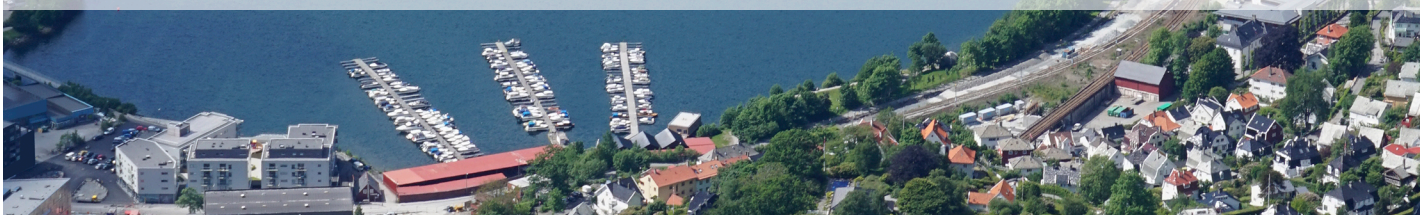
Antimicrobial Resistance in Selected Environments in Norway:

Occurrence of Antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARG) associated with wastewater treatment plants (WWTPs)

M-736|2017



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Occurrence of Antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARG) associated with wastewater treatment plants (WWTPs)

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Background

Development of bacterial resistance to antibiotics is a growing problem in the world. The national strategy of the Norwegian government against antibiotic resistance for 2015-2020 highlights that this issue must be considered in a holistic perspective, where it is acknowledged that human and animal health and the environment interact and must be seen in context to each other. The national strategy is based on the report "*Antibiotikaresistens-kunnskapshull og aktuelle tiltak (2014*)" prepared by an expert group. In the report, the identification and monitoring of the presence of antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARG) in different environments is highlighted as one of the areas where more information is needed. The presence of resistant bacteria in different natural environments, such as soil, fresh water, sea sediments and wild animals, has however only been sporadically studied, although they may contribute to the development of resistance of clinical importance. This implies that there is therefore a need for more knowledge about antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARGs) in different natural environments in Norway.

GenØk – Centre for Biosafety (www.genok.no) is an independent research institute founded in 1998 and located in Tromsø, Norway. GenØk is engaged in the field of biosafety and gene ecology research on modern biotechnology, nanotechnology, synthetic biology and other technologies emerging from these. The institution also works on capacity building and advisory activities related to biosafety. GenØk takes a precautionary, holistic and interdisciplinary approach to biosafety. In 2007, GenØk was appointed national competence center on biosafety by Norwegian authorities.

Acknowledgements

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Contents

Occurrence of antimicrobial resistance in Norwegian environments

Summary

In this study, we have used culture-based methods combined with molecular techniques, in addition to metagenomics studies, to investigate the prevalence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in wastewater treatments plants (WWTPs) from two different locations in Norway. In total, six WWTPs located in Tromsø and Bergen were chosen to establish a baseline frequency of ARB and ARG connected to these treatment plants (TPs).

We determined phenotypic resistance to the following antibiotics: ampicillin (AMP), amoxicillin (AMX), ciprofloxacin (CIP), dicloxacin (DCX), erythromycin (ERI), kanamycin (KM), streptomycin (STP), sulfamethoxazole (SMX), tetracycline (TET) and trimethoprim (TMT). Resistant colonies as well as total DNA extracted from the WWTPS were examined for the presence of clinically-relevant ARGs including *blaTEM, mecA, qnrS, ErmB, aph(3`)-IIa, aph(3`)-IIIa, aac(6`)/aph(2``), sull, tetA,* and *dfrA1*.

Overall, the level of total colony forming units (CFU), hence, antimicrobial resistant bacteria (ARB) were higher in samples from Tromsø compared to Bergen. Samples from Tromsø contained approximately 10^7 CFU per gram sludge whereas the samples from Bergen contained between 10^3- 10^4 CFU per gram sludge. The percentage of bacteria that could grow in the presence of the different antibiotics selected varied between the different antibiotics. The highest resistance percentages were observed for sulfamethoxazole and trimethoprim and the lowest were observed for amoxicillin and ampicillin. Among the antimicrobial resistance phenotypes examined, sulfamethoxazole exhibited the widest variations, from 7-86 %. A comparison between the samples tested from Tromsø and from Bergen did not give an observed sharp variation in resistance percentage.

Up to 96 isolates representing the antimicrobial resistant population for each antibiotic tested and each field were further screened by polymerase chain reaction (PCR) for the presence of specific ARGs. For single colonies, PCR results were positive for resistance genes known to confer resistance to beta-lactams, aminoglycosides, tetracycline, macrolides, trimethoprim as well as sulfonamides in a few of the tested colonies.

DNA samples extracted directly from the WWTP samples were also analysed by the same PCRs. For total DNA ezxtracted from samples, five out of the 10 considered ARGs (bla_{TEM}, *aph(3`)-IIIa, aac(6`)/aph(2``), qnrS* and *ermB*) were detected in two of the WWTPs in Tromsø (Hamna and Breivika) In total DNA extracted from samples from Strandveien, three out of 10 considered ARGs (*aph(3`)-IIIa, aac(6`)/aph(2``)* and *qnrS*) were detected. The sample material from Bergen is different from the sample material from Tromsø, and there were substances that inhibited the PCR present in the eluated DNA from Bergen, since the 16S rRNA PCR failed. These samples were not analyzed by the ARG specific PCRs

In the second module, we did metagenomics analysis of DNA purified from the microbial fraction of the WWTP and WTP samples to investigate the occurrence of a broader set of ARG. Bioinformatics tools and a manually-curated antibiotic resistance gene database with comprehensive ARG-ontology was applied to the sequence data for identification of coding regions with perfect or high similarity to known antibiotic resistance genes. We observed a higher occurence of antibiotic resistance genes in untreated sewage solids (Tromsø) relative to fjord surface sediments proximal to municipal wastewater treatment discharge locations (Bergen). Furthermore, genes encoding efflux pumps, which can confer broad-spectrum biocide and antimicrobial resistance, were the most common ARG identified in the metagenomic data, indicating the relevance of these genes in future investigations of ecological links between natural and clinical environments.

Norsk sammendrag

For å kartlegge forekomsten av antibiotikaresistente bakterier (ARB) og antibiotikaresistensgener (ARG) i tilknytning til ulike renseanlegg har vi i dette prosjektet benyttet dyrkningsbaserte metoder i kombinasjon med molekylærbiologiske metoder, i tillegg har vi utført metagenomikk studier. Totalt ble seks kloakkrenseanlegg (WWTPs), hhv. tre i Tromsø og tre i Bergen, valgt for å studere forekomsten av ARB og ARG.

Prøver fra de ulike områdene i Tromsø og Bergen ble testet for mikrober med antimikrobiell resistens overfor følgende antibiotika: ampicillin (AMP), amoxicillin (AMX), ciprofloxacin (CIP), dicloxacin (DCX), erythromycin (ERI), kanamycin (KM), streptomycin (STP), sulfamethoxazole (SMX), tetracyklin (TET) og trimethoprim (TMT). Videre ble DNA fra resistente kolonier i tillegg til total DNA fra WWTPs, undersøkt for forekomsten av relevante ARG inkludert *blaTEM, mecA, qnrS, ermB, aph(3`)-IIa, aph(3`)-IIIa, aac(6`)/aph(2``), sulI, tetA* og *dfrA1.*

Resultatene viser at nivået av total CFU og derav ARB var høyere i prøver fra Tromsø, sammenlignet med prøvematerialet fra Bergen. I prøvene fra Tromsø var total CFU ca. 10^7 CFU per gram slam og i prøvene fra Bergen var total CFU ca. 10^3 til 10^4 CFU per gram slam. Prosentandelen av bakterier som vokste i nærvær av de forskjellige antibiotika varierte. Den høyeste prosentandel ble observert for sulfametoksazol og trimetoprim og den laveste for amoxicillin og ampicillin. Det ble ikke registrert store forskjeller mellom prøvene fra Tromsø og Bergen. Av de ulike antibiotikaresistens fenotypene som ble analysert var den største variasjonen mellom områdene for sulfamethoaxole. Her varierte prosentandelen resistente fra 7-86% mellom de ulike prøvetakingsområdene.

Omtrent 96 resistente bakterieisolater for hvert antibiotikum og fra hvert område ble videre analysert ved hjelp av PCR for spesifikke ARG. DNA ekstrahert direkte fra prøvene fra renseanleggene ble også analysert ved hjelp av de samme PCR metodene. PCR resultatene var positive for resistensgener som er kjent for å gi resistens mot beta-laktamer, aminoglykosider, makrolider, tetracyklin, trimetoprim samt sulfonamider i noen av de testede kolonier. Av prøvene som ble analysert for total DNA kunne vi påvise 5 av 10 utvalgte ARGS (*blaTEM, aph(3`)-IIIa, aac (6`) / APH (2``), qnrS og ermB*) fra WWTPs i Tromsø (Hamna og Breivika) og 3 av 10 utvalgte ARGs (*aph(3`)-IIIa, aac(6`)/aph(2``), og qnrS*) fra prøvene fra Strandveien. Total DNA fra Bergen ble ikke fullstendig analysert på grunn av tekniske vanskeligheter med PCR inhiberende substanser i prøvematerialet.

I den andre delen av studiet ble forekomsten av ARG kartlagt ved bruk av metagenomanalyser. Sekvenseringsdataene fra de ulike områdene ble analysert ved hjelp av bioinformatiske verktøy basert på en antibiotikaresistens-database, i tillegg til multivariable statistiske analyser. De to viktigste resultatene av denne delen av studiet er: (1) ubehandlet kloakk har høyere forekomst av ARG enn prøver fra overflatefjordsedimenter, og (2) gener som koder for antimikrobielle effluksпumper viser seg å være dominerende i det prøvematerialet vi har analysert.

Aims of the project

The mandate of the call for this project was mapping of ARB and ARG in Norwegian natural environments in order to increase knowledge about diversity and prevalence of AMR outside clinical environments. The overall goal was to provide a better basis for the identification of the relationships between resistance in the environment and the spread of AMR not only from clinical settings to the environment but also from the environment to human and animal pathogens. Such knowledge will be important for the understanding of the antimicrobial resistome within the context of historical and current antimicrobial usage for human health and in agriculture. This study is therefore of importance for efforts to limit the transmission of antimicrobial resistance in Norway, but also elsewhere as antimicrobial resistance is a global problem. The call text specified that a survey of the prevalence of bacteria resistant to clinically relevant antibiotics in different natural environment in Norway should be carried out.

Our approach, within the project period for six months, has been to map the prevalence of bacteria resistant to ten clinically relevant antibiotics in samples associated with wastewater treatments plants (WWTP) or water treatment plants (WTP) from two different locations in Norway (Tromsø and Bergen) with the following objectives:

1) To determine the prevalence, distribution and characteristics of ARB and ARG in selected environments in Norway by cultivation dependent- and cultivation independent molecular approaches.

2) Identify knowledge gaps and areas for further research.

Sources of information

The main sources of information used in this report are:

- Publicly available literature, mostly scientific peer-reviewed articles, reports and book chapters.
- Data produced, based on the environmental samples collected, as part of the project.

Abbreviations/descriptions

AB	antibiotics
AMP	ampicillin
AMR	antimicrobial resistance
AMX	amoxicillin
ARB	antimicrobial resistant bacteria
ARG	antimicrobial resistance gene
ARMG	antimicrobial resistance marker gene
ARO	antibiotic resistance ontology
APH	aminoglycoside phosphotransferase (protein)
bp	base pair
CARD	Comprehensive Antibiotic Resistance Database
CFU	colony forming unit
CIP	ciprofloxacin
DCX	dicloxacillin
DNA	deoxyribonucleic acid
ERI	erythromycin
EtOH	ethanol
g	gram
GM	genetically modified
HGT	horizontal gene transfer
KM	kanamycin
NCS	Norwegian sequencing centre
NeLS	Norwegian e-Infrastructure for Life sciences
NORM-VET	Nasjonalt overvåkningssystem for antibiotikaresistens hos mikrober fra fôr, dyr og næringsmidler
MIC	minimum inhibitory concentration
Ml	millilitre
PCR	polymerase chain reaction
RGI	Resistance Gene Identifier
rRNA	ribosomal ribonucleic acid
STP	sewage treatment plant
SMX	sulfamethoxazole
TET	tetracycline
TP	treatment plants
URE	Uni Research Environment
VKM	Vitenskapskomiteen for mattrygghet
WHO	World Health Organization
WWT	waste water treatment
WWTP	waste water treatment plants
WTP	water treatment plants
WW	waste water
µl	microliter

«One Health» - The term is a recognition of the relationship between human and animal health and diseases, and the environment we work in. In order to understand the origin and spread of infectious diseases, it requires an comprehensive perspective on the interplay between humans, animals and the environment (Folkehelseinstituttet, 2014).

1. Introduction

Antimicrobial resistance (AMR) is present all over the world and has been found in people, animals, food and the environment (Allen et al., 2010; Alonso, Sanchez, & Martinez, 2001; Aminov, 2009; Berendonk et al., 2015). Antimicrobial resistance occurs when microorganisms such as bacteria, viruses, fungi and parasites are changed in ways that render the medications used to cure the infections they cause ineffective. The development and spread of bacterial resistance to antibiotics is a growing problem worldwide now limits our ability to treat common infectious diseases. In 2012, the World Health Organization (WHO) stated that antimicrobial resistance is one of the greatest health threats the world faces. The national strategy of the Norwegian Government against antibiotic resistance for 2015-2020 highlights that this issue must be considered in a "One health" perspective, where it is acknowledged that human and animal health and the environment interact and must be seen in context (Regjeringen, 2015).

Antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARGs) have existed in the environment since before the introduction of commercially-produced antibiotics as treatment of infections. However, human activities have increased the prevalence of resistant bacteria in different natural environments (Allen et al., 2010). The usage levels, not only in clinical settings, but also the use of antibiotics (AB) in agriculture and aquaculture and food production has contributed to the increased level of ARB in different environments (O`Neill, 2015). Although the presence of ARB in different natural environments (such as soil, fresh water, sea sediments and wild animals), has only been sporadically studied it has been demonstrated that phenotypic resistance to clinically relevant antibiotics can be found more or less in all environments investigated (Munck et al., 2015; Volkmann, Schwartz, Bischoff, Kirchen, & Obst, 2004; Williams, Stedtfeld, Guo, & Hashsham, 2016; Yang, Li, Zou, Fang, & Zhang, 2014).

It is well known that some bacterial species are intrinsically resistant to some antibiotics (Alonso et al., 2001; Davies & Davies, 2010). However, bacteria with transferable resistance traits as well as the resistance genes in the environment are increasingly seen as an environmental problem (Kummerer, 2004). Resistance genes have been detected in environments like e.g. surface water, ground water, drinking water, sediments and soil (Martinez, 2009; Martinez et al., 2009; Schwartz, Kohnen, Jansen, & Obst, 2003; Segura, Francois, Gagnon, & Sauve, 2009; Séveno N. A., 2002).

Knowledge of antimicrobial resistance (both acquired/transferable and phenotypic/intrinsic) and antimicrobial agents and disinfectants in Norwegian nature is sporadic and inadequate. The NORM-VET (Norsk overvåkingssystem for antibiotikaresistens hos mikrober fra fôr, dyr og næringsmidler) has for instance monitored resistance in a limited number of samples of intestinal *E. coli* bacteria in red fox, deer and reindeer. The results indicate an overall low incidence of antimicrobial resistance. However, multidrug-resistant *E. coli* has been detected in foxes (NORM/NORM-VET, 2015). In addition, a few studies have determined the non-clinical distribution of phenotypic antimicrobial resistance patterns and potential natural reservoir of antimicrobial resistance genes in Norwegian environments (Brusetti et al., 2008; Glad, Bernhardsen, et al., 2010; Glad, Kristiansen, et al., 2010; Kruse & Sorum, 1994; Nielsen K. M., 2005; Nordgård, 2016). In general, the wide distribution of ARGs and the potential threats to human and animal health arising from horizontal gene transfer (HGT) of these genes

highlights the importance of identification and monitoring of the presence and level of ABs and ARGs in the environments, as it can function as a source and sink of transferable resistance.

In this study, we have investigated samples associated with WWTPs two different locations in Norway (Tromsø and Bergen), for phenotypic AMR to different clinical relevant antibiotics and for the prevalence of a selection of ARGs representing the main classes of antibiotics.

1.1 Selective pressure and the environmental resistome

Human actions, namely the use and misuse of antibiotics, have led to an increase in AMR occurring within the clinical setting. Nevertheless the phenomenon of AMR predates mass production and clinical use of antibiotics by some 30 000 years. The diverse vast reservoir of antimicrobial resistance genes in the environment is likely the origin of many clinical antimicrobial resistance traits (D'Costa et al., 2011; Hughes & Datta, 1983; Julie Perry, Nicholas Waglechner, & Gerard Wright, 2016). The capacity of microorganisms to share genetic material via HGT means that genes present in the environmental resistome have the potential to move between bacterial communities, potentially transferring antimicrobial resistance genes to human and animal pathogens. Two key questions, in the light of the worsening situation of clinical resistance arise. Firstly, whether human activities significantly influence antimicrobial resistance levels in the environment by dissemination of resistant microbes and pollution with pharmaceutically produced antibiotics. Secondly, what is the nature of the interaction between the environmental resistome and clinical settings, and when and how antibiotic resistance of environment can make a difference in clinical resistance patterns (Berkner, Konradi, & Schonfeld, 2014; Rita L Finley et al., 2013; Larsson, 2014). Anthropogenic activities and the environment intersect in several ways when considering the issue of AB, ARBs and ARGs, as illustrated in figure 1.

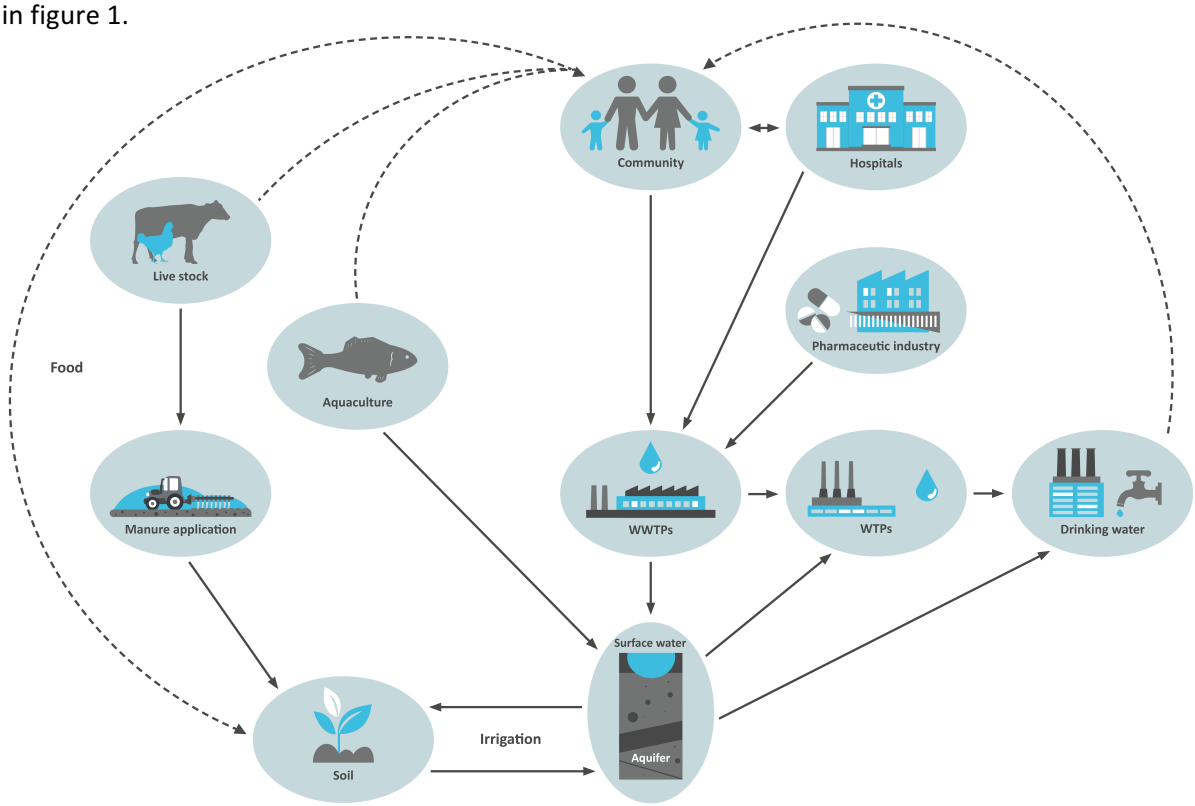

Figure 1: A representation of the intersections between human activity and environmental compartments in terms of the spread of AB, ARB and antimicrobial resistance determinants (ARD) (Ill. By Cathrine Brynjulfsen, GenØk. All icons are designed by designers at Freepik.com: Macrovector, Ibrandify, Zirconicusso)

Occurrence of antibiotics in low concentrations can be expected in environments exposed to sewage, runoff from livestock/manure, landfill leaching, industrial effluent, as well as from other sources where antibiotics are used in human and veterinary medicine (Andersson & Hughes, 2012; Gullberg et al., 2011; K.-R. Kim et al., 2010; Sandegren, 2014; Wellington et al., 2013). WWTPs are of particular concern, since they bring antibiotics, pathogens, environmental bacteria and resistance genes in close proximity to each other. Aside from antibiotics, biocides and heavy metals also present in these conditions and may act as an additional layer selecting resistant phenotypes (Berglund, Fick, & Lindgren, 2015; Pal, Bengtsson-Palme, Kristiansson, & Larsson, 2015; VKM, 2016).

1.2 Antibiotic resistance in the environments

Antimicrobial resistance hotspots are found not only in medical settings but also in environmental compartments that are subject to some anthropogenic activities. Over the last decade, increased resistance against antibiotics among bacteria in many different ecological niches like soil, wastewater treatment plants, river water, drinking water, seawater, sediments etc. has emerged (Berglund et al., 2015; Berglund, Khan, Lindberg, Fick, & Lindgren, 2014; D'Costa, McGrann, Hughes, & Wright, 2006; Lindberg, Wennberg, Johansson, Tysklind, & Andersson, 2005; Rizzo et al., 2013; Segura et al., 2009; Xi et al., 2009; S. Zhang et al., 2015; X. H. Zhang et al., 2016). In these different environments, AB, ARB and ARGs have routinely been observed. The reasons for this are complex, but the increased use of antibiotics in general combined with exposure to additional resistance promoting substances, such as disinfectants, biocides and some heavy metals is significant (Alonso et al., 2001; Berglund et al., 2015; Martinez, 2009).

It is important to note that the occurrence of antimicrobial resistance in the environment may also result from the production of antimicrobials by certain groups of microorganisms (R. L. Finley et al., 2013; Séveno N. A., 2002; Waksman & Woodruff, 1940). The first antibiotics that were introduced in the 1940-50`s were actually naturally occurring microbial products that had been discovered by laboratories screening for the pharmaceutical industry (Mazel & Davies, 1999). Most antibiotics in medical- and/or agricultural use are derived from or produced by a group of soil bacteria called *Actinomycetes* (J. Perry, N. Waglechner, & G. Wright, 2016).

Resistance to antimicrobials is a natural consequence of bacterial adaption as a result of exposure to antibiotics and other drivers for resistance (Séveno N. A., 2002; VKM, 2016). This means that all use of antimicrobials, as well as some biocides and heavy metals, in human and veterinary medicine including aquaculture, increase pressure for development of resistance in a wide range of bacterial groups (Davies & Davies, 2010; G. A. Khan, Berglund, Khan, Lindgren, & Fick, 2013; S. Khan, Beattie, & Knapp, 2017; Kruse & Sorum, 1994). The potential for anthropogenically-produced antibiotics to augment the basal level of resistance genes present in environmental compartments was demonstrated by Knapp, Dolfing, Ehlert, and Graham (2009), who studied archived soil samples spanning various intervals between 1940 and 2008 in the Netherlands. This study documented a general increase in the presence of ARG over time, especially for tetracycline resistance genes, some of which were found at levels 15 times higher in 2008 than in the period between 1970-1979.

It was thought that dissemination of resistance genes mainly occurs from anthropogenic communities to natural environments. However, recent studies also suggest that bacteria in nature can be a source

of resistance, which can spread to pathogenic bacteria in clinical environments. Direct evidence, how this may occur and to which extent is still not clear (Canica et al., 2015; Hiltunen, Virta, & Laine, 2017; Manaia, 2017; Martinez, Coque, & Baquero, 2015; Perry, Westman, & Wright, 2014; Perry & Wright, 2013; Williams et al., 2016). The general lack of studies and data in this filed can be explained by methodological limitations to retrospectively assign directions and sources of HGT events. HGT events are only detectable in larger bacterial populations after they have reaches proportions that are defined by the scale of sampling scheme selected (Nielsen, Bohn, & Townsend, 2014).

1.3 Antibiotic resistance in waste water treatment plants

Antimicrobial resistance genes (ARGs), in association with ARB, have been identified as widespread contaminants of wastewaters and treated drinking water. The WWTPs receive wastewater from household and hospitals where antibiotics are applied and the prevalence of ARB and ARG might therefore increase in such environments (Baquero, Martinez, & Canton, 2008; Roca et al., 2015; R. Szczepanowski et al., 2009). This means that municipal WWTPs can serve as important reservoir of ARB and ARG (J. S. Kim et al., 2016; Laht et al., 2014; Munck et al., 2015; Rafraf et al., 2016; Rizzo et al., 2013; R. Szczepanowski et al., 2009; Williams et al., 2016; Yang et al., 2014). The WWTPs may be considered hotspots for ARB and ARG spread into the environment, not only because of the potential presence of substances with selective pressure in the WWTP system (Michael et al., 2013; Plosz, Leknes, Liltved, & Thomas, 2010; K. V. Thomas, Dye, Schlabach, & Langford, 2007; Xi et al., 2009), but also because the WWTPs offers nutrient rich growth conditions to microorganisms during WWT process that may favour events of horizontal gene transfer of AMR.

A number of studies show that AB, ARB and ARG can be detected in samples from WWTPs around the world (Bondarczuk, Markowicz, & Piotrowska-Seget, 2016; Marathe, Shetty, Shouche, & Larsson, 2016; Munck et al., 2015; Narciso-da-Rocha & Manaia, 2017; Rahube et al., 2016; Rizzo et al., 2013; Yang et al., 2014). Since much of the sewage sludge produced by WWTPs is recycled as fertilizer products and used as soil conditioner on cultivated areas, and on green areas including parks, private gardens etc. concerns of increased spread of AMR has been questioned (Bondarczuk et al., 2016; Rahube et al., 2016; Rizzo et al., 2013). In a report published by VKM in 2009, the risk of increased occurrence of ARB and ARG in soil following application of sewage sludge as soil conditioner in Norway was assessed (VKM, 2009). Based on their literature study and findings the conclusion by VKM in the report is that it is unlikely that AMR may be promoted in the sewage treatment plant water (STPW), in the sludge or in the soil following application of sewage sludge as fertilizer.

Drinking water often originates from surface water, which is also the discharge point for wastewater (Baquero et al., 2008; Berry, Xi, & Raskin, 2006; Williams et al., 2016; Xi et al., 2009). This means that also the aquatic environment may be important when it comes to introduction and dissemination of AMR through the environment. The main concern about drinking water and the quality has mainly been on the presence of pathogens, but the increasing number of studies demonstrating antibiotics and ARB in the surrounding water sources and the finished drinking water is an emerging issue (Armstrong, Calomiris, & Seidler, 1982; Baquero et al., 2008; Berry et al., 2006; Dodd, 2012; Jones, 1986; Schwartz et al., 2003; X. X. Zhang, Zhang, & Fang, 2009).

1.3.1 Waste water treatment

Sewage water coming from the different wastewater treatment plants (WWTPs) is a mixture of wastewater from households, industries, hospitals and runoff waters from urban areas. The conventional wastewater treatment generally consists of a primary, secondary and sometimes a tertiary treatment stage ((VKM), 2009; Michael et al., 2013; Tromsø kommune, 2015) that are associated with certain emission standards. Primary treatment is mechanical cleaning which intends to reduce the solid contents of the wastewater. The secondary treatment intend to remove organic matter and/or nutrients. This may be achieved by both chemical and biological treatment. In tertiary treatment nutrients such as phosphorus and nitrogen are also removed and this can be done by precipitation or use of filters (Michael et al., 2013). Most of the WWTPs in Norway were built after 1970 as a result of pollution and algal blooms caused by phosphorus discharges. The treatment plants in Norway today use mechanical, chemical or biological treatment, or a combination of these, depending on whether the emissions are into fresh water, sea water or an area designated as sensitive or less sensitive (Norsk Vann Rapport, 2013; Tromsø kommune, 2015; VKM, 2009).

Municipal WWTPs are designed and dimensioned to mainly remove organic matter, phosphorus and possibly nitrogen, but not primarily to remove antibiotics or other pharmaceuticals. In fact, it has been shown that many antibiotics enter aquatic environments after being discharged in municipal and hospital wastes, and studies have shown that WWTPs are not necessarily efficient at removing pharmaceuticals from the water (B. Berglund, G. A. Khan, S. E. Weisner, et al., 2014; Hendricks & Pool, 2012; Lindberg et al., 2005; Segura et al., 2009). In addition, there are studies that demonstrate that the purification process is not appropriate to fully remove resistance genes in the different compartments of WWTPs, meaning that also the resistance genes end up in the environment (Hendricks & Pool, 2012).

1.4 Antibiotics and resistance

Antibiotics may either kill or inhibit growth of bacteria. Different antibiotics can be divided into main groups according to their target (Davies & Davies, 2010; Lewis, 2013; Mazel & Davies, 1999). The main mechanisms of action of the antibiotics are:

- Inhibition of cell wall synthesis
- Inhibition of protein synthesis
- Inhibition of nucleic acid synthesis
- Inhibition of folic acid synthesis
- Interference with cell membrane function

Today, the microbes that were once susceptible to antibiotics are becoming more and more difficult to treat as results of acquired antibiotic resistance (Davies & Davies, 2010; Lewis, 2013; Mazel & Davies, 1999; Schmieder & Edwards, 2012). Resistance can be caused by four major types of mechanisms:

- Inactivation or modification of the antibiotics
- Alteration in the target site
- Modification of metabolic pathways to circumvent the antibiotic effect
- Decreased accumulation

The general targets for antibiotics as well as the mechanism of resistance are summarized in figure 2.

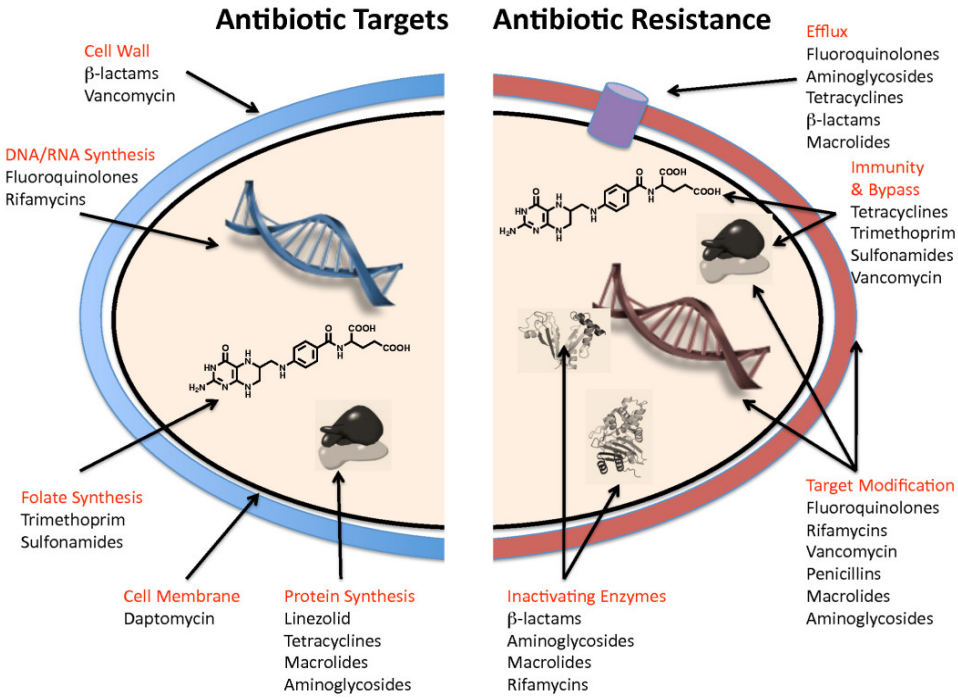

Figure 2: Antibiotic targets and mechanisms of resistance (Source: Wright, G.D. (2010) Antibiotic targets and mechanisms of resistance. Copyright BMC Biology 2010 8:123 doi:10.1186/1741-7007-8-123. Licenced under Creative Commons Attribution 2.0 generic licence. http://www.biomedcentral.com/content/figures/1741-7007-8-123-1-l.jpg

1.4.1 Selected antibiotics in this study

In this study, we wanted to evaluate the prevalence of antimicrobial resistance to ten antibiotics that are relevant in human/veterinary medicine in Norway. The selection of antibiotics was done on the basis of data gathered in the period 2011-2015 by NORM/NORM-VET 2015. A short overview of the mechanism of action as well as the resistance mechanism of the relevant antibiotics used in this study is listed in table 1 (Davies & Davies, 2010; Lewis, 2013; Mazel & Davies, 1999; Schmieder & Edwards, 2012).

Table 1: Selected antibiotics used in this study, their mechanisms of action and resistance mechanisms

Class	Examples	Target	Resistance mechanisms
B-lactams	Ampicillin (AMP) Amoxicillin (AMX)	Targets: Penicillin binding proteins (PBPs). Inhibition of cell wall synthesis. Bactericidal.	Beta-lactamases, modification of penicillin binding proteins (PBPs), efflux pumps and membrane impermeability.
B-lactamase resistant penicillin	Dicloxacillin (DCX)	Targets: Penicillin binding proteins (PBPs). Inhibition of cell wall synthesis. Dicloxacillin is stable against hydrolysis by a variety of beta-lactamases. Bactericidal.	Modification of penicillin binding proteins (PBPs), efflux pumps and membrane impermeability.
Aminoglycosides	Kanamycin (KM) Streptomycin (STP)	Targets: Binding of 30 ribosomal subunit.Disrupts translation, inhibits protein synthesis. Bactericidal.	Modification of antibiotic by acylation, phosphorylation or adenylation.
Tetracycline's	Tetracycline (TET)	Target: Binding of 30 ribosomal subunit. Inhibits protein synthesis. Bacteriostatic.	Efflux, ribosomal protection and enzymatic inactivation.
Fluoroquinolones	Ciprofloxacin (CIP)	Targets the GyrA subunit of DNA gyrase, and topoisomerase IV. Inhibition of DNA synthesis. Bacteriicidal.	Target modification, efflux pumps.
Macrolides	Erythromycin (ERI)	Targets: 50S ribosomal subunit. Inhibits protein synthesis. Bacteriostatic.	Target modification, mutations in 23S rRNA, efflux pumps and enzymatic inactivation. Phosphotransferases: phosphorylation of hydroxyl group. Glycotransferases: glycosylation of hydroxyl group. Esterases: hydrolyzation of lactone ring.
Trimethoprim	Trimethoprim (TMT)	Targets: dihydrofolate reductase (DHFR). Inhibits folate synthesis. Bacteriostatic.	**Resistant** forms of the DHFR enzyme. M**utations** in gene promoter and upstream genetic elements lead to overexpression of intrinsic DHFR enzyme.
Sulfonamides	Sulfamethoxazole (SMX)	Targets: dihydropteroate synthase (DHPS). Inhibits folate synthesis. Bacteriostatic.	Resistant forms of DHPS enzymes, mutations in *dhp* gene.

2. Materials and methods

2.1 Sampling area and sampling methods

Six different WWTPs located in Tromsø and Bergen were chosen to establish a baseline frequency of ARB and ARG connected to these treatment plants (TPs). The sampling areas/points were selected with the intention to analyze areas with different exposure of antibiotics and other resistance promoting substances, such as disinfectants, biocides and heavy metals that have the potential to co-selective resistance. The samples were all obtained from already established sampling area in collaboration with Tromsø and Bergen municipalities. The sampling period was September and October of 2016. The characteristics of the different sampling areas are summarized in table 2. Figure 3 shows sampling of bottom sediment with an van Veen grabb in Byfjorden near Bergen.

All samples from Tromsø were taken from WWTPs connected to households and one was also connected to the reginal hospital (Breivika). These samples represents areas with relatively high exposure of antibiotics, according to literature. The samples from Byfjorden near Bergen were taken at eight different sampling stations, previously established for regular monitoring of biodiversity in fjord sediments. These samples were not taken directly from the WWTPs as the samples from Tromsø, but are bottom samples from the fjord with variable distance to different WWTPs in Bergen, (Figure 1, appendix A). These latter samples represent exposures from residential and industrial areas.

Table 2: Description of the different sampled areas

WWTPs and WTP	Type of treatment		Established/ renovated	Characteristics	Sample
Strandveien (Tromsø)	Mechanical treatment (Salsnes filters)	Primary treatment	1995/ 2008	Close to residential area	Sewage
Breivika (Tromsø)	Mechanical treatment (Salsnes filters)	Primary treatment	2003/ 2007/2008	Close to residential area and hospital	Sewage
Hamna (Tromsø)	Mechanical treatment (Salsnes filters)	Primary treatment	2005/ 2006	Close to residential area	Sewage
Holen (LYR 7) (Bergen)	Mechanical, biological and chemical treatment	Tertiary treatment	1997/ 2015	Close to residential and industrial areas	Bottom sample
Kvernevik (KVR1) (Bergen)	Mechanical and biological treatment	Secondary treatment	1978/ 2015	Close to residential area	Bottom sample
Kjøkkelvik (St. 5) (Bergen)	Mechanical treatment (Salsnes filters)	Primary treatment	1990	Close to residential area	Bottom sample

Figure 3: *Sampling of bottom sediment by van Veen grabb in Byfjorden near Bergen. Sampling in Byfjorden was done in conjunction with another ongoing project to quantify microplastic particles in fjord sediments, led by Marte Haave (URE) and funded by Bergen Municipality. In this photograph, technician Ragni Torvanger (Fishguard) prepares a van Veen grab for deployment at Station 13 in Byfjorden. Photograph by Jessica Louise Ray.*

2.2 Enumeration of cultivable bacteria

For the culture-based approach the three sampling areas from Tromsø: Strandveien, Breivika and Hamna, and three of the sampling points from Bergen: Lyr2, Kvr1 and St. 5 were selected. Colony-forming units (CFU) were determined for the total cultivable aerobic bacteria and for the total cultivable antimicrobial resistant aerobic bacteria for all sampling areas in Tromsø and Bergen. Ten-fold dilutions made in saline were plated on R2A agar (Merck, Darmstadt, Germany) in triplicates. The media were supplemented with 50 µg/ml cycloheximide to avoid growth of fungi. For the corresponding antimicrobial resistant subpopulation, the same media were supplemented with 20 µg/ml of the specific antibiotic (Table 1). All antibiotics were obtained from Sigma Aldrich. The agar plates were incubated for 5 days at room temperature before enumeration. After incubation and enumeration, the bacteria were collected separately for each antibiotic used for selection and colonies were stored in 20% glycerol at – 20°C until further use, and at -80°C for long term storage.

2.3 DNA extraction from bacterial isolates

DNA was isolated from re-streaked antibiotic resistant colonies using Quickextract^{tm} DNA extraction Solution 1.0 (Epicenter Biotechnologies) according to the manufacturer`s instructions. The eluted DNA extracts were stored at -20°C until further analysis, and at -80°C for long term storage. Four µl of a 10^{-2} dilution of the resulting DNA solution served as template for the 16S rRNA PCR assay as well as for the resistance gene specific PCRs.

2.4 Total DNA extraction from samples from the different sampling area.

Total DNA was extracted using the QIAamp DNA stool kit (Qiagen) according to the manufacturer's instructions. Quantity and quality of the purified DNA were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The eluted DNA extracts were stored at -20°C until further analysis. Approximately 100 ng of the DNA solution served as template for the 16S rRNA PCR assay as well as for the antibiotic resistance gene specific PCRs.

2.5 PCR based detection of 16S rRNA and ARGs in antimicrobial resistant bacterial isolates and total DNA

PCR experiments for the amplification of specific resistance genes were performed with single isolates of phenotypic resistant bacteria. The genes that were tested for were *blaTEM, mecA, qnrS, ErmB, aph(3`)-IIa, aph(3`)-IIIa, aac(6`)/aph(2``)sulI, tetA* and *dfrA1.* The 16S rRNA gene was amplified as a control of the extracted DNA to confirm the general absence of PCR inhibitors.

In general, all reactions were performed in a total volume of 20 µl containing the following: 1 µl of each specific primer (Sigma Aldrich) at 10 µM concentration, 10 µl mastermix (DreamTaq PCR Mastermix, Thermo Fisher), 4 µl water and 4 µl template DNA. The PCR conditions were as follows: 1 cycle of initial denaturation at 95˚C for 2 min, 30 cycles of denaturation at 95 ˚C for 30 s and annealing at x ˚C for 30 s (see table 1, appendix B for specific annealing temperature) and elongation at 72 ˚C for x s (see table 1, appendix B for specific elongation period), one cycle of final elongation at 72˚C for 5 min. All PCRs were carried out on a BioRad ThermoCycloS1000. The PCR products were run on 2% agarose well E-gels, using E-Gel® 1 Kb plus DNA ladder (all supplied by Invitrogen, Norway), before visualization. All PCR analyses included no-template controls and positive controls. Primers, controls and the details for the PCR conditions are listed in Table 1 in appendix B.

2.6 Metagenomic approach

2.6.1 Total DNA extraction for metagenomic studies

Tromsø: Untreated solid waste was collected into sterile 50 ml PP tubes, and kept frozen during shipment to Bergen. Samples were stored at -20°C until preparation for DNA extraction. Byfjorden in Bergen: Mixed surface sediment samples were immediately collected from van Veen grabs using a metal spatula wiped clean with EtOH, and transferred into a sterile 50 ml PP tube. Samples were immediately frozen to -20°C and kept frozen until DNA extraction.

One day prior to DNA extraction, sediment samples were briefly thawed so that ten replicate 0.5 g (wet weight) subsamples could be taken using EtOH-cleaned and flamed metal dissection tools (Tromsø and Bergen). Subsamples were weighed into PowerBead lysis tubes (MoBio) then stored again at -20°C overnight. Lab coats, particle masks, safety goggles and clean gloves were worn at all times during sampling to minimize the risk of transfer between samples and laboratory users.

The MoBio PowerSoil kit is commonly utilized for DNA extraction of microbial communities in soils and sediments (Lekang, Thompson, & Troedsson, 2015). To improve sequencing evenness and coverage, ten replicate subsamples per sampling station were subjected to DNA extraction (Lekang et al. 2014). Because of the higher processing load when including ten replicates per sample, DNA was extracted

using an automated nucleic acid extraction platform. Furthermore, to improve lysis efficiency, with particular emphasis on Gram positive bacteria (Guo & Zhang, 2013) the initial lysis step of the PowerSoil protocol was combined with additional enzymatic and thermic lysis steps to improve lysis efficiency. It should be noted that sediment samples were not pre-treated with DNAse prior to DNA extraction, therefore the DNA preparations generated here reflect both the cellular and environmental DNA fractions.

In brief, 0.5 g samples were added to PowerBead tubes (MoBio) with 60 µl Solution C1. Bead-beating was performed on a Tissue Lyzer (Precellys) using three cycles of 6000 rpm for 40 seconds with 2 min rests between. After homogenization, samples were briefly spun to remove liquid from the tube caps. Enzymatic lysis was then performed by adding 50 µl of a 20 mg ml^{-1} lysozyme solution (Sigma Aldrich) in 20 mM Tris-Cl, 2 mM EDTA, pH 8.0, 1.2% Triton-X to samples, shaking to distribute, and incubating at 37°C for 30 min. Lysozyme facilitates lysis of Gram positive bacterial cells by destabilizing the peptidoglycan in cell walls. Finally, samples were incubated at 70°C for 30 minutes. Lysed samples were then spun at 10,000 x *g* for 60 seconds to sediment solids. Three hundred microliters of lysis supernatant was transferred to a 2 ml tube compatible with QIAsymphony SP sample loading racks. DNA from lysed samples was performed on the QIAsymphony SP using reagents from the QIAsymphony DSP virus/pathogen mini kit (QIAGEN) together with the Complex200_V6_DSP_default_IC protocol (QIAGEN). Carrier RNA (poly-A) was included in all samples to improve DNA recovery. Purified DNA was eluted in 110 µl RNAse-free water containing 0.04% sodium azide. DNA samples were stored at -20°C. The remaining lysates in PowerBead tubes were kept at -20°C. This protocol is suitable for simultaneous extraction of genomic DNA and plasmid DNA.

Carrier RNA was digested from DNA preparations (pooled by sample) by adding 2 µl 20 mg ml^{-1} RNAse A (QIAGEN) and incubating at 37°C for 30 min. DNA was purified from RNAse-treatments using the DNA Clean & Concentrator-5 kit (Zymo) with all centrifugation steps at 10,000 x *g* for 30 sec. A 2:1 binding buffer to sample volume ratio was used, and samples were eluted in 31 µl 65°C Elution Buffer (Zymo). DNA concentration in samples was measured using Qubit HS dsDNA assay (Life Technologies). DNA content was visually assessed using agarose gel electrophoresis. DNA samples were sent to the Norwegian Sequencing Centre (NSC) at the University of Oslo for metagenomic library preparation using the THRUplex kit (Rubicon) and sequencing on an Illumina MiSeq PE300 platform.

2.6.2 Metagenome sequencing and AMR gene identification

Raw sequence data was archived on the Norwegian Bioinformatics Platform (StoreBioInfo) via the Norwegian e-Infrastructure for Life Sciences (NeLS). Primer and adapter removal, merging of mate pairs, quality trimming and unsupervised assembly of clean reads was performed using CLC Genomics Workbench (QIAGEN Bioinformatics) with standard parameters. The assembly process joins related sequence fragments into longer, contiguous sequences ("contigs"). These contigs were then analyzed using a local installation of the open source Resistance Gene Identifier (RGI) tool from the Comprehensive Antibiotic Resistance Database (CARD) version 1.1.3 (McArthur et al., 2013), allowing for only "perfect" and "strict" matches. Visualization of resistance profiles for each sample were generated by the RGI tool in JSON format and visualized using the CARD webserver (accessed at https://card.mcmaster.ca/).

3. Results

3.1 Bacterial enumeration and level of antimicrobial resistance

In total, samples connected to six municipal wastewater treatment plants (WWTPS) were investigated for the occurrence of antimicrobial resistant bacteria. Microbiological analysis of the samples from Tromsø indicated that the level of total aerobic bacteria growing on the media used was approximately 10^7 CFU per gram sludge. In the samples from Bergen the levels were about 10^3- 10^4 CFU per gram sludge, meaning that there was a lower microbial abundances in the samples from Bergen compared to the samples from Tromsø.

The percentage of bacteria that could grow in the presence of the different antibiotics selected varied between the different sampling areas. The highest resistance percentages were observed for sulfamethoxazole (SUL) and trimethoprim (TMT) and the lowest was observed for amoxicillin and ampicillin. Among the antimicrobial resistance phenotypes examined, sulfamethoxazole (SUL) exhibited the widest variations, from 7-86%. A particular variation in the resistance percentage pattern between the samples from Tromsø compared to the samples from Bergen was not observed (Table 3).

Table 3: Detection of antibiotic resistance in sludge samples from selected area in Tromsø and Bergen

		Resistance (%)									
AREA	**Total CFU**	**AMP**	**AMX**	**DCX**	**KM**	**STP**	**TET**	**CIP**	**ERI**	**TMP**	**SMX**
Strandveien	1,1E+07	4	5	47	6	46	18	13	33	45	30
Hamna	3,0E+07	2	6	27	7	8	ND	42	8	44	52
Breivika	2,1E+07	3	12	22	9	10	ND	22	3	34	7
KVR1	4,3E+03	ND	ND	20	62	16	3	32	37	33	86
LYR	2,9E+03	ND	40	29	13	12	ND	42	30	40	32
ST. 5	1,4E+04	ND	2	6	12	2	1	8	7	12	14

ND = not detected, AMP = ampicillin, AMX = amoxicillin, DCX = dicloxacillin, KM = kanamycin, STP = streptomycin, TET = tetracycline, CIP = ciprofloxacin, ERI = erythromycin TMP = trimetroprim, SMX = sulfamethoxazole

3.2 Detection of target resistance genes in resistant bacterial isolates from WWTP samples

To detect ARGs present in bacteria growing on media supplemented with antibiotics, PCR analysis using 10 specific primer sets were carried out. Total DNA preparations from antimicrobial resistant colonies were used as template DNA in the PCRs. The targeted resistance genes confer resistance to the different classes of antibiotics used in this study. In general, 250 to 550 resistant colonies per antibiotic were analyzed for 16S rRNA. If the PCR was able to amplify the 16S rRNA, the colonies were further screened by PCR for specific ARGs. The screen detected specific resistance genes known to confer resistance to beta-lactams, aminoglycosides, tetracycline, macrolides, trimetoprim as well as sulfonamides in a minor proportion of the colonies examined (Table 4)

Table 4. PCR based detection of target genes in antimicrobial resistant bacterial isolates

	B-lactams						Aminoglycosides		Tetracycline	Fluoroquinolones	Macrolides	Trimetoprim	Sulfonamides
AB ARGs	*AMP (n) blaTem*	*AMP (n) mec A*	*AMX (n)blaTem*	*AMX (n) mec A*	*DXC (n) blaTEM*	*KM (n) aph(3`)-IIa*	*KM (n) aph(3`)-IIIa*	*STP(n) aac(6\) /Aph(2``)*	*TET (n) tetA*	*CIP (n) qnrS*	*ERI (n) erm (b)*	*TMP (n) dfrA1*	*SMX (n) sulI*
Strandveien	(90) 0	(90) 4	(87) 0	(87) 8	(89) 0	(77) 0	(77) 2	(22) 0	(89) 4	NG	(75) 0	(93) 3	(92) 2
Hamna	(84) 2	(84) 6	(91) 1	(91) 8	(93) 0	(93) 0	(93) 11	(93) 3	(88) 42	NG	(89) 15	(83) 0	(91) 0
Breivika	(89) 5	(89) 7	(82) 1	(82) 6	(79) 0	(76) 0	(76) 1	(77) 0	0	NG	(75) 0	(84) 4	(84) 1
KVR1	0	0	0	0	(92) 0	(75) 0	(75) 4	(83) 1	(11) 4	NG	(48) 0	(88) 0	(91) 0
LYR	0	0	(73) 0	(73) 4	(71) 0	(84) 0	(84) 0	(90) 1	(14) 0	NG	(40) 0	(93) 55	(92) 0
ST. 5	0	0	(72) 1	(72) 3	(65) 0	(78) 0	(78) 0	(83) 4	(56) 3	NG	67 (1)	(88) 4	(92) 4
Total number of colonies	**(263) 7**	**(263) 17**	**(405) 3**	**(405) 29**	**(430) 0**	**(483) 0**	**(483) 18**	**(448) 9**	**(258) 53**	**ND**	**(394) 16**	**(260) 66**	**(542) 7**

n= number 16S positive colonies, NG= no growth after 5 days, ND = not determined

Table 5: PCR based detection of target genes in total DNA from environmental samples.

	B-lactams		Aminoglycosides			Tetracycline	Fluoroquinolones	Macrolide	Trimetoprim	Sulfonamides
ARGs	***blaTEM***	***mecA***	***aph(3`)-IIa***	***aph(3`)-IIIa***	***aac(6\) /Aph(2``)***	***tetA***	***qnrS***	***erm (b)***	***dfrA1***	***sulI***
Strandveien	-	-	-	+	+	-	+	-	-	-
Hamna	+	-	-	+	+	-	+	+	-	-
Breivika	+	-	-	+	+	-	+	+	-	-
KVR1 /LYR/ST.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND = not determined

3.3 PCR based detection of target genes in total DNA isolated from sample areas

The yield of total DNA was lower in the samples from Bergen compared to the samples from Tromsø. In addition, the amplification of 16S rRNA in the total DNA from the samples from Bergen was unsuccessful and these samples were not screened for ARGs. The results from the total DNA analysis from Tromsø demonstrates that the PCR results were positive for resistance genes known to confer resistance to beta-lactams, aminoglycosides, fluoroquinolones and macrolides (Table 5).

3.4 Metagenomic analysis

This report contains metagenome sequence information from three out of six sampling points associated with WWTPs. Because of impurities and difficulties in the DNA extraction process the library preparation for the samples from Hamna (Tromsø), Breivika (Tromsø) and Lyr (Bergen) did not succeed. The source of contamination, such that it could be reduced or removed, was not identified during the project period. URE has, however, procured the remaining DNA samples from the NSC and will test various purification methods on them with the expressed goal of completing the metagenomic sequence analysis for all samples.

Metagenome assembly

Using default quality-trimming and assembly parameters in the CLC Genomics Workbench, the metagenomes generated in this report bespeak highly complex and diverse communities in the sediments sampled. Numbers of contigs assembled per sample ranged from 10062 (St. 13) to 43541 (Strandvegen) with mean contig lengths of approximately 900 bp for all samples from Byfjorden, and 1100 bp for the Strandvegen sample (Table 6). Surprisingly, only 5-10% of sequence reads from Byfjorden samples could be mapped back to contigs (Table 6), indicating high prevalence of low-abundance sequences which typically cannot be joined to contigs during an unsupervised assembly process (Howe et al., 2014). For the Strandvegen sample, approximately 40% of reads mapped to contigs (Table 6).

Resistance Gene Identifier (RGI) results

The RGI algorithm is a freely-available open-source algorithm designed to search the Comprehensive Antibiotic Resistance Database (CARD) antibiotic resistance ontology (ARO) for the presence of antimicrobial resistance genes in DNA sequences (McArthur et al., 2013). In this report, a local installation of the RGI and accompanying CARD (v.1.1.3) was utilized to search the eight sediment metagenomes for the presence of AMR genes.

Assembled metagenomes (contigs) were input as query sequences into the RGI tool for two reasons: 1) assembly reduces the size of metagenome datasets through contig formation, making AMR gene identification less computational intensive, and 2) assembly decreases sequence errors through increased coverage of individual nucleotide positions. In total, the RGI was able to identify 110 matches to the CARD (v.1.1.3, December 2016) among the eight assembled sediment metagenome datasets (Table 3 and Figure 4). Of these 110 matches, three “Perfect” matches were identified in the Strandvegen metagenome (Table 1, Appendix C). Two of these matches, to the CARD reference genes *msrE* and *ermB*, encode an efflux pump and modification enzyme, respectively, that mediate resistance

to macrolide antibiotics (Figure 4). The third "Perfect" match in the Strandvegen metagenome was to a CARD reference gene encoding the APH(6)-Id aminoglycoside inactivating enzyme (Figure 4), The remaining 107 "Strict" matches were distributed across all eight metagenomes presented in this report (Table 1, Appendix C), and represent a diversity of AMR target classes (Appendix 1).

The two main findings of the metagenomics module in this study can be summarized accordingly:

1) Untreated sewage solids may have higher AMR gene prevalence than surface fjord sediments, and 2) Genes encoding antimicrobial efflux pumps represent the majority of AMR genes and AMR functional types in the analyzed metagenomes.

Table 6. Summary metrics for sediment/sludge DNA recovery, metagenome sequencing, assembly and AMR gene identification. RGI, resistance gene identifier (McArthur et al., 2013).

Municipality	**Tromsø**	**Bergen**						
Station	Strandvegen	St. 4	St. 5	St. 11	St. 13	Kvr1	Lyr2	Ny01
DNA concentration (ng g^{-1} wet weight)	493	189	236	251	262	131	163	267
Number of sequence reads (raw data)	3166603	3176440	3023248	2853527	2075938	2613747	2658705	1864991
Number of contigs	43541	18375	12088	21930	10062	28356	32998	20693
Number of reads mapped to contigs	1295912	206157	150562	251842	151196	624697	442491	233985
Mean contig length ± sd (base pairs)	1146 ± 963	876 ± 332	851 ± 278	929 ± 328	924 ± 587	914 ± 401	941 ± 420	985 ± 433
Lowest mean contig coverage	1	1	1	1	1	1.1	1	1
Highest mean contig coverage	50906	16356	5063	12314	8525	17189	26027	7528
Median, mean contig coverage	3.50	2.47	2.39	2.51	2.28	2.80	2.90	2.46
Number of «Perfect» RGI matches	3	0	0	0	0	0	0	0
Number of «Strict» RGI matches	65	3	1	5	1	15	12	5

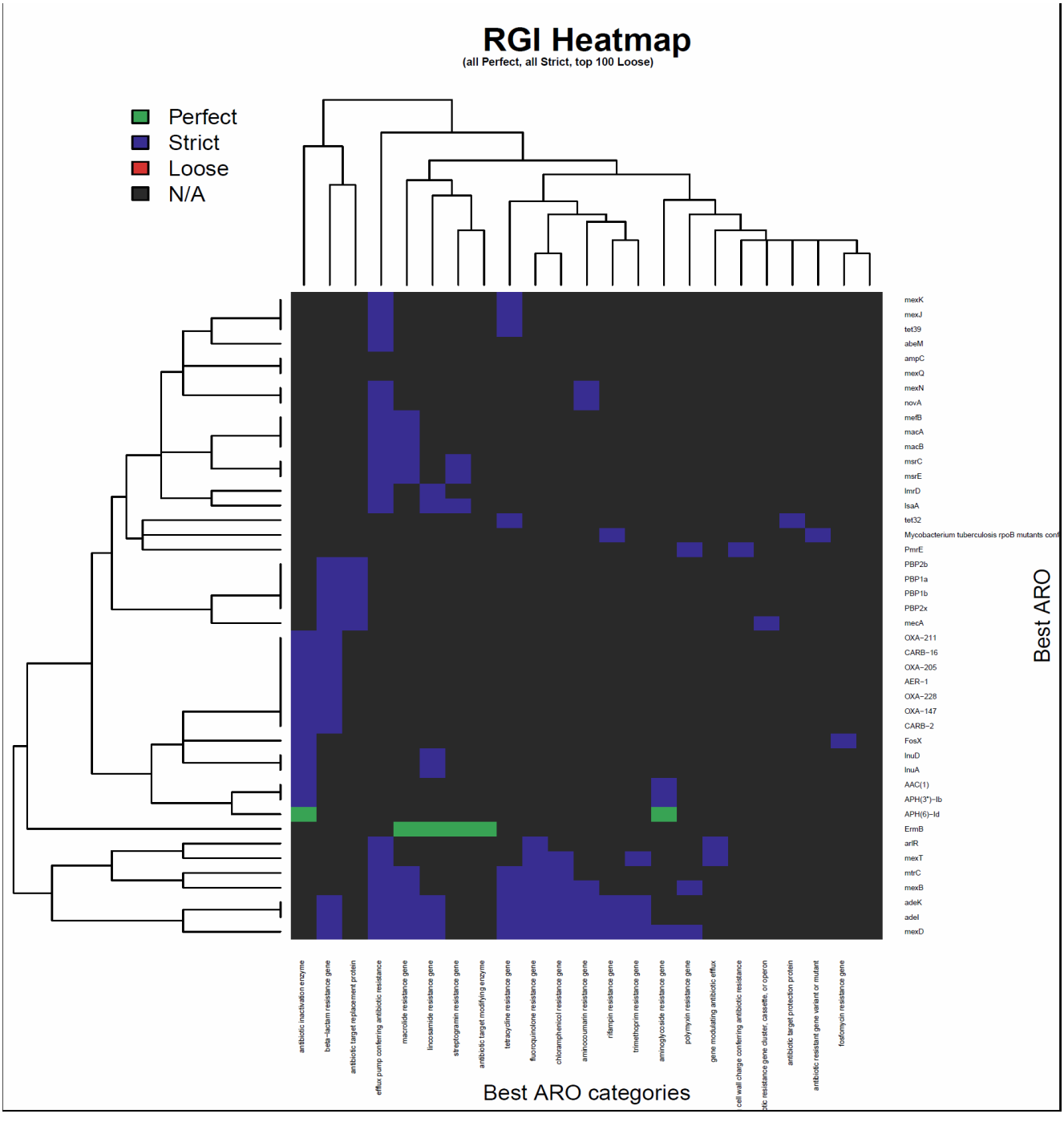

Figure 4: Heatmap from Strandveien that illustrates the prevalence and diversity of AMR and AMR-like genes in this sample by metagenomics analysis

4. Discussion

Several studies have detected different important classes of antibiotics in low concentrations in different environmental compartments, e.g. hospital effluent, municipal waste water, effluent from sewage treatment plants surface water and also ground water. This might suggest that e.g. WWTP effluents may be important sources of ARB and ARGs (Bouki, Venieri, & Diamadopoulos, 2013; Karkman et al., 2016; S. Zhang et al., 2015; T. Zhang, Shao, & Ye, 2012). The efficiency of waste water treatment in removing AB, ARB and ARG is not clear. Since most of the published data are from other countries it is important to assess the prevalence of ARB and ARGs in samples connected to WWTPs in Norway as well. In this study, we assessed the prevalence of antimicrobial resistance in samples connected to WWTPs in two Norwegian cities. Data of the most used human antibacterial agents for systemic use in Norway 2009-2014 gathered by NORM/NORM-VET 2015 was used as the basis for selection of the antibiotics used in this study. The following antibiotics from different classes were selected because of their high prescription levels: ampicillin (AMP), amoxicillin (AMX), ciprofloxacillin (CIP), dicloxicillin (DCX), erythromycin (ERI), kanamycin (KM), streptomycin (STP), sulfamethoxazole (SMX), tetracycline (TET) and trimethoprim (TMT). Kanamycin was chosen due to its use as a selective agent for ARMG.

Bacterial enumeration and percentage of antimicrobial resistance

The first objective of this study was to evaluate the prevalence of antimicrobial resistance in environments related to different treatment plants (TPs) in two Norwegian cities by culture-dependent methods. In this study R2A medium, which is a general medium for culturing and isolation of aerobic bacteria in soil and water, was used. The use of this medium with low temperature and increased incubation time makes it a suitable media for isolating bacteria from different environments (Volkmann et al 2004, Xi et al 2009). Bacteria from the same environment that are not able to grow on the selected media was not analyzed.

The CFUs of the total and the antimicrobial resistant fraction of aerobic bacteria were determined in all samples. Total CFU were higher in the samples from Tromsø compared to the samples from Bergen. As the samples from Tromsø consisted of untreated sewage solids, the microbial biomass in this sample is therefor expected to be higher than in the Byfjorden samples, which consisted of marine surface sediments. This result is corroborated by higher DNA concentration and higher incidence of ARG in the metagenome from sewage solids from Tromsø relative to the fjord sediment metagenomes from Byfjorden in Bergen. It might also be that the selected media in this study was not the most appropriate for the sediment samples. In a study by Lunestad and Goksøyr, media containing 70% seawater has been recommended as a standard for testing antibiotic resistance of marine bacteria (Lunestad, 1990; Samuelsen, Torsvik, & Ervik, 1992). The samples from Bergen were taken in October 2016, the climate is quite cold, and there is little sunlight, which also might affect the bacteria that have the best growth conditions. In our experiment the bacteria were cultivated at room temperature, which may not be optimal for the bottom samples from Bergen. In addition, it is well known that different bacteria may have different growth requirements, which means that the nutrient supply and other growth conditions can be optimized to improve the growth from the different samples (Birošová et al., 2014; Novo, Andre, Viana, Nunes, & Manaia, 2013).

The percentage of bacteria that could grow in the presence of the selected antibiotics in our study varied between the different sampling areas and between the different classes of antibiotics. A clear trend based on the sampling area was difficult to identify. Three different β-lactam antibiotics were used in this study and, surprisingly, the resistance percentage was in general higher on the media supplemented with dicloxacillin (13-42%) compared to ampicillin (ND-4%) and amoxicillin (ND-40%).

For the group of aminoglycosides two different antibiotics was used. The growth on streptomycin was in general higher in the samples from Tromsø (8-46%), compared to Bergen (2-16%) and in the samples from Bergen the growth on kanamycin (12-62%) was higher compared to the samples from Tromsø (6-9%). For the other groups of antibiotics the percentage of growth on antibiotic containing media ranged between 3-42%.

For the determination of aerobic resistant bacteria, 20 µg/ml concentration of each specific antibiotic was used in the agar plates. The selected concentration was based on previous environmental studies, where culture-based studies of antimicrobial resistance in soil bacteria have defined antimicrobial resistance as growth at 20 µg/ml (Bhullar et al., 2012; Novo et al., 2013; Smith, Hiney, & Samuelsen, 1994; R. Szczepanowski et al., 2009; Walsh, 2013a, 2013b). This definition is based on the use of 20 µg/ml as the breakpoint concentration in the initial soil resistome study of *Streptomyces* species. The definition is used for all bacteria and all classes of antibiotics (Walsh, 2013a). The antibiotic breakpoint for each of the isolates in our study is not defined and the use of 20 µg/ml as the breakpoint concentration for non-identified species has some limitations. In the published literature, a variety of concentrations have been used on different environmental samples, and the rationale for their use has rarely been presented. In general, it is well known that culture-dependent methods haves some limitations and will underrepresent the microbial diversity in most environments. Culture-based methods are only able to sample a small fraction, perhaps as little as 1% of the total microbial diversity is soil (McLain, Cytryn, Durso, & Young, 2016; Munck et al., 2015; Wright, 2010) and 5-10 % in WWTPs (Kummerer, 2004). In studies on sediments it has been estimated that approximately only 0.3% of the total microbial diversity are cultivable (Samuelsen et al., 1992). This low percentage will not reflect the real variability or the actual amount of the ARB or ARGs. New genetically based methods have been developed which in combination with cultivation based methods, gives more information.

Detection of target resistance genes in resistant bacterial isolates from WWTPs samples

Polymerase chain reaction (PCR) was applied to detect target ARGs in bacteria identified as resistant. The classes of the selected genes are representatives from most all classes of antibiotics used in this study. PCR results were positive for resistance genes known to confer resistance to beta-lactams, aminoglycosides, tetracycline, macrolides, trimethoprim as well as sulfonamides in a few of the tested colonies.

For the group of beta-lactams, two specific ARGs were tested for. The proportion of bla_{TEM} genes in the culturable amp^r isolates was < 3% in the samples from Tromsø and the percentage of *mecA* genes were < 7%. Amx^R isolates as well as dxc^R isolates were also tested for the precence of bla_{TEM} and *mecA* gene and < 1% of the cultivable amx^R isolates were positive for bla_{TEM} and ~7% were positive for *mecA*. For the dxc^R colonies we could not detect the target genes. Bla_{TEM} has been described as one of the most clinically important ARGs and a few studies have determined the distribution of this gene in WWTPs as well as potential natural environments (Brusetti et al., 2008; Di Cesare et al., 2016; Gilliver,

Bennett, Begon, Hazel, & Hart, 1999; Glad, Bernhardsen, et al., 2010; Glad, Kristiansen, et al., 2010; Osterblad, Norrdahl, Korpimaki, & Huovinen, 2001; Rafraf et al., 2016; Volkmann et al., 2004). Bla_{TEM} has previously been detected in samples from WWTPs (Di Cesare et al., 2016; Rafraf et al., 2016; Volkmann, Schwartz, Kirchen, Stofer, & Obst, 2007). It is well known that the *mecA* gene is widely distributed in *Staphylococcus* strains and our results demonstrates the presence of *mecA* in a limited number of bacterial isolates from WWTPs. In a study by Volkmann et al (2004) the *mecA* gene was not detected, compared to a study by Börjesson et al (2009) were the *mecA* gene was detected in samples from WWTPs.

For the group of aminoglycosides, three different ARGs were tested for. We were not able to detect the *aph(3`)-IIa* gene in any of the colonies. The *aph(3`)-IIa* gene, encodes the neomycin phosphotransferase enzyme which confers resistance to the aminoglycoside antibiotics neomycin and kanamycin. This is the most commonly used antimicrobial resistance marker gene (ARMG) in genetically modified (GM) plants (Goldstein et al., 2005; Ramessar et al., 2007; Rosellini, 2012). Only a few studies have investigated the prevalence of *aph(3`)-IIa* genes in the environment (Leff, Dana, McArthur, & Shimkets, 1993; Ma, Blackshaw, Roy, & He, 2011; Nordgård, 2016; Smalla, van Overbeek, Pukall, & van Elsas, 1993; Woegerbauer et al., 2015). These studies show only a very low prevalence of *aph(3`)-IIa* genes among bacteria in non-clinical environments like soil, river water, sewage and manure. *Aph(3`)-IIIa* was selected for prevalence analysis due to its reported status as an abundant aminoglycoside phosphotransferase gene in clinical settings with broad substrate-inactivation spectrum, including amikacin (Becker & Cooper, 2013; Shaw, Rather, Hare, & Miller, 1993; Woegerbauer et al., 2015; Woegerbauer et al., 2014). Of the 483 colonies tested, approximately 4% were positive for the *aph(3`)-IIIa gene*. Gene prevalence data for *aph(3`)-IIIa* are rare and Woegerbauer and colleagues has reported that this genes were moderately prevalent in a pool of clinically important pathogens (Woegerbauer et al., 2015). In a study by Nordgård et al (2016) different environmental samples were investigated for the prevalence of *aph(3`)-IIIa,* it was not detected (Nordgård, 2016). The *aac(6`)/aph(2``)* is wide spread and conserved throughout different bacterial groups and encodes enzymes inactivation most aminoglycosides (Fluit, Visser, & Schmitz, 2001). Of 448 colonies screened for this gene, 2% were positive. The prevalence of *aac(6`)/aph(2``)*have previously been reported in samples connected to WWTPs and other environments (Borjesson et al., 2009; Heuer et al., 2002).

In this study we found that approximately 20% of the colonies screened for *tetA* were positive. *TetA* is commonly found on plasmids in a wide range of gram-negative bacteria (Chopra & Roberts, 2001), and encodes resistance to efflux pumps. *TetA* has shown to be more common in different types of waste water samples and other environmental samples compared to other tetracycline resistance mechanisms (Aubertheau et al., 2017; Berglund et al., 2015; Borjesson et al., 2009; Borjesson, Mattsson, Lindgren, & Lindgren, 2010)..

After re-streaking the bacteria that were picked form the media supplemented with ciprofloxacin, we were not able to see any growth after five days and no colonies were therefor screened for *qnrS*. However, previous studies have demonstrated both the absence of and the occurrence of the *qnrS* gene in wastewater samples (Berglund et al., 2015; B. Berglund, G. A. Khan, R. Lindberg, et al., 2014; Björn Berglund et al., 2014; Proia et al., 2016).

The *ermB* gene was detected in 4% of the colonies screened. The *ermB* often reside on mobile genetic elements and is harbored by gram positive bacteria (Di Cesare et al., 2016). The prevalence of this gene

has been described in many environmental samples as well as samples connected to WWTPs (J. Chen, Z. Yu, F. C. Michel, Jr., T. Wittum, & M. Morrison, 2007; Chen et al., 2016).

Of the trimethoprim resistant colonies, the *drfA1* gene was detected in 25%. Several studies has shown that trimethoprim (as well as fluoroquinolones and sulfonamides), are poorly removed during waste water treatment processes, and genes conferring resistance to these classes of antibiotics has been shown to be prevalent in samples connected to WWTPs (R. Szczepanowski et al., 2009). There are more than 30 known *dfr* genes, and a few types seems to predominate in most parts of the world. Especially the *dfrA1* predominates in some studies of large collections of bacteria (M. Grape, A. Motakefi, S. Pavuluri, & G. Kahlmeter, 2007).

Among the bacteria growing on sulfamethoxazole, 1,3% were positive for *sulI*. This resistance gene has been found in gram negative bacteria (Sköld, 2000) in animals and humans and environmental samples such as natural water, animal and human wastewaters, river sediments and other areas (Pei, Kim, Carlson, & Pruden, 2006). *SulI* is often associated with *intI1* which could contribute to its dissemination (Laht et al., 2014; Mazel, 2006; Wang, Ben, Yang, Zhang, & Qiang, 2016).

The selection of the different ARGs targeted by PCR (*blaTEM, mecA, qnrS, ermB, aph(3`)-IIa, aph(3`)-IIIa, aac(6`)/aph(2``)sulI, tetA, and dfrA1)* was done based in previous studies according to their clinical and environmental relevance (B. Berglund, G. A. Khan, S. E. Weisner, et al., 2014; Di Cesare et al., 2016). The classes of the selected genes are representatives from all classes of antibiotics used in this study. Only a limited number of genes was tested due to a limited project period. Many resistance genes can confer resistance to the same and different classes of antibiotics and it can be difficult to decide which genes to screen for in large collections of isolates. This might imply that the selected ARGs may not represent the major resistance determinants among the bacteria within the samples. As an example, in recent years at least 39 different genes encoding resistance for tetracycline, and more than 30 classes of different *erm* genes that encodes resistance for macrolides has been identified and detected in different environmental samples (J. Chen et al., 2007; Chopra & Roberts, 2001; Wen, Yang, Duan, & Chen, 2016). Another reason for failure to detect specific ARGs in the resistant colonies may be because some bacteria are intrinsically resistant to the particular antibiotic. This means they have either an impermeable membrane or they lack the antibiotic target (Proia et al., 2016). However, in a follow up study, other relevant ARGs could be included for testing. To verify the identity of the PCR products obtained in the analysis described above it will important to follow up randomly selected amplicons for sequencing. Sequencing data can describe the exact nucleotide sequence of the resistance gene.

PCR based detection of target genes in total DNA isolated from sample

The overall ARG occurence was determined by ARGs specific primers of DNA extracted directly from the samples. Given the difficulties in cultivating most of the bacteria in the environment, DNA based techniques, especially PCR, is a preferred technique for examination of resistance genes present in total DNA extracted from environmental samples. In our study, the yield of the total DNA extracted from the WWTPs samples from Tromsø and Bergen differed. The yield of total DNA was lower in the samples from Bergen compared to the samples from Tromsø, and in addition the detection of 16S r RNA in the samples from Bergen was unsuccessful. The sample material is different and there were

substances that inhibited the PCR present in the eluated DNA from Bergen, since the 16S rRNA PCR failed. These samples were not analyzed by the ARG specific PCRs.

However, 5 out 10 selected ARGs (*bla*$_{TEM}$, *aph(3`)-IIIa, aac(6`)/aph(2``), qnrS* and *ermB*) were detected in the total DNA in samples from two of the WWTPs in Tromsø (Hamna and Breivika) and 3 out of 10 selected ARGs (*aph(3`)-IIIa, aac(6`)/aph(2``)* and *qnrS*) were detected in the samples from Strandveien. As with the positive PCRs of the total DNA isolated from bacterial isolates, the positive results need to be verified by sequencing. Our results do not say anything about the quantities of the different ARGs. This can be followed up by a quantitative PCR analysis. A quantitative PCR would add more information about the extent of the total ARG reservoir in the different WWTP samples.

Metagenomic analysis

Metagenomics is a modern approach that overcome the challenges and limitations of culture-dependent methods and amplification (Schmieder & Edwards, 2012). By this approach direct genetic analysis of genomes contained with environmental samples is possible (Franzosa et al., 2015; T. Thomas, Gilbert, & Meyer, 2012). In our samples, some DNA eluates had a yellow tint, presumably due to co-purifying contaminants. Spin-column purification after RNAse treatment eliminated most colored substances from DNA preparations, however Illumina MiSeq sequencing library preparation was still unsuccessful for 3 of 11 samples from Tromsø and Bergen. The NSC could not explain the reason for this difficulty but did speculate that persistence of co-purifying contaminants (guanidinium salts, humic substances) might be a plausible explanation. This report therefore does not contain metagenome sequence information from Hamna (Tromsø), Breivika (Tromsø) and Lyr 7 (Bergen), as library preparation for these samples did not succeed within the limited time frame of this project. The source of contamination, such that it could be reduced or removed, was not identified during the project period. URE has, however, procured the remaining DNA samples from the NSC and will test various purification methods on them with the express goal of completing metagenomic sequence analysis for all samples. This finding provides important information about the challenges of purifying DNA from “dirty” environmental samples, and highlights the potential need for additional purification steps when molecular analytical tools are applied downstream. Co-purifying contaminants that challenge sequencing library preparation may also have inhibitory effects on other types of direct downstream molecular analyses of sediment DNA, such as PCR and quantitative PCR. Appropriate controls to test for and if possible, mitigate, the effects of contaminants are strongly advised.

We observed low-abundance reads that did not map to contigs in the metagenomes from Byfjorden. This may be a consequence of artefacts that occur during metagenome library preparation in the laboratory, or they may arise as a consequence of the choice of bioinformatic tool used for metagenome assembly. For example, some assembly programs have been designed specifically to tackle the informational complexity of highly uneven sediment metagenomes (Alneberg et al., 2014; Howe et al., 2014; Peng, Leung, Yiu, & Chin, 2012). The noteworthy loss of sequence information during the assembly process (reads not mapped to contigs are not included in the “finished” metagenomes) employed in this report indicates that optimization is advisable in order to maximize the informational content of the metagenomes analyzed, thereby increasing the likelihood of identification of AMR genes.

From the results, untreated sewage solids (Strandveien) was shown to have higher AMR gene prevalence than surface fjord sediments (Byfjorden). The Strandvegen metagenome contains a higher prevalence of AMR and AMR-like genes compared with the Byfjorden metagenomes (Table 1, Appendix C). Furthermore, the only "Perfect" AMR genes identified in the metagenomes generated for this report occurred in the Strandvegen metagenome. As the Strandvegen sample uniquely consisted of untreated sewage solids, the (human gut microbiota) microbial biomass in this sample is higher than in the Byfjorden samples, which consisted of marine surface sediments. Indeed, the highest DNA recovery was observed for the Strandvegen sample (Table 6). A previous study of soil bacterial resistomes found that the AMR potential observed was highly correlated with the taxonomic content of the soils analyzed (Forsberg et al., 2014). The presumably higher load of human gut microbiota in the Strandvegen sample may provide a partial explanation for the higher occurence and diversity of AMR and AMR-like genes in this sample (see heatmap of RGI results for the Strandvegen metagenome in Fig. 4). Without an investigation of the taxonomic content of metagenomes, however, an assessment of the reasons for the higher AMR content of the Strandvegen sample would be speculative.

The majority of AMR genes and AMR functional types in the analyzed metagenomes were genes encoding antimicrobial efflux pumps represent. Of the 110 matches to the CARD identified by the RGI tool, 99 matches encoded genes in the *mex* family of efflux pumps. Although these genes have been associated with clinically-relevant resistance to a broad spectrum of antibiotics and antimicrobials, they may also function to provide resistance to naturally-produced antagonistic compounds or even promote persistence of bacteria during colonization of host organisms (Piddock, 2006). The same study goes on to suggest that efflux pumps may actually play an important role in bacterial pathogenesis (Piddock 2006). The near universal presence of this class of AMR gene in the present study suggests that efflux pumps play an important role in both human and environmental microbiology.

As the individual sediment metagenomes were assembled separately, i.e. reads in one metagenome sample were only compared with other reads within the same metagenome sample, the degree of sequence "overlap" between metagenome samples is uncharacterized. It is therefore not possible to speculate about overlapping AMR gene content between the different samples analyzed. We did not observe a relationship between strength of RGI match ("Perfect" or "Strict" matches) and mean contig coverage. The range of mean coverage depth for all contigs to which RGI matches were identified (N =110) was 1.36 - 140.9 with a median of 5.80.

One critical aspect of AMR and its importance for human health is the risk of horizontal gene transfer of AMR genes between bacteria. The localization of AMR genes on mobile genetic elements, for example plasmids or transposons, may enhance rates of transfer and thus spread of AMR in bacterial assemblages. Localization of the identified AMR genes, i.e. whether they are chromosomally located or located on mobile genetic elements, falls outside the scope of the present study. A more extensive metagenomic analysis of AMR genes might however include the implementation of bioinformatic tools designed to assess whether AMR genes bear DNA signatures that may indicate taxonomic origin (e.g. (Chor, Horn, Goldman, Levy, & Massingham, 2009; Forsberg et al., 2014). Further studies utilizing a metagenomic approach might also include a supervised assembly process, i.e. assembly using reference genomes as scaffolds for contig formation. This may also increase knowledge about localization of identified AMR genes through comparison to full genomic complement, including plasmids and extrachromosomal elements, of known bacterial vectors of AMR genes.

5. Limitations

There are many limitations/uncertainties related to environmental studies and antimicrobial resistance (AMR). Many of the uncertainties arise due to technical limitations, data limitations and lack of standardized protocols. In this study, some of the listed limitations are also due to the limited project period and can easily be followed up. This means that this study represents a snapshot of the environments tested, and cannot provide all details. Moreover, resistance patterns are expected to change over time, and no "true" stable value can be expected that will be robust to scale and time.

- Cultivation based approaches will only recover a small proportion of bacterial species from specific environments/samples. In this study, the nutrient medium and other growth conditions including temperature can probably be optimized to improve the growth of the bacteria especially from the sediment samples.

- Isolation of total DNA for PCR and metagenomic sequencing may need optimization. Low yields, discoloration, and issues with amplification suggest the presence of inhibitors which may have reduced the probability of detecting genes present in those samples. Both the methods of DNA isolation and PCR conditions with this DNA require more optimization, especially from the sediment samples.

- Positive resistance-gene-specific amplicons has not been confirmed due to time constraint by sequencing, and can be included in follow up studies.

- The identity of the different bacterial isolates has not been characterized. In a follow up study, identification of the isolates where ARG amplicons have been confirmed should be done by sequencing and the minimum inhibitory concentrations (MIC) for specific ABs should be determined.

- Our results do not attempt to determine the relative abundance of the different ARGs. This can be followed up by a quantitative PCR. A quantitative PCR would add more information about the total ARG reservoir in the different WWTP samples.

- In the metagenomic part, loss of sequence information during the assembly process indicates that further optimization is advisable to maximize the informational content of the metagenomes analyzed, thereby increasing the likelihood of identification of AMR genes.

- RGI presents only results from perfect and strict hits. RGI can also be used for discovery of new AMR genes with lower amino acid similarity to know AMR genes by activating lower-threshold "loose" matches. In this study, "loose" matches were not included as these datasets were cumbersome and difficult to visually analyze. For identification of emerging or novel AMR, the acceptance of "loose" matches to the CARD may be an appropriate analysis tool.

- It is advisable to determine the occurrence of key antibiotics, biocides and heavy metals in the samples from the studied environments. Such information can give us insight into the type of selective pressure the microorganisms may be facing.

- It is difficult to compare the outcome of the study with several other published studies because different methods are applied and there is limited standardization within the field.

6. Follow up, recommendation

There are new knowledge gaps, as well as uncertainties, emerging from the investigation of ARB and ARG in natural environments. With the limited scope and number of experimental studies available to resolve remaining uncertainties regarding ARB and ARG in the environment, we suggest some areas that are of specific importance to follow up in further studies. These studies should include:

- **Experimental limitations and reducible uncertainties** from the completed project should be followed up to enable stronger conclusions to be drawn.

- **WWTPs**: Additional research in needed to further characterize the ARB in municipal wastewater as well as the ability the different treatment processes entails to eliminate these bacteria.

- **Selectors and co-selectors for resistance development**: Among the chemical substances that are known to contribute to the development of resistance to antimicrobial are disinfectants, biocides, pesticides and heavy metals. Even very low environmental levels of antimicrobial residues derived from human usage, may exert selection pressure and increase the risks of antimicrobial resistance (AMR) in the environment. More knowledge about the presence and quantities of different classes of antibiotics and other substances that are known as co-selectors for resistance is needed.

- **Prevalence of antibiotic resistance in different environments**: More information is needed on the prevalence of ARB and ARG in environments with different exposure to human activities.

- **Origin of ARG:** Further studies are needed to assess the origins of detected ARGs to determine whether transfer and selection for ARGs occurs in the wastewater treatment process or whether the ARGs seen in the recipient waste comes from other sources (e.g. hospitals).

- **Possibility for spread of resistance from the environment to pathogenic bacteria**: At present it is not clear to what extent environmental ARB and ARG promote the acquisition and spread of antibiotic resistance among clinically relevant bacteria, or whether ARGs that are acquired by both clinically relevant bacteria and strictly environmental bacteria originate from the same reservoir (Berendonk et al., 2015).

7. Concluding remarks

This study examined the occurrence of ARB and ARGs in samples connected to different WWTPs and WTP. The sampling points represent areas with different levels of exposure to human activities and industrial influences that might have an impact on the development of ARB and ARGs.

The main conclusions that can be drawn from our study are:

- Different ARB and ARGs can be found in samples connected to different WWTPs. Both culture-dependent and culture-independent methods the results indicates that the bacteria in the samples are resistant to different antibiotics.

- The metagenomics data indicates a much higher occurrence of AMR and AMR-like genes in the untreated samples from Tromsø compared with the Byfjorden metagenomes. The majority of AMR genes and AMR functional types were encoding antimicrobial efflux pumps.

- The combination of the different approaches in this study, culture-based or molecular based, clearly demonstrates advantages and drawbacks for each approach, which highlights the importance of combining different tools for characterizing ARB and ARG in different environments.

- Given the risk posed by ARGs in the environment, further research on ARGs distribution in selected area should be conducted.

According to the reviewed studies referred to in this report:

- There is still a lack of data from environmental hot spots like, WWTPs, on the prevalence and fate of ARB and ARGs.

- The available studies comes from “small” research studies and not long-term monitoring programs.

- Cultivation conditions, DNA extraction methods, targeted resistance phenotypes and genotypes or primers sets used in different environmental studies varies and make it difficult to compare data. More standardized guidelines for resistance testing in different environments would enable comparison between different environmental studies worldwide more easily.

- Bacteria in nature can be a source of resistance, which can spread to pathogenic bacteria in clinical environments. However, how this occurs and to which extent is still not clear.

As a final concluding remark, the results demonstrate a snapshot of the prevalence of ARB and ARG in the selected environments. The results might indicate that environments outside the clinical settings also possibly plays an important role in the dissemination of antibiotic resistance. It is also important to highlight that the presented data in this report are limited scale and time and continued research is needed to fully understand the occurrence and development of antibiotic resistance in samples connected to WWTPs and other natural environments in Norway.

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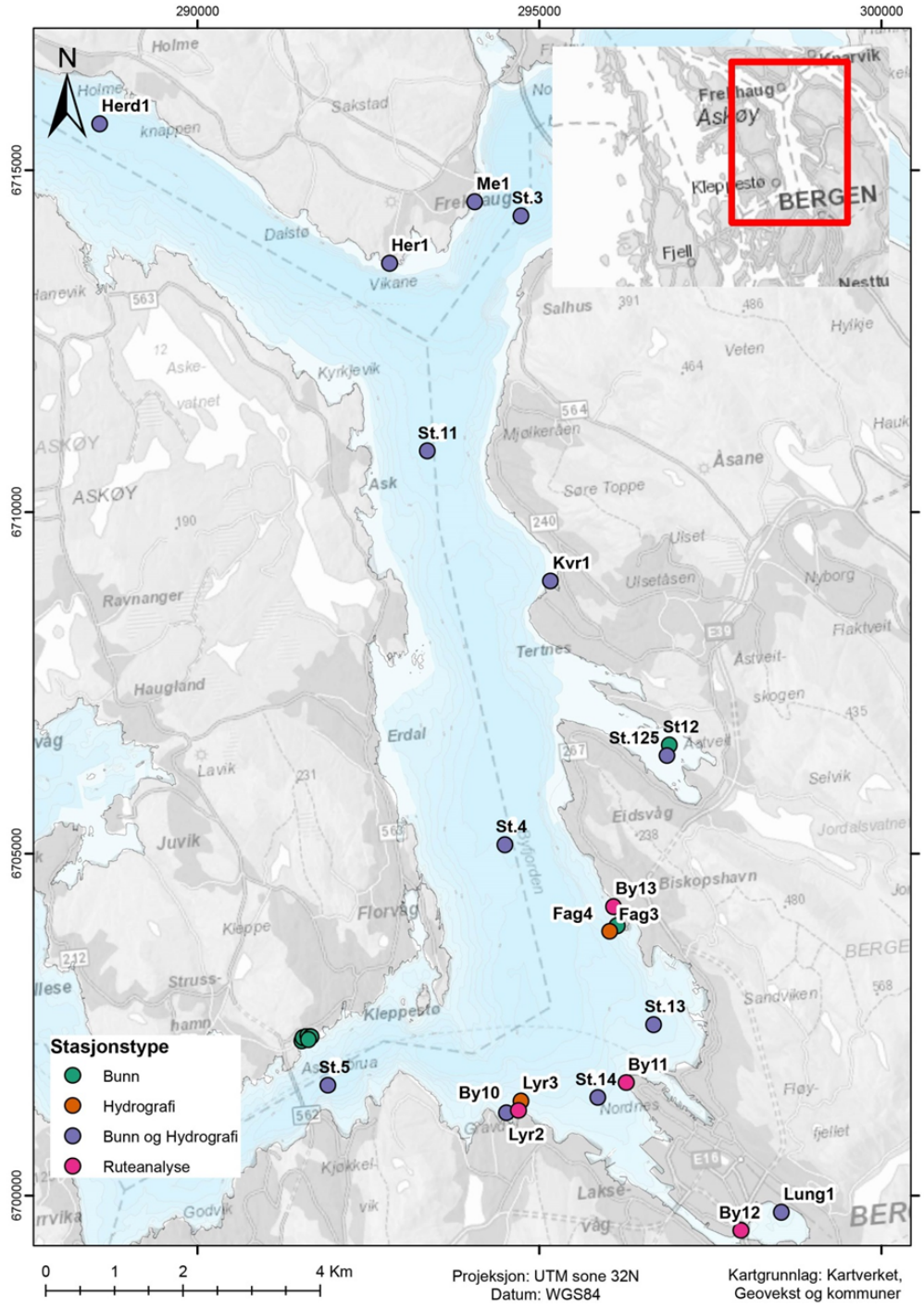
Appendix A. Map

Figure 1: Map of the sampling area in Byfjorden in Bergen



Appendix B: Primers and controls

Table 1: PCR conditions, primers and controls used in this study

AB	Gene	Primer sequence (5`-3`)	Annealing temp (°C)/sek	Elongation temp (°C)/sek	Amplicon size (bp)	Controls/Accession number	Reference
AMP	*mecA*	F: AAA AAG ATG GCA AAG ATA TTC AA R: TTC TTC GTT ACT CAT GCC ATA CA	56		185	KC243783.1 (McArthur et al., 2013)	(Rafael Szczepanowski et al., 2009)
AMX	*BlaTem*	F. CAT TTC CGT GTC GCC CTT ATT CC R: GGC ACC TAT CTC AGC GAT CTG TCT A	61/30	72/45	828	pGEM32F IHB101 B	(Ehlers et al., 1997)
DXC							
KM	*aph(3`)-IIa*	F: ATGATTGAACAAGATGGATTGC R: TCAGAAGAACTCGTCAAGAAGG	60/30	72/40	795	*A. baylyi* BD413 JV28-Km^R	(Woegerbauer et al., 2014)
	aph(3`)-IIIa	F: ATGGCTAAAATGAGAATATCACCG R: CTAAAACAATTCATCCAGTAAAATATAA	60/30	72/40	795	*A. baylyi* ADP1200Com+Km_+	
STP	*aac(6')-Ie + aph(2'')*	F: CATTATACAGAGCCTTGGGAAGA R: GCCCTCGTGTAATTCATGtTC	55/30	72/45	364	NC_005024.1 (McArthur et al., 2013)	(Börjesson et al., 2009)
TET	*tetA*	F: CCT GAT TAT GCC GGT GCT R: TGG CGT AGT CGA CAG CAG	63/30	72/30	200	NC_004840 (removed), X61367 (Ng, Martin, Alfa, & Mulvey, 2001)	(Rafael Szczepanowski et al., 2009)
CIP	*qnrS*	F: ATCAAGTGAGTAATCGTATGTACT R: CACCTCGACTTAAGTCTGAC	61		171	DQ485529.1 (Gay et al., 2006)	(Björn Berglund et al., 2014)
ERI	*Erm(B)*	F: GATACCGTTTACGAAATGG R: GAATCGAGACTTGAGTGTGC	58		364	K00551.1 (McArthur et al., 2013)	(J. Chen, Z. Yu, F. C. Michel, T. Wittum, & M. Morrison, 2007)
TMT	*dfrA1*	F: ATGGAGTGCCAAAGGTGAAC R: TATCTCCCCACCACCTGAAA	63/30	72/30	241	AJ400733.1 (McArthur et al., 2013)	(M Grape, A Motakefi, S Pavuluri, & G Kahlmeter, 2007)
SUL	*sulI*	F: GACGAGATTGTGCGGTTCTT R: GAGACCAATAGCGGAAGCC	63/30	72/30	185	JF969163.1 (McArthur et al., 2013)	(Rafael Szczepanowski et al., 2009)

Appendix C: Metagenomics results: RGI raw data

Table 1: RGI raw data is available at: http://genok.no/appendix-c-metagenomics-results-rgi-raw-data/