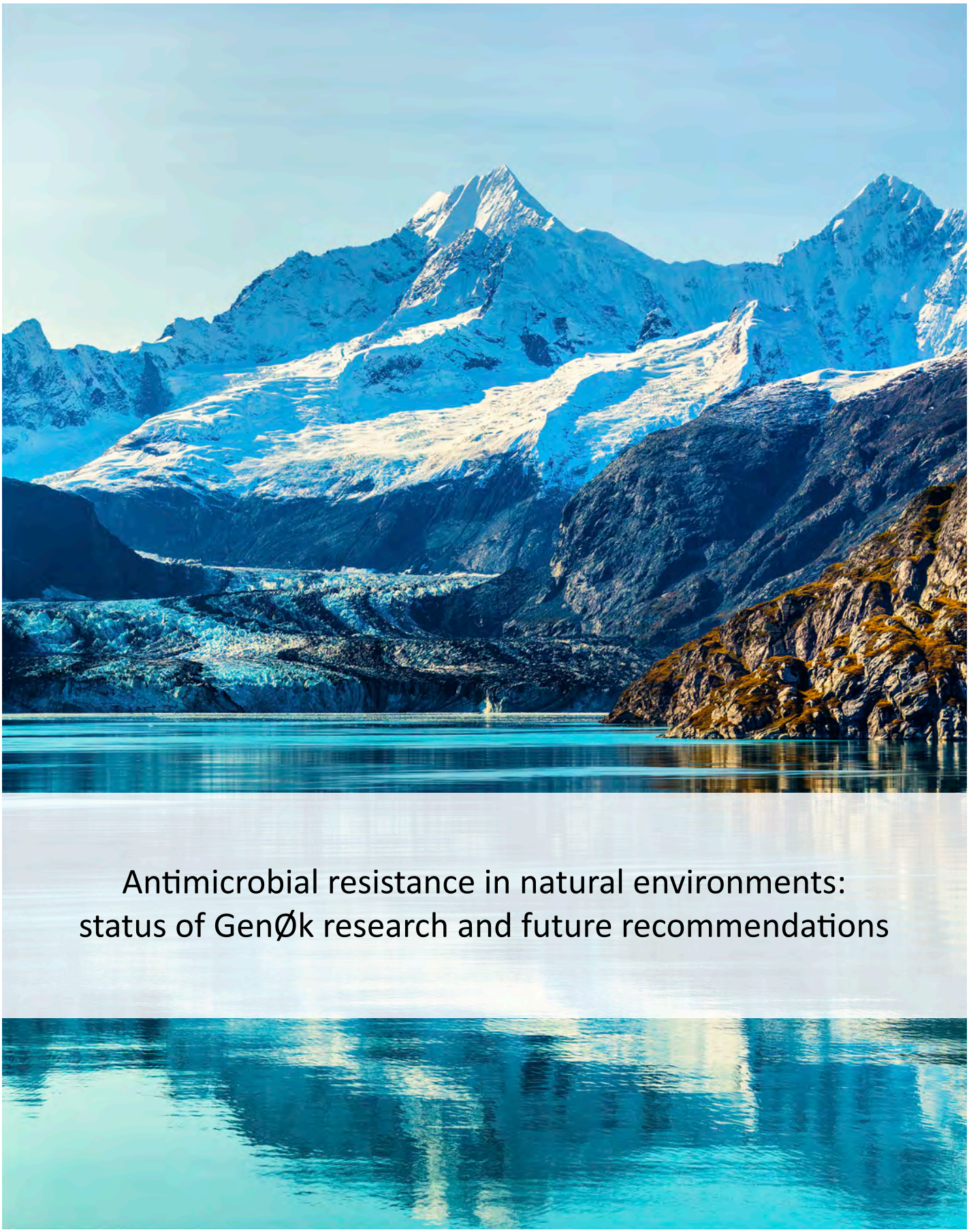

Antimicrobial resistance in natural environments: status of GenØk research and future recommendations

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Summary

At the request of The Norwegian Environment Agency, this report briefly discusses and sums up GenØk's activities and experiences by screening environmental samples, from sediments, soils, manure and from terrestrial and aquatic animals, for antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARG). The progression from culture based to molecular based methods to direct PCR/sequencing and metagenomic sequencing, discusses and exemplifies the strengths and weaknesses of these different methods. Culture based methods have limitations for in depth comprehensive analysis, and even though direct PCR may help overcome these, full metagenomic sequencing, possibly combined with direct gene sequencing/detection, have emerged as preferred methods for environmental monitoring of antibiotic resistance genes. However, in order to understand the dynamics of ARGs in microbial populations, it is necessary to develop lacking standardization of sampling procedures, methods for how to measure chemical (antibiotic/other drivers) impact, and initiate studies on how to describe genetic context (plasmids etc). There is an emerging indication that biofilm formation and dynamics also play a role in dissemination of ARGs.

Introduction

Antimicrobial resistance (AMR), particularly antibiotic resistance among bacteria, is globally recognized as a threat to modern medicine, and thus a threat to human and animal health (World Health Organization, 2015). There is increasing appreciation for the fact that the environment plays a dynamic role in the issue of antibiotic resistance. It has become clear that the environment is both a source and a recipient of antimicrobial resistance genes (ARGs) and antimicrobial resistant bacteria (ARB). The national strategy of the Norwegian government against antibiotic resistance for 2015-2020 highlights that this issue must be considered in a holistic perspective. It is aligned with the One Health principle, and acknowledges that human and animal health and the environment interact and must be seen in context to each other. The identification and monitoring of the presence of ARB and ARGs 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 a need for more knowledge about ARB and ARGs in different natural environments in Norway.

Status of research at GenØk

GenØk has in the period from 2016 to 2019 investigated different environments in Norway with the aim of describing AMR and ARGs. The findings have been published in 5 reports.

Report 1 (2016): Prevalence of antibiotic resistance marker genes (ARMG) in selected environments in Norway.

In 2016, we focused on two antibiotic resistance genes, neomycin phosphotransferase II (*nptII*) and neomycin phosphotransferase III (*nptIII*), which both confer resistance to the aminoglycosides kanamycin and neomycin. *nptIII* also confers resistance to amikacin, lividomycin and isepamicin, which are deemed highly relevant for human therapy by WHO. In addition to being used in human and veterinary medicine, kanamycin also has a history of use in the production of genetically modified organisms (GMOs). Some GM plants harbor the *nptII* gene as a marker gene to be used under the modification process. This implies that there may be a probability for transfer to soil bacteria if such a GM plants is approved for cultivation.

One of the main gaps of knowledge, has been the understanding of how the resistance profile of *nptII* are in the receiving environment. If the frequency of *nptII* genes already is high in soil, then introducing the gene through GM crops, may be lower than if the opposite was true.

We used both culture-based and molecular methods (PCR) to investigate different environments in Norway to get a better understanding of the reservoirs of *nptII/III* resistance. The samples were collected from grassland and from pig farms in Tromsø municipality. Soil and faeces were sampled and bacteria were recovered and tested for phenotypical resistance against kanamycin. In addition, total DNA were extracted from soil and tested with PCR for presence of *nptII/III*. Phenotypic resistance against kanamycin grown at 100 ug/ml, ranged from 0,4 to 14-15%, but PCR with several different sets of primers did not confirm the presence of either *nptII* or *nptIII* in the phenotypic kanamycin resistant colonies. Also, PCR on total DNA failed to detect the *nptII/III* genes.

In our study, we investigated different fields with different exposure of organic fertilizers (manure), to see if it could provide condition that could favour bacteria harbouring *nptII/nptIII* genes. Concentrations of antibiotics were not determined in our soil or faeces samples, but a strong selection pressure can be assumed to be absent given the limited use of aminoglycosides in Norway (NORM/NORM-VET, 2015). We could not observe a difference between the different samples sites as Woegerbauer and colleagues (2015) did in a similar study in Austria.

Report 2 (2017): Antimicrobial resistance in selected environments in Norway. Occurrence of antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARG) associated with wastewater treatment plants (WWTPs).

Other than manure, low levels of antibiotics can be expected from runoff from usage in human and veterinary medicine. Sewage, treated at wastewater treatment facilities are of particular concern since they create an interface where environmental bacteria, antibiotics, pathogens, co-selectors and resistant genes are mixed together.

In 2017, GenØk published a report investigating the pool of resistant genes at two wastewater treatment facilitates, one in Bergen and one in Tromsø. Similarly to the first report from 2016, this study was a combination of culture based and non-culture based methods, but PCR detection was expanded by much more comprehensive metagenomic analyses. Additionally, the range of antibiotics tested was much larger then compared to the previous study.

	GenØk 2016	GenØk 2017
Antibiotics tested by growing	1	10
Types of samples	Soil and manure	Bottom sediments and sludge
Molecular methods	Gene specific PCR	Gene specific PCR and metagenome
Levels of AB during cultivation	100 ug/ml	20 ug/ml

In summary, the culture-based approach revealed phenotypic resistance to all tested antibiotics. Gene specific PCR directly on a selection of resistant colonies did reveal the specific resistant genes tested for in a varying number of colonies. Only three antibiotic resistant phenotypes were not confirmed by PCR.

The samples have also undergone metagenomic analyses which is part of a submitted publication including samples from fresh water. This report will be updated after publication of that study.

From these two studies (report 1 and 2), it is clear that phenotypic resistance does not necessarily correspond to the expected genetic composition. The vast majority of phenotypic resistant colonies tested, could not be explained by the known genetic resistant mechanism. This is maybe not unexpected, but clearly show the usefulness and necessity of molecular verification of resistant mechanism in order to understand the resistome (i.e. pool of resistant genes) makeup.

Report 3 (2017): Prevalence of antibiotic resistance marker genes (ARMG) in selected environments in Norway – Reindeer.

Free roaming domesticated animals, such as reindeers, are interesting subjects for mapping the antimicrobial resistant pool. The reason is that they may be exposed to varying degrees of human activity and the environment in a unique way, providing both input of human activities as well as long term exposure to the natural environment through migration and natural feeding/grazing. In 2017, we published a report from a 3 years study, screening 76 faecal samples from reindeers collected from 3 different reindeer grazing areas. For this study, total DNA was extracted from the faecal samples and analysed with gene specific PCR with focus on *nptII/III* (kanamycin resistance).

The different grazing areas were selected based on different interface with humans, with Tønsvika in closest proximity to human activities (settlements, tourists, industry), Røros as intermediate contact and Varangerhalvøya as the most remote grazing area. Over the course of three years, 76 samples were analysed with two different primer sets for the nptII and nptIII genes. We did not detect the *nptII* gene in any samples, but we detected three positive samples for the *nptIII* gene.

As with many other published studies on environmental antimicrobial resistance, the sample size in our study is limited. Still, even with only 76 sample tested, we did discover the *nptIII* gene in faeces of 3 reindeers. As discussed in the report, *nptIII* has been found previously, most notably in wild birds from Poland and wild animals from Spain.

Report 4 (2019): Antimicrobial resistance in the marine environment: MIC profiles of bacteria isolated from whale and seal faeces

Migratory animals are of particular interest as reservoirs for antimicrobial resistant bacteria and genes because they may play a role as vector for spreading these bacteria and genes between different environments. Minke whales are examples of such highly mobile animals that, due to its marine environment, are exposed to antibiotic resistance drivers released into the oceans (ex: heavy metals, antibiotics) and covers vast distances in its migratory pattern. Since it is likely that antimicrobial resistance has its origin in environmental reservoirs, these animals are very relevant for mapping the resistome.

The chosen method for this study was cultivating bacteria for determining the MIC (minimum inhibitory concentration). With this approach, 600 isolates (10 pr. Sample) from 55 whales and 5 seals were tested for MIC against 8 antibiotics. In addition, all 600 isolates were screened with PCR for the presence of resistance genes against the antibiotics tested. The results show several phenotypic

resistance profiles, some with quite high MIC values, but with low correspondence to the tested resistance genes. Since we only tested one resistant gene for each antibiotic, this could be explained by the presence of other resistance genes than the one we chose. Also, due to the low number of isolates tested (only 10 from each animal), the picture is far from complete as an overview of the resistome.

Nevertheless, in the total sample collection, the PCR approach detected all resistance genes except *nptII* (kanamycin resistance). In particular, the number of isolates with a positive result for *vanA* (Vancomycin) is surprisingly high when compared to the other antibiotic resistance genes. 81 out of 600 samples contained a PCR product corresponding to the correct size for *vanA*. Vancomycin is a last resort, broad-spectrum antibiotic, and resistance against vancomycin are therefor of particular concern. We found that only seal samples showed a high phenotypic resistance to vancomycin, which, due to the low number of individual animals (five), should be interpreted with caution.

Gene	Number of amplicons (Whale isolates)	Number of amplicons (Seal isolates)	Total
TetA	5	0	5
mecA	10	1	11
nptII	0	0	0
Erm(B)	2	0	2
qnrS	1	0	1
dfrA1	5	5	10
VanA	54	27	81
acrB	7	27	34
mexD	3	12	15

Table 1: Summary of PCR results after amplification with primers targeting selected ARGs

Report 5 (2020): Antimicrobial resistance in marine mammals: Targeted PCR and metagenomic analysis

Given the difficulties of cultivating a representative population of bacteria from any environmental source, molecular methods such as PCR of total DNA or metagenomic analyses would give a more complete overview over antimicrobial resistance genes. We used targeted PCR (i.e. used PCR primers targeting a predetermined set of resistance genes) on ten whale samples targeting known resistance mechanisms. We found a that all of the individual whale samples were positive for one of the 14 tested

ARGs, all in the category of efflux pumps. Unfortunately, *vanA* coding for vancomycin resistance, was not tested.

Because this testing method is a PCR based approach, we have no information on the sequence of the PCR products, nor the abundance of these genes. Further work on sequencing the PCR products and qPCR (quantitative PCR) must be undertaken to understand the abundance of resistance genes and verify the identity of such genes.

We then proceeded to do a full metagenomic sequencing of the total DNA from eight of the whale samples. We chose an Illumina based metagenomic approach, which gives great coverage but requires assembly due to short sequence reads. The assembled metagenomes gave results for 237 matches to sequences with 75% homology to reference sequence for resistance genes. It is noteworthy to mention that several variants of genes coding for vancomycin resistance was discovered. These results also underline another advantage with metagenomic approaches vs qPCR/targeted PCR. Because the homology was at 75%, this could potentially hinder a positive PCR result, if those sequences diverged from the reference sequence in the primer binding areas, or probe binding area in case of qPCR. Further experiments are required to understand if, and to what degree, those genes give rise to resistance against antibiotics, but it clearly indicates an antibiotic resistance potential in the bacterial metagenomes of free roaming Minke whales.

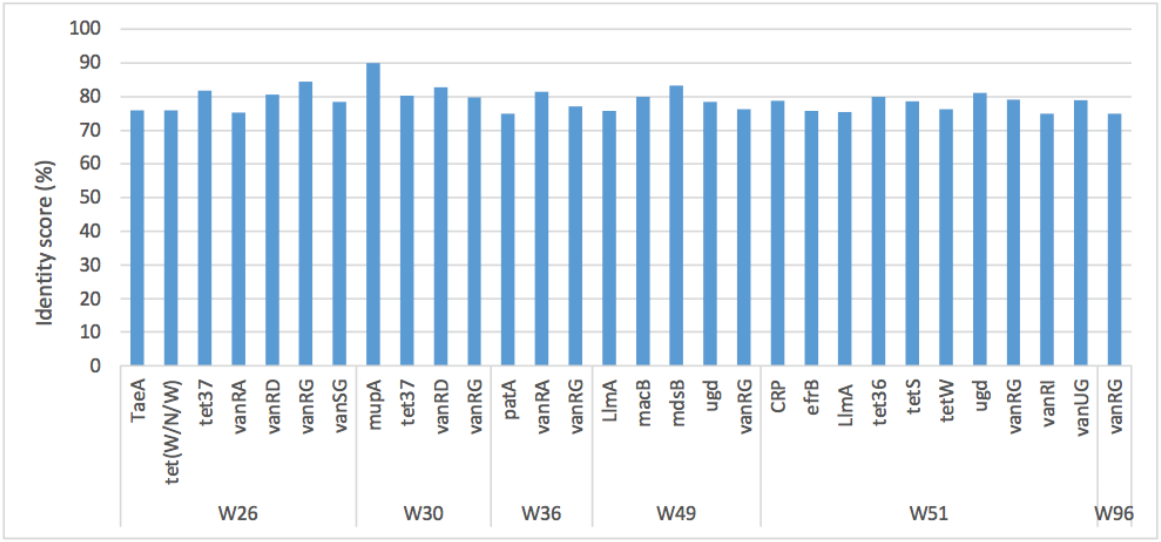

Table 2: Overview the ARG (excluding mutations) with an identity score above 75% compared to homologue in CARD from six of the whale metagenomes.

By testing the abundancy of sequences in metagenomic samples, we did discover that variants of van genes, conferring resistance to vancomycin, were detected in all six metagenomes reported. This is in

accordance with the results presented found in the total DNA PCR analyses (Table 1), and the identification of phenotypic vancomycin resistance in cultivated isolates from whale faeces.

Conclusions from GenØk's activities

Cultivation and subsequent testing of growing bacteria on media containing antibiotics have several limitations. The most important are that only a small part of the total pool of bacteria are able to grow under any given growing conditions. The samples are also subjected to various storing and transportation conditions, which for samples taken from animals in the remote wilderness or in the marine/aquatic environments, may be a huge problem to the quality of the samples. This is because it is difficult to freeze and keeping the samples frozen at sufficient low temperatures, when the location is remote. This method is also very labor intensive, in particular if there are more than one samples to be investigated. For faecal samples from whales and seals, we only picked 10 colonies from each animal, and quickly ended up with testing more than 600 colonies on 8 different antibiotics, in total almost 5000 individual tests.

In addition to the bias in sampling and growing bacteria, results indicating resistance to antibiotics must be verified genetically. It is important to identify what the resistance mechanisms are, and what the potential for transfer to other bacteria is. For instance, an increase of efflux pumps may be a general resistance mechanism with little potential for transfer to human pathogens, while plasmid born antibiotic inactivation genes may have high transfer potential.

Targeted PCR combined with qualitative PCR may help understand and monitor specific resistance mechanisms. Screening with PCR for a high number of possible resistance genes, are labor intensive and requires prior knowledge and verification by sequencing. Variants of the same genes are hard to detect and may give false negative results. Our metagenomic approach in whales revealed several sequence variants of vancomycin (*vanA*) and tetracyclin (*tet* genes) resistant genes.

Metagenome sequencing gives a wealth of information, but require extensive post-sequencing work, such as assembly and annotations of genes. Our experiences are, however, that metagenomic analyses did give much more information than the other methods. As mentioned above, several resistance genes had sequence variations that potentially could give false negatives in a targeted PCR screen (primers fail to bind or give variable sized PCR-products). We were able to verify that the whales do indeed carry a set of vancomycin resistance genes, although to what degree those genes and sequence variants give rise to vancomycin resistance should be further tested.

Other clear advantages from metagenomic analyses are that the method is less labor intensive and sequences, once processed, are available for reanalyzing and data mining in hindsight.

Based on our current knowledge we would have started each project with metagenomics since this gives an overview of what can be found in the environmental sample. Then moved on to other analyses for further identification.

Current AMR/ARG projects at GenØk

MicroPlastResist

Microplastics in wastewater as a carrier and dispersal route of antibiotic resistance in oceans (MicroPlastResist) – Funded by the Norwegian Research Council and the South African National Research Foundation (SANOCEAN program).

In 2018 the first publications appeared that described the link between antibiotic resistance and microplastics particles (Arias-Andres et al., 2018; Eckert et al., 2018). Up to now this novel and complex challenge had been overlooked, and there is thus very little understanding of the dynamics of this association in various aquatic environments. This first studies dealt with freshwater systems, and at present no published data could be accessed that describe research that was done on antibiotic resistant bacteria (ARB), antibiotic resistant genes (ARG) and microplastics in the ocean environment. Considering the dispersal potential of the ocean and the fact that large quantities of plastic material, treated wastewater and other anthropogenic material find its way into this environment, suggests that data is urgently needed to investigate these links. The study is thus timeously for the data to make contributions towards policy with regards to disposal of treated wastewater in ocean outfalls.

The specific research questions are:

1. How does seasonal variations and different national context affect the types and amounts of released microplastics through wastewater treatment plants?
2. What types of bacterial diversity and type/amount of antibiotic resistance genes exists in association with microplastics?
3. Do different environmental-relevant types of microplastics affect the associated bacteria with regards to biofilm formation, DNA uptake and spread?

We are sampling from wastewater treatment facilities in Tromsø and in South Africa, in a comparative manner, our collaborators are North West University.

shAMRock (Shocking antimicrobial resistance)

This project is funded by Norway and EEC grants for bilateral collaboration between Norway and Czech Republic, and undertaken in collaboration with University of Chemistry and Technology, Prague. shAMRock is working with establishing rapid molecular methods for surveillance of AMR at wastewater treatment facilities. Specifically, molecular methods such as MinIon sequencing techniques (Oxford Nanopore) are being established and deployed to quickly screen for antimicrobial resistance genes in wastewater. See project website for more information (https://tvp.vscht.cz/research/projects-grants/shamrock/news)

JPIAMR Surveillance Network

GenØk is one of several partners in an international network on antimicrobial resistance in the environment with funding from JPIAMR (JPIAMR Surveillance Network). In this network, efforts are being made to systematize methods and map relevant indicators for environmental studies of antibiotic resistance. The project manager is William Gaze (University of Exeter).

Knowledge gaps

Processes and substances that leads to development and spread of antimicrobial resistance and antimicrobial resistance genes, are still not fully understood. We do know that environmental antimicrobial resistance can spread to human pathogens, particularly in association with genetic mobile elements, but the conditions needed that promote mobilization are not fully understood. We need much more information about under which conditions antimicrobial resistance is selected for. For instance, what types of environment, which animals may contribute to spread and what kind of human activities and substances contribute to this development.

Current projects at GenØk focuses on microplastics as a newly discovered driver or environment for promoting resistance spread. Environments where environmental resistance genes, drivers and human pathogens have the potential to meet, such as wastewater treatment facilities or industrial production areas, are particularly interesting to investigate. Also domesticated and/or free roaming wild animals are relevant to monitor for antimicrobial resistance genes due to being exposed both to different environments and humans/human activities.

One of the major obstacles of comparing scientific work already done on environmental antimicrobial resistance, is the lack of standardization of methods for surveying and for monitoring. Burning issues are what types of antibiotics are good indicators, what types of bacteria are most relevant, and what

are relevant breakpoints/concentrations and MIC for the different bacteria? What are the best sampling methods and how is such sampling conducted? It is also important to determine what types of environments would be important to survey.

Recommendations for future screening and monitoring

- Microbiomes (bacterial diversity) and metagenomic analyses with a focus on antimicrobial resistance genes are the most effective method for generation large datasets for surveying purposes. Illumina based techniques, such as utilized in GenØk report 3 and 6, are maybe the most used technique, but recently methods based on MinIon also have emerged. Reinterpretation of MinIon sequencing data with the platform ARGpore has recently been proven effective for antimicrobial resistance gene mapping (Białasek and Miłobędzka, 2020). These platforms combine rapid sequencing (particularly MinIon) with reliable AMR detection in huge datasets and a combination of these techniques could be utilized more for complimentary datasets.

- Developing good and reliable techniques for environmental sampling. This implies methods using for example GPS or similar to ensure that sample sites are not decided on random and to facilitate the possibility for taking samples for monitoring purposes (identify changes over time). How to take samples involves good training to avoid contamination and break down of the material.

- Biofilms on particles, such as plastics, may contribute to accumulation and spread of antibiotic resistance genes (i.e. Arias-Andres et al., 2018; Eckert et al., 2018; Radisic et al., 2020). Monitoring biofilm formation and ABR association on relevant surfaces will increase our understanding on how biofilms contribute to ABR spread and persistence.

- Potential for ABR spread is linked to genetic context. ABR genes located on mobile genetic elements are considered more likely to spread horizontally and crossing the species barrier. Understanding single cell dynamics, which plasmids may spread to different hosts and what is the capacity for carrying ABR genes, are generally difficult but new techniques, such as single cell sequencing, could generate the needed data.

Key References

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Report 3: December 2017: Prevalence of antibiotic resistance marker genes (ARMG) in selected environments in Norway – Reindeer.

Report 4: February 2019: Antimicrobial resistance in the marine environment: MIC profiles of bacteria isolated from whale and seal faeces.

Report 5: February 2020: Antimicrobial resistance in marine mammals: Targeted PCR and metagenomic analysis.