

Prevalence of Antibiotic Resistance Marker Genes (ARMG) in Selected Environments in Norway

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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 human and animal health and the environment interact and must be seen in context to each other. The presence of resistant bacteria in different natural environments, such as soil, fresh water, sea sediments and wild animals, has only been sporadically studied, although they may contribute to the development of resistance of clinical importance. There is therefore a need for more knowledge about antibiotic-resistant bacteria in different natural environments in general and in Norway in particular. The national strategy is based on the report "Antibiotikaresistens-kunnskapshull og aktuelle tiltak (2014)" prepared by an expert group. In this report, the need to assess the risk of a possible deliberate release of genetically modified organisms (GMOs) with antibiotic resistance marker genes (ARMG) is identified as one of the areas where more information is necessary.

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. This 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.

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# **Summary**

We have investigated the natural occurrence of two antibiotic resistance marker genes (ARMG) – neomycin phosphotransferase II (*nptlII*) and neomycin phosphotransferase III (*nptlII*) in selected environments in Norway. Three different agricultural fields in Tromsø differing in exposure to manure were analyzed in addition to samples of soil and faeces taken from four different pig farms in the Tromsø area of Northern Norway. In total, seven sampling sites were analyzed, three from agriculture fields and four from pig farms.

The prevalence of *nptll* and *nptlll* were determined in bacterial communities from each environmental sample site, by cultivation dependent- and cultivation independent molecular approaches. From the different environmental samples, approximately  $5.7 \times 10^4$  to  $3.1 \times 10^6$  bacterial cells per gram soil and  $1.5 \times 10^6$  to  $2.1 \times 10^8$  bacterial cells per gram faeces were recovered. 0 to 12% of the cultivable bacteria from the different samples tested were resistant to kanamycin (100 µg/ml) on both growth media tested.

A randomly picked set of approximately 2000 kanamycin resistant bacterial isolates were further purified and screened by PCR. None of the isolates could be identified as harboring the *nptll* or *nptlll* gene. In addition, total DNA extracted from either soil or fecal samples was analyzed in triplicates by PCR for the prevalence of *nptll* (129 bp and 795 bp targets) and *nptlll* (82 bp and 795 bp targets). The efficiency of DNA extraction and purification was evaluated in a test soil and faeces sample spiked with a defined concentration of maize DNA. No inhibition was detectable at a 10<sup>-1</sup> dilution of the DNA extract. None of the tested samples were positive for these aminoglycoside phosphotransferase genes. The detection limit in this study was approximately 150 copies per gram fresh soil.

Although a small overall number of environmental samples have been tested, the results indicate that the prevalence of *nptll* and *nptlll* genes in local environments in Tromsø is very low and below the detection limit of the methods used. It is noted that sampling was done over a limited time. The low prevalence of these specific genes in the tested environments suggests that the introduction of external sources of such genes, including ARM genes present in GM plants, may add new genetic sources of such resistance determinants to environments in Norway.

# Norsk sammendrag

I dette prosjektet har vi kartlagt forekomsten av to antibiotikaresistensmarkørgener (ARMG) - neomycin phosphotransferase II (*nptIII*) and neomycin phosphotransferase III (*nptIII*) i ulike naturlige miljø i Tromsø. Jord fra tre områder med ulik eksponering av kunstgjødsel ble undersøkt i tillegg til jord fra fire ulike grisegårder i Tromsø og faeces prøver fra grisebesetningene der. Totalt ble det innhentet prøver fra syv steder, tre fra jord og fire fra grisegårder.

Fra hvert miljø har vi bestemt forekomsten av kanamycin-resistente bakterieisolater gjennom dyrkningsbasert metoder og videre karakterisering av et utvalg av de kanamycin-resistente bakteriene ved bruk av. PCR for spesifikk deteksjon av de spesifikke antibiotikaresistensgener (nptII og nptIII). I de ulike miljøene viste det seg at 5,7 x  $10^4$  til 3,1 x  $10^6$  bakterier per gram jord og at 1,5 x  $10^6$  til 2,1 x  $10^8$  bakterier per gram feces var dyrkbare. Av disse var 0 til 12% resistente mot kanamycin ( $100 \mu g/mI$ ) på de to dyrkingsmediene benyttet.

Videre ble ca. 2000 kanamycin-resistente bakteriekolonier plukket for videre karakterisering. Det viste seg at ingen av disse var positive for hverken *nptll* eller *nptlll* ved PCR analyse. Total DNA ble ekstrahert fra de ulike miljøprøvene og disse ble analysert i triplikater for tilstedeværelse av *nptll* (129 bp and 795 bp) og *nptlll* (82 bp and 795 bp) ved PCR. Heller ikke disse prøvene var positive for aminoglykosid fosfotransferase genene.

Selv om kun et begrenset antall bakterier og mengde totalt DNA er blitt analysert fra de ulike miljøprøvene, så er den naturlige forekomsten/bakgrunnen av *nptll* og *nptlll* gener i de utvalgte miljøene ikke påvisbar og under deteksjonsgrensen. Disse dataene indikerer at tilføring av eksterne kilder for disse genene, som f. eks ARMG fra genmodifiserte (GM) planter, vil kunne bidra til økt forekomst i disse miljøene.

# Aims of the project

This project seeks to determine the possible occurrence of and prevalence level of *nptll* and *nptlll* in selected environments in Norway. The project included establishing the methodological basis for detection of *nptll* and *nptlll* genes in environmental samples. The major objectives are:

- 1) To determine the prevalence, distribution and characteristics of ARMG (*nptII* and *nptIII* genes) used in GMOs 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.
- The publicly available part of the technical dossiers of GMO applications assessed by GenØk in the period 2010-2016.
- GenØk's policy briefs and reports.

#### **Abbreviations**

Abbreviation	Description

APH aminoglycoside phosphotransferase (protein)

ARG antibiotic resistance gene
ARM antibiotic resistance marker
ARMG antibiotic resistance marker gene

BIOHAZ EFSA Biohazard Panel CFU colony forming unit

 $\begin{array}{ccc} \mu l & microliter \\ ml & millilitre \\ g & gram \\ bp & base pair \end{array}$ 

DNA deoxyribonucleic acid
rRNA ribosomal ribonucleic acid
EFSA European Food Safety Authority
EMA European Medicines Agency

GM genetically modified

GMO genetically modified organism
HGT horizontal gene transfer

Cmr chloramphenicol

Amp ampicillin
Str streptomycin
Km kanamycin
Neo neomycin

nptII neomycin phosphotransferase II (gene)
nptIII neomycin phosphotransferase III (gene)

PCR polymerase chain reaction
WHO World Health Organization

#### 1. Introduction

Over the last decade, there have been growing concerns about the effects of pharmaceuticals and antibiotics on bacterial populations in the environment. Accumulating evidence of increased resistance against antibiotics among bacteria in soil and other natural environments including wastewater treatment plants, river water, drinking water, seawater, sediments has emerged (Berglund et al., 2014, Lindberg et al., 2005, Abuin et al., 2006, Segura et al., 2009). These studies show that both antibiotics and antibiotic resistance genes (ARGs) are prevalent in many different environments.

In addition, genes conferring resistance to antibiotics have also been widely used as markers for the selection of transformed cells in the development of genetically modified (GM) plants (Miki and McHugh, 2004, Nap et al., 1992). In some instances, the antibiotic resistance marker (ARM) gene remains in the finished commercial plant product (Ramessar et al., 2007). The most commonly used ARM gene for plant cell selection is *nptll* (neomycin phosphotransferase II, also referred to as aph-3`-II or aminoglycoside phosphotransferase 3`-II) (EFSA 2004, 2009). Several commercialized GM plants are carriers of this ARM gene (Nap et al., 1992, Miki and McHugh, 2004, EFSA, 2009).

The presence of ARM gene in GM plants and large-scale release in the environment, or use as food or feed, has raised concerns over the past years regarding possible risks for human health and the environment (Nielsen et al., 1997, Kay et al., 2002, Badosa et al., 2004, Breyer et al., 2014). One of the main environmental concerns is that the cultivation of GM plants and its use in food, feed and industrial purposes might provide a source of AR genes that will contribute to the development of new drug-resistant bacteria (Woegerbauer et al., 2014, Woegerbauer et al., 2015b, Ramessar et al., 2007, EFSA, 2009, Bennett et al., 2004). The risk of horizontal gene transfer (HGT) of plant-derived ARM genes to soil or gut bacteria resulting in a reduced antimicrobial treatment of animal and human infectious diseases have been claimed to be very low but cannot be excluded (Gay and Gillespie, 2005, Goldstein et al., 2005).

Phenotypic resistance to kanamycin in soil bacterial communities is quite common (Leff et al., 1993, Ma et al., 2011, Woegerbauer et al., 2015b, Smalla et al., 1993). However, it is well known that some bacterial species are intrinsically resistant to some antibiotics. Concerns emerge over the increasing prevalence of resistance in previously susceptible species and the increased mobility of such traits. At present, there are only a few studies that have been conducted to determine the prevalence of specific ARMG in bacterial populations in natural environments. The wide distribution of antibiotic resistance genes (ARGs) in general, and the potential threats to the human and animal health arising from horizontal gene transfer highlights the importance of identification and monitoring of the presence and level of antibiotics and AMRG in the environment, as it can function as reservoirs for transferable resistance. Bacterial resistance levels and usage levels of antibiotics in agricultural and aquaculture varies dramatically between countries even within Europe (VKM, 2005, NORM/NORM-VET, 2015).

We have investigated different environments in Northern Norway with different expected exposure of antibiotics for the phenotypic resistance to kanamycin and the prevalence of natural analogues to the most commonly used ARMG. We focused on soil samples with different exposure to manure and soil from grazing area for livestock in addition to fecal samples form the pigs in areas close to Tromsø city. In general, the use of antibiotics in these environments can be considered as low, according to NIBIO

and the pig-farmers history of use of antibiotics. Antibiotics are not allowed as growth promoters in Norway.

# 1.1 Antibiotic resistance marker genes (ARMG)/GMO

The combination of antibiotic resistance genes and antibiotics is an important tool for selection in genetic engineering in general and in plant biotechnology in particular. During the process of genetic modification of plants or other organisms, marker genes are used to facilitate identification of the cells/seeds that has been successfully modified from those that did not undergo transformation. These marker genes most often confer herbicide tolerance or antibiotic resistance (Bennett et al., 2004, Ramessar et al., 2007). Some alternatives to ARMGs for *in vitro* selection of GM plants have been developed, but ARMGs are in many cases preferred because they have shown to be efficient for selection. In addition, the selection is cost-efficient and applicable to a large number of plant species (Breyer et al., 2014, Ferradini et al., 2011, Gay and Gillespie, 2005, Rosellini, 2012). Some of the new plant-breeding techniques do not necessarily rely on the use of ARMGs including nuclease-based techniques (e.g. CRISPR), and oligonucleotide-directed mutagenesis. These techniques will however also genetically alter only a minor fraction of targeted plant cells and thus face similar challenges in selection of the few cells that have undergone successful genetic modification.

#### 1.1.1 Different types

In 2004, The European food safety authority (EFSA) divided the ARMGs used in GM plants into three groups using mainly two criteria (EFSA, 2004) to aid the risk assessment of these. The first considered whether the given gene is already widely distributed in the environment. The second criteria was whether the given gene confers resistance to antibiotics that has therapeutic relevance towards human and veterinary medicine. From the EFSA 2004 report:

**Group 1:** contains resistance genes considered to be already widely distributed in the environment and confer resistance to antibiotics that have no or minor therapeutic relevance for both human and veterinary purposes. Group 1 includes *nptll* and *hpt* that confer resistance to the antibiotics kanamycin, neomycin, paromycin, butirosin, gentamicin B, geneticin or hygromycin, respectively.

**Group 2:** contains resistance genes considered to be widely distributed in microorganisms in the environment and confers resistance towards antibiotics that are used in defined areas of human and veterinary medicine. Group 2 contains following genes *Cmr (cat)*, *Amp (bla<sub>TEM-1</sub>)* and *str (aadA)*, which confers resistance to the antibiotics chloramphenicol, or ampicillin or streptomycin/spectinomycin, respectively.

**Group 3:** contains resistance genes considered highly relevant for human therapy. Group 3 contains genes *nptlll* and *tetA*, which confer resistance to the antibiotics amikacin or tetracycline's, respectively.

On the background of these criteria, the EFSA GMO panel considered there was no need for restricting the use of the ARMGs in group 1, whereas the ARMGs in group 2 should be restricted to field trial purposes only. Group 3 should be avoided in GM plants to ensure the highest standard of preventative care.

The 2004 opinion from EFSA was subsequently replaced by the 2009 Opinion that is less categorical, but retains the natural prevalence levels, likelihood of horizontal gene transfer and selection as a basis for the assessment (EFSA, 2009).

#### 1.1.2 nptII and nptIII

*Nptll*, that encodes the neomycin phosphotransferase enzyme conferring resistance to the aminoglycoside antibiotics neomycin and kanamycin, is the most commonly used ARM gene used in GM plants (Goldstein et al., 2005, Ramessar et al., 2007, Rosellini, 2012). This gene was first discovered as part of the transposon Tn5 in *E. coli* (Garfinkel et al., 1981, Smalla et al., 1993) and its gene product works primarily by inactivating kanamycin and neomycin by phosphorylation (Davies and Wright, 1997, Shaw et al., 1993).

NptIII is among the most prevalent aminoglycoside phosphotransferases in Gram-positive bacteria and therefore more clinically relevant than *nptII* (Woegerbauer et al., 2015a). The reason is that *nptIII* also gives resistance to amikacin, lividomycin and isepamicin, which are antibiotics that according to WHO are "highly relevant for human therapy" (Shaw et al., 1993, EFSA, 2009, EFSA, 2004). Both the *nptII* and *nptIII* are present in a range of synthetic vectors and also in naturally occurring plasmids and transposons, and may be horizontally transferable between different bacterial cells and strains (Miki and McHugh, 2004, Wright and Thompson, 1999, Trieu-Cuot and Courvalin, 1983, Shaw et al., 1993, Fong and Berghuis, 2002, Becker and Cooper, 2013).

#### 1.1.3 Clinical use of aminoglycosides

Overall, due to strict prescription policies and guidance the total use of aminoglycosides in Norway is low. The phenotypic resistance to a few aminoglycoside antibiotics is routinely monitored by NORM/NORMVET in the bacterial species *E. coli, Salmonella* sp., *E. coli* and *S. aureus* (NORM/NORM-VET, 2015). Low usage level is also related to general low usage levels of antibiotics in Norway for veterinary purposes, strong emphasis on prescription guidance, the use limited spectrum antibiotics, focus on animal welfare, and lack of financial incentives for prescription for veterinarians and doctors.

The gene product of *nptll* inactivates both neomycin and kanamycin (Mingeot-Leclercq et al., 1999). In Norway neomycin is only used topically in eye and ear drops, and there is only one licensed product, maxitrol, on the market in Norway (http://legemiddelhandboka.no/). For veterinary use, only one product, Colivet Vet, used for the treatment of mastitis and enteritis in pigs and calves, is registered for veterinary use (http://xn--veterinrkatalogen-xrb.no/ir/medisin-vet/colivet-vet-vetpharma-a-s-547604, 2015). Kanamycin is presently not licensed for the treatment of infectious diseases in humans or animals in Norway, development of resistance to other antibiotics may however make it more important in the future.

Amikacin, which is inactivated by the *nptlII* encoded aminoglycoside phosphotransferase (3`)-IIIa, is not approved for marketing in Norway. It is however used intravenously in hospitals to treat Pseudomonas meningitis, and it is exempt from approval (http://www.felleskatalogen.no/medisin/godkjenningsfritak/preparatliste,

http://legemiddelhandboka.no/terapi/1849, http://legemiddelhandboka.no/terapi/1849paratliste, NORM/NORM-VET, 2015). In veterinary medicine, amikacin may be used for the treatment of septic arthritis in animals (https://lovdata.no/dokument/SF/forskrift/2007-01-16-50).

A WHO expert working group has listed Amikacin under "critically important antibiotics". Neomycin and Kanamycin, which were previously listed as "highly important" antimicrobials have now been included in the "critically important" category (WHO, 2012). This is due to a constant increase of bacteria resistant to various different classes of antibiotics. Treatments of some infections will in the future rely on older (aminoglycosides) antibiotics that are not preferred today because of unfavourable side effects and ADME (absorption, distribution, metabolism and excretion) properties.

#### **1.1.4 Risks**

All GM plants are required to undergo safety and risk assessment before commercialization in Europe. There are several criteria in use for evaluating risks related to the use of ARGs, like the medical importance and the distribution of microorganisms already possessing these genes in different environments (EFSA, 2004). In EFSA 2004, *nptll* was initially classified as a selection marker that does not pose any risk to human and animal health or the environment. In 2009, EFSA evaluated ARMG in plants and the panel concluded again that "according to information currently available, adverse effects on human health and the environment resulting from the transfer of the two antibiotic resistance marker genes, *nptll* and aadA, from GM plants to bacteria, associated with use of GM plants, are unlikely".

The same assessment contained minority opinions (EFSA, 2009), in addition a member of the panel objected to the conclusion and suggested some changes. One example for suggested change was:

"The transfer of antibiotic resistance markers genes from GM plants to bacteria, appear to be either not occurring, or occurring below detection limits or at very low levels (10<sup>-9</sup> probability of a transfer per exposure). Other suggestion for changes in the conclusion included highlighting the possibility for horizontal gene transfer, and that the emerging pandemic of antibiotic resistance poses a serious threat to public and human health. Even though antibiotics such as kanamycin and streptomycin have historically not been used frequently in clinical settings for decades, they are now becoming more relevant again since they can be used as the 2<sup>nd</sup> or last line drug against some life threatening infections.

There is therefore at present a disagreement among scientists about the possible consequences on human health and the environment by the use of ARMGs in GM plants. Horizontal transfer of genes to new recipients with undesired outcomes has been identified as a potential risk associated with the use of GM plants (Conner et al., 2003, Nielsen and Townsend, 2004). GM plants with ARMGs that are cultivated might provide a source of ARGs that might be taken up by bacteria in the environment or by bacteria present in the gastrointestinal tract (Netherwood et al., 1999, Nordgard et al., 2007, EFSA, 2009). It has not been shown that ARMGs has been transferred from GM plants to bacteria in the environment or the gut (EFSA, 2009). However, although the event may be rare, it may have an ecological impact if the transferred gene alters the fitness of the recipient bacteria or cell (Nielsen and Townsend, 2004, Woegerbauer et al., 2014). In general, the specific conditions (environmental location and time) that have given rise to new troublesome resistance in the clinic are rarely identified. Thus, the mechanisms behind past events of horizontal gene transfer of ARGs are not understood at the detailed level that are necessary to inform specific assessments of the effects of potential ARMG transfers.

As mentioned, the sources of the genes used as ARMGs are originally isolated from naturally occurring bacteria in the environment (Garfinkel et al., 1981, Smalla et al., 1993). The large-scale release of GM plants containing these genes may therefore not necessarily introduce new ARGs into a particular environment, but it may change the concentration and increase the exposure level of the bacteria to certain ARGs. A higher concentration of such genes and continual exposure might increase the likelihood of horizontal gene transfer. Data on the prevalence, diversity and the ecology of AMRGs analogues in naturally occurring bacterial populations in the environment are few but important when one should evaluate the possible health and environmental consequences of introduced ARMGs (Smalla et al., 1993, Demaneche et al., 2008, EFSA, 2009, Woegerbauer et al., 2015b).

#### 1.1.5 Prevalence of *nptII* in the environment

The observation of phenotypic antibiotic resistance as a proportion of the overall number of bacterial cells in a bacterial community in soil, aquatic systems and habitats associated with animals and humans is common (EFSA 2004). This will always be the case as not all bacterial species are naturally susceptible to a given antibiotic. There is therefore an important distinction to be made between intrinsic/nontransferable resistance, and acquired/transferable resistance. EFSA states that there is a widespread presence of the nptll gene in different environments (EFSA, 2009, EFSA, 2004). A review of the scientific literature retrieves, however, only few studies that have investigated the prevalence of nptll genes in the environment (Leff et al., 1993, Smalla et al., 1993, Ma et al., 2011, Woegerbauer et al., 2015b). These studies show only a very low prevalence of nptll genes among bacteria in nonclinical environments like soil, river water, sewage and manure. In the two most recent studies (Woegerbauer et al., 2015b, Ma et al., 2011), the nptll prevalence in soils was investigated. Ma et al. could not find nptll copies in total soil bacterial DNA extractions (Ma et al., 2011). Also by a metagenomic approach, this group did not identify positive nptll PCR results from total soil bacterial DNA. In a very recent study by Wogerbauer et al. (2015) the prevalences of nptll and nptlll in soil from 100 fields selected to represent Austrian maize and potato growing regions were determined. Of all fields, 6% of the total DNA extracts were positive for nptll and 85% for nptll. Of the cultivable kanamycin resistant soil bacteria, none were positive for nptll and 1,8 % were positive for nptlll, demonstrating a low background level of *nptll* in these specific environments.

#### 1.1.6 ARMGs on the market today

A number of GM plants intended for placing on the market as food or feed contains the *nptll* gene. Examples are listed in table 1. These products are currently under consideration by EFSA or have already been approved for commercialization in the EU. They are all still in the assessment process in Norway, and no final decision on approval or not has been made (\*).

In a period from 2008-2014, the fish feed industry in Norway was given a dispensation allowing the industry to be able to import 19 genetically modified GM fish feed ingredients. Eight of these products contained the *nptll* gene. In 2014, the Norwegian Food Safety Authority withdrew this dispensation since the industry did not use it.

 Table 1: Examples of GMOs containing nptll gene that are approved or under approval in the EU

Maize	Cotton	Potato
MON863xNK603 (Scope of	MON1445 (Scope of Application: food	EH92-527-1 (Scope of Application:
Application: Food, feed, import and	and feed)-Approved	Import, processing and cultivation) –
processing) -Approved		Under approval
MON863xNK603xMON810 (Scope of	MON15585x15985 (Scope of	EH92-527-1 (Scope of Application:
Application: Food, feed, import and	Application: food and feed)	food, feed, import and processing) –
processing) - Approved		Under approval
MON863xMON810 (Scope of	MON15585x15985 (Scope of	
Application: Import and processing) -	Application: Food, feed, import and	
Approved	processing) –Approved	
MON863xMON810 (Scope of	MON531 (Scope of Application: food	
Application: food and feed) -	and feed) - Approved	
Approved		
MON863 (Scope of Application:	MON531xMON1445 (Scope of	
Import and processing) - Approved	Application: food and feed) -	
	Approved	
MON87460 (Scope of Application:	MON15985xMON1445 (Scope of	
Food, feed, import and processing) –	Application: food, feed, import and	
Under approval	processing) – Under approval	
	MON88913xMON15985 (Scope of	
	Application: food, feed, import and	
	processing) – Under approval	

<sup>\*</sup> Examples of databases used: http://en.biosafetyscanner.org/index.php, http://www.euginius.eu/euginius/pages/gmo\_index.jsf, http://www.euginius.eu/euginius/pages/gmo\_modified\_traits.jsf, http://cera-gmc.org/GMCropDatabase

#### 2. Material and Methods

# 2.1 Description of the different sampling area in Tromsø

Different areas were chosen to establish a baseline frequency of *nptll* and *nptlll* in local environments in Tromsø community. None of these areas have been intentionally exposed to any ARM gene containing DNA from GM plants.

Three different fields of soil with different history of use of organic fertilizers (manure) were selected in collaboration with NIBIO (Norwegian Institute of Bioeconomy Research, Tromsø). The manure used as fertilizer was also included in this study.

In addition, four pig farms with different types of farming were included. From these pig farms both soil and pig faeces were sampled. The characteristics of the different sampling areas are summarized in table 2.

The history of use of fertilization was also clarified for all sampling areas. In field soil 1 and 2, manure from horses was used or had been used. In the different pig farms, mainly urine from pigs was used. The contact person for each sampling area was also asked about the history of use regarding antibiotics. In all sampling area, the use of antibiotics for veterinary use was considered as low.

Table 2: Sources and characteristics of samples used in this study

Field	Characteristic	Plants	Sample	Field (n = number of pigs)	Characteristic	Plants	Sample
SOIL 1	Yearly fertilization with manure	Grass	Soil	Pigfarm1* (n = 20)	Meat farm	Grass	Soil and faeces
SOIL 2	No fertilization the last 7 years	Grass	Soil	Pigfarm 2* (n = 11)	Meat farm, Ecological farming	Grass	Soil and faeces
SOIL 3	No use of fertilizers	Grass	Soil	Pigfarm 3** (n =3 00)	Meat farm		Soil
Manure	Faeces from horses	-	Faces	Pigfarm 4* (n = 240)	Meat farm	Grass	Soil and faeces







Figure 1: Pictures that illustrates some of the sampling fields. A) Soil 1, B) Pigfarm 2, and C) Pigfarm 4

## 2.2 Sampling and processing

A modified sample scheme, based on Andersen et al (2012) and Woegerbauer et al (2015), using a grid model was used to ensure random soil sampling (figure 2). From each test field, a composite soil sample comprising of 13 single soil subsamples (extracted 1-10 cm depth using a cut with a spade) within a 35x50 meters rectangle was collected. Only for one field (pigfarm 2), the sampling scheme had to be adopted because of a smaller size of area that could be sampled. However, 13 subsamples were also taken in this field.

The composite soil samples, were mixed, homogenized and stored in a beaker before sample processing done the same day. Each crude batch of sample were homogenized and used for cultivation of soil bacteria and extraction of total DNA.

Strict protocols to avoid cross contamination throughout each step were enforced. The protocols included e.g. decontamination of all equipment used and use of plastic gloves.

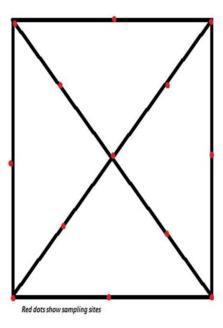


Figure 2: Sample scheme for soil illustration the locations of the 13 different subsample points.

# 2.3 Determination of total CFU and kanamycin resistant bacteria

From one g of sample material (soil/faeces/manure), colony-forming units (CFU) were determined for the total cultivable aerobic bacteria and for the total cultivable kanamycin resistant aerobic bacteria. Ten-fold dilutions made in saline were plated on Standard I Nutrient (complete medium) and R2A Agar (minimal medium) (Merck, Darmstadt, Germany). The media were supplemented with 50  $\mu$ g/ml cycloheximide (Sigma-Aldrich) and for the cultivation of kanamycin resistant strains, the agar was also supplemented with 100  $\mu$ g/ml kanamycin disulphate (Sigma-Aldrich).

After three days at room temperature the total number cultivable aerobic bacteria and the total number of cultivable kanamycin resistant aerobic bacteria was counted.

For further characterization of kanamycin resistant strains, colonies were picked and re-streaked to obtain pure cultures. A suspension of three to six colonies of the pure cultures was stored in 20% glycerol at – 80 until further use and DNA extraction. Positive and negative controls (listed in table 4) were included in this protocol.

# 2.4 DNA isolation

#### 2.4.1 Total DNA isolation from soil

One gram of fresh weight samples (4 x 250 mg) was used to isolate the total DNA from composite soil and faeces. DNA from the four aliquots was extracted using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions. In addition, PowerClean DNA Clean-Up Kit (MoBio Laboratories, Carlsbad, CA USA) was used 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.

Two  $\mu$ l of the resulting DNA solution served as template for the *nptll/nptlll* screening as well as for the 16S rRNA PCR assay.

#### 2.4.2 DNA isolation from bacterial colonies

Re-streaked kanamycin resistant bacterial isolates was extracted using Quickextract<sup>tm</sup> DNA Extraction Solution 1.0 (Epicentre Biotechnologies) according to the manufacturer's instructions. The eluted DNA extracts were stored at -20°C until further analysis. Two  $\mu$ l of a 10<sup>-1</sup> dilution of the resulting DNA solution served as template for the *nptll/nptlll* screening as well as for the 16S rRNA PCR assay.

# 2.5 Amplification and DNA extraction control

The eluted DNA from environmental samples and bacterial colonies served as template for the bacterial 16S rRNA gene to confirm the general absence of PCR inhibitors. The reactions were performed in a total volume of 20  $\mu$ l containing the following: 1  $\mu$ l of each primer (Eurogentech) at 10  $\mu$ M concentration, 10  $\mu$ l mastermix (DreamTaq PCR Mastermix, Thermo Fisher), 4 $\mu$ l water and 4  $\mu$ l 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 60°C for 30 s and elongation at 72°C for 40 s, one cycle of final elongation at 72°C for 5 min. Primers and controls are listed in Table 4. The PCR products were run on 2% agarose well E-gels, using E-Gel® Low Range Quantitative DNA Marker. All supplied by Invitrogen, Norway, before visualization.

# 2.6 PCR amplification of *nptII* and *nptIII* genes in the Km<sup>R</sup> isolates

To determine if *nptll* and/or *nptlll* genes were present in the total DNA from the environmental samples and the kanamycin resistant bacterial colonies four different sets of primers were used (Table 3). The reactions were performed in a total volume of 20  $\mu$ l containing the following: 1  $\mu$ l of each primer at 10  $\mu$ M concentration, 10  $\mu$ l mastermix (DreamTaq PCR Mastermix, Thermo Fisher), 4 $\mu$ l water and 4  $\mu$ l 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 60°C for 30s and elongation at 72°C for 40 s, one cycle of final elongation at 72°C for 5 min. Primers and controls are listed in Table X. The PCR products were run on 2% agarose well E-gels, using E-Gel® Low Range Quantitative DNA Marker. All supplied by Invitrogen, Norway, before visualization. Positive and negative controls (listed in table 4) were included in each PCR set-up.

Table 3. PCR primers (Eurogentech)

Target	Name	Size	Primer sequence (5'-3' direction)	Reference
16S rRNA	16S_F 16S_R	571 bp	TGGAGAGTTTGATCMTGGCTCAG	(Woegerbauer
			CTTTACGCCCARTRAWTCC	et al 2014)
nptll short	NptII_F	129 BP	GATCTCCTGTCATCTCACCTTGCT	(Woegerbauer
	NptII_R		TCGCTCGATGCGATGTTTC	et al 2014)
nptII long	Nptll:Full_F	795 bp	ATGATTGAACAAGATGGATTGC	(Woegerbauer
	NptII:FuII_R		TCAGAAGAACTCGTCAAGAAGG	et al 2014)
nptIII short	Nptlll_F	82 bp	ACATATCGGATTGTCCCTATACGAA	(Woegerbauer
	NptIII_R		TCGGCCAGATCGTTATTCAGTA	et al 2014)
nptIII long	Nptlll_Full_F	795 bp	ATGGCTAAAATGAGAATATCACCG	(Woegerbauer
	NptIII_Full_R		CTAAAACAATTCATCCAGTAAAATATAA	et al 2014)

# 2.7 Controls used in this study

Table 4. Bacterial strains used as controls in this study

Strain	Relevance	Control	_
A. baylyi ADP1200 Com+ (1200)	Km (S)	Negative control	(Utnes et al., 2015)
A. baylyi BD413	Km (S)	Negative control	(de Vries et al., 2003)
A. baylyi ADP1200 Com + Km <sup>R</sup>	Km (R), nptll	Positive control	(Utnes et al., 2015)
<i>A. baylyi</i> BD413 JV28-Km <sup>R</sup>	Km (R), nptll	Positive control	(de Vries et al., 2003)
A. baylyi ADP1200Com+Km <sub>+</sub>	Km (R), nptIII	Positive control	(Utnes et al., 2015)
Water	DNAse/RNAse free water (Sigma)	Negative control	-

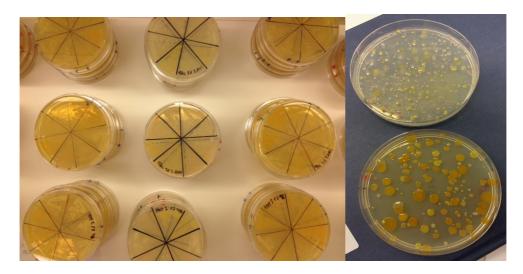
<sup>\*</sup>all our bacterial strains controls were kindly provided from the Department of Pharmacy, UiT The Arctic University of Norway

#### 3. Results

#### 3.1 Bacterial Counts and Antibiotic Resistance

On average, the total aerobic bacterial counts (total CFU) for the soil samples with different exposure to manure ranged between 1,5 E+6 to 3,1E+6 cfu/g soil on complete medium and the prevalence of aerobic kanamycin resistant isolates ranged between 0,4 to 12 %. On minimal medium between 0 to 12% of the cells tolerated the concentration of kanamycin used.

The average total aerobic bacterial counts (total CFU) for the soil samples in the pig farm was between 5,7E+4 to 1,4E+6 cfu/g soil and the average prevalence of aerobic kanamycin resistant isolates ranged between 1,0 % to 10,5 %. In feces samples from the pig farms the average total aerobic bacterial counts (total CFU) ranged between 1,5E+6 to 2,1E+8 cfu/g feces and the average prevalence of kanamycin resistance ranged between 0,2% and 2%. The results are summarized in table 5.



**Figure 3**: Representative picture of the petri dishes used for bacterial counting and appearance of different colony morphologies.

**Table 5:** Proportion of phenotypic kanamycin resistance in bacterial populations from soil and pig farms on different growth media.

	CFU/g soil	Kanamycin	CFU/g soil (R2A)	Kanamycin
	Complete medium	resistance % (SN1)		resistance % (R2A)
Soil 1	3,1E+6	4	3,5E+6	12
Soil 2	1,7E+6	12	3,9E+6	11
Soil 3	1,5E+6	3	2,2E+6	4
Manure	4,4E+6	2	3,7E+6	1
Pig farm 1				
Soil	3,3E+4	4,5	1,2E+5	4,4
Feces	6,6E+7	3,3	1,6E+8	0,7
Pig farm 2				
Soil	1,0E+6	1	2,8E+6	0,9
Feces	2,2E+6	1	8,2E+5	1
Pig farm 3				
Soil	1,3E+5	15	2,4E+5	4,9
Feces	-	-	-	-
Pig farm 4				
Soil	3,3E+4	11	8,2E+4	10
Feces	3,9E+8	0,4	2,4E+7	0,01

# 3.2 Prevalence of nptII and nptIII genes in kanamycin resistant bacteria

Bacterial strains resistant to kanamycin were recovered from glycerol stocks on nutrient agar plates before DNA isolation and PCR. All bacteria that were re-streaked and were growing were included in the PCR (table 6).

Table 6: PCR based detection of nptll and nptlll genes in Km resistant bacterial isolates

Test field/sample	Number of colonies included in PCR (total number bacteria re-streaked)	16S positive	<i>nptII</i> positive	nptIII positive
Soil <sup>a</sup> 1,2 and 3	553 (576)	530	0	0
Manure	192 (192)	188	0	0
Soil from pig farms	638 (672)	570	0	0
Faeces from pigs	564 (576)	516	0	0

# 3.3 Prevalence of *nptII* and *nptIII* in different environmental samples

Each of the individual samples was analyzed for the presence of *nptll* and *nptlll* genes by PCR. Two different primer sets were used for both *nptll* and *nptlll* (table 7).

Table 7: PCR based detection of *nptll* and *nptlll* genes in different environmental samples

Test field/sample	Number of sample tested*	16S positive (571 bp)	<i>nptll</i> positive (129 bp / 795bp)	<i>nptIII</i> positive (82 bp / 795 bp)
Soil <sup>a</sup>	9	9	0/0	0/0
Fertilizer	3	3	0/0	0/0
Soil from pig farms	12	12	0/0	0/0
Faeces from pig farms	9	9	0/0	0/0

<sup>\*</sup>Three parallels from each field

## 4. Discussion

In the present study we analyzed the natural occurrence of aminoglycoside phosphotransferase genes aph(3`)-IIIa and aph(3`)-II, (nptII and nptIII), in selected environments in the northern part of Norway. Phenotypic antibiotic resistance is common in soil bacterial communities and up to  $10^5$  bacteria per gram soil were reported to be resistant to kanamycin in a study by Smalla et al (Smalla et al., 1993). This was also the case in our study. 0 to 12% of the cultivable bacteria from the different samples tested were resistant to kanamycin at a concentration of  $100~\mu g/ml$ . This is also in accordance with other studies published (Gebhard and Smalla, 1999, Ma et al., 2011, Woegerbauer et al., 2015b, Woegerbauer et al., 2014).

The *nptll* or *nptlll* genes could not be detected in the kanamycin resistant bacterial colonies examined or in our total DNA sample from the different sample sites. These results demonstrate low prevalence and is comparable to other studies (Woegerbauer et al., 2015b, Smalla et al., 1993, Ma et al., 2011, Leff et al., 1993). Thus, the Km<sup>R</sup> bacterial isolates examined in this study will either be intrinsically resistant of have already acquired other mechanisms to overcome Km exposure.

NptII is used as a selectable marker gene in GM-plants. However, none of our sample sites are affected by GM plant cultivation or release. As previously discussed, recombinant DNA released by GM plants used at a large scale level could enhance the potential for horizontal gene transfer from plants to soil and soil microorganisms (Conner et al., 2003, Nielsen and Townsend, 2004, Woegerbauer et al., 2014). Several groups have studied the presence of recombinant DNA in soil cultivated with GM plants containing the nptll gene (Bonadei et al., 2009, Gebhard and Smalla, 1999, Hay et al., 2002, Ma et al., 2011). Gebhard and Smalla (Gebhard and Smalla, 1999) demonstrated long-term persistence of transgenic DNA from GM sugar beets under field conditions (up to 2 years). In studies with GM poplar, containing nptll gene, Hay and colleagues (Hay et al., 2002) demonstrated long term persistence of transgenic DNA from decomposing GM poplar trees. Bondei and colleagues (Bonadei et al., 2009) detected recombinant DNA sequences in total DNA extracted from soil samples taken at different times in a period of 20-months, after planting GM poplar. In a study published in 2011 by Ma et al. in Canada, a 3-year field trial was set up to assess the occurrence of gene transfer from a GM corn line containing the nptll gene compared to a near isogenic conventional line. The nptll gene was not detected in any of the Km<sup>R</sup> isolates screened by PCR and seemed to appear relatively rare in total DNA isolated from the same area that was also analyzed by PCR. None of these studies could demonstrate an increase of resistance in the fields upon cultivation of GM plants containing ARM genes. Gene transfer from GM plants to bacteria via natural transformation remains undetected under field conditions (Heinemann and Traavik, 2004, Nielsen and Townsend, 2004, Badosa et al., 2004, Demaneche et al., 2008, Conner et al., 2003). However, it is clear from mathematical analyses that the different methodological approaches used so far have many limitations that exclude the opportunity to estimate the occurrence and impact of horizontal gene transfer in short time frames (Nielsen and Townsend, 2004, Nielsen et al., 2014). Factors contributing to gene transfer reported in the literature includes the long-term DNA persistence in soil (Lerat et al., 2005, Lerat et al., 2007) and the presence of competent bacterial cells in soil (Sikorski et al., 2002).

It is evident that high concentrations of antibiotics can select for resistant bacterial cells (Drlica, 2003). Recent studies have also discussed the importance of low antibiotic concentrations and how such concentrations can enrich and maintain resistance traits in bacterial populations, in many natural environments (Gullberg et al., 2011, Andersson and Hughes, 2012, Sandegren, 2014, Martinez, 2009).

Low concentrations of antibiotics in the environment may be due to antibiotic-producing microorganisms or it may be present in animal/human feces because of therapy or prohylaxis. In our study, we investigated different fields with different exposure of organic fertilizers, to see if it could provide (co)selective condition that could favour bacteria harbouring <code>nptll/nptlll</code> 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 et al (Woegerbauer et al., 2015b) did in a similar study in Austria. A positive correlation between the prevalence of <code>nptlll</code> in the soils samples and the application of organic fertilizers of animal origin was observed by Woegerbauer.

# **4.1 Uncertainties in assaying ARMGs**

There are many uncertainties connected to assaying ARMGs in natural environments. Many of these uncertainties are due to technical limitations and data interpretation, e.g. due to resource limited sampling and detection efforts (Heinemann and Traavik, 2004, Nielsen et al., 2014).

**In depth analysis of soil parameters:** To be able to cover a large range of bacterial diversity in soil samples thoroughly, in depth analysis of soil parameters should be included.

**Sampling and sample size**: Representative sampling of soil is important for studying microbial diversity. Despite a good and systematic sampling strategy, the high number of bacterial cells per gram and sampled material limit the capacity to achieve a representative sampling of agricultural fields and animal herds/farm systems.

**Cultivation based methods**: Culturable bacteria, obtained from plate screening, represent only a minor fraction of the bacterial populations of the tested habitats. It is methodologically challenging to assign a positive PCR result to a particular bacterial host in a highly composite sample.

**Molecular methods**: The detection limit of a given molecular method is rarely zero. Thus, given that the sample size is limited per analysis, current analyses are not scaled to detect prevalence levels below 1 *nptll* copy per 0,1-1 g of soil or feces sample.

**Estimate exposure levels:** The extent to which microbial communities are exposed to plant material over a crop-growing season is at present not understood at the quantitative or qualitative level.

#### 5. Conclusions

The samples tested in this study suggests that the naturally occurring background of *nptll* and *nptlll* genes in local environments in Tromsø appears to be very low and not detectable. We also conclude that given the low levels of these specific genes we cannot exclude that the introduction of new/external sources of such genes will not increase the prevalence level over time.

# 6. Follow up/recommendation

There are still significant knowledge gaps, as well as uncertainties, in assaying ARMGs in natural environments. With the limited number of experimental studies available to resolve the uncertainties arising from introduction of ARMGs, we suggest some areas that are important to follow up in further studies. These areas should include:

Sampling area: Further information on the natural occurrence of *nptll/nptlll* in different relevant Norwegian environments is necessary to be able to provide quantitative assessment of the possible risk of introducing ARMGs into Norwegian environments. In addition, more information about the presence of ARMGs in soil cultivated with GM plants containing the *nptll* gene with soil cultivated with a non-GM counterpart is desirable. Samples from soil cultivated with GM plants are not available in Norway and need to be collected outside Norway.

**Antibiotic concentration**: Knowledge of the concentration of relevant antibiotics and biocides that can select for resistant bacteria in the environment need to be determined.

**Identification of selective conditions**: Knowledge of different selective conditions for rare bacterial transformants is important in order to understand and predict the ecological and clinical outcomes.

**Genetic barriers:** Understanding leakage of genetic barriers to the horizontal gene transfer of transgenic DNA between unrelated donors and recipient bacteria.

More experimental and epidemiological data in general, are needed on the distribution of ARMGs, mechanisms for resistance development and antibiotic usage pattern from Scandinavia and Europe.

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