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Prevalence of *nptII* genes in three different soil fields in Tromsø

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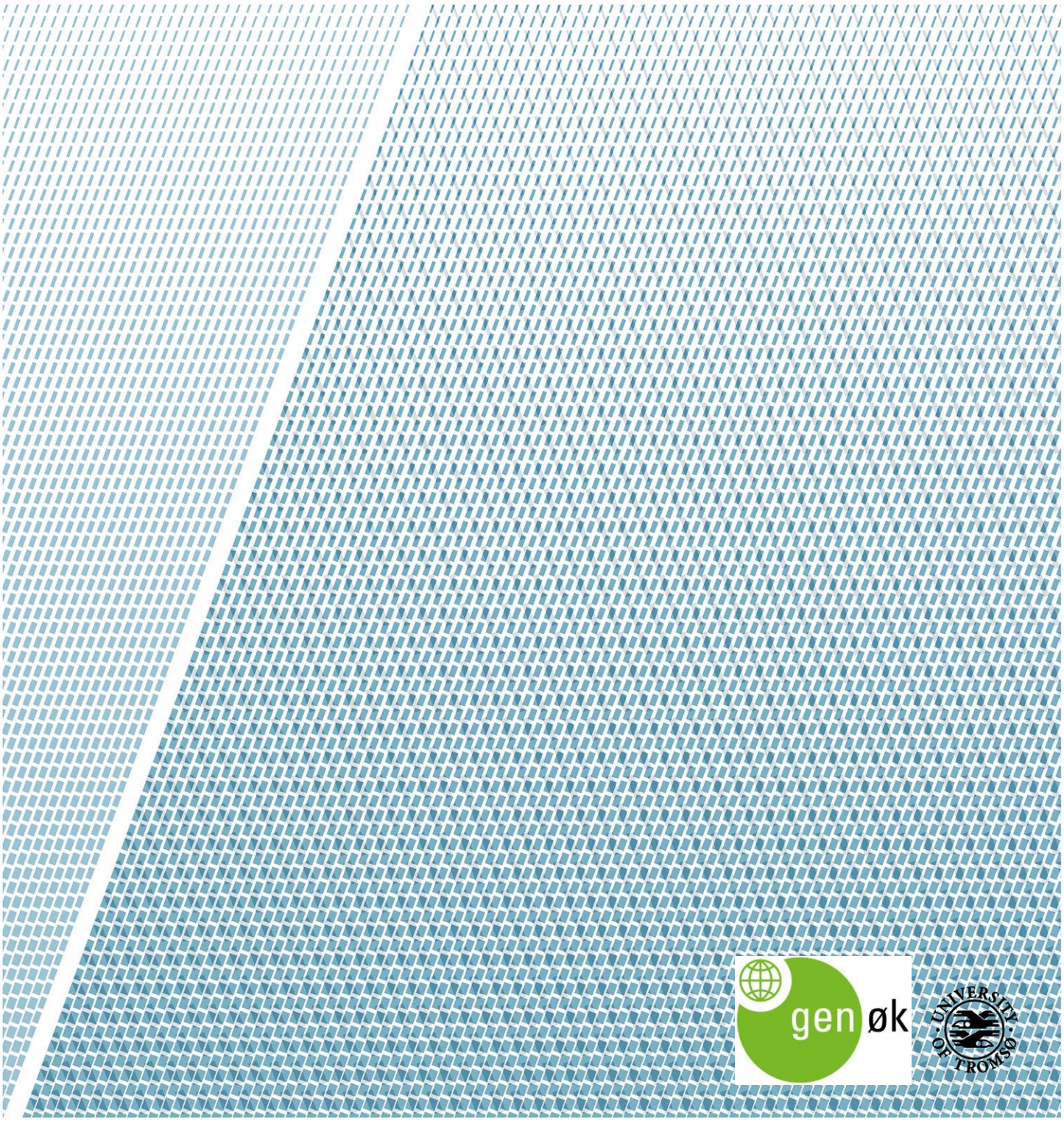

MASTER THESIS FOR THE DEGREE MASTER OF PHARMACY

Prevalence of *nptII* genes in three different soil fields in Tromsø

BY
ANDREAS ELLINGSEN STEFFENSEN
MAY 2015

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Abstract (English)

The Worlds health organisation (WHO) stated in 2012 that antibiotic resistance is one of the three biggest health threats the world faces. Generally there is little knowledge about the rate and span of antibiotic resistance in different environments in Norway. Natural environments like soil, fresh water, sea water and sediments are today not examined with emphasis on antibiotic resistance. There is also little knowledge as far as antibiotic resistance marker genes (ARMG) used in the manufacturing of GM plants can have an impact on the current levels of antibiotic resistance.

ARMGs are used as a selection marker in the manufacturing of GM plants by identifying seeds that successfully incorporated the DNA granting them the wanted traits.

The most used ARMG is *nptII* that confers a aminoglycoside phosphotransferase that grants resistance towards aminoglycosides like kanamycin, neomycin, paromomycin, butirosin, gentamicin B and genticin. EUs own health and environment authority (EFSA) have in a report concluded that *nptII* can be used without restrictions. This conclusion is partly based on the already high findings of *nptII* in bacterial populations.

To be able to evaluate the potential impact the release of GMO that contains *nptII* as a selective marker, it is important to study the incidence of *nptII* in soil with varying exposure to antibiotics, e.g. grazing lands for livestock, woodlands etc including wildlife, birds and water. There are just a few studies available that say anything about the rate of *nptII* in different environments in Europe, but most of the peer-reviewed studies shows low findings of *nptII* in bacteria in non-clinical settings.

In this study, the main goal has been to establish methods to be able to study the rate of *nptII* in soil in Tromsø, in aerobe cultivation of bacteria and in soil DNA. Soil is a complex and diverse environment for bacteria, and it has all the qualities required to be a potential reservoir for antibiotic resistance genes. In collaboration with Bioforsk Tromsø we have gathered soil samples from soil where it has been used manure yearly, soil where it has been approx. seven years since it was used manure and soil where it has never been used manure as fertilizer.

Out of totally 10^6-10^7 cultivable bacteria from the different soils, our results show that approx. 3-12% of the bacteria are Km resistant but none of the approx. 550 Km resistant bacteria we have analysed carry the *nptII* gene. Since only 1-2% of soil bacteria can be grown we also did a direct analysis of soil DNA from the three separate fields by isolating and purifying DNA from soil before running qualitative and quantitative PCR. We did not detect any *nptII* in any of the soil samples.

The conclusion of our pilot study is that there is no or low rate of the *nptII* gene in bacteria and soil DNA in the three separate soils we have examined in Tromsø. Further continuation of this pilot study would be to expand it and examine different relevant environments considering the rate of *nptII* and also to examine the concentration of kanamycin in the same environments.

Keywords: *nptII*, soil, kanamycin, ARMG

Abstract (Norwegian)

Verdens helseorganisasjon (WHO) slo i 2012 fast at antibiotikaresistens er en av de tre største helsetruslene verden står overfor. Generelt eksisterer det lite kunnskap om tilstedeværelse og omfang av resistens i ulike miljø i Norge. Naturmiljø som jord, ferskvann, sjø og sedimenter er i dag lite undersøkt med hensyn på forekomst av resistens. Videre er det liten kunnskap om i hvilken grad antibiotikaresistensgener (ARMG)brukt i framstillingen av genmodifiserte planter vil kunne påvirke antibiotikaresistens situasjonen. ARMG brukes som seleksjonsmarkør under produksjonen GM planter til å identifisere frø som har lykkes i og ta til seg nye gener. Det mest brukte ARMG er *nptII* genet koder for en aminoglykoside fosfotransferase som gir resistens mot antibiotikum som kanamycin, neomycin, paromycin, butirosin, gentamycin B og genticin. EUs eget helse- og miljørisikovurderingsorgan EFSA har i en rapport konkludert med at *nptII* kan brukes uten restriksjoner. Konklusjonen er bl.a. basert på at det allerede er høy forekomst
av *nptII* i bakteriepopulasjoner. For å kunne evaluere hvilken potensiell betydning utslipp av GMO som inneholder nptII som markørgen vil ha, er det viktig å studere forekomsten av *nptII* i jord med ulik grad av eksponering for antibiotika, eks. beiteområder for husdyr, skog, etc. i tillegg til viltlevende pattedyr, fugler og vann. Det er veldig få studier tilgjengelig som sier noe om forekomsten av *nptII* i miljø i Europa men de fleste tilgjengelige fagfellevurderte studiene viser til lav forekomst av *nptII*-genet hos bakterier i ikke kliniske miljøer.

I dette sudiet har hensikten vært å etablere metoder for å kunne studere forekomsten av nptII i jord i Tromsø, hos aerobekultiveringsbare bakterier, og i jord-DNA. Jord er et komplekst og mangfoldig miljø for bakterier og har alle kvaliteter for å kunne være et potensielt reservoar for resistensgener. I samarbeid med Bioforsk Tromsø har vi samlet inn jordprøver fra jord hvor det i dag er bruk av husdyrgjødsel, jord hvor det tidligere har vært bruk av husdyrgjødsel (ca 7 år siden) og jord hvor der ikke har vært noen bruk av husdyrgjødsel
Av totalt 10^6-10^7 kultiverbare bakterier i de ulike jordområdene viser resultatene våre at ca 3-12% av bakteriene er Kmresistente, men at ingen av de ca 550 Km-resistente bakteriene vi har analysert er bærere av nptII-genet. Siden kun 1-2% av jordbakterier kan dyrkes har vi også gjort direkte analyse av jord-DNA fra de tre ulike feltene, ved å isolere og rense DNA fra jord og videre kjøre kvalitativ- og kvantitativ PCR. Heller ikke her har vi observert forekomst av nptII i noen av prøvene fra de ulike jordområdene.

Konklusjonen av vårt pilotstudie er at det er ingen eller lav forekomst av nptII genet i bakterier og jord-DNA i de tre ulike jordområdene vi har undersøkt i Tromsø. Videre oppfølging av dette pilotstudiet vil være å utvide studien og undersøke
ulike relevante miljø med tanke på forekomst av nptII genet og også studere konsentrasjon av kanamycin i de samme miljøene.

Nøkkelord: *nptII*, jord, kanamycin, ARMG

1. INTRODUCTION

1.1 Antibiotic resistance marker genes (ARMG)

The combination of antibiotic resistance genes and antibiotic is an important tool in genetic engineering in general and in plant biotechnology in particular (1).

During the process of genetic modification of plants or other organisms, marker genes is used to facilitate the selection and identification of the cells/seeds that has been successfully modified from those who did not undergo transformation (Figure 1). These marker genes are most often either herbicide tolerance or antibiotic resistance genes (ARMG) (2).

After the transfer of DNA to the plant cells the plant culture media, to verify which cells had successfully transformed, the plant culture media would have antibiotics added to it if an antibiotic resistance gene was used as a marker. The cells that survived the addition of antibiotics have at least had uptake of the resistance gene, and likely the rest of the genes. Moreover, this is a part of the debate is the fact that beyond the laboratory the marker gene serves no purpose. However the use of ARM has been one of the go-to methods in order to separate the completed transformed cells and those who failed.

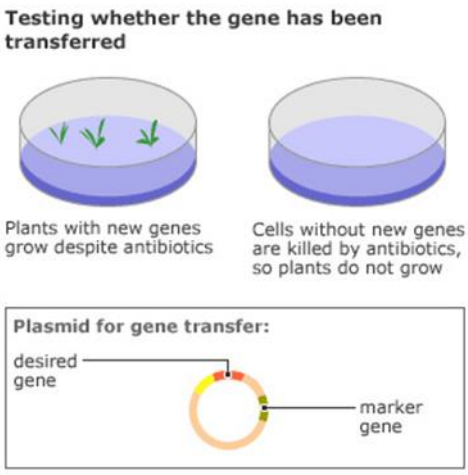


Figure 1: Antibiotic resistance marker gene concept.
http://news.bbc.co.uk/2/shared/spl/hi/pop_ups/03/sci_nat_how_a_plant_is_genetically_modified/html/5.stm

1.1.1 Types of ARMGs used in GM plants

The European food safety authority (EFSA) has divided the ARMGs used in GMO into three groups using two criteria. The first considers whether or not the given gene is already widely distributed amongst soil, water, plant and enteric bacteria. The second criteria is whether or

not it confers resistance to anything that has therapeutic relevance towards human and veterinary medicine.

Group one contains genes that are considered to be already widely distributed in soil and enteric bacteria and confer resistance to antibiotics that have no or minor therapeutic relevance for both human and veterinary purposes. These genes are considered extremely unlikely to impact the spread of resistance towards antibiotics and in turn have no significant impact on human or veterinary medicine.
Group one contains genes *nptII* and *hpt,* that confer resistance to the antibiotics kanamycin,neomycin, paromycin, butirosin, gentamicin B,geneticin or hygromycin, respectively.

Group two contains resistance genes that are widely distributed in microorganisms in the environment and confers resistance towards antibiotics that are used in defined areas of human and veterinary medicine. They are therefore likely to have a minimal effect on the spread of these resistance genes and minimal impact on human and veterinary medicine, if any at all.
Group two contains following genes *Cmr (cat), Ampr* (*bla*TEM-1) and *str* (*aadA),* which confers resistance to the antibiotics chloramphenicol, or ampicillin or streptomycin/spectinomycin, respectively.

Group three contains resistance genes that are considered highly relevant for human therapy and regardless of the realistic value of the threat should be avoided in transgenic plants to ensure the highest standard of preventative care.
Group three contains genes *nptIII* and *tetA,* which confer resistance to the antibiotics amikacin or tetracyclines, respectively (3, 4).

1.1.2 Main traits introduced in GM plants using ARMG

Some of the main traits that have been transferred to plants since the introduction of GM crops in the mid 1990's is insect resistance based on a toxin from *Bacillus thuringiensis* (*Bt*), herbicide (glyphosate) resistance, resistance towards viruses, oil quality and male sterility (5). These traits have been inserted into crops such as maize, soy, cotton, canola and sugar beets. More than 80% of the global transgenic crops grown annually contains glyphosate resistance,

and until glufosinate resistance was introduced, all GM soy in the US contained only glyphosate resistance (6). Introducing resistance towards the herbicide glyphosate allowed farmers to apply herbicide to their entire crops and killing only weeds in the process that allowed for more efficient farming. Another problem addressed with the introduction of transgenic crops was one concerning insect pests infecting crops. With the introduction of *Bt* toxins, pests that eat the crops would die when the toxin would be activated by low pH in the midgut of the gluttonous insect causing pore formation and death (7).

Transgenic crops are not limited to just one trait. If there is more than one trait introduced to a plant, it is 'stacked'. Stacking is the term used when several traits are applied to the same crop. The percentage of crops with stacked traits has increased rapidly. GM maize with stacked traits grew in USA from 1 % of maize acres in 2000 to 71 % in 2013. Stacked GM cotton accounted for 67 % of cotton acres in 2013 (8).

1.1.3 *nptII*, kanamycin/neomycin resistance gene in GMO

The *nptII* (also called *aph-(3)`-IIa*) gene confers resistance towards kanamycin and neomycin is the most used ARM in transgenic plants (9). *nptII* was first discovered on the Gram-negative transposon Tn5 (10), and gives the bacteria the ability to produce an enzyme called neomycin phosphotransferase II (Figure 2) that can inactivate the aminoglycosides kanamycin and neomycin through phosphorylation.

The genes *nptI*(*aph-3'-I*) and *nptIII*(*aph-3'-III*) also confers resistance to other aminoglycosides but have not been used as much in transgenic plants after EFSA put *nptIII* in group three, stating that it was '*Highly relevant for human therapy'* (3, 11, 12).

nptII is seen in transgenic plants such as MON 863, the potato EH92-127-1 and cotton MON 531 (13-15).

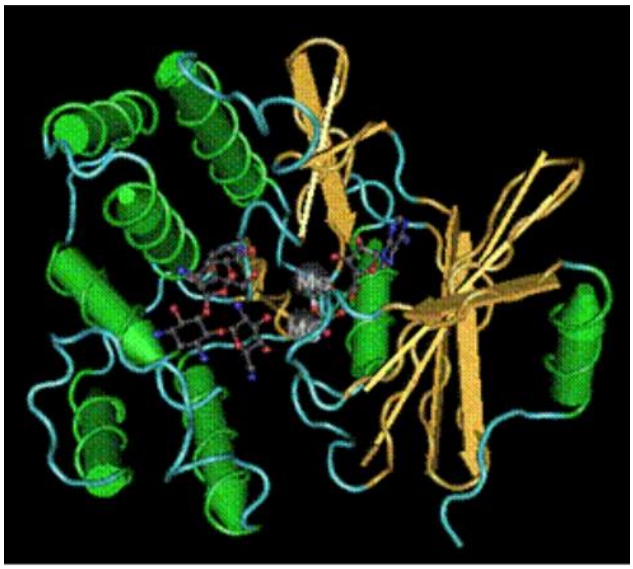


Figure 2: 3D structure of the neomycin phosphotransferase II(11)

1.1.4 Aminoglycosides and their mechanism of action

Aminoglycosides (several are shown in Figure 3) are composed of one or several aminated sugars joined in glycosidic to a dibasic cyclitol. Of these gentamicin, tobramycin and amikacin are among the most commonly used. In Norway streptomycin, gentamicin and tobramycin are the only ones currently registered (slv.no May 2015).

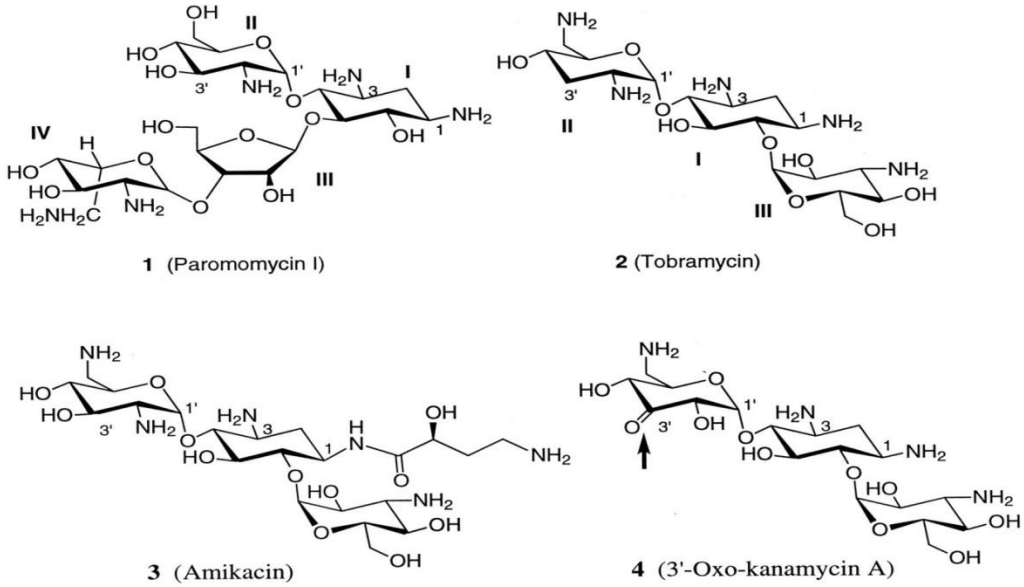


Figure 3: A selection of aminoglycosides
http://aac.asm.org/content/44/12/3249/F1.expansion.html

Aminoglycosides have a broad spectrum of activity covering gram-negative (for example *Enterobacteriaceae, Pseudomonas spp, Acinetobacter spp, and Haemophilus influenza*) and gram positive bacteria (*Staphylococcus aureus, Streptococci*), as well as some mycobacteria. Against gram-positive bacteria it is seldom viewed as efficient enough for monotherapy, and

will usually be used in synergy with another cell-wall active drug, such as beta-lactams and vancomycin. However anaerobic bacteria have an intrinsic resistance to aminoglycosides. They penetrate gram-negative cells by disrupting Mg^{2+} bridges between neighbouring lipopolysaccharide channels, this is the synergic point with other cell-wall active antibiotics like vancomycin.

Aminoglycosides work by impairing protein synthesis within the bacteria by binding to prokaryotic ribosomes such as the aminoacyl I binding site on the 30S subunit (Figure 4). This causes misreading of its genetic code, a bactericidal effect. It does not stop the binding of the 50S subunit or the binding of mRNA. The first cycle goes as it should, but elongation fails due to the failure of ensuring translational accuracy (16, 17).

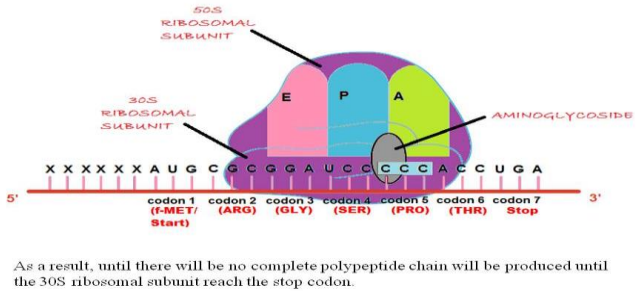


Figure 4: Aminoglycosides mechanism of action
https://www.webmedcentral.com/articlefiles/0a1d02abf8a991924c2d6e08e1289e8e.jpg

1.1.5 Clinical uses of aminoglycosides

The use of aminoglycosides in Norway are mostly seen in hospitalized patients, especially ones with severe infections such as sepsis, severe pneumonia, intra-abdominal infections and some cases of meningitis where they are given intravenously since gentamicin and tobramycin can't be absorbed in the GI tract (18, 19).

Tobramycin is used to prevent or treat eye infections and inflammation post cataract surgery.

Kanamycin (Figure 5), which we used in this study, has not been used much in a clinical setting due to its toxicity profile and that other aminoglycosides has had a broader spectrum, a more researched profile and less severe side effects. Kanamycin has been used in veterinary medicine, and European medicines agency (EMEA) has established a dosage table for bovine, chicken and rabbits (20). It was moved into the class of 'critically important' by WHO in 2011 due to increasing cross-resistance within the group of antibiotic group of aminoglycosides as well as the fact it has was adopted into the program against multi-drug

resistant (MDR) tuberculosis (21). In some countries it is first choice injectable aminoglycoside due to its price and because it is readily available.

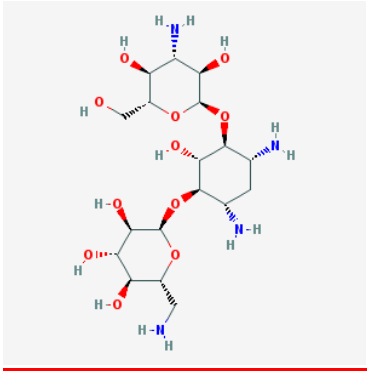


Figure 5: Structure of kanamycin
http://pubchem.ncbi.nlm.nih.gov/compound/kanamycin#section=2D-Structure

Aminoglycosides have two major toxicity points, ototoxicity and nephrotoxicity. Ototoxicity ('ear' toxic) includes a general loss of hearing, tinnitus and balance disorders. These effects can occur unpredictably, after a single dose or even months after a completed treatment with aminoglycoside antibiotics (22). Due to the groups' nephrotoxicity ('kidney toxic)' the patients' renal functions should ideally be unimpaired, but a physician should make individual assessments in each case.

1.1.6 Antimicrobial resistance towards aminoglycosides

Resistance towards aminoglycosides is a problem, especially since they have such a broad spectre of effect. Norway does not have the same problems regarding antibiotic resistant microbes as seen in other parts of the world. The latest NORM (Norwegian surveillance system for antibiotic resistance in microbes) report stated "*Antimicrobial resistance is still a limited problem among clinically important microbes in Norway. The relatively low usage of antimicrobial agents as well as appropriate patterns of use must be maintained to preserve this rather favourable situation* (23)."

There are three ways a bacteria can be resistant towards antibiotics; natural resistance, environmental resistance and acquired resistance (24). Natural resistance can simply be that it does not have the antibiotic attack site or is unable to absorb the antibiotic. Environmental resistance covers outside factors like temperature or pH that hinders the antibiotic effect. Aminoglycosides can be affected by lack of oxygen, pH and divalent cations. Acquired resistance is mainly a result of horizontal gene transfer (25).

There are several means of acquired resistance towards aminoglycosides; modifying enzymes such as neomycin phosphotransferase II gained through the gene *nptII/aph(3)-II*, alteration of chromosome binding sites and altered aminoglycoside uptake/efflux pumps which confers cross-resistance and change in cell membrane due to e.g. mutations. Figure eight shows points where aminoglycoside modifying enzymes can deactivate kanamycin B with a different array of enzymes; acetyltransferases (AAC), phosphotransferases (APH), and nucleotidyltransferases (ANT)(26). The above-mentioned enzymes all have different regiospecificities (Figure 6) and different versions of the enzyme. The *nptII* gene is said to be found everywhere in nature since the resistance it grants is natural, but Shaw et al only found *nptII* in 2.5 % of its isolates (27).

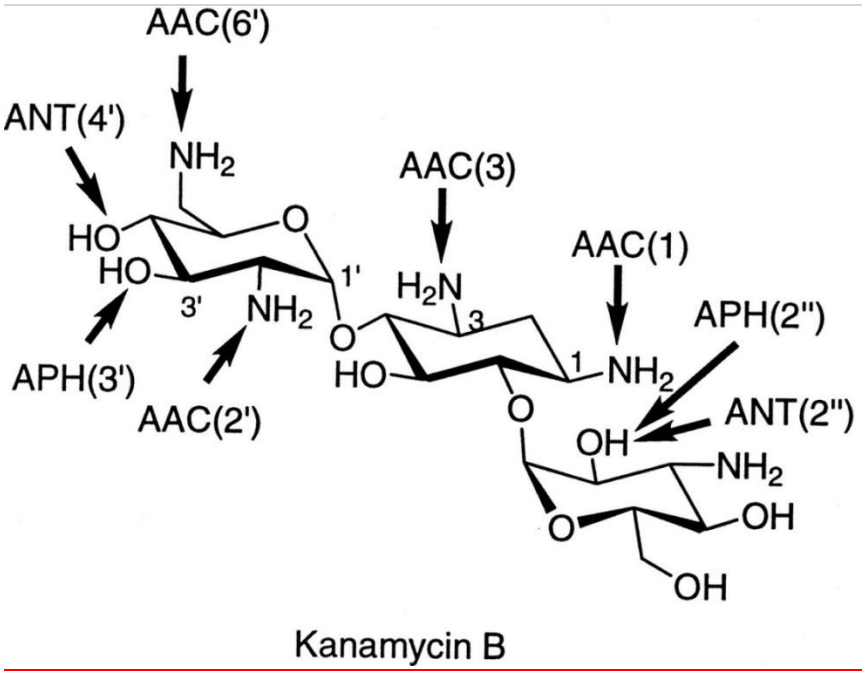


Figure 6: Points of enzymatic modifications conveying resistance
http://aac.asm.org/content/44/12/3249/F5.expansion.html

Figure 6 shows kanamycin B that has an NH_2 group replacing an OH group where it indicates a site for AAC(2'). Figure 2 showed 3-oxo kanamycin, where the OH group at APH(3') has been replaced with a double bonded O. We have used kanamycin A (Figure 5) in our study.

1.2 Prevalence of *nptII* in the environment

There have been some studies done to check whether or not *nptII* is to be found everywhere as it seems to be expected. A study done on 355 bacteria isolated from river water (Rhine), soil, pig manure and sewer spillage from the Netherlands. They found a low percentage of *nptII* in sewage and pig manure. Those were *Pseudomonas* (9,1 %), *Xanthomonas* (10 %),

and the families *Enterobacteriaceae* (30 %) of *Vibrionaceae* (33,3 %) (10). Leff et al. found *nptII* genes in 3 out of 184 kanamycin-resistant isolates in river water from The Runs Creek, USA. Their environmental DNA screening found no *nptII*, but it was abundant with *nptII* like sequences (28). De Vries found that the *nptII* was quite abundant in DNA derived from soil as it varied from 2 x 10^5 and 3 x 10^8 molecules per gram of (dry weight) soil (29). In Austria during the period 2008-2011, 10 429 clinical isolates were tested for both *nptII*, where the found prevalence was 0.0096% and for *nptIII* 10 440 clinical isolates were tested and 1.62% harboured *nptIII (30).*

A study covering 26 Europan bacterial habitats which included bulk and rhizosphere soils, manure from farm animals, activated sludge from wastewater treatment plants and seawater, where they looked for streptomycin resistance found that the phosphotransferase gene *aph(6)-1d* was the most prevalent whereas the presence of the other four (*aph (3")-1*, *aph (6)-1c*, *ant (3")-1* and *ant (6)-1*) genes were found in 58% of the tested habitats. Only 8% did not possess any of the selected genes (31).

A study in China found ARMGs in synthetic plasmid vectors, used widespread in laboratory setting, in six rivers (32). Another study conducted ion the Baltic Sea on 49 bacteria isolated from surface water when exposed to 11 antibiotics found that 88 % showed resistance towards tetracyclins, 71 % had resistance towards NAL, 69 % towards streptomycin and 65% for both ampicillin and kanamycin (33).

The prevalence of *nptII* in the environment seems to be less than anticipated. The studies mentioned all finds resistance towards kanamycin in varying degrees, but resistance towards kanamycin is not necessarily due to the *nptII*. If anything from the mentioned studies it seems *nptI* is more frequent in the environment.

1.3 Main concerns with respect to the presence of antibiotic resistance marker genes in GM plants

Some of the questions regarding the use of ARMG:

Is the gene itself harmful to humans or animals?

Is the protein produced by the gene harmful to humans or animals?

Can ARMGs be transferred over to bacteria and impact antimicrobial therapy?

‘Regular genes,' genes that are found un-altered are not dangerous to consume, so it follows that ARMGs that have been designed by scientists are considered equally as harmless. The ARMG DNA will be digested and processed in the gut just like any natural DNA (34). The produced proteins from the ARMG are not fundamentally different from other dietary proteins and will like the DNA be processed like other naturally occurring proteins.

The third question is where today’s debate lies. Attempts to confirm horizontal gene transfer (HGT) from plants to soil microorganisms in the environment has failed or been inconclusive (35, 36), but HGT from a transgenic organism to a recipient organism has been detected, but not without constructs (e.g. a modified *nptII* with an internal deletion) that has significantly similar sequences to plant transgenes (e.g. *nptII).* This influences the event by homologous recombination to boost its detection of transfer (37).
Risk assessments of GM organisms also consider the possibility of HGT of recombinant DNA inserts (38). So large-scale cultivation of GM plants on approximately 170 million hectares worldwide gives multitudinous opportunities for bacterial exposure to recombinant DNA and following opportunities for unintended HGT (39, 40).
But given the generally low mechanistic probability of HGT of non-mobile DNA in complex environments such as soil, HGT events will initially be present at such a low frequency in the overall bacterial population that it might take months or years for the first transformed cells to divide, and out-compete non-transformed bacteria so that it can be efficiently detected by a sampling design (41). So regulatory instances regard HGT as a possibility and as a possible threat to health but given the aforementioned very low possibility of HGT, and that HGT research suffers from significant methodological limitations, knowledge gaps and model uncertainty (42) leads to what some consider a false sense of security (43). The study done in six rivers in China showed that *blà* genes(ampicillin resistance) from synthetic plasmids found in one of the rivers gave resistance towards third and fourth generation cephalosporin drugs. The antibiotic marker-containing plasmids had been used extensively before ARM-free plasmids were introduced. They concluded ‘*The data from our study suggest that pollution of synthetic plasmid vectors-sourced drug resistance genes in rivers may be another cause of drug resistance in animals and humans. Therefore, the potential hazards of environment release of synthetic plasmid vectors and genetically modified products containing the vector components should be given more attention (32).’*
There has also been shown that natural competent bacteria can take up and incorporate fragmented/damaged DNA segments as short as 20bp from a 43 000 year old wooly

mammoth. It seems that short DNA does not encounter the same barriers as longer DNA fragments, so that they are easier incorporated into the genome of competent bacteria (44). One side wants the usage of ARMGs and transgenes in general put on hold until science has caught up and has a good grasp on what effects usage of ARMGs and other transgenes has on different parts of the environment, whereas the other side seems to think it is so rare that it's hard to give concrete evidence to legislators.

1.3.1 Horizontal gene transfer (HGT)

There are three ways bacteria can exchange genetic material (Figure 7), one of which is through transformation. During transformation, exogenous DNA is taken up by competent bacteria and transported through its cell membrane and incorporated into their genome. This DNA can come from animal excrements, decaying plants or just other dead microorganisms. DNA from GM plants was shown to be detectable two years after release (45). In order for the bacteria to transport the genetic material into the cell it has to be what is called genetically competent, which far from all bacteria are (46). At least 70 bacterial species distributed through all taxonomic groups have been found to be naturally transformable (25, 47).
A bacteria being competent is a temporary state, in order for a bacteria to be competent it has to express a set of proteins that's dedicated to uptake and following homologous recombination of the transforming DNA, e.g. a type IV pili as seen in gram-negative bacteria. To enter the competent state, the bacteria has to be exposed to certain factors or growth conditions, such as pheromones, antibiotic stress, high cell density or nutrient limitations. These signals then get interpreted and activate the expression of genes required for DNA uptake (47).

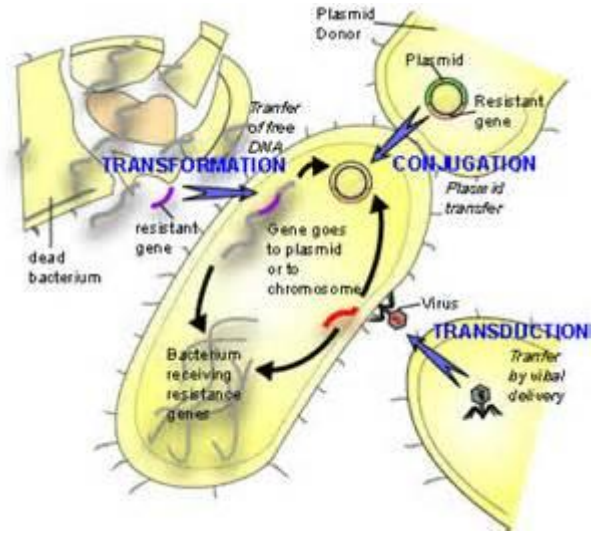


Figure 7: Concepts of transformation, conjugation and transduction
http://ts3.mm.bing.net/th?id=JN.OwUdxbyACejAbAAN%2BW5sYw

The second possibility is conjugation, where the bacteria through specialized means connect and a single DNA strand from a plasmid is transferred from a donor to a recipient. The donor bacteria would carry an F-plasmid, the plasmid that will be transferred. The donor then attaches itself to the recipient with a pilus. An enzyme called relaxosome then splits the plasmid into two strands, one of which the relaxosome is still attached to. The relaxosome then breaks down, and a part of it is still attached to the DNA strand and is called relaxase. The relaxase is recognized by a coupling factor and transferred over to the recipient through the pilus. When it is transferred, the relaxase then binds the ends of the T-DNA (the DNA strand transferred to the cell) into a circular DNA before both the donor and recipient synthesize the complementary strand and can continue to spread the plasmid.
Transduction is the third method is where DNA is transferred between bacteria without physical contact between the bacteria, through bacteriophages, viruses that infect bacteria. The bacteriophage infects the bacteria, breaks down it's bacterial genome and replicates its phage DNA. When it then makes new bacteriophages within the infected bacteria, bacterial DNA fragments may be packed in a phage. This phage containing bacterial DNA will then infect new bacteria where the DNA will be incorporated.
HTG in between prokaryotes was viewed as common, but now the growing evidence that HGT can happen between sexually incompatible is not only more widespread than accounted for but it also occurs between bacteria and plants (48).

1.4 Are there alternatives to ARMG?

The debate on ARMG in GM plants brings forth the question of alternatives to ARMG as selection markers, are there any? One alternative is to just remove the ARMG after it has done its job. This can be done by co-transformation, post-transformation, excision with recombinase systems or by only allowing transient expression. Maker removal techniques have limitations, they necessitate either more work and time or the preparation of more complex genetic structures or more difficult regeneration protocols (9).

Another alternative is marker free genetic transformation. It is the most straightforward strategies to obtain marker free GM plants because the need for preparation of complex vectors, recombinase or transposase enzymes can be disregarded. Its only problem is the exact reason selective marker genes such as *nptII* are used, a low transformation rate.
Agrobacterium is the method applied One of the highest reported transformation frequencies is 7 %.

The effort of screening without a ARMG is so time consuming and costly, however marker less transformation and co-transformation are capable of generating marker-free transgenic plants but the methods should be much improved to be practically applicable (49).

Reporter genes are another option, where fluorescent proteins have become by far the most successful reporter gene type and their scope of applications have become large. They have been isolated from many different organisms and mutated versions adapted to more specific experimental systems continue to appear (9).

1.5 Hypothesis

nptII genes are widely distributed in soil and can be detected through cultivation-based and DNA-based techniques.

1.6 Objectives

1.6.1 Major objectives

To determine the prevalence, distribution and characteristics of *nptII* genes among bacteria in three different soils in the western part of the tromsø island.

1.6.2 Minor objectives

1. Determine the prevalence of kanamycin resistance bacteria through cultivation dependent selection of Km resistant bacteria from soil samples.
2. Determine the prevalence of *nptII* genes responsible for kanamycin resistance in the culturable bacterial fraction through plating and PCR.
3. Determine if there are any differences between the prevalence of phenotypic and genotypic kanamycin resistance in the fields given their different fertilizing history.

2. MATERIAL AND METHODS

Table 1: Bacterial growth Media

Product	Brand	Product Code	Lot number
Standard Nutrient Broth no.1	Fluka	S4681-500G	BCBF2060V
R2A Agar	Merck	1.00416.0500	VM545416324
Brain Heart Infusion Broth	Fluka	53286-500G	BCBN2163V
Agar, Bacteriological Grade	Amresco	J367	1743C028

Table 2: Reagents, chemicals, kits and enzymes

Product	Brand	Product Code	Lot number
DNAse/RNAsefree water	Sigma	W4502	RNBD1580
Kanamycin	Sigma	K1876-5G	BCBC5819V
Cycloheximide	Sigma	C7698-5G	MKBR7099V
Sodium Chloride	Merck	1.06404.1000	K39301104842
PowerSoil	MoBio	12888-100	PS14D14
PowerClean	MoBio	12997-50	PCD14J7
DyNAzyme II	Thermo	F-508	00148904
Trizma Base	Sigma	93350-1KG	0001430002
Boric Acid	Sigma	B0394-1Kg	SZBD3090V
IDRANAL III (EDTA)	Fluka	34549	SZBA1380
SeaKem Agarose	Lonza	50004L	0000338935
Gel-Red	Biotium	41003	13G1218
Loading Dye 6X	Biolabs	B7021S	31302
Gycerol	Sigma	G5516-500ml	SHBD3108V
Sodium hydroxide	Sigma	30620	SZBA1880
Hydrochloric acid	Sigma	30721-1L	SZBB1490V
Taq-Man Environmental mix	Applied Biosystems	4398044	1410048
DNA Molecular Weight marker	Amresco	K180-205µl	30224C014
Quick Extract solution	Epicentre	QE09050	40457
Absolute Alkohol Prima	Kemetyl	256103	601439
Water, milliq grade			

Table 3: Experimental equipment

Use	Name	Brand
Thermal Cycler	S1000 Thermal Cycler	Bio-RAD
UV hood	Universal Hood II	Bio-RAD
Electrophoresis power supply	Consort EV231	Sigma
Nano Drop	NanoDrop 2000C	Therm
Colony counter	Starcount STC 1000	VWR
Desktop centrifuge	MiniSpin	Eppendorf
Vortexer		VWR
Mini centrifuge	Galaxy, Mini Star	VWR
Microwave		Whirlpool

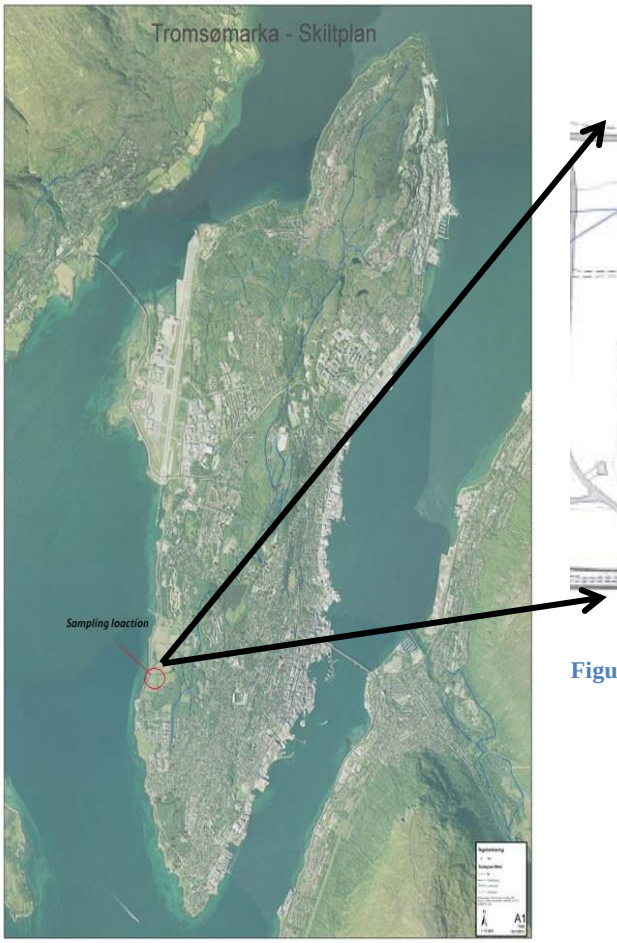
Table 4: Primers and probes

Target gene	Amplicon size	Primers/Probes	Gene Size	Sequence (5' - 3')	Ref	Producer
nptII	129 bp	NptII_F	795 bp	GATCTCCTGTCATCTCACCTTGCT	(30)	Eurogentec
		NptII_R		TCGCTCGATGCGATGTTTC		
		NptII_P		FAM-TCATGGCTGATGCAATGCGGC-BHQ-1	(30)	
	795 bp	NptII_Full_F		ATGATTGAACAAGATGGATTGC	(30)	
		NptII_Full_R		TCAGAAGAACTCGTCAAGAAGG	(30)	
16S rRNA	571 bp	16S_ F	1541bp	TGGAGAGTTTGATCMTGGCTCAG	(30)	
		16S_R		CTTTACGCCCARTRAWTCCG	(30)	

2.1 Soil sampling and processing

2.1.1 Selection of test fields

Three different local field of soil from the south-west past of tromsøya were selected in collaboration with BioForsk Holt (Figure 8 and 9).



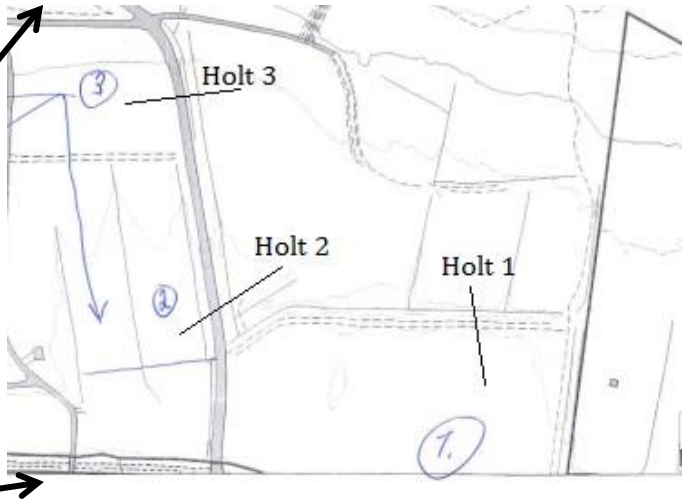


Figure 8: Holt 1-3 located at the encircled locations

Figure 9: Sampling location

These fields had different fertilizer history as listed below. In addition other data like soil characteristics, weather conditions during sampling and type of plants growing were noted.

Table 5: Field soil characteristics

Field	Characteristics	Plants	Fertilizer status	Date of sampling	Weather conditions
HOLT 1	Dark and dense	Grass	Yearly fertilization with animal waste	7/10-14 21/10-14	Sunny, slightly clouded and wet 5^0C
HOLT 2	Higher clay content	Grass	No fertilization the last seven years	9/10-14 21/10-14	Clear, frosty 3^0C
HOLT 3	Light, grainy and higher sand content	Grass	No use of fertilizer	10/10-14 21/10-14	Clear, frosty 3^0C **Clear, frosty, 2^0C**

Bold conditions apply to triplicate sampling

2.1.2 Soil sampling and processing

For determining the sampling locations a sample scheme using a grid model, described Andersen et al. was used in this study (50) (Figure10).

A rectangle was measured in the field 50 meters by 35-50 meters varying by the fields' size. Then from the corners, sides and diagonals of the rectangle, a total of 13 samples of approximately 2 ml were collected from about 5 cm depth with a sterile 10 ml modified syringe (50). The samples were stored in a beaker during transportation for approximately 5 minutes. Back in the lab the composite samples were weighed, and larger debris removed (roots, pieces of wood, etc).

One field was sampled a day initially, and those samples were the ones used validating our soil DNA protocols and screened for *nptII* using PCR with both *nptII* primers(soil samples set one). On a later occasion all three fields were sampled on the same day and these samples then got treated as a triplicate set with a corresponding pair of blanks (soil samples set two), undergoing all protocols together.

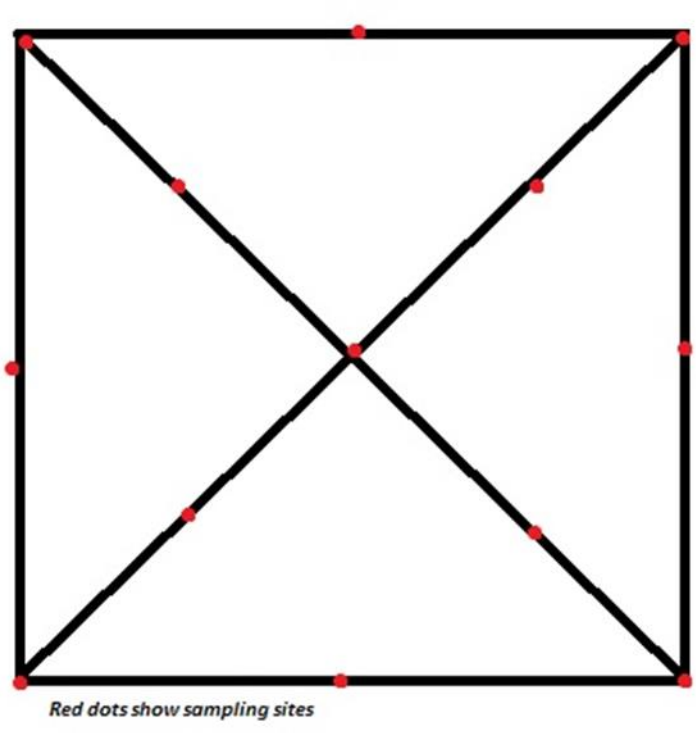


Figure 10: Sampling scheme. The center of the field and the sampling points are indicated in red.

2.1.3 Safety protocol to avoid cross contamination

To avoid cross-contamination throughout the whole soil sampling and processing procedure, which may lead to false positive results in the following sensitive TaqMan quantitative PCR analyses a strict safety protocol was followed. All the equipment used in the field (sampling syringes, beaker, spades) had been bleached (1:10 chlorine with distilled water) and wiped with disinfectant (70 % EtOH) and vinyl gloves were used during sampling.

2.1.4 Dry weight determination

Dry weight was measured in triplicate by heating one gram of soil in a heating cabinet at 70^0C for 24 hours. This was done for all the composite samples shortly after arriving at the lab.

2.2 Plating and testing for Km resistant strains

2.2.1 Media

The media used in this study was the rich media complete standard nutrition agar no.1 (Fluka), suitable for a broad range of bacteria and the low nutrient R2A broth (Merck), where a wide spectrum of bacteria can grow without being suppressed by fast-growing bacteria. Four agar solutions (two R2A and two SN1 solutions) were made, all of which contained 100 µg/ml cycloheximide (Sigma) to prevent the growth of fungi and one of each media were added 100 µg/ml kanamycin (Sigma) for selection of kanamycin(Km)-resistant bacteria.

By using different nutritional profiles in our agar, we want to grow a wider selection of different bacteria based on nutritional needs and increase our chances of finding the *nptII* gene.

The SN1 media was a broth and agar had to be added in order to make plates.

Table six and seven shows the composition of our agar solutions.

Table 6: Standard Nutrient No.1 agar composition

Components	Amount
Standard nutrient broth no.1 (SN1 broth)	25 grams
Agar	15 grams
H2O (Milliq)	1000 ml
Cycloheximide stock 10 mg/ml	10 ml (100 mg)
Kanamycin stock 50 mg/ml	2 ml (100 mg)*

*Only added to one of the solutions

The SN1 broth and the agar were weighed in, added to a one-litre bottle and then dissolved in 1000 ml water. It was then autoclaved, the filter sterilized kanamycin (Sigma), and cycloheximide (Sigma) was added and the bottle then stirred before pouring the plates. It was made one solution without kanamycin (SN1-) and one solution with kanamycin (SN1+).

Table 7: R2A agar composition

Components	Amount
R2A Agar	18,2 grams
H2O (Milliq)	1000 ml
Cycloheximide stock 10 mg/ml	10 ml (100 mg)
Kanamycin stock 50 mg/ml	2 ml (100 mg)*

*Only added to one of the solutions

The R2A complete media (Merck) were weighed in, added to a one-litre bottle and then dissolved in 1000 ml water. It was then autoclaved, the filter sterilized kanamycin (Sigma) and cycloheximide (Sigma) was added and the bottle then stirred, before pouring the plates. It was made both with and without kanamycin (R2A+, R2A-).

2.2.2 Stock Solutions

30 ml stock solution of 50 mg/ml kanamycin was made by dissolving 1, 5 grams of kanamycin (Sigma) in 30 ml of water and then filtering it sterile through 0.22 µm filters before it was stored at -20 ^{0}C in 15 ml falcon tubes.

200 ml stock solution of 10 mg/ml cycloheximide was made by dissolving 2 grams of cycloheximide (Sigma) in 200 ml of water and then sterilized using 0.22 µm filters. Due to its low solubility it was made in a 10 mg/ml concentration. It was then stored at -20 ^{0}C in 50 ml falcon tubes.

Cycloheximide is among the most used laboratory compounds to inhibit eukaryotic protein synthesis as it has been shown to inhibit the elongation phase of translation. It does so by binding to the ribosome and inhibits the eukaryotic elongation factor 2 (eEF2) surprisingly after one complete translocation instead of immediate total inhibition as one would expect (51).

2.2.3 Plating out

Only the soil samples that were collected on 21/10-14 were plated
Samples collected 7-10/10-14 were not plated.

2.2.3.1 Preparation of soil dilutions

- Three dilution series (F 1-3) from each field (Holt 1-3), a total of nine dilution series plated out
- 10^{-1} solution: one gram of soil was diluted to 10 ml in a 15 ml falcon tube with 0.9 % NaCl
- Further dilution to 10^{-6} was done by adding 100 µl of the 10^{-1} solution with 900 µl 0.9 % NaCl to give us 1000µl of 10^{-2}. This step was repeated with each new dilution to reach dilution of 10^{-6}. As shown in figure 11.

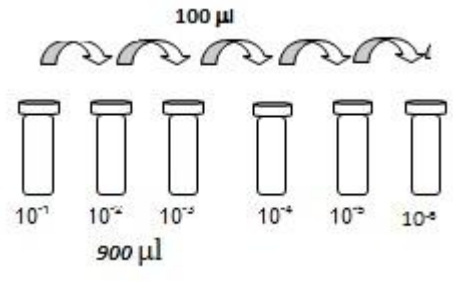


Figure 11: Serial dilution

- 50 µl per dilution was plated on both media with and without the added 100 µg/ml kanamycin (SN1-, SN1+, R2A- and R2A+)

- Plating of general aerobic cultivation was done with dilutions 10^{-1} to 10^{-6}
- Plating of kanamycin resistant cultivation was done with dilutions 10^{-1} to 10^{-5}
    - Ex; F 1 → Five SN1+, five R2A+ , six SN1- and six R2A-.
      A total of 22 plates plated for Holt 1, F1
- 50 µl of the 0.9 % NaCl solution was plated on SN1-, SN1+, R2A- and R2A+ as a contamination control

25 µl of our control strain was plated on each half of four plates (SN1-, SN1+, R2A- and R2A+) as a control to the effect of kanamycin.

2.2.3.2 Cultivation conditions

The plates were then stored at 23 ^{0}C for incubation for three days before counting

2.2.3.3 Counting colonies & Streaking/Incubation

Bacteria were counted with the help of a colony counter that provided a good background light as well as counting checkpoints during the count. The amount of bacteria on the plates went from too many to none. In cases where there were too many bacteria to get a reliable count the plate would be divided into half or into four and the count from the section multiplied accordingly.

When the plates had been counted 40 bacteria from each dilution series per media per field were streaked in an effort to separate the different bacteria. That gave 120 streaks from each media for Holt 1. The bacteria streaked were from the 10^{-1} to 10^{-3} plates.

Not all bacteria got streaked but a selection that reflected each fields bacterial diversity. Streaking is a technique where the goal is to isolate a pure strain from e.g. bacteria. It is done by picking a colony and smear it on an agar plate in several strokes. The goal is as stated previously to separate the bacteria by dragging them apart on the agar so that they grow as single pure colonies. Usually it is done in sectors, three or four lines, based on the amount of bacteria picked and when a new sector is streaked the first line will cross at least one line of the previous sector. Figure 12 outlines the idea. At the end of the streaking it will hopefully

grow pure colonies.

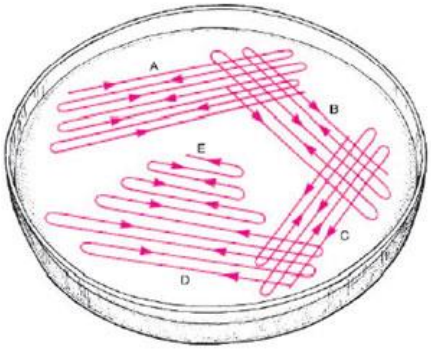


Figure 12: Example of streaking.

http://www.biocyclopedia.com/index/microbiology_methods/basic_techniques_biotechnologies/streaking_technique_obtain_pure_cultures.php

The plates were divided into eight and a picked colony got streaked on each part. Again they were five days to grow before growth and possible re-streaking due to lack of growth or if there were more than once type of bacteria in the streak.

2.2.3.4 Storing colonies

After the bacteria had been streaked to pure colonies they were picked with 10 µl tips and storied in Brain heart infusion broth (Fluka) with 20 % glycerol (Sigma) solution at -20 ^{0}C. 96 colonies was stored from each media and field (Holt 1-3), giving 192 stored samples per field, a total of 576 samples from all three fields.

2.3 Controls used in this study

All our controls strains were kindly provided by Julia Maria Kloos from the institute of pharmacy, UiT.

Strain *A. baylyi ADP1200Com* + Kan(R) /+ used as positive control (52).

Strain *A. baylyi ADP1200Com* + Kan(S) /- used as negative control.

A. baylyi Jv28KmR used as positive control for PCR screening of Km resistant bacteria and as positive control for soil samples in qPCR (37).

2.4 Preparation of Template DNA for PCR

2.4.1 Template DNA from bacteria

The Quick Extract (QE) solution breaks down the cell and the second round of heating inactivates the added enzymes as well as the enzymes released from the cell so that the DNA is intact for further downstream application.

- 96 out of 576 bacteria were streaked from the stored colonies at a time
- Incubated for 5 days in room temperature

- Then extracted with QE
    - Colonies picked with white sterile 1µl loops
    - Suspended in 100 µl QE
    - Vortex 15 seconds
    - Heated to 65^{0}C and incubated for 6 minutes
    - Vortexed for 15 seconds
    - Heated to 98 ^{0}C and incubated for 2 minutes
- No more than 4 PCR strips put through the QE protocol at the same time (32 samples) in order to prevent eventual breakdown of the DNA.
- One control *JV28 KmR* (one control if all samples were isolated the same day, if split over two days add a control for the second day)
- Stored at -20^{0}C until downstream application

If the bacteria were contaminated with other bacteria or did not grow after being streaked from the storage broth, the process was repeated. If they did not grow on the second try they were excluded.

2.4.2 Template DNA from soil

The template DNA for soil sample PCR was isolated with QE solution from *Acinetobacter Baylyi* and served as control for both 16S and *nptII*.

2.5 DNA preparation of total DNA from Soil

The Power Soil kit (MoBio) was used to remove PCR inhibiting substances from our soil samples such as humic acids, physical debree (small rocks etc), proteins and other possible contaminants while preserving the DNA in our soil sample.

Eight samples from each field (Holt 1-3) from soil sample set one plus two blanks were isolated with the PowerSoil kit (MoBio) the same day the sampling was done.

Soil sample set two, where all three fields was sampled the same day, here DNA was isolated from two samples from Holt 1-3 along with two blanks the same day as the sampling was done.

DNA was extracted directly from soil with the Power Soil kit (MoBio) as instructed by the protocol with two exceptions. After step four we included the troubleshooting step of incubation at 70^{0}C for 10 minutes and in step five we used FastPrep at sig m/s for 40 seconds. Apart from that the protocol was followed for all our soil samples (53). The isolated DNA was then stored at -20^{0}C.

2.6 DNA purification

After the initial PCR setups checking the different dilutions of the Power Soil DNA solutions it was decided that we purify the DNA, which was done with the Power Clean Kit (MoBio). One of the risks of purifying DNA is that there is a chance to reduce the total amount of DNA in our solution, but in our case it was assessed as safe as our target was *nptII* and loss of small DNA segments would not hurt our experiment.

The idea behind this step is to precipitate contaminants, spin them down into a pellet before fixating the DNA to a filter where it is rinsed before it is eluded from the filter into a clean tube.

The producers' protocol was followed with two exceptions (54). All spin times 1 min> was set to 1 minute, and the incubation at step 13 was extended to three minutes at 70^0C.

The purified DNA was then stored at -20^0C.

2.7 Measurement of DNA concentration by NanoDrop

The Nano Drop works as a spectrophotometer and uses absorbance of UV and visible light to assess the purity of different solutions, in our case the purity of our DNA solutions.

NanoDrop is a spectrophotometry appliance where the sample is held in between to optical surfaces with the help of surface tension and fibre optic technology. Its spectral range is 190nm to 860nm, but the wavelengths 260nm, used for DNA/RNA and 280nm, used for proteins are the ones we utilized in our study.

The software coupled with the NanoDrop (standard software by Thermo) uses different programs for materials. The ratio of absorbance at the wavelengths of 260nm, 280nm and the ratio of these (260/280 nm) is used to assess the purity of DNA. If the ratio is approximately 1.8, it is generally accepted as 'pure'. If the ratio is significantly lower it is usually due to contaminants that absorb strongly at 280 nm such as proteins and phenol.

DNA isolates from the Power Soil kit were run through Nano Drop measurement before the initial PCR.

2.8 PCR

The polymerase chain reaction (PCR) is one of the most, if not the most used technique within molecular biology. The PCR is used to amplify and detect a specified gene or genomic sequence with the help of primers. The PCR works in three steps (Figure 13), the first one being denaturation that separates the DNA strands. This is done by heating the DNA up to 95 ^{0}C which causes the hydrogen bindings to break, giving two strands of DNA instead of one double helix. The second step is annealing phase in which the primers bind. For the primers to

bind it usually requires lower temperatures but the specific temperature varies with the primer from 45 up to 72 ^{0}C depending on length and nucleotide build.

After the primers annealed the temperature is then raised to 72 ^{0}C, the working temperature of the heat stable polymerase. It binds to the primers and adds nucleotides to the primers eventually copying the selected gene. That concludes one PCR cycle, a PCR usually runs for 20-35 cycles generating more than enough DNA for downstream applications or just so that it can be detected.

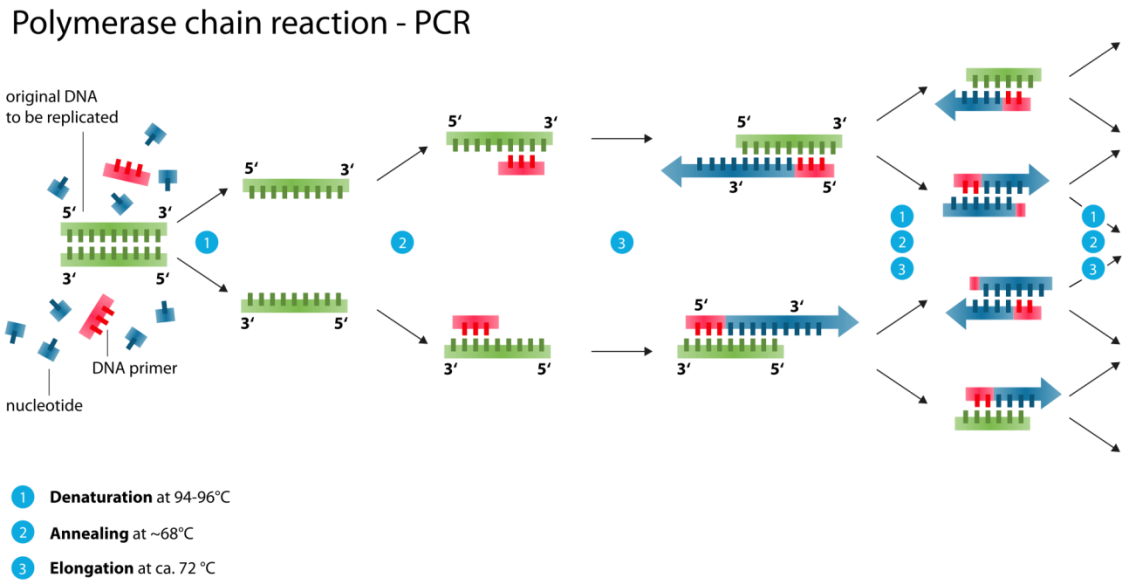


Figure 13: Illustration of PCR principle
http://upload.wikimedia.org/wikipedia/commons/thumb/9/96/Polymerase_chain_reaction.svg/2000px-Polymerase_chain_reaction.svg.png

In our study we ran 550 bacteria through two PCRs in our search for the *nptII* gene. They all followed the same setup after the decision of using four µl, as shown in table eight.

In all our PCR setups it was used positive controls using genomic DNA isolated from our *JV28 KmR* strain. Screening for the *nptII* gene in our selected bacteria we also used DNA from our *JV28* that was run through our QE protocol alongside the given bacteria.

Table 8: PCR mix

PCR Mix	Mastermix
10 µl Dynazyme	Dynazyme 50%
1 µl Primer R (16S)	Primer 16S R 5%
1 µl Primer F (16S)	Primer 16S F 5%
4 µl DNA $10^{0\ -4*}$ (Soil/Bacterial)	DNA 20%
4 µl H_2O	H_2O 20%
=20 µl	= 100 %

*DNA $^{0\ -(-4)}$ refers to testing of PCR response to extracted soil DNA from undiluted to diluted to tenth to the fourth.

All DNA added to our PCRs were a 10^{-1} dilution, if not it will be stated in the actual procedure.

There was one exception with one of our soil isolates that had to be diluted to 10^{-2} to give clear PCR bands.

Our positive control for all our PCR setups was *JV28 KmR* (37).

Table 9: PCR program

Part	Temperature	Time	Cycles
Initial denaturation	95^0C	2 minutes	Once
Denaturation	95^0C	30 seconds	35 cycles
Annealing	60^0C	30 seconds	
Extension/elongation	72^0C	40 seconds*	
Final elongation	72^0C	5 minutes	Once
End/Hold	10^0C	∞	

* 30 seconds were used if the reaction only used 16S primers

2.8.1 PCR of soil samples

The PCR part of this project had to parts. One focused on the bacteria selected through cultivation on 100 µg/ml kanamycin containing agar and one focused on the DNA isolated directly from our soil samples.

2.8.1.1 Degree of inhibition of soil samples

In order to check whether or not the samples needed to be purified half of two samples and two blanks was run through the PowerClean kit and then compared in a PCR setup. The

samples were run through PCR in a dilution series of $10^{0 - -4}$ using 16S primers. With *A. baylyi ADP1200Com* + Kan(S) /- used positive control.

2.8.1.2 PCR on purified soil samples

PCR on our purified soil samples were done with three reactions per sample; 16S, NPT_II and NPTII_Full. The soil DNA samples used in this protocol was from the three initial samplings from Holt 1-3. Eight samples from each field with their corresponding two blanks were used in this PCR.

2.9 Quantitative PCR

Quantitative PCR (qPCR) works the same way as the traditional PCR but in between the cycles it emits light of a specific wavelength and measures the fluorescence, if there is any. This allows for detecting and measuring the results in real time. This is done either with none specific dyes that intercalate with double-stranded DNA or with more specific DNA probes that is quite similar to primers in their specificity. The probes take advantage of the *taq* polymerases exonuclease activity which allows it to cut nucleotides and replace them. This allows it to separate the fluorescence part from the quencher since they will be read as a mismatch to the opposing base when the polymerase starts elongation.

The samples subjected to qPCR was from a triplicate sampling in which the samples were sampled at the same day, undergone every step together and the control blanks showed no sign of contamination in the PCR.

In our study it was used qPCR as well as traditional PCR on our soil samples as the amount of DNA in our soil samples might have been so low that detecting it might be undetectable with a PCR.

It was done three separate qPCRs with 16 samples from each of our fields with 4 no template controls and 4 positive controls.

It was used DNA probes specific to the *nptII* gene in our real time PCR. The probe is made up of the corresponding DNA sequence to our target gene, a fluorescent marked oligonucleotide (reporter) and a quencher. While the reporter and the quencher are in close proximity to the reporter, the quencher absorbs the light the reporter emits. During the annealing phase the probe binds to its target sequence and during the extension the *taq* polymerases exonuclease activity separates the reporter from the quencher, allowing the qPCR to measure it, as shown in figure 14.

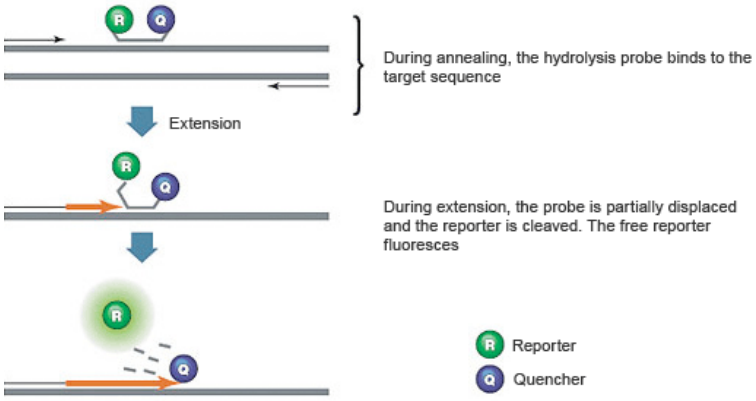


Figure 14: The binding and hydrolyzation of the probe.
http://www.bio-rad.com/webroot/web/images/lsr/solutions/technologies/gene_expression/qPCR_real-time_PCR/technology_detail/real-time-pcr-detection-standard-pcr-primer-and-hydrolysis-probe.gif

As our samples came from soil we opted to use an environmental mastermix (Applied Biosystems) that would have a higher tolerance for contaminants that could inhibit our reaction even though the DNA had been purified.

Only soil DNA from Holt sample set two were used in qPCR.

Table 10: qPCR Mix

qPCR Mix	**Mastermix**
10 µl Environmental Master Mix	Environmental Master Mix 50%
1 µl *nptII* Assay Mix	*nptII* Assay mix 5%
5 µl H_2O	H_2O 25%
4 µl DNA 10^{-1} (Soil/*JV28*)	DNA 20%
=20 µl	= 100 %

Table 11: Duplex qPCR Mix

qPCR Mix	**Mastermix**
10 µl Environmental Master Mix	Environmental Master Mix 50%
1 µl *nptII* Assay Mix	*nptII* Assay mix 5%
1 µl H_2O	H_2O 5%
4 µl DNA 10^{-1} (Soil)	DNA 20%
4 µl DNA 10^{-1} (*JV28*)	DNA 20 %
=20 µl	= 100 %

Table 12: qPCR setup

Real Time PCR Setup	Temperature	Time	Cycles
Initial Hot Start	95 ^{0}C	10 min	1
Denaturation	95 ^{0}C	15 sec	45 Cycles
Annealing and extension	60 ^{0}C	1 min	

The probe in use was a single colour hydrolysis probe so the FAM settings on the light cycler were used.

First we ran a standard curve setup using a DNA dilution series from our purified *JV28 KmR* strain where it was based on NanoDrop measurements and the knowledge of its genome was estimated how many copies there would be per dilution in a dilution series.

4 µl from each dilution would give reactions with 300 000 copies down to 3 copies.

To make our standard curve we made two dilution series with our control DNA so that any errors in the dilution would be discovered and ran them both as a triplicate during the same qPCR.

Table 13: Our know DNA dilution series from JV28 KmR

bp/copy	g/bp	g/copy	genomic DNA g/µl
3,60E+06	1,096E-21	3,95E-15	2,82E-08

This is the measurements taken before diluting it to a starting point for the dilution series.

Table 14: Our known DNA dilution from JV28 KmR

copy number	in grams	in g/µl	final vol, µl	µl DNA	µl water
3000000	1,18E-08	1,18E-08	100	42,0	58,0

Table 15: The makeup of our dilution series based on the DNA solution of table 14

copy number	in grams	in g/µl	final volume, µl	µl DNA	µl water
300000	1,18E-09	2,96E-10	100	2,5	97,5
30000	1,18E-10	2,96E-11	100	10,0	90,0
3000	1,18E-11	2,96E-12	100	10,0	90,0
300	1,18E-12	2,96E-13	100	10,0	90,0
30	1,18E-13	2,96E-14	100	10,0	90,0
3	1,18E-14	2,96E-15	100	10,0	90,0

As shown in Table 11 we also ran a duplex, wanting to compare the reactions without soil DNA to those with soil DNA whether or not there was a significant amount of inhibition.

The final setup looking for the *nptII* gene used soil DNA from the set of samples collected the same day as well as put through all protocols together.
In each setup 16 samples from each field was run with positive and negative controls. This setup was run three separate times.

2.10 Agarose gel electrophoresis

Gel electrophoresis is a technique used for separation and analysis of macromolecules e.g. DNA in this case, based on their size and charge. It is done by applying an electric field to the gel, often agarose, while it is submerged in a buffer that supplies ions to keep the current going through the gel. In our study a TBE buffer was used. GelRed was also added to the agarose gel so that the DNA could be observed in the gel by UV light after the electrophoresis. The GelRed compound binds to the DNA through intercalation, binding in between the base pairs and is fluorescent so that the DNA can be observed in a UV hood.

- 2 grams of agar was added to an Erlenmeyer flask
- 100 ml water(Milliq grade) was added
- Put in microwave for approximately 90 seconds
- 10 µl GelRed added
    - Flask was stirred to distribute the GelRed
- Poured into electrophoresis tub
    - Removed/busted big bubbles with pipette tips
- Placed two 24 well combs in designated slots
- Allowed to set for about 30 minutes

While the gel was setting the 4 µl 6X loading dye was added to our samples and mixed well before loading the gel.
Loading of gel:

- Submerged the agarose gel in 0.5X TBE buffer
- Added 6 µl 6X loading dye (BioLabs) to our 20 µl post-PCR mix
- Stirred the mix before loading 6 µl of the post-PCR mix into the wells

Table 16: Electrophoresis conditions

Parameter	Value
Voltage	90 V
Time	90 minutes
Amperage	Ca 48 mA*

*The power supply adjusts the amperage accordingly to the set voltage

The gel was then put into the UV hood and exposed to UV light, the hoods auto settings was used to determine exposure time.

2.11 Gel reading

The electrophoresis gels were put in the UV hood right after the electrophoresis was done using the BioRad universal hood and the accompanying software.

If there was no band for the 16S reaction on any sample during the screening for *nptII* from bacterial DNA that bacteria would be put through another round of QE and PCR. If the sample still gave no bands at 16S it would be deemed as a negative.

3. RESULTS

3.1 Cultivation based results

3.1.1 Total CFU

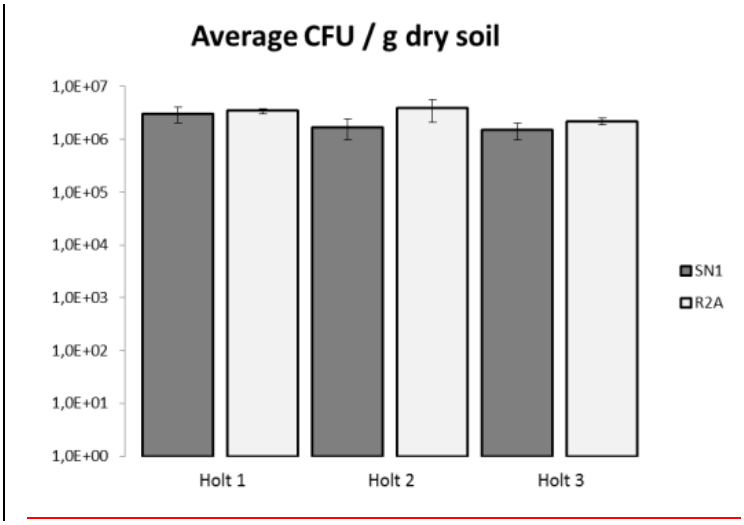


Figure 15: Total CFU findings SN1 and R2A

3.1.2 Phenotypic Km-resistance

Table 17: Phenotypic resistance in selected soil bacteria

Field	%KmR SN1	%KmR R2A
Holt 1	4	10.6
Holt 2	10.5	9.6
Holt 3	2.9	4.2

3.2 Genotypic resistance in selected bacteria

Table 18: Results from SN1 bacteria PCR screening

Field	Included in PCR Screening	Positive 16S band	Positive *nptII* bands
Holt 1	91	82	0
Holt 2	96	92	0
Holt 3	96	93	0

Table 19: Bacteria from R2A included in screening

Field	Amount	Positive 16S bands	Positive *nptII* bands
Holt 1	89	85	0
Holt 2	89	86	0
Holt 3	92	92	0

We were unable to find any bacteria containing the *nptII* gene among any of the 530 bacteria that was successfully screened with PCR.

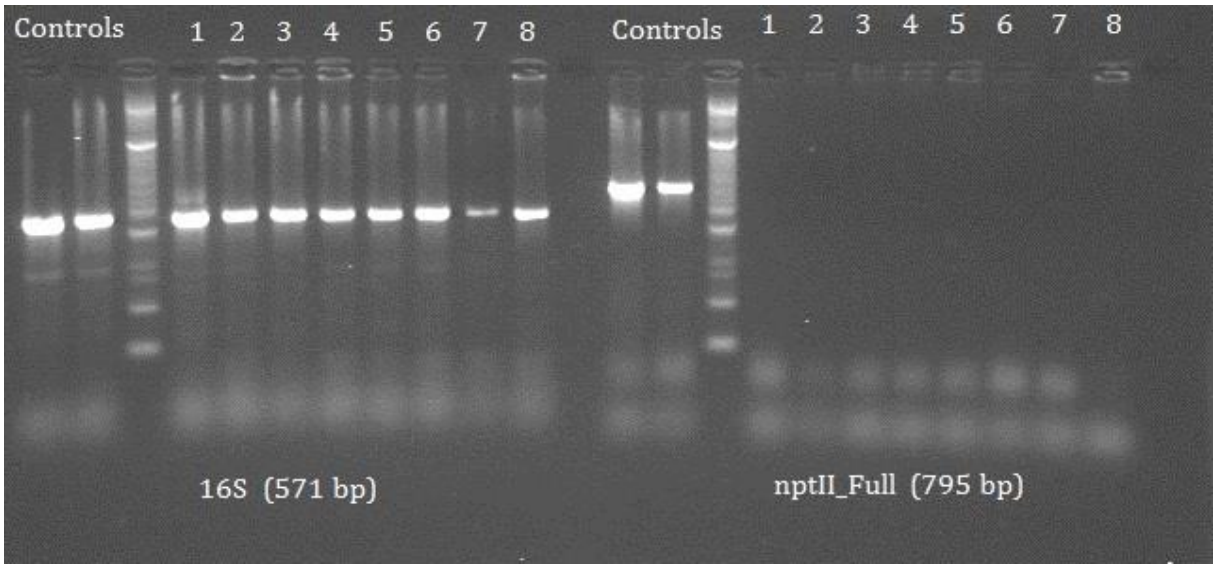


Figure 16: PCR of screened R2A bacteria

This is a representation of our findings with PCR on DNA isolated with QE from the R2A plates from Holt 1-3. The control to the right comes from the same QE session.

3.4 PCR on soil DNA

This is a representation of our findings with PCR on soil samples. To the left of the ladder is the controls, one was extracted with QE at the same time as the samples, the other was purified with kit components. We got bands for housekeeping bacterial DNA from the soil samples but no hit on our *nptII* gene.

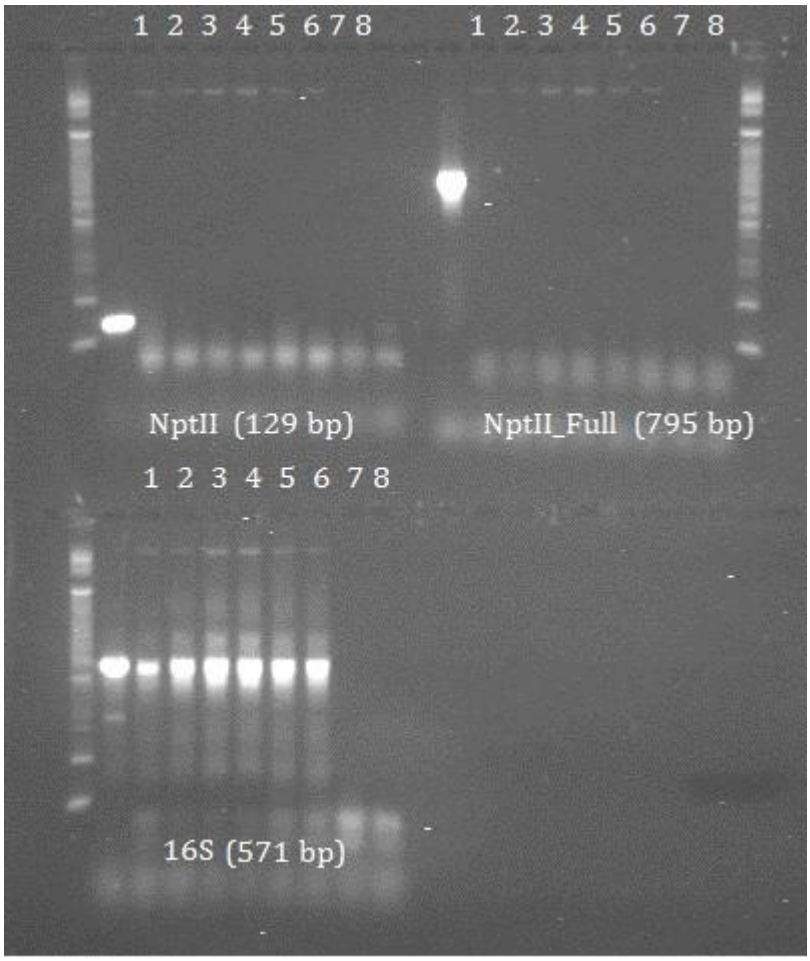


Figure 17: PCR on DNA extracted from Soil, sampled 21/10-14

Sample 7 and 8 are blanks.

3.5 qPCR results

A standard curve was made using our isolated *JV28 KmR* DNA.

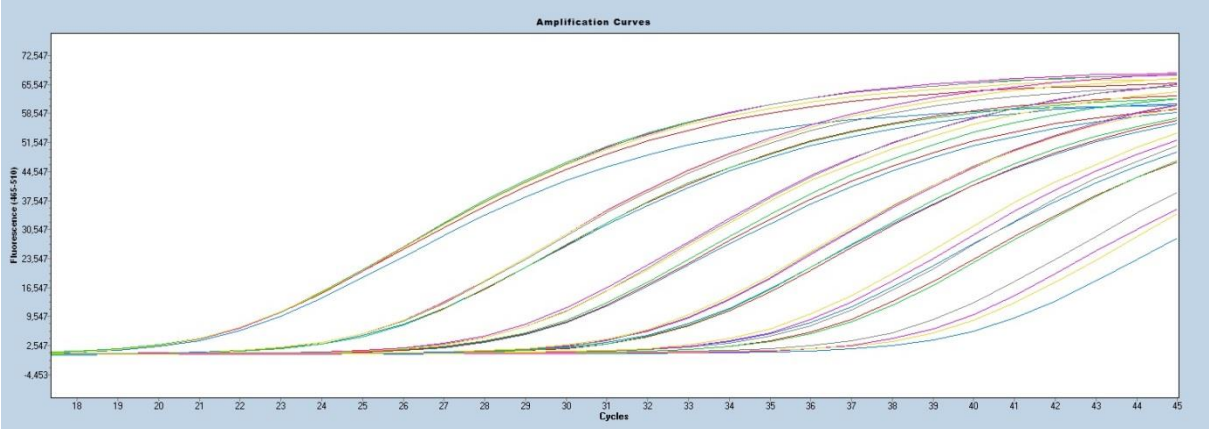


Figure 18: Results with our known DNA

Above is the response to our two separately diluted DNA dilution series. The space between the different readings is consistent but trails somewhat off at the lower parts of the series, at 30 copies and 3 copies but that is not surprising.

The six clearly separated curve trends goes from 300 000 copies per reaction well down to three copies per well.

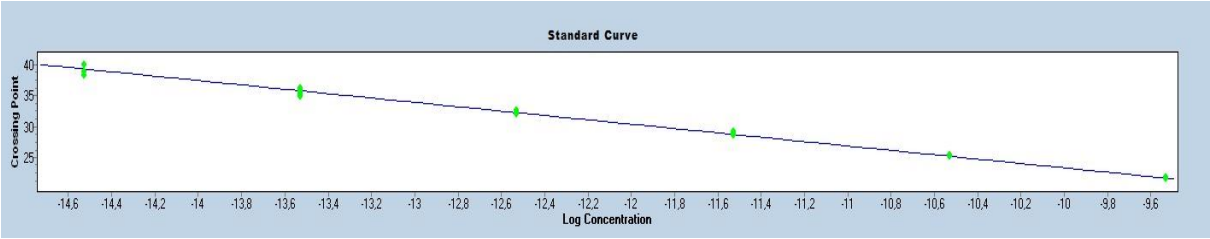


Figure 19: Standard curve from known DNA

Using the results from our known DNA dilution series a standard curve could be made.
The standard curve being this flat shows that our known DNA solution has been acceptable, e.g. there are no big fluctuations of our green dots.

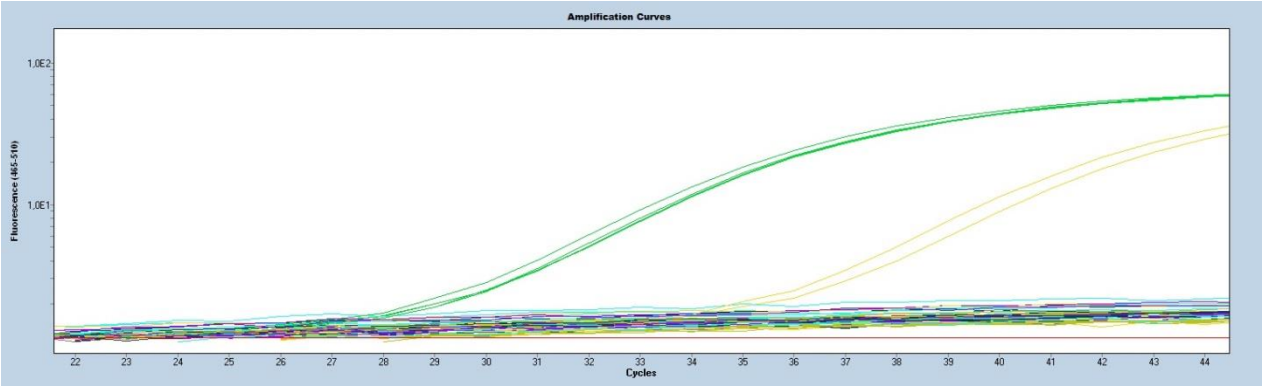


Figure 20: Soil samples with 3000 copies control

This is representative of our QPCR findings. In 48 reactions from each field the positive responses were not consistent enough to interpret as positive results as well as the fact they showed so late in the cycles. Our three copies controls showed somewhat earlier in the cycles and they were not really consistent (two of six gave no response).
Using our known concentrations of DNA and its detection at three copies per qPCR we've been able to set a lower detection limit of copies *nptII* per gram of soil.

Table 20: Lowest detectable copies per gram dry soil

Field	Lowest detectable copies per gram of dry weight soil
Holt 1	247
Holt 2	506
Holt 3	257

4. DISCUSSION

NptII antibiotic resistance marker gene is used for selection of genetically modified (GM) plants. The possibility of opening for import of GM plants has been a point of debate recently in Norway (55, 56). Given the strict regulations Norway has lead towards GM plants and GMOs in general it is ideal to establish a baseline before the release of GMOs.
To be able to evaluate the potential impact the release of GMO that contains *nptII* as a selective marker it is important to study the incidence of *nptII* in different environments like soil with varying exposure to antibiotics, e.g. grazing lands for livestock, woodlands etc including wildlife, birds and water. In this study, methods for detecting *nptII* in soil samples have been established.

The selection of sample sites, had a detailed history regarding what the fields had been exposed to and when. The fields had different history regarding livestock manure as fertilizer, allowing us to see whether or not it had an impact on the results. If there had been antibiotic treatment applied to the animals we could have compared to soil samples that no exposure to antibiotics. There is a possibility of applying this to fields on farms actually producing livestock or maybe sample from a more isolated environment in woods that are not normally traversed in by humans. Sampling from aquatic environments and especially wastewater exits sediments are also areas of interest. Given the activity due to bio-prospecting in the northern parts of Norway it might interesting to see if there is any resistance genes in the areas where potential new antibiotics can be found. Results from any of the above mentioned environments will add valuable data.

4.1 Results from cultivable bacteria

4.1.1 Total CFU and kanamycin resistance

It is known that not nearly all bacteria can be grown in cultures, only about one percent of observable bacteria can be grown on cultivate media and that again can be affected of the nutrition in the selected media (57).
In our study we opted to use SN1 for fast growing bacteria and the less nutritious R2A that would help slower growing bacteria not to get overrun by the faster-growing ones. When it has been seen that one gram soil can contain more than 10^{10} bacterial cells (57) and less than 10 % of these can be grown in a lab setting (41), and a minority of soil bacteria show high level of intrinsic resistance to many common antibiotics including kanamycin. So it was not necessarily given that the Km resistant bacteria that grew on the media would contain *nptII*.

Our bacterial count of aerobic bacteria is consistent with that found in Small et al., lower than in the manure samples and soil samples but quite similar to their sewage findings (10). Leff et al. showed a much lower count of cultivable bacteria, but they examined water samples and the general amount of bacteria found in that water is less than what's expected in soil (28, 57). Esiobu et al. had a higher CFU from soil approx. $1*10^8$ this was soil from an active farm, so the soil contained manure from cow.

Our findings of phenotypic resistance towards kanamycin seem to be as expected from other literature. Smalla et al. found resistance from 2.9 % - 33.3 % in varying habitats (highest in sewer) but they had small sample sizes and urges to use caution regarding the numbers (10). Esiobu et al. had a higher range of kanamycin resistance 38 % - 59 % but they were limited to two of seven environments tested. The two environments were a hospital lake and an active dairy farm, which might explain the higher interval (58).

4.1.2 *nptII* and 16S amplification

The Quick Extract solution we used for DNA extraction from bacteria worked quite well, but there was the potential of picking too many bacteria and overload the PCR so the reaction would not give the necessary amplification.

We had no findings of the *nptII* gene in our selected bacteria. Smalla et al. found *nptII* in 44 out of 355 kanamycin-resistant strains (10) where *Enterobacteriaceae* had the biggest chance of containing *nptII*, 47.6% of the tested *Enterobacteriaceae* from sewage were *nptII* positive. Leff et al. found *nptII* in three out of 184 bacterial isolates (28). It seems that resistance towards kanamycin is due to *nptII* in just a few cases.

Had we encountered *nptII* we would have run DNA sequencing to identify which bacteria that carried it and if it has pathogenic properties.

4.2 Results from non-culturable methods

4.2.1 DNA extraction

In our study we used a soil DNA extraction kit from MoBio. Soil is a complex media containing living and dead bacteria, fungi, plant material and possibly other material as well as humic acids that can inhibit DNA amplification (59). Even though it was possible to run PCR on our isolated DNA we purified parts of it and compared them in a PCR reaction across several dilutions. The purified version did not have to be as diluted to give bands and gave significantly clearer bands. We also considered loss of DNA when we decided to purify our

soil DNA samples, and if there were any loss of DNA it would be short, fragmented DNA <70 bp. Since the shortest segment of *nptII* we tested for was 129 bp, so we did not fear it would be lost in the purification process.

4.2.2 PCR

Since it has been shown that fragmented DNA and damaged DNA from exposure and natural decomposition can persist in soil for several years we decided to use both the primer for the full *nptII* gene (795 bp) as well as the primer for a smaller part (129 bp) of the gene on the DNA we isolated and purified from soil (44, 45). If there were bacteria containing *nptII* the primer for the full gene would give us a confirmation of that, since it is unlikely that that big a DNA fragment would be absorbed into soil particles, even though it has been seen adsorption of DNA fragments of 2,69 kbp (60). It is also highly likely that it has been degraded past 795 bp and then we intended to find it using the primer for the 129 bp sequence.
When screening on bacteria we used DNA extracted from bacteria using QE and had them stored at -20^{0}C so DNA breakdown was not a concern if the protocol was followed. That's why we used only the NPTII_Full primer on the bacterial DNA extractions.

4.2.3 qPCR

In our setup of the qPCR the standard curve showed that we were able to detect an amount as low as three copies per reaction but we had no consistent reads on our isolated soil DNA. Our detection limit was established at 247, 257 and 506 copies per gram of dry soil from Holt 1-3. We also used a mastermix that would have a higher tolerance of potential inhibitors from soil samples so if there is *nptII* in our soil DNA then it is present at less than the limits above.

4.3 Sample size

The sample size is very important when investigating rare events such as the possibility of HGT of *nptII*, due to the low frequency of natural transformation in soil that is said to occur below 10^{-7} per recipient (35). Our cultivation was done on a total of nine grams of soil and we screened 553 kanamycin-resistant bacteria. There is however the problem that of the few bacteria, if any that contain *nptII*, will get outgrown by other kanamycin resistant bacteria since a fraction of soil bacteria is known to have intrinsic resistance towards kanamycin (41). The amount of DNA in one qPCR from Holt 1 equalled 1,07x10^5 bacteria, Holt 2 equalled 7.52x10^4 and Holt 3 5.92x10^4.

4.2.4 DNA Sequencing:

A sample of our isolated soil DNA has been sent to a sequencing centre where the total DNA content will represented as millions of different reads of approx. 100 bp. These reads can then be scanned up against databases of known antibiotic resistance gene sequences e.g. ResFinder to see if it contains other resistance genes than *nptII*. This was done to be extra thorough so if we did not find any *nptII* through the means in our study we would now more than likely find out if the gene is present in our soil after all, just in an amount we cannot detect using our DNA extraction from soil and apparatus. If the *nptII* gene is found in our sequencing data then we know that we were unable to find it due to its scarcity.

5. CONCLUSION

The *nptII* gene is assumed to be ubiquitous by many instances (3) even though it a limited amount of studies done regarding the prevalence of *nptII* (4). In 1993 Smalla K et al. found that resistance towards kanamycin were found in all habitats tested but not very resistant bacteria harboured the *nptII* gene (10). In Austria, a study on clinical isolates found that *nptII* was rare (0.0096%)(30). Leff et al. examined a stream in South Carolina for kanamycin resistance and found kanamycin resistance in all tested habitats, but only a few of the bacteria tested positive for *nptII*. Our results supplement previous findings that soil bacteria is resistant to kanamycin, but none of the screened bacteria contained *nptII* as source of that resistance. This goes against EFSAs statement calling it ubiquitous and in turn puts the *nptII* gene in a classification group where it can be used freely (3) where they also refer to studies that are now becoming of age, both from 1993.

6. FURTHER CONSIDERATIONS

Determining the prevalence of kanamycin resistance and *nptII* in other environments e.g. farmlands with livestock, woodlands further away from human influence and aquatic environments will be of help establishing a baseline. Including determining if there is any kanamycin in the soil as it would provide carriers of kanamycin resistance advantages in the soil environment and most likely increase the amount of resistant bacteria (61).

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