al.*, 1987). The quantitative data also can be used to test for correlation between the abundances of ARGs and mobile elements. For this common correlation analysis or simple linear regression can be used to model the relationship between the ARGs and mobile elements in different samples (Yan, 2009).



2.

AIMS OF THE STUDY

2. Aims of the Study

The main aim of this research project was to determine the abundance and diversity of antibiotic resistance genes (ARGs) and mobile elements in sediments impacted by the fish farming process. Another aim was to investigate the source of ARGs in the farm sediments. In addition, this study aimed to see relationships between mobile elements and the ARGs in the fish farming environments. Understanding the antibiotic resistome profiles in fish farming environments is essential to predict the ARG emergence in fish farming facilities. Emergence of ARGs at fish farm facilities causes ineffectiveness of antibiotic treatment against bacterial infections of fish, which leads to production losses. The results of this research project also permit managing the potential risks of ARG spread from the farms to the surrounding environments. The information from the research results is expected to improve the fish farming management and contributing to healthier water environment.

The research project is formulated around the following questions:

1. Does fish farming impact the antibiotic resistome and mobile elements in sediments below the farms compared to the sediments outside the farms?
   Question 1 is discussed and answered in article **I**
2. What is the long-term impact of fish farming in the farm sediments?
3. Do the ARGs spread from the farm sediments to the outside sediments?
   Question 2 and 3 are discussed and answered in articles **II** & **III**
4. What is the plausible source of ARGs in the farm sediments?
   Question 4 is discussed and answered in article **IV**
5. Are there any correlation between the abundances of ARGs and mobile elements?
   Question 5 is discussed and answered in article **II** and **IV**

3.

SUMMARY OF METHODS

3. Summary of Methods

Study sites

The study sites are located in the Northern Baltic Sea, which has a brackish water environment. The study was carried out at two fish farms (FIN1 and FIN2), which are separated geographically by tens of kilometers, and two control sites from each farm in the Turku archipelago, Finland (Figure 3). Both FIN1 and FIN2 farms use open-cage systems which allow free flow of water from the fish farms to the surrounding water environment and eventually to sediments. Each cage was 20 m in diameter and 5 m deep.

The farms raise European whitefish (*Coregonus lavaretus* (Linnaeus)) and rainbow trout (*Oncorhynchus mykiss* (Walbaum)). The FIN1 and FIN2 farms each produce approximately 50 tons of fish annually. Fingerlings of juvenile rainbow trout (ca. 100 g) and whitefish (ca. 250 g) are typically raised for growing periods of two years. Occasionally, the farms also raise mature fish for one growing period.

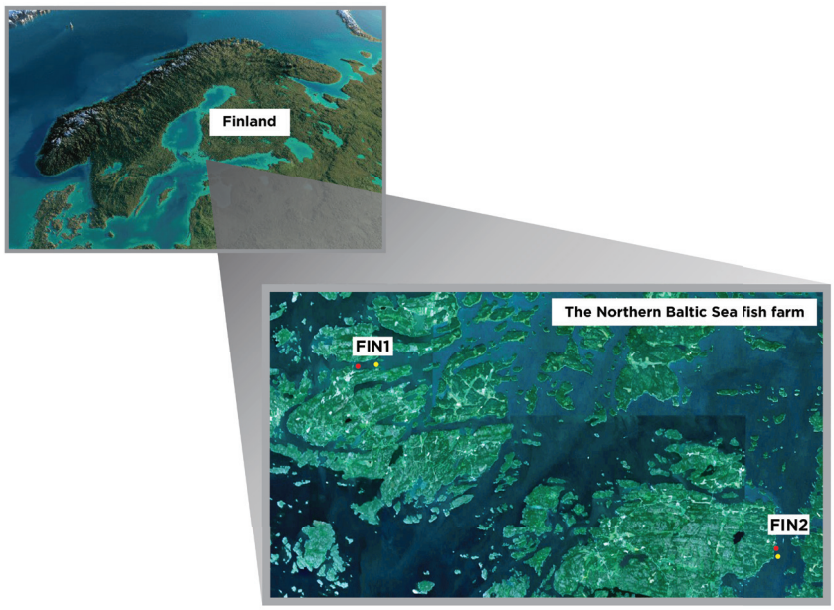


Figure 3. The two fish farms, FIN1 and FIN2 (red-circles) and control sites (yellow-circles) from each farm in Turku archipelago in the Northern Baltic Sea, Finland. Figure by Santika Januaruddin.

Sampling

Sediment samples were collected below fish cages at the FIN1 and FIN2 farms and from control sites from each farm during summers in 2006 to 2012 (Table 5). In addition, transect interval samples were collected along the shoreline at a distance of 200 m up to 1000 m from the FIN1 farm in 2006 to 2008. Three biological replicates from each site were pooled in 2006 to 2009. In 2011 and 2012, the three replicates were individually collected from each site to see the variance within the biological replicates. Each 3-10 cm of surface sediments was collected using a Limnos sediment probe (Limnos Ltd., 100 Turku, Finland). Each sample was homogenized manually inside a zipper storage plastic bag and immediately frozen on dry ice. The transport from sampling sites in Turku archipelago is around 6 hours away by car from the laboratory in Helsinki, Finland.

Table 5. The description of sampling sites (**I, II & III**)

Sites	**Mean value at sampling times***			**Locations**
	depth (m)	**T (°C)**	**pH**	
FIN1 farm	6	15.3	7.6	Located in the middle of a 400-m-wide strait
FIN1 control	8	15.1	4.2	A site 1000-m distance from the FIN1 farm. In addition, a transect was sampled along the strait of the FIN1 farm at 200-m intervals up to 1000 m
FIN2 farm	7.4	16	7.9	Located next to the seashore in an 800-m-wide strait
FIN2 control	5.1	16.5	8.1	A site 200-m distance from the FIN2 farm

*Mean values of depth, temperature (T) and pH were measured from bottom seawater at sampling sites located in the archipelago area in the northern Baltic Sea.

Fish samples were collected directly from the FIN1 and FIN2 farms during summer in September 2014 (**IV**). The average surface water temperature was 14.2 °C during the sampling time. Five fish of each small and big rainbow trout and small and big whitefish were sampled. In total 20 farmed fish were analyzed in this study. The fish samples, which were healthy and sacrificed at the farms, were kept in ice boxes during the transport within 6 h from the farms to the laboratory. At the same day, the fish were measured and weighted at the laboratory. Swabs were collected from gill filaments and skin mucus of each fish. The fish were also directly incised to collect the intestinal contents. All the swabs and the fish intestinal content samples were stored at -80 °C until DNA extraction.

DNA extraction

The sediments were stored at -80 °C until DNA extraction. The environmental genomic DNA was extracted using a commercial FastDNA Spin kit for soil (MP Biomedicals). An extra washing step with 5.5 M guanidine thiocyanate was done during the DNA extraction to remove organic matter from the sediments (**I, II,** & **III**). The environmental genomic DNA from the swabs of gills and skin mucus was extracted using a commercial Cador Pathogen Mini Kit (QIAGEN) and pre-treatment B2 was done to deal with the difficult-to-lyse bacteria (**IV**). The environmental genomic DNA from the intestinal content samples was extracted using QIAamp DNA Stool Mini Kit (QIAGEN) and pre-treatment using FastPrep method (MP Biomedicals). The modifications of the standard protocols were performed according to the manufacturer's instructions to improve the yield and quality of DNA.

qPCR and qPCR array

In this thesis, a PCR-based analysis with specific primer sets has been employed to determine the environmental resistome in the Baltic Sea fish farms. qPCR (**II** & **III**) and a recent high throughput qPCR array (**I** & **IV**) were used for the detection and quantification of known ARGs and MGE associated genes. The study of MGE is focused on class 1 integrons (**II**) and transposons (**I** & **IV**). The qPCR array used the latest 5184-well format with the parallel combinations of 16 samples with 296 assays (**I**) and 12 samples with 384 assays (**IV**).

Data analysis

The qPCR array data were explored using Microsoft Excel and statistically analyzed by R using RStudio frontend to provide a user-friendly graphical interface. Multivariate analysis of ecological communities in R (Vegan) package (Oksanen *et al.*, 2015) is used to compute the PCoA (**I**) and NMDS (**IV**). The statistical analyses performed in this thesis include permutational multivariate analysis of variance (**I**), student t-test (**II**) and linear regression (**II** & **IV**). Box-

plot (**I** & **II**) and notched box-plot (**IV**) are used to visualize summary statistics of the data.

The methods used to study ARGs and mobile elements in fish farming environments are listed in Table 4. A more detailed description of each method has been described in the original articles (**I, II, III** and **IV**).

Table 6. The methods used in this study.

No	Method	Manuscript
1	Sediment sampling	**I, II, III**
2	Fish sampling	**IV**
3	Total DNA extraction from sediments	**I, II, III**
4	Total DNA extraction from fish intestinal contents	**IV**
5	Total DNA extraction from swabs of fish skin mucus and gill filaments	**IV**
6	Determination of abiotic parameters from sediments	**I, II, III**
7	Determination of sulfonamides and trimethoprim residues in sediment	**II**
8	Determination of tetracycline residues in sediments	**III**
9	PCR analysis	**II, III**
10	Primer design for trimethoprim resistance gene, *dfrA*	**II**
11	Primer design for *intI1* gene of class 1 integron	**II**
12	Quantitative PCR (qPCR) measurements	**II, III**
13	Quantitative PCR (qPCR) array measurements	**I, IV**
14	Data analysis using Microsoft Excel	**I, II, III, IV**
15	Statistical analysis using RStudio	**I, II, IV**

4.

RESULT AND DISCUSSION

4. Results and Discussion

4.1. Impact of fish farming on the antibiotic resistome and mobile elements in sediments

To observe the impact of fish farming on the abundance and diversity of antibiotic resistance genes (ARGs) and mobile elements in sediments, we compared sediment samples below each of the two fish farms (FIN1 and FIN2) and control sediment samples taken 1000 m outside of the FIN1 farm in the Northern Baltic Sea, Finland. The qPCR arrays used 285 primer sets to detect and quantify ARGs, 9 primer sets for transposases of transposon and a primer set for the 16S rRNA gene. Altogether 66 ARGs and 5 transposases were detected and quantified (Figure 4, **I**).

Our results showed that particular ARGs and transposases were enriched in the sediments below the two farms. The enriched ARGs included genes encoding resistance to tetracycline, sulfonamide, trimethoprim and aminoglycosides. Tetracycline and a combination of sulfonamide-trimethoprim are used or have had been used at the two farms. Two classes of tetracycline resistance genes, ribosomal protection proteins (*tet(32), tetM,* and *tetO*) and tetracycline efflux pumps (*tetA, tetE, tetG,* and *tetH*) are likely enriched because they confer resistance to tetracycline, and *sul2* and *dfrA1* were likely enriched in the farm sediment because they confer resistance to sulfonamide and trimethoprim. The aminoglycoside resistance gene, *aadA,* and the quaternary ammonium compound resistance gene, *qacEΔ1*, were co-enriched in the farm sediments despite the fact that corresponding antibiotics were not used at the farms. Moreover, the composition of the genes detected in the farm sediments and outside sediments was significantly different based on permutational multivariate analysis of variance (p-value < 0.01; $R^2 = 0.62$).

This study shows that fish farming has a significant impact on the antibiotic resistome composition in the sediments and that the enrichment is mainly limited to the ARGs associated with the antibiotics which are/have been used at the farms. In addition, the presence of transposons in the farm sediments may lead to the prevalence of certain ARGs in the fish farming environments, and their potential for mobilizing the ARGs to other bacteria, including fish and human pathogens.

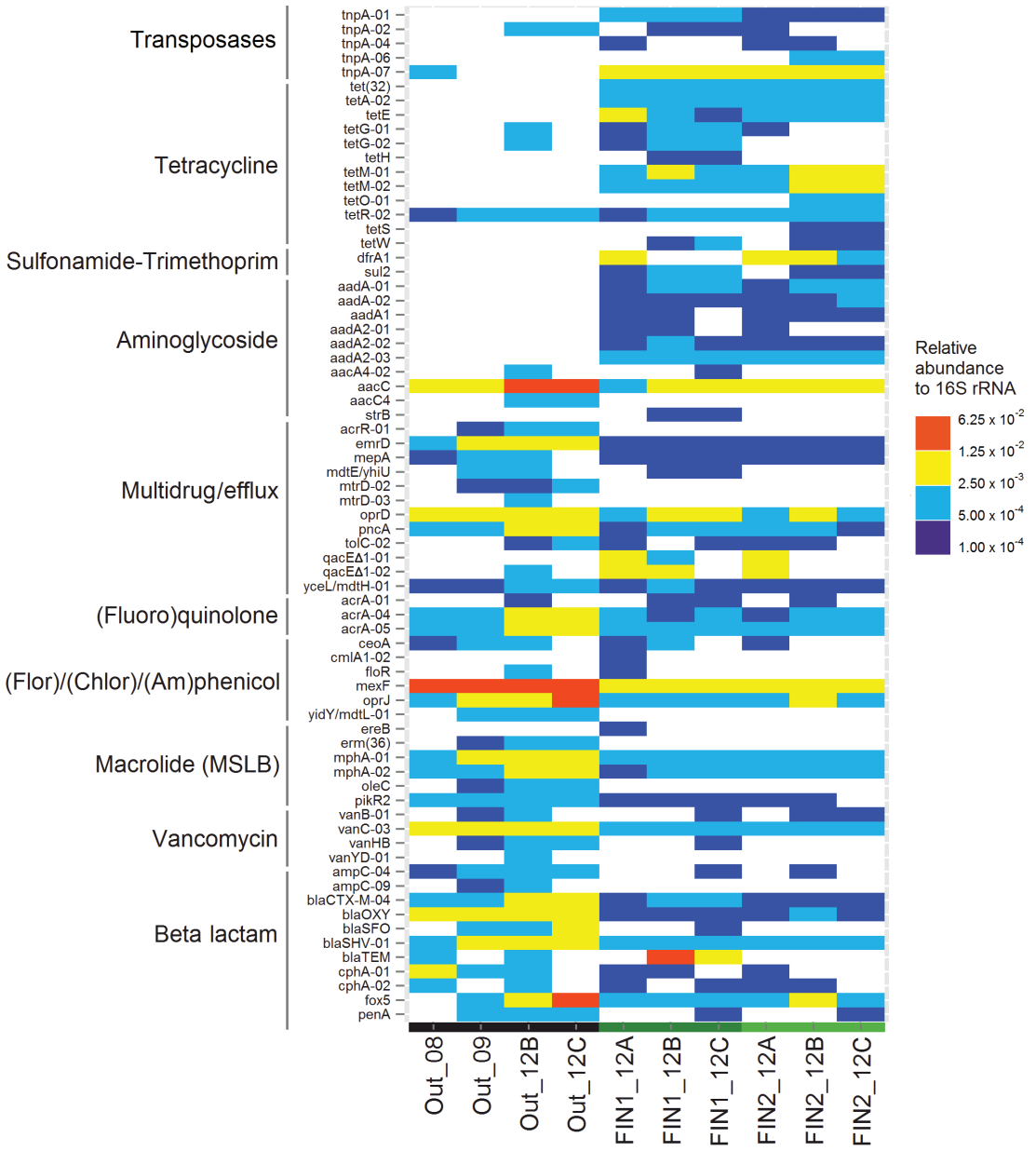


Figure 4. Composition of ARGs and transposases detected in the Northern Baltic Sea sediments. Y-axis presents the gene assays of qPCR grouped by transposases and by classification of the antibiotics the genes confer resistance to. X-axis presents the sampling locations organized by the sediment sample type: "Out" represents sediments outside the fish farms (black), "FIN1" (dark green) and "FIN2" (green) represent the sediments below the two fish farms. The color scale indicates the five-fold changes of genes' relative abundance in proportion the 16S rRNA gene. White indicates the respective gene was not detected or below the detection limit of each assay (C_T cut-off was at cycle 27) in the qPCR array.

Until now most studies on antibiotic resistance in sediments associated with fish farming have been based on bacterial cultivation that may introduce a bias in the assessment due to the inability to cultivate most of environmental bacteria (Amann *et al.*, 1995). Previous studies using culture-independent methods have investigated only 15 or fewer ARGs in farm sediments (Gao *et al.*, 2012, Xiong *et al.*, 2015). In this study, by using a considerably wider selection of primer sets to investigate 285 ARGs, our results also show that ARGs are ubiquitous in the Baltic Sea sediment. It seems that there is a background resistome in the Baltic Sea sediments which mainly consists of efflux-pump resistance genes (Figure 4, **I**). The relative contribution of ARGs attributed to fish farming and the original resistome in sediments is not simple to evaluate due to the variable composition of the environmental resistome in sea sediments, even without influence from human activities (Chen *et al.*, 2013). It is therefore important to determine the possible sources of ARGs and minimize the introduction of ARGs to environment, especially those ARGs which are carried by mobile elements such as transposons.

4.2. Long term observation of ARGs and their spread in the sediments

At the Baltic Sea fish farms, sulfonamides, trimethoprim and tetracycline are/have been used to treat the fish diseases. We therefore examined the persistence of ARGs associated with sulfonamides and trimethoprim (**II**) as well as tetracycline (**III**) in sediments below the FIN1 and FIN2 farms by using manual qPCR over several years of observation from 2006 to 2012. We also examined the distance to which the ARGs spread from the farm sediments. For this, qPCR was used to quantify the ARG copy numbers in sediments at 1000 m outside the two farms. We also sampled transects in 200-m intervals ranging from 200-m to 1000-m distance from the FIN1 farm. We quantified the copy numbers of sulfonamide resistance genes, *sul1* and *sul2*, a trimethoprim resistance gene, *dfrA1*, tetracycline resistance genes, *tetA, tetC, tetH* and *tetM* in proportion to the 16S rRNA gene copies in the sediment samples. Also, an integrase gene of class 1 integron, *intI1* was measured in the sediments. New qPCR primers were designed for quantification *dfrA1* and *intI1* which can be found in article **II**.

Figure 5 shows that the *sul1, sul2, dfrA1* and *intI1* genes were present during the 6-year observation period (**II**). Our results showed that class 1 integrase gene and the ARGs associated with sulfonamides, trimethoprim and tetracycline persist in the two farm sediments but are not detected in the sediments outside the farms even at 200 m distance from the farm (**II** & **III**). The ARGs persist for several years of observation even though the concentrations of sulfonamide, trimethoprim and tetracycline were very low in the sediment samples, approximately ca. 1 – 100 ng per gram of sediments (**II** & **III**). Our results may indicate a local impact of fish farming on ARGs present in the Northern Baltic Sea sediments, even when a clear antibiotic selection pressure is not present. To our knowledge, no studies have reported the long-term observation of the impact of fish farming on the surrounding environments.

Because the ARGs were not detected in the sediments outside of the farms, in the current environmental conditions, we consider the risk of ARG spread from the fish farms to the surrounding aquatic environments in the Northern Baltic Sea low. In contrast, in other studies fish farming-associated ARGs are detected in the surrounding sediments outside the fish farm facilities in Japan (Nonaka *et al.*, 2007) and in Chile (Buschmann, *et al.*, 2013). The difference between these results is most likely because the different geographical conditions between the shallow water with slow currents in the Baltic Sea and deeper coastal sea with strong currents in Japan and Chile. The relatively small amounts of the antibiotics used in the Baltic Sea fish farms also may explain that the ARGs were not detected in the sediments outside the farms. This suggests that in the Northern Baltic Sea, the ARGs are potentially a problem for the fish farming-industry, but have less impact on the surrounding environment.

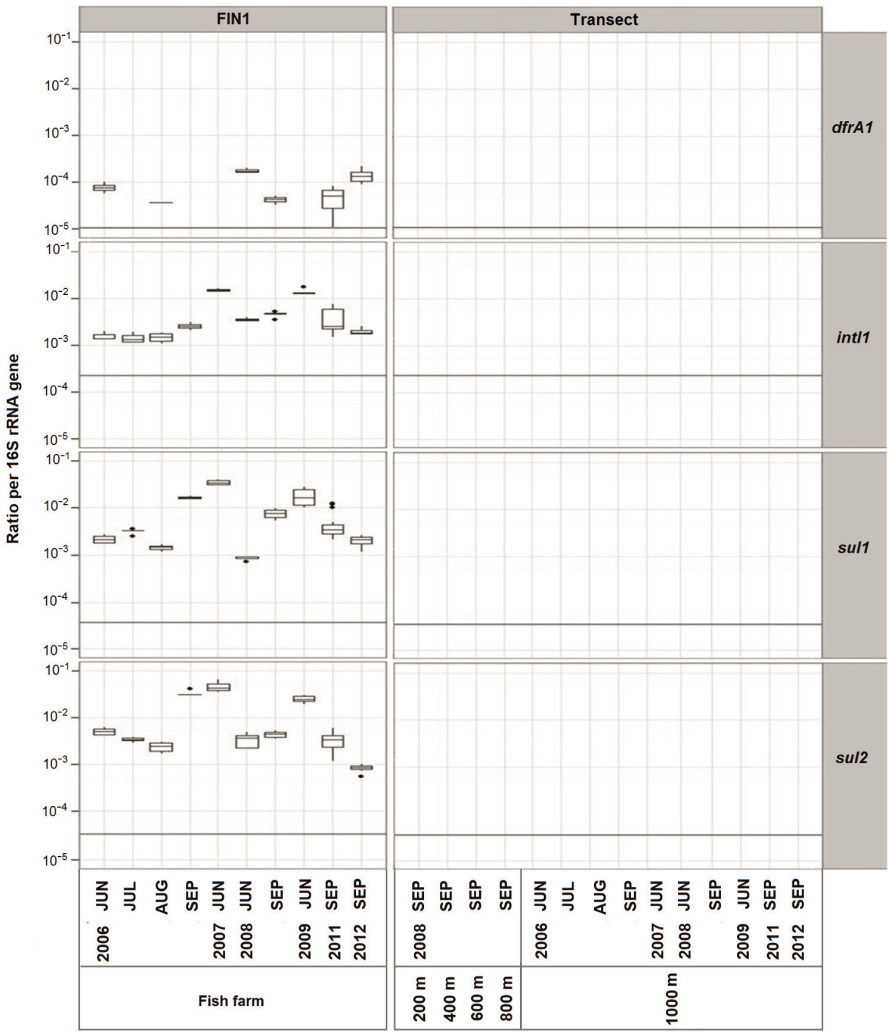


Figure 5. The ratios of the trimethoprim resistance gene (*dfrA1*), sulfonamide resistance genes (*sul1* and *sul2*) and an integrase gene of class 1 integron (*intI1*) to the 16S rRNA gene copies in the sediments below the FIN1 farm and in the sediments at 200 m to 1000 m away from the farm. The missing data values in the plot mean that the respective gene copy numbers were below the limit of detection (LOD) in the qPCR (for *dfrA1, sul1 and sul2* = 102 and *intI1* = 103). The grey line indicates the gene quantification limit (LOQ) normalized to the average numbers of the 16S rRNA gene copies.

4.3. Source of ARGs in the fish farm sediments

Sources of the ARGs in the sediments below the Baltic fish farms have yet to be elucidated. Since there was no clear antibiotic selection pressure in the farm sediments, the development of ARGs may occur in the fish intestine under antibiotic treatment at the farms (Giraud *et al.*, 2006). The ARGs in the fish intestine may be excreted through fish feces and eventually enter the sediments (Kümmerer, 2009). Alternatively, the enrichment of ARGs could be caused by co-selection with heavy metals (Baker-Austin *et al.*, 2006). Mercury can be found in fish feed (Choi & Cech, 1998) and may enter the farm sediments through uneaten feed. However, the concentration of mercury in the farm sediments is very low and therefore co-selection with mercury is unlikely (Pitkänen *et al.*, 2011). We, therefore, hypothesized that the plausible source of ARG enrichment in the farm sediments was a constant influx of farmed fish feces into the farm sediments.

To test out our hypothesis, we analyzed ARG composition and mobile elements in the intestinal contents of rainbow trout and whitefish taken from the Baltic Sea fish farms, using the qPCR array with 363 primer sets. We found that most of the detected genes in the intestinal contents of the farmed fish were the same genes that were enriched in the farm sediments (**I, II, III** & **IV**). Figure 6 shows the genes that were detected in the fish intestinal contents are also found in the farm sediments. These genes included transposases (*tnpA*) associated with IS21, IS6100, IS1216 and ISEcp1, an integrase gene of class 1 integron (*intI1*), a gene encoding resistance to antiseptic which is also known as a backbone gene of class 1 integron (*qacEΔ1*), a sulfonamide resistance gene (*sul1*), a trimethoprim resistance gene (*dfrA1*), tetracycline resistance genes (*tet(32), tetM, tetO, tetT* and *tetW*), aminoglycoside resistance genes (*aadA1* and *aadA2*). These findings indirectly provide evidence to conclude that the farmed fish feces are a plausible source of certain genes enriched in the sediments below fish farms in the Northern Baltic Sea, Finland.

On the other hand, not all sediment-enriched ARGs were detected in the intestinal contents. This might be due to the amount of the genes in the intestinal contents that were below the detection limit of the qPCR array or might be coming from other sources such as uneaten fish feed (Kerry *et al.*, 1995). It might also have been caused by the enrichment of sediment bacteria which results from the deposition of fish feces and uneaten feed that increases the amount of organic matter in the farm sediments (Tamminen *et al.*, 2011b, Buschmann *et al.*, 2013).

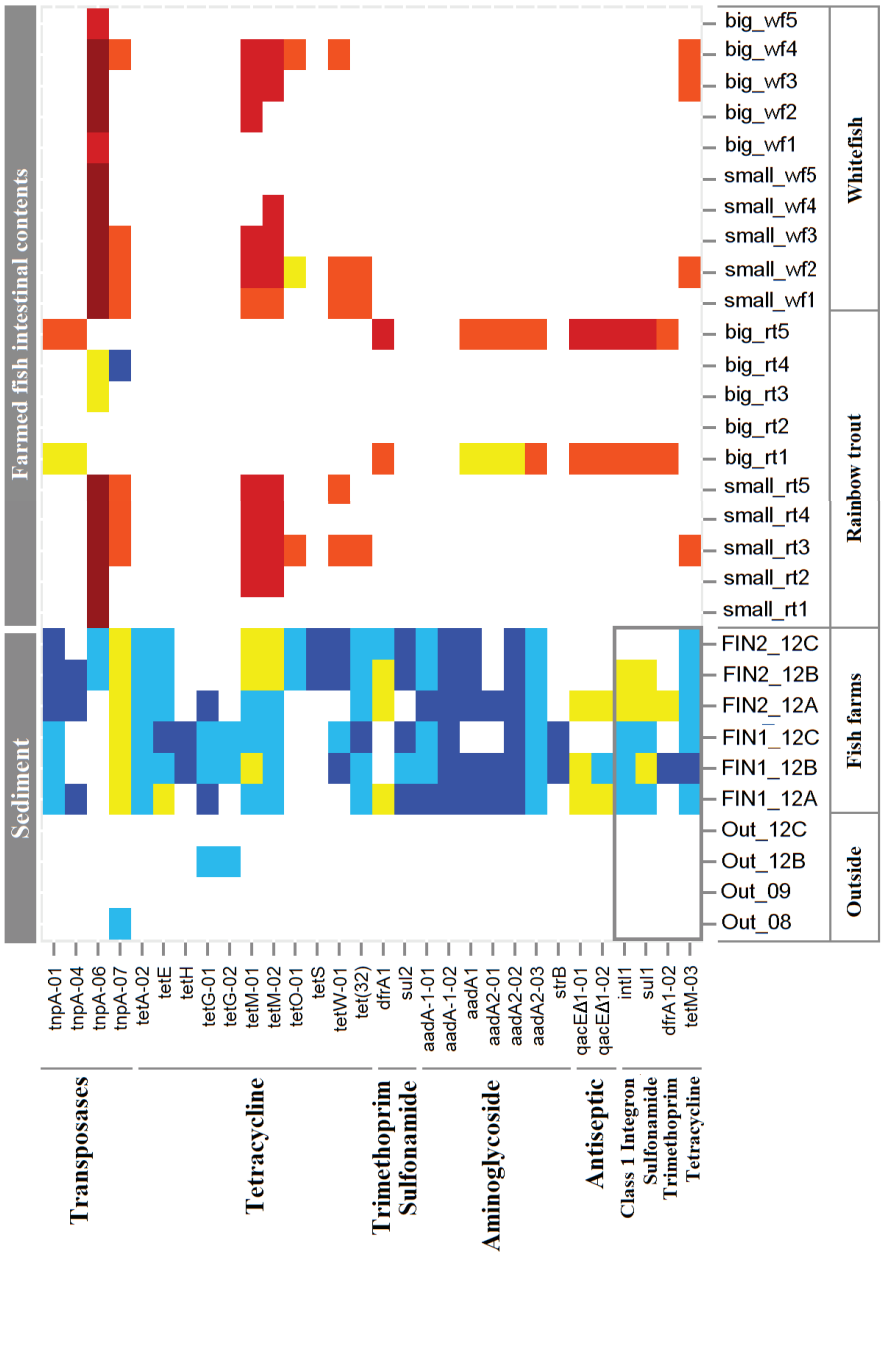


Figure 6. The genes found to be enriched in the farm sediments and were found in the intestinal contents of the farmed fish. Y-axis presents the gene assays of qPCR grouped by tranposases and by classification of the antibiotics the genes confer resistance to. X-axis presents the sampling locations organized by the sediment sample type and the intestinal contents organized by the farmed fish type. The color scale indicates the five-fold changes of the genes' relative abundance in proportion to the 16S rRNA gene. White indicates that the respective gene was not detected or below the detection limit of each assay (C_T cut-off was at cycle 27) in the qPCR array (**I** & **IV**). The grey box presents the genes' abundance were quantified using manual qPCR (**II, III**).

While many studies have reported the presence of ARGs in the intestinal contents using culture-dependent method (Furushita *et al.*, 2003, Akinbowale *et al.*, 2007, Shah, *et al.* 2012). Our results showed that the farmed fish resistome was far less diverse than the sediment resistome (**IV**). In the farmed fish resistome, the cellular protection resistance genes were the most abundant ARGs, however, in the farm sediment resistome the efflux-pump resistance genes were the most abundant (**I**). To our knowledge, this is the first study reporting the resistome of farmed fish using a culture-independent method.

4.4. Correlation between ARGs and mobile elements

To test whether the genes associated with mobile elements are involved in the prevalence and abundance of ARGs, we analyzed the correlation between (A) the copy numbers of the *intI1* of class 1 integrase gene and ARGs detected in the farm sediments (*sul1, sul2* and *dfrA1*) (**II**) and (B) the relative abundances of the total transposases, *tnpA* and tetracycline resistance genes, *tet* in the intestinal contents of the farmed fish (**IV**). We used linear regression model and the model is considered to be significant with the p-value less than 0.05.

Our results show a significant correlation between the *intI1-sul1* ($F_{1,22}$ = 19.39; p-value = < 0.001; $R2$ =0.47; Figure 7A, **II**) in the sediment samples. Correlation between class 1 integron and sulfonamide resistance gene *sul1* is expected since *sul1* is known as one of the backbone genes of class 1 integron. Similar correlation between *intI1-sul1* has been observed also in river sediments in China (Luo *et al.*, 2010) and in USA (Pruden et al., 2012). In addition, both of *intI1* and *sul1* were always found together in the farmed fish intestinal contents (Figure 6, **IV**).

A significant correlation also was observed between the *tnpA-tet* ($F_{1,9}$ = 20.3; p-value = 0.001; R^2 = 0.71; Figure 7B, **IV**) in the fish intestinal contents. The transposases were prevalent in the intestinal contents of rainbow trout and whitefish (**IV**). The tetracycline resistance gene, *tetM* was the most prevalent among the detected ARGs in the intestinal contents. The *tetM* was also the most frequently found in the fish intestinal bacteria (Furushita *et al.*, 2003, Akinbowale *et al.*, 2007). These results are expected since *tetM* has the widest host range of all known tet genes and is well known to be associated with a very wide range of conjugative transposons (Roberts, 2005). This suggests that the transposons may be associated with the abundance of tetracycline resistance

genes in the intestinal contents of the farmed fish.

Correlation between the transposases and tetracycline resistance genes is also expected since the *tet* genes are known to be carried by transposons, especially the *tetM* with conjugative transposons (Roberts, 2005, Hegstad, 2010). Similar significant correlation between the abundances of transposases and tetracycline resistance genes also has been observe in swine intestine (Zhu *et al.*, 2013). The spreading of ARGs in the environment is mediated by mobile elements via horizontal gene transfer (HGT) which may lead to the prevalence of ARGs in the environment, and their potential for mobilizing the ARGs to other bacteria (Blahna *et al.*, 2006). These observations indicate that the presence of class 1 integrons and transposons may play a big role in the prevalence of certain ARGs in fish farming environments in the Northern Baltic Sea.

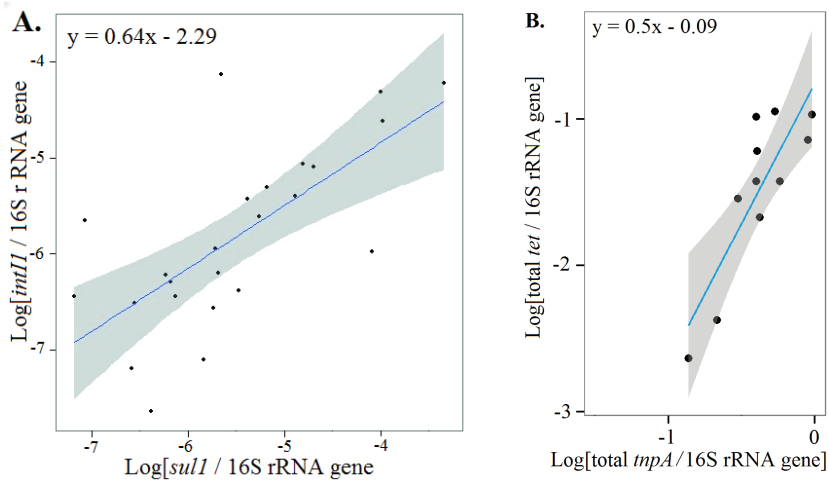


Figure 7. Correlation analysis and linear regression model with log transformed variables between: **A**. the integrase gene of class 1 integron (*intI1*) and sulfonamide resistance gene (*sul1*) in the farm sediments ($F_{1,22}$ = 19.39; p-value = < 0.001; R^2 =0.47) and **B**. the transposases (*tnpA* genes) and tetracycline resistance genes (*tet* genes) in the intestinal contents of the Baltic Sea farmed fish ($F_{1,9}$ = 20.3; p-value = 0.001; R^2 = 0.71). Each point presents the genes' abundances in proportion to the 16S rRNA gene in every DNA sample. The blue line indicates the regression model and the grey area the 95% confidence intervals.

5.

CONCLUSION AND FUTURE PROSPECTS

5. Conclusion and Future Prospects

Observing the impact of fish farming on the composition of antibiotic resistome and mobile elements, and finding a plausible source of the antibiotic resistance genes at the Baltic fish farm sediments, are the two main topics in this thesis. In addition, the relationships between mobile elements and the ARGs in the fish farming environments is also examined.

This thesis presents evidence suggesting that fish farming locally impacts the resistome composition in the Northern Baltic Sea sediments without causing a clear antibiotic selection pressure. The enrichment is mainly limited to the ARGs encoding resistance to tetracycline, sulfonamide and trimethoprim which are antibiotics that have been used at the farms. The enrichment of transposons in the farm sediments may contribute to the enrichment of certain ARGs in the fish farming environment, and may cause their mobility to new bacterial hosts including fish pathogens. The antibiotic resistome associated with fish farms can be due to the antibiotic use at the farms or introduction of ARGs and mobile elements by the fish farming process. Since the enriched ARGs are not detected in sediments outside the farms, even at 200 m away from the farm, the impact of fish farming to the surrounding aquatic environments is not likely to be a serious concern in the current environmental conditions.

The resistance genes for sulfonamide, trimethoprim and tetracycline are persistent at the Baltic Sea fish farms and remain enriched in the farm sediments over the observation period of six years. Determining a plausible source of the ARGs in the fish farm sediments is important for the management strategies used at the farms. It was evident from this study that a plausible source of ARGs in the farm sediments is the intestinal contents of the farmed fish. The ARGs from fish intestinal contents are most likely carried by fish feces to the sediments.

Because ARGs were detected in the farmed fish intestinal contents even before the fish had been exposed to antibiotics and the Baltic Sea farms, it is possible that these ARGs have already been selected in the fish intestine before entering the brackish farms - possibly during hatching or rearing the juvenile fish. This does not rule out the selection caused by antibiotics at the farms but the juvenile fish or fingerlings may have a greater role as the source of ARGs than has been expected before. It is important to control the antibiotic treatments of the juvenile fish during their growing time in freshwater ponds to minimize the potential risk of ARG emergence in the brackish farm environments. If the

source of the ARGs at the farms is from an external source, it may be possible for the farms to minimize the introduction of ARGs to the farm facilities.

The qPCR array provides a promising high-throughput method for detecting and quantifying the antibiotic resistome and its association with mobile elements in environmental samples. For instance, by using primer sets to target hundreds known ARGs, this study reveals that there is a natural environmental resistome in the Northern Baltic Sea mainly consisting of efflux-pumps. In addition to providing comprehensive presence/absence data of antibiotic resistance genes, the quantitative data from qPCR can be used to model the relationships between ARGs and mobile genetic elements. However, qPCR only detects those genes for which PCR primers have been designed and included in the analysis. To completely cover the resistome of a given sample, qPCR analyses can be complemented with metagenomic sequencing that provides a semi-quantitative information of the resistome in an untargeted manner, provided sufficient sequencing depth.

Significant correlations between class 1 integron and a sulfonamide resistance gene as well the transposases and tetracycline resistance genes were detected in this study. This suggests that the detected ARGs could be embedded in mobile elements, although direct evidence for this is not provided in this study. This indicates a potential economical and health risk since ARGs in mobile elements could move further to bacteria that are fish or human pathogens. The results of this thesis contribute to more efficient management practices at Baltic Sea fish farms to reduce the amount of antibiotic resistant fish infections.

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