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Frequency of Environmental Antibiotic Resistance

Baseline prevalence of neomycin phosphotransferase genes II and III in maize and potato fields, feed and human bacterial pathogens in Austria

Final Report

Impressum

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Executive Summary – English

The naturally occurring prevalence of two antibiotic resistance marker genes – neomycin phosphotransferase II and neomycin phosphotransferase III – was determined by TaqMan real time PCR in three environments - human pathogens, soil, and feed - in Austria.

The clinically relevant environment was represented by an analysis of the nptII/nptIII carrier status of *Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus* isolates which were collected prospectively in 2010 and 2011 from representative clinical laboratories located in all Austrian federal states. Additionally, a strain repository of *Salmonella enterica* subsp. *enterica* isolates collected from 2008 – 2010 at the Austrian Reference Centre for Salmonella was retrospectively analysed. In total nptII and nptIII resistance gene prevalence data of more than 10 400 samples were compiled. A single *Salmonella* strain could be identified as carrier for nptII. All other isolates were nptII negative. Within a confidence limit of 95% (depicted in brackets) the calculated prevalence of nptII was below 0.70% in *Escherichia coli* [0%; 0.70%], and below 0.75%, 0.73%, 0.32%, and 0.058% in enterococci [0%; 0.75%], *Staphyloccocus aureus* [0%; 0.73%], *Pseudomonas aeruginosa* [0%; 0.32%] and *Salmonella* spp. [0%; 0.058%] isolates, respectively. For nptIII the prevalence - within a confidence limit of 95% - was below 1.47% in *E. coli* [0%; 1.47%], and below 42.4%, 5.02%, 0.32%, and 0.037% with enterococci [32.84%; 42.4%], *S. aureus* [1.51%; 5.02%], *Pseudomonas aeruginosa* [0%; 0.32%], and *Salmonella enterica* subsp. *enterica* [0%; 0.037%] isolates, respectively. The obtained results support – with a high grade of confidence - the hypothesis of a low prevalence of nptII in the analysed human pathogenic strains collected representatively all over Austria.

Soil bacterial communities were tested for the prevalence of nptII and nptIII by cultivation-dependent and cultivation-independent metagenomic approaches analysing 100 composite soil samples representing 1000 single soil extractions from 50 maize and 50 potato fields. Total soil DNA analysis revealed 6% of the fields positive for the presence of nptII genes (95% confidence interval [2.2%; 12.6%]). A mean concentration of 340 nptII gene copies/g soil (minimum: 31 copies/g soil; maximum: 850 copies/g soil) were detected in these fields. A study published in 2011 by Ma et al. in Canada attained similar results regarding the low prevalence and the low copy number of nptII in agricultural soils. 85% of all soil samples were positive for nptIII (95% confidence interval [76.5%; 91.4%]) representing average background loads of 4750 copies/g soil (range: 13 – 61,600 copies/g soil) of the positively tested fields. Comprehensive supplemental data packages for each test field were compiled. A strong correlation between fertilization of organic origin and high prevalence of nptIII was obtained. On average 8.29% of all cultivable bacteria from ten reference fields were resistant to kanamycin (range: 0.47 – 19.12%). 396 bacterial strains resistant to kanamycin were isolated and taxonomically characterized, none could be identified as carrier for nptII (95% confidence interval: [0%; 0.8%]). In a few fields, some strains with potential to cause human disease (e.g. *Klebsiella pneumoniae, Stenotrophomonas maltophilia*) were detected. The prevalence of nptIII positive strains was 1.8% on average ([0%; 3.3%]). Microbial biodiversity of these ten reference fields was established by pyrosequencing. Between 194 and 268 genera could be identified and their relative quantities could be established per field. Genera containing human pathogens were present. Compared to nptIII the naturally occurring background load of nptII resistance genes in agricultural habitats used for the cultivation of maize and potatoes in Austria was low. To our knowledge this was the most comprehensive effort to collect resistance gene specific and additional data from soil environments to date by covering 100 fields of different soil types cultivated with different crops.

The feed/plant environment was represented by maize and potato samples routinely collected for federal control measurements in Austria. Forty two total DNA extracts were analysed for the prevalence of nptII and nptIII. None of them were positive for both aminoglycoside phosphotransferases (95% confidence interval: [0%; 6.9%]). Up to 73.05% of the bacterial population recovered from maize were resistant to kanamycin, potato associated bacteria showed a maximum kanamycin resistance of 6.6%. A single bacterial isolate carried nptII (prevalence estimator: 1.1%; 96% confidence interval: [0%; 4,9%]). Although only a small number of bacterial samples was tested the obtained results support the hypothesis of a low naturally occurring background load of nptII and nptIII genes in maize and potatoes cultivated and used as feed in Austria during the testing period.

In silico analysis of nptII gene sequences showed no extended regions of DNA similarity with other aminoglycoside phosphotransferase genes. The identified contiguous stretches of identical sequence were extremely short. This observation indicates that homologous recombination between nptII and the other aminoglycoside phosphotransferase genes under investigation will not be the primary route for the exchange of DNA fragments and sequence evolution. However, a remaining possibility for exchange of DNA sequences between aminoglycoside phosphotransferase genes is illegitimate recombination due to identification of regions with microhomologies. Sequence alignments using standard parameters of the BLAST algorithm did not reveal the presence of nptII mosaic genes of natural origin in GenBank sequence entries. Data retrieved from the *in silico* analysis available at the time of query did not provide substantial support to the hypothesis of an involvement of the nptII gene in the formation of mosaic genes with altered resistance patterns within the family of aminoglycoside phosphotransferases. However, there were indications from experiments performed in the course of the project that nptII containing DNA might recombine with certain aminoglycoside phosphotransferase genes cloned into *Acinetobacter baylyi* during natural genetic transformation. These results could not be confirmed by sequence analysis of the putative target regions.

A computer model simulating the horizontal transfer of plant derived antibiotic resistance genes into soil bacterial communities expanding pivotal work from Townsend et al. indicates that fixation of the new trait is a long term process extremely dependent on the selection pressure prevailing in the respective habitat. Taking plant degradation processes into account the frequency of horizontal gene transfers plays also a role to a certain extent concerning the fixation of a new phenotype in the population.

The effects of selection pressure mediated by antibiotics in soils were comprehensively discussed. Analysis of recent literature revealed that antibiotics at drug concentrations several hundred-fold below the minimum inhibitory concentrations of susceptible bacteria may select effectively for resistant bacteria and that low antibiotic concentrations found in natural environments are supposed to be important for the enrichment and maintenance of resistance in bacterial populations.

Conclusion

The naturally occurring background load of nptII resistance genes in the tested bacterial habitats in Austria appeared to be low during the testing period. The obtained quantitative results indicated that the resistance gene pools under investigation were not saturated with nptII gene copies. The baseline frequency of nptII in these natural habitats was usually distinctly lower compared to the prevalence of nptIII in the same habitats. In summary the resistance gene pools under investigation

appear to be not saturated but receptive for the input of exogenous DNA carrying aminoglycoside phosphotransferase (3')-IIa gene homologues. A long-term and constant exposure of these habitats with exogenous nptII carrying DNA might be capable of elevating the abundance of this resistance determinant in these environments.

Some of the obtained data can be found in the following publication:

Woegerbauer M, Zeinzinger J, Springer B, Hufnagl P, Indra A, Korschineck I, Hofrichter J, Kopacka I, Fuchs R, Steinwider J, Fuchs K, Nielsen KM, Allerberger F. 2014. Prevalence of the aminoglycoside phosphotransferase genes aph(3')-IIIa and aph(3')-IIa in Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Salmonella enterica subsp. enterica and Staphylococcus aureus isolates in Austria. J Med Microbiol **63:**210-217.

Executive Summary – Deutsch

Die natürlich vorkommende Prävalenz von zwei Antibiotikaresistenzmarkergenen - Neomycinphosphotransferase II und Neomycinphosphotransferase III – wurde mit TaqMan real time PCR in den Habitaten „Menschliche Krankheitserreger“, „Boden“ und „Futtermittel“ in Österreich bestimmt.

In der klinisch relevanten Umgebung wurden folgende humanpathogene Bakterien auf nptII und nptIII Resistenzgene untersucht, welche prospektiv in den Jahren 2010 - 2011 von klinischen Labors repräsentativ in allen österreichischen Bundesländern gesammelt wurden: *Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus.* Zusätzlich wurden *Salmonella enterica* subsp. *enterica* Isolate der Stammsammlung des österreichischen Referenzlabors für Salmonella retrospektiv für den Zeitraum 2008 – 2010 analysiert. Von insgesamt 10 400 auf nptII und nptIII Resistenzgene untersuchten Isolaten wurde ein einzelner Salmonellenstamm positiv als Träger von nptII getestet. Alle anderen Isolate waren nptII negativ. Mit einer Sicherheit von 95% war die Prävalenz von nptII bei *Escherichia coli* niedriger als 0.70% [0%; 0.70%], bei Enterokokken niedriger als 0.75% [0%; 0.75%], bei *Staphylococcus aureus* niedriger als 0.73% [0%; 0.73%], *bei Pseudomonas aeruginosa* niedriger als 0.32% [0%; 0.32%] und bei *Salmonella* spp. niedriger als 0.058% [0%; 0.058%]. In Klammer ist das 95% Konfidenzintervall angeführt. Bei nptIII lagen die Prävalenzen – innerhalb eines 95% Konfidenzlimits - bei *Escherichia coli* unter 1.47% [0%; 1.47%], bei Enterokokken unter 42.4% [32.84%; 42.4%], bei *Staphylococcus aureus* unter 5.02% [1.51%; 5.02%], bei *Pseudomonas aeruginosa* unter 0.32% [0%; 0.32%] und bei *Salmonella enterica* subsp. *enterica* unter 0.037% [0%; 0.037%]. Die erzielten Ergebnisse bestätigen mit einer Sicherheit von 95% die Hypothese einer niedrigen Prävalenz von nptII in den repräsentativ in Österreich gesammelten und analysierten humanpathogenen Bakterienisolaten.

Bodenbakterienpopulationen wurden auf die Prävalenz von nptII und nptIII mit kultivierungsabhängigen und –unabhängigen metagenomischen Verfahren getestet. Es wurden 100 Bodenmischproben von jeweils 50 Mais- und 50 Kartoffelfeldern untersucht. Insgesamt wurden 1000 Einzelbodenprobenextrakte gezogen. Die Analyse der Gesamt-Boden-DNA zeigte, dass 6% der Bodenproben positiv auf das Vorhandensein von nptII Genen (95% Konfidenzintervall [2,2%; 12,6%]) waren. Im Mittel konnten 340 nptII Genkopien/g Boden (Minimum: 31 Kopien/g Boden; Maximum: 850 Kopien/g Boden) in den positiv getesteten Feldern detektiert werden. Eine im Jahr 2011 publizierte Studie von Ma et al. in Kanada kommt hinsichtlich der niedrigen Prävalenz und der geringen Kopienanzahl von nptII in landwirtschaftlich genutzten Anbauflächen zu einem ähnlichen Ergebnis. 85% aller Bodenproben wurden positiv auf nptIII getestet [76,5%; 91,4%], welche eine durchschnittliche Hintergrundbelastung von 4750 Kopien/g Boden (Bereich 13 bis 61.600 Kopien/g Boden) in den positiv getesteten Feldern aufwiesen. Für die einzelnen Felder wurden umfangreiche Zusatz-Daten gesammelt und ausgewertet. Es wurde eine starke Korrelation zwischen organischer Düngung und der Prävalenz von nptIII Genen festgestellt. Im Durchschnitt waren 8.29% aller kultivierbaren Bakterien aus zehn Referenzfeldern resistent gegen Kanamycin (Bereich: 0,47 bis 19,12%). 396 gegen Kanamycin resistente Bakterienstämme wurden isoliert und taxonomisch charakterisiert. Keiner dieser Stämme konnte als Träger für nptII identifiziert werden (95% Konfidenzintervall [0%; 0,8%]). Die durchschnittliche Prävalenz der nptIII positiven Stämme betrug 1,8% ([0%, 3,3%]). In einigen Feldern konnten humanpathogene Stämme (z.B. *Klebsiella pneumoniae, Stenotrophomonas maltophilia*) identifiziert werden. Weiters wurde die mikrobielle Diversität dieser zehn Referenzfelder mittels Pyrosequenzierung untersucht. Es konnten zwischen 194 und 268

Gattungen in den Bodenproben identifiziert und ihre relativen Anteile ermittelt werden. Humanpathogene Gattungen kommen dabei ebenfalls vor. Im Vergleich zu nptIII ist die natürliche Hintergrundbelastung von nptII Resistenzgenen in für Mais- und Kartoffelanbau genutzten landwirtschaftlichen Habitaten in Österreich gering. Unseres Wissens war dies der bisher umfassendste Ansatz, um Daten über die Prävalenz von Resistenzgenen in 100 Bodenproben mit verschiedenen Bodentypen zu sammeln und mit umfangreichen zusätzlichen spezifischen Bodendaten auszuwerten.

Das Habitat Pflanze bzw. Futtermittel wird repräsentiert durch Mais- und Kartoffelproben, welche routinemäßig im Rahmen von Kontrollen des Bundesamtes für Ernährungssicherheit in Österreich gesammelt werden. Zweiundvierzig Gesamt-DNA-Extrakte wurden auf die Prävalenz von nptII und nptIII analysiert. Keine Probe war positiv auf eine der beiden Aminoglykosidphosphotransferasen (95% Konfidenzintervall: [0%; 6,9%]). Bis zu 73,05% der aus Mais gewonnenen Bakterienpopulation waren resistent gegen Kanamycin, während aus Kartoffeln gewonnene Bakterien eine maximale Kanamycin-Resistenz von 6,6% aufwiesen. Ein einzelnes Bakterienisolat wurde nptII positiv getestet (Prävalenz Schätzer: 1,1%; 96%-Konfidenzintervall: [0%, 4,9%]). Obwohl nur eine kleine Anzahl von bakteriellen Proben getestet wurde, unterstützen die erhaltenen Ergebnisse die Hypothese einer niedrigen natürlichen Hintergrundbelastung von nptII und nptIII Genen in Mais und Kartoffeln, welche im Untersuchungszeitraum in Österreich angebaut und als Futtermittel verwendet wurden.

Eine *in silico* Analyse der nptII Gensequenz mit anderen Aminoglycosidphosphotransferasegenen zeigt keine ausgeprägten Bereiche mit DNA Ähnlichkeit. Die identifizierten zusammenhängenden DNA Abschnitte mit totaler Sequenzidentität sind extrem kurz. Das deutet darauf hin, dass homologe Rekombination zwischen nptII und den anderen untersuchten Aminoglycosidphosphotransferasegenen nicht der primäre Weg zum Austausch von DNA Fragmenten und zur Sequenzevolution ist. Es verbleibt jedoch die Möglichkeit eines DNA Austausches zwischen Aminoglycosidphosphotransferasegenen durch illegitime Rekombination, da Bereiche mit Microhomologien auftreten. BLAST Sequenzalignments mit Standardparametern zeigen, dass sich keine nptII Mosaikgene natürlichen Ursprungs in GenBank befinden. Die aus der *in silico* Analyse gewonnenen Daten unterstützen nicht wesentlich die Hypothese einer Beteiligung von nptII an der Bildung von Mosaikgenen mit anderen Aminoglycosidphosphotransferasegenen, die zur Veränderung des Resistenzmusters führen können. Es gibt jedoch Hinweise aus im Rahmen des Projekts durchgeführten Experimenten, dass nptII codierende DNA mit ausgewählten Aminoglycosidphosphotransferasegenen, die zuvor in *Acinetobacter baylyi* kloniert worden waren, nach natürlicher genetischer Transformation rekombinieren. Die Ergebnisse konnten durch Sequenzierung der vermutlichen Zielregionen nicht bestätigt werden.

Ein Computer-Modell zur Simulation des horizontalen Gentransfers von aus Pflanzen freigesetzten Antibiotikaresistenzgenen auf die Bakteriengemeinschaften im Boden erweitert das Schlüsselwerk von Townsend et al. und bestätigt, dass die Fixierung eines neu eingebrachten Gens ein langfristiger Prozess ist, welcher extrem vom Selektionsdruck im jeweiligen Habitat abhängig ist. Bezieht man die Abbauprozesse von pflanzlichem Material ins Model mit ein, zeigt sich dass auch die Häufigkeit des horizontalen Gentransfers bei der Fixierung eines neuen Genotyps in einem gewissen Ausmaß eine Rolle spielt.

Die Wirkung des durch Antibiotika in Böden erzeugten Selektionsdrucks wird umfangreich diskutiert. Die Analyse aktueller Literatur zeigt, dass Antibiotikamengen, die um das Hundertfache unter den

tatsächlichen minimalen Hemmkonzentrationen von empfindlichen Bakterien liegen, effektiv resistente Bakterien selektieren und dass diese niedrigen, in natürlicher Umgebung zu findenden Antibiotikakonzentrationen wichtig für Anreicherung und Erhalt von Resistenzen in Bakterienpopulationen sind.

Schlussfolgerung

Die natürlich vorkommende Hintergrundbelastung von nptII Resistenzgenen in den in Österreich getesteten bakteriellen Habitaten scheint niedrig zu sein. Die erzielten quantitativen Ergebnisse zeigen, dass die untersuchten Resistenzgen-Pools nicht mit nptII Genen gesättigt sind. Die Baseline-Frequenz von nptII in diesen natürlichen Lebensräumen ist in der Regel deutlich niedriger im Vergleich zur Prävalenz von nptIII in den gleichen Habitaten. Die Ergebnisse zusammenfassend betrachtet deuten darauf hin, dass die untersuchten Resistenzgen-Pools nicht mit nptII Genen gesättigt und daher aufnahmefähig für den Input exogener DNA mit Aminoglycosidphosphotransferase (3 ')-IIa-Gen Homologen sind. Eine langfristige und konstante Exposition dieser Lebensräume mit exogener nptII codierender DNA könnte in der Lage sein, die Häufigkeit dieser Resistenz-Determinante in diesen Ökosystemen zu erhöhen.

Einige der erzielten Ergebnisse sind in folgender Publikation zu nachzulesen:

Woegerbauer M, Zeinzinger J, Springer B, Hufnagl P, Indra A, Korschineck I, Hofrichter J, Kopacka I, Fuchs R, Steinwider J, Fuchs K, Nielsen KM, Allerberger F. 2014. Prevalence of the aminoglycoside phosphotransferase genes aph(3')-IIIa and aph(3')-IIa in Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Salmonella enterica subsp. enterica and Staphylococcus aureus isolates in Austria. J Med Microbiol **63:**210-217.

Table of Contents and Parts of the Final Report

The final report for the project

„Frequency of Environmental Antibiotic Resistance - Baseline prevalence of neomycin phosphotransferase genes II and III in maize and potato fields, feed and human bacterial pathogens in Austria" (Bestimmung der Hintergrundbelastung von natürlichen Habitaten mit Aminoglykosidphosphotransferase Genen (nptII/nptIII) zur verbesserten Risikoabschätzung von Antibiotikaresistenzmarkergenen aus gentechnisch veränderten Organismen. FEAR-Projekt BMG-70420/0089-II/B/9/2010 und BMLFUW Dafne Nr. 100598/1)

contains the following parts:

Each Part is prepared with a separate Table of Contents, List of Tables and Figures, separate Annexes and a Summary of the respective part of the project.

Abbreviations

Abbreviation	Description
µl	microliter
µM	micromolar
µm	micrometer
AGES	Austrian Agency for Health and Food Safety - Agentur für Gesundheit und Ernährungssicherheit
Amk	amikacin
APH	aminoglycoside phosphotransferases (protein)
ARM	antibiotic resistance marker
BIOHAZ	EFSA Biohazard Panel
BLAST	Basic local alignment search tool
But	butirosin
CP	crossing point; real time PCR
CFU	colony forming unit
ct	threshold cycle; real time PCR
CV	coefficient of variation
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
EMA	European Medicines Agency
FAM	fluorescein amidite; 6-FAM phosphoroamidite; real time PCR
g	gram
GM	genetically modified
GmB	gentamicin B
GMO	genetically modified organism
HBV	hepatitis B virus
HCV	hepatitis C virus
HEX	hexa-chloro-fluorescein; real time PCR
HGT	horizontal gene transfer
HIV	human immunodeficiency virus

IMED	Institute for Medical Microbiology & Hygiene - Institut für med. Mikrobiologie und Hygiene
INVEKOS	Integriertes Verwaltung- und Kontrollsystem - Integrated Administration and Control System (IACS), e.g. an identification system covering all agricultural areas
Isp	isepamicin
Km	kanamycin
LOD	limit of detection
LOQ	limit of quantification
Lvdm	lividomycin
MIC	minimum inhibitory concentration
NCBI	National Center for Biotechnology Information
Neo	neomycin
nM	nanomolar
nptII	neomycin phosphotransferase II (gene)
nptIII	neomycin phosphotransferase III (gene)
Nts	nucleotide(s)
pbp	penicillin binding protein
PCR	polymerase chain reaction
PEI	Paul Ehrlich Institute
Prm	paromomycin
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
Rsm	ribostamycin
SD	standard deviation
TaqMan	5' nuclease assay; real time PCR
YYE	fluorescence dye for 5' probe labelling; real time PCR

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Frequency of Environmental Antibiotic Resistance

Part A:

NptII and nptIII Prevalence in Human Pathogens

Final Report

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1 Prevalence of nptII and nptIII in bacterial pathogens of human origin in Austria

1.1 Summary

Five bacterial species (*Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus)* were collected prospectively from representative clinical laboratories located in all Austrian federal states. The strains were sampled without any antimicrobial pre-selection and were analysed for the presence of nptII and/or nptIII resistance genes with TaqMan real time PCR on the LightCycler LC480 device. Additionally, a strain repository of *Salmonella enterica* subsp. *enterica* isolates collected from 2008 – 2010 at the Austrian Reference Centre for Salmonella was retrospectively analysed for susceptibility to kanamycin and the resistant strains were tested for their nptII/nptIII carrier status. In total nptII and nptIII resistance gene prevalence data of more than 10 400 samples were compiled. Only a single isolate (*Salmonella)* could be identified as nptII carrier .

Within a confidence limit of 95% (depicted in brackets) the prevalence of nptII was below 0.70% in *Escherichia coli* [0%; 0.70%], and below 0.75%, 0.73%, 0.32%, and 0.058% in enterococci [0%; 0.75%], *Staphylococcus aureus* [0%; 0.73%], *Pseudomonas aeruginosa* [0%; 0.32%] and *Salmonella* spp. [0%; 0.058%] isolates, respectively.

For nptIII the prevalences - within a confidence limit of 95% - were below 1.47% in *E. coli* [0%; 1.47%], and below 42.4%, 5.02%, 0.32%, and 0.037% with enterococci [32.84%; 42.4%], *S. aureus* [1.51%; 5.02%], *Pseudomonas aeruginosa* [0%; 0.32%] and *Salmonella enterica* subsp. *enterica* [0%; 0.037%] isolates, respectively.

The prevalence of nptII in human pathogenic bacterial strains in Austria was low and clinically irrelevant. NptIII was distinctly more prevalent in the sample compilation analysed during the test period compared to the frequency of nptII. However, no nptIII carriers could be found in *Pseudomonas* and *Salmonella* isolates.

The presented results support – with a high grade of confidence - the hypothesis of a low prevalence of nptII in the analysed human pathogenic strains collected representatively all over Austria.

1.2 Zusammenfassung

Fünf Bakterienspecies (*Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus)* wurden prospektiv von repräsentativen klinisch-diagnostischen Laboratorien aus jedem österreichischen Bundesland gesammelt. Die Stämme wurden ohne antibiotische Vorselektion isoliert und mittels TaqMan Real time PCR auf dem LightCycler 480 auf die Anwesenheit von nptII und/oder nptIII Resistenzgenen untersucht. Zusätzlich wurde eine *Salmonella enterica* subsp. *enterica* Stammsammlung aus den Jahren 2008 – 2010 auf Kanamyin-Empfindlichkeit getestet und die resistenten Stämme auf ihren nptII/nptIII Trägerstatus hin überprüft. Insgesamt wurden so die nptII und nptIII Resistenzgenprävalenzdaten von mehr als 10 400 Proben ermittelt.

Mit einer Sicherheit von 95% war die Prävalenz von nptII bei *Escherichia coli* niedriger als 0.70% [0%; 0.70%], bei Enterokokken niedriger als 0.75% [0%; 0.75%], bei *Staphylococcus aureus* niedriger als 0.73% [0%; 0.73%], *bei Pseudomonas aeruginosa* niedriger als 0.32% [0%; 0.32%] und bei *Salmonella* spp. niedriger als 0.058% [0%; 0.058%]. In Klammer ist das 95% Konfidenzintervall angeführt.

Bei nptIII lagen die Prävalenzen – innerhalb eines 95% Konfidenzlimits - bei *Escherichia coli* unter 1.47% [0%; 1.47%], bei Enterokokken unter 42.4% [32.84%; 42.4%], bei *Staphylococcus aureus* unter 5.02% [1.51%; 5.02%], bei *Pseudomonas aeruginosa* unter 0.32% [0%; 0.32%] und bei *Salmonella enterica* subsp. *enterica* unter 0.037% [0%; 0.037%].

Die Prävalenz von nptII in humanpathogenen Bakterien in Österreich war niedrig und klinisch irrelevant. NptIII kam im Vergleich zu nptII im untersuchten Bakterienkollektiv im Testzeitraum deutlich häufiger vor. Trotzdem konnten bei *Pseudomonas* und *Salmonella* Isolaten keine nptIII Träger ermittelt werden.

Die vorliegenden Untersuchungsergebnisse unterstützen mit einer hohen Sicherheit die Hypothese einer geringen Häufigkeit von nptII in den analysierten humanpathogenen Stämmen, die repräsentativ für ganz Österreich gesammelt worden sind.

1.3 Aims of the project

In the presented part of the project the following objectives were pursued:

1. Establishment of the baseline frequency of nptII and nptIII in pathogenic bacterial populations in Austria:

   To get an overview about the prevalence of neomycin phosphotransferase II and III genes in pathogenic bacteria the baseline frequency of these two representatives of antibiotic resistance marker (ARM) genes and their wild type homologs in bacterial pathogens in Austria should be established. These data provide a valuable pool of information for the following two reasons:

   a) The relevance of nptII and nptIII genes in the development of aminoglycoside resistance in pathogenic bacterial strains in Austria will be elucidated.

   b) These baseline frequency data provide a convenient reference line of the nptII and nptIII abundance in a relevant gene pool before exposure of this gene pool to external sources like ARM genes of transgenic plants. The impact of exogenously introduced antibiotic resistance genes via transgenic plants into microbial ecosystems can be evaluated in the future by comparison of nptII and nptIII frequencies after the introduction of ARM containing transgenic plants with the pristine baseline established in the course of this project.

2. The selected monitoring species should be of clinical relevance i.e. they should be responsible for a substantial burden of infectious disease of the testing area (Austria).

1.4 Introduction

Facing a global crisis in the treatment of infectious diseases with increasing morbidity and mortality rates, resistant and multiresistant strains of bacterial pathogens constitute a major burden for health systems (18, 22). Impeding the transfer of antibiotic resistance determinants is the ultimate objective in hospital and community settings since the delivery of new antimicrobial compounds has not been sufficient nor adequate during the past decades to get this situation under control (36). Under these circumstances a possible release of DNA from antibiotic resistance marker (ARM) gene containing transgenic plants into the environment over long periods of time should be the subject of an in depth risk analysis.

The neomycin phosphotransferase II gene (nptII; aph(3')-IIa) is the most abundantly used antibiotic resistance marker gene in plant gene technology today and many commercialized transgenic plants are carriers of this ARM gene (10, 26, 28). When the transgenic plants are used as food or feed, resistance encoding plant DNA has the opportunity for getting into contact with competent bacteria residing in the gastrointestinal tracts of the consumers. Transgenic plants cultivated on the field may provide soil bacteria with antibiotic resistance determinants encoded in the transgenic plant DNA (20). In both instances these resistance determinants might be taken up either directly by pathogens or by commensal bacteria passing them on to human or animal pathogens via horizontal gene transfer which consequently may impede effective treatment of bacterial infections (21, 29). There have been several attempts to provide a holistic risk assessment of the nptII ARM gene, but none of them considered a quantitative approach as appropriate (4, 9, 11, 13, 21). However, for estimations of the impact of an additional introduction of ARM genes into the naturally occurring resistance gene pool via genetically modified plants information about the original ecology of the respective resistance genes is a prerequisite (7, 25). This also includes the estimation of the frequency of nptII carrying strains in certain bacterial populations and the quantitative determination of nptII gene copy numbers in certain environments.

This project tries to help to diminish this knowledge gap by providing prevalence data of two antibiotic resistance marker genes - nptII and nptIII – for pristine environments not yet exposed to ARM gene carrying DNA of transgenic plants.

1.4.1 The aminoglycoside phosphotransferase gene aph(3')-IIa as a risk factor

Horizontal transfer of plant-derived ARM genes to soil or gut bacteria resulting in an impaired antimicrobial treatment of animal and human infectious diseases is unlikely but cannot be excluded a priori (12, 19, 21). However, the additional input of DNA which is coding for antibiotic resistance determinants derived from transgenic organisms over extended periods of time via plant decaying processes or root exsudates in soil or uptake by food or feed would increase the likelihood of contact between resistance encoding DNA and competent bacteria (29).

At present it is unclear whether this increased exposure rate is actually capable of inducing changes in the composition of the global bacterial antibiotic resistance gene pool. According to the consolidated scientific opinion of the EFSA GMO and the BIOHAZ Panels it is unlikely that disseminated ARM genes result in an increased failure rate in treatment of infectious diseases (12). However, a quantitative analysis or other direct experimental evidence backing this conclusion are lacking. Additionally, the authors identify a certain degree of uncertainty (like limitations related to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source), which have had to be considered during the preparation of the opinion (12). Two members of the BIOHAZ Panel did not concur with the final consolidated conclusion and expressed minority opinions concerning the likelihood of adverse effects of ARM genes (nptII, aadA) to human health and the environment (12).

The neomycin phosphotransferase nptII (= aph(3')-IIa) inactivates the aminoglycoside antibiotics kanamycin, neomycin, paromomycin, ribostamycin, butirosin, gentamicin B, and geneticin (= G418) by phosphorylation of the hydroxyl at position C3 in the first aminoglycan ring (27). *In vitro* inactivation of amikacin was reported, but only a combination of a chromosomal mutation, which reduces the general uptake of aminoglycosides and a second mutation, which increases the copy number of the plasmid carrying the aph(3')-IIa gene, leads to high level amikacin resistance (31).

The nptII variant currently used in transgenic plants inactivates only kanamycin, neomycin and geneticin (32, 33).

A number of nptII containing GMOs intended for cultivation and/or placing on the market as food or feed are currently in the risk assessment pipeline of EFSA or have already been approved for commercialization in the EU (see Appendix C, Table 32)(10, 15, 16). MON 863, a maize variety carrying nptII as ARM gene, has been licensed for placing on the market already in 2005 (17). The nptII containing potato line EH92-527-1 was approved for cultivation and marketing by the European Commission in 2010 (15).

It is supposed that natural environments harbour resistance determinants inactivating kanamycin and neomycin to a large extent and that DNA transfers from plant to bacteria are extremely rare events under naturally occurring conditions (12). At present approx. 50 different enzymes with the potential to inactivate aminoglycoside antibiotics are known and may be categorized into three distinct classes: aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferases (ANT) and aminoglycoside phosphotransferases (APH) (27, 34).

1.4.2 Resistance to kanamycin/neomycin in clinical isolates

Resistance to kanamycin or neomycin in general is a known phenomenon in clinical environments, although the frequency is varying considerably between locations and bacterial strains, possibly reflecting variable selection pressure. NptII was shown to be located on transposon Tn5 (3) and is supposed to be spread within bacterial communities. However, phenotypical resistance to kanamycin is only rarely mediated by nptII in clinical isolates (34). In theses rare cases *Pseudomonas*, *Aeromonas* and *Escherichia coli* have been shown to be major nptII carriers (35). Alvarez et al. demonstrated a low prevalence of nptII genes in clinical samples (1). In Austria kanamycin resistance of major pathogens like *Salmonella enterica* subsp. *enterica* and *Campylobacter* spp. is usually rare and/or clinically insignificant (5).

1.4.3 Clinical relevance of kanamycin/neomycin in Austria

Neomycin and kanamycin are both inactivated by the gene product of nptII (27). In Austria neomycin is applied only topically for the treatment of superficial skin and mucosal infections in humans (30). Currently, 12 preparations containing neomycin are licensed and on the local market (see Appendix C, Table 33) (2). Eleven neomycin preparations are available for veterinary applications. Predominantly neomycin is used for the treatment of mastitis and enteritis in cattle, pigs, and poultry, but also pets (e.g. cats, dogs, etc...) with eczema and dermatitis of microbial origin receive a therapy relying on neomycin preparations (see Appendix C, Table 34).

In Austria kanamycin is presently not licensed for the treatment of infectious diseases in humans (30). Nevertheless, this aminoglycoside is a second line antibiotic for the treatment of multidrug resistant tuberculosis (especially for the extremely difficult therapy of extensively drug-resistant tuberculosis (XDR-TB) (12, 23, 37). In Austria three kanamycin preparations are licensed for treatment and prophylaxis of a broad range of infections in cattle, sheep, pigs, and poultry (see Appendix C, Table 35).

Amikacin, which is inactivated by the nptIII encoded aminoglycoside phosphotransferase (3')-IIIa, is an important aminoglycoside antibiotic exclusively restricted for the treatment of serious infections in man. Amikacin is applied for the treatment of bacterial strains, which have been proven to be resistant to other classes of aminoglycoside drugs (e.g. gentamicin) (23, 30). Moreover, amikacin is an important second line antibiotic for the treatment of multidrug resistant tuberculosis (23, 30). There is no indication for amikacin application in veterinary medicine (11).

Amikacin has been classified as "critically important antibiotic" by a WHO expert working group; neomycin and kanamycin were characterized as "highly important" antimicrobials (38). EMA is also of the opinion that neomycin and kanamycin continue to play a role in clinical and veterinary applications (14).

Due to a constant increase of bacteria resistant to various different classes of antibiotics during the past decades, treatment of some infections will rely on older (aminoglycoside) antibiotics with unfavorable pharmacologic properties in the future (14).

1.5 Sampling plan

To achieve a precision of +/- 1% with a statistical power of 50% at least 384 bacterial isolates per species have had to be collected and validly analysed (for an in depth analysis of project specific random sampling and the development of a sampling plan see section 3.1). The sampling procedure of the isolates guaranteed a representative distribution of the collecting laboratories (Table 1 and e.g. Figure 2). The strains were stored at the central strain collection at AGES-IMED in Graz frozen at -80°C using commercial cryovials.

Province	Inhabitants	% Total population	Number of strains
Burgenland	283,965	3.4%	**13**
Kärnten	559,315	6.7%	**26**
Niederösterreich	1,607,976	19.2%	**74**
Oberösterreich	1,411,238	16.9%	**65**
Salzburg	529,861	6.3%	**24**
Steiermark	1,208,372	14.4%	**55**
Tirol	706,873	8.4%	**32**
Vorarlberg	368,868	4.4%	**17**
Wien	1,698,822	20.3%	**78**
Austria	**8,375,290**	**100%**	**384**

Table 1. Sampling plan

1.6 Selection of bacterial samples for monitoring nptII and nptIII prevalences – reference strains

To get an overview about the prevalence of nptII and nptIII resistance genes in human pathogens in Austria, certain representative bacterial species have had to be selected for monitoring. For this purpose the following bacterial species already on the monitoring list of the European Antibiotic Resistance Surveillance System (EARS) and of the German Network for Antibiotic Resistance Surveillance (GENARS) were chosen: *Escherichia coli, Enterococcus faecalis*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Table 2).

Strains of these species were actively collected for the present research project in collaboration with clinical diagnostic laboratories located representatively all over Austria (for participating laboratories see Table 31). At least one laboratory from each of the nine provinces of Austria took part in the survey. The samples were collected on a first in - first out basis under routine conditions in the participating laboratories, identified on site and sent to the research project specific collection centre in Graz (AGES-IMED, Graz).

There was no pre-selection of the isolates on kanamycin containing selective media before forwarding them to the collection centre or before PCR analysis. Thus, the PCR results represent the prevailing nptII/nptIII status in the surveyed bacterial populations from clinical and community environments without any obvious selection bias.

This approach had to be pursued because the frequency of resistance to kanamycin in the respective bacterial species is known to be low in Austria. A collection of the statistically significant number of **resistant** isolates would not have been possible within a reasonable time frame of the project.

For the respective sampling periods please see Table 2.

Bacterial Strain	Sampling procedure	Sampling period
Escherichia coli	active sampling	February – July 2011
Enterococcus faecalis, E. faecium	active sampling	February – July 2011
Pseudomonas aeruginosa	active sampling	July – November 2010
Salmonella enterica subsp. *enterica*	existing local strain collection	2008 – 2010
Staphylococcus aureus	active sampling	February – July 2011

Table 2. Bacterial pathogens used for nptII/nptIII prevalence analysis

Additionally, a section of the local strain collection for *Salmonella* isolates from the Austrian Reference Centre for *Salmonella* (AGES-IMED, Graz) was screened for the prevalence of nptII and nptIII resistance genes. In Austria all *Salmonella* isolated (including human, animal, food, feed and environmental origins) have to be submitted to AGES. The strains of human origin have not been collected specifically for the present research project but are part of the national routine sampling efforts. This *Salmonella* strain collection provides a representative overview about the resistance status of Salmonella strains prevalent in Austria. The project specific nptII/nptIII screening approach covered over 8200 *Salmonella* isolates sampled during the period from 2008 – 2010 (see section 2.1.7, Table 14). Only kanamycin resistant samples were subjected for PCR analysis.

1.7 Clinical relevance of the selected reference strains

Escherichia coli

Escherichia coli is the most abundant cause for bacteremia and for hospital- and community-aquired urinary tract infections. *E. coli* can be identified as etiological agent in a broad spectrum of other infectious diseases e.g. peritonitis, wound- and gastrointestinal-infections. In 2010, 51% of the *E. coli* isolates in Austria showed resistance to aminopenicillins, 5.5% were resistant to aminoglycosides (5).

Enterococcus faecalis, Enterococcus faecium

Enterococci are Gram-positive inhabitants of the normal bacterial flora of the human gastrointestinal tract. However, under certain adverse circumstances (e.g. immunocompromised host) these commensals can induce severe infections (e.g. endocarditis, bacteremia, wound- and urinary tract-infections, peritonitis and intraabdominal abscesses). Enterococci are intrinsically resistant to a broad spectrum of antimicrobials and easily transferable between humans, constituting a substantial hazard for nosocomial infections. The incidence for bacteremia is 11.8 cases per 100,000 inhabitants in Austria. In 2010, 32.1% of all *E. faecalis* and 41.8% of all *E. faecium* isolates showed high level resistance to aminoglycosides (5).

Pseudomonas aeruginosa

P. aeruginosa, a Gram-negative non-fermenter, can cause nosocomial infections and chronic infections in patients suffering from cystic fibrosis. Due to co-morbidities of the patients and/or resistance to various antibiotics lethality in *P. aeruginosa* induced bacteremia is high; the incidence is 6.6 cases per 100,000 inhabitants. A maximum of 14.4% of the isolates were resistant to carbapenems in 2010 in Austria, approx. 10% were resistant to aminoglycosides (5).

Salmonella enterica subsp. *enterica*

Salmonella enterica subsp. *enterica* is still one of the main leading etiologic agents inducing gastroenteritis worldwide. Although reporting of disease was decreasing in Austria over the past decade, salmonellosis remains a public health concern. In 2010 the incidence was 26 cases per 100,000 inhabitants in Austria. In the past few years a shift towards higher resistance rates in Austria was observed, which was caused by a decline of fully susceptible *S.* Enteritidis isolates. In 2010 the highest resistance rates were found against ampicillin (13.8%), streptomycin (12.4%), sulfonamides (13.4%) and tetracyclines (14.9%; i.e. a resistance pattern typical for multiresistant *S.* Typhimurium strains). Resistance against nalidixic acid (10.4%) is typical for several *S.* Enteritidis phage-types. Resistance to kanamycin was infrequently observed (0.7%)(5).

Staphylococcus aureus

The Gram-positive bacterium *S. aureus* colonizes the skin of approx. 30% of the healthy population without doing any harm. Under certain conditions *S. aureus* can cause severe infections which are of major concern in hospital settings, especially if methicillin resistant (=MRSA) strains are involved. *S. aureus* infections prolong the duration of hospitalisation and increase mortality and the financial burden of the health care system. The incidence of *S. aureus* induced bacteremia is 24.2 cases per

100,000 inhabitants. The proportion of MRSA isolates in Austria for 2010 was 7.4%. Antibiotic susceptibility data for Austria concerning kanamycin are not available (5).

1.8 Identification of isolated bacterial strains

The five bacterial species actively collected for the project were identified at the collaborating peripheral laboratories (see section 3.10; Table 31), usually the one with the highest sample turnover rate in the respective province, using the locally established and validated routine procedures (API identification test strips, VITEK, MALDI-TOF).

At the central collection centre in Graz (AGES-IMED, Graz) the correct assignment of the sample and the accompanying data sheet from the peripheral laboratory were checked and the isolates were controlled for contaminations by single colony plating on Columbia blood agar plates (Becton Dickinson, Heidelberg, Germany) or on CPS3 agar plates (Chromogenagar, bioMérieux, Marcy l'Etoile, France). Identification of *E. coli* samples were routinely verified at the collection centre by using an indol test (James-Reagent).

Colonies with unclear results were re-tested by VITEK at the central collection centre. For a detailed description of the handling procedure for incoming external samples and characterization of actively sampled isolates at the collection centre see section 3.2.

1.9 Real time PCR analysis

All samples which were subjected for nptII/nptIII resistance gene prevalence analysis were sent as cryostocks from the collection centre in Graz to the real time PCR testing facility at AGES-IMED, Vienna. The strains were directly inoculated from the cryostock into 100 µl BHI medium and incubated overnight at 37°C. After centrifugation the pellet was resuspended in 100 µl QuickExtract DNA Extraction Solution 1.0 (Epicentre, USA) and incubated at 65°C and 98°C (for detailed procedure see section 3.4). A 10^{-1} dilution served as template (2 µl/assay) for all TaqMan PCR assays (nptII/nptIII TaqMan Double Screening and 16S TaqMan Single Assay). A summary of the PCR parameters is documented in Table 3.

	nptII	nptIII	16S
Probes			
Label:	FAM	5'YYE	Cy5
Concentration:	0.2 µM	0.2 µM	0.2 µM
Primers			
Forward	0.6 µM	0.6 µM	0.5 µM primer mix (degenerated, universal 16S)
Revers	0.6 µM	0.6 µM	
PCR conditions			
A) Initial denaturation	95°C/10 min/1x	95°C/10 min/1x	95°C/10 min/1x
B) Cycling	45 x	45 x	45 x
denaturation	95°C/10 s	95°C/10 s	95°C/10 s
annealing + elongation	60°C/20 s	60°C/20 s	60°C/1 min
C) Cool down	40°C/continuous	40°C/continuous	40°C/continuous
Assay			
Template volume	2 µl	2 µl	2 µl
PCR mix volume	8 µl	8 µl	8 µl
Total PCR assay volume	10 µl	10 µl	10 µl

Table 3. LightCycler LC480 real time TaqMan PCR parameters

1.9.1 Screening: nptII/nptIII TaqMan PCR Double Assay

All real time PCR assays were implemented on the LightCycler LC480 real time PCR platform (Roche, Austria) using 96-well microtiter plates. NptII and nptIII gene targets were detected simultaneously in a single well. For validation data on the PCR systems see section 3.7.

Of the 10^{-1} diluted bacterial Quickextract DNA solution 1.0 (Epicentre, USA), 2 µl were transferred into 8 µl of the real time PCR TaqMan assay mix resulting in total PCR assay volume of 10 µl. PCR TaqMan Double Assays were prepared according to the recommendations of the manufacturer (Ingenetix, Vienna). For details see section 3.5.

Each 96-well microtiter plate contained two negative controls (H_2O as template) and nptII and nptIII plasmid positive controls in duplicates. The DNA extraction solution from each sample was tested once with the nptII/nptIII TaqMan Double Assay and once with the 16S TaqMan DNA extraction/amplification control assay.

1.9.2 DNA extraction and amplification control: 16S TaqMan PCR Assay

Each sample was tested for amplifiable DNA and for PCR inhibition by analysis of the respective Epicenter DNA extract with an "internal" control 16S TaqMan Assay. NptII and nptIII PCR results were valid only if the 16S TaqMan PCR assay was positive (Cp cut off: ≤ 27), i.e. the assay was indicative for

the presence of a sufficient number of target molecules from a single copy gene in the PCR assay. For details on the interpretation of the TaqMan PCR results see section 1.10.3.

Samples were handled as described in sections 3.4 and 3.5. 16S rRNA TaqMan PCR specific details concerning assay composition and PCR conditions are presented in section 3.6.

1.10 PCR screening results

1.10.1 Prevalence of nptII in human pathogenic bacteria in Austria

Five bacterial species (*Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus)* were collected prospectively for this study and analysed for the presence of nptII and/or nptIII. Additionally, *Salmonella enterica* subsp. *enterica* isolates collected from 2008 – 2010 were evaluated for resistance to kanamycin resulting in 60 isolates resistant to this aminoglycoside antibiotic. These 60 kanamycin resistant isolates were tested by TaqMan PCRs.

A single *Salmonella* isolate (MRS-10/00765; serotype *S.* Typhimurium, lysotype 120, antigen formula: 1,4,5,12:i:1,2) could be identified as nptII positive (for details: Table 13). This resulted in a prevalence estimation for nptII in this genus of 0.013%. All other isolates were negative for nptII (Table 4).

The prevalence for nptII in human pathogenic *Escherichia coli* during the test period was - with a confidence level of 95% - below 0.70%. For enterococci the prevalence of nptII was calculated to be below 0.75%. For *S. aureus*, *Pseudomonas aeruginosa*, and *Salmonella* isolates the prevalence of nptII was - with a confidence level of 95% - below 0.73%, 0.32%, and 0.06%, respectively (Figure 1).

To achieve a precision of +/- 1% with a statistical power of 50% at least 384 bacterial isolates per monitoring species have had to be collected and validly analysed. This was accomplished with each monitoring species (see Table 4).

The regional sources of the tested samples and their distribution are depicted in Figure 2 showing the results exemplarily for *Pseudomonas aeruginosa* isolates.

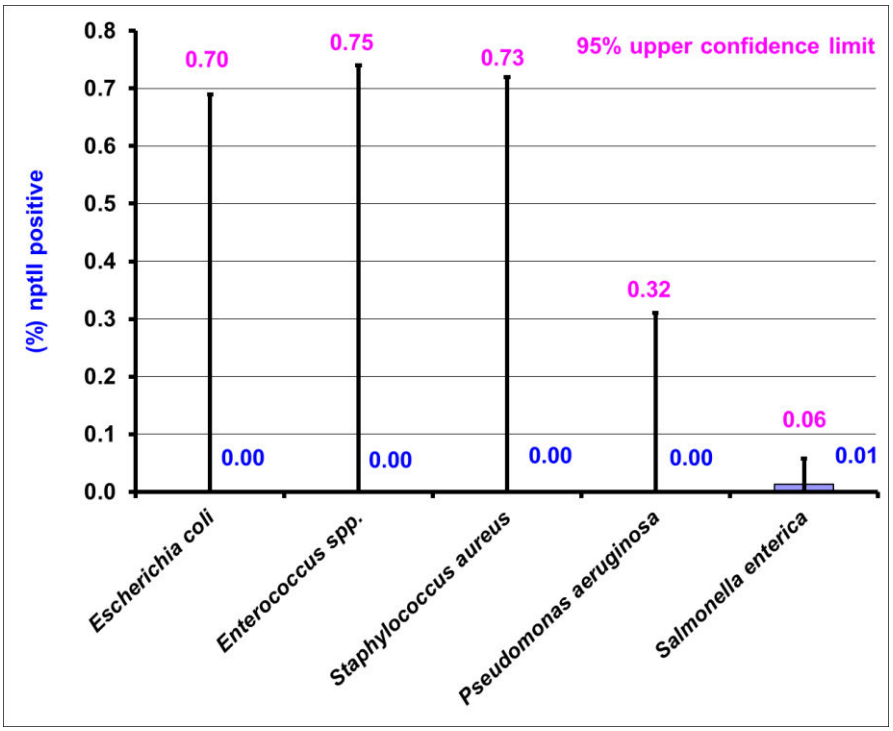


Figure 1. Prevalence of nptII in human pathogenic strains in Austria

Prevalence estimator and 95% upper confidence limit error bars are shown. The 95% confidence interval was calculated according to Clopper-Pearson.

Strain	No. of isolates[1)]	No. isolates nptII positive	% isolates nptII positive[2)]	95% confidence[3)]
Escherichia coli	428	0	0	0 – 0.70
Enterococcus spp.[4)]	403	0	0	0 – 0.75
Staphylococcus aureus	412	0	0	0 – 0.73
Pseudomonas aeruginosa	951	0	0	0 – 0.32
Salmonella enterica	8,235[5)]	1	0.013[6)]	0 – 0.058
Total:	10,429	1	0.0096	0 – 0.046

Table 4. Prevalence of nptII in human pathogenic bacteria in Austria.

1) Number of isolates which were egligible for statistical evaluation. Included were isolates with positive nptII or negative nptII results in combination with a positive 16S TaqMan PCR result (= valid PCR result). Excluded were samples with negative nptII and negative 16S TaqMan PCR results (possible false negative nptII results due to insufficient amounts of target DNA in the assay indicated by a negative 16S PCR result; = invalid PCR result)

2) prevalence estimator

3) 95% confidence interval according to Clopper-Pearson

4) *Enterococcus faecalis* and *Enterococcus faecium* isolates combined

5) In total 8,235 samples were tested for resistance to kanamycin. Sixty isolates appeared to be resistant and were analysed by TaqMan real time PCR. If only these 60 PCR results were included for statistical analysis the corresponding results were: 1 strain was nptII positive resulting in a prevalence estimator of 1.67 % and a 95% confidence interval of 0 – 7.67%.

6) Using this approach non-expressed nptII genes and gene variants producing no active enzyme were not detectable

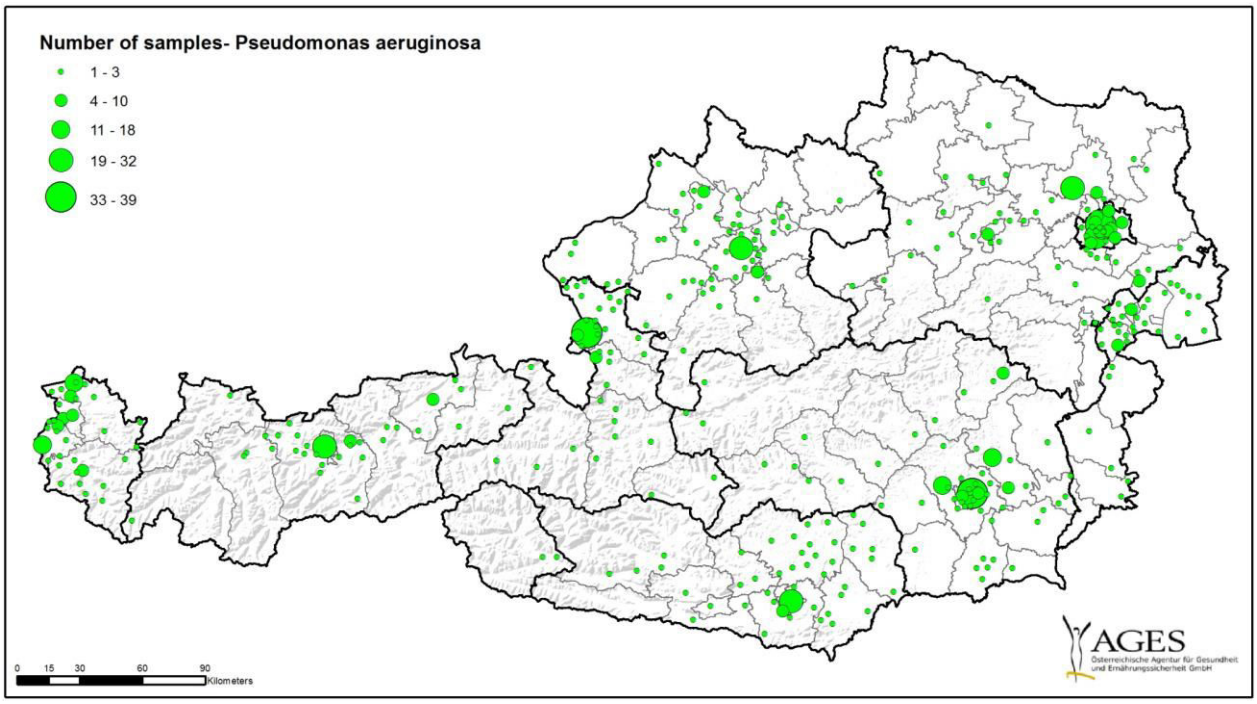


Figure 2. Localisation and source of the *Pseudomonas aeruginosa* isolates tested validly for the presence of nptII

1.10.2 Prevalence of nptIII in human pathogenic bacteria in Austria

Five bacterial species (*Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus)* were collected prospectively for this study and analysed for the presence of nptII and/or nptIII. Additionally, *Salmonella enterica* isolates collected from 2008 – 2010 were retrospectively evaluated for resistance to kanamycin resulting in 60 isolates resistant to this aminoglycoside antibiotic. These 60 kanamycin resistant isolates were tested by TaqMan PCRs.

Of the collected *E. coli* isolates, 0.47% tested nptIII positive during the test period (95% unilateral confidendence interval [0%; 1.47%]). Enterococci and *S. aureus* isolates were nptIII positive in 37.53% (bilateral 95% confidendence interval [32.84%; 42.4%]) and 2.9% [1.51%; 5.02%] of the analyses. No nptIII positive isolates could be retrieved from *Pseudomonas aeruginosa* and from *Salmonella enterica* samples (Table 5).

The prevalence for nptIII in human pathogenic *Escherichia coli* during the test period was - with a confidence level of 95% - below 1.46%. For enterococci the prevalence of nptIII was calculated to be below 42.4%. For *S. aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica* isolates the prevalence of nptII was - with a confidence level of 95% - below 5.02%, 0.31%, and 0.037%, respectively (Figure 3).

To achieve a precision of +/- 1% with a statistical power of 50% at least 384 bacterial isolates per monitoring species have had to be collected and validly analysed. This was accomplished with each monitoring strain (Table 5).

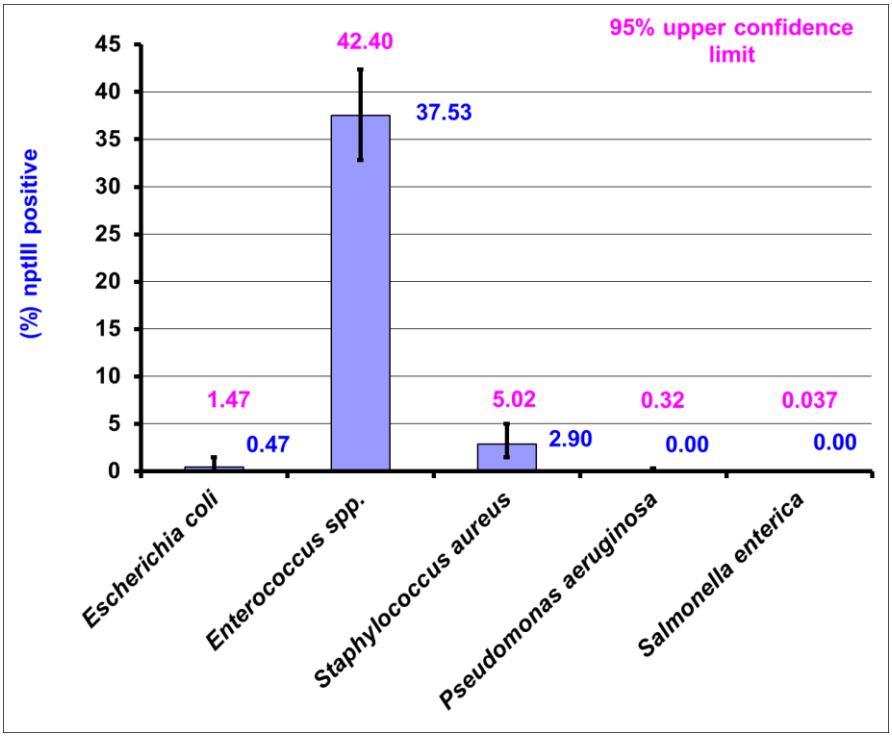


Figure 3. Prevalence of nptIII in humanpathogenic strains in Austria

Prevalence estimator and 95% upper confidence limit error bars. The 95% confidence interval was calculated according to Clopper-Pearson.

Strain	No. of isolates[1)]	No. isolates nptIII positive	% isolates nptIII positive[2)]	95% confidence[3)]
Escherichia coli	428	2	0.47	0 – 1.47
Enterococcus spp.[4)]	413	155	37.53	32.84 – 42.40
Staphylococcus aureus	413	12	2.90	1.51 – 5.02
Pseudomonas aeruginosa	951	0	0	0 – 0.32
Salmonella enterica	8,235[5)]	0	0	0 – 0.037
Total:	10,440	169	1.62	1.38 – 1.88

Table 5. Prevalence of nptIII in human pathogenic bacteria in Austria

1) isolates with positive nptIII or negative nptII and positive 16S TaqMan PCR result (for a detailed explanation see Table 4)

2) prevalence estimator

3) 95% confidence interval according to Clopper-Pearson

4) *Enterococcus faecalis* and *Enterococcus faecium* isolates combined

5) In total 8235 samples were tested for resistance to kanamycin (disk diffusion test: inhibition diameter: ≤13 mm; according to CLSI clinical breakpoints). Sixty isolates appeared to be resistant and were analysed by TaqMan real time PCR. If only the PCR results of this 60 isolates were analysed statistically the corresponding results are: None of the strains was nptIII positive resulting in a prevalence estimator of 0% and a 95% confidence interval of 0 – 4.88% for these 60 samples.

Number and localisation of the collected isolates are exemplarily shown for enteroccoci in Figure 4. The sources for the nptIII positive enterococci isolates are depicted in Figure 5.

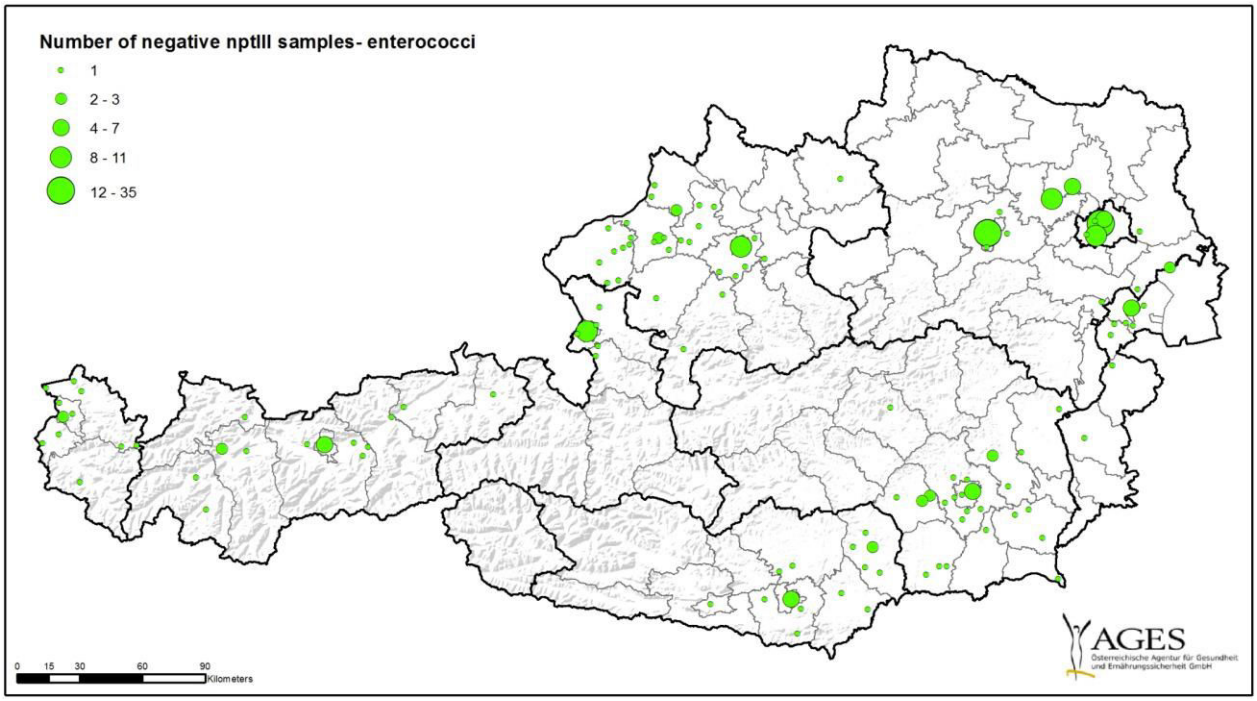


Figure 4. Localisation and source of the enterococci isolates tested negative for the presence of nptIII

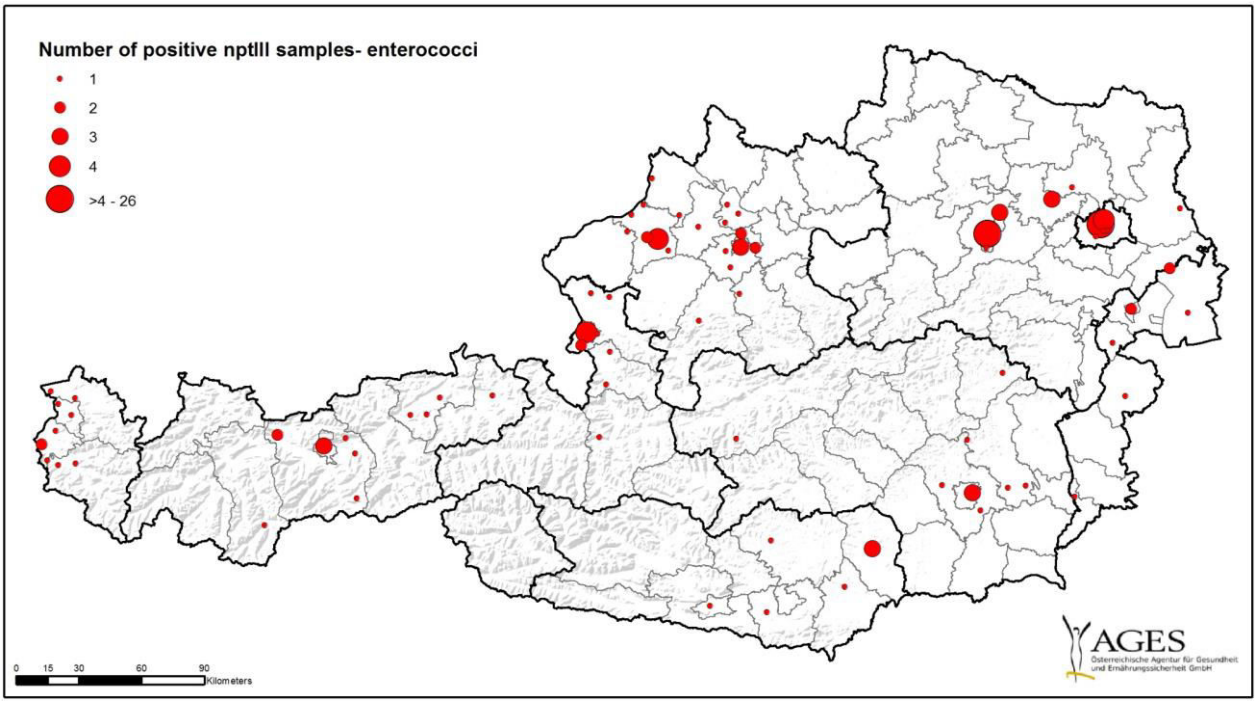


Figure 5. Localisation and source of the enterococci isolates tested positive for the presence of nptIII

1.10.3 Interpretation of the PCR results: characteristics of the applied screening system

The nptII, nptIII and 16S TaqMan PCR systems were validated thoroughly. For details see section 3.7. The 95% detection limit (as determined by probit regression analysis) for the nptII assay was 7.9 and for nptIII 11.4 copies/assay. A detection limit for the 16S TaqMan PCR assay was not applicable due to the background load of assay components with 16S rDNA. The 16S TaqMan assay produced an amplification signal in the no template controls around crossing point (Cp) 34. Cp 34 corresponds to 34 PCR amplification cycles.

NptII or nptIII TaqMan PCRs were rated negative if no amplification occurred before Cp 45 and the corresponding 16S TaqMan assay was positive (see *Amplification control* below). Samples which produced an amplification curve between Cp 35 and Cp 45 were reanalysed. All samples with an amplification signal < Cp 35 were rated ARM gene positive. As the pellet of a 100 µl overnight pure culture served as PCR template nptII and/or nptIII positive strains produced amplification curves usually around Cp 22 – Cp 24.

The PCR run was only valid if all negative controls on the 96-well microtiter plate were negative and all positive controls were positive. Otherwise the run was repeated.

nptII/nptIII Crosstalk in the TaqMan Double Assay:

This was no issue in the test setting since there was no negative impact on the amplification of each template if the concentration of both targets differed by a factor of 100.

Amplification control:

Samples with a negative 16S TaqMan assay result (Cp > 27) were excluded from statistical analysis if the nptII and/or the nptIII PCR result was negative. If the sample showed a 16S PCR result with a Cp value of 27 or lower and the nptII/nptIII PCR assay was negative then the bacterial strain under investigation was negative for the tested ARM genes under any circumstance. Additionally, the 16S amplicon was approx. 5 x longer (see section 3.5) compared to the nptII and nptIII amplicons: a functional (= positive) 16S TaqMan PCR must lead to the amplification of the shorter nptII and nptIII amplicons - if the sample is resistance gene positive – taking into consideration DNA degradation and PCR inhibitors.

Due to the combination of a stable and sensitive nptII/nptIII TaqMan PCR system and the low 16S PCR cut off value for the internal DNA extraction and amplification control, the negative results of the nptII/nptIII prevalence analysis were of high confidence.

1.11 Sequencing of nptII/nptIII positive strains

Strains which were nptII and/or nptIII positive were submitted for full length gene sequencing to gather information of resistance gene sequence variability in naturally occurring populations of pathogenic bacteria in Austria and to confirm the positive screening results. For details on the applied procedure see section 3.8, for the retrieved sequences see section 2.2.

Due to a single direct sequencing approach using the forward primer for the full-length gene amplicon a sequence coverage of approx. 750 bp could be achieved. Most of the retrieved sequences

showed complete homology to the respective wild type reference sequences. Those resistance gene carrier strains which showed sequence aberrations are listed in Table 6.

There were two silent mutations (positions 564 and 597 with EK-360 and ST-405). All other mutations led to an exchange of an amino acid in the aminoglycoside phosphotransferase enzyme (Table 6).

Position (reference: V01547 aph(3')-IIIa)	**Nucleotide exchange**	**Amino acid exchange**	**Strains**
322	C → A	proline → threonine (non polar → polar)	EK_287 EK_270 EK_264 EK_259 EK_233 EK_013
392	C → T	proline → histidine (non polar → basic)	EK_399
414	C → A	serine → arginine (non polar → basic)	EK_300
538	C → T	proline → threonine (non polar → polar)	EK_252
564	C → T	histidine → histidine	EK_360
597	A → G	lysine → lysine	ST_405

Table 6. NptIII gene sequence mutations

1.12 MIC determination of nptII/nptIII positive strains

Minimum inhibitory concentrations (MIC) of kanamycin were determined for all nptII and nptIII positive strains using the Epsilon-Test (for details on the procedure see section 3.9). Additionally, some PCR negative stains were randomly chosen as controls for MIC analysis.

The two nptIII carriers of *E. coli* were sensitive to kanamycin (MIC: 1.5 µg/ml; see Appendix A, Table 7). Most of the nptIII positive enterococci were high-level resistant to kanamycin (MIC >256 µg/ml; EUCAST, 2013), with the exception of EK-11/403 (MIC: 64 µg/ml) and EK-11/423 (eTest: 48 µg/ml; rounded up MIC: 64 µg/ml; see Appendix A, Table 9) which were low-level resistant. All of the nptIII positive *S. aureus* strains were resistant to kanamycin (MIC >256µg/ml; see Appendix A, Table 15; CLSI, 2009). The nptII carrying *Salmonella* isolate was resistant to kanamycin (MIC >256 µg/ml; see Appendix A, Table 13). The nptII and nptIII negative control strains were all sensitive to kanamycin (Table 9).

With the exception of both nptIII *E. coli* and the indicated enterococci carriers the MIC analysis supported the PCR screening results. Inhibition of gene expression due to a promoter mutation or a mutation inactivating the aminoglycoside phosphotransferase enzyme would be possible explanations for these aberrant results.

1.13 Conclusions: Prevalence of nptII and nptIII resistance genes in bacterial pathogens of human origin in Austria

The prevalence of nptII in the tested collection of human pathogenic strains from Austrian sources is low. From a total of more than 10,400 samples only one isolate could be identified as carrier of a functional aminoglycoside phosphotransferase (3')-IIa gene. This result is indicative for a resistance gene pool depleted for nptII. Under such conditions this resistance gene pool might be receptive for additional nptII gene copies from external sources. This observation is of significance since already a minute number of additional external nptII gene copies will alter the availability of nptII genes for competent bacteria to some extent - compared to a situation where a gene pool already endogenously saturated with nptII gene copies would not be substantially disturbed by the availability of minute amounts of external nptII genes of plant origin. At least at the time of the testing period the analysed resistance gene pool of human pathogenic bacteria in Austria appeared to be receptive for external copies of nptII.

Kanamycin resistance rates for most of the investigated bacteria are low in Austria. Although plant to bacteria gene transfer rates are extremely infrequent under naturally occurring conditions long term and constant exposure of bacteria with ARM gene containing DNA will increase the contact frequency between competent bacteria and resistance gene fragments. Under certain selection conditions this scenario might be capable to induce changes in the frequency of nptII gene copies in bacterial populations with a low endogenous nptII baseline. In this case it cannot be entirely excluded that an introduction of ARM genes into the accessible gene pool of the investigated bacteria will not lead to an increase in resistant bacteria.

Compared to the situation with neomycin phosphotransferase II genes nptIII resistance gene copies were distinctively more abundant in the analysed human pathogenic bacteria in Austria, cumulating in a worst case scenario of up to 42% of all enterococci being carriers of an aminoglycoside (3')-IIIa gene homolog. However, taking also into account that resistance gene transfer is substantially more efficient between bacteria compared to DNA uptake from plant material, an exogenous input of additional copies of nptIII from these sources into environments populated by enterococci would certainly be of minor importance compared to the current ecological status of nptII. In these environments enterococci may serve as effcient nptIII donors spreading this resistance determinant more rapidly via conjugation and transduction to potential other receptor bacteria compared to ARM gene transfer from plants to bacteria via transformation.

2 Appendix A: Individual Test Results

2.1 NptII/nptIII prevalence in bacterial pathogens in Austria

2.1.1 Prevalence of nptII/nptIII in *Escherichia coli* samples collected in Austria

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EC-11/001	-	-	+	n.d.		n.a.
EC-11/002	-	-	+	n.d.		n.a.
EC-11/003	-	-	+	n.d.		n.a.
EC-11/004	-	-	+	n.d.		n.a.
EC-11/005	-	-	+	n.d.		n.a.
EC-11/006	-	-	+	n.d.		n.a.
EC-11/007	-	-	+	n.d.		n.a.
EC-11/008	-	-	+	n.d.		n.a.
EC-11/009	-	-	+	n.d.		n.a.
EC-11/010	-	-	+	n.d.		n.a.
EC-11/011	-	-	+	n.d.		n.a.
EC-11/012	-	-	+	n.d.		n.a.
EC-11/013	-	-	+	n.d.		n.a.
EC-11/014	-	-	+	n.d.		n.a.
EC-11/015	-	-	+	n.d.		n.a.
EC-11/016	-	-	+	n.d.		n.a.
EC-11/017	-	-	+	n.d.		n.a.
EC-11/018	-	-	+	n.d.		n.a.
EC-11/019	-	-	+	n.d.		n.a.
EC-11/020	-	-	+	n.d.		n.a.
EC-11/021	-	-	+	n.d.		n.a.
EC-11/022	-	-	+	n.d.		n.a.
EC-11/023	-	-	+	n.d.		n.a.
EC-11/024	-	-	+	n.d.		n.a.
EC-11/025	-	-	+	n.d.		n.a.
EC-11/026	-	-	+	n.d.		n.a.
EC-11/027	-	-	+	n.d.		n.a.
EC-11/028	-	-	+	n.d.		n.a.
EC-11/029	-	-	+	n.d.		n.a.
EC-11/030	-	-	+	n.d.		n.a.
EC-11/031	-	-	+	n.d.		n.a.
EC-11/032	-	-	+	n.d.		n.a.
EC-11/033	-	-	+	n.d.		n.a.
EC-11/034	-	-	+	n.d.		n.a.
EC-11/035	-	-	+	n.d.		n.a.
EC-11/036	-	-	+	n.d.		n.a.
EC-11/037	-	-	+	n.d.		n.a.
EC-11/038	-	-	+	n.d.		n.a.
EC-11/039	-	-	+	n.d.		n.a.
EC-11/040	-	-	-	n.d.		n.a.
EC-11/041	-	-	+	n.d.		n.a.
EC-11/042	-	-	+	n.d.		n.a.
EC-11/043	-	-	+	n.d.		n.a.
EC-11/044	-	-	+	n.d.		n.a.
EC-11/045	-	-	+	n.d.		n.a.
EC-11/046	-	-	+	n.d.		n.a.
EC-11/047	-	-	+	n.d.		n.a.
EC-11/048	-	-	+	n.d.		n.a.
EC-11/049	-	-	+	n.d.		n.a.
EC-11/050	-	-	+	n.d.		n.a.
EC-11/051	-	-	+	n.d.		n.a.
EC-11/052	-	-	+	n.d.		n.a.
EC-11/053	-	-	+	n.d.		n.a.
EC-11/054	-	-	+	n.d.		n.a.
EC-11/055	-	-	+	n.d.		n.a.
EC-11/056	-	-	+	n.d.		n.a.
EC-11/057	-	-	+	n.d.		n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EC-11/058	-	-	+	n.d.		n.a.
EC-11/059	-	-	+	n.d.		n.a.
EC-11/060	-	-	+	n.d.		n.a.
EC-11/061	-	-	+	n.d.		n.a.
EC-11/062	-	-	+	n.d.		n.a.
EC-11/063	-	-	+	n.d.		n.a.
EC-11/064	-	-	+	n.d.		n.a.
EC-11/065	-	-	+	n.d.		n.a.
EC-11/066	-	-	+	n.d.		n.a.
EC-11/067	-	-	+	n.d.		n.a.
EC-11/068	-	-	+	n.d.		n.a.
EC-11/069	-	-	+	n.d.		n.a.
EC-11/070	-	-	+	n.d.		n.a.
EC-11/071	-	-	+	n.d.		n.a.
EC-11/072	-	-	+	n.d.		n.a.
EC-11/073	-	-	+	n.d.		n.a.
EC-11/074	-	-	+	n.d.		n.a.
EC-11/075	-	-	+	n.d.		n.a.
EC-11/076	-	-	+	n.d.		n.a.
EC-11/077	-	-	+	n.d.		n.a.
EC-11/078	-	-	+	n.d.		n.a.
EC-11/079	-	-	+	n.d.		n.a.
EC-11/080	-	-	+	n.d.		n.a.
EC-11/081	-	-	+	n.d.		n.a.
EC-11/082	-	-	+	n.d.		n.a.
EC-11/083	-	-	+	n.d.		n.a.
EC-11/084	-	-	+	n.d.		n.a.
EC-11/085	-	-	+	n.d.		n.a.
EC-11/086	-	-	+	n.d.		n.a.
EC-11/087	-	-	+	n.d.		n.a.
EC-11/088	-	-	+	n.d.		n.a.
EC-11/089	-	-	+	n.d.		n.a.
EC-11/090	-	-	+	n.d.		n.a.
EC-11/091	-	-	+	n.d.		n.a.
EC-11/092	-	-	+	n.d.		n.a.
EC-11/093	-	-	+	n.d.		n.a.
EC-11/094	-	-	+	n.d.		n.a.
EC-11/095	-	-	+	n.d.		n.a.
EC-11/096	-	-	+	n.d.		n.a.
EC-11/097	-	-	+	n.d.		n.a.
EC-11/098	-	-	+	n.d.		n.a.
EC-11/099	-	-	-	n.d.		n.a.
EC-11/100	-	-	+	n.d.		n.a.
EC-11/101	-	-	+	n.d.		n.a.
EC-11/102	-	-	+	n.d.		n.a.
EC-11/103	-	-	+	n.d.		n.a.
EC-11/104	-	-	+	n.d.		n.a.
EC-11/105	-	-	+	n.d.		n.a.
EC-11/106	-	-	+	n.d.		n.a.
EC-11/107	-	-	+	n.d.		n.a.
EC-11/108	-	-	+	n.d.		n.a.
EC-11/109	-	-	+	n.d.		n.a.
EC-11/110	-	-	+	n.d.		n.a.
EC-11/111	-	-	+	n.d.		n.a.
EC-11/112	-	-	+	n.d.		n.a.
EC-11/113	-	-	+	n.d.		n.a.
EC-11/114	-	-	+	n.d.		n.a.
EC-11/115	-	-	+	n.d.		n.a.
EC-11/116	-	-	+	n.d.		n.a.
EC-11/117	-	-	+	n.d.		n.a.
EC-11/118	-	-	+	n.d.		n.a.
EC-11/119	-	-	+	n.d.		n.a.
EC-11/120	-	-	+	n.d.		n.a.
EC-11/121	-	-	+	n.d.		n.a.
EC-11/122	-	-	+	n.d.		n.a.
EC-11/123	-	-	+	n.d.		n.a.
EC-11/124	-	-	+	n.d.		n.a.
EC-11/125	-	-	+	n.d.		n.a.
EC-11/126	-	-	+	n.d.		n.a.
EC-11/127	-	-	+	n.d.		n.a.
EC-11/128	-	-	+	n.d.		n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EC-11/129	-	-	+	n.d.		n.a.
EC-11/130	-	-	+	n.d.		n.a.
EC-11/131	-	-	+	n.d.		n.a.
EC-11/132	-	-	+	n.d.		n.a.
EC-11/133	-	-	+	n.d.		n.a.
EC-11/134	-	-	+	n.d.		n.a.
EC-11/135	-	-	+	n.d.		n.a.
EC-11/136	-	-	+	n.d.		n.a.
EC-11/137	-	-	+	n.d.		n.a.
EC-11/138	-	-	+	n.d.		n.a.
EC-11/139	-	-	+	n.d.		n.a.
EC-11/140	-	-	+	n.d.		n.a.
EC-11/141	-	-	+	n.d.		n.a.
EC-11/142	-	-	+	n.d.		n.a.
EC-11/143	-	-	+	n.d.		n.a.
EC-11/144	-	-	+	n.d.		n.a.
EC-11/145	drop out					
EC-11/146	-	-	+	n.d.		n.a.
EC-11/147	-	-	+	n.d.		n.a.
EC-11/148	-	-	+	n.d.		n.a.
EC-11/149	-	-	+	n.d.		n.a.
EC-11/150	-	-	+	n.d.		n.a.
EC-11/151	drop out					
EC-11/152	-	-	+	n.d.		n.a.
EC-11/153	-	-	+	n.d.		n.a.
EC-11/154	-	-	+	n.d.		n.a.
EC-11/155	-	-	+	n.d.		n.a.
EC-11/156	-	-	+	n.d.		n.a.
EC-11/157	drop out					
EC-11/158	-	-	+	n.d.		n.a.
EC-11/159	-	-	+	n.d.		n.a.
EC-11/160	drop out					
EC-11/161	-	-	+	n.d.		n.a.
EC-11/162	-	-	+	n.d.		n.a.
EC-11/163	-	-	+	n.d.		n.a.
EC-11/164	-	-	+	n.d.		n.a.
EC-11/165	-	-	+	n.d.		n.a.
EC-11/166	-	-	+	n.d.		n.a.
EC-11/167	-	-	+	n.d.		n.a.
EC-11/168	-	-	+	n.d.		n.a.
EC-11/169	-	-	+	n.d.		n.a.
EC-11/170	-	-	+	n.d.		n.a.
EC-11/171	-	+	+	1.5	sensitive	overlaid
EC-11/172	-	-	+	n.d.		n.a.
EC-11/173	-	-	+	n.d.		n.a.
EC-11/174	-	+	+	1.5	sensitive	wild type
EC-11/175	-	-	+	n.d.		n.a.
EC-11/176	-	-	+	n.d.		n.a.
EC-11/177	-	-	+	n.d.		n.a.
EC-11/178	-	-	+	n.d.		n.a.
EC-11/179	-	-	+	n.d.		n.a.
EC-11/180	-	-	+	n.d.		n.a.
EC-11/181	-	-	+	n.d.		n.a.
EC-11/182	-	-	+	n.d.		n.a.
EC-11/183	-	-	+	n.d.		n.a.
EC-11/184	-	-	+	n.d.		n.a.
EC-11/185	-	-	+	n.d.		n.a.
EC-11/186	-	-	+	n.d.		n.a.
EC-11/187	-	-	+	n.d.		n.a.
EC-11/188	-	-	+	n.d.		n.a.
EC-11/189	-	-	+	n.d.		n.a.
EC-11/190	-	-	+	n.d.		n.a.
EC-11/191	-	-	+	n.d.		n.a.
EC-11/192	-	-	+	n.d.		n.a.
EC-11/193	-	-	+	n.d.		n.a.
EC-11/194	-	-	+	n.d.		n.a.
EC-11/195	-	-	+	n.d.		n.a.
EC-11/196	-	-	+	n.d.		n.a.
EC-11/197	-	-	+	n.d.		n.a.
EC-11/198	-	-	+	n.d.		n.a.
EC-11/199	-	-	+	n.d.		n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EC-11/200	-	-	+	n.d.		n.a.
EC-11/201	-	-	-	n.d.		n.a.
EC-11/202	-	-	+	n.d.		n.a.
EC-11/203	drop out					
EC-11/204	-	-	+	n.d.		n.a.
EC-11/205	-	-	+	n.d.		n.a.
EC-11/206	-	-	+	n.d.		n.a.
EC-11/207	-	-	+	n.d.		n.a.
EC-11/208	-	-	+	n.d.		n.a.
EC-11/209	-	-	+	n.d.		n.a.
EC-11/210	-	-	+	n.d.		n.a.
EC-11/211	-	-	+	n.d.		n.a.
EC-11/212	-	-	+	n.d.		n.a.
EC-11/213	-	-	+	n.d.		n.a.
EC-11/214	-	-	+	n.d.		n.a.
EC-11/215	-	-	+	n.d.		n.a.
EC-11/216	-	-	+	n.d.		n.a.
EC-11/217	-	-	+	n.d.		n.a.
EC-11/218	drop out					
EC-11/219	-	-	+	n.d.		n.a.
EC-11/220	-	-	+	n.d.		n.a.
EC-11/221	-	-	+	n.d.		n.a.
EC-11/222	-	-	+	n.d.		n.a.
EC-11/223	-	-	+	n.d.		n.a.
EC-11/224	-	-	+	n.d.		n.a.
EC-11/225	drop out					
EC-11/226	-	-	+	n.d.		n.a.
EC-11/227	drop out					
EC-11/228	-	-	+	n.d.		n.a.
EC-11/229	-	-	+	n.d.		n.a.
EC-11/230	-	-	+	n.d.		n.a.
EC-11/231	-	-	+	n.d.		n.a.
EC-11/232	-	-	+	n.d.		n.a.
EC-11/233	-	-	+	n.d.		n.a.
EC-11/234	-	-	+	n.d.		n.a.
EC-11/235	-	-	+	n.d.		n.a.
EC-11/236	-	-	+	n.d.		n.a.
EC-11/237	-	-	+	n.d.		n.a.
EC-11/238	-	-	+	n.d.		n.a.
EC-11/239	-	-	+	n.d.		n.a.
EC-11/240	-	-	+	n.d.		n.a.
EC-11/241	-	-	+	n.d.		n.a.
EC-11/242	-	-	+	n.d.		n.a.
EC-11/243	-	-	+	n.d.		n.a.
EC-11/244	-	-	+	n.d.		n.a.
EC-11/245	-	-	+	n.d.		n.a.
EC-11/246	-	-	+	n.d.		n.a.
EC-11/247	-	-	+	n.d.		n.a.
EC-11/248	-	-	+	n.d.		n.a.
EC-11/249	-	-	+	n.d.		n.a.
EC-11/250	-	-	+	n.d.		n.a.
EC-11/251	-	-	+	n.d.		n.a.
EC-11/252	-	-	+	n.d.		n.a.
EC-11/253	-	-	+	n.d.		n.a.
EC-11/254	-	-	+	n.d.		n.a.
EC-11/255	-	-	+	n.d.		n.a.
EC-11/256	-	-	+	n.d.		n.a.
EC-11/257	-	-	+	n.d.		n.a.
EC-11/258	-	-	+	n.d.		n.a.
EC-11/259	-	-	+	n.d.		n.a.
EC-11/260	-	-	+	n.d.		n.a.
EC-11/261	-	-	+	n.d.		n.a.
EC-11/262	-	-	+	n.d.		n.a.
EC-11/263	-	-	+	n.d.		n.a.
EC-11/264	-	-	+	n.d.		n.a.
EC-11/265	-	-	+	n.d.		n.a.
EC-11/266	-	-	+	n.d.		n.a.
EC-11/267	-	-	+	n.d.		n.a.
EC-11/268	-	-	+	n.d.		n.a.
EC-11/269	-	-	+	n.d.		n.a.
EC-11/270	-	-	+	n.d.		n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EC-11/271	-	-	+	n.d.		n.a.
EC-11/272	-	-	+	n.d.		n.a.
EC-11/273	-	-	+	n.d.		n.a.
EC-11/274	-	-	+	n.d.		n.a.
EC-11/275	-	-	+	n.d.		n.a.
EC-11/276	-	-	+	n.d.		n.a.
EC-11/277	-	-	+	n.d.		n.a.
EC-11/278	-	-	+	n.d.		n.a.
EC-11/279	-	-	+	n.d.		n.a.
EC-11/280	-	-	+	n.d.		n.a.
EC-11/281	-	-	+	n.d.		n.a.
EC-11/282	-	-	+	n.d.		n.a.
EC-11/283	-	-	+	n.d.		n.a.
EC-11/284	-	-	+	n.d.		n.a.
EC-11/285	-	-	+	n.d.		n.a.
EC-11/286	-	-	+	n.d.		n.a.
EC-11/287	-	-	+	n.d.		n.a.
EC-11/288	-	-	+	n.d.		n.a.
EC-11/289	drop out					
EC-11/290	-	-	+	n.d.		n.a.
EC-11/291	-	-	+	n.d.		n.a.
EC-11/292	-	-	+	n.d.		n.a.
EC-11/293	-	-	+	n.d.		n.a.
EC-11/294	-	-	+	n.d.		n.a.
EC-11/295	-	-	+	n.d.		n.a.
EC-11/296	-	-	+	n.d.		n.a.
EC-11/297	-	-	+	n.d.		n.a.
EC-11/298	-	-	+	n.d.		n.a.
EC-11/299	-	-	+	n.d.		n.a.
EC-11/300	-	-	+	n.d.		n.a.
EC-11/301	-	-	+	n.d.		n.a.
EC-11/302	-	-	+	n.d.		n.a.
EC-11/303	-	-	+	n.d.		n.a.
EC-11/304	-	-	+	n.d.		n.a.
EC-11/305	-	-	+	n.d.		n.a.
EC-11/306	-	-	+	n.d.		n.a.
EC-11/307	-	-	+	n.d.		n.a.
EC-11/308	-	-	+	n.d.		n.a.
EC-11/309	-	-	+	n.d.		n.a.
EC-11/310	-	-	+	n.d.		n.a.
EC-11/311	-	-	-	n.d.		n.a.
EC-11/312	-	-	+	n.d.		n.a.
EC-11/313	-	-	+	n.d.		n.a.
EC-11/314	-	-	+	n.d.		n.a.
EC-11/315	-	-	+	n.d.		n.a.
EC-11/316	-	-	+	n.d.		n.a.
EC-11/317	-	-	+	n.d.		n.a.
EC-11/318	-	-	+	n.d.		n.a.
EC-11/319	-	-	+	n.d.		n.a.
EC-11/320	-	-	+	n.d.		n.a.
EC-11/321	-	-	+	n.d.		n.a.
EC-11/322	-	-	+	n.d.		n.a.
EC-11/323	-	-	+	n.d.		n.a.
EC-11/324	-	-	+	n.d.		n.a.
EC-11/325	-	-	+	n.d.		n.a.
EC-11/326	-	-	+	n.d.		n.a.
EC-11/327	-	-	+	n.d.		n.a.
EC-11/328	-	-	+	n.d.		n.a.
EC-11/329	-	-	+	n.d.		n.a.
EC-11/330	-	-	+	n.d.		n.a.
EC-11/331	-	-	+	n.d.		n.a.
EC-11/332	-	-	+	n.d.		n.a.
EC-11/333	-	-	+	n.d.		n.a.
EC-11/334	-	-	+	n.d.		n.a.
EC-11/335	-	-	+	n.d.		n.a.
EC-11/336	-	-	+	n.d.		n.a.
EC-11/337	-	-	+	n.d.		n.a.
EC-11/338	-	-	+	n.d.		n.a.
EC-11/339	-	-	+	n.d.		n.a.
EC-11/340	-	-	+	n.d.		n.a.
EC-11/341	-	-	+	n.d.		n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EC-11/342	-	-	+	n.d.		n.a.
EC-11/343	-	-	+	n.d.		n.a.
EC-11/344	-	-	+	n.d.		n.a.
EC-11/345	-	-	+	n.d.		n.a.
EC-11/346	-	-	+	n.d.		n.a.
EC-11/347	-	-	+	n.d.		n.a.
EC-11/348	-	-	+	n.d.		n.a.
EC-11/349	-	-	+	n.d.		n.a.
EC-11/350	-	-	+	n.d.		n.a.
EC-11/351	-	-	+	n.d.		n.a.
EC-11/352	-	-	+	n.d.		n.a.
EC-11/353	drop out					
EC-11/354	-	-	+	n.d.		n.a.
EC-11/355	-	-	+	n.d.		n.a.
EC-11/356	-	-	+	n.d.		n.a.
EC-11/357	-	-	+	n.d.		n.a.
EC-11/358	-	-	+	n.d.		n.a.
EC-11/359	-	-	+	n.d.		n.a.
EC-11/360	-	-	+	n.d.		n.a.
EC-11/361	-	-	+	n.d.		n.a.
EC-11/362	-	-	+	n.d.		n.a.
EC-11/363	-	-	+	n.d.		n.a.
EC-11/364	-	-	+	n.d.		n.a.
EC-11/365	-	-	+	n.d.		n.a.
EC-11/366	-	-	+	n.d.		n.a.
EC-11/367	-	-	+	n.d.		n.a.
EC-11/368	-	-	+	n.d.		n.a.
EC-11/369	-	-	+	n.d.		n.a.
EC-11/370	-	-	+	n.d.		n.a.
EC-11/371	-	-	+	n.d.		n.a.
EC-11/372	-	-	+	n.d.		n.a.
EC-11/373	-	-	+	n.d.		n.a.
EC-11/374	-	-	+	n.d.		n.a.
EC-11/375	-	-	+	n.d.		n.a.
EC-11/376	-	-	+	n.d.		n.a.
EC-11/377	-	-	+	n.d.		n.a.
EC-11/378	-	-	+	n.d.		n.a.
EC-11/379	-	-	+	n.d.		n.a.
EC-11/380	-	-	+	n.d.		n.a.
EC-11/381	-	-	+	n.d.		n.a.
EC-11/382	-	-	+	n.d.		n.a.
EC-11/383	-	-	+	n.d.		n.a.
EC-11/384	-	-	+	n.d.		n.a.
EC-11/385	-	-	+	n.d.		n.a.
EC-11/386	-	-	+	n.d.		n.a.
EC-11/387	-	-	+	n.d.		n.a.
EC-11/388	-	-	+	n.d.		n.a.
EC-11/389	-	-	+	n.d.		n.a.
EC-11/390	-	-	+	n.d.		n.a.
EC-11/391	-	-	+	n.d.		n.a.
EC-11/392	-	-	+	n.d.		n.a.
EC-11/393	-	-	+	n.d.		n.a.
EC-11/394	-	-	+	n.d.		n.a.
EC-11/395	-	-	+	n.d.		n.a.
EC-11/396	-	-	+	n.d.		n.a.
EC-11/397	-	-	+	n.d.		n.a.
EC-11/398	-	-	+	n.d.		n.a.
EC-11/399	-	-	+	n.d.		n.a.
EC-11/400	-	-	+	n.d.		n.a.
EC-11/401	-	-	+	n.d.		n.a.
EC-11/402	-	-	+	n.d.		n.a.
EC-11/403	-	-	+	n.d.		n.a.
EC-11/404	-	-	+	n.d.		n.a.
EC-11/405	-	-	+	n.d.		n.a.
EC-11/406	-	-	+	n.d.		n.a.
EC-11/407	-	-	+	n.d.		n.a.
EC-11/408	-	-	+	n.d.		n.a.
EC-11/409	drop out					
EC-11/410	-	-	+	n.d.		n.a.
EC-11/411	-	-	+	n.d.		n.a.
EC-11/412	-	-	+	n.d.		n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EC-11/413	-	-	+	n.d.		n.a.
EC-11/414	-	-	+	n.d.		n.a.
EC-11/415	-	-	+	n.d.		n.a.
EC-11/416	-	-	+	n.d.		n.a.
EC-11/417	-	-	+	n.d.		n.a.
EC-11/418	-	-	+	n.d.		n.a.
EC-11/419	-	-	+	n.d.		n.a.
EC-11/420	-	-	+	n.d.		n.a.
EC-11/421	-	-	+	n.d.		n.a.
EC-11/422	-	-	+	n.d.		n.a.
EC-11/423	-	-	+	n.d.		n.a.
EC-11/424	-	-	+	n.d.		n.a.
EC-11/425	-	-	+	n.d.		n.a.
EC-11/426	-	-	+	n.d.		n.a.
EC-11/427	-	-	+	n.d.		n.a.
EC-11/428	-	-	+	n.d.		n.a.
EC-11/429	-	-	+	n.d.		n.a.
EC-11/430	-	-	+	n.d.		n.a.
EC-11/431	-	-	+	n.d.		n.a.
EC-11/432	-	-	+	n.d.		n.a.
EC-11/433	-	-	+	n.d.		n.a.
EC-11/434	-	-	+	n.d.		n.a.
EC-11/435	-	-	+	n.d.		n.a.
EC-11/436	-	-	+	n.d.		n.a.
EC-11/437	-	-	+	n.d.		n.a.
EC-11/438	-	-	+	n.d.		n.a.
EC-11/439	-	-	+	n.d.		n.a.
EC-11/440	-	-	+	n.d.		n.a.
EC-11/441	-	-	+	n.d.		n.a.
EC-11/442	-	-	+	n.d.		n.a.
EC-11/443	-	-	+	n.d.		n.a.
EC-11/444	-	-	-	n.d.		n.a.

Table 7. *Escherichia coli*: TaqMan PCR results

1) minimum inhibitory concentrations for kanamycin (Epsilon test; Biomerieux)

2) Interpretation: sensitive/resistant according to CLSI (Clinical Laboratory Standards Institute)

3) Sequence of nptIII of positive strains

n.d. not done

n.a. not available

wild type homolog to aph(3')-IIIa reference sequence V01547

overlaid chromatogram showed two or more sequcences overlapping

drop out no *E. coli* or no growth on agar plate (for details see Table 19)

	Number of isolates nptII	Number of isolates nptIII
Samples collected	444	444
Drop outs	11	11
Samples PCR tested	433	433
Samples not valid (16S and Screen negative)	5	5
Samples for statistical analysis	428	428
NptII/nptIII positive	0	2
Prevalence estimator	0.00%	0.47%
Confidence interval (95%)	0.00% - 0.6975%	0.00% - 1.4637%

Table 8. Data for statistical analysis; *Escherichia coli*

2.1.2 Source and localisation of *E. coli* samples

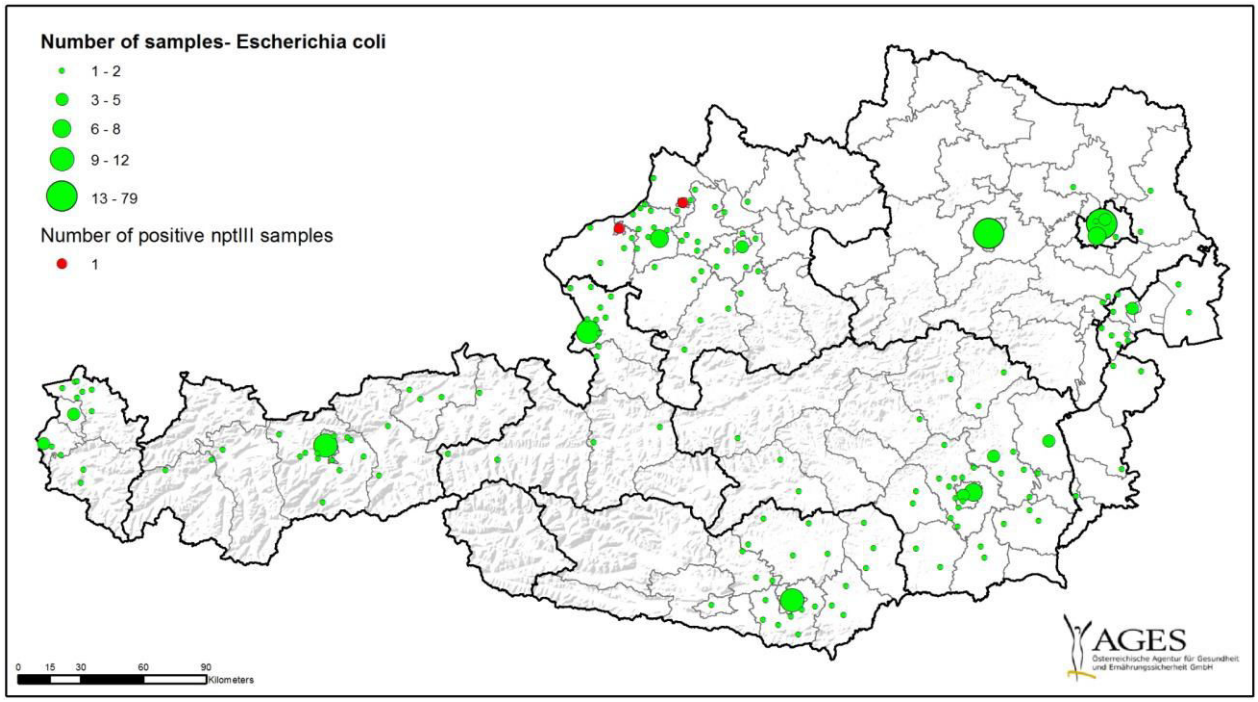


Figure 6. Distribution of *E. coli* sampled in Austria

2.1.3 Prevalence of nptII/nptIII in *Enterococcus* spp. samples collected in Austria

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EK-11/001	-	-	+	n.d.	n.a.	n.a.
EK-11/002	-	+	+	>256	resistant	wild type
EK-11/003	-	+	+	>256	resistant	wild type
EK-11/004	-	+	+	>256	resistant	wild type
EK-11/005	-	-	+	n.d.	n.a.	n.a.
EK-11/006	-	-	+	n.d.	n.a.	n.a.
EK-11/007	-	+	+	>256	resistant	wild type
EK-11/008	-	-	+	n.d.	n.a.	n.a.
EK-11/009	-	-	+	n.d.	n.a.	n.a.
EK-11/010	-	+	+	>256	resistant	wild type
EK-11/011	-	-	+	n.d.	n.a.	n.a.
EK-11/012	-	-	+	n.d.	n.a.	n.a
EK-11/013	-	+	+	>256	resistant	mutation
EK-11/014	-	-	+	n.d.	n.a.	n.a.
EK-11/015	-	-	+	n.d.	n.a.	n.a.
EK-11/016	-	+	+	>256	resistant	wild type
EK-11/017	-	-	+	n.d.	n.a.	n.a.
EK-11/018	-	-	+	n.d.	n.a.	n.a.
EK-11/019	-	-	+	n.d.	n.a.	n.a.
EK-11/020	-	-	-	n.d.	n.a.	n.a.
EK-11/021	-	-	+	n.d.	n.a.	n.a.
EK-11/022	-	+	-	>256	resistant	wild type
EK-11/023	-	+	-	>256	resistant	wild type
EK-11/024	-	-	-	n.d.	n.a.	n.a.
EK-11/025	-	-	+	n.d.	n.a.	n.a.
EK-11/026	-	-	+	n.d.	n.a.	n.a.
EK-11/027	-	-	+	n.d.	n.a.	n.a.
EK-11/028	-	-	-	n.d.	n.a.	n.a.
EK-11/029	-	-	+	n.d.	n.a.	n.a.
EK-11/030	-	-	-	n.d.	n.a.	n.a.
EK-11/031	-	+	+	>256	resistant	wild type
EK-11/032	-	-	-	n.d.	n.a.	n.a.
EK-11/033	-	+	-	>256	resistant	wild type
EK-11/034	-	-	+	n.d.	n.a.	n.a.
EK-11/035	-	-	+	n.d.	n.a.	n.a.
EK-11/036	-	+	+	>256	resistant	wild type
EK-11/037	-	-	+	n.d.	n.a.	n.a.
EK-11/038	-	-	+	n.d.	n.a.	n.a.
EK-11/039	-	-	-	n.d.	n.a.	n.a.
EK-11/040	-	-	+	n.d.	n.a.	n.a.
EK-11/041	-	-	+	n.d.	n.a.	n.a.
EK-11/042	-	-	+	n.d.	n.a.	n.a.
EK-11/043	-	+	+	>256	resistant	wild type
EK-11/044	-	-	+	n.d.	n.a.	n.a
EK-11/045	-	-	-	n.d.	n.a.	n.a
EK-11/046	-	+	-	>256	resistant	wild type
EK-11/047	-	-	+	n.d.	n.a.	n.a.
EK-11/048	-	-	-	n.d.	n.a.	n.a.
EK-11/049	-	-	+	n.d.	n.a.	n.a.
EK-11/050	-	-	+	n.d.	n.a.	n.a.
EK-11/051	-	-	+	n.d.	n.a.	n.a.
EK-11/052	-	-	+	n.d.	n.a.	n.a.
EK-11/053	-	+	+	>256	resistant	wild type
EK-11/054	-	-	+	32	low-level res	n.a.
EK-11/055	-	-	+	n.d.	n.a.	n.a.
EK-11/056	-	-	-	n.d.	n.a.	n.a.
EK-11/057	-	+	+	>256	resistant	wild type
EK-11/058	-	-	+	n.d.	n.a.	n.a.
EK-11/059	-	-	+	n.d.	n.a.	n.a.
EK-11/060	-	-	+	n.d.	n.a.	n.a.
EK-11/061	-	-	+	n.d.	n.a.	n.a.
EK-11/062	-	+	+	>256	resistant	wild type
EK-11/063	-	+	-	>256	resistant	wild type
EK-11/064	-	+	-	>256	resistant	wild type
EK-11/065	-	-	+	n.d.	n.a.	n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EK-11/066	-	-	+	n.d.	n.a.	n.a.
EK-11/067	-	-	+	n.d.	n.a.	n.a.
EK-11/068	-	-	-	n.d.	n.a.	n.a.
EK-11/069	-	-	+	n.d.	n.a.	n.a.
EK-11/070	-	-	+	n.d.	n.a.	n.a.
EK-11/071	-	-	-	n.d.	n.a.	n.a.
EK-11/072	-	-	+	n.d.	n.a.	n.a.
EK-11/073	-	+	+	>256	resistant	wild type
EK-11/074	-	-	+	n.d.	n.a.	n.a.
EK-11/075	-	+	+	>256	resistant	wild type
EK-11/076	-	-	+	n.d.	n.a.	n.a.
EK-11/077	-	+	+	>256	resistant	wild type
EK-11/078	-	+	+	>256	resistant	wild type
EK-11/079	-	+	+	>256	resistant	wild type
EK-11/080	-	+	-	>256	resistant	wild type
EK-11/081	-	+	-	>256	resistant	wild type
EK-11/082	-	-	+	n.d.	n.a.	n.a.
EK-11/083	-	+	+	>256	resistant	wild type
EK-11/084	-	+	+	>256	resistant	wild type
EK-11/085	-	-	+	n.d.	n.a.	n.a.
EK-11/086	-	-	+	n.d.	n.a.	n.a.
EK-11/087	-	-	+	32	low-level res	n.a.
EK-11/088	-	-	+	n.d.	n.a.	n.a.
EK-11/089	-	+	+	>256	resistant	wild type
EK-11/090	-	-	+	n.d.	n.a.	n.a.
EK-11/091	-	+	+	>256	resistant	wild type
EK-11/092	-	-	+	n.d.	n.a.	n.a.
EK-11/093	-	+	+	>256	resistant	wild type
EK-11/094	-	-	+	n.d.	n.a.	n.a.
EK-11/095	-	-	+	n.d.	n.a.	n.a.
EK-11/096	-	-	+	n.d.	n.a.	n.a.
EK-11/097	-	+	+	>256	resistant	wild type
EK-11/098	-	+	+	>256	resistant	wild type
EK-11/099	-	-	+	n.d.	n.a.	n.a.
EK-11/100	-	+	+	>256	resistant	wild type
EK-11/101	-	+	+	>256	resistant	wild type
EK-11/102	-	-	+	n.d.	n.a.	n.a.
EK-11/103	-	-	+	n.d.	n.a.	n.a.
EK-11/104	-	-	+	n.d.	n.a.	n.a.
EK-11/105	-	-	+	n.d.	n.a.	n.a.
EK-11/106	-	-	+	n.d.	n.a.	n.a.
EK-11/107	-	-	+	n.d.	n.a.	n.a.
EK-11/108	-	+	+	>256	resistant	wild type
EK-11/109	-	+	+	>256	resistant	wild type
EK-11/110	-	-	+	n.d.	n.a.	n.a.
EK-11/111	-	+	+	>256	resistant	wild type
EK-11/112	-	-	+	n.d.	n.a.	n.a.
EK-11/113	-	+	+	>256	resistant	wild type
EK-11/114	-	+	+	>256	resistant	wild type
EK-11/115	-	+	+	>256	resistant	wild type
EK-11/116	-	-	+	n.d.	n.a.	n.a.
EK-11/117	-	+	+	>256	resistant	wild type
EK-11/118	-	-	+	n.d.	n.a.	n.a.
EK-11/119	-	+	+	>256	resistant	wild type
EK-11/120	-	-	+	n.d.	n.a.	n.a.
EK-11/121	drop out					
EK-11/122	-	-	+	n.d.	n.a.	n.a.
EK-11/123	-	-	+	n.d.	n.a.	n.a.
EK-11/124	-	+	+	>256	resistant	wild type
EK-11/125	-	-	+	n.d.	n.a.	n.a.
EK-11/126	-	-	+	n.d.	n.a.	n.a.
EK-11/127	-	-	+	n.d.	n.a.	n.a.
EK-11/128	drop out					
EK-11/129	-	-	+	n.d.	n.a.	n.a.
EK-11/130	-	-	+	n.d.	n.a.	n.a.
EK-11/131	-	+	+	>256	resistant	wild type
EK-11/132	-	-	+	n.d.	n.a.	n.a.
EK-11/133	-	-	+	n.d.	n.a.	n.a.
EK-11/134	-	-	+	n.d.	n.a.	n.a.

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EK-11/135	-	-	+	n.d.	n.a.	n.a.
EK-11/136	drop out					
EK-11/137	drop out					
EK-11/138	-	-	+	n.d.	n.a.	n.a.
EK-11/139	-	-	+	n.d.	n.a.	n.a.
EK-11/140	-	+	+	>256	resistant	wild type
EK-11/141	-	+	+	>256	resistant	wild type
EK-11/142	-	-	+	n.d.	n.a.	n.a.
EK-11/143	-	-	+	n.d.	n.a.	n.a.
EK-11/144	-	+	+	>256	resistant	wild type
EK-11/145	-	-	+	n.d.	n.a.	n.a.
EK-11/146	-	-	+	n.d.	n.a.	n.a.
EK-11/147	-	-	+	n.d.	n.a.	n.a.
EK-11/148	-	-	+	n.d.	n.a.	n.a.
EK-11/149	-	-	+	n.d.	n.a.	n.a.
EK-11/150	-	+	+	>256	resistant	wild type
EK-11/151	-	-	+	n.d.	n.a.	n.a.
EK-11/152	-	+	+	>256	resistant	wild type
EK-11/153	-	+	+	>256	resistant	wild type
EK-11/154	-	+	+	>256	resistant	wild type
EK-11/155	-	+	+	>256	resistant	wild type
EK-11/156	-	+	+	>256	resistant	wild type
EK-11/157	-	-	+	n.d.	n.a.	n.a.
EK-11/158	-	+	+	>256	resistant	wild type
EK-11/159	-	-	+	n.d.	n.a.	n.a.
EK-11/160	-	-	+	n.d.	n.a.	n.a.
EK-11/161	-	-	+	n.d.	n.a.	n.a.
EK-11/162	-	+	+	>256	resistant	n.a.
EK-11/163	-	-	+	n.d.	n.a.	n.a.
EK-11/164	-	+	+	>256	resistant	wild type
EK-11/165	-	-	+	n.d.	n.a.	n.a.
EK-11/166	-	-	+	n.d.	n.a.	n.a.
EK-11/167	-	-	+	n.d.	n.a.	n.a.
EK-11/168	-	-	+	n.d.	n.a.	n.a.
EK-11/169	-	-	+	n.d.	n.a.	n.a.
EK-11/170	-	+	+	>256	resistant	wild type
EK-11/171	-	-	+	>256	resistant	n.a.
EK-11/172	-	-	+	n.d.	n.a.	n.a.
EK-11/173	-	+	+	>256	resistant	wild type
EK-11/174	-	-	+	n.d.	n.a.	n.a.
EK-11/175	-	-	+	n.d.	n.a.	n.a.
EK-11/176	-	-	+	n.d.	n.a.	n.a.
EK-11/177	-	-	+	n.d.	n.a.	n.a.
EK-11/178	-	-	+	n.d.	n.a.	n.a.
EK-11/179	-	-	+	n.d.	n.a.	n.a.
EK-11/180	-	-	+	n.d.	n.a.	n.a.
EK-11/181	-	-	+	n.d.	n.a.	n.a.
EK-11/182	-	+	+	>256	resistant	wild type
EK-11/183	-	-	+	n.d.	n.a.	n.a.
EK-11/184	-	+	+	>256	resistant	wild type
EK-11/185	-	-	+	n.d.	n.a.	n.a.
EK-11/186	-	-	+	n.d.	n.a.	n.a.
EK-11/187	-	+	+	>256	resistant	wild type
EK-11/188	drop out					
EK-11/189	-	-	+	n.d.	n.a.	n.a.
EK-11/190	-	-	+	n.d.	n.a.	n.a.
EK-11/191	-	-	+	n.d.	n.a.	n.a.
EK-11/192	-	-	+	n.d.	n.a.	n.a.
EK-11/193	-	-	+	n.d.	n.a.	n.a.
EK-11/194	-	-	+	n.d.	n.a.	n.a.
EK-11/195	-	-	+	n.d.	n.a.	n.a.
EK-11/196	-	-	+	n.d.	n.a.	n.a.
EK-11/197	-	+	+	>256	resistant	wild type
EK-11/198	-	-	+	n.d.	n.a.	n.a.
EK-11/199	-	-	+	n.d.	n.a.	n.a.
EK-11/200	-	-	+	n.d.	n.a.	n.a.
EK-11/201	-	-	+	n.d.	n.a.	n.a.
EK-11/202	-	-	+	n.d.	n.a.	n.a.
EK-11/203	-	+	+	>256	resistant	wild type

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EK-11/204	-	-	+	n.d.	n.a.	n.a.
EK-11/205	-	+	+	>256	resistant	wild type
EK-11/206	-	+	+	>256	resistant	wild type
EK-11/207	-	-	+	n.d.	n.a.	n.a.
EK-11/208	-	-	+	n.d.	n.a.	n.a.
EK-11/209	-	+	+	>256	resistant	wild type
EK-11/210	-	-	+	n.d.	n.a.	n.a.
EK-11/211	-	+	+	>256	resistant	wild type
EK-11/212	-	-	+	n.d.	n.a.	n.a.
EK-11/213	-	-	+	n.d.	n.a.	n.a.
EK-11/214	-	-	+	n.d.	n.a.	n.a.
EK-11/215	-	+	+	>256	resistant	wild type
EK-11/216	-	+	+	>256	resistant	wild type
EK-11/217	-	+	+	>256	resistant	wild type
EK-11/218	-	+	+	>256	resistant	wild type
EK-11/219	-	-	+	n.d.	n.a.	n.a.
EK-11/220	-	-	+	n.d.	n.a.	n.a.
EK-11/221	-	-	+	n.d.	n.a.	n.a.
EK-11/222	-	-	+	n.d.	n.a.	n.a.
EK-11/223	-	-	+	n.d.	n.a.	n.a.
EK-11/224	-	+	+	>256	resistant	wild type
EK-11/225	-	-	+	n.d.	n.a.	n.a.
EK-11/226	-	-	+	n.d.	n.a.	n.a.
EK-11/227	-	-	+	n.d.	n.a.	n.a.
EK-11/228	-	-	+	n.d.	n.a.	n.a.
EK-11/229	-	-	+	n.d.	n.a.	n.a.
EK-11/230	-	-	+	n.d.	n.a.	n.a.
EK-11/231	-	-	+	n.d.	n.a.	n.a.
EK-11/232	-	-	+	n.d.	n.a.	n.a.
EK-11/233	-	+	+	>256	resistant	mutation
EK-11/234	-	-	+	n.d.	n.a.	n.a.
EK-11/235	-	+	+	>256	resistant	wild type
EK-11/236	-	+	+	>256	resistant	wild type
EK-11/237	-	+	+	>256	resistant	wild type
EK-11/238	-	-	+	n.d.	n.a.	n.a.
EK-11/239	-	-	+	n.d.	n.a.	n.a.
EK-11/240	-	-	+	48 (64)[5)]	low-level res	n.a.
EK-11/241	-	-	+	n.d.	n.a.	n.a.
EK-11/242	-	+	+	>256	resistant	wild type
EK-11/243	-	+	+	>256	resistant	wild type
EK-11/244	-	-	+	n.d.	n.a.	n.a.
EK-11/245	-	-	+	n.d.	n.a.	n.a.
EK-11/246	-	-	+	n.d.	n.a.	n.a.
EK-11/247	-	-	+	n.d.	n.a.	n.a.
EK-11/248	-	+	+	>256	resistant	wild type
EK-11/249	-	-	+	n.d.	n.a.	n.a.
EK-11/250	-	+	+	>256	resistant	wild type
EK-11/251	-	+	+	>256	resistant	wild type
EK-11/252	-	+	+	>256	resistant	mutation
EK-11/253	-	-	+	n.d.	n.a.	n.a.
EK-11/254	drop out					
EK-11/255	-	-	+	n.d.	n.a.	n.a.
EK-11/256	-	-	+	n.d.	n.a.	n.a.
EK-11/257	-	+	+	>256	resistant	wild type
EK-11/258	-	-	+	n.d.	n.a.	n.a.
EK-11/259	-	+	+	>256	resistant	mutation
EK-11/260	-	+	+	>256	resistant	wild type
EK-11/261	-	-	+	n.d.	n.a.	n.a.
EK-11/262	drop out					
EK-11/263	-	-	+	n.d.	n.a.	n.a.
EK-11/264	-	+	+	>256	resistant	mutation
EK-11/265	-	-	+	n.d.	n.a.	n.a.
EK-11/266	-	-	+	n.d.	n.a.	n.a.
EK-11/267	-	-	+	n.d.	n.a.	n.a.
EK-11/268	-	-	-	n.d.	n.a.	n.a.
EK-11/269	-	-	+	n.d.	n.a.	n.a.
EK-11/270	-	+	+	>256	resistant	mutation
EK-11/271	-	-	+	n.d.	n.a.	n.a.
EK-11/272	-	+	+	>256	resistant	wild type

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EK-11/273	-	-	+	n.d.	n.a.	n.a.
EK-11/274	-	+	+	>256	resistant	wild type
EK-11/275	-	-	+	n.d.	n.a.	n.a.
EK-11/276	-	+	+	>256	resistant	wild type
EK-11/277	-	-	+	n.d.	n.a.	n.a.
EK-11/278	-	-	+	n.d.	n.a.	n.a.
EK-11/279	-	+	+	>256	resistant	wild type
EK-11/280	-	-	+	n.d.	n.a.	n.a.
EK-11/281	-	-	+	n.d.	n.a.	n.a.
EK-11/282	-	-	+	n.d.	n.a.	n.a.
EK-11/283	-	-	+	n.d.	n.a.	n.a.
EK-11/284	-	-	+	n.d.	n.a.	n.a.
EK-11/285	-	-	+	n.d.	n.a.	n.a.
EK-11/286	-	-	+	n.d.	n.a.	n.a.
EK-11/287	-	+	+	>256	resistant	mutation
EK-11/288	-	-	+	n.d.	n.a.	n.a.
EK-11/289	-	-	+	n.d.	n.a.	n.a.
EK-11/290	-	-	+	n.d.	n.a.	n.a.
EK-11/291	-	-	+	n.d.	n.a.	n.a.
EK-11/292	-	+	+	>256	resistant	wild type
EK-11/293	-	+	+	>256	resistant	wild type
EK-11/294	-	-	+	n.d.	n.a.	n.a
EK-11/295	-	+	+	>256	resistant	wild type
EK-11/296	-	-	+	n.d.	n.a.	n.a.
EK-11/297	-	+	+	>256	resistant	wild type
EK-11/298	-	-	+	n.d.	n.a.	n.a.
EK-11/299	-	-	+	n.d.	n.a.	n.a.
EK-11/300	-	+	+	>256	resistant	wild type
EK-11/301	-	-	+	n.d.	n.a.	n.a.
EK-11/302	-	-	+	n.d.	n.a.	n.a.
EK-11/303	-	-	+	n.d.	n.a.	n.a.
EK-11/304	-	+	+	>256	resistant	wild type
EK-11/305	-	-	+	n.d.	n.a.	n.a.
EK-11/306	-	+	+	>256	resistant	wild type
EK-11/307	-	+	+	>256	resistant	wild type
EK-11/308	-	+	+	>256	resistant	wild type
EK-11/309	-	+	+	>256	resistant	wild type
EK-11/310	-	-	+	n.d.	n.a.	n.a.
EK-11/311	-	-	+	n.d.	n.a.	n.a.
EK-11/312	-	+	+	>256	resistant	wild type
EK-11/313	-	-	+	n.d.	n.a.	n.a.
EK-11/314	-	-	+	48 (64)[5)]	low-level res	n.a.
EK-11/315	-	+	+	>256	resistant	wild type
EK-11/316	-	-	+	n.d.	n.a.	n.a.
EK-11/317	-	+	+	>256	resistant	wild type
EK-11/318	-	+	+	>256	resistant	wild type
EK-11/319	-	+	+	>256	resistant	wild type
EK-11/320	-	+	+	>256	resistant	wild type
EK-11/321	-	+	-	>256	resistant	wild type
EK-11/322	drop out					
EK-11/323	-	-	+	n.d.	n.a.	n.a.
EK-11/324	-	-	+	n.d.	n.a.	n.a.
EK-11/325	-	-	+	n.d.	n.a.	n.a.
EK-11/326	-	+	+	>256	resistant	wild type
EK-11/327	-	-	+	n.d.	n.a.	n.a.
EK-11/328	-	-	+	n.d.	n.a.	n.a.
EK-11/329	-	+	+	>256	resistant	wild type
EK-11/330	-	-	+	n.d.	n.a.	n.a.
EK-11/331	-	-	+	n.d.	n.a.	n.a.
EK-11/332	-	-	+	n.d.	n.a.	n.a.
EK-11/333	-	-	+	n.d.	n.a.	n.a.
EK-11/334	drop out					
EK-11/335	drop out					
EK-11/336	-	+	+	>256	resistant	wild type
EK-11/337	-	-	+	n.d.	n.a.	n.a.
EK-11/338	-	-	+	n.d.	n.a.	n.a.
EK-11/339	-	-	+	n.d.	n.a.	n.a.
EK-11/340	-	+	+	>256	resistant	wild type
EK-11/341	-	+	+	>256	resistant	wild type

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EK-11/342	-	+	+	>256	resistant	wild type
EK-11/343	-	+	+	>256	resistant	wild type
EK-11/344	-	-	+	n.d.	n.a.	n.a.
EK-11/345	-	+	+	n.d.[4)]	n.a.	n.a.
EK-11/346	-	-	+	n.d.	n.a.	n.a.
EK-11/347	-	-	+	n.d.	n.a.	n.a.
EK-11/348	-	+	+	>256	resistant	wild type
EK-11/349	-	-	+	n.d.	n.a.	n.a.
EK-11/350	-	-	+	96 (128)[5)]	low-level res	n.a.
EK-11/351	-	-	+	n.d.	n.a.	n.a.
EK-11/352	-	-	+	n.d.	n.a.	n.a.
EK-11/353	-	-	+	n.d.	n.a.	n.a.
EK-11/354	-	+	+	>256	resistant	wild type
EK-11/355	-	-	+	n.d.	n.a.	n.a.
EK-11/356	-	-	+	n.d.	n.a.	n.a.
EK-11/357	-	-	+	n.d.	n.a.	n.a.
EK-11/358	-	+	+	>256	resistant	wild type
EK-11/359	-	+	+	>256	resistant	wild type
EK-11/360	-	+	+	>256	resistant	mutation
EK-11/361	-	+	+	>256	resistant	wild type
EK-11/362	-	+	+	>256	resistant	wild type
EK-11/363	-	+	+	>256	resistant	wild type
EK-11/364	-	+	+	>256	resistant	wild type
EK-11/365	-	-	+	n.d.	n.a.	n.a.
EK-11/366	-	-	+	n.d.	n.a.	n.a.
EK-11/367	-	+	+	>256	resistant	wild type
EK-11/368	-	+	+	>256	resistant	wild type
EK-11/369	-	+	+	>256	resistant	wild type
EK-11/370	-	-	-	n.d.	n.a.	n.a.
EK-11/371	-	+	+	>256	resistant	wild type
EK-11/372	-	-	+	n.d.	n.a.	n.a.
EK-11/373	-	+	+	>256	resistant	wild type
EK-11/374	-	-	+	n.d.	n.a.	n.a.
EK-11/375	-	-	+	n.d.	n.a.	n.a.
EK-11/376	-	+	+	>256	resistant	wild type
EK-11/377	-	+	+	>256	resistant	wild type
EK-11/378	-	-	+	>256	resistant	n.a.
EK-11/379	-	-	+	n.d.	n.a.	n.a.
EK-11/380	-	+	+	>256	resistant	wild type
EK-11/381	-	-	+	n.d.	n.a.	n.a.
EK-11/382	-	-	+	n.d.	n.a.	n.a.
EK-11/383	-	-	+	n.d.	n.a.	n.a.
EK-11/384	-	-	+	n.d.	n.a.	n.a.
EK-11/385	-	-	+	n.d.	n.a.	n.a.
EK-11/386	-	-	+	n.d.	n.a.	n.a.
EK-11/387	-	+	+	64	low-level res	wild type
EK-11/388	-	+	+	>256	resistant	wild type
EK-11/389	-	+	+	>256	resistant	wild type
EK-11/390	-	-	+	n.d.	n.a.	n.a.
EK-11/391	-	+	+	>256	resistant	wild type
EK-11/392	-	-	+	n.d.	n.a.	n.a.
EK-11/393	-	-	+	n.d.	n.a.	n.a.
EK-11/394	-	-	+	n.d.	n.a.	n.a.
EK-11/395	-	-	+	n.d.	n.a.	n.a.
EK-11/396	-	-	+	n.d.	n.a.	n.a.
EK-11/397	-	-	+	n.d.	n.a.	n.a.
EK-11/398	-	+	+	>256	resistant	wild type
EK-11/399	-	+	+	>256	resistant	mutation
EK-11/400	-	+	+	>256	resistant	wild type
EK-11/401	-	-	+	n.d.	n.a.	n.a.
EK-11/402	-	-	+	n.d.	n.a.	n.a.
EK-11/403	-	+	+	64	low-level res	wild type
EK-11/404	-	-	+	n.d.	n.a.	n.a.
EK-11/405	-	+	+	>256	resistant	wild type
EK-11/406	-	-	+	n.d.	n.a.	n.a.
EK-11/407	-	+	+	>256	resistant	wild type
EK-11/408	-	+	+	>256	resistant	wild type
EK-11/409	-	-	+	n.d.	n.a.	n.a.
EK-11/410	-	+	+	>256	resistant	wild type

Isolate	npt II	npt III	16S	MIC Kan (µg/ml)[1)]	MIC Interpretation[2)]	Sequence[3)]
EK-11/411	-	-	+	n.d.	n.a.	n.a.
EK-11/412	-	-	+	n.d.	n.a.	n.a.
EK-11/413	-	+	+	>256	resistant	wild type
EK-11/414	-	-	+	n.d.	n.a.	n.a.
EK-11/415	-	-	+	n.d.	n.a.	n.a.
EK-11/416	-	-	+	n.d.	n.a.	n.a.
EK-11/417	-	+	+	>256	resistant	wild type
EK-11/418	-	-	+	n.d.	n.a.	n.a.
EK-11/419	-	-	+	n.d.	n.a.	n.a.
EK-11/420	-	-	+	n.d.	n.a.	n.a.
EK-11/421	-	-	+	n.d.	n.a.	n.a
EK-11/422	-	+	+	>256	resistant	wild type
EK-11/423	-	+	+	48 (64)[5)]	low-level res	wild type
EK-11/424	-	-	+	n.d.	n.a.	n.a.
EK-11/425	-	-	+	n.d.	n.a.	n.a.
EK-11/426	-	-	+	n.d.	n.a.	n.a.
EK-11/427	-	-	+	n.d.	n.a.	n.a.
EK-11/428	-	-	+	n.d.	n.a.	n.a.
EK-11/429	-	+	+	>256	resistant	wild type
EK-11/430	-	+	-	>256	resistant	wild type
EK-11/431	-	-	-	n.d.	n.a.	n.a.
EK-11/432	-	-	+	n.d.	n.a.	n.a.
EK-11/433	-	-	+	n.d.	n.a.	n.a.
EK-11/434	-	-	+	n.d.	n.a.	n.a.
EK-11/435	-	-	+	n.d.	n.a.	n.a.
EK-11/436	-	+	+	>256	resistant	wild type
EK-11/437	-	+	+	>256	resistant	wild type

Table 9. Enterococci: PCR results, MIC and sequence interpretation of positive strains

1) minimum inhibitory concentrations for kanamycin (Epsilon test; Biomerieux)

2) Interpretation: sensitive/resistant according to EUCAST, 2013; low-level res: low-level resistant; resistant: high-level resistant

3) Sequence of nptIII of positive strains

4) Isolate did not arrive in the testing laboratory

5) First number: original e-test value; rounded up MIC value in brackets

n.d. not done

n.a. not available

wild type homolog to aph(3')-IIIa reference sequence V01547

overlaid chromatogram showed two or more sequences overlapping

drop out no enterococci or no growth on agar plate (for details see Table 19)

	Number of isolates nptII	Number of isolates nptIII
Samples collected	437	437
Drop outs	10	10
Samples PCR tested	427	427
Samples not valid (16S and Screen negative)	24	14
Samples for statistical analysis	403	413
NptII/nptIII positive	0	155
Prevalence estimator	0.00%	37.53%
Confidence interval (95%)	0.00% - 0.7407%	32.84% - 42.40%

Table 10. Data for statistical analysis for enterococci

2.1.4 Source and localisation of enterococci samples

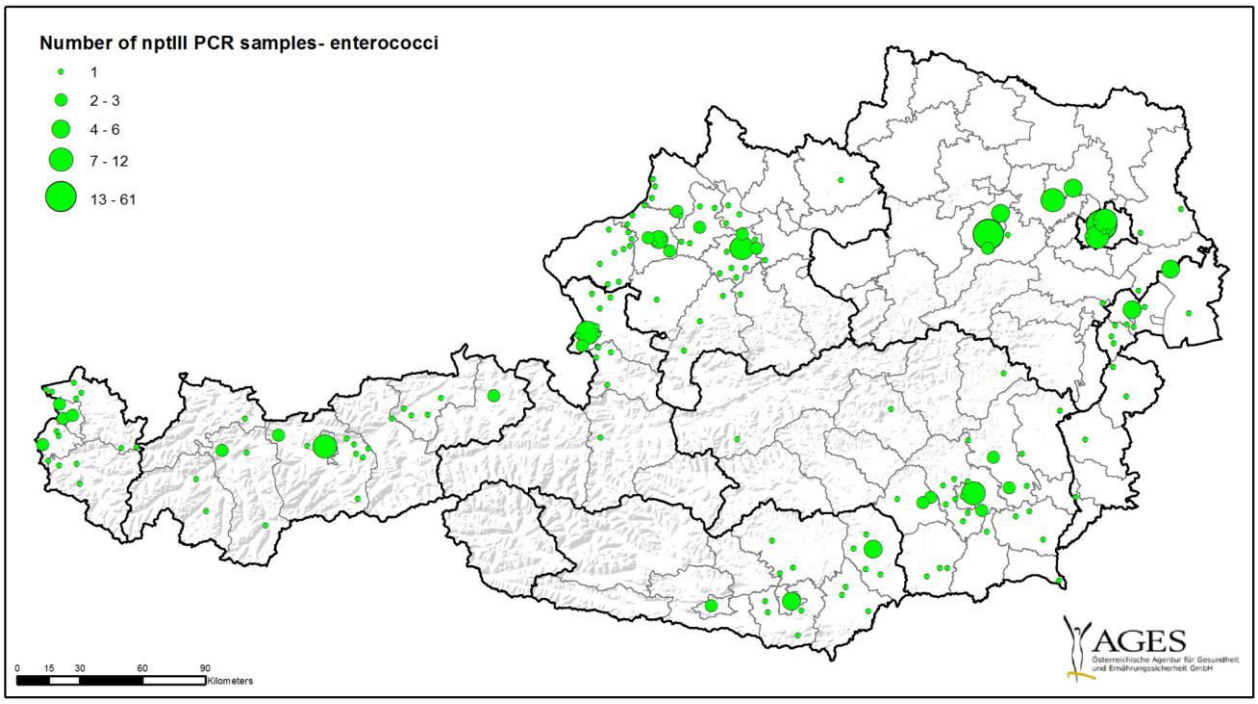


Figure 7. Distribution of enterococci sampled in Austria

2.1.5 Prevalence of nptII/nptIII in *Pseudomonas aeruginosa* collected in Austria

Isolate	nptII	nptIII	16S
P-10/001	-	-	+
P-10/002	-	-	+
P-10/003	-	-	+
P-10/004	-	-	+
P-10/005	-	-	+
P-10/006	-	-	+
P-10/007	-	-	+
P-10/008	-	-	+
P-10/009	-	-	+
P-10/010	-	-	+
P-10/011	-	-	+
P-10/012	-	-	-
P-10/013	-	-	+
P-10/014	-	-	+
P-10/015	-	-	+
P-10/016	-	-	+
P-10/017	-	-	+
P-10/018	-	-	+
P-10/019	-	-	-
P-10/020	-	-	+
P-10/021	-	-	+
P-10/022	-	-	+
P-10/023	drop out		
P-10/024	-	-	+
P-10/025	-	-	+
P-10/026	-	-	-
P-10/027	-	-	+
P-10/028	-	-	+
P-10/029	-	-	+
P-10/030	-	-	+
P-10/031	-	-	+
P-10/032	-	-	+
P-10/033	-	-	+
P-10/034	-	-	+
P-10/035	-	-	+
P-10/036	-	-	+
P-10/037	-	-	+
P-10/038	-	-	+
P-10/039	-	-	+
P-10/040	-	-	+
P-10/041	-	-	+
P-10/042	-	-	+
P-10/043	-	-	+
P-10/044	-	-	+
P-10/045	-	-	+
P-10/046	-	-	+
P-10/047	-	-	+
P-10/048	-	-	+
P-10/049	-	-	+
P-10/050	-	-	+
P-10/051	-	-	+
P-10/052	-	-	+
P-10/053	-	-	+
P-10/054	-	-	+
P-10/055	-	-	+
P-10/056	-	-	+
P-10/057	-	-	+
P-10/058	-	-	+
P-10/059	-	-	+
P-10/060	-	-	+
P-10/061	-	-	+
P-10/062	-	-	+
P-10/063	-	-	+
P-10/064	-	-	+
P-10/065	-	-	+
P-10/066	-	-	+
P-10/067	-	-	+

Isolate	nptII	nptIII	16S
P-10/068	-	-	+
P-10/069	-	-	+
P-10/070	-	-	-
P-10/071	-	-	+
P-10/072	-	-	+
P-10/073	-	-	+
P-10/074	-	-	+
P-10/075	-	-	+
P-10/076	-	-	+
P-10/077	-	-	+
P-10/078	-	-	+
P-10/079	-	-	+
P-10/080	-	-	+
P-10/081	-	-	+
P-10/082	-	-	+
P-10/083	-	-	+
P-10/084	-	-	+
P-10/085	-	-	+
P-10/086	-	-	+
P-10/087	-	-	+
P-10/088	-	-	+
P-10/089	-	-	+
P-10/090	-	-	+
P-10/091	-	-	+
P-10/092	-	-	+
P-10/093	-	-	+
P-10/094	-	-	+
P-10/095	-	-	+
P-10/096	-	-	+
P-10/097	-	-	+
P-10/098	-	-	+
P-10/099	-	-	+
P-10/100	-	-	+
P-10/101	-	-	+
P-10/102	-	-	+
P-10/103	-	-	+
P-10/104	-	-	+
P-10/105	-	-	+
P-10/106	-	-	+
P-10/107	-	-	+
P-10/108	-	-	+
P-10/109	-	-	+
P-10/110	-	-	+
P-10/111	-	-	+
P-10/112	-	-	+
P-10/113	-	-	+
P-10/114	-	-	+
P-10/115	-	-	+
P-10/116	-	-	+
P-10/117	-	-	+
P-10/118	-	-	+
P-10/119	-	-	+
P-10/120	-	-	+
P-10/121	-	-	+
P-10/122	-	-	+
P-10/123	-	-	+
P-10/124	-	-	-
P-10/125	-	-	+
P-10/126	-	-	+
P-10/127	-	-	+
P-10/128	-	-	+
P-10/129	-	-	+
P-10/130	-	-	+
P-10/131	-	-	+
P-10/132	-	-	+
P-10/133	-	-	+
P-10/134	-	-	+
P-10/135	-	-	+
P-10/136	-	-	+
P-10/137	-	-	+
P-10/138	-	-	+
P-10/139	-	-	+

Isolate	nptII	nptIII	16S
P-10/140	-	-	+
P-10/141	-	-	+
P-10/142	-	-	+
P-10/143	-	-	+
P-10/144	-	-	+
P-10/145	-	-	+
P-10/146	-	-	+
P-10/147	-	-	+
P-10/148	-	-	+
P-10/149	-	-	+
P-10/150	-	-	+
P-10/151	-	-	+
P-10/152	-	-	+
P-10/153	-	-	+
P-10/154	-	-	+
P-10/155	-	-	+
P-10/156	-	-	+
P-10/157	-	-	+
P-10/158	-	-	-
P-10/159	-	-	+
P-10/160	-	-	+
P-10/161	-	-	+
P-10/162	-	-	+
P-10/163	-	-	+
P-10/164	-	-	+
P-10/165	-	-	+
P-10/166	-	-	+
P-10/167	-	-	+
P-10/168	-	-	+
P-10/169	-	-	+
P-10/170	-	-	+
P-10/171	-	-	+
P-10/172	-	-	+
P-10/173	-	-	+
P-10/174	-	-	+
P-10/175	-	-	+
P-10/176	-	-	+
P-10/177	-	-	+
P-10/178	-	-	+
P-10/179	-	-	+
P-10/180	-	-	+
P-10/181	-	-	+
P-10/182	-	-	+
P-10/183	-	-	+
P-10/184	-	-	+
P-10/185	-	-	+
P-10/186	-	-	+
P-10/187	-	-	+
P-10/188	-	-	+
P-10/189	-	-	+
P-10/190	-	-	+
P-10/191	-	-	+
P-10/192	-	-	+
P-10/193	-	-	+
P-10/194	-	-	+
P-10/195	-	-	+
P-10/196	-	-	+
P-10/197	-	-	+
P-10/198	-	-	+
P-10/199	-	-	+
P-10/200	-	-	+
P-10/201	-	-	+
P-10/202	-	-	+
P-10/203	-	-	+
P-10/204	-	-	+
P-10/205	-	-	+
P-10/206	-	-	+
P-10/207	-	-	+
P-10/208	-	-	+
P-10/209	-	-	+
P-10/210	-	-	+
P-10/211	-	-	+

Isolate	nptII	nptIII	16S
P-10/212	-	-	+
P-10/213	-	-	+
P-10/214	-	-	+
P-10/215	-	-	+
P-10/216	-	-	+
P-10/217	-	-	+
P-10/218	-	-	+
P-10/219	-	-	+
P-10/220	-	-	+
P-10/221	-	-	+
P-10/222	-	-	+
P-10/223	-	-	+
P-10/224	-	-	+
P-10/225	-	-	+
P-10/226	-	-	+
P-10/227	-	-	+
P-10/228	-	-	+
P-10/229	-	-	+
P-10/230	-	-	+
P-10/231	-	-	+
P-10/232	-	-	+
P-10/233	-	-	+
P-10/234	-	-	+
P-10/235	-	-	+
P-10/236	-	-	+
P-10/237	-	-	+
P-10/238	-	-	+
P-10/239	-	-	+
P-10/240	-	-	+
P-10/241	-	-	+
P-10/242	-	-	+
P-10/243	-	-	-
P-10/244	-	-	+
P-10/245	-	-	+
P-10/246	-	-	+
P-10/247	-	-	+
P-10/248	-	-	+
P-10/249	-	-	+
P-10/250	-	-	+
P-10/251	-	-	+
P-10/252	-	-	+
P-10/253	-	-	+
P-10/254	-	-	+
P-10/255	-	-	+
P-10/256	-	-	+
P-10/257	-	-	+
P-10/258	-	-	+
P-10/259	-	-	+
P-10/260	-	-	+
P-10/261	-	-	+
P-10/262	-	-	+
P-10/263	-	-	+
P-10/264	-	-	+
P-10/265	-	-	+
P-10/266	-	-	+
P-10/267	-	-	+
P-10/268	-	-	+
P-10/269	-	-	+
P-10/270	-	-	+
P-10/271	-	-	+
P-10/272	-	-	+
P-10/273	-	-	+
P-10/274	-	-	+
P-10/275	-	-	+
P-10/276	-	-	+
P-10/277	-	-	+
P-10/278	-	-	+
P-10/279	-	-	+
P-10/280	-	-	+
P-10/281	-	-	+
P-10/282	-	-	+
P-10/283	-	-	+

Isolate	nptII	nptIII	16S
P-10/284	-	-	+
P-10/285	-	-	+
P-10/286	-	-	+
P-10/287	-	-	+
P-10/288	-	-	+
P-10/289	-	-	+
P-10/290	-	-	-
P-10/291	-	-	+
P-10/292	-	-	+
P-10/293	-	-	+
P-10/294	-	-	+
P-10/295	-	-	+
P-10/296	-	-	+
P-10/297	-	-	+
P-10/298	-	-	+
P-10/299	-	-	+
P-10/300	-	-	+
P-10/301	-	-	+
P-10/302	-	-	+
P-10/303	-	-	+
P-10/304	-	-	+
P-10/305	-	-	+
P-10/306	-	-	+
P-10/307	-	-	+
P-10/308	-	-	+
P-10/309	-	-	+
P-10/310	-	-	+
P-10/311	-	-	+
P-10/312	-	-	+
P-10/313	-	-	+
P-10/314	-	-	+
P-10/315	-	-	+
P-10/316	-	-	+
P-10/317	-	-	+
P-10/318	-	-	+
P-10/319	-	-	+
P-10/320	-	-	+
P-10/321	-	-	+
P-10/322	-	-	+
P-10/323	-	-	+
P-10/324	-	-	+
P-10/325	-	-	+
P-10/326	-	-	+
P-10/327	-	-	+
P-10/328	-	-	+
P-10/329	-	-	+
P-10/330	-	-	+
P-10/331	-	-	+
P-10/332	-	-	+
P-10/333	-	-	+
P-10/334	-	-	+
P-10/335	-	-	+
P-10/336	-	-	+
P-10/337	-	-	+
P-10/338	-	-	+
P-10/339	-	-	+
P-10/340	-	-	+
P-10/341	-	-	+
P-10/342	-	-	+
P-10/343	-	-	+
P-10/344	-	-	+
P-10/345	-	-	+
P-10/346	-	-	+
P-10/347	-	-	+
P-10/348	-	-	+
P-10/349	-	-	+
P-10/350	-	-	+
P-10/351	-	-	+
P-10/352	-	-	-
P-10/353	-	-	+
P-10/354	-	-	+
P-10/355	-	-	+

Isolate	nptII	nptIII	16S
P-10/356	-	-	+
P-10/357	-	-	+
P-10/358	-	-	+
P-10/359	-	-	+
P-10/360	-	-	+
P-10/361	-	-	+
P-10/362	-	-	+
P-10/363	-	-	+
P-10/364	-	-	+
P-10/365	-	-	+
P-10/366	-	-	+
P-10/367	-	-	+
P-10/368	-	-	+
P-10/369	-	-	+
P-10/370	-	-	+
P-10/371	-	-	+
P-10/372	-	-	+
P-10/373	-	-	+
P-10/374	-	-	+
P-10/375	-	-	+
P-10/376	-	-	+
P-10/377	-	-	+
P-10/378	-	-	+
P-10/379	-	-	+
P-10/380	-	-	+
P-10/381	-	-	+
P-10/382	-	-	+
P-10/383	-	-	+
P-10/384	-	-	+
P-10/385	-	-	+
P-10/386	-	-	+
P-10/387	-	-	+
P-10/388	-	-	+
P-10/389	-	-	+
P-10/390	-	-	+
P-10/391	-	-	+
P-10/392	-	-	+
P-10/393	-	-	+
P-10/394	-	-	+
P-10/395	-	-	+
P-10/396	-	-	+
P-10/397	-	-	+
P-10/398	-	-	+
P-10/399	-	-	+
P-10/400	-	-	+
P-10/401	-	-	+
P-10/402	-	-	+
P-10/403	-	-	+
P-10/404	-	-	+
P-10/405	-	-	+
P-10/406	-	-	+
P-10/407	-	-	-
P-10/408	-	-	+
P-10/409	-	-	+
P-10/410	-	-	+
P-10/411	-	-	+
P-10/412	-	-	+
P-10/413	-	-	+
P-10/414	-	-	+
P-10/415	-	-	+
P-10/416	-	-	+
P-10/417	-	-	+
P-10/418	-	-	+
P-10/419	-	-	+
P-10/420	-	-	+
P-10/421	-	-	+
P-10/422	-	-	+
P-10/423	-	-	+
P-10/424	-	-	+
P-10/425	-	-	+
P-10/426	-	-	+
P-10/427	-	-	+

Isolate	nptII	nptIII	16S
P-10/428	-	-	+
P-10/429	-	-	+
P-10/430	-	-	+
P-10/431	-	-	+
P-10/432	-	-	+
P-10/433	-	-	+
P-10/434	-	-	+
P-10/435	-	-	+
P-10/436	-	-	+
P-10/437	-	-	+
P-10/438	-	-	+
P-10/439	-	-	+
P-10/440	-	-	+
P-10/441	-	-	+
P-10/442	-	-	+
P-10/443	-	-	+
P-10/444	-	-	+
P-10/445	-	-	+
P-10/446	-	-	+
P-10/447	-	-	+
P-10/448	-	-	+
P-10/449	-	-	+
P-10/450	-	-	+
P-10/451	-	-	+
P-10/452	-	-	+
P-10/453	-	-	+
P-10/454	-	-	+
P-10/455	-	-	+
P-10/456	-	-	+
P-10/457	-	-	+
P-10/458	-	-	+
P-10/459	-	-	+
P-10/460	-	-	+
P-10/461	-	-	+
P-10/462	-	-	+
P-10/463	-	-	+
P-10/464	-	-	+
P-10/465	-	-	+
P-10/466	-	-	+
P-10/467	-	-	+
P-10/468	-	-	+
P-10/469	-	-	+
P-10/470	-	-	+
P-10/471	-	-	+
P-10/472	-	-	+
P-10/473	-	-	+
P-10/474	-	-	-
P-10/475	-	-	+
P-10/476	-	-	+
P-10/477	-	-	+
P-10/478	-	-	+
P-10/479	-	-	+
P-10/480	-	-	+
P-10/481	-	-	+
P-10/482	-	-	+
P-10/483	-	-	+
P-10/484	-	-	+
P-10/485	-	-	+
P-10/486	-	-	+
P-10/487	-	-	+
P-10/488	-	-	+
P-10/489	-	-	+
P-10/490	-	-	+
P-10/491	-	-	-
P-10/492	-	-	+
P-10/493	-	-	+
P-10/494	-	-	+
P-10/495	-	-	+
P-10/496	-	-	+
P-10/497	-	-	+
P-10/498	-	-	+
P-10/499	-	-	+

Isolate	nptII	nptIII	16S
P-10/500	-	-	-
P-10/501	-	-	-
P-10/502	-	-	+
P-10/503	-	-	+
P-10/504	-	-	+
P-10/505	-	-	+
P-10/506	-	-	+
P-10/507	-	-	+
P-10/508	-	-	+
P-10/509	-	-	+
P-10/510	-	-	+
P-10/511	-	-	+
P-10/512	drop out		
P-10/513	-	-	+
P-10/514	-	-	+
P-10/515	-	-	+
P-10/516	-	-	+
P-10/517	-	-	-
P-10/518	-	-	+
P-10/519	-	-	-
P-10/520	-	-	+
P-10/521	-	-	+
P-10/522	-	-	+
P-10/523	-	-	+
P-10/524	-	-	+
P-10/525	-	-	+
P-10/526	-	-	+
P-10/527	-	-	+
P-10/528	-	-	+
P-10/529	-	-	+
P-10/530	-	-	+
P-10/531	-	-	+
P-10/532	-	-	+
P-10/533	-	-	+
P-10/534	-	-	+
P-10/535	-	-	+
P-10/536	-	-	+
P-10/537	-	-	+
P-10/538	-	-	+
P-10/539	-	-	+
P-10/540	-	-	+
P-10/541	-	-	+
P-10/542	-	-	+
P-10/543	-	-	+
P-10/544	-	-	+
P-10/545	-	-	+
P-10/546	-	-	+
P-10/547	-	-	+
P-10/548	-	-	+
P-10/549	-	-	-
P-10/550	-	-	-
P-10/551	-	-	+
P-10/552	-	-	+
P-10/553	-	-	+
P-10/554	-	-	+
P-10/555	-	-	+
P-10/556	-	-	+
P-10/557	-	-	+
P-10/558	-	-	-
P-10/559	-	-	+
P-10/560	-	-	+
P-10/561	-	-	+
P-10/562	-	-	+
P-10/563	-	-	+
P-10/564	-	-	+
P-10/565	-	-	-
P-10/566	-	-	+
P-10/567	-	-	+
P-10/568	-	-	+
P-10/569	-	-	+
P-10/570	-	-	+
P-10/571	-	-	+

Isolate	nptII	nptIII	16S
P-10/572	-	-	+
P-10/573	-	-	+
P-10/574	-	-	+
P-10/575	-	-	+
P-10/576	-	-	+
P-10/577	-	-	+
P-10/578	-	-	+
P-10/579	-	-	+
P-10/580	-	-	+
P-10/581	-	-	+
P-10/582	-	-	+
P-10/583	-	-	+
P-10/584	-	-	+
P-10/585	-	-	+
P-10/586	-	-	+
P-10/587	-	-	+
P-10/588	-	-	+
P-10/589	-	-	+
P-10/590	-	-	+
P-10/591	-	-	+
P-10/592	-	-	+
P-10/593	-	-	+
P-10/594	-	-	+
P-10/595	-	-	+
P-10/596	-	-	+
P-10/597	-	-	+
P-10/598	-	-	+
P-10/599	-	-	+
P-10/600	-	-	+
P-10/601	-	-	-
P-10/602	-	-	+
P-10/603	-	-	-
P-10/604	-	-	+
P-10/605	-	-	+
P-10/606	-	-	+
P-10/607	-	-	+
P-10/608	-	-	-
P-10/609	-	-	+
P-10/610	-	-	+
P-10/611	-	-	+
P-10/612	-	-	+
P-10/613	-	-	+
P-10/614	-	-	+
P-10/615	-	-	+
P-10/616	-	-	+
P-10/617	-	-	+
P-10/618	-	-	+
P-10/619	-	-	+
P-10/620	-	-	+
P-10/621	-	-	+
P-10/622	-	-	+
P-10/623	-	-	+
P-10/624	-	-	+
P-10/625	-	-	+
P-10/626	-	-	+
P-10/627	-	-	+
P-10/628	-	-	+
P-10/629	-	-	+
P-10/630	-	-	+
P-10/631	-	-	+
P-10/632	-	-	+
P-10/633	-	-	+
P-10/634	-	-	+
P-10/635	-	-	+
P-10/636	-	-	+
P-10/637	-	-	+
P-10/638	-	-	+
P-10/639	-	-	+
P-10/640	-	-	-
P-10/641	-	-	-
P-10/642	-	-	+
P-10/643	-	-	+

Isolate	nptII	nptIII	16S
P-10/644	-	-	+
P-10/645	-	-	+
P-10/646	-	-	+
P-10/647	-	-	+
P-10/648	-	-	+
P-10/649	-	-	+
P-10/650	-	-	+
P-10/651	-	-	+
P-10/652	-	-	-
P-10/653	-	-	+
P-10/654	-	-	+
P-10/655	-	-	+
P-10/656	-	-	+
P-10/657	-	-	+
P-10/658	-	-	+
P-10/659	-	-	+
P-10/660	-	-	+
P-10/661	-	-	+
P-10/662	-	-	+
P-10/663	-	-	+
P-10/664	-	-	+
P-10/665	-	-	+
P-10/666	-	-	+
P-10/667	-	-	+
P-10/668	-	-	+
P-10/669	-	-	+
P-10/670	-	-	+
P-10/671	-	-	+
P-10/672	-	-	+
P-10/673	-	-	+
P-10/674	-	-	+
P-10/675	-	-	+
P-10/676	-	-	+
P-10/677	-	-	+
P-10/678	-	-	+
P-10/679	-	-	+
P-10/680	-	-	+
P-10/681	-	-	+
P-10/682	-	-	+
P-10/683	-	-	+
P-10/684	-	-	+
P-10/685	-	-	+
P-10/686	-	-	+
P-10/687	-	-	+
P-10/688	-	-	+
P-10/689	-	-	+
P-10/690	-	-	+
P-10/691	-	-	+
P-10/692	-	-	-
P-10/693	-	-	-
P-10/694	-	-	+
P-10/695	-	-	+
P-10/696	-	-	+
P-10/697	-	-	+
P-10/698	-	-	+
P-10/699	-	-	+
P-10/700	-	-	+
P-10/701	-	-	+
P-10/702	-	-	+
P-10/703	-	-	+
P-10/704	-	-	+
P-10/705	-	-	+
P-10/706	-	-	+
P-10/707	-	-	+
P-10/708	-	-	+
P-10/709	-	-	+
P-10/710	-	-	+
P-10/711	-	-	+
P-10/712	-	-	+
P-10/713	-	-	+
P-10/714	-	-	+
P-10/715	-	-	+

Isolate	nptII	nptIII	16S
P-10/716	-	-	+
P-10/717	-	-	+
P-10/718	-	-	+
P-10/719	-	-	+
P-10/720	-	-	+
P-10/721	-	-	+
P-10/722	-	-	+
P-10/723	-	-	+
P-10/724	-	-	+
P-10/725	-	-	+
P-10/726	-	-	+
P-10/727	-	-	+
P-10/728	-	-	+
P-10/729	-	-	+
P-10/730	-	-	+
P-10/731	-	-	+
P-10/732	-	-	+
P-10/733	-	-	-
P-10/734	-	-	+
P-10/735	-	-	+
P-10/736	-	-	+
P-10/737	-	-	+
P-10/738	-	-	+
P-10/739	-	-	+
P-10/740	-	-	+
P-10/741	-	-	+
P-10/742	-	-	+
P-10/743	-	-	+
P-10/744	-	-	+
P-10/745	-	-	+
P-10/746	-	-	+
P-10/747	-	-	+
P-10/748	-	-	+
P-10/749	-	-	+
P-10/750	-	-	+
P-10/751	-	-	+
P-10/752	-	-	+
P-10/753	-	-	+
P-10/754	-	-	+
P-10/755	-	-	-
P-10/756	-	-	+
P-10/757	-	-	+
P-10/758	-	-	+
P-10/759	-	-	+
P-10/760	-	-	+
P-10/761	-	-	+
P-10/762	-	-	+
P-10/763	-	-	+
P-10/764	-	-	+
P-10/765	-	-	+
P-10/766	-	-	+
P-10/767	-	-	+
P-10/768	-	-	+
P-10/769	-	-	+
P-10/770	-	-	+
P-10/771	-	-	-
P-10/772	-	-	+
P-10/773	-	-	-
P-10/774	-	-	+
P-10/775	-	-	+
P-10/776	-	-	+
P-10/777	-	-	+
P-10/778	-	-	+
P-10/779	-	-	+
P-10/780	-	-	+
P-10/781	-	-	+
P-10/782	-	-	+
P-10/783	-	-	+
P-10/784	-	-	+
P-10/785	-	-	+
P-10/786	-	-	+
P-10/787	-	-	+

Isolate	nptII	nptIII	16S
P-10/788	-	-	+
P-10/789	-	-	+
P-10/790	-	-	+
P-10/791	-	-	+
P-10/792	-	-	+
P-10/793	-	-	+
P-10/794	-	-	+
P-10/795	-	-	+
P-10/796	-	-	+
P-10/797	-	-	+
P-10/798	-	-	+
P-10/799	-	-	+
P-10/800	-	-	+
P-10/801	-	-	+
P-10/802	-	-	+
P-10/803	-	-	+
P-10/804	-	-	+
P-10/805	-	-	+
P-10/806	-	-	+
P-10/807	-	-	+
P-10/808	-	-	+
P-10/809	-	-	+
P-10/810	-	-	+
P-10/811	-	-	+
P-10/812	-	-	+
P-10/813	-	-	+
P-10/814	-	-	+
P-10/815	-	-	+
P-10/816	-	-	+
P-10/817	-	-	+
P-10/818	-	-	+
P-10/819	-	-	+
P-10/820	-	-	+
P-10/821	-	-	+
P-10/822	-	-	+
P-10/823	-	-	+
P-10/824	-	-	+
P-10/825	-	-	+
P-10/826	-	-	+
P-10/827	-	-	+
P-10/828	-	-	+
P-10/829	-	-	+
P-10/830	-	-	+
P-10/831	-	-	+
P-10/832	-	-	+
P-10/833	-	-	+
P-10/834	-	-	+
P-10/835	-	-	+
P-10/836	-	-	+
P-10/837	-	-	+
P-10/838	-	-	+
P-10/839	-	-	+
P-10/840	-	-	+
P-10/841	-	-	+
P-10/842	-	-	+
P-10/843	-	-	+
P-10/844	-	-	+
P-10/845	-	-	+
P-10/846	-	-	+
P-10/847	-	-	+
P-10/848	-	-	+
P-10/849	-	-	+
P-10/850	-	-	+
P-10/851	-	-	+
P-10/852	-	-	+
P-10/853	-	-	+
P-10/854	-	-	+
P-10/855	-	-	+
P-10/856	-	-	+
P-10/857	-	-	+
P-10/858	-	-	+
P-10/859	-	-	+

Isolate	nptII	nptIII	16S
P-10/860	-	-	+
P-10/861	-	-	+
P-10/862	-	-	+
P-10/863	-	-	+
P-10/864	-	-	+
P-10/865	-	-	+
P-10/866	-	-	+
P-10/867	-	-	+
P-10/868	-	-	+
P-10/869	-	-	+
P-10/870	-	-	+
P-10/871	-	-	+
P-10/872	-	-	+
P-10/873	-	-	+
P-10/874	-	-	+
P-10/875	-	-	+
P-10/876	-	-	+
P-10/877	-	-	+
P-10/878	-	-	+
P-10/879	-	-	+
P-10/880	-	-	+
P-10/881	-	-	+
P-10/882	-	-	+
P-10/883	-	-	+
P-10/884	-	-	+
P-10/885	-	-	+
P-10/886	-	-	+
P-10/887	-	-	+
P-10/888	-	-	+
P-10/889	-	-	+
P-10/890	-	-	+
P-10/891	-	-	+
P-10/892	-	-	+
P-10/893	-	-	+
P-10/894	-	-	+
P-10/895	-	-	+
P-10/896	-	-	+
P-10/897	-	-	+
P-10/898	-	-	+
P-10/899	-	-	+
P-10/900	-	-	+
P-10/901	-	-	+
P-10/902	-	-	+
P-10/903	-	-	+
P-10/904	-	-	+
P-10/905	-	-	+
P-10/906	-	-	+
P-10/907	-	-	+
P-10/908	-	-	+
P-10/909	-	-	+
P-10/910	-	-	+
P-10/911	-	-	+
P-10/912	-	-	+
P-10/913	-	-	+
P-10/914	-	-	+
P-10/915	-	-	+
P-10/916	-	-	+
P-10/917	-	-	+
P-10/918	-	-	+
P-10/919	-	-	+
P-10/920	-	-	+
P-10/921	-	-	+
P-10/922	-	-	+
P-10/923	-	-	+
P-10/924	-	-	+
P-10/925	-	-	+
P-10/926	-	-	+
P-10/927	-	-	+
P-10/928	-	-	+
P-10/929	-	-	+
P-10/930	-	-	+
P-10/931	-	-	+

Isolate	nptII	nptIII	16S
P-10/932	-	-	+
P-10/933	-	-	+
P-10/934	-	-	+
P-10/935	-	-	+
P-10/936	-	-	+
P-10/937	-	-	+
P-10/938	-	-	+
P-10/939	-	-	+
P-10/940	-	-	+
P-10/941	-	-	+
P-10/942	-	-	+
P-10/943	-	-	+
P-10/944	-	-	+
P-10/945	-	-	+
P-10/946	-	-	+
P-10/947	-	-	+
P-10/948	-	-	+
P-10/949	-	-	+
P-10/950	-	-	+
P-10/951	-	-	+
P-10/952	-	-	+
P-10/953	-	-	+
P-10/954	-	-	+
P-10/955	-	-	+
P-10/956	-	-	+
P-10/957	-	-	+
P-10/958	-	-	+
P-10/959	-	-	+
P-10/960	-	-	+
P-10/961	-	-	+
P-10/962	-	-	+
P-10/963	-	-	+
P-10/964	-	-	+
P-10/965	-	-	+
P-10/966	-	-	+
P-10/967	-	-	+
P-10/968	-	-	+
P-10/969	-	-	+
P-10/970	-	-	+
P-10/971	-	-	+
P-10/972	-	-	+
P-10/973	-	-	+
P-10/974	-	-	+
P-10/975	-	-	-
P-10/976	-	-	+
P-10/977	-	-	+
P-10/978	-	-	+
P-10/979	-	-	+
P-10/980	-	-	+
P-10/981	-	-	+
P-10/982	-	-	+
P-10/983	-	-	+
P-10/984	-	-	+
P-10/985	-	-	+
P-10/986	-	-	+

Table 11. *Pseudomonas aeruginosa*: TaqMan PCR results

drop out no *Pseudomonas aeruginosa* or no growth on agar plate (for details see Table 19)

	Number of samples nptII	Number of samples nptIII
Samples collected	986	986
Drop outs	2	2
Samples PCR tested	984	984
Samples not valid (16S and Screen negative)	33	33
Samples for statistical analysis	951	951
nptII/nptIII positive	0	0
Prevalence estimator	0.00%	0.00%
Confidence interval (95%)	0.00% - 0.3146%	0.00% - 0.3146%

Table 12. Data for statistical analysis; *Pseudomonas aeruginosa*

2.1.6 Source and localisation of *Pseudomonas aeruginosa* samples

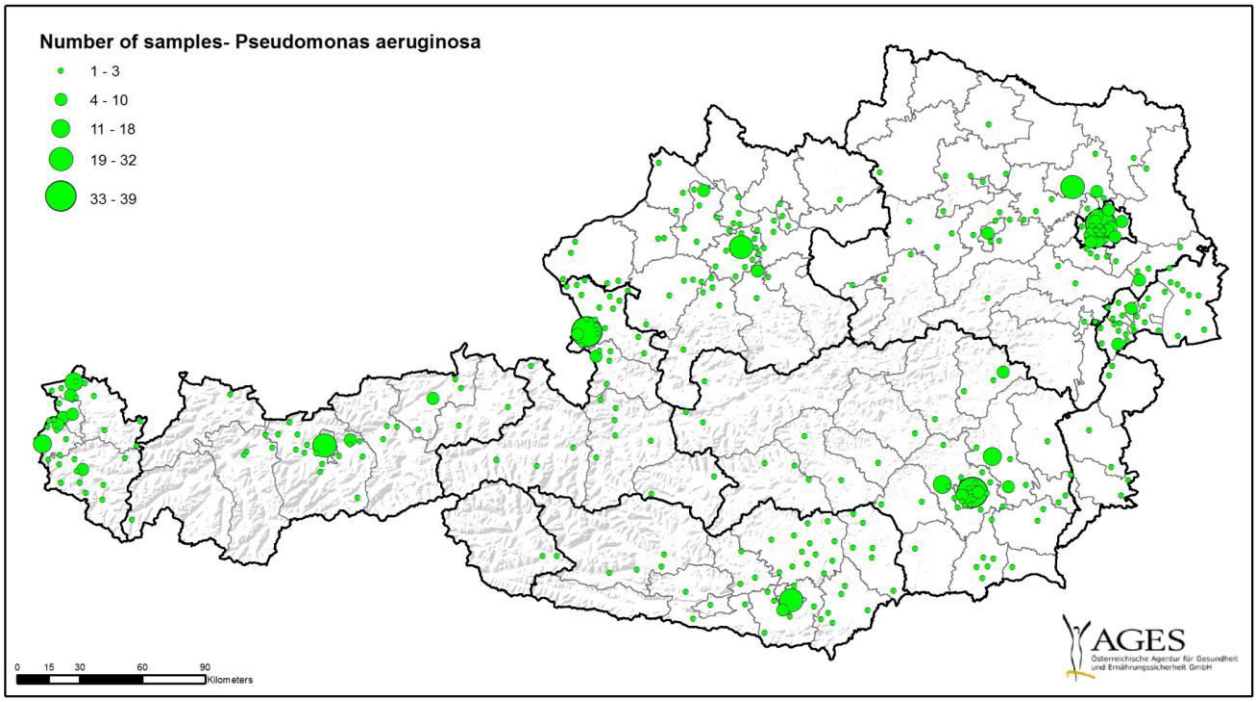


Figure 8. Distribution of *Pseudomonas aeruginosa* sampled in Austria

2.1.7 Prevalence of nptII/nptIII in *Salmonella enterica* subsp. *enterica* collected in Austria

Isolate	nptII	nptIII	16S	MIC Kana µg/ml[1]	MIC Interpretation[2]	Sequence[3]
MRS-10/00755	-	-	+	n.d.		n.a.
MRS-10/00765	+	-	+	> 256	resistant	wild type
MRS-10/00930	-	-	+	n.d.		n.a.
MRS-10/00980	-	-	+	n.d.		n.a.
MRS-10/01334	-	-	+	n.d.		n.a.
MRS-10/01349	-	-	+	n.d.		n.a.
MRS-10/01984	-	-	+	n.d.		n.a.
MRS-10/02018-2	-	-	+	n.d.		n.a.
MRS-10/02430	-	-	+	n.d.		n.a.
MRS-10/02508	-	-	+	n.d.		n.a.
MRS-10/02590-2	-	-	+	n.d.		n.a.
MRS-10/02638	-	-	+	n.d.		n.a.
MRS-10/02714	-	-	+	n.d.		n.a.
MRS-10/02969	-	-	+	n.d.		n.a.
MRS-10/03290	-	-	+	n.d.		n.a.
09/00000038	-	-	+	n.d.		n.a.
09/00000318-2	-	-	+	n.d.		n.a.
09/00001417	-	-	+	n.d.		n.a.
09/00001462	-	-	+	n.d.		n.a.
09/00001505-2	-	-	+	n.d.		n.a.
09/00002496	-	-	+	n.d.		n.a.
09/00002818	-	-	+	n.d.		n.a.
09/00002839	-	-	+	n.d.		n.a.
09/00003083	-	-	+	n.d.		n.a.
09/00003860	-	-	+	n.d.		n.a.
09/00005271	-	-	+	n.d.		n.a.
08/00000162	-	-	+	n.d.		n.a.
08/00000208	-	-	+	n.d.		n.a.
08/00000717	-	-	+	n.d.		n.a.
08/00000744-2	-	-	+	n.d.		n.a.
08/00001075	-	-	+	n.d.		n.a.
08/00001526	-	-	+	n.d.		n.a.
08/00002140-1	-	-	+	n.d.		n.a.
08/00002645	-	-	+	n.d.		n.a.
08/00002762	-	-	+	n.d.		n.a.
08/00003022-2	-	-	+	n.d.		n.a.
08/00003052	-	-	+	n.d.		n.a.
08/00003107	-	-	+	n.d.		n.a.
08/00003142	-	-	+	n.d.		n.a.
08/00003145	-	-	+	n.d.		n.a.
08/00003389	-	-	+	n.d.		n.a.
08/00003415	-	-	+	n.d.		n.a.
08/00003501	-	-	+	n.d.		n.a.
08/00003691-2	-	-	+	n.d.		n.a.
08/00004653	-	-	+	n.d.		n.a.
08/00004722	-	-	+	n.d.		n.a.
08/00004892	-	-	+	n.d.		n.a.
08/00005174	-	-	+	n.d.		n.a.
08/00005387	-	-	+	n.d.		n.a.
08/00005429	-	-	+	n.d.		n.a.
3235/08	-	-	+	n.d.		n.a.
1213/08I	-	-	+	n.d.		n.a.
2053/08II	-	-	+	n.d.		n.a.
883/08	-	-	+	n.d.		n.a.
318/09	-	-	+	n.d.		n.a.
4354/09	-	-	+	n.d.		n.a.
330/09	-	-	+	n.d.		n.a.
5278/08	-	-	+	n.d.		n.a.
4366/09	-	-	+	n.d.		n.a.
4001/10	-	-	+	n.d.		n.a.

Table 13. *Salmonella enterica* subsp. *enterica:* PCR results, MIC and sequence interpretation of positive strains

1) minimum inhibitory concentrations for kanamycin (Epsilon test; Biomerieux)

2) Interpretation: sensitive/resistant according to CLSI

3) Sequence of nptIII of positive strains

n.d. not done

n.a. not available

wild type homolog to aph(3')-IIa reference sequence V00618

Year	Total	No. kanamycin resistant	% resistant
2000	7,417	50	0.67
2001	7,684	77	1
2002	8,403	56	0.67
2003	8,250	45	0.55
2004	7,286	27	0.37
2005	5,615	34	0.61
2006	5,379	35	0.65
2007	4,050	19	0.47
2008	3,196	29	0.91
2009	2,829	15	0.53
2010	2,210	16	0.72
2008 – 2010	8,235	60	0.73
Total (2000- 2010)	62,319	406	0.65

Table 14. Kanamycin resistant *Salmonella* among the tested isolates in Austria (2008 – 2010)

Resistance was analysed by the disk diffusion test: an isolate was determined as resistant to the antimicrobial if the diameter of the inhibition zone was ≤13 mm (according to clinical breakpoints/CLSI).

2.1.8 Prevalence of nptII/nptIII in *Staphylococcus aureus* collected in Austria

Isolate	nptII	nptIII	16S	MIC Kana µg/ml	MIC Interpretation	Sequence
ST-11/001	-	-	+			
ST-11/002	-	-	+			
ST-11/003	-	-	+			
ST-11/004	-	-	+			
ST-11/005	-	-	+			
ST-11/006	-	-	+			
ST-11/007	-	+	+	>256	resistant	overlaid
ST-11/008	-	-	+			
ST-11/009	-	-	+			
ST-11/010	-	-	-			
ST-11/011	-	-	+			
ST-11/012	-	-	-			
ST-11/013	-	-	-			
ST-11/014	-	-	-			
ST-11/015	-	-	-			
ST-11/016	-	-	-			
ST-11/017	-	-	+			
ST-11/018	-	-	+			
ST-11/019	-	-	+			
ST-11/020	-	-	+			
ST-11/021	-	-	-			
ST-11/022	-	-	-			
ST-11/023	-	-	-			
ST-11/024	-	-	-			
ST-11/025	-	-	+			
ST-11/026	-	-	+			
ST-11/027	-	-	+			
ST-11/028	-	-	+			
ST-11/029	-	-	+			
ST-11/030	-	-	+			
ST-11/031	-	-	+			
ST-11/032	-	-	-			
ST-11/033	-	-	+			
ST-11/034	-	-	+			
ST-11/035	-	-	+			
ST-11/036	-	-	+			
ST-11/037	-	-	+			
ST-11/038	-	-	+			
ST-11/039	-	-	-			
ST-11/040	-	-	+			
ST-11/041	-	-	+			
ST-11/042	-	-	+			
ST-11/043	-	-	+			
ST-11/044	-	-	+			
ST-11/045	-	-	+			
ST-11/046	-	-	+			
ST-11/047	-	-	-			
ST-11/048	-	-	-			
ST-11/049	-	-	+			
ST-11/050	-	-	+			
ST-11/051	-	-	+			
ST-11/052	-	-	+			
ST-11/053	drop out					
ST-11/054	drop out					
ST-11/055	-	-	+			
ST-11/056	-	-	+			
ST-11/057	-	-	+			
ST-11/058	-	-	+			
ST-11/059	-	-	+			
ST-11/060	-	-	+			
ST-11/061	-	-	+			
ST-11/062	-	-	+			
ST-11/063	-	-	+			
ST-11/064	-	-	-			
ST-11/065	-	-	-			
ST-11/066	-	-	-			

Isolate	nptII	nptIII	16S	MIC Kana µg/ml	MIC Interpretation	Sequence
ST-11/067	-	-	+			
ST-11/068	-	-	+			
ST-11/069	-	-	+			
ST-11/070	-	-	+			
ST-11/071	-	-	+			
ST-11/072	-	-	-			
ST-11/073	-	-	+			
ST-11/074	-	-	+			
ST-11/075	-	-	+			
ST-11/076	-	-	+			
ST-11/077	-	-	+			
ST-11/078	-	-	+			
ST-11/079	-	-	+			
ST-11/080	-	-	+			
ST-11/081	-	-	+			
ST-11/082	-	-	+			
ST-11/083	-	-	+			
ST-11/084	-	-	+			
ST-11/085	-	-	+			
ST-11/086	-	-	+			
ST-11/087	-	-	+			
ST-11/088	-	-	+			
ST-11/089	-	-	+			
ST-11/090	-	-	+			
ST-11/091	-	-	+			
ST-11/092	-	-	+			
ST-11/093	-	-	+			
ST-11/094	-	-	+			
ST-11/095	-	-	+			
ST-11/096	-	-	+			
ST-11/097	-	-	+			
ST-11/098	-	-	+			
ST-11/099	-	-	+			
ST-11/100	-	-	+			
ST-11/101	-	-	+			
ST-11/102	-	-	+			
ST-11/103	-	-	+			
ST-11/104	-	-	+			
ST-11/105	-	-	+			
ST-11/106	-	-	+			
ST-11/107	-	-	+			
ST-11/108	-	-	+			
ST-11/109	-	-	+			
ST-11/110	-	-	+			
ST-11/111	-	-	+			
ST-11/112	-	-	+			
ST-11/113	-	-	+			
ST-11/114	-	-	+			
ST-11/115	-	-	+			
ST-11/116	-	-	+			
ST-11/117	-	-	+			
ST-11/118	-	-	+			
ST-11/119	-	-	+			
ST-11/120	-	-	+			
ST-11/121	-	-	+			
ST-11/122	-	-	+			
ST-11/123	-	-	+			
ST-11/124	-	-	+			
ST-11/125	-	-	+			
ST-11/126	-	-	+			
ST-11/127	-	-	+			
ST-11/128	-	-	+			
ST-11/129	-	-	+			
ST-11/130	-	-	+			
ST-11/131	-	-	+			
ST-11/132	-	-	+			
ST-11/133	-	-	+			
ST-11/134	-	-	+			
ST-11/135	-	-	+			
ST-11/136	-	-	+			
ST-11/137	-	-	+			

Isolate	nptII	nptIII	16S	MIC Kana µg/ml	MIC Interpretation	Sequence
ST-11/138	-	-	+			
ST-11/139	-	-	+			
ST-11/140	-	-	+			
ST-11/141	-	-	+			
ST-11/142	-	-	+			
ST-11/143	-	-	+			
ST-11/144	-	-	+			
ST-11/145	-	-	+			
ST-11/146	-	-	+			
ST-11/147	-	-	+			
ST-11/148	-	-	+			
ST-11/149	-	-	+			
ST-11/150	-	-	+			
ST-11/151	-	-	+			
ST-11/152	-	-	+			
ST-11/153	-	-	+			
ST-11/154	-	-	+			
ST-11/155	-	-	+			
ST-11/156	-	-	+			
ST-11/157	-	-	+			
ST-11/158	-	-	+			
ST-11/159	-	-	+			
ST-11/160	-	-	+			
ST-11/161	-	-	+			
ST-11/162	-	-	+			
ST-11/163	-	-	+			
ST-11/164	-	-	+			
ST-11/165	-	-	+			
ST-11/166	-	-	+			
ST-11/167	-	+	-	>256	resistant	wild type
ST-11/168	-	-	+			
ST-11/169	-	-	+			
ST-11/170	-	-	+			
ST-11/171	-	-	+			
ST-11/172	-	-	+			
ST-11/173	-	-	+			
ST-11/174	-	-	+			
ST-11/175	-	+	+	>256	resistant	wild type
ST-11/176	-	-	+			
ST-11/177	-	-	+			
ST-11/178	-	-	+			
ST-11/179	-	-	+			
ST-11/180	-	-	+			
ST-11/181	-	-	+			
ST-11/182	-	-	+			
ST-11/183	-	-	+			
ST-11/184	-	-	+			
ST-11/185	-	-	+			
ST-11/186	-	-	+			
ST-11/187	-	-	+			
ST-11/188	-	-	+			
ST-11/189	-	-	+			
ST-11/190	-	-	+			
ST-11/191	-	-	+			
ST-11/192	-	-	+			
ST-11/193	-	-	+			
ST-11/194	-	+	+	>256	resistant	wild type
ST-11/195	-	-	+			
ST-11/196	-	-	+			
ST-11/197	-	-	+			
ST-11/198	-	-	+			
ST-11/199	-	-	+			
ST-11/200	-	-	+			
ST-11/201	-	-	+			
ST-11/202	drop out					
ST-11/203	-	-	+			
ST-11/204	-	-	+			
ST-11/205	-	-	+			
ST-11/206	-	-	+			
ST-11/207	-	-	+			
ST-11/208	-	-	+			

Isolate	nptII	nptIII	16S	MIC Kana µg/ml	MIC Interpretation	Sequence
ST-11/209	-	+	+	>256	resistant	wild type
ST-11/210	-	-	+			
ST-11/211	-	-	+			
ST-11/212	-	-	+			
ST-11/213	-	-	+			
ST-11/214	-	-	+			
ST-11/215	-	-	+			
ST-11/216	-	-	+			
ST-11/217	-	-	+			
ST-11/218	-	-	+			
ST-11/219	-	-	+			
ST-11/220	-	-	+			
ST-11/221	-	-	+			
ST-11/222	-	-	+			
ST-11/223	-	-	+			
ST-11/224	-	-	+			
ST-11/225	-	-	+			
ST-11/226	-	-	+			
ST-11/227	-	-	+			
ST-11/228	-	-	+			
ST-11/229	-	-	+			
ST-11/230	-	-	+			
ST-11/231	-	-	+			
ST-11/232	-	-	+			
ST-11/233	-	-	+			
ST-11/234	-	-	+			
ST-11/235	-	-	+			
ST-11/236	-	-	+			
ST-11/237	-	-	+			
ST-11/238	-	-	+			
ST-11/239	-	-	+			
ST-11/240	-	-	+			
ST-11/241	-	-	+			
ST-11/242	-	-	+			
ST-11/243	-	-	+			
ST-11/244	-	-	+			
ST-11/245	-	-	+			
ST-11/246	-	-	+			
ST-11/247	-	-	+			
ST-11/248	-	-	+			
ST-11/249	-	-	+			
ST-11/250	-	-	+			
ST-11/251	-	-	+			
ST-11/252	-	-	+			
ST-11/253	-	-	+			
ST-11/254	-	-	+			
ST-11/255	-	-	+			
ST-11/256	-	-	+			
ST-11/257	-	-	+			
ST-11/258	-	-	+			
ST-11/259	-	-	+			
ST-11/260	-	-	+			
ST-11/261	-	-	+			
ST-11/262	-	-	+			
ST-11/263	-	-	+			
ST-11/264	-	-	+			
ST-11/265	-	-	+			
ST-11/266	-	-	+			
ST-11/267	-	-	+			
ST-11/268	-	-	+			
ST-11/269	-	-	+			
ST-11/270	-	-	+			
ST-11/271	-	-	+			
ST-11/272	-	-	+			
ST-11/273	-	-	+			
ST-11/274	-	-	+			
ST-11/275	-	-	+			
ST-11/276	-	-	+			
ST-11/277	-	-	+			
ST-11/278	-	-	+			
ST-11/279	-	-	+			

Isolate	nptII	nptIII	16S	MIC Kana µg/ml	MIC Interpretation	Sequence
ST-11/280	-	-	+			
ST-11/281	-	-	+			
ST-11/282	-	-	+			
ST-11/283	-	-	+			
ST-11/284	-	-	+			
ST-11/285	-	-	+			
ST-11/286	-	-	+			
ST-11/287	-	-	+			
ST-11/288	-	-	+			
ST-11/289	-	-	+			
ST-11/290	-	-	+			
ST-11/291	-	-	+			
ST-11/292	-	+	+	>256	resistant	wild type
ST-11/293	-	-	+			
ST-11/294	-	-	+			
ST-11/295	-	-	+			
ST-11/296	-	-	+			
ST-11/297	-	-	+			
ST-11/298	-	-	+			
ST-11/299	-	-	+			
ST-11/300	-	-	+			
ST-11/301	-	-	+			
ST-11/302	-	-	+			
ST-11/303	-	-	+			
ST-11/304	-	-	+			
ST-11/305	-	-	+			
ST-11/306	-	-	+			
ST-11/307	-	-	+			
ST-11/308	-	-	+			
ST-11/309	-	-	+			
ST-11/310	-	-	+			
ST-11/311	-	-	+			
ST-11/312	-	-	+			
ST-11/313	-	-	+			
ST-11/314	-	-	+			
ST-11/315	-	-	+			
ST-11/316	-	-	+			
ST-11/317	-	-	+			
ST-11/318	-	-	+			
ST-11/319	-	-	+			
ST-11/320	-	-	+			
ST-11/321	-	-	+			
ST-11/322	-	-	+			
ST-11/323	-	-	+			
ST-11/324	-	-	+			
ST-11/325	-	-	+			
ST-11/326	-	-	+			
ST-11/327	-	-	+			
ST-11/328	-	-	+			
ST-11/329	-	-	+			
ST-11/330	-	-	+			
ST-11/331	-	-	+			
ST-11/332	-	-	+			
ST-11/333	-	-	+			
ST-11/334	-	-	+			
ST-11/335	-	-	+			
ST-11/336	-	-	+			
ST-11/337	-	-	+			
ST-11/338	-	-	+			
ST-11/339	-	-	+			
ST-11/340	-	-	+			
ST-11/341	-	-	+			
ST-11/342	-	-	+			
ST-11/343	-	-	+			
ST-11/344	-	+	+	>256	resistant	wild type
ST-11/345	-	-	+			
ST-11/346	-	-	-			
ST-11/347	-	-	+			
ST-11/348	drop out					
ST-11/349	-	-	+			
ST-11/350	-	-	+			

Isolate	nptII	nptIII	16S	MIC Kana µg/ml	MIC Interpretation	Sequence
ST-11/351	-	-	+			
ST-11/352	-	-	+			
ST-11/353	-	-	+			
ST-11/354	-	-	+			
ST-11/355	-	+	+	>256	resistant	overlaid
ST-11/356	-	-	+			
ST-11/357	-	-	+			
ST-11/358	-	-	+			
ST-11/359	-	+	+	1	sensitive	wild type
ST-11/360	-	+	+	>256	resistant	wild type
ST-11/361	-	-	+			
ST-11/362	-	-	+			
ST-11/363	-	-	+			
ST-11/364	-	-	+			
ST-11/365	-	+	+	>256	resistant	wild type
ST-11/366	-	-	+			
ST-11/367	-	-	+			
ST-11/368	-	-	+			
ST-11/369	-	-	+			
ST-11/370	-	-	+			
ST-11/371	-	-	+			
ST-11/372	-	-	+			
ST-11/373	-	-	+			
ST-11/374	-	-	+			
ST-11/375	-	-	+			
ST-11/376	-	-	+			
ST-11/377	-	-	+			
ST-11/378	-	-	+			
ST-11/379	-	-	+			
ST-11/380	-	-	+			
ST-11/381	-	-	+			
ST-11/382	-	-	+			
ST-11/383	-	-	+			
ST-11/384	-	-	+			
ST-11/385	drop out					
ST-11/386	-	-	+			
ST-11/387	-	-	+			
ST-11/388	-	-	+			
ST-11/389	-	-	+			
ST-11/390	-	-	+			
ST-11/391	-	-	+			
ST-11/392	drop out					
ST-11/393	-	-	+			
ST-11/394	-	-	+			
ST-11/395	-	-	+			
ST-11/396	-	-	-			
ST-11/397	-	-	+			
ST-11/398	-	-	+			
ST-11/399	-	-	+			
ST-11/400	-	-	+			
ST-11/401	-	-	+			
ST-11/402	-	-	+			
ST-11/403	-	-	+			
ST-11/404	-	-	+			
ST-11/405	-	+	+	>256	resistant	mutation
ST-11/406	-	-	+			
ST-11/407	-	-	+			
ST-11/408	-	-	+			
ST-11/409	-	-	+			
ST-11/410	-	-	+			
ST-11/411	-	-	+			
ST-11/412	-	-	+			
ST-11/413	-	-	+			
ST-11/414	-	-	+			
ST-11/415	-	-	+			
ST-11/416	-	-	+			
ST-11/417	-	-	+			
ST-11/418	-	-	+			
ST-11/419	-	-	+			
ST-11/420	-	-	+			
ST-11/421	-	-	+			

Isolate	nptII	nptIII	16S	MIC Kana µg/ml	MIC Interpretation	Sequence
ST-11/422	-	-	+			
ST-11/423	-	-	+			
ST-11/424	-	-	+			
ST-11/425	-	-	+			
ST-11/426	-	-	+			
ST-11/427	-	-	+			
ST-11/428	-	-	+			
ST-11/429	-	-	+			
ST-11/430	-	-	+			
ST-11/431	-	-	+			
ST-11/432	-	-	+			
ST-11/433	-	-	+			
ST-11/434	-	-	+			
ST-11/435	-	-	+			
ST-11/436	-	-	+			
ST-11/437	-	-	+			
ST-11/438	-	-	+			
ST-11/439	-	-	+			

Table 15. *Staphylococcus aureus*: PCR results, MIC and sequence interpretation of positive strains

1) minimum inhibitory concentrations for kanamycin (Epsilon test; Biomerieux)

2) Interpretation: sensitive/resistant according to CLSI

3) Sequence of nptIII of positive strains

n.d. not done

n.a. not available

wild type homolog to aph(3')-IIIa reference sequence V01547

drop out no *S. aureus* or no growth on agar plate (for details see Table 19)

	Number of isolates nptII	Number of isolates nptIII
Samples collected	439	439
Drop outs	6	6
Samples PCR tested	433	433
Samples not valid (16S and Screen negative)	21	20
Samples for statistical analysis	412	413
NnptII/nptIII positive	0	12
Prevalence estimator	0.00%	2.90%
Confidence interval (95%)	0.00% - 0.73%	1.51% - 5.02%

Table 16. Data for statistical analysis; *Staphylococcus aureus*

2.1.9 Source and localisation of *S. aureus* samples

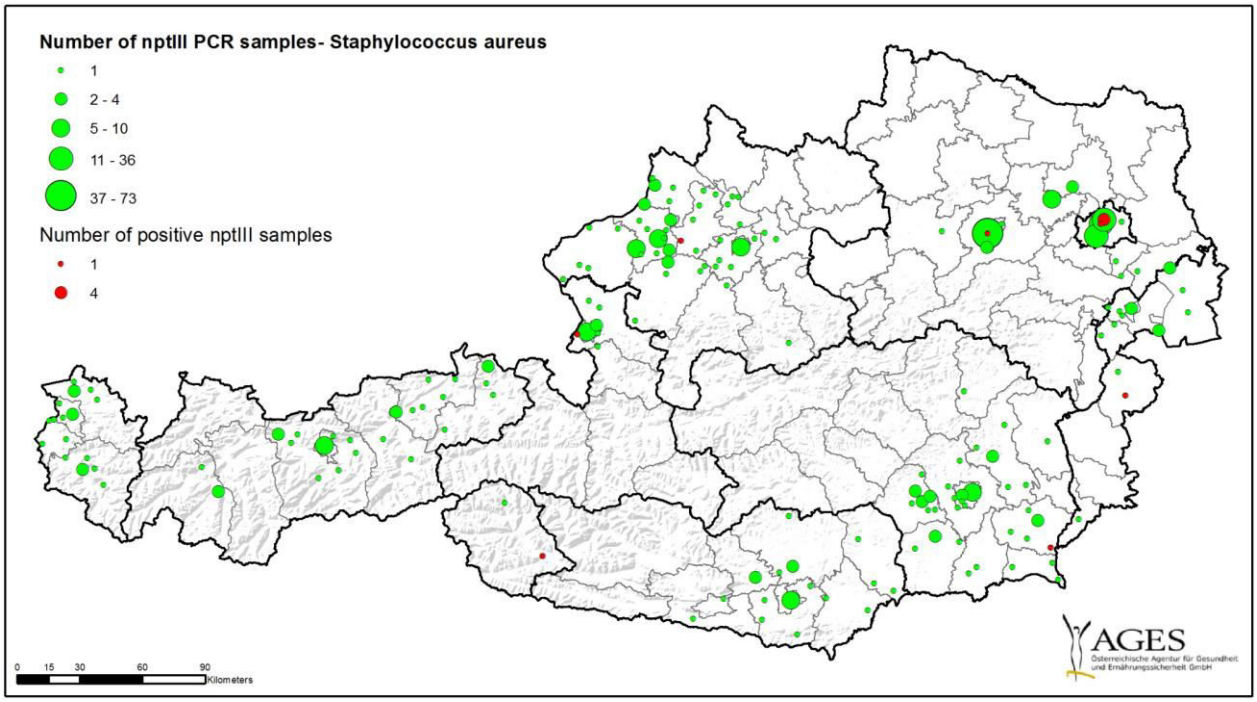
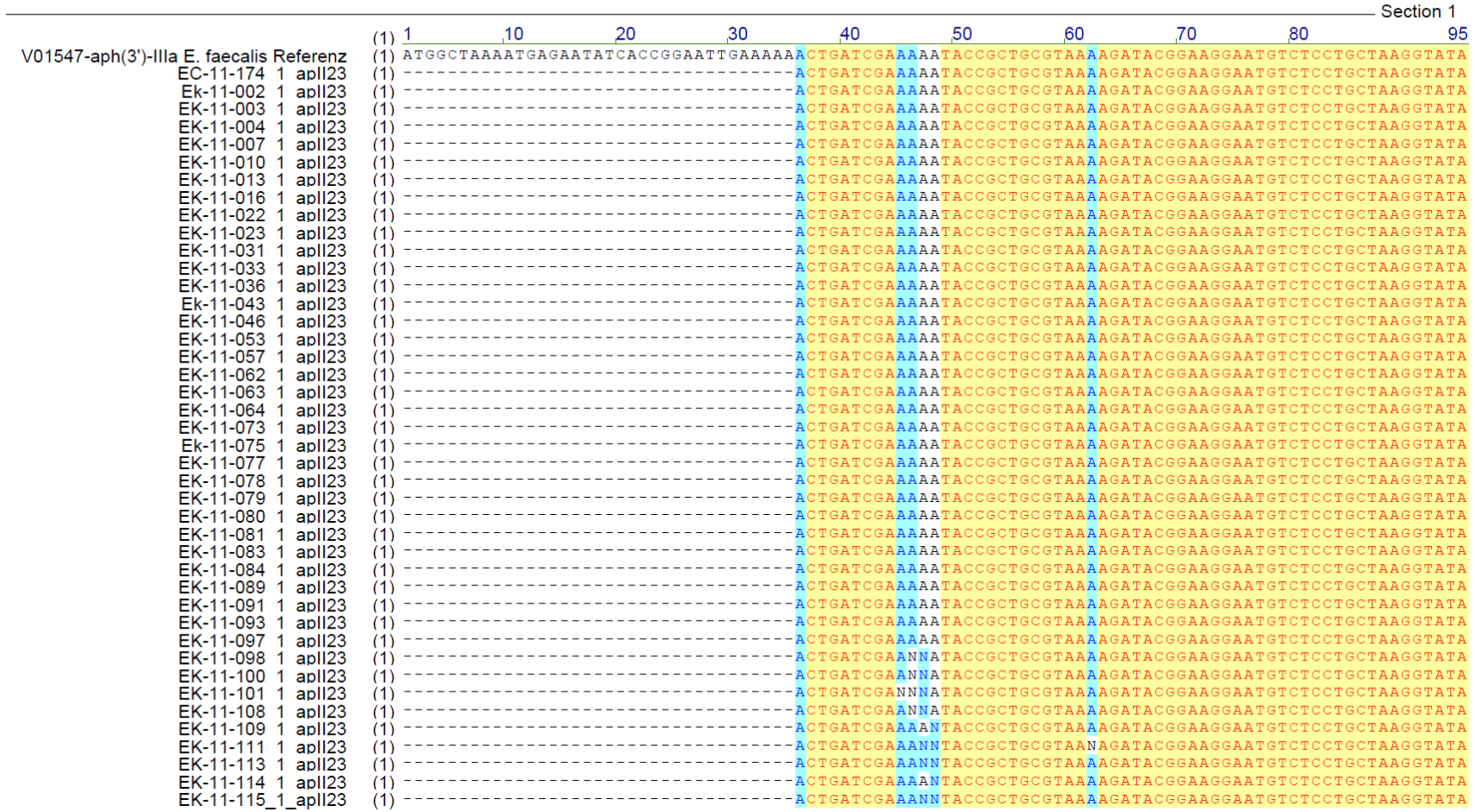
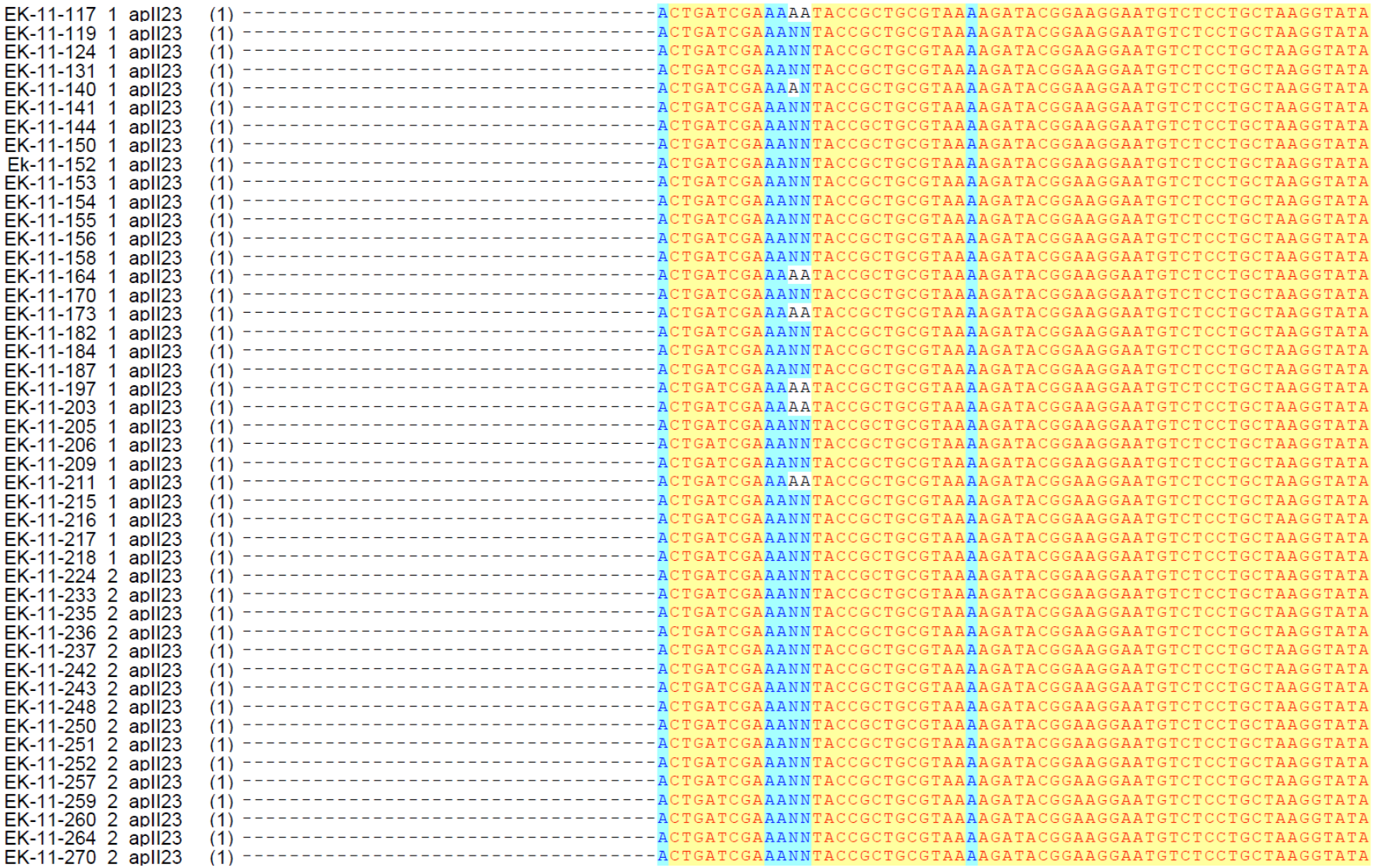


Figure 9. Distribution of *S. aureus* sampled in Austria

2.2 Sequence analysis of nptII/nptIII positive bacterial strains

nptIII alignment human pathogenic bacteria_final_7.8.2012.apr




EK-11-117 1 apII23 (1) ----------ACTGATCGAAAATACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-119 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-124 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-131 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-140 1 apII23 (1) ----------ACTGATCGAAAANTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-141 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-144 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-150 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
Ek-11-152 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-153 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-154 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-155 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-156 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-158 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-164 1 apII23 (1) ----------ACTGATCGAAAAATACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-170 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-173 1 apII23 (1) ----------ACTGATCGAAAAATACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-182 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-184 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-187 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-197 1 apII23 (1) ----------ACTGATCGAAAAATACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-203 1 apII23 (1) ----------ACTGATCGAAAAATACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-205 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-206 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-209 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-211 1 apII23 (1) ----------ACTGATCGAAAAATACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-215 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-216 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-217 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-218 1 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-224 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-233 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-235 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-236 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-237 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-242 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-243 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-248 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-250 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
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EK-11-257 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-259 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-260 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
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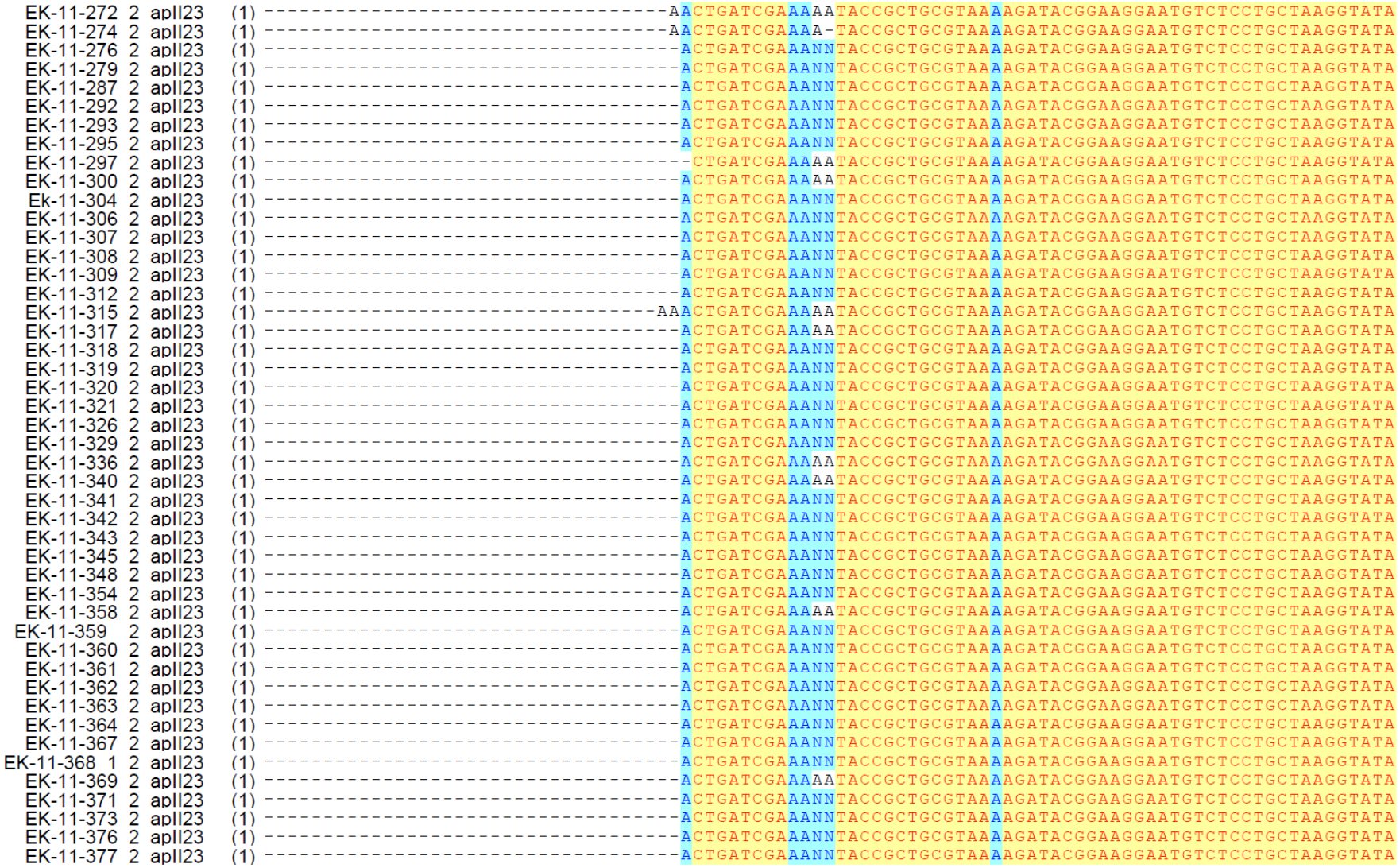
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EK-11-279 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
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EK-11-292 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
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EK-11-297 2 apII23 (1) ----------CTGATCGAAAAATACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
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EK-11-309 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-312 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
EK-11-315 2 apII23 (1) ----------AAACTGATCGAAAAATACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
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EK-11-369 2 apII23 (1) ----------ACTGATCGAAAAATACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA
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EK-11-377 2 apII23 (1) ----------ACTGATCGAAANNTACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATA

Figure 10. Sequences of nptIII positive strains

5’ termini of nptIII gene sequences; remaining sequences are available upon request. CLUSTAL W analysis, VectorNTI 7.1

3 Appendix B: Material and Methods

3.1 Random sampling plan – theoretical considerations

The project plan originally contained a sample size of 100. Statistical analysis however concluded that the resulting degree of variation of the result would exceed an acceptable level. For the abovementioned sample size and an assumed prevalence of 1%, the 95%-confidence interval ranges from 0.04% to 5.4%. Due to this wide margin of variation, the need for more detailed planning of the sampling strategy arises.

The aim of this project is to estimate the prevalence p of nptII in the environment. The prevalence is to be estimated according to a predefined accuracy. The sample size required to achieve said accuracy strongly depends on the actual prevalence p. The required sample size is smallest, when p is close to 0% or 100%. It increases as the prevalence gets closer to 50%.

Whether the predefined accuracy is achieved or not depends on the sample and is a random variable. The probability of achieving the target accuracy is determined by the so-called “power” and can be incorporated into the design of the sampling plan. A power of 50% results in samples for which the desired accuracy is achieved 50% of the time. For 50% of the samples, the estimate may, however, exceed the desired accuracy.

Table 17 shows the required sample sizes for prevalences ranging from 2% to 10% and an accuracy of ±1%. The sample sizes were computed for a power of 50% and 80%. For an assumed prevalence of 2%, an accuracy of ±1% and a power of 50%, the required sample size amounts to 889 isolates. In order to achieve an increased power of 80%, 1,858 isolates are required.

Prevalence (in %)	n for Power = 50%	n for Power = 80%
2	889	1,858
3	1,242	2,599
4	1,593	3,323
5	1,961	4,038
6	2,281	4,734
7	2,608	5,417
8	2,952	6,082
9	3,267	6,723
10	3,579	7,347

Table 17: Required sample sizes for an accuracy of ±1%, powers of 50% and 80% and varying prevalence values.

For small prevalences, one might consider specifying the accuracy with respect to an increase of the prevalence only. The main point of interest of such a study is to determine whether the prevalence lies below a certain threshold, i.e., we are only interested in the upper limit of the confidence interval. As the lower limit of the confidence interval is relaxed, the required sample size is reduced.

Table 18 contains the sample sizes for an assumed prevalence of 1% and values of the accuracy ranging from 0.2% to 1.4%. The required sample sizes are given for powers of 50% and 80%. In order to achieve an accuracy of 1% at an assumed prevalence of 1% (corresponding to an upper limit of the confidence interval below 2%) and a power of 50%, a sample size of 384 is necessary. For a power of 80%, the sample size lies at 905 isolates.

On the basis of the present analysis, it was decided that for the entire region of Austria 384 isolates are to be collected for each bacterial species under consideration. The sampling of the isolates must be done in such a way that a representative distribution of the collecting laboratories can be guaranteed. The samples are to be stored centrally in Graz. For each of the samples, cryo-isolates are to be generated.

Accuracy (in %)	n for power = 50%	n for Power = 80%
1.4	195	520
1.2	258	670
1.0	384	905
0.8	537	1,311
0.6	917	2,153
0.5	1,245	3,011
0.4	1,905	4,514
0.3	3,282	7,685
0.2	7,139	12,680

Table 18: Required sample size for an assumed prevalence of 1%, powers of 50% and 80% and varying values for the accuracy.

3.2 Handling procedure of incoming bacterial isolates forwarded by peripheral laboratories

The following steps were performed at the collection centre at AGES-IMED, Graz:

1. Allocation of an identification number (= sample number). If inconsistencies were identified the sender was contacted and/or the sample was discarded (i.e. identified as "drop out" and excluded from further analyses; for details please see Table 19)
2. Single colony plating of the sample on blood agar (*Staphylococcus, Pseudomonas*, and enterococci) or CPS3 agar (*E. coli*; James-Reagent)
3. Check for purity of the spread colonies on agar plates. Visually aberrant isolates were re-tested by VITEK. If the result did not confirm the initial identification of the strain, the sample was discarded and marked as drop out.
4. A single colony from the subcultivation was cryopreserved and stored at -80°C for further analyses.

Bacterial species	Reason for exclusion
Escherichia coli	10 x no *Escherichia coli* 1 x no growth (11)
enterococci	2 x no enterococci 7 x no growth 1 x discrepancy with sender certificate (danger of mix-ups) (10)
Pseudomonas aeruginosa	1 x drop out (at the initial phase of the project no reason recorded) 1 x discrepancy with sender data sheet (danger of mix-ups) (2)
Staphylococcus aureus	2 x no *Staphylococcus aureus* 1 x no growth 3 x discrepancy with sender certificate (danger of mix-ups) (6)

Table 19. List of bacterial drop out samples

3.3 Identification procedures of the strains actively sampled at the central collection centre in Graz (AGES-IMED, Graz) and at the participating laboratory in Vienna (AGES-IMED, Vienna)

All incoming samples were treated on a routine basis and handled as any other bacterial isolate in the usual identification pipeline. The usually applied identification procedures for each respective species are described below.

3.3.1 *Escherichia coli*

E. coli from specimens of human origin (e.g. urine/Uricult, feces, etc…) were identified by growth on solid media (e.g. MacConkey or Columbia blood agar plates) and in liquid broth (e.g. brain heart

infusion (BHI)). Ambiguous colonies were biochemically characterized by VITEK or API 20 E. If EHEC/VTEC positivity was assumed the presence of Shiga-toxins was verified by ELISA or PCR. An antimicrobial susceptibility testing was performed with all isolates (except for EHEC/VTEC strains).

3.3.2 *Enterococcus faecalis, Enterococcus faecium*

Enterococci from specimen of human origin (e.g. urine/Uricult) were identified by growth on solid media (e.g. Columbia blood or enterococci selective agar plates) and in liquid broth (e.g. brain heart infusion (BHI)). Colonies were biochemically identified by VITEK. Antimicrobial susceptibility testing was performed with clinically relevant isolates.

3.3.3 *Pseudomonas aeruginosa*

P. aeruginosa from specimens of human origin (e.g. swab samples from the external ear) were identified by growth on solid media (e.g. MacConkey or Columbia blood agar plates) and in liquid broth (e.g. brain heart infusion (BHI)). For a quick differentiation from enterobacteriaceae an oxidase test was applied, which induces an intense blue colour in positive strains (*Pseudomonas* produces cytochrome c oxidase which oxidizes the applied tetramethyl-p-phenylendiaminhydrochloride reagent leading to blue coloration of the bacterial colony). A biochemical identification was performed with VITEK or API 20 NE strips. Antimicrobial susceptibility testing was performed with clinically relevant isolates.

3.3.4 *Salmonella enterica* subsp. *enterica*

For the identification of *Salmonella* spp. from feces, blood or swab specimens, the following procedures were applied: All isolates were serotyped and biochemically characterized according to the White-Kauffmann-Le Minor-scheme. *S.* Enteritidis and *S.* Typhimurium (most prevalent serotypes in Austria) were additionally characterized by phage typing according to the recommendations of the Health Protection Agency (HPA), Colindale, UK. Antimicrobial susceptibility testing was performed with all isolates using agar diffusion testing according to CLSI recommendations. Indifferent results were re-analysed for MIC determination with the E-test (Biomerieux).

3.3.5 *Staphylococcus aureus*

S. aureus from specimens of human origin (e.g. swab samples from wound infections) were identified by growth on solid media (e.g. blood agar plates) and in liquid broth (e.g. brain heart infusion (BHI)). For *S. aureus* colonies a catalase test was performed routinely (*S. aureus* specific catalase dissociates H_2O and O_2 from the applied drops of H_2O_2 which leads to spontaneous formation of gas and bubbling). To discriminate the strain from other, coagulase negative, staphylococci the presence of clumping factor and protein A (both specific for *S. aureus*) were verified. A final biochemical identification was done by VITEK or Rapidec if necessary. An antimicrobial susceptibility testing was performed with all isolates.

3.4 Cultivation and DNA-extraction of the bacterial isolates for real time PCR analysis

All samples which were subjected for nptII/nptIII prevalence analysis were received at the real time PCR testing site AGES-IMED, Vienna, as cryostocks from the collection center in Graz. The strains were thawed, directly inoculated from the cryostock into 100 µl BHI medium using an inoculation loop and incubated over night at 37°C on a shaker. The bacterial over night culture was pelleted by centrifugation at 500 rpm for 5 min at room temperature. The supernatant was removed carefully with a pipet tip avoiding to touch the surface of the pellet. Only samples with a clearly visible pellet were processed further.

The cell pellet was resuspended in 100 µl of QuickExtract™ DNA extraction solution 1.0 (Epicentre Biotechnologies, Madison, USA). The resulting suspension was vortexed vigorously and then incubated at 65°C for 7 min. After an additional incubation at 98°C for 5 min a 10^{-1} dilution in H_2O (microbiological grade; Sigma , Austria) was prepared and used as template for the PCR assays.

3.5 Screening: nptII/nptIII real time PCR TaqMan Double Assay

All real time PCR assays were implemented on the LightCycler LC480 real time PCR platform (Roche, Austria) using 96-well microtiter plates. All pipetting steps were performed manually under strict adherence to good laboratory practices (distinct sample preparation and PCR mastermix pipetting rooms, separated PCR platform and post-PCR analysis room, separate laboratory equipment and gowns in each area, routine decontamination of the equipment with 10% sodium hypochlorite, pipetting exclusively in laminar airflow hoods and daily decontamination of these hoods with 10% sodium hypochlorite and/or UV radiation).

NptII and nptIII gene targets were detected simultaneously in a single well.

Of the 10^{-1} diluted bacterial Quickextract DNA solution 1.0 (Epicentre; Madison, USA), an amount of 2 µl was transferred into 8 µl of the real time PCR TaqMan assay mix resulting in total PCR assay volume of 10 µl. PCR TaqMan Double Assays were prepared according to the recommendations of the manufacturer (Ingenetix, Vienna). For details see Table 20.

Each 96-well microtiter plate contained two negative controls (H_2O as template) and nptII and nptIII plasmid positive controls in duplicates. The PCR run was only valid if all negative controls were negative and all positive controls positive. Otherwise the run was repeated. The amplification conditions are depicted in Table 21. For PCR primer sequences see Table 22. The amplicon lengths for the nptII, nptIII and 16S specific TaqMan PCRs were 129 bp, 82 bp, and 571 bp, respectively. The position of the nptII amplicon and TaqMan probe relative to the nptII reference sequence (GenBank accession No. V00618) is depicted in Figure 11. The characteristics of the nptIII amplicon (reference sequence: V01547) can be found in Figure 12.

The DNA extraction solution from each sample was tested once with the nptII/nptIII TaqMan Double Assay and once with the 16S TaqMan DNA extraction/amplification control assay. Samples with a negative 16S TaqMan assay result (Cp > 27) were excluded from statistical analysis if the nptII and/or the nptIII PCR result was negative.

Reagent	Volume
LC480 Probemaster (Roche, Austria):	5 µl
nptII mix (Ingenetix, Austria): primer concentration: 0.6 µM probe concentration: 0.2 µM probe label : FAM	0.5 µl
nptIII mix (Ingenetix; Austria): primer concentration: 0.6 µM probe concentration: 0.2 µM probe label : 5'YYE	0.5 µl
H_2O (Sigma; molecular biology grade):	2 µl
Template:	2 µl
Total:	10 µl

Table 20. PCR TaqMan Double Screening Assay: composition

Item	Temperature	Time	Cycles
Intitial denaturation	95°C	10 min	1 x
Amplification: Denaturation Annealing and elongation	 95°C 60°C	 10 s 20 s	45 x
Cooling	40°C	continuous	1 x

Table 21. PCR TaqMan nptII/nptIII Double Screening Assay: PCR conditions (LC480 96-well microplate format)

Primer	Sequence (5' → 3')	Length
np2-F:	GAT CTC CTG TCA TCT CAC CTT GCT	24 nts
np2-R	TCG CTC GAT GCG ATG TTT C	19 nts
nptIII-F	ACA TAT CGG ATT GTC CCT ATA CGA A	25 nts
nptIII-R	TCG GCC AGA TCG TTA TTC AGT A	22 nts
16S_F	TGG AGA GTT TGA TCM TGG CTC AG	23 nts
16S_R	CTT TAC GCC CAR TRA WTC CG	20 nts

Table 22. PCR primer for TaqMan nptII/nptIII Double Screening and 16S Single Assay

Probe	Sequence (5' → 3')	Length
nptII	FAM-TCATGGCTGATGCAATGCGGC-BHQ-1	21 nts
nptIII	YYE -AGACAGCCGCTTAGCCGAATTGGATT-BHQ-1	26 nts
16S	confidential business information	

Table 23. TaqMan probe sequences

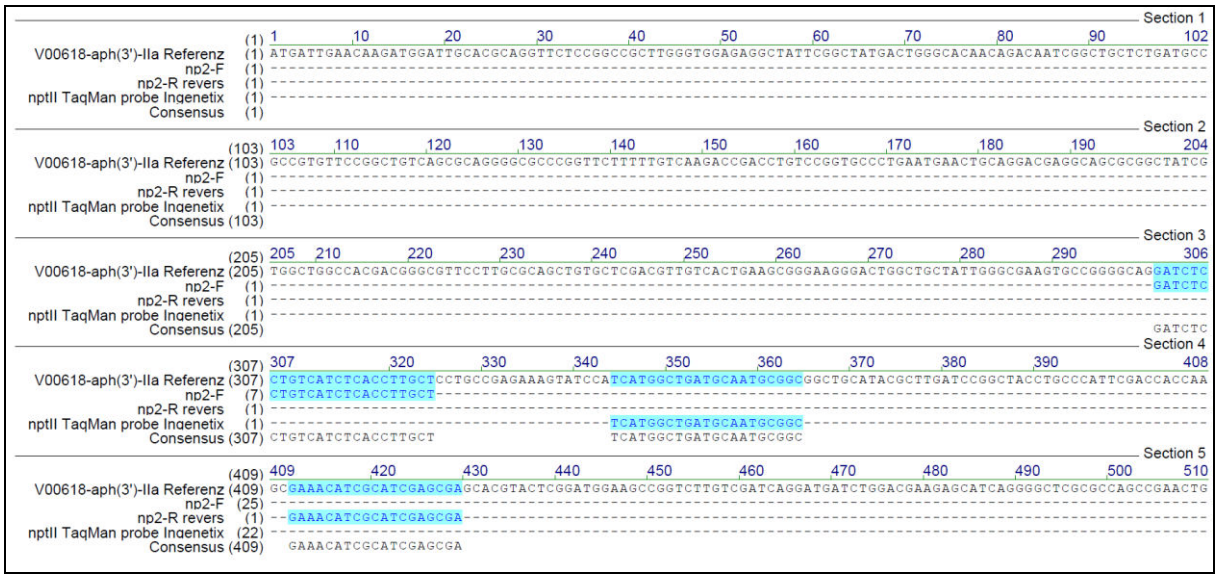

Figure 11. nptII TaqMan amplicon: primers and probe

AlignX modul, VectorNTI 7.1.

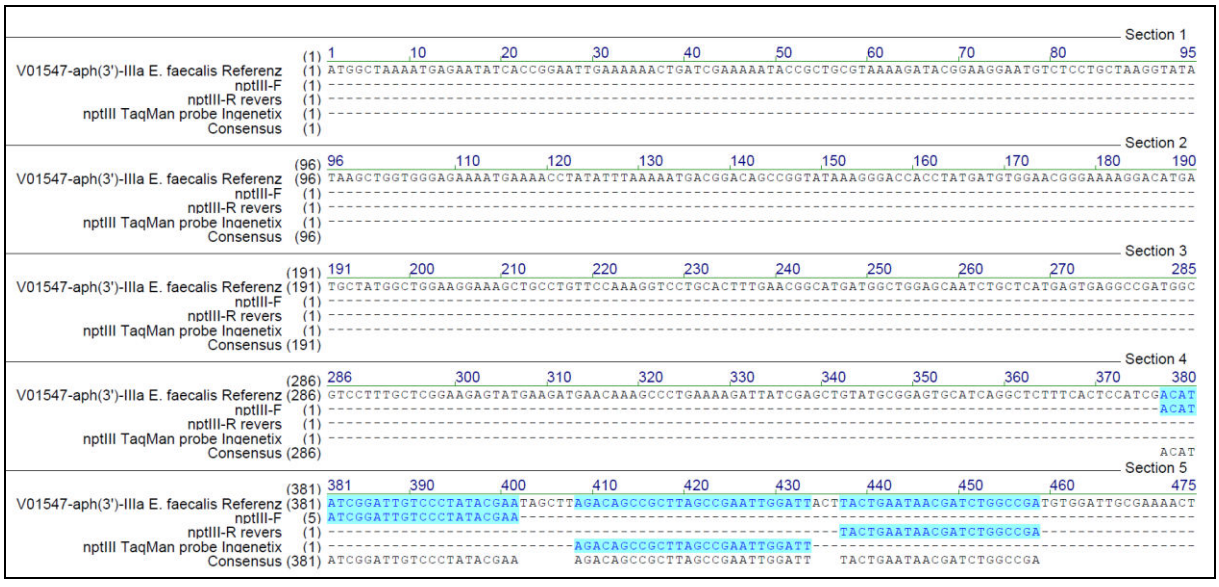

Figure 12. nptIII TaqMan amplicon: primers and probe

AlignX modul, VectorNTI 7.1.

3.6 Amplification and DNA extraction control: 16S rRNA TaqMan Single Screening Assay

Each sample was tested for amplifiable DNA and for PCR inhibition by analysing the respective Epicenter DNA extract with an “internal” control 16S TaqMan Assay.

Samples were handled as described in sections 3.4 and 3.5. 16S rRNA TaqMan PCR specific details concerning assay composition and PCR conditions are depicted in Table 24 and Table 25.

Reagent	Volume
LC480 Probemaster (Roche, Austria):	5 µl
Primer 16S (degenerated, mix) (Ingenetix, Austria): primer concentration: 0.5 µM	0.28 µl
probe concentration: 0.2 µM probe label : Cy5	0.5 µl
H_2O (Sigma, molecular biolgy grade):	2.22 µl
Template:	2 µl
Total:	10 µl

Table 24. PCR TaqMan 16S Single Assay: composition

Item	Temperature	Time	Cycles
Intitial denaturation	95°C	10 min	1 x
Amplification: Denaturation Annealing and elongation	 95°C 60°C	 10 s 1 min	45 x
Cooling	40°C	continuous	1 x

Table 25. PCR TaqMan 16S Single Assay: PCR conditions (LC480 96-well microplate format)

3.7 Validation of the PCR Assays

Data acquisition for the validation of the TaqMan PCR assays was performed as follows:

A semilogarithmic limited dilution of a pCR2.1 plasmid containing the full length nptII or nptIII or 16S rDNA of *E. coli* was prepared (750,000 – 0.38 copies/assay). The PCR measurement of each dilution step was performed in at least 8 replicates per run. A statistical analysis of at least 3 independent PCR runs resulting in at least 24 data points for each dilution step was performed. 95% detection limit was calculated by probit regression analysis. The linear quantification limit was established by the following formula: LOQ = 10* sigma/S (sigma = standard deviation of the response variables; S = slope of the calibration curve). Inter and Intra-assay variability was characterized by the calculation of the standard deviation and the coefficient of variation of each dilution step.

For details on the validation procedure see part E of the report.

TaqMan system	**95% detection limit** (copies/assay; median)	**Linear quantification limit** (copies/assay)
nptII	7.9	43
nptIII	11.4	318.1
16S	-[1)]	56.2

Table 26. Detection and linear quantification limits of the TaqMan PCRs

1) not applicable due to 16S rDNA background in PCR reagents

nptII TaqMan Assay	**Intra-assay variability**	**Inter-assay variability**
SD min	0.05	0.13
SD max	0.7	1.07
CV (%) min	0.19	0.60
CV (%) max	1.97	2.13
Measurements	216	216

Table 27. Reproducibility of the nptII TaqMan PCR system

Analysis range: 48 – 750,000 copies/assay.

nptIII TaqMan Assay	Intra-assay variability	Inter-assay variability
SD min	0.02	0.27
SD max	1.51	1.11
CV (%) min	0.10	1.34
CV (%) max	5.15	3.46
Measurements	253	253

Table 28. Reproducibility of the nptIII TaqMan PCR system

Analysis range: 48 – 750,000 copies/assay

16S TaqMan Assay	Intra-assay variability	Inter-assay variability
SD min	0.04	0.17
SD max	0.24	0.54
CV (%) min	0.16	0.58
CV (%) max	0.70	2.14
Measurements	168	168

Table 29. Reproducibility of the 16S TaqMan PCR system

Analysis range: 48 – 750,000 copies/assay

SD: standard deviation

CV: coefficient of variation

3.8 Sequencing of nptII and nptIII positive bacterial strains

As templates for sequencing the 10^{-1} dilutions of the DNA Quickextract solution of the positive samples were used. PCR Amplification was performed using HotStarTaq DNA Polymerase (Qiagen; #203205, Lot 139274539). The concentration of the primer was 0.5 µM. The final volume for PCR was 20 µl. Two microliter of template were used for each reaction. PCR was run on a DNA Engine Dyad Peltier Thermal Cycler from BioRad. PCR was performed according to the parameters as depicted in Table 30.

Item	Temperature	Time	Cycles
Initial denaturation:	95°C	5 min	1 x
Amplification: Denaturation Annealing Elongation	 95°C 53°C 72°C	 1 min 1 min 30 s 2 min	40 x
Final elongation:	72°C	10 min	1 x

Table 30. PCR conditions for the production of amplicons for sequencing

PCR products were purified by an enzymatic treatment to remove excess of primers and nucleotides. Sequencing reactions were then performed using BigDye V1.1 from Applied Biosystems/Life Technologies. Sequencing reactions were further analysed on an ABI 3730xl instrument. The whole procedure was performed at Ingenetix, Vienna.

Sequence alignment was performed with the CLUSTAL W algorithmus of the VectorNTI 7.1 software.

3.9 MIC determination of nptII/nptIII positive strains

Minimum inhibitory concentrations of nptII and nptIII positive strains for kanamycin were determined using the E-Test (Biomerieux, Marcy- l'Etoile, France).

One hundred and seventy human isolates obtained from the strain collection of the Austrian Agency for Health and Food Safety Vienna including 12 *Staphylococcus aureus*, 155 enterococci, 2 *E. coli* strains and one *Salmonella* species strain were tested for *in vitro* susceptibility to kanamycin.

The isolates were stored at -80°C in cryobank vials (Mast Diagnostics, Bootle Merseyside, UK) until testing. Isolates were recultured on Columbia blood agar plates (BioMerieux, Marcy- l'Etoile, France); all were tested for susceptibility to kanamycin by kanamycin Epsilon-test (E-test) (BioMerieux, Marcy-l'Etoile, France).

Kanamycin E-Tests (MIC range: 0.016 – 256 µg/ml) were performed according to the manufacturer's instructions (BioMerieux) using Müller Hinton agar plates (Oxoid, Basingstoke, UK; custom-made product).

We used the CLSI interpretative criteria for *Enterobacteriaceae* and *Staphylococcus aureus* for *in vitro* resistance: sensitive: MIC ≤16µg/ml; intermediate =32 µg/ml and resistant: MIC ≥64 µg/ml.

For enterococci, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) classification for high-level aminoglycoside resistance (MIC >128 mg L^{-1}) was applied (EUCAST, 2013). Enterococcal strains showing MICs <8 mg L^{-1} were classified as sensitive and as low-level resistant if the MIC was >16 mg L^{-1} and ≤128 mg L^{-1}.

3.10Peripheral Clinical Microbiology Laboratories: participants

For laboratories which contributed bacterial samples for the project see **Table 31**.

Laboratory	Reference	Province / State
Krankenhaus Barmherzige Brüder Eisenstadt	interne.sekretariat@bbeisen.at Prof. Dr. Karl Silberbauer	Burgenland
Landeskrankenhaus Klagenfurt	Institut für Labordiagnostik und Mikrobiologie evelyn.grund@kabeg.at	Kärnten
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Table 31. Prevalence of nptII/nptIII resistance genes in human pathogens: participating laboratories

1) project central collection centre and actively contributing samples

4 Appendix C: Additional Information

4.1 GMOs containing antibiotic resistance marker genes

Tag	Dossier No.	Organism	ARM gene	Reference
MON87460	EFSA/GMO/NL/2009/70	maize	nptII	(10)
PL73	EFSA/GMO/FR/2008/61	bacterial biomass	nptII*)	(10)
MON15985 x MON1445	EFSA/GMO/UK/2008/58	cotton	nptII	(10)
MON 15985 x MON 88913	EFSA-GMO-UK-2007-42	cotton	nptII	(10)
EH92-527-1	EFSA/GMO/UK/2005/14	potato	nptII	(10)
MON 1445 x MON 531	EFSA-GMO-UK-2005-09	cotton	nptII	(10)
MON863 x MON810 X NK603	EFSA/GMO/BE/2004/07	maize	nptII	(10)
MON863 x NK603	EFSA/GMO/UK/2004/06	maize	nptII	(10)
MON863 x MON810	EFSA/GMO/DE/2004/03	maize	nptII	(10)
MON863	EFSA-GMO-RX-MON863	maize	nptII	(10)
MON531	EFSA-GMO-RX-MON531	cotton	nptII	(10)
MON 1445	EFSA-GMO-RX-MON1445	cotton	nptII	(10)
FP967		lineseed	nptII	(24)
HCN92		rape seed	nptII	(6)
A5-15		sugar beet	nptII	(8)

Table 32. Genetically modified organisms carrying antibiotic resistance marker genes

*) NptII carrier status unclear. EFSA GMO panel request for proof of absence in the final product not provided by applicant as of August 2012.

4.2 Neomycin: Clinical relevance in Austria

Preparation	Active Ingredient	Marketing Authorisation Holder	Indications[1)]
Baneocin pro instillatione	Neomycin Sulfate Bacitracin (20:1)	Sandoz GmbH Kundl, Austria	instillation as lavage or as aerosol inhalation, lavage for fistula, for surgical interventions; sinusitis, otitis media, wound infections; aerosol inhalation as supplemental therapy for upper respiratory tract infections
Baneocin powder	Neomycin Sulfate Bacitracin (20:1)	Sandoz GmbH Kundl, Austria	topical applications; skin infections (small areas), prophylaxis for umbilical infections; after surgical interventions (skin)
Baneocin ointment	Neomycin Sulfate Bacitracin (20:1)	Sandoz GmbH Kundl, Austria	topical applications; focal bacterial skin infections: furunculosis, carbuncle (after surgical treatment), folliculitis barbae, folliculitis profunda, hidradenitis suppurativa, periporitis, paronychia; impetigo contagiosa, infected ulcera cruris, secondary infections of eczema
Betnesol N eye, ear and nosedrops	Betamethason Neomycin Sulfate	Defiante Farmaceutica SA Funchal, Madeira (PT)	eye: non-infected inflammatory diseases, at risk for bacterial infections ear: otitis externa and other inflammatory diseases with manifest or expected bacterial infection nose: inflammatory diseases at risk for bacterial infections
Betnovate N creme	Betamethason Neomycin Sulfate	GlaxoSmithKline Pharma GmbH, Vienna, Austria	bacterial secondary infections of the skin with pathogens sensitive to neomycin: • eczema: children (from 2 years) and adults (including atopic and discoid eczema) • prurigo nodularis • psoriasis (except for psoriasis with extended Plaques) • seborrhoic dermatitis • contact dermatitis • intertrigo analis und genitalis

Preparation	Active Ingredient	Marketing Authorisation Holder	Indications[1)]
Betnovate N ointment	Betamethason Neomycin Sulfate	GlaxoSmithKline Pharma GmbH, Vienna, Austria	see Betnovate N - creme
Hydoftal 1,5 % eye ointment	Hydrocortisone, Hydrocortisone 21- acetate, Neomycin Sulfate	AGEPHA, Söding, Austria	blepharitis (non purulent) conjuctivitis (non purulent) especially if cause by allergy keratitis without defect of the epithel (do not apply with Sjögrenschic kerato-conjunctivitis and yperite induced keratitis iritis, iridozyclitis, scleritis, episkleritis postsurgical non-contagious irritations
Hydoftal 2,5 % eye drops	Hydrocortisone Neomycin Sulfate	AGEPHA, Söding, Austria	see Hydoftal 1,5 % - eye ointment
Nebacetin pro instillatione	Neomycin Sulfate Bacitracin	Sandoz GmbH Kundl, Austria	instillations and/or lavages with head and neck diseases, surgical, urologic, dermatologic and ophthalmologic applications; inhalation of aerosols with respiratory diseases
Nebacetin powder	Neomycin Sulfate Bacitracin	Sandoz GmbH Kundl, Austria	bacterial skin and small superficial wound infections and their prophylaxis; burns, scalding; Herpes zoster and H. simplex induced blisters with bacterial infections; follow up treatment of perineal lacerations, episisotomy, mastitis after incisions, prophylaxis of mastitis an umbilical infections, bacterially induced diaper dermatitis
Nebacetin ointment	Neomycin Sulfate Bacitracin	Sandoz GmbH Kundl, Austria	topical application; focal bacterial skin infections: furunclosis, carbuncle (after surgical treatment), folliculitis barbae, folliculitis profunda, hidradenitis suppurativa, periporitis, paronychia; impetigo contagiosa, infected ulcera cruris, secondary infections of eczema
Otosporin ear drops	Neomycin, Polymyxin B, Hydrocortisone	GlaxoSmithKline Pharma GmbH, Vienna, Austria	topical applications: bacterial infections of the external auditory canal

Table 33. Available neomycin preparations in Austria

(as of: March 2010)(2, 30)

1) Product information from the manufacturer

4.3 Neomycin: Relevance for veterinary applications in Austria

Preparation	Active Ingredient	Marketing Authorisation Holder	Indications[1)]
Ani-Neopre drug premix for feed applications for pigs and calves	Neomycin Sulfate	Animed Service AG Graz, Austria	therapy and supplemental live stock treatment of bacterially induced diseases of the gastrointestinal tract: enteritis caused by *E. coli, Salmonella, Pasteurella* etc… for piglets and non-ruminating calves; oedema disease; MMA complex of breeding sows
Cloxagel forte injectors for animals	Cloxacillin Benzathin, Neomycin Sulfate	Virbac Laboratoires, Carros, France	prophylaxis and therapy of mastitis for cattle at the dry stage
Cloxagel injectors for animals	Natrium Cloxacillinat, Neomycin Sulfat	Virbac Laboratoires, Carros, France	treatment of mastitis for lactating cows
Enteran powder for animals	Colecalciferol, Neomycin Sulfat, Phthalylsulfathiazol, Retinol, Sulfadimidin, Tanninum albuminatum	aniMedica GmbH, Senden-Bösensell, Germany	calves, pigs and piglets with infectious diarrhoea caused by neomycin sensitive bacteria
Lincocin forte solution for intramammary application in cattle	Lincomycin Hydrochloride, Neomycin Sulfate	Pfizer Corporation Austria GmbH, Vienna Austria	Inflammation of the udder induced by lincomycin and neomycin sensitive pathogens; for lactating cows
Mastitar udder injector for cattle	α-Tocopherolacetat, Benzylpenicillin Kalium, Neomycin Sulfate, Procain Benzylpenicillin	Virbac Laboratoires, Carros, France	for drying of a clinically healthy udder of dairy cows taking into account the clinical condition of the udder and the resistance pattern of the herds
Neo-Mix drug premix for feed applications for animals	Neomycin Sulfate	AniMed Service AG Graz, Austria	therapy and supplemental live stock treatment of bacterially induced diseases of the gastrointestinal tract: enteritis caused by *E. coli, Salmonella, Shigella, Pasteurella, Vibrio* etc… pigs: diarrhoea, oedema disease,

Preparation	Active Ingredient	Marketing Authorisation Holder	Indications[1)]
			salmonellosis, MMA complex of sows hens and turkeys: *Vibrio* induced hepatitis, enteritis induced by *E. coli*, salmonellosis
Neo-Mix powder for feeding animals	Neomycin Sulfate	AniMed Service AG Graz, Austria	see Neo-Mix - drug premix for feed applications for animals
Neomycin-Penicillin injectable suspension for pigs and cattle	Neomycin Sulfate, Procain Benzylpenicillin	Intervet GmbH, Vienna, Austria	infectious diseases induced by multiple penicillin and neomycin sensitive bacterial pathogens (especially *E. coli*) rearing diseases of calves and piglets, coli-induced bacillosis, MMA complex of sows mastitis, endometritis of cows
Neomycinsulfat "Chevita" 70 % powder for animals	Neomycin Sulfate	Chevita Tierarzneimittel - Gesellschaft m.b.H., Wels, Austria	for the treatment of bacterially induced enteritis caused by neomycin sensitive *E. coli, Salmonella* or *Vibrio* strains
Panolog ointment for dogs and cats	Neomycin Sulfate, Nystatin, Thiostrepton, Triamcinolon Acetonid	Novartis Animal Health GmbH, Kundl, Austria	for the treatment of acute and chronic otitis externa, interdigital eczema, inflamation of anal glands; therapy of inflammatory dermatosis, dry and exsudative dermatitis in combination with bacteria or mycotic infections, eczema

Table 34. Neomycin preparations for veterinary applications available in Austria

(as of: March 2010). Information about veterinary medicinal products in Austria, Federal Ministry of Health, Austria. Published:17/04/2009.
http://www.bmgfj.gv.at/cms/site/standard.html?channel=CH0723&doc=CMS1216820062496

1) Product information from the manufacturer

4.4 Kanamycin: Veterinary applications in Austria

Preparation	Active Ingredient	Marketing Authorisation Holder	Indications[1)]
Kanamycin "Virbac" puncture bottle for animals	Kanamycin Sulfate	Virbac Laboratoires, Carros, Frankreich	contagious organ and general diseases caused by pathogens sensitive to kanamycin; for cattle, sheep, pigs, poultry
Ubrolexin suspension for intramammary application with lactating cows	Cefalexin, Kanamycin Sulfate	Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Deutschland	for the treatment of clinically apparent mastitis caused by pathogens sensitive to kanamycin and cephalexin
Vanakan 10% injection solution for animals	Kanamycin Disulfate	Vana GmbH, Vienna, Austria	therapy and prophylaxis of infectious diseases caused by pathogens sensitive to kanamycin for cattle, sheep, pigs, poultry

Table 35. Kanamycin preparations for veterinary applications in Austria

(as of: March 2010).

1) see Table 34

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Frequency of Environmental Antibiotic Resistance

Part B:

NptII and nptIII Prevalence in Soil

Final Report

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1 Prevalence of nptII and nptIII in the soil of maize and potato fields in Austria

1.1 Summary

Some of the commercialized genetically modified plants carry antibiotic resistance marker (ARM) genes which may constitute a source for resistance determinants in soil or gut bacteria. Although horizontal transfer of intact ARM genes from transgenic plants to prokaryotes has been observed only under optimized laboratory conditions so far, ARM gene transfer from plant to bacteria in natural habitats cannot be excluded entirely, introducing an option for interference with animal and human antimicrobial chemotherapy.

The neomycin phosphotransferase II gene – one of the most abundantly used ARM gene in plant gene technology - is present in the transgenic maize variety MON863 and the potato line EH92-527-1. MON863 is approved in the European Union for food and feed use but also for cultivation. The nptII antibiotic resistance marker gene was in the focus of several risk assessment attempts by EFSA and other working groups, all concluding that the use of nptII as a selection marker in plants did not pose a risk to the environment or to human and animal health. None of these evaluations considered it necessary to back up their conclusions with quantitative information on the prevalence of nptII in natural habitats. However, data on the prevalence, diversity and ecology of ARM gene homologues in naturally occurring bacterial populations in soils are prerequisites for the evaluation of the consequences of a possible transfer of plant derived antibiotic resistance marker genes to bacterial soil communities.

Within the framework of the present project we provide quantitative information on the naturally occurring background load of nptII and nptIII resistance gene homologues in agricultural soils in Austria. These data may be used as nptII and nptIII copy number baseline in habitats not yet exposed to transgenic plant DNA. In cultivation-dependent and cultivation-independent metagenomic approaches the prevalence of nptII and nptIII in maize and potato fields - according to potentially cultivable transgenic plant lines – were representatively obtained from the respective Austrian agricultural regions. The nptIII ARM gene was included as comparator in the present project. Extensive data packages for each of 100 fields comprising a total of 1000 single soil extractions were compiled. To our knowledge this is the most comprehensive attempt for quantification of resistance genes in agricultural soils to date.

NptII and nptIII in total soil DNA

Composite soil samples from 50 maize and 50 potato fields consisting of 10 single soil extractions per field were collected shortly before harvest (6.8. - 9.9.2011) and analysed by TaqMan real time PCR for the presence of nptII and nptIII genes in the total DNA extracted from these soils.

From these 100 composite soil samples 6% tested positive for the presence of nptII genes (95% confidence interval [2.2%; 12.6%]). 85% of these soil samples were positive for nptIII (95% confidence interval [76.5%; 91.4%]). Taking into consideration the upper 95% confidence level for nptII 12.6% of all maize and potato fields in Austria could harbour nptII. But up to 91.4% may contain nptIII resistance genes above the detection limit. A subsample analysis showed that 6% of the maize fields were nptII positive ([1.3%; 16.6%] but 92% of them carried nptIII genes ([80.8%; 97.8%]). The

situation was similar with soil samples from potato fields where 6% ([1.3%; 16.6%]) of them were nptII and 78% ([64.0%; 88.5%]) were nptIII positive.

Using quantitative TaqMan real time PCR the determination of the nptII background load of the six nptII positive fields resulted in a mean concentration of approx. 340 nptII copies per gram soil. The field with the lowest nptII concentration showed approx. 31 copies per gram soil and was cultivated with potatoes. The maximum nptII concentration observed during the project was approx. 850 copies per gram soil, found in a maize field.

A study published in 2011 by Ma et al. in Canada attained similar results regarding the low prevalence and the low copy number of nptII in agricultural soils.

The nptIII background load of the analysed fields was distinctly higher. A mean concentration of approx. 4750 nptIII copies per gram soil (range: 13 – 61600) was encountered in nptIII positive agricultural fields. The maximum nptIII concentration was observed in a maize field.

Soil parameters routinely used for characterization of soil samples (pH, phosphor, potassium, humus, total organic carbon, nitrogen; particle size, soil type, humidity) were determined for all samples. Additionally, the following information was collected for each location during soil sampling: crop, preceding crop, organic fertilizer of animal origin, inorganic fertilizer, soil humidity at time of collection, weather conditions, and ambient temperature. In a multivariate analysis a significant correlation between nptIII prevalence and the usage of “organic fertilizer of animal origin” was detected. No other correlations concerning nptII or nptIII prevalence or quantities with the tested parameters could be revealed.

16S rRNA gene copy number in total soil DNA

The copy number of 16S rRNA genes was obtained from total soil DNA extracts of all tested fields by quantitative TaqMan real time PCR. It was used as surrogate marker for the total cell number per gram soil, for normalisation of the nptII and nptIII quantitative results, and as amplification inhibition control in the screening assays. The mean concentration of 16S rRNA genes in the 50 maize fields was 1.74×10^9 copies per g soil (range: $3.04 \times 10^8 – 5.48 \times 10^9$). A mean copy number of 1.28×10^9 per gram soil was retrieved from the soils of the 50 potato fields (range: $5.88 \times 10^7 – 3.52 \times 10^9$). For all maize and potato fields analysed a mean copy number of 1.51×10^9 per g soil (range: $5.88 \times 10^7 – 5.48 \times 10^9$) was obtained. The 16S rRNA gene copy number is a surrogate marker for the approximate number of bacterial cells in 1 g of the soil sample under investigation. The results indicate a good correlation with the cultivation approach (see below) and with expected values (approx. 10^9 cells/g soil; 1% of them cultivable).

Cultivation of kanamycin resistant bacterial strains

Ten reference soil samples representing major soil types in the study (maize and potato growing regions, five of each) were analysed in detail as to cultivation and taxonomic characterization of kanamycin resistant strains, the acquisition of additional soil parameters and bacterial biodiversity. Bacterial strains from these maize and potato reference fields were grown on nutrient (full medium) and R2A (minimal medium) agar supplemented with or without kanamycin. From all maize and potato reference soils analysed approx. 1.65×10^7 cells per g soil were recovered on complete medium (minimal medium: 2.16×10^7) with 8.29% of them being resistant to kanamycin (range: 0.47 – 19.12%). On minimal medium approx. 5.78% were resistant (range: 0.14 – 16%). From 1 g soil of the tested reference maize fields on average 2.3×10^7 cells (range: $5.65 \times 10^6 – 6.05 \times 10^7$) grew on complete medium. Approximately 11% of this population showed a high level resistance to 100 µg/ml kanamycin (range: 0.54 – 19.12%). From 3×10^7 cells recovered on minimal medium 6.22% on

average were resistant (range: 0.34 – 16%). Approx. 1 x 10^7 cells from 1 g potato soil could be cultivated on complete medium; 5,78 % of them on average were resistant to kanamycin (range: 0.47 – 9.51%). A comparable situation was encountered on minimal medium with 5.42% of the total cultivable bacterial population being resistant (range: 0.14 – 10.94%). Similar kanamycin resistance rates of soil bacteria are described in literature.

Kanamycin resistant strains were taxonomically classified via 16S rDNA sequencing. In a few fields, some strains with a potential to cause human disease (e.g. *Klebsiella pneumoniae, Stenotrophomonas maltophilia*) were detected. No strains at the species level known to be capable of developing competence for DNA uptake could be identified so far.

NptII and nptIII in kanamycin resistant bacterial strains

396 bacterial isolates resistant to kanamycin were analysed for nptII and nptIII in real time TaqMan PCR assays. NptII could not be detected in the tested sample collection (95% confidence interval: [0%; 0.8%]). The prevalence of nptIII positive strains was 1.8% on average ([0%; 3.3%]). In the subsample analysis, at a significance level of 0.05, a maximum of 1.5% of the strains isolated from soils with maize cultivation ([0%; 3.9%]) and 2% from potato soils ([0%; 4.6%]) were nptIII positive. The prevalence of nptIII was again higher than that of nptII supporting the data retrieved in other parts of the project. The baseline frequency of nptII genes appears to be low in bacterial populations obtained from Austrian agricultural soils used for potato and maize cultivation. Moreover, the results indicated that the resistance phenotype of the cultivable fraction of soil bacteria insensitive to kanamycin did not rely predominantly on the presence of nptII and nptIII resistance genes.

Bacterial biodiversity in reference soils

The bacterial biodiversity was assessed in the ten reference fields using pyrosequencing of the 16S soil metagenome. Between approx. 31 000 and 49 000 sequences per field could be retrieved and classified into 11 to 13 phyla. Approx. 30% of the sequences could not be classified at the phylum level. The ten soil samples showed a high correlation with respect to the frequency of bacteria at the phylum level. Between 194 and 268 genera could be identified per field. All genera detected during the cultivation approach could be re-detected in the metagenomic assays. Phylogenetic analysis showed the formation of two soil clusters on the genus level, however, according to the analysis of several diversity indexes (Shannon, Shannon evenness, richness) the bacterial populations from the tested soils appear to be similar.

Conclusions

The naturally occurring background load of nptII resistance genes in agricultural habitats used for the cultivation of maize and potatoes in Austria appears to be low. The obtained quantitative results indicate that the resistance gene pools of the maize and potato fields under investigation are not saturated with nptII gene copies. The baseline frequency of nptII in these natural habitats is distinctly lower compared to the prevalence of nptIII in the same habitats. NptII could not be detected in bacterial strains isolated from ten reference soils supporting also the observation of a low prevalence of nptII in agricultural environments used for the cultivation of the crops mentioned above. In summary the abundance of nptII appears to be low in the resistance gene pools under investigation. It is likely that an anthropogenic input of exogenous DNA carrying aminoglycoside phosphotransferase (3')-IIa gene homologues is of relevance: A long-term and constant exposure of these ecosystems with exogenous nptII carrying DNA via root exsudates or plant cell decay might be capable of elevating the abundance of this resistance determinant in the relevant environments.

The naturally occurring background load of nptIII in maize and potato growing fields is usually higher compared to the situation with nptII reaching peak concentrations in some maize fields of up to 62 000 copies per gram soil. But the nptIII copy number is still several orders of magnitude lower compared to the abundance of e.g. tetracycline resistance determinants in pig manure (10^7 – 10^9 copies per g) used for soil fertilization as could be shown by Hölzel et al. in 2012. Tetracycline resistance genes are usually encountered in substantially higher concentrations in soils from fields which were fertilized with organic manure. In the present setting statistical evaluation showed a significant correlation between nptIII prevalence and the application of organic fertilizer of animal origin in Austrian maize and potato fields. Out of 396 bacterial isolates cultivated from the soils of ten reference fields 7 isolates appeared to be carriers of nptIII. This observation also supports the conclusion that nptIII is more prevalent in naturally occurring soil bacterial populations of agricultural origin compared to the situation with nptII. An additional input of exogenous DNA carrying aminoglycoside phosphotransferase (3')-IIIa genes into the analysed ecosystems would probably be of less significance than an additional input of nptII into the same habitats. We would like to point to the fact that the presented results were a snapshot view of the resistance gene status of the analysed fields. The data were obtained from a soil extraction performed once at a defined point of time (shortly before harvest) per field. Soil extractions performed at different times points during the growth season of the crops under investigation may result in different antibiotic resistance gene abundances because antibiotic resistance is a dynamic phenomenon and may change over time according to the selection pressure in the respective environment.

1.2 Zusammenfassung

Einige transgene Pflanzen, die für den kommerziellen Gebrauch zugelassen sind, enthalten Antibiotikaresistenzmarkergene, auf die Boden- oder Darmbakterien zugreifen können. Obwohl der horizontale Transfer von ARM Genen aus transgenen Pflanzen auf Prokaryoten bisher nur unter optimierten Laborbedingungen nachgewiesen wurde, kann man diesen Vorgang in natürlichen Habitaten nicht a priori völlig ausschließen. Eine derartige Übertragung von Resistenzgenen, die entweder direkt oder über Zwischenwirte in humanpathogenen Keimen landen, würde jedoch unter Umständen negative Auswirkungen auf eine antimikrobielle Chemotherapie im Erkrankungsfall bei Mensch und Tier haben.

Das Neomycinphosphotransferase II Gen - jenes Antibiotikaresistenzmarkergen, das mit Abstand am häufigsten in der Pflanzengentechnologie eingesetzt wird – ist auch in der gentechnisch veränderten Maislinie MON863 und in der Kartoffelsorte EH92-527-1 vorhanden. Beide transgene Pflanzen sind sowohl als Lebens-und Futtermittel als auch für den Anbau in der Europäischen Union zugelassen. Das nptII ARM Gen wurde bereits mehrmals von der EFSA und anderen Fachgremien auf Sicherheitsrisiken für die Gesundheit von Mensch und Tier evaluiert, wobei alle zu dem einheitlichen Schluss gelangen, das dieses Resistenzgen in transgenen Pflanzen keine negativen Auswirkungen auf Mensch, Tier und Umwelt hat. Keiner der Beteiligten hat es jedoch für notwendig erachtet, quantitative Daten zur Prävalenz von nptII in natürlichen Habitaten d.h. jenen Ökosystemen, die mit exogenen nptII Kopien exponiert werden, zur Unterstützung der gemachten Schlussfolgerungen heranzuziehen. Dies, obwohl Daten zur Prävalenz, Diversität und der Ökologie von zu diesen Antibiotikaresistenzen homologen Genen in natürlich im Boden vorkommenden Bakterienpopulationen eine Voraussetzung dafür sind, die Auswirkungen eines potentiell möglichen Transfers von ARM Genen in Bodenbakterien richtig einzuschätzen.

Im gegenständlichen Projekt liefern wir quantitative Daten zur nptII und nptIII Hintergrundbelastung von landwirtschaftlich genutzten Flächen in Österreich. Diese Informationen können als „Baseline" für die Häufigkeit des Vorkommens dieser Gene in Habitaten, die bisher noch keine Exposition mit transgener Pflanzen-DNA erfahren haben, herangezogen werden. Mit kultivierungsabhängigen und –unabhängigen metagenomischen Ansätzen wird die Prävalenz von nptII und nptIII Genen in Mais-

und Kartoffelfeldern – entsprechend der potentiell in Österreich anbaubaren und bereits von der EU Kommission zugelassenen transgenen Pflanzensorten – ermittelt. Die Testfelder wurden repräsentativ aus den österreichischen Anbauregionen ausgewählt. Das nptIII Gen wurde als Komparator, und weil es bei zahlreichen Freisetzungsexperimenten als ARM Gen verwendet wurde, in die Studie mit aufgenommen. Umfangreiche Datenpakete wurden zu jedem der hundert getesteten Felder – was insgesamt einer Menge von 1000 einzelnen Bodenextraktionen entspricht – kompiliert. Unserem Wissen nach handelt es sich um die zurzeit umfangreichste Studie im Bereich landwirtschaftlicher Böden.

NptII und nptIII in Gesamt-DNA Extrakten aus Böden

50 Bodenmischproben von Mais- und 50 Bodenmischproben von Kartoffelfeldern wurden kurz vor der Ernte im Sommer 2011 (6.8. – 9.9.2011) gesammelt und auf die Präsenz von nptII und nptIII Genen in der aus der Bodenmischprobe gewonnenen Gesamt-DNA mittels TaqMan Real Time PCR untersucht. 1 Bodenmischprobe setzte sich dabei aus 10 Teilproben pro Feld zusammen. Dem Projekt stand somit Bodenmaterial aus insgesamt 1000 verschiedenen Entnahmestellen zur Verfügung.

Von diesen 100 Bodenmischproben waren sechs nptII positiv (95% Konfidenzintervall: [2,2%; 12,6%]). NptIII war in 85% der Testfelder nachweisbar (95% Konfidenzintervall: [76,5%; 91,4%]). Das bedeutet, dass mit 95%iger Sicherheit weniger als 12,6% aller Mais- und Kartoffelfelder in Österreich eine über dem Detektionslimit liegende nptII Kopienanzahl aufweisen, aber man mit maximal bis zu 91, 4% nptIII positiven Felder rechnen muss. Eine Teilanalyse zeigte, dass 6% der Maisfelder nptII (95% Konfidenzintervall: [1,3%; 16,6%], aber 92% von ihnen nptIII positiv (95% Konfidenzintervall: [80,8%; 97,8%]) waren. Eine ähnliche Situation bot sich für die untersuchten Kartoffelfelder: 6% waren nptII positiv (95% Konfidenzintervall: [1,3%; 16,6%)], aber 78% trugen nptIII Gene (95% Konfidenzintervall: [64,0%; 88,5%]).

Mittels quantitativer Real Time TaqMan PCR konnte eine mittlere Belastung der getesteten Böden von ca. 340 nptII Kopien pro Gramm Boden ermittelt werden. Die minimalste nptII Belastung von ca. 31 Kopien/g Boden wurde in einem Kartoffelfeld festgestellt, die höchste im Projekt gemessene nptII Kopienanzahl war 850 Gene/g Boden aus einem Maisfeld.

Die Hintergrundbelastung mit nptIII Genen war deutlich höher. Eine mittlere Konzentration von ca. 4750 Kopien pro Gramm Boden (Bereich: 13 – 61 600 Kopien/g Boden) wurde über alle getesteten Felder ermittelt. Die höchste nptIII Konzentration war wieder in einem Maisfeld zu beobachten.

Eine im Jahr 2011 publizierte Studie von Ma et al. in Kanada kommt hinsichtlich der niedrigen Prävalenz und der geringen Kopienanzahl von nptII in landwirtschaftlich genutzten Anbauflächen zu einem ähnlichen Ergebnis.

Bodenparameter die routinemäßig zur Charakterisierung von Bodenproben herangezogen werden wie pH-Wert, Phosphor-, Kalium-, gesamter organischer Kohlenstoff-, und Stickstoffgehalt, sowie Partikelgröße, Bodentyp und Humidität wurden bei allen Bodenproben erhoben. Zusätzlich wurden folgende Informationen für jede Bodenprobeentnahmestelle gesammelt: Feldfrucht, Vorfrucht, organische Düngung tierischen Ursprungs, anorganische Düngung, Bodenfeuchtigkeit, Wetter, Lufttemperatur. In einer Multivarianzanalyse konnte eine signifikante Korrelation zwischen der Prävalenz von nptIII und dem Parameter „organische Düngung tierischen Ursprungs“ ermittelt werden. Sonst gab es keine signifikanten Korrelationen zwischen den gesammelten Bodenparametern und nptII/nptIII Prävalenz bzw. quantitativer Belastung des untersuchten Habitats mit diesen Resistenzgenen.

16S rRNA Genkopienanzahl in der Gesamt-DNA aus Böden

Die Anzahl der 16S rRNA Genkopien wurde ebenfalls in allen Gesamt-DNA-Extrakten aus den Bodenmischproben mittels quantitativer Real Time PCR ermittelt. Die Zahlenwerte dienten einerseits als Surrogat-Marker für die Gesamtzellanzahl pro Gramm Boden, andererseits wurden sie zur Normalisierung der nptII und nptIII Genkopienanzahl herangezogen, um einen quantitativen Vergleich zwischen den einzelnen Bodenproben zu ermöglichen. Die mittlere Anzahl von 16S rRNA Genen in den 50 Maisfeldern war 1,74 x 10^9 pro Gramm Boden (Bereich: 3,04 x 10^8 – 5,48 x 10^9), in den Kartoffelfeldern 1,28 x 10^9 (Bereich: 5,88 x 10^7 – 3,52 x 10^9). Über alle 100 Testfelder gemittelt wurden 1,51 x10^9 16S rRNA Genkopien/g Boden (Bereich: 5,88 x 10^7 - 5,48 x 10^9) ermittelt. Diese Ergebnisse korrelieren gut mit den Kultivierungsdaten (siehe unten) und mit den Erwartungswerten (ca. 10^9 Zellen pro Gramm Boden; 1% davon kultivierbar).

Kultivierung von Kanamycin resistenten Bakterienstämmen

Zehn Referenzbodenmischproben (jeweils fünf von Mais und fünf von Kartoffelfeldern mit unterschiedlichen Bodentypen bzw. pH-Werten) wurden im Detail (Kultivierung und taxonomische Charakterisierung von Kanamycin resistenten Stämmen, Ermittlung zusätzlicher Bodenparameter, bakterielle Biodiversitätsuntersuchung) analysiert.

Insgesamt betrachtet konnten von allen Böden im Mittel 1,65 x 10^7 Zellen pro Gramm Boden kultiviert werden (Minimalmedium: 2,16 x 10^7), wobei im Mittel 8,29% gegen Kanamycin resistent waren (Bereich: 0,47 – 19,12%). Auf Minimalmedium waren durchschnittlich 5,78% resistent (Bereich: (0,14 – 16%). Aus 1 g Boden von Maisfeldern konnte eine mittlere Anzahl von 2,3 x 10^7 Zellen (Bereich: 5,65 x 10^6 – 6,05 x 10^7) auf Vollmedium angezüchtet werden. Ungefähr 11% dieser Population war im Mittel resistent gegen 100 µg/ml Kanamycin (Bereich: 0,54 – 19,12%). Auf Minimalmedium wuchsen 3 x 10^7 Zellen pro Gramm Boden, von denen im Durchschnitt 6,22% resistent waren (Bereich: 0,34 – 16%). Aus 1 g Erde aus Kartoffelfeldern konnten ca. 1 x 10^7 Zellen auf Vollmedium angezüchtet werden. Im Mittel waren 5,78% davon Kanamycin resistent (Bereich: 0,47 – 9,51%). Eine ähnliche Situation war auf Minimalmedium anzutreffen, wo durchschnittlich 5,42% Resistenz zeigten (Bereich: 0,14 – 10,94%). Ähnliche Kanamycin Resistenzraten werden auch in der relevanten Literatur beschrieben.

Die Kanamycin resistenten Stämme wurden via 16S rRNA Gensequenzierung taxonomisch charakterisiert. In einigen Feldern konnten humanpathogene Stämme (z.B. *Klebsiella pneumoniae, Stenotrophomonas maltophilia*) identifiziert werden. Bakterienarten, die bekannt dafür sind, leicht exogene DNA aufzunehmen, konnten auf Species-Ebene nicht entdeckt werden.

NptII und nptIII in Kanamycin resistenten Bakterienstämmen

396 Kanamycin resistente Bakterienisolate wurden mittels nptII und nptIII TaqMan Real Time PCR auf die Präsenz dieser Resistenzgene getestet. Keines dieser Isolate trug nptII (95% Konfidenzintervall: [0%; 0,8%]). Sieben dieser Isolate waren positiv für nptIII was einer mittleren Trägerrate von 1,8% entspricht (95% Konfidenzintervall: [0%; 3,3%]). Die Untergruppenanalyse deutet daraufhin, dass man mit einer Sicherheit von 95% davon ausgehen kann, dass durchschnittlich 1,5% der Isolate die aus Maisfeldern gewonnen wurden nptIII positiv waren (95% Konfidenzintervall: [0%; 3,9%]). Durchschnittlich 2% der Isolate aus Kartoffelfeldern waren nptIII positiv (95% Konfidenzintervall: [0%; 4,6%]). Die Prävalenz von nptIII in Bakterienisolaten war erneut höher im Vergleich zur Häufigkeit von nptII, was die Beobachtungen aus den anderen Teilen des Projekts unterstützt. Die Prävalenz von nptII in Bakterienpopulation aus Böden auf denen Mais oder Kartoffel in Österreich angebaut werden ist niedrig. Insgesamt deuten die Ergebnisse darauf hin, dass die phänotypisch beobachtete

Kanamycinresistenz in den Bakterien aus den analysierten Habitaten nicht vorwiegend auf dem Vorhandensein der untersuchten Aminoglycosidphosphotransferasegene beruht.

Bakterielle Biodiversität in Referenzböden

Eine bakterielle Biodiversitätsanalyse wurde für die zehn Referenzfelder mittels Pyrosequenzierung des 16S rRNA Metagenoms durchgeführt. Zwischen 31 000 und 49 000 Sequenzen konnten pro Feld erhalten und 11 – 13 Phyla zugeordnet werden. Ca. 30% der Sequenzen konnten auf Phylum-Ebene nicht ausgewertet werden. Überdies konnten zwischen 194 und 268 Genera pro Feld identifiziert werden. Alle Genera aus dem Kultivierungsversuch konnten auch in dieser Metagenomanalyse wiedergefunden werden. Bei der phylogenetischen Stammbaumanalyse zeigte sich die Entwicklung von zwei Bodenclustern auf Genus-Ebene, insgesamt weisen jedoch die Ergebnisse der ermittelten Biodiversitätsindexe (Shannon-Weaver, Shannon Evenness, Richness Index) darauf hin, dass die Zusammensetzung der untersuchten Bakterienpopulationen aus den zehn Referenzfeldern doch sehr ähnlich sein dürfte. Unserem Wissen nach wurde eine derartig umfangreiche mikrobiologische Biodiversitätsanalyse im Bereich unterschiedlicher Ackerböden erstmals durchgeführt.

Schlussfolgerungen

Die natürliche Hintergrundbelastung mit nptII Resistenzgenen in den Ackerböden, die in Österreich für den Anbau von Mais und Kartoffeln verwendet werden, scheint niedrig zu sein. Die ermittelten quantitativen Ergebnisse deuten darauf hin, dass die Resistenzgenpools von Bodenbakterienpopulationen in Mais- und Kartoffelfeldern nicht mit nptII Genkopien gesättigt sind. Die „Baseline“ für die nptII Häufigkeit in diesen Habitaten ist deutlich niedriger im Vergleich zur Situation mit nptIII. NptII konnte in Kanamycin resistenten Bakterien, die aus den 10 Referenzfeldern isoliert worden waren, überhaupt nicht detektiert werden. Dies unterstützt ebenfalls die in anderen Teilen des Projekts gemachte Beobachtung einer niedrigen Verbreitung von nptII in natürlich vorkommenden Bakterienpopulationen aus Ackerböden mit Mais und Kartoffelanbau. Insgesamt betrachtet bedeutet das, dass die Häufigkeit von nptII in den untersuchten Resistenzgenpools niedrig ist. Es ist daher nicht unwahrscheinlich, dass eine künstliche Einbringung von nptII von Relevanz ist: Eine permanente Exposition dieser Ökosysteme mit freier nptII codierender DNA über Wurzelexkretion oder während des Zerfalls von pflanzlichem Material könnte durchaus zu einer Veränderung in der Häufigkeit dieses Resistenzgens in den betroffenen Lebensräumen führen.

Die Häufigkeit von nptIII in den untersuchten Habitaten ist durchwegs höher, die natürliche Hintergrundbelastung erreicht in manchen Maisfeldern eine Kopienanzahl von bis zu 62 000 pro Gramm Boden. Trotzdem ist diese Kopienanzahl immer noch um einige Größenordnungen niedriger als bei anderen Resistenzgenen. Hölzel et al. konnten zum Beispiel 2012 zeigen, dass bis zu 10^7 - 10^9 Tetrazyklinresistenzgenkopien pro Gramm in organischem Dünger tierischen Ursprungs, der zur Bodenfertilisierung aufgebracht wird, vorkommen. Im vorliegenden Versuchssetting zeigte eine Multivarianzanalyse eine signifikante Korrelation zwischen Prävalenz von nptIII und der Düngung von Böden mit Gülle oder Stallmist. 7 Kanamycin resistente Isolate von 396 erwiesen sich als Träger von nptIII. Auch diese Beobachtung unterstützt das Ergebnis einer höheren Verbreitungshäufigkeit von nptIII in natürlich vorkommenden Bakterienpopulationen aus Mais und Kartoffelfeldern. Ein zusätzlicher Eintrag von Aminoglycosidphosphotransferase (3‘)-IIIa Genen in diese dürfte weniger Auswirkungen haben als bei nptII. Wir möchten darauf hinweisen, dass es sich bei den präsentierten Ergebnissen um eine Momentaufnahme bezüglich der Resistenzgenbelastung der untersuchten Felder handelt. Die Bodenproben wurden ein einziges Mal zu einem definierten Zeitpunkt (kurz vor der Ernte) während der Anbauphase der Feldfrüchte entnommen. Da es sich bei der Antibiotikaresistenzentwicklung um ein äußerst dynamisches Phänomen handelt, das vom jeweils herrschenden Selektionsdruck im untersuchten Habitat abhängt, ist es möglich, dass bei zu anderen

Zeitpunkten durchgeführten Probenentnahmen andere nptII und nptIII Resistenzgenhäufigkeiten beobachtet werden können.

1.3 Aims of the project

The objectives of the present project were:

1. Determination of the frequency of nptII and nptIII gene copies in relevant soil habitats before exposure of these habitats to exogenous plant derived ARM gene containing DNA. The naturally occurring background load with nptII and nptIII resistance genes in certain soil bacterial populations should be established.

2. The obtained data serve as reference baseline for comparison if the impact of cultivation of ARM gene containing transgenic plants on resistance gene frequencies in these natural habitats will have to be evaluated in the future.

3. The approach should provide quantitative information about gene copy numbers to allow subsequently a quantitative comparison between nptII and nptIII gene copy numbers already residing in the soil bacterial community and the number of nptII ARM gene copies which might be introduced into the soil antibiotic resistance gene pool via transgenic plant material decay or active secretion.

4. Soil samples should be extracted from relevant test sites – in the present case from maize and potato growing regions in Austria corresponding to ARM gene carrying transgenic maize and potato varieties with approval for marketing and cultivation in the European Union – in a representative fashion.

5. Soil samples should be analysed by classical cultivation methods for characterization of residing bacterial species. Their total cultivable cell number, the phenotypic kanamycin resistance ratios, and those bacterial species which would be preferred bacterial hosts for nptII and nptIII genes should be determined.

6. Microbiological biodiversity in ten reference fields should be established by a metagenomic approach to get an overview about the distribution of potential DNA acceptor strains in the analysed maize and potato agricultural soil bacteria populations in Austria. This approach would also provide insight whether and to which extent human pathogens reside in these habitats.

1.4 Introduction

Some of the commercialized genetically modified plants carry antibiotic resistance marker (ARM) genes which may constitute a source for resistance determinants in soil or gut bacteria. On a global scale the cultivation of transgenic crops has rapidly increased over the past 15 years (28), which increases the potential for contacts between transgenic DNA and bacteria capable for uptake of free DNA (44).

The neomycin phosphotransferase II gene is the most abundantly used ARM gene in plant gene technology and also maize MON863 and the potato variety EH92-527-1 – both approved for cultivation in the European Union - are carriers of nptII (14, 40, 43).

Facing a global crisis in the therapy of infectious diseases caused by antibiotic resistant bacteria (20, 24) the superfluous presence of the antibiotic resistance gene nptII in each plant cell of the transgenic crops has led to concerns (30, 46) because a horizontal transfer of the plant derived ARM genes to soil or gut bacteria cannot be excluded a priori (13). A potential uptake of plant derived resistance genes by human pathogens which subsequently may interfere with antimicrobial therapy has to be evaluated (23) and the overall impact of horizontal gene transfer from transgenic plants to soil or gut bacteria have to be taken carefully into consideration for risk assessment of antibiotic resistance marker genes (25, 45).

The EFSA GMO Panel released an Opinion on the use of antibiotic resistance genes in GM plants in 2004 providing a 3-tiered classification scheme for antibiotic resistance genes (category 1: no risk for human and animal health and the environment; category 2: should only be used for authorized field trials; category 3: should not be used in transgenic crops in general). The Panel concluded that the use of nptII as a selection marker did not pose a risk to the environment or to human and animal health (15). EFSA provided two additional statements including a “Joint Scientific Opinion of the GMO and BIOHAZ Panels“ on the use of ARM genes in GM plants in 2009, which resulted in the same conclusions (13, 16). Two Members of the BIOHAZ Panel expressed a critical minority opinion. Additionally this Joint Opinion pointed to limitations related among others to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source and stressed the importance of taking those and other uncertainties described in that Opinion into account (13). Two notable reviews on the risk assessment of antibiotic resistance marker genes also concluded on the innocuousness of nptII (5, 23). None of these evaluations considered it necessary to back up their conclusions with quantitative information on the prevalence of nptII in natural habitats. However, data on the prevalence, diversity and the ecology of antibiotic resistance genes in naturally occurring bacterial populations in soils are a prerequisite for evaluation of the consequences of a possible transfer of plant derived antibiotic resistance marker genes to bacterial soil communities (12).

It was the intention to reduce this knowledge gap and diminish uncertainties by providing gene copy numbers for nptII and nptIII in Austrian agricultural soils.

1.4.1 Plant derived ARM genes and soil bacteria

Factors positively contributing to a potential ARM gene containing plant DNA transfer to environmental bacteria are i) the long-term persistence of DNA in soil (32, 33), ii) the presence of competent bacterial cells in the receiving habitat (55), iii) the heterogeneous soil structure favouring contact between bacterial cells and free DNA (9), and iv) the prokaryotic origin of the relevant ARM genes facilitating homologous recombination with bacterial genomes during natural genetic transformation (23). On fields under continuous cultivation of genetically modified crops transgenic

DNA in soil samples was detectable throughout the year (32, 33). Plant DNA is released by plant decay or root exudates (6, 11, 39, 66) and is usually rapidly degraded in soil environments by biotic and abiotic factors (34). However, long-term persistence was reported if adsorbed to soil particles (8, 36, 47, 52, 66). Free DNA associated on mineral surfaces is protected against DNase-mediated degradation (47, 66) and appears to maintain its biological active status for transformation (9).

Plant derived ARM gene uptake by soil bacteria was established in soil microcosm and green house studies if homologous recombination with the recipient genome was possible (13). However, such transfer events remain to be undetected under field conditions (4, 12). There are many reasons why gene transfer by genetic transformation under naturally occurring environmental conditions may be impeded (60), but there may also be technical limitations in experimental study designs, insufficient detection limits of the applied procedures in combination with an extremely low gene transfer frequency in natural habitats which renders HGT from plant to bacteria undetectable (25, 45).

1.4.2 Resistance to kanamycin/neomycin in soil

Phenotypic resistance to kanamycin is common in soil bacterial communities. It was reported that up to 10^5 bacteria per gram soil were resistant to kanamycin (56). Gebhard et al. provided evidence for high-level resistance in 0.6 – 5.2% and for low-level resistance to kanamycin in 4 – 30% of the total analysed bacterial population of agricultural soils (22). In loam and sandy soils Smalla et al. found a bacterial fraction of 0,01 – 0,56% to be resistant to kanamycin (56). In agricultural soils Ma et al. detected resistance to kanamycin in 2.3 – 15.6% of the bacterial population (37). Concerning the analysis of soil samples usually only a minor fraction of approx. 1 % of the bacteria are cultivable under laboratory conditions (12).

A survey of the scientific literature retrieved 4 publications which were dealing explicitly with the prevalence of nptII genes in natural environments (31, 37, 56, 69). Three of them document a low-level presence of nptII in naturally occurring bacterial populations of non-clinical origin: Leff et al. could detect nptII only in samples from river water (3 out of 184 isolates) (31), Smalla et al. found nptII positive isolates in sewage, and manure (3 out of 350) but none in soil (56). Both studies were performed in 1993 and were semi-quantitative at best. A study by Zhu et al. analysed samples from Canada for the presence of nptII in river water (69). The analyses over a 2 years period showed extremely variable nptII concentrations ranging from 0 to 4,36 x 10^6 copies per litre of water. The experimental setting was not representative for the relevant European conditions and environmental exposure pathways (i.e. transfer of plant derived transgenic DNA to soil or gut bacteria).

In the most recent study dealing with the detection of neomycin phosphotransferase II genes in agricultural soils Ma et al. could not find nptII copies in total soil bacterial DNA extractions (37). The authors compared kanamycin and neomycin resistant soil bacterial populations from fields cultivated with GM maize or its near isogenic non-GM line in a 3 year trial. In the cultivation approach they determined the proportion of soil bacteria naturally resistant to one of these aminoglycoside antibiotics and analysed a total of 3000 resistant bacterial isolates for the presence of nptII retrieving no nptII positive clones. In a metagenomic approach total soil bacterial DNA was isolated by Nycodenz density gradient centrifugation from 48 soil samples from GM and non-GM cultivated fields, each, achieving also no positive nptII PCR result. The study design of the Canadian group was similar to the efforts of the present project concerning the determination of nptII genes but Ma et al. focused on the detection of horizontal gene transfers from plant to bacteria and a comparison of GM and non GM fields over several years (37). The present project on the contrary was designed to acquire the naturally occurring nptII/nptIII resistance background load at a single time point in GMO free agricultural environments, encompasses the analysis of substantially more soil samples of various soil types and does not provide any information about horizontal gene transfer processes from plant to bacteria.

No systematic analysis concerning the prevalence of nptIII in environmental samples is available (15).

The presented data below are compiled from bacterial cultivation-dependent (isolation of kanamycin resistant bacterial strains) and cultivation-independent (total soil DNA extraction) approaches to determine the baseline frequency of nptII and nptIII gene copies in agricultural soils representative for the Austrian maize and potato growing regions. The data are a snapshot view retrieved from a single composite soil sample collection at a distinct point of time (summer season; shortly before harvest of the crop). The collection period was carefully chosen and reduced to an absolute minimum to provide approximately comparable environmental conditions for evaluation of bacterial biodiversity and abundance of resistance genes.

1.5 Sampling plan

Two areas of interest – maize and potato growing regions - according to the commercialized transgenic plant varieties MON863 and EH92-427-1 which carry antibiotic resistance markers genes and have the approval to be cultivated in the European Union were chosen to establish the baseline frequency of nptII and nptIII in Austrian soil samples before exposure to exogenous ARM gene containing DNA from transgenic plants.

Since soil sampling readily exceeds financial and staff capacities only a representative fraction of the relevant test sites could be analysed in practice.

For the following reasons it was decided to use maize fields from the BINATS project (48, 49):

1. These fields had been already stratified and were established to be representative for the Austrian maize growing regions.
2. There was a bulk of data already available for these fields which would be supplemented with soil and resistance gene analysis data from the present project.
3. The locations of the fields were known to the project field workers, thus, saving a substantial amount of time usually necessary to localize unknown test areas on site.
4. Farmers and project field workers were familiar to each other and have had already proven to be capable of co-operation.

For details on the sampling procedure of maize test areas see section 1.5.1., for details on the selection of potato test areas see section 1.5.2.

Composite soil samples were actually collected from 50 maize and 50 potato fields. A pool of an additional 50 substitute areas (25 per each crop type) were necessary to maintain representativity if the land owner did not allow entering one of the 50 core fields.

1.5.1 Sampling plan for areas used for the cultivation of maize

Fifty sample areas with maize cultivation had been determined as part of the BINATS project (48, 49). This sampling plan was adopted for the current project. In addition, 25 substitute areas were sampled in a manner similar to the sampling of the potato areas (see section 1.5.2) totalling in 75 test areas representatively selected for Austrian maize growing regions. A pool of substitute areas was necessary to avoid a shortage of representative test fields if the entry on the originally planned test site was prohibited by the land owner. The spatial distribution of the core and the substitute sampling areas for soils from maize fields is provided in Figure 1.

For details on the stratification and selection procedure of maize test fields see section 3.1.1.

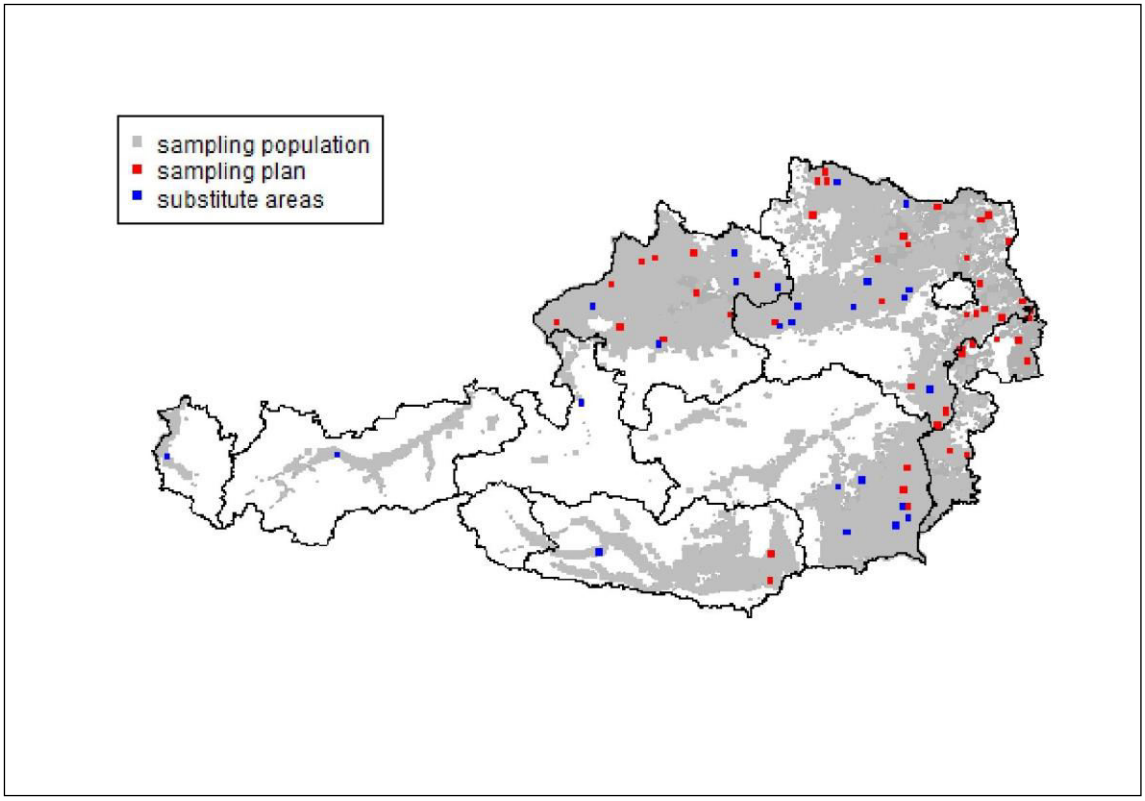

Figure 1: Spatial distribution of sampling plan and substitute areas for maize fields in Austria.

1.5.2 Sampling plan for areas used for the cultivation of potatoes

Fifty representative sample areas with potato cultivation were determined for the present project. In addition, 25 substitute areas were sampled totalling in 75 test areas representatively selected for Austrian potato growing regions. A pool of substitute areas was necessary to avoid a shortage of representative test fields if the entry on the originally planned test site was prohibited by the land owner. The spatial distribution of the areas included in the sampling plan and the locations of the substitute sampling areas are provided in Figure 2: For details on the stratification and selection procedure of potato test fields see section 3.1.2.

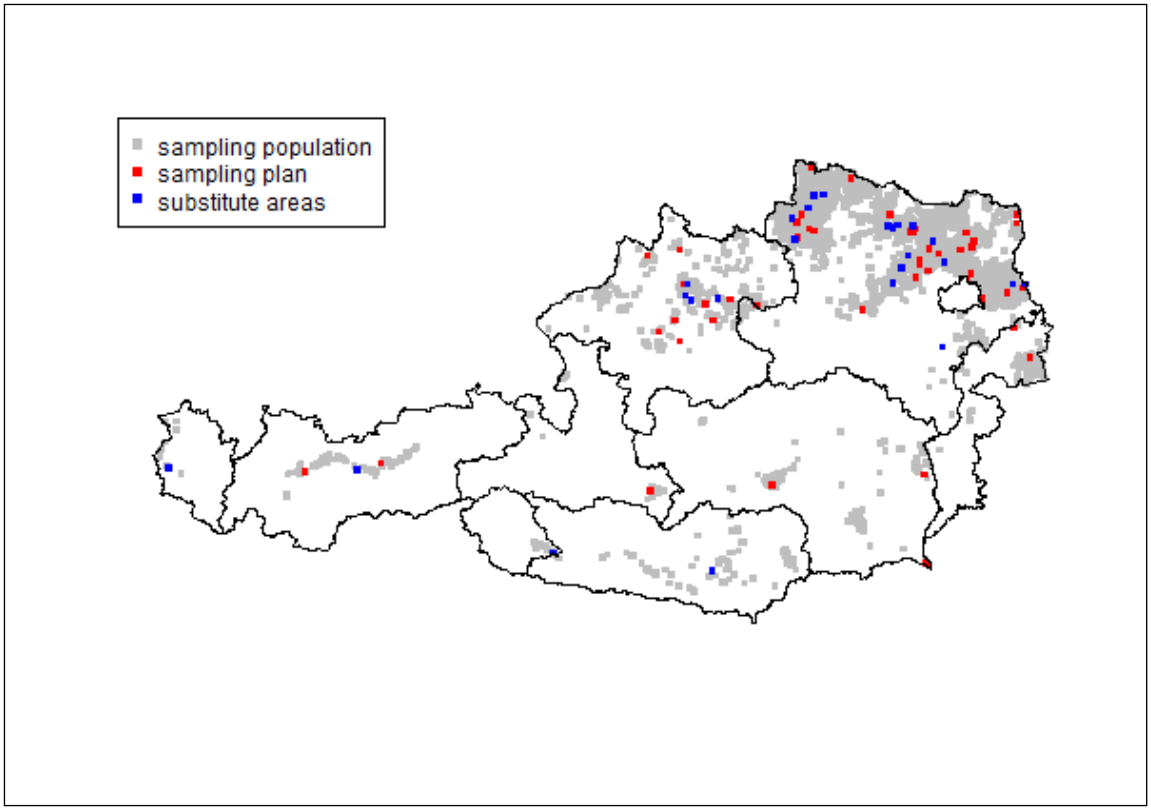

Figure 2: Spatial distribution of sampling plan and substitute areas for potato fields in Austria.

1.5.3 Reference areas for maize and potato cultivating regions

Five reference areas for maize and five reference areas for potato growing regions were determined from a total of 100 test areas (see sections 1.5.1 and 1.5.2) - in addition to the nptII/nptIII prevalence analysis - for an in depth analysis of soil parameters, bacterial biodiversity in the rhizosphere (sensu latu) and circumjacent soil, cultivation, characterization and kanamycin resistance testing of bacterial strains. A selection according to soil types (following the German soil systematic) was chosen. The aim was to cover a large range of possible bacterial pedodiversity by choosing at least one sample per most abundant occurring soil types followed by differences in acidity. The selection criteria for the reference areas, thus, were (weighted from high to low importance): 1. high abundance of the soil type, 2. different soil types to cover a wide array of biodiversity, and 3. different pH cluster. For details on the selection process of reference areas see section 3.1.2.1.

This approach was chosen according to the dependency and the feasibility related to quantitative biodiversity studies.

The selected soil types for the reference areas are shown in Table 1.

Maize growing regions				Potato growing regions			
Soil type	**pH**	**Test area code[1)]**	**Internal number[2)]**	**Soil type**	**pH**	**Test area code[1)]**	**Internal number[2)]**
Fluvisol	acidic	M34_0009	M1	Fluvisol	alkaline	E4648N2800	K1
Cambisol	acidic	M34_0020	M2	Cambisol	alkaline	E4800N2840	K2
Chernozem	alkaline	M34_0055	M3	Chernozem	neutral	E4834N2816	K3
Relict soil	acidic	M34_0124	M4	Cambisol	acidic	E4594N2825	K4
Fluvisol	neutral	E4574N2632	M5	Chernozem	alkaline	E4824N2812	K5

Table 1: Soil types for reference areas.

1) Selected test area with respective soil type

2) Field/composite soil sample number

1.6 Implementation of the fieldwork – selection of test areas, identification of test fields, soil sample collection, and sample preparation for analyses

1.6.1 Selection of the 100 test areas and localisation of potato and maize test fields

From a total of 150 preliminary statistically selected test areas a final set of 100 core test areas was chosen for the actual acquisition of composite soil samples (for details on the random sampling procedure for the selection of these test areas see section 3.1).

Approx. 2500 farmers who cultivated potato and/or maize within the 150 selected test areas in 2010 were identified, contacted, informed about the project and asked for co-operation by the Federal Ministry of Agriculture, Forestry, Environment and Water Management. A total of 455 farmers responded positively. 248 of them indicated to grow maize also during the project test period in 2011. Fields of 152 of these farmers resided within the 50 core test areas for maize, 96 farmers were available for the remaining 25 substitute areas. Concerning potatoes 207 farmers agreed to cooperate in 2011. 147 farmers indicated the availability of potato fields located within the 50 core test areas for potatoes, 60 were owners of fields in the 25 substitute areas.

For each of the 150 randomly selected test areas in the Austrian maize and potato cultivation regions, a map of the location of the test area (Austrian map, Figure 3 a) as well as an aerial photograph were received. In the aerial photographs the potato fields as well as the maize fields cultivated in 2010 with entering permission were marked in green and yellow, respectively (Figure 3 b). Areas which farmers pronounced not to be entered were framed in red colour. The 150 selected test areas - 75 selected in the Austrian maize cropping areas, 75 in potato cultivation areas - were validated concerning their suitability for the purpose of the present study. The existence of a maize- or potato-field within each test area was checked as well as entering prohibitions. Finally, a set of 50 suitable test areas for each of the two crops was defined.

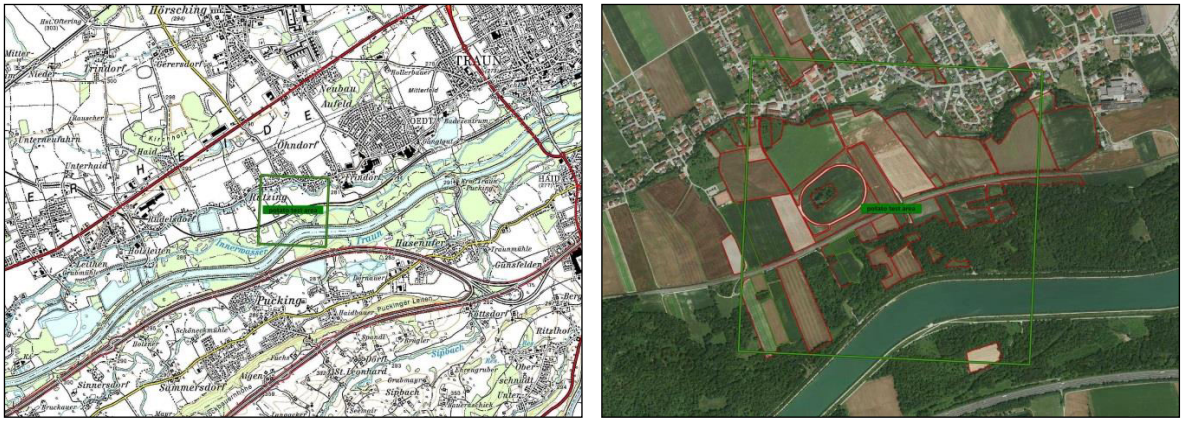

Figure 3: Maps and aerial photographs for the localisation of test fields.

a) Austrian map (OEK) and b) map of a typical test area (1 km2) in the Austrian potato cultivation region. The potato fields cultivated in 2010 are framed in green, entering prohibitions are marked in red colour.

Each maize or potato field within the 100 selected core test areas was designated with a nine-digit number code and a field name on the aerial photograph (this information is not shown in Figure 3 b due to privacy data restrictions concerning the collaborating farmers). In each test area a single potato or maize test field was chosen. The number code of the selected field was looked up in a list containing fields with entering permission, and the names as well as the contact addresses of the respective farmers of these fields were then identified. Each selected farmer of all 100 test fields was contacted personally in order to identify the current position of his potato and/or maize field because the data on the available map showed cultivation data from 2010 and did not reflect the actual situation at the time of soil sample collection (summer 2011). Due to the unavailability of the INVEKOS-data 2011 in electronic form at that project stage - spring 2011 - data from the preceding year had to be used. In those cases where no test field was located within the test area in the sampling year 2011, the field of that farmer which was located next to the test area within a maximum distance of 2 kilometres was chosen for soil sampling. This was based on the assumption that within this area the soil type and climatic conditions would be the same with a high probability. Thus a possible difference between the soil types should be negligible and a representative sample should be given. Concerning this issue a typical situation for maize fields is displayed in Figure 4. For potato fields refer to Figure 5.

Concerning maize, 24 of a total of 50 selected test fields lay within the test area, 26 were situated outside the test area (Table 2 and Table 4). Only 19 potato-fields were within the test area, 31 had their position outside the test area. This was due to the fact that in comparison to maize, potatoes were regionally cultivated to a lesser extent which was also reflected in a smaller number of fields in Austria (Table 2 and Table 4). Consequently, in these cases farmers had to provide exact information about the new location of the field to be eligible for analysis. The new position was marked in the aerial photograph and in the Austrian map, respectively, which showed a bigger section of the map. Also the name of the new field was requested and noted on the field protocol.

For the localisation of the actually tested maize fields see Figure 6. The actually sampled potato fields are depicted in Figure 7.

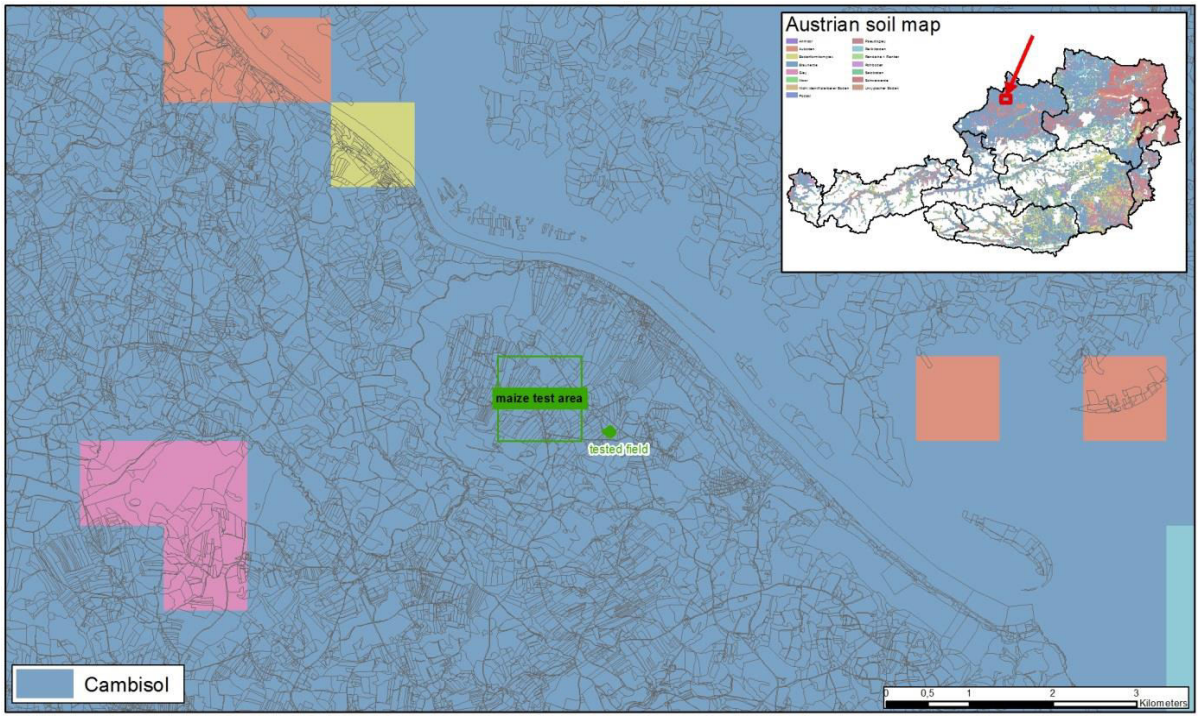

Figure 4: Maize. Test field outside maize test area.

Areas shaded in light blue represent Cambisol as predominant soil type. Areas shaded in orange yellow and magenta represent soil types different from Cambisol.

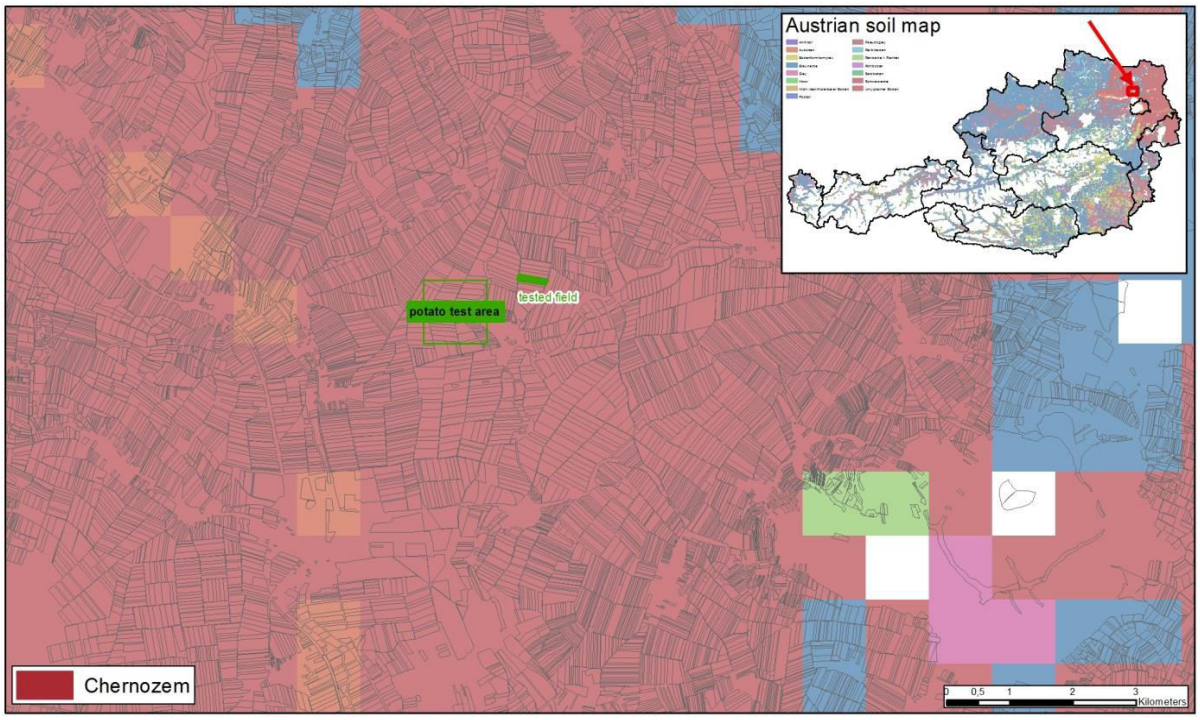

Figure 5: Potatoes. Test field outside potato test area.

Areas shaded in light red represent Chernozem as predominant soil type. Areas shaded in blue indicate Cambisol. Areas shaded in orange, green white and magenta represent soil types different from Chernozem.

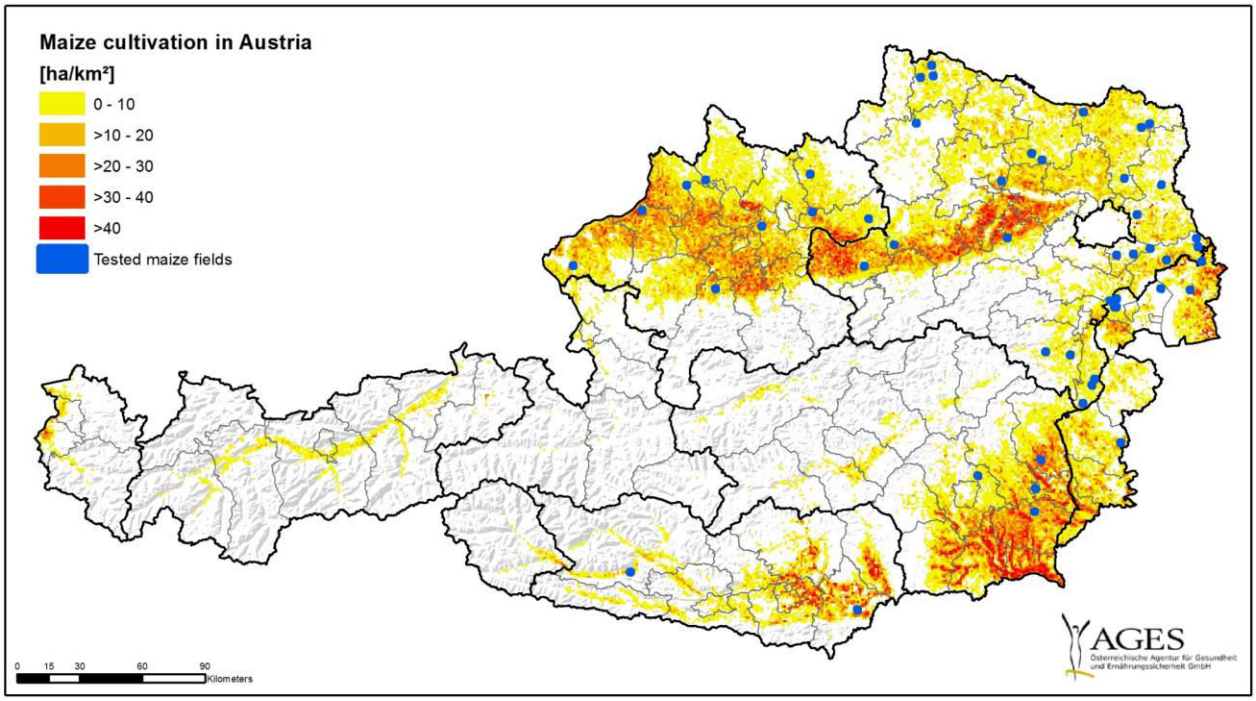

Figure 6: Maize cultivation in Austria (INVEKOS, 2010) and tested maize fields.

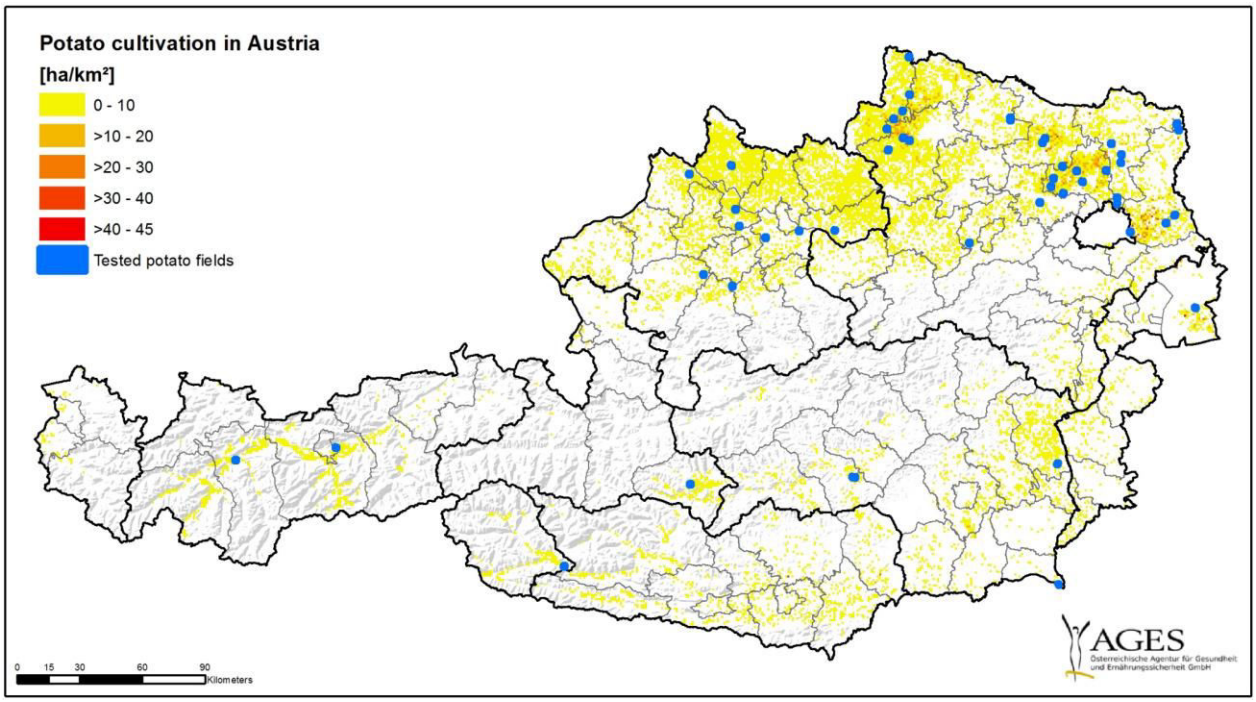

Figure 7: Potato cultivation in Austria (INVEKOS, 2010) and tested potato fields.

1.6.2 Information about the agricultural management of the test fields

In a second phone call circle, the farmers of all 100 test areas were asked for the performed fertilization procedure (inorganic fertilizer/organic fertilizer) on the test field, the previously grown crop, the usage of the crop (e. g. potatoes for industrial purposes or food), the field size and the estimated date of harvest. The information was summarized in Table 2, Table 3, Table 4, and Table 5. In those tables also the field code, the field name, the locality outside or inside of the test area, the name of the farmer and his contact addresses including telephone numbers as well as soil moisture, weather and air temperature were included. For privacy reasons personal data of the landowners are not shown.

1.6.3 Testing of the methodology for soil sampling – 'pilot study'

In order to test the methodology for taking soil samples in maize and potato fields, a basic approach was developed and tested in May 2011 on a fodder maize field in Styria. For details on the pilot study see section 2.1. The experiences gained during this exercise were implemented into the practical execution of the sampling of the 100 composite soil samples.

Number of test area	Number of the test field	Location relative to the test area	Application of fertilizers on the test field	
			Organic fertilizer	Inorganic fertilizer
E4574N2632	M5	Inside	cattle muck	commercial fertilizer (Handelsdünger): N/P/potassium 15/15/15, N27% (Nitromonkal)
E4652N2827	FM39	Inside	cattle muck, cattle liquid manure before sowing	artificial fertilizer (during sowing), NAC-nitrogen application
E4654N2809	FM40	Inside	no organic fertilizer of animal excrements	only inorganic fertilizer
E4681N2807	FM43	Inside	cattle muck before sowing	NAC
E4694N2795	FM41	Inside	pig liquid manure	urea, Vollkorndünger (NPK)
E4740N2686	FM44	Outside	cattle muck before sowing, cattle liquid manure (big quantities)	no inorganic fertilizer
E4767N2670	FM45	Inside	no information	no information
E4781N2746	FM42	Inside	2x liquid manure (spring)	commercial fertilizer (100kg)
M31_0000	FM1	Inside	biogas manure (consisting of fowl muck and cattle manure): 20m^3/ha	nitrogen (21), phosphor (9), potassium (9)
M31_0035	FM6	Inside	pig liquid manure (20m^3/ha)	DAP (100kg), AHL (N27%, 100l)
M31_0046	FM7	Inside	no organic fertilizer of animal excrements	inorganic fertilizer: phosphor/potassium/nitrogen: 15/15/15, Nitromonkal (nitrogen)
M31_0048	FM4	inside	no organic fertilizer of animal excrements	inorganic fertilizer: Volldünger 15/15/15 (500kg/ha), urea (200kg/ha)
M31_0074	FM5	outside	no organic fertilizer of animal excrements	inorganic fertilizer: DAP (200kg), Vollkorn gelb (400kg), NAC
M31_0080	FM39	outside	no organic fertilizer of animal excrements	essential nutrients: phosphor (50-55kg), N (110-120kg), potassiumum (60-70kg)
M31_0087	FM2	outside	cattle manure 45m^3/ha	inorganic fertilizer: NAC
M31_0092	FM8	outside	cattle muck, cattle liquid manure	Diamonphosphat
M34_0009	M1	inside	no organic fertilizer of animal excrements	inorganic fertilizer: Volldünger (MPK) 15/15/15, Nitromonkal 27%
M34_0012	FM9	inside	fowl muck	inorganic fertilizer: nitrogen NAC
M34_0013	FM10	inside	no organic fertilizer of animal excrements	artificial fertilizer: 012-20er, N 27%
M34_0017	FM11	outside	cattle muck (spring)	Vollkorngelb (ca. 300kg)
M34_0019	FM12	inside	cattle liquid manure (spring)	NAC/nitrogen (ca. 100kg, during sowing)
M34_0020	M2	outside	cattle manure (24m^3/ha), cattle muck (15t/ha)	nitrogen (26kg)
M34_0022	FM13	inside	cattle muck	artificial fertilizer: phosphor-potassium
M34_0026	FM14	outside	no organic fertilizer of animal excrements	no inorganic fertilizer
M34_0029	FM15	inside	turkey muck (autumn)	Nitromonkal (spring)
M34_0031	FM16	outside	no organic fertilizer of animal excrements	artificial fertilizer
M34_0032	FM17	inside	muck (autumn)	potassiumum, nitrogen (spring)
M34_0038	FM18	outside	no organic fertilizer of animal excrements	no inorganic fertilizer, only green manuring
M34_0039	FM19	inside	no organic fertilizer of animal excrements	no inorganic fertilizer
M34_0050	FM20	outside	pig liquid manure	Nitromonkal, DAP
M34_0051	FM21	outside	no organic fertilizer of animal excrements	only nitrogen fertilizer
M34_0054	FM22	outside	no organic fertilizer of animal excrements?	NAC, urea, DAP, nitrogen, phosphor, N_2O
M34_0055	M3	outside	no organic fertilizer of animal excrements	phosphor, potassium, nitrogen
M34_0063	FM23	outside	no organic fertilizer of animal excrements	Nitromonkal, no inorganic fertilizer
M34_0064	FM24	outside	no organic fertilizer of animal excrements	inorganic fertilizer: phosphor, potassium, nitrogen
M34_0069	FM25	inside	pig liquid manure (25m^3)	150 Diamon (nitrogen fertilizer and sulphur)
M34_0070	FM26	inside	no organic fertilizer of animal excrements	since ten years no application of inorganic fertilizer
M34_0083	FM27	outside	no organic fertilizer of animal excrements	nitrogen? (spring), no fertilization in autumn
M34_0090	FM28	outside	no organic fertilizer of animal excrements	nitrogen
M34_0093	FM29	inside	no organic fertilizer of animal excrements	inorganic fertilizer
M34_0100	FM30	outside	cattle manure	fertilization in spring ?
M34_0102	FM31	outside	no organic fertilizer of animal excrements	2x urea (during sowing and 2-3 weeks after that)
M34_0104	FM32	outside	no organic fertilizer of animal excrements	biogas liquid manure, artificial fertilizer
M34_0110	FM33	outside	no organic fertilizer of animal excrements	inorganic fertilizer NAC
M34_0111	FM34	outside	no organic fertilizer of animal excrements	Nitromonkal 27%, urea (commercial fertilizer)
M34_0112	FM35	outside	no information	no information
M34_0118	FM36	outside	no organic fertilizer of animal excrements	NAC, biogas liquid manure, maize spring water
M34_0124	M4	inside	cattle liquid manure	Vollkorn gelb (artificial fertilizer)
M34_0125	FM37	inside	sheep muck	urea
M34_0126	FM38	outside	no organic fertilizer of animal excrements	Diamonphosphat, Nitromonkal

Table 2: Maize fields: Test areas and agricultural field management.

Number of test area	Number of the test field	Additional information			Conditions at sampling time		
		Previous crop	Field size	Comment	Soil moisture	Weather	Air temperature (°C)
E4574N2632	M5	summer barley	2.20 ha	silo maize	moist	sunny	16-20
E4652N2827	FM39	rye	0.36 ha	silo maize	moist	clear	21-25
E4654N2809	FM40	maize	3.00 ha	grain maize	wet	clear	16-20
E4681N2807	FM43	maize	0.75 ha	silo maize	moist	cloudy	16-20
E4694N2795	FM41	winter barley	0.40 ha	grain maize	moist	clear	16-20
E4740N2686	FM44	spelt	0.50 ha	silo maize, seeds (old varieties)	moist	sunny	16-20
E4767N2670	FM45	no information	n.a.	no information, contact data of farmer incorrect	moist to wet	sunny	26-30
E4781N2746	FM42	alternating meadow	0.90 ha	silo maize	partly moist/partly dry	clear	21-25
M31_0000	FM1	maize	1.20 ha	grain maize	dry	sunny	21-25
M31_0035	FM6	winter barley	0.60 ha	grain maize	moist	clear	26-30
M31_0046	FM7	maize	1.00 ha	silo maize	moist	sunny	26-30
M31_0048	FM4	maize	3.36 ha	grain maize	wet	cloudy/rainy	21-25
M31_0074	FM5	sugar beet	5.14 ha	maize for seed production	moist	sunny	26-30
M31_0080	FM39	wheat	6.50 ha	maize for seed production: 4 rows female, 3 rows male	wet	cloudy	21-25
M31_0087	FM2	summer barley/mustard	1.25 ha	silo maize	moist	cloudy	16-20
M31_0092	FM8	winter barley	1.00 ha	silo maize	moist	sunny	26-30
M34_0009	M1	maize	1.06 ha	grain maize	wet	sunny	26-30
M34_0012	FM9	maize	0.56 ha	grain maize	moist	clear	16-20
M34_0013	FM10	wheat, before: soybean (2009)	0.18 ha	grain maize	partly moist/partly dry	sunny	21-25
M34_0017	FM11	alternating meadow	1.76 ha	silo maize	partly moist/partly dry	cloudy	16-20
M34_0019	FM12	alternating meadow/clover	3.15 ha	silo maize	partly moist/partly dry	sunny	21-25
M34_0020	M2	barley	0.75 ha	silo maize	dry	sunny	21-25
M34_0022	FM13	winter barley/clover (2x)	1.75 ha	silo maize for cattle feeding	partly moist/partly dry	clear to sunny	16-20
M34_0026	FM14	rye	7.00 ha	fodder maize, irrigated	moist	cloudy	16-20
M34_0029	FM15	winter wheat	0.98 ha	grain maize	partly moist/partly dry	sunny	21-25
M34_0031	FM16	winter wheat, before: squash	7.50 ha	grain maize	partly moist/partly dry	clear	21-25
M34_0032	FM17	soybean	0.48 ha	grain maize	partly moist/partly dry	clear	16-20
M34_0038	FM18	yellow mustard, before: winter wheat	3.07 ha	waxy corn (organic)	dry	sunny	16-20
M34_0039	FM19	winter triticale	4.20 ha	grain maize (organic)	dry	sunny	16-20
M34_0050	FM20	winter durum	2.39 ha	no information	dry	cloudy	16-20
M34_0051	FM21	winter wheat	1.60 ha	grain maize (wet-dry)	dry	cloudy	16-20
M34_0054	FM22	sugar beet, before: winter wheat	2.60 ha	grain maize	dry	cloudy	16-20
M34_0055	M3	wheat	2.64 ha	grain maize/wet maize	dry	cloudy	16-20
M34_0063	FM23	wheat	4.40 ha	grain maize	dry	cloudy	16-20
M34_0064	FM24	wheat	1.30 ha	grain maize, wet maize, flour maize	dry	cloudy	16-20
M34_0069	FM25	winter barley	0.36 ha	grain maize	moist	cloudy	16-20
M34_0070	FM26	winter wheat (two preceding years)	2.90 ha	grain maize, export as starch	dry	cloudy	16-20
M34_0083	FM27	wheat	3.00 ha	wet maize	dry	sunny	26-30
M34_0090	FM28	wheat	1.25 ha	grain maize	dry	cloudy	16-20
M34_0093	FM29	winter barley	4.00 ha	waxy maize/maize for consumption	dry	sunny	>30
M34_0100	FM30	wheat	0.09 ha	silo maize	dry	sunny	>30
M34_0102	FM31	wheat	3.39 ha	grain maize	dry	cloudy	16-20
M34_0104	FM32	winter wheat	1.66 ha	silo maize for biogas plant	dry	sunny	>30
M34_0110	FM33	fallow land (quite old)	0.04 ha	silo maize	moist (soil surface)	cloudy	16-20
M34_0111	FM34	winter wheat	3.18 ha	industry maize	dry	cloudy	16-20
M34_0112	FM35	no information	n.a.	no information, farmer inaccessible	dry	clear	>30
M34_0118	FM36	sugar beet	3.31 ha	grain maize for pig feeding	dry, partly moist	clear	26-30
M34_0124	M4	winter barley	7.00 ha	silo maize	dry	sunny	>30
M34_0125	FM37	triticale	n.a.	silo maize	dry	sunny	>30
M34_0126	FM38	winter wheat	3.00 ha	silo maize	dry	sunny	>30

Table 3: Maize fields: Table 2 continued.

Number of test area	Number of the test field	Field location relative to the test area	Application of fertilizers on the test field	
			Organic fertilizer	Inorganic fertilizers
E4382N2678	FK28	inside	muck (autumn)	Patentkali
E4430N2686	FK38	outside	no organic fertilizer of animal excrements	Novatex-Suprem (N, P, K: 450 kg), Extrad. Kalisulfat (300kg/ha)
E4542N2634	FK37	inside	cattle manure: 30m^3/ha	Ammonsulfat, Superphosphat, 60er Kali
E4594N2825	K4	inside	no organic fertilizer of animal excrements	Patentkali
E4602N2676	FK43	outside	muck (spring)	no inorganic fertilizer
E4603N2777	FK34	inside	muck (autumn)	no inorganic fertilizer
E4614N2830	FK16	inside	muck (autumn), liquid manure (15-20 m^3, spring)	Basta (1,5l/ha)
E4617N2772	FK35	inside	no organic fertilizer of animal excrements	Vollkorn rot
E4618N2808	FK14	outside	no organic fertilizer of animal excrements	Biofert (spring)
E4619N2801	FK27	outside	no organic fertilizer of animal excrements	nitrogen (11), phosphor (5), potassium (20)
E4632N2796	FK25	inside	no organic fertilizer of animal excrements	Mikromokal-Bor (spring)
E4648N2800	K1	outside	pig muck (40t, autumn)	potassium (750kg), artificial fertilizer Linzer Top (spring)
E4665N2797	FK11	outside	no organic fertilizer of animal excrements	60% potassium, NAC (300kg) in spring
E4679N2683	FK36	inside	no organic fertilizer of animal excrements	inorganic fertilizer: N (87kg), P (67kg), K (67kg)
E4680N2683	FK45	outside	cattle muck	NAC (100kg), Kaltnitrogen (100kg)
E4688N2851	FK41	outside	cattle muck	Kaltnitrogen, Nitromonkal, foliar fertilizer
E4689N2841	FK1	outside	no organic fertilizer of animal excrements	no inorganic fertilizer
E4691N2856	FK42	inside	no organic fertilizer of animal excrements	no inorganic fertilizer
E4695N2860	FK6	outside	muck (25t)	NAC27%, Linzer Top 270kg/ha
E4696N2847	FK44	inside	muck (15t/ha)	no inorganic fertilizer
E4696N2886	FK40	outside	cattle muck (ca. 25t)	no inorganic fertilizer
E4698N2868	FK39	inside	cattle muck	Vollkorn rot (artificial fertilizer): 200kg
E4699N2846	FK24	inside	pig liquid manure (autumn)	urea (180-200 kg)
E4732N2797	FK19	outside	no organic fertilizer of animal excrements	urea (commercial fertilizer)
E4747N2858	FK18	outside	cattle manure, muck	potassium, nitrogen, urea, wheat straw
E4747N2859	FK23	outside	no organic fertilizer of animal excrements	the last ten years no application of inorganic fertilizers
E4761N2848	FK8	outside	no organic fertilizer of animal excrements	nitrogen, NAC (spring), DC45 (artificial fertilizer), phosphor-potassium fertilizer
E4762N2848	FK2	outside	no organic fertilizer of animal excrements	phosphor, potassium, NAC
E4764N2850	FK7	inside	no organic fertilizer of animal excrements	NAC, 60er Kali, hypophosphat
E4765N2819	FK3	outside	muck of cattles, fowl and pigs (small part)	no inorganic fertilizer
E4767N2828	FK21	outside	no organic fertilizer of animal excrements	straw, mustard planting, NAC
E4767N2830	FK26	outside	no organic fertilizer of animal excrements	artificial fertilizer
E4773N2824	FK10	inside	cattle manure	planting of suitable plants for nutrient supply, NAC 27%
E4773N2838	FK5	inside	no organic fertilizer of animal excrements	NAC-Kombi, urea, foliar fertilizer
E4776N2694	FK31	outside	liquid manure	Vollkorn rot
E4779N2835	FK15	outside	no organic fertilizer of animal excrements	NAC, no phosphor-potassium
E4780N2638	FK17	outside	chicken muck	artificial fertilizer: nitrogen
E4783N2830	FK20	outside	no organic fertilizer of animal excrements	commercial fertilizer (Handelsdünger), urea, planting of mustard, scorpionweed, clover, buckwheat
E4793N2838	FK30	outside	no organic fertilizer of animal excrements	commercial fertilizer: phosphor-potassium, nitrogen: NAC
E4796N2849	FK12	inside	no organic fertilizer of animal excrements	inorganic fertilizer
E4798N2820	FK13	outside	no organic fertilizer of animal excrements	potassium 60, PK45/1836, NAC, N, Chlor/NTec ground fertilizer 9718
E4800N2823	FK4	inside	no organic fertilizer of animal excrements	Entek (NTK, spring)
E4800N2840	K2	inside	no organic fertilizer of animal excrements	mixed fertilizer (Mischdünger) after seeding: nitrogen-phosphor-potassium
E4801N2844	FK9	inside	no organic fertilizer of animal excrements	urea, planting of peas
E4808N2808	FK32	outside	no organic fertilizer of animal excrements	phosphor-potassium, nitrogen, urea; application against Phytophthora
E4824N2812	K5	outside	no organic fertilizer of animal excrements	inorganic fertilizer
E4828N2857	FK29	outside	no organic fertilizer of animal excrements	NAC
E4828N2862	FK33	outside	no organic fertilizer of animal excrements	commercial fertilizer: nitrogen-phosphor-potassium
E4834N2816	K3	outside	no organic fertilizer of animal excrements	DiRot, 120 NAC
E4840N2772	FK22	outside	no organic fertilizer of animal excrements	artificial fertilizer (spring)

Table 4: Potato fields: Test areas and agricultural field management.

Number of test area	Number of the test field	Additional information			Conditions at sampling time		
		Previous crop	Field size	Comment	Soil moisture	Weather	Air temperature (°C)
E4382N2678	FK28	triticale	0.25 ha	potatoes for consumption	moist	sunny	21-25
E4430N2686	FK38	red radish	0.768 ha	potatoes for consumption	moist	sunny	21-25
E4542N2634	FK37	grain	2.35 ha	potatoes for seed propagation	moist	sunny	10-15
E4594N2825	K4	grain	2.00 ha	potatoes for consumption	moist	cloudy	16-20
E4602N2676	FK43	grassland (meadow)	0.08 ha	potatoes for consumption for home requirements	moist	sunny	21-25
E4603N2777	FK34	summer oat	0.90 ha	potatoes for consumption	moist	sunny	26-30
E4614N2830	FK16	winter rye, before: mustard	1.00 ha	potatoes for consumption	wet	rainy	10-15
E4617N2772	FK35	winter grain, before: raygrass	1.04 ha	potatoes for consumption	dry	cloudy	26-30
E4618N2808	FK14	florence fennel	0.30 ha	potatoes for consumption (more or less early potato variety)	dry	sunny	26-30
E4619N2801	FK27	maize	0.50 ha	potatoes for consumption	dry	clear	21-25
E4632N2796	FK25	triticale	1.00 ha	potatoes for consumption	dry	sunny	26-30
E4648N2800	K1	winter wheat	2.95 ha	potatoes for consumption (early potatoes)	dry	sunny	>30
E4665N2797	FK11	winter barley	2.30 ha	starch potatoes	wet	rainy	16-20
E4679N2683	FK36	summer barley	8.68 ha	potatoes for consumption	moist	sunny	26-30
E4680N2683	FK45	silo maize	1.00 ha	potatoes for consumption	partly moist/partly dry	sunny	26-30
E4688N2851	FK41	silo maize	1.00 ha	potatoes for consumption	dry	sunny	>30
E4689N2841	FK1	vetches as green manuring	0.30 ha	potatoes for consumption	dry	sunny	>30
E4691N2856	FK42	mixture of clover and grasses	0.48 ha	potatoes for consumption industry	dry	sunny	26-30
E4695N2860	FK6	oat	n.a.	potatoes for consumption as well as starch potatoes	dry	sunny	>30
E4696N2847	FK44	oat	3.30 ha	organic: potatoes for seed production and for consumption	dry	sunny	>30
E4696N2886	FK40	mixture of clover and grasses	1.55 ha	organic: starch potatoes	dry	sunny	>30
E4698N2868	FK39	grain: oat or rye	2.10 ha	potatoes for starch industry	dry	sunny	>30
E4699N2846	FK24	triticale	0.73 ha	starch potatoes	dry	clear	>30
E4732N2797	FK19	maize	2.50 ha	starch potatoes	moist	cloudy/rainy	10-15
E4747N2858	FK18	winter wheat	4.86 ha	potatoes for consumption	dry	sunny	>30
E4747N2859	FK23	Wheat	5.00 ha	potatoes for consumption, organic	dry	sunny	>30
E4761N2848	FK8	summer barley	1.38 ha	potatoes for starch industry	dry	sunny	26-30
E4762N2848	FK2	wheat or barley	2.00 ha	potatoes for consumption: two varieties	dry	clear	26-30
E4764N2850	FK7	winter wheat	1.75 ha	mainly starch potatoes, additional potatoes for consumption	dry	sunny	26-30
E4765N2819	FK3	spelt	1.65 ha	potatoes for starch industry	dry	sunny	21-25
E4767N2828	FK21	winter wheat	2.73 ha	potatoes for consumption	dry	sunny	26-30
E4767N2830	FK26	sugar beet	1.44 ha	potatoes for consumption	dry	sunny	26-30
E4773N2824	FK10	winter wheat	0.60 ha	starch potatoes	moist	cloudy	16-20
E4773N2838	FK5	sugar beet	1.60 ha	potatoes for consumption	dry	cloudy	21-25
E4776N2694	FK31	grain	0.10 ha	potatoes for consumption	dry	cloudy	21-25
E4779N2835	FK15	winter wheat	2.79 ha	potatoes for consumption	dry	cloudy	16-20
E4780N2638	FK17	maize	0.51 ha	potatoes for consumption	moist	sunny	26-30
E4783N2830	FK20	summer barley	2.60 ha	potatoes for consumption	dry	rainy	16-20
E4793N2838	FK30	winter wheat	5.17 ha	potatoes for consumption and starch industry	dry	sunny/cloudy	16-20
E4796N2849	FK12	winter wheat	0.15 ha	potatoes for consumption	moist	cloudy	16-20
E4798N2820	FK13	wheat	1.10 ha	starch potatoes	dry	sunny	26-30
E4800N2823	FK4	winter wheat	3.00 ha	potatoes for consumption	dry	sunny	>30
E4800N2840	K2	sugar beet	4.20 ha	potatoes for consumption	dry	cloudy	10-15
E4801N2844	FK9	winter wheat	15.00 ha	starch/industry potatoes	dry	cloudy	16-20
E4808N2808	FK32	wheat	5.00 ha	potatoes for consumption	dry	sunny/cloudy	16-20
E4824N2812	K5	part with maize / part with baby carrots	12.40 ha	potatoes for consumption	dry	sunny	26-30
E4828N2857	FK29	winter wheat	6.00 ha	starch potatoes	moist	cloudy/rainy	10-15
E4828N2862	FK33	winter wheat	2.68 ha	starch potatoes	moist	cloudy/rainy	10-15
E4834N2816	K3	wheat	4.64 ha	industry potatoes for consumption	dry	sunny/clear	21-25
E4840N2772	FK22	wheat	2.40 ha	potatoes for consumption, irrigated	partly wet/partly moist	cloudy	16-20

Table 5: Potato fields: Table 4 continued.

1.6.4 Implementation of test field soil sampling

In principle, the sampling methodology developed for the pilot study proved to be practicable. However, during the implementation of soil sampling on the 100 test areas some adaptations of the basic sampling plan were necessary. Soil samples were collected from August 6th to September 9th, 2011. This time frame was chosen to cover a defined period shortly before harvest of the plants. The collection procedure and the modifications are described below:

A composite soil sample - consisting of ten single soil extractions from the rooting zone of ten individual plants - of the test field was collected following the predefined sampling scheme shown in Figure 9 (see section 1.6.4.1). This scheme proved to be appropriate to be applied to different field sizes. The sampling followed strictly the shown scheme and did not respect different soil qualities of the field which were visible.

Soil was extracted directly from the rhizosphere (sensu lato) of the plants usually in a layer zone between 0 – 25 cm below the surface using a cut with a spade. Individual maize and potato plants were not removed from the field as originally intended. This modification was necessary because at the time of sampling (6. 8. – 9. 9. 2011) the maize plants had already reached a maximum size up to 3.5 m in some fields and showed intense rootedness in the soil (Figure 8 a and b). It was also not possible to remove an adult potato plant from the soil without substantial efforts and time delays (Figure 8 c and d). Digging down as low as the tuber was done instead and soil material was taken directly beneath a tuber.

Figure 8: Soil extraction on the test site.

a) Soil sampling in maize fields with an average maize plant size, b) maize roots of a mature maize plant, c) large potato test-field, d) adult potato plant.

The ten single soil extractions per field were collected in a clean and dry plastic bucket and intensely homogenized by hands wearing medical gloves. Approximately 750 g of the composite soil sample were transferred into a plastic bag. This plastic bag was labelled twofold (area code, the field number, date of collection), firstly with a water-resistant pen directly on the label of the plastic bag and secondly on a tesa-stripe in order to prevent possible illegibility due to effects of moisture. To protect the soil sample from external soil contamination, the plastic bag filled with the soil sample was coated with another plastic bag and stored at 8 - 10°C in a car refrigerator or in a cooling box until the samples were deployed at the soil sample collection center at AGES, Vienna. The soil samples were forwarded to the collection center as soon as possible (usually within 1 – 2 days) for further processing.

A strong focus was put on the avoidance of soil, bacterial and DNA cross contaminations throughout each step of the collection procedure and from field to field: all equipment was decontaminated with a sodium hypochlorite containing solution and the field workers wore medical plastic gloves during the soil sample extraction procedure (see protocol for contamination-free handling of the soil samples in section 3.9).

The sieving procedure of humid soil proved to be very time consuming because the soil had to be pressed through the fine meshes of the sieve. Hence, to save working time in the field, sieving of nearly all of the 100 soil samples was performed in the laboratory at the collection center at AGES, Vienna. Working time saving was also achieved by sieving only a small proportion of the collected soil sample. After the performed homogenization of the composite sample, the proportion of each soil sample was sieved through the 2 mm sieve which equalled approximately with the content of a 50 ml plastic tube. The remainder of the composite soil sample which was used for the analysis of soil parameters was transferred into a plastic bag (Toppits).

For each composite soil sample an accompanying sheet containing all relevant data was filled out and handed over at the soil sample collection center (for details on the collected information see section 3.7).

1.6.4.1 Standardized soil sample collection scheme

Per test field the soil of the rooting zone of ten individual plants were collected to constitute a field specific composite soil sample which was used for further analysis. The extraction scheme is shown in Figure 9.

↓ Lines → rows

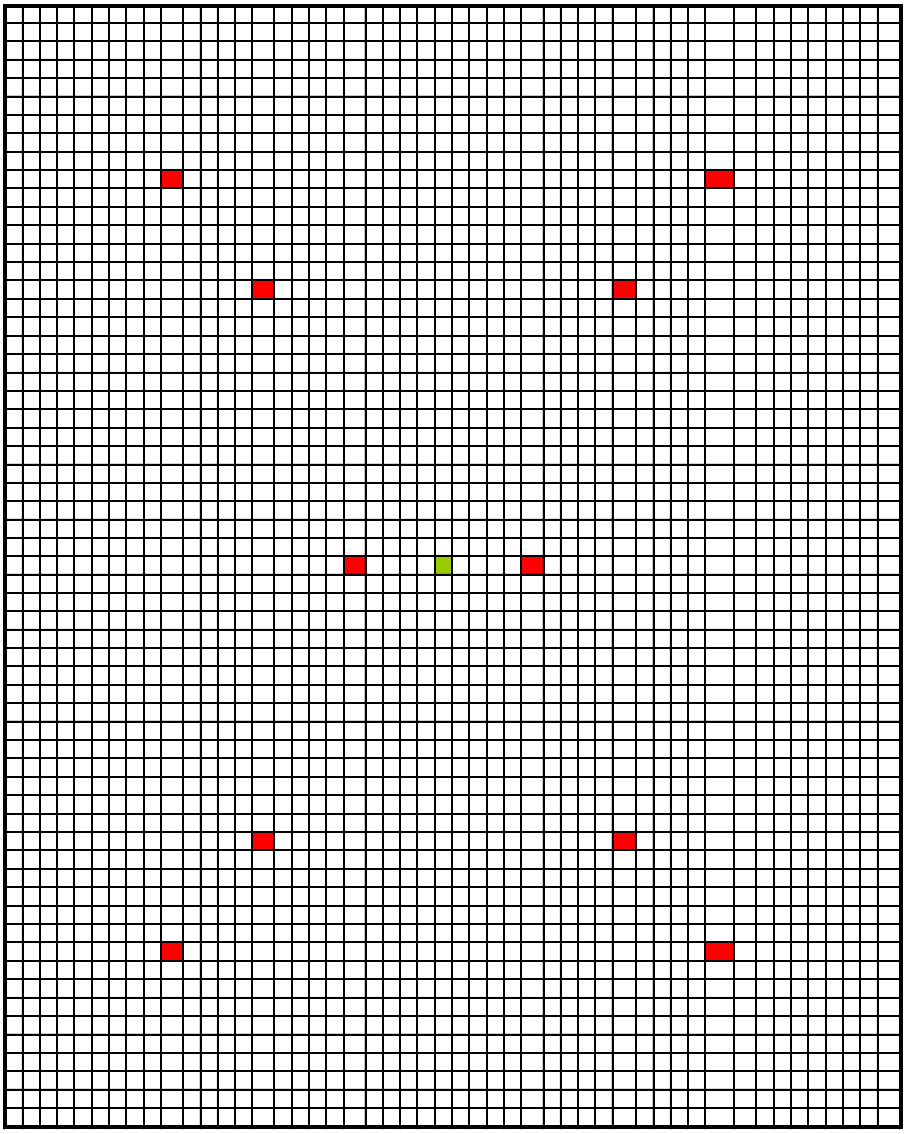

Figure 9: Sampling design for the composite soil samples consisting of ten single soil extractions. The center of the field is labelled in green; the sampled plants are marked with red squares.

The exact positions of the test plants as depicted in **Figure 9** is described below:

Position of test plant 1: starting from the left upper field corner: go 10 rows down and 10 lines (crop plants) to the right

Position of test plant 2: starting from the right upper field corner: go 10 rows down and 10 lines (crop plants) to the left

Position of test plant 3: from the field centre (green): go to the left: 10 rows, 15 lines (crop plants) up

Position of test plant 4: from the field centre (green): go to the right: 10 rows, 15 lines (crop plants) up

Position of test plant 5: from the field centre (green): go to the left: 5 rows

Position of test plant 6: from the field centre (green): go to the right: 5 rows

Position of test plant 7: from the field centre (green): go to the left: 10 rows, 15 lines (crop plants) down

Position of test plant 8: from the field centre (green): go to the right: 10 rows, 15 lines (crop plants) down

Position of test plant 9: starting from the left field corner on the bottom: go 10 rows up and 10 lines to the right

Position of test plant 10: starting from the right field corner on the bottom: go 10 rows up and 10 lines to the left.

The sampling scheme does not consider different compositions of the field soil which could be differentiated optically. In most cases the developed sampling design of the ten plants tested in the pilot study could be abided to all 100 test fields which differed in size between 0.04 and 15 ha. Only a few deviations were necessary. For very small fields, especially potato fields, consisting of only a few crop rows, the sampling scheme had to be adopted. In those cases - a small field with only a few crop rows is shown (Figure 10) - the middle row was chosen for soil sampling to avoid field edge effects. First of all the distance of the whole field length was measured and divided into eleven equal parts. Soil samples were taken from ten positions in the same distances.

Figure 10: Small potato field in Lower Austria consisting of only a few crop rows.

1.7 Determination of soil parameters

The samples (bulk soil, not sieved part of the composite soil sample) were handed over to the Department for Sustainable Plant Production, AGES, Vienna. This Department is officially certified for soil parameter analysis in Austria and is producing this kind of data on a routine basis according to relevant national (ÖNORM) and international guidelines (http://www.ages.at/landwirtschaft/produkte-und-tarife/tarife-des-geschaeftsfeldes-ernaehrungssicherung/boden/).

All soil samples got a certificate for analysis. This certificate may be used officially for interactions with federal authorities.

1.7.1 Soil sample parameters: results

Forty five composite soil samples from maize and 44 composite soil samples from potato fields were available for analysis for the following – routinely collected - soil parameters: pH, phosphor, potassium, carbon, nitrogen, and particle size (sand, silt, clay).

The 10 reference fields were analysed additionally for boron, cadmium, copper, zinc, iron, and manganese content. The data for composite soil sample of field FK17 are missing due to unavailability of the plastic bag with the corresponding bulk soil at the time of testing. However, 50 g of sieved FK17 soil were available and submitted to nptII and nptIII prevalence analysis.

The results of the analysis of the core test fields are recorded in Table 6. Table 7 presents soil parameter results of the reference test fields. A statistical evaluation of the results separated into maize and potato fields can be found in Table 8 and Table 9 for the core test fields and in Table 10 and Table 11 for the reference fields.

			pH	pH	pH	Phosphate	Phosphor	Potassium oxide	Potassium	Humus	TOC	Nitrogen (total)	Sand	Slit	Clay
			-	-	-	mg/100g	mg/kg	mg/100g	mg/kg	%	%	%	%	%	%
Number of test area	**Number of test field**	**Crop**	pH $CaCl_2$-MW Acidity acc. ÖNORM L 1083	pH in $CaCl_2$	In Ca-acetate (for estimation of liming demand in acid soils)	CAL-MW	Plant available P in CAL acc. ÖNORM L 1087	CAL-MW	Plant available K in CAL acc. ÖNORM L 1087	TOC * 1,72	Total organic carbon acc. ÖNORM L 1080	total nitrogen acc. ÖNORM L 1095	Particle size < 2 µm acc. ÖNORM L 1061-2	Particle size 2-63 µm acc. ÖNORM L 1061-2	Particle size 63-2000 µm acc. ÖNORM L 1061-2
E4574N2632	M5	maize	7.21	7.2		8	35	5.1	42	2.4	1.37	0.143	46	49.5	4.5
E4652N2827	FM39	maize	5.88	5.9		30.9	142	24.3	202	3.7	2.17	0.232	64.6	29.6	5.8
E4654N2809	FM40	maize	6.42	6.4		17.4	76	21.4	177	3.3	1.89	0.202	13.6	52.1	34.4
E4681N2807	FM43	maize	5.15	5.2	6.2	18.4	89	16.1	134	5	2.92	0.283	48	41.4	10.5
E4694N2795	FM41	maize	5.5	5.5		11.1	58	14.1	117	2.3	1.31	0.154	30.2	57.9	11.9
E4740N2686	FM44	maize	5.47	5.5	6.37	9.2	50	17.1	142	2.7	1.59	0.201	27.1	52.7	20.2
E4767N2670	FM45	maize	6.23	6.2		13.8	60	17.6	146	2.1	1.21	0.156	31.7	44.9	23.4
E4781N2746	FM42	maize	6.25	6.3		13.4	58	32.9	273	6.3	3.65	0.352	43.7	47.2	9.2
M31_0000	FM1	maize	5.69	5.7		24.7	116	24.6	204	3.6	2.11	0.213	40.4	48.1	11.5
M31_0035	FM6	maize	5.97	6		6	36	16.1	134	2.4	1.39	0.163	37.7	45.6	16.6
M31_0046	FM7	maize	6.13	6.1		7	30	11.6	97	2.8	1.62	0.19	33.1	55.3	11.6
M31_0048	FM4	maize	6.64	6.6		25.8	112	34	282	2.2	1.29	0.161	10.4	77.5	12.2
M31_0074	FM5	maize	7.37	7.4		37.6	164	38.8	322	2.5	1.45	0.177	8	71	21
M31_0080	FM3	maize	6.95	7		39.9	174	18.9	157	2	1.17	0.162	22.1	60.9	17.1
M31_0087	FM2	maize	5.13	5.1	6.12	18.3	88	38.9	323	6	3.49	0.294	52.6	36.9	10.6
M31_0092	FM8	maize	6.04	6		18.3	80	31.5	261	3.9	2.24	0.212	53.6	38.8	7.6
M34_0009	M1	maize	5.59	5.6	6.4	4.5	30	10.6	88	3.7	2.15	0.266	9.4	62.1	28.5
M34_0012	FM9	maize	6.32	6.3		30.4	133	9.7	81	2.8	1.63	0.186	39.5	50.7	9.8
M34_0013	FM10	maize	6.53	6.5		25.6	112	30.3	252	2.8	1.65	0.196	28.2	44.3	27.5
M34_0017	FM11	maize	6.74	6.7		12.4	54	19.1	158	5.7	3.34	0.362	40.6	50.6	8.8
M34_0019	FM12	maize	6.47	6.5		16.8	73	47.4	394	4.8	2.78	0.282	47.5	44.8	7.7
M34_0020	M2	maize	6.06	6.1		15.4	67	39.1	324	3.7	2.16	0.211	51.5	42.6	5.9
M34_0022	FM13	maize	7.18	7.2		32.7	143	15.7	130	3.7	2.18	0.231	33.5	56.9	9.6
M34_0026	FM14	maize	7.44	7.4		23.3	102	24.2	201	3.1	1.83	0.189	45.7	37.5	16.8
M34_0029	FM15	maize	7.43	7.4		56.5	247	44.1	366	4.5	2.61	0.288	32.7	36.7	30.6
M34_0031	FM16	maize	7.64	7.6		17.9	78	19.3	161	5	2.89	0.292	32.8	48.8	18.4
M34_0032a	FM17	maize	7.56	7.6		13.3	58	20.6	171	2.9	1.7	0.172	38.2	42.5	19.3
M34_0038	FM18	maize	7.68	7.7		17.3	75	44.3	368	3.3	1.9	0.2	23.1	51.4	25.4
M34_0039	FM19	maize	7.56	7.6		9.1	40	20.3	169	3.7	2.15	0.216	19.5	61.4	19
M34_0050	FM20	maize	7.6	7.6		20	87	23.6	196	3.1	1.8	0.169	17.7	63.4	19
M34_0051	FM21	maize	7.62	7.6		3.9	17	4.9	41	6.3	3.68	0.376	34.3	49	16.7
M34_0054	FM22	maize	7.44	7.4		55.6	242	57.1	474	3.6	2.1	0.226	26.6	48.3	25
M34_0055	M3	maize	7.63	7.6		11.9	52	19.7	164	3	1.72	0.165	37.1	44	19
M34_0063	FM23	maize	7.56	7.6		23.6	103	33.1	275	2.6	1.51	0.162	22.8	56.5	20.7
M34_0064	FM24	maize	7.59	7.6		13.8	60	13.8	115	3	1.75	0.188	26.4	57.9	15.7

			pH	pH	pH	Phosphate	Phosphor	Potassium oxide	Potassium	Humus	TOC	Nitrogen (total)	Sand	Slit	Clay
			-	-	-	mg/100g	mg/kg	mg/100g	mg/kg	%	%	%	%	%	%
Number of test area	**Number of test field**	**Crop**	pH $CaCl_2$-MW Acidity acc. ÖNORM L 1083	pH in $CaCl_2$	In Ca-acetate (for estimation of liming demand in acid soils)	CAL-MW	Plant available P in CAL acc. ÖNORM L 1087	CAL-MW	Plant available K in CAL acc. ÖNORM L 1087	TOC * 1,72	Total organic carbon acc. ÖNORM L 1080	total nitrogen acc. ÖNORM L 1095	Particle size < 2 µm acc. ÖNORM L 1061-2	Particle size 2-63 µm acc. ÖNORM L 1061-2	Particle size 63-2000 µm acc. ÖNORM L 1061-2
M34_0069	FM25	maize	7.06	7.1		8.3	36	14	117	2.5	1.45	0.174	38.6	45.8	15.6
M34_0070	FM26	maize	7.52	7.5		59.8	261	14.6	121	2.7	1.55	0.153	45.4	46.5	8.1
M34_0083	FM27	maize	7.08	7.1		16	70	16	133	2	1.14	0.117	53.8	32.9	13.3
M34_0090	FM28	maize	7.66	7.7		17.8	78	15.6	130	2.3	1.34	0.134	20.7	55.9	23.5
M34_0093	FM29	maize	7.31	7.3		20.5	90	42	349	2.5	1.46	0.174	25.2	58.5	16.3
M34_0100	FM30	maize	7.71	7.7		17.3	75	32.4	269	1.7	0.96	0.104	13.5	71.5	15
M34_0102	FM31	maize	7.46	7.5		20.6	90	40.2	333	3.1	1.79	0.177	13.1	67.7	19.2
M34_0104	FM32	maize	7.67	7.7		21.6	94	35.5	294	2.6	1.52	0.164	10.8	68	21.2
M34_0110	FM33	maize	7.44	7.4		33.5	146	20.4	169	2.4	1.41	0.135	30.6	44.7	24.7
M34_0111	FM34	maize	7.65	7.7		19.4	84	23.3	193	3.1	1.83	0.174	12.6	65.3	22.1
M34_0112	FM35	maize	5.06	5.1	6.17	17.8	86	23.3	194	2.7	1.55	0.178	45.8	43	11.3
M34_0118	FM36	maize	7.54	7.5		34.1	149	55.8	463	3.2	1.87	0.206	51.1	28.7	20.2
M34_0124	M4	maize	5.17	5.2	6.31	18.3	88	43.4	360	2.9	1.7	0.191	28.7	53.4	17.9
M34_0125	FM37	maize	6.17	6.2		12.3	54	39.9	331	2.9	1.68	0.186	71	25.2	3.8
M34_0126	FM38	maize	6.18	6.2		34.8	152	16.1	134	2.9	1.66	0.171	43.5	46.4	10.1
E4382N2678	FK28	potato	7.26	7.3		32.4	141	23.8	197	6.2	3.58	0.348	48.1	44.4	7.5
E4430N2686	FK38	potato	7.5	7.5		35.4	155	42.9	356	3.7	2.15	0.219	40.5	52.1	7.4
E4542N2634	FK37	potato	7.17	7.2		45.8	200	9	75	2.4	1.38	0.147	63.6	32.7	3.7
E4594N2825	K4	potato	6.26	6.3		18.4	80	50	415	5	2.93	0.273	36.5	50.4	13
E4602N2676	FK43	potato	6.02	6		2.1	9	7	58	6.2	3.62	0.351	50.1	44.9	4.9
E4603N2777	FK34	potato	6.99	7		21.4	93	29.7	247	4.7	2.76	0.304	43.5	44.2	12.3
E4614N2830	FK16	potato	5.81	5.8		12.9	65	41.2	342	3.2	1.87	0.191	53.5	37.9	8.5
E4617N2772	FK35	potato	6.58	6.6		10.4	45	24.3	202	2.2	1.28	0.152	10.4	78.8	10.8
E4618N2808	FK14	potato	7.49	7.5		27.5	120	21.6	179	1.8	1.06	0.122	64.8	31.3	3.9
E4619N2801	FK27	potato	5.36	5.4	6.55	8.7	47	11.3	94	1.6	0.94	0.126	20.8	55.7	23.5
E4632N2796	FK25	potato	7.43	7.4		20.2	88	14.3	119	3.1	1.79	0.18	45.7	48.6	5.7
E4648N2800	K1	potato	7.42	7.4		63.7	278	29.4	244	2.5	1.48	0.168	39.2	47.9	12.9
E4665N2797	FK11	potato	7.49	7.5		18.2	80	25	208	3.2	1.87	0.206	23.1	69.6	7.3
E4679N2683	FK36	potato	7.04	7		31.5	137	16	133	3.3	1.93	0.21	45.4	45.6	9
E4680N2683	FK45	potato	6.29	6.3		13.1	57	36.1	300	3.6	2.11	0.222	44.2	48.1	7.7
E4688N2851	FK41	potato	4.97	5	6.17	15.4	76	34.1	283	4.7	2.74	0.273	56.8	33.9	9.3
E4689N2841	FK1	potato	5.79	5.8		0.6	13	14.9	124	4.7	2.71	0.272	51.5	37.4	11.1
E4691N2856	FK42	potato	5.03	5	6.17	12.3	63	38.2	317	5.1	2.94	0.293	60.8	31.8	7.4
E4695N2860	FK6	potato	5.52	5.5		9.8	52	19.8	164	2.8	1.61	0.17	51.4	38.6	10
E4696N2847	FK44	potato	5.37	5.4		11	57	32.4	269	3.7	2.15	0.212	58.9	32.8	8.3
E4696N2886	FK40	potato	6.29	6.3		8.6	38	12.1	101	4.2	2.43	0.223	44.2	47.3	8.6
E4698N2868	FK39	potato	4.58	4.6	6.16	9.2	49	18.9	157	4.3	2.51	0.237	66.7	25.9	7.4
E4699N2846	FK24	potato	5.13	5.1	6.3	14.7	73	11.2	93	2.5	1.47	0.158	54.6	37.1	8.3

			pH	pH	pH	Phosphate	Phosphor	Potassium oxide	Potassium	Humus	TOC	Nitrogen (total)	Sand	Slit	Clay
			-	-	-	mg/100g	mg/kg	mg/100g	mg/kg	%	%	%	%	%	%
Number of test area	**Number of test field**	**Crop**	pH $CaCl_2$-MW Acidity acc. ÖNORM L 1083	pH in $CaCl_2$	In Ca-acetate (for estimation of liming demand in acid soils)	CAL-MW	Plant available P in CAL acc. ÖNORM L 1087	CAL-MW	Plant available K in CAL acc. ÖNORM L 1087	TOC * 1,72	Total organic carbon acc. ÖNORM L 1080	total nitrogen acc. ÖNORM L 1095	Particle size < 2 µm acc. ÖNORM L 1061-2	Particle size 2-63 µm acc. ÖNORM L 1061-2	Particle size 63-2000 µm acc. ÖNORM L 1061-2
E4732N2797	FK19	potato	6.8	6.8		7.9	35	7.6	63	1.8	1.05	0.133	7.1	67.7	25.2
E4747N2858	FK18	potato	7.47	7.5		26.8	117	21.2	176	2.1	1.22	0.149	13.9	60.7	25.4
E4747N2859	FK23	potato	7.37	7.4		9	39	11.7	97	2.3	1.32	0.152	19.6	57.4	23
E4761N2848	FK8	potato	7.62	7.6		4.9	21	8.2	68	2.9	1.68	0.183	16.9	60.4	22.7
E4762N2848	FK2	potato	7.65	7.7		18.3	80	20.9	174	1.9	1.08	0.128	24.8	48.7	26.6
E4764N2850	FK7	potato	7.53	7.5		4.3	19	8.5	70	2.6	1.53	0.173	10	62.8	27.2
E4765N2819	FK3	potato	7.32	7.3		9.1	40	18.3	152	4.3	2.51	0.264	17.5	46	36.5
E4767N2828	FK21	potato	7.65	7.7		4.3	19	7.9	65	2.5	1.46	0.154	23.8	52	24.2
E4767N2830	FK26	potato	7.65	7.7		3.3	15	3.9	32	2	1.16	0.124	16.7	66.7	16.6
E4773N2824	FK10	potato	7.59	7.6		28.1	122	9.8	81	1.7	0.99	0.099	38.7	54.6	6.7
E4773N2838	FK5	potato	7.57	7.6		54.4	237	29.7	247	2.8	1.62	0.153	23.8	60.6	15.5
E4776N2694	FK31	potato	6.54	6.5		21.3	93	29.4	244	2.1	1.24	0.139	57.5	29.7	12.8
E4779N2835	FK15	potato	7.6	7.6		15.1	66	23	191	3.2	1.84	0.191	11.9	66.2	21.8
E4783N2830	FK20	potato	7.52	7.5		20.5	89	19.6	163	3	1.74	0.191	17.2	62.2	20.6
E4793N2838	FK30	potato	7.63	7.6		13.1	57	17.8	148	1.8	1.06	0.1	19	64	17
E4796N2849	FK12	potato	7.6	7.6		21	92	15.2	126	1.9	1.09	0.116	11.5	66.7	21.9
E4798N2820	FK13	potato	7.61	7.6		29.7	130	23.3	193	3	1.74	0.162	37.2	51.2	11.6
E4800N2823	FK4	potato	6.38	6.4		17.4	76	17.8	148	1.7	1	0.108	58.3	30	11.7
E4800N2840	K2	potato	7.55	7.6		35.7	155	22.7	189	2.1	1.23	0.121	29.4	52.1	18.5
E4801N2844	FK9	potato	7.56	7.6		13.4	58	13	108	2.4	1.4	0.129	12	64.2	23.8
E4808N2808	FK32	potato	7.63	7.6		24.3	106	20.4	169	2.7	1.56	0.151	29	57.4	13.6
E4824N2812	K5	potato	7.91	7.9		18.3	80	19.8	164	3.3	1.89	0.148	29.5	50.8	19.7
E4828N2857	FK29	potato	7.44	7.4		33.9	148	19.1	159	2.1	1.21	0.119	44.7	41.4	13.9
E4828N2862	FK33	potato	7.35	7.4		8	35	14.5	120	2.5	1.43	0.141	14.4	61.8	23.8
E4834N2816	K3	potato	7.44	7.4		20.1	88	11.3	93	1.6	0.91	0.101	59.3	26.8	13.9
E4840N2772	FK22	potato	7.35	7.4		13.7	60	13.1	109	3.1	1.82	0.176	36.3	40.8	22.9
Minimum:			4.58	4.6	6.12	0.6	9	3.9	32	1.6	0.91	0.099	7.1	25.2	3.7
Maximum:			7.91	7.9	6.55	63.7	278	57.1	474	6.3	3.68	0.376	71	78.8	36.5
Mean:			6.83	6.8	6.27	20	89	23.1	191	3.2	1.83	0.192	34.6	49.8	15.5
Median:			7.31	7.3	6.2	17.8	78	20.3	169	2.9	1.68	0.177	34.3	48.8	15

Table 6: Soil parameters of tested fields - basic analysis data used for routine characterization of soils.

CAL: Calcium-Acetate-Lactate-Extract

				Boron	Boron	Cadmium	Copper	Zinc	Iron	Manganese
				mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	g/kg	g/kg
Number of test area	**Number of test field**	**Crop**	**Soil type**	Boron in soils MW	Plant available Boron acc. ÖNORM L 1090	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085
E4574N2632	M5	maize	Fluvisol/neutral	0.41	0.4	0.4	35.5	85.1	35	0,51
M34_0009	M1	maize	Fluvisol/acidic	0.38	0.4	0.3	30.7	90.7	46	0,85
M34_0020	M2	maize	Cambisol/acidic	0.34	0.3	0.2	11.7	54	27	0,5
M34_0055	M3	maize	Chernozem/alkaline	1.65	1.7	0.3	14.1	37.7	18	0,35
M34_0124	M4	maize	Relict soil/acidic	0.26	0.3	0.4	38	66.3	31	1,1
E4594N2825	K4	potato	Cambisol/acidic	0.47	0.5	0.4	28.3	106.3	33	1
E4648N2800	K1	potato	Fluvisol/alkaline	1.27	1.3	0.3	24.9	103.8	22	0,57
E4800N2840	K2	potato	Cambisol/alkaline	1.3	1.3	0.3	17.5	45.9	22	0,59
E4824N2812	K5	potato	Chernozem/alkaline	2.33	2.3	0.3	15.2	36.8	18	0,37
E4834N2816	K3	potato	Chernozem/neutral	0.9	0.9	0.3	13	35.1	17	0,43
Minimum:				0,26	0,3	0.2	11.7	35.1	17	0.35
Maximum:				2,33	2,3	0.4	38	106.3	46	1.1
Mean:				0,93	0,9	0.3	22.9	66.2	27	0.63
Median:				0,69	0,7	0.3	21.2	60.1	24	0.54

Table 7: Soil parameters of five reference soil types with cultivation of maize and five reference soil types with cultivation of potatoes.

Additional analysis data.

Maize fields FM1 - FM45	pH	pH	pH	Phosphate	Phosphor	Potassium oxide	Potassium	Humus	TOC	Nitrogen (total)	Sand	Slit	Clay
	-	-	-	mg/100g	mg/kg	mg/100g	mg/kg	%	%	%	%	%	%
	pH $CaCl_2$-MW Acidity acc. ÖNORM L 1083	pH in $CaCl_2$	In Ca-acetate (for estimation of liming demand in acid soils)	CAL-MW	Plant available P in CAL acc. ÖNORM L 1087	CAL-MW	Plant available K in CAL acc. ÖNORM L 1087	TOC * 1,72	Total organic carbon acc. ÖNORM L 1080	total nitrogen acc. ÖNORM L 1095	Particle size < 2 µm acc. ÖNORM L 1061-2	Particle size 2-63 µm acc. ÖNORM L 1061-2	Particle size 63-2000 µm acc. ÖNORM L 1061-2
Minimum:	5.06	5.10	6.12	3.90	17.00	4.90	41.00	1.70	0.96	0.10	8.00	25.20	3.80
Maximum:	7.71	7.70	6.40	59.80	261.00	57.10	474.00	6.30	3.68	0.38	71.00	77.50	34.40
Mean:	6.77	6.77	6.26	21.12	93.88	25.85	214.62	3.28	1.91	0.20	33.49	50.25	16.28
Median:	7.07	7.10	6.26	18.10	82.00	22.35	185.00	2.95	1.71	0.19	32.95	48.90	16.65

Table 8: Soil parameters results: maize fields.

Potato fields FK1 - FK45	pH	pH	pH	Phosphate	Phosphor	Potassium oxide	Potassium	Humus	TOC	Nitrogen (total)	Sand	Slit	Clay
	-	-	-	mg/100g	mg/kg	mg/100g	mg/kg	%	%	%	%	%	%
	pH $CaCl_2$-MW Acidity acc. ÖNORM L 1083	pH in $CaCl_2$	In Ca-acetate (for estimation of liming demand in acid soils)	CAL-MW	Plant available P in CAL acc. ÖNORM L 1087	CAL-MW	Plant available K in CAL acc. ÖNORM L 1087	TOC * 1,72	Total organic carbon acc. ÖNORM L 1080	total nitrogen acc. ÖNORM L 1095	Particle size < 2 µm acc. ÖNORM L 1061-2	Particle size 2-63 µm acc. ÖNORM L 1061-2	Particle size 63-2000 µm acc. ÖNORM L 1061-2
Minimum:	4.58	4.60	6.16	0.60	9.00	3.90	32.00	1.60	0.91	0.10	7.10	25.90	3.70
Maximum:	7.91	7.90	6.55	63.70	278.00	50.00	415.00	6.20	3.62	0.35	66.70	78.80	36.50
Mean:	6.90	6.91	6.27	18.76	83.53	20.22	167.88	3.02	1.76	0.18	35.80	49.39	14.81
Median:	7.37	7.40	6.17	15.40	76.00	19.10	159.00	2.80	1.61	0.16	37.20	48.70	12.90

Table 9: Soil parameters results: potato fields.

Maize fields M1 – M5	Boron	Boron	Cadmium	Copper	Zinc	Iron	Manganese
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	g/kg	g/kg
	Boron in soils MW	Plant available Boron acc. ÖNORM L 1090	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085	Extraction in Aqua regia acc. ÖNORM L 1085
Minimum:	0.26	0.30	0.20	11.70	37.70	18.00	0.35
Maximum:	1.65	1.70	0.40	38.00	90.70	46.00	1.10
Mean:	0.61	0.62	0.32	26.00	66.76	31.40	0.66
Median:	0.38	0.40	0.30	30.70	66.30	31.00	0.51

Table 10: Soil parameters additional data results: maize fields.

Potato fields K1 – K5	Boron mg/kg	Boron mg/kg	Cadmium mg/kg	Copper mg/kg	Zinc mg/kg	Iron g/kg	Manganese g/kg
	Boron in soils MW	Boron in soils	Heavy metals in KW	Heavy metals in KW	Heavy metals in KW	Iron-manganese in KW	Iron-manganese in KW
Minimum:	0.47	0.50	0.30	13.00	35.10	17.00	0.37
Maximum:	2.33	2.30	0.40	28.30	106.30	33.00	1.00
Mean:	1.25	1.26	0.32	19.78	65.58	22.40	0.59
Median:	1.27	1.30	0.30	17.50	45.90	22.00	0.57

Table 11: Soil parameters additional data results: potato fields.

1.8 DNA extraction and microbiological analysis of soil samples

For the determination of the baseline frequency of nptII and nptIII in relevant agricultural sites in Austria culture dependent and culture independent approaches were performed. The quantity of nptII and nptIII genes in total soil DNA was determined (for details see sections 1.9.6 and 1.9.7). Additionally a range of fastidious and potential heterotrophic bacteria resistant to kanamycin were grown by standard microbiological cultivation techniques. These isolates were checked for the presence of nptII and nptIII genes in order to link the kanamycin resistance to a distinct bacterial species. Furthermore, bacterial biodiversity studies on the soils of the 10 reference fields representing the variety of soil type clusters combined with varying soil acidity for maize and potato fields in Austria were performed (M1 – M5 and K1 – K5; see Table 1). These soil types were representative of all collected soil types concerning the respective cultivated field crops in Austria. The studies should throw light on the composition of bacterial taxa of these different soil types in order to allow a correlation of the abundance of nptII and nptIII genes to certain groups of bacteria.

1.8.1 Total DNA extraction from soil with the PowerSoil™ DNA Isolation Kit.

The total DNA from soil (mixed and sieved to 2 mm) was isolated from the amount of 1 g fresh weight per sample. This amount was described to be adequate for having insignificant variations in genetic community structure within replicates when assessing the bacterial community structure of forest soils (18). DNA from four soil aliquots with 0.25 g each were extracted with the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions and the eluted DNA of the four aliquots was pooled. The obtained total soil DNA was kept at approx. -20^{o}C for further analyses.

1.8.2 Isolation and characterization of kanamycin tolerant bacterial strains from selected soil samples

1.8.2.1 Soil sample preparation for plating

Soil samples of selected soils covering the range of varying soil type clusters for maize and potato sites were processed according to Steven et al. (57) described in the chapter "Microbial enumeration and characterization", using 0.75 – 1 mm glass beads (Carl Roth GmbH, Karlsruhe, Germany). Decimal dilution series were plated on Standard I Nutrient Agar and R2A (Merck, Darmstadt, Germany). The media were provided with fungicides benomyl (Dr. Ehrestorfer, Augsburg, Germany) 20 µg/ml, nistadin (Merck, Darmstadt, Germany) 20 µg/ml and cycloheximide (Sigma-Aldrich, Steinheim Germany) 50 µg/ml) whereas another lot of plates were additionally supplied with 100µg/ml kanamycin disulphate (Sigma-Aldrich, Steinheim Germany). The fungicide concentrations were chosen according to following publications (17, 18, 50, 58, 59) and personal experience in the laboratory.

1.8.2.2 Determination of the kanamycin resistance ratio

After counting bacterial cells on the two media with and without kanamycin the total number was determined counted and a resistance index was calculated. This index consists of the total number of counted bacteria per number of kanamycin (100 µg/ml) resistant bacteria (see Table 12, Table 13, and Table 14).

From 1 g soil of the tested reference maize fields a mean fraction of 2.3×10^7 cells (range: 5.65×10^6 – 6.05×10^7) grew on complete medium. Approximately 11% of this population showed a high level resistance to kanamycin (range: 0.54 – 19.12 %). From 3×10^7 cells recovered on minimal medium 6.22% on average were resistant (range: 0.34 – 16 %) (Table 12). Approx. 1×10^7 cells from 1 g potato soil could be cultivated on complete medium; 5,78 % of them on average were resistant to kanamycin (range: 0,47 – 9,51 %). A similar situation was encountered on minimal medium with 5.42% of the total cultivable bacterial population being resistant (range: 0.14 – 10.94 %) (Table 13).

From all agricultural soils analysed approx. 1.65×10^7 cells per g soil were recovered on complete medium (minimal medium: 2.16×10^7) with 8.29% of them being resistant to kanamycin (range: 0.47 – 19.12 %). On minimal medium approx. 5.78% were resistant (range: 0.14 – 16 %) (Table 14).

Test field	**Complete medium** (nutrient agar)				**Minimal medium** (R2A agar)			
	CFU/g soil	+ kanamycin (CFU/g soil)	Index[1)]	Kanamycin resistance (%)[2)]	CFU/g soil	+ kanamycin (CFU/g soil)	Index[1)]	Kanamycin resistance (%)[2)]
M1	2.23E07	3.06E06	7.29	13.72	1.37E+07	1.08E+06	12.69	7.88
M2	1.35E07	2.54E06	5.31	18.81	[3)]	[3)]	-	-
M3	1.28E07	2.28E05	56.14	1.78	2.89E+07	9.83E+04	294.00	0.34
M4	6.05E07	3.29E05	183.89	0.54	7.25E+07	4.87E+05	148.87	0.67
M5	5.56E06	1.08E06	5.23	19.12	4.85E+06	7.76E+05	6.25	16.00
Minimum	5.65E+06	2.28E+05	5.23	0.54	4.85E+06	9.83E+04	6.25	0.34
Maximum	6.05E+07	3.06E+06	183.89	19.12	7.25E+07	1.08E+06	294.00	16.00
Mean	2.30E+07	1.45E+06	51.57	10.80	3.00E+07	6.10E+05	115.45	6.22
Median	1.35E+07	1.08E+06	7.29	13.72	2.13E+07	6.32E+05	80.78	4.28
SD	2.18E+07	1.29E+06	77.10	9.06	3.00E+07	4.18E+05	135.99	7.39

Table 12: Maize reference fields. Cultivable cell number and kanamycin resistance on full and minimal medium.

1) Kanamycin resistance quotient,

2) kanamycin resistant strains per analysed population,

3) excluded due to implausible data submitted by the laboratory (mixing the counting plates)

CFU: colony forming unit

M1-M5: bacterial strains from reference soils for maize growing fields

Test field	**Complete medium** (nutrient agar)				**Minimal medium** (R2A agar)			
	CFU/g soil	+ Kanamycin CFU/g soil)	Index	Kanamycin resistance (%)	CFU/g soil	+ Kanamycin (CFU/g soil)	Index	Kanamycin resistance (%)
K1	1.68E+07	9.91E+05	16.95	5.90	2.79E+07	9.47E+05	29.46	3.39
K2	7.65E+06	3.60E+04	212.50	0.47	2.20E+07	3.15E+04	698.41	0.14
K3	5.85E+06	2.56E+05	22.85	4.38	5.10E+06	5.58E+05	9.14	10.94
K4	9.35E+06	8.89E+05	10.52	9.51	1.12E+07	6.10E+05	18.36	5.45
K5	1.03E+07	8.91E+05	11.56	8.65	7.95E+06	5.70E+05	13.95	7.17
Minimum	5.85E+06	3.60E+04	10.52	0.47	5.10E+06	3.15E+04	9.14	0.14
Maximum	1.68E+07	9.91E+05	212.50	9.51	2.79E+07	9.47E+05	698.41	10.94
Mean	9.99E+06	6.13E+05	54.88	5.78	1.48E+07	5.43E+05	153.86	5.42
Median	9.35E+06	8.89E+05	16.95	5.90	1.12E+07	5.70E+05	18.36	5.45
SD	4.17E+06	4.35E+05	88.25	3.62	9.71E+06	3.28E+05	304.50	4.05

Table 13: Potato reference fields. Cultivable cell number and kanamycin resistance on full and minimal medium.

Maize + potato fields	**Complete medium** (nutrient agar)				**Minimal medium** (R2A agar)			
	CFU/g soil	+ Kanamycin (CFU/g soil)	Index	Kanamycin resistance (%)	CFU/g soil	+ Kanamycin (CFU/g soil)	Index	Kanamycin resistance (%)
Minimum	5.65E+06	3.60E+04	5.23	0.47	4.85E+06	3.15E+04	6.25	0.14
Maximum	6.05E+07	3.06E+06	212.50	19.12	7.25E+07	1.08E+06	698.41	16.00
Mean	1.65E+07	1.03E+06	53.22	8.29	2.16E+07	5.73E+05	136.79	5.78
Median	1.16E+07	8.90E+05	14.26	7.27	1.37E+07	5.70E+05	18.36	5.45
SD	1.63E+07	1.01E+06	78.14	7.02	2.12E+07	3.47E+05	231.75	5.37

Table 14: All agricultural soils analysed. Cultivable cell number and kanamycin resistance on full and minimal medium.

1.8.2.3 Isolation of kanamycin resistant bacterial colonies

From the bacterial colonies growing on the plates supplemented with kanamycin per selected soil sample 20 isolates were picked from the Standard I Nutrient Agar and 20 isolates were picked from the R2A plates. These isolates were again plated to obtain pure cultures. An aliquot of the suspensions of these pure cultures were conserved in 20% glycerol at approx. -80°C, another aliquot was boiled and stored at -20°C for further molecular analyses.

1.8.2.4 Characterization of bacterial strains resistant to kanamycin

Aqueous dilutions (1:50) from the boiled bacteria suspensions were used as a template for a bacteria specific PCR on 16S rDNA (64). The resulting PCR amplicons were sequenced with the 0907R primer (described in (42)) by Sanger sequencing (LGC Genomics GmbH, Berlin, Germany). Sequences were aligned with the GenBank database sequences by BLAST search (2). Taxonomic classification is presented in Table 37.

Some strains with a potential to cause disease in humans (e.g. *Klebsiella pneumoniae, Stenotrophomonas maltophilia*) were detected in a few fields (1, 35, 41). These strains were highlighted in Table 37. No strains at the species level known to be capable of developing competence for DNA uptake could be identified.

1.8.2.5 NptII/nptIII prevalence in kanamycin resistant strains from agricultural soils

For more information please see section 1.12.

1.9 Real time PCR

1.9.1 Total DNA extraction from soil samples

DNA was isolated from 1 g of soil (= composite soil sample comprising of 10 soil subsamples from a single test field, mixed and sieved to 2 mm) using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions (for details please see 1.8.1).

1.9.2 16S TaqMan real time PCR assay - Check for the presence of amplifiable DNA

Each of the 100 soil sample DNA isolates were checked for the presence of amplifiable DNA by adding 2 µl of a 10^{-1} dilution of the original PowerSoil DNA eluate to the 16S TaqMan real time PCR assay. For PCR assay and amplification conditions see section 3.3. All samples were analysed at least in duplicates. Both assays had to be positive to achieve a positive 16S TaqMan assessment.

16S TaqMan PCR results were classified as positive, if the crossing point (Cp) value was <27. If the amplification curve showed a Cp ≥27, the assay was classified as negative. A sample retrieving a single negative 16S PCR result was re-analysed and – if again negative – excluded from statistical analysis if nptII and/or nptIII PCRs were also negative.

1.9.3 NptII/nptIII TaqMan double screening assay for total soil DNA

Each of the 100 soil sample DNA isolates were checked for the presence of nptII and nptIII genes by adding 2 µl of a 10^{-1} dilution of the original PowerSoil DNA eluate to the nptII/nptIII TaqMan real time PCR double assay. For PCR conditions please see section 3.2. All of the samples were tested at least in duplicates, some of them four times. A single positive replicate was sufficient to classify the sample as positive.

The same DNA extraction solution from each sample was tested with the nptII/nptIII TaqMan Double Assay and with the 16S TaqMan DNA extraction/amplification control assay. Samples with a negative 16S TaqMan assay result (Cp > 27) were excluded from statistical analysis if the nptII and/or the nptIII PCR result were negative. According to the results of the quantitative PCR assays (Table 23) and taking into account a DNA recovery rate from soil of 50 – 60 % as established in the pilot study for the PowerSoil procedure (section 2.1.2, Table 34) the detection limit for nptII and nptIII was approx. 500 – 700 copies per gram soil. All samples were checked for inhibition by analysing 1:5 and 1:25 serial dilutions.

A sample was classified as nptII and/or nptIII positive if PCR amplification occurred before Cp 40. Single replicates producing an amplification curve with a Cp ≥40 were re-analysed. A sample was classified as nptII and/or nptIII negative only, if the 16S rRNA TaqMan assay was positive and all nptII/nptIII replicates showed no amplification in the respective PCR assay.

1.9.4 Results: Screening for nptII/nptIII in total DNA isolated from the tested soil sample collection

From 100 soil samples six tested positive for the presence of nptII genes. 85 soil samples were positive for nptIII (Table 15). A statistical evaluation retrieved a prevalence estimator for nptII of 6 % in the analysed soil sample collection within a 95% confidence interval of 2.2% and 12.6%. A prevalence estimator of 85% was calculated for nptIII (95% confidence interval: 76.5% - 91.4%; Table 15). Within a confidence interval of 95% less than 12.6% of maize and potato fields in Austria harbour nptII and up to 91.4% contain nptIII resistance genes above the detection limit.

For detailed results of the real time TaqMan PCR assays see section 2.2, Table 35.

	Number of soil samples	Number of positive soil samples	Prevalence estimator	Confidence interval (95%)[1)]
nptII	100	6	6%	[2.2%;12.6%]
nptIII	100	85	85%	[76.5%;91.4%]

Table 15: Abundance of nptII/nptIII positive maize and potato fields in Austria.

1) Two-sided confidence interval according to Pearson-Clopper.

The results show a striking difference between nptII and nptIII prevalences in Austrian soil samples with nptII being substantially less frequently detectable in soils compared to nptIII.

	Number of soil samples	Number of positive soil samples	Prevalence estimator	Confidence interval (95%)[1)]
nptII	50	3	6%	[1.3%;16.6%]
nptIII	50	46	92%	[80.8%;97.8%]

Table 16: Abundance of nptII/nptIII positive maize fields in Austria.

1) Two-sided confidence interval according to Pearson-Clopper.

Within a confidence interval of 95% less than 16.6% of maize fields in Austria harbour nptII and up to 97.8% contain nptIII resistance genes (Table 16).

	Number of soil samples	Number of positive soil samples	Prevalence estimator	Confidence interval (95%)[1)]
nptII	50	3	6%	[1.3%;16.6%]
nptIII	50	39	78%	[64.0%;88.5%]

Table 17: Abundance of nptII/nptIII positive potato fields in Austria.

1) Two-sided confidence interval according to Pearson-Clopper.

Within a confidence interval of 95% less than 16.6% of potato fields in Austria harbour nptII and up to 88.5% contain nptIII resistance genes (Table 17).

1.9.5 Quantification of the 16S rRNA gene copy number in soil samples

The quantitative 16S TaqMan assay was performed as described in section 3.4, Table 64.

Quantitative data were obtained by absolute quantification using an external standard curve which was prepared by a semi logarithmic dilution of a plasmid with an *E. coli* 16S gene insert.

For the establishment of the rRNA gene copy number 2 µl of a 10^{-2} dilution of the original PowerSoil total DNA eluate were used as template for the quantitative PCR assay. The samples were tested in quadruples. Copy number calculations were performed using the second derivative maximum algorithm provided in the LightCycler LC480 software package (version 1.5) with default parameters.

1.9.5.1 Results of the 16S rRNA gene copy number determination

A mean concentration of 16S rRNA genes in the 50 maize fields was 1.74E+09 copies per g soil (range 3.04E+08 - 5.48E+09; Table 18). The results of the 50 soil samples from potato fields can be found in Table 19. For all fields analysed a mean copy number of 1.51E+09 per g soil was obtained (Table 20). The 16S rRNA gene copy number is a surrogate marker for the approximate number of bacterial cells in 1 g of the soil sample under investigation. An overall analysis of the results indicates a good correlation with available data in the literature (approx. 10^9 cells/g soil) (21). The detailed PCR results can be found in section 2.5

16S rRNA	Copy number/g soil	SD[1)]
Minimum[2)]	3.04E+08	-
Maximum[3)]	5.48E+09	-
Mean	1.74E+09	9.29E+08
maize fields[4)]	50	-

Table 18: 16S rRNA gene copy numbers: maize fields

1) Standard deviation 2) field with the lowest 16S rRNA copy number

3) Field with the highest 16S rRNA copy number 4) number of maize fields tested

16S rRNA	Copy number/g soil	SD[1)]
Minimum[2)]	5.88E+07	-
Maximum[3)]	3.52E+09	-
Mean	1.28E+09	8.13E+08
potato fields[4)]	50	-

Table 19: 16S rRNA gene copy numbers: potato fields

1) Standard deviation 2) field with the lowest 16S rRNA copy number

3) Field with the highest 16S rRNA copy number 4) number of potato fields tested

16S rRNA	Copy number/g soil	SD[1)]
Minimum[2)]	5.88E+07	-
Maximum[3)]	5.48E+09	-
Mean	1.51E+09	8.97E+08
maize + potato fields[4)]	100	-

Table 20: 16S rRNA gene copy numbers: summary maize and potato fields

1) Standard deviation 2) field with the lowest 16S rRNA copy number

3) Field with the highest 16S rRNA copy number 4) number of all fields tested

1.9.6 Quantification of the nptII gene copy number in soil samples

DNA of each of the 6 nptII positive soil sample was concentrated by vacuum centrifugation of 100 µl of the original PowerSoil eluate for 30 min. The dried pellet was resuspended in 20 µl of H_2O (Sigma, Austria, molecular biology grade) and stored at -20°C for further analysis. Two µl of the concentrated solution were used as template for the quantitative nptII TaqMan single assay. Sample eluates which showed inhibition were re-purified with the Powerclean Kit (MoBio Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions.

The background load with nptII gene copies of the analysed fields is low showing a mean concentration of approx. 340 nptII copies per gram soil (Table 23). The highest concentrations at approx. 850 copies per gram soil were found in a potato field (Table 21). For detailed results see section 2.6.

NptII	Copy number/g soil	SD[1)]
Minimum[2)]	1.34E+02	-
Maximum[3)]	8.56E+02	-
Mean	3.76E+02	4.16E+02
maize fields[4)]	3	-

Table 21: NptII gene copy numbers: maize fields

2) Standard deviation 2) field with the lowest nptII gene copy number

3) Field with the highest nptII gene copy number 4) number of maize fields tested

NptII	Copy number/g soil	SD[1)]
Minimum[2)]	3.12E+01	-
Maximum[3)]	7.16E+02	-
Mean	3.03E+02	3.64E+02
potato fields[4)]	3	-

Table 22: NptII gene copy numbers: potato fields

1) Standard deviation 2) field with the lowest nptII gene copy number

3) Field with the highest nptII gene copy number 4) number of potato fields tested

NptII	Copy number/g soil	SD[1)]
Minimum[2)]	3.12E+01	-
Maximum[3)]	8.56E+02	-
Mean	3.40E+02	3.52E+02
maize + potato fields[4)]	6	-

Table 23: NptII gene copy numbers: summary maize and potato fields

1) Standard deviation 2) field with the lowest nptII gene copy number

3) Field with the highest nptII gene copy number 4) number of all fields tested

1.9.7 Quantification of the nptIII gene copy number in soil samples

DNA of each of the 85 nptII positive soil sample was concentrated by vacuum centrifugation of 100 µl of the original PowerSoil eluate for 30 min. The dried pellet was resuspended in 20 µl of H_2O (Sigma, Austria, molecular biology grade) and stored at -20°C for further analysis. Two µl of the concentrated solution were used as template for the quantitative nptIII TaqMan single assay. Sample eluates which showed inhibition were re-purified with the Powerclean Kit (MoBio Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions.

The background load of the analysed fields is distinctly higher concerning nptIII. A mean concentration of approx. 4750 nptIII copies per gram soil can be expected in nptIII positive agricultural fields (Table 26). The highest concentration at approx. 62 000 copies per gram soil was encountered in a maize field (Table 24). For detailed results see section 2.7.

NptIII	Copy number/g soil	SD[1)]
Minimum[2)]	4.68E+01	-
Maximum[3)]	6.16E+04	-
Mean	5.92E+03	1.19E+04
maize fields[4)]	46	-

Table 24: NptIII gene copy numbers: maize fields

1) Standard deviation 2) field with the lowest nptIII gene copy number

3) Field with the highest nptIII gene copy number 4) number of maize fields tested

NptIII	Copy number/g soil	SD[1)]
Minimum[2)]	1.32E+01	-
Maximum[3)]	2.91E+04	-
Mean	3.37E+03	5.40E+03
potato fields[4)]	39	-

Table 25: NptIII gene copy numbers: potato fields

1) Standard deviation 2) field with the lowest nptIII gene copy number

3) field with the highest nptIII gene copy number 4) number of potato fields tested

NptIII	Copy number/g soil	SD[1)]
Minimum[2)]	1.32E+01	-
Maximum[3)]	6.16E+04	-
Mean	4.75E+03	9.49E+03
maize + potato fields[4)]	85	-

Table 26: NptIII gene copy numbers: summary maize and potato fields

1) Standard deviation 2) field with the lowest nptIII gene copy number

3) field with the highest nptIII gene copy number 4) number of all fields tested

1.9.8 Relative quantification for comparison of gene copy numbers between fields

For a quantitative comparison of the NptII/nptIII gene copy background load between the test fields a normalisation of the gene copy numbers retrieved in sections 1.9.6 and 1.9.7 was performed. This procedure was necessary to compensate for different DNA recovery rates from the soils of the different test fields. The goal was achieved by referencing the NptII/nptIII gene copy number to the obtained 16S rRNA gene copy number of each soil sample. The resulting quotient is independent of the absolute amount of DNA in the 2 µl template solution for the quantitative PCR assays and, thus, an indicator for the gene load which can be used for a quantitative gene load comparison between the test fields. Details on the relative quantification can be found in section 2.8, Table 41. The results are graphically displayed for nptII in Figure 11, Figure 12, and Figure 13. Results for the nptIII background load of the fields under investigation can be found in Figure 14, Figure 15, and Figure 16.

1.9.8.1 Quantitative distribution of nptII/nptIII concentrations on the Austrian map

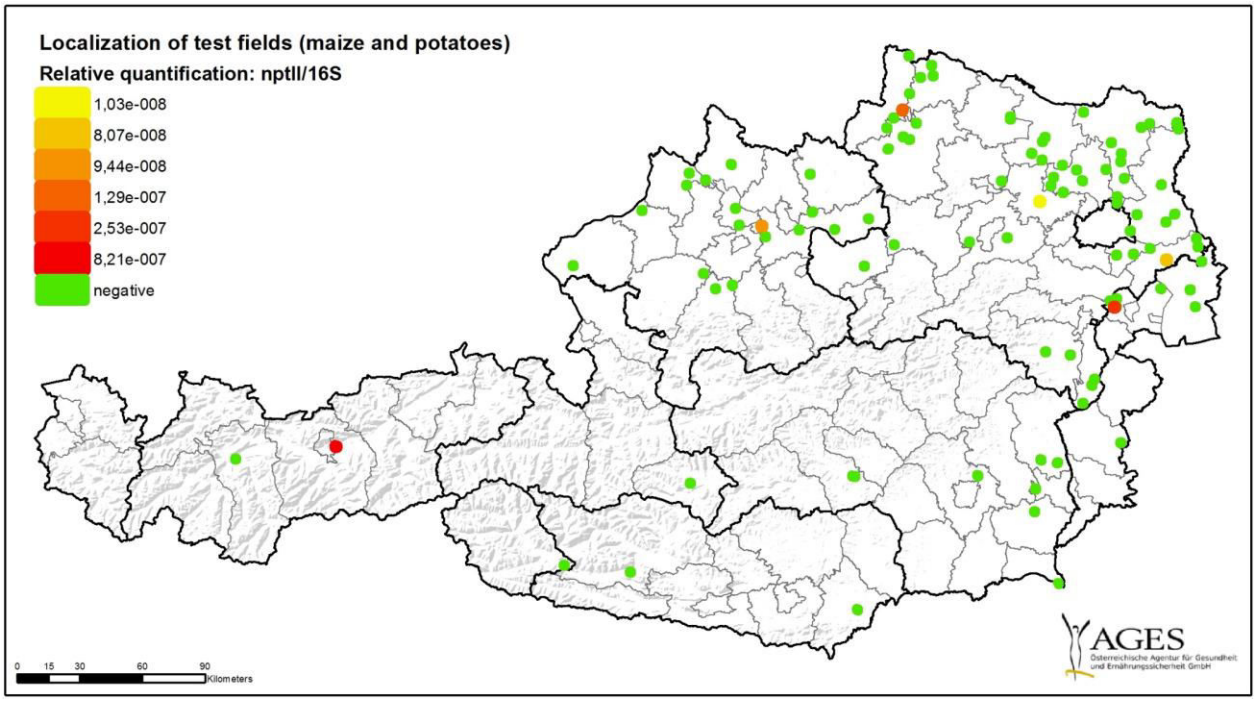

Figure 11: Relative quantification: comparison nptII/maize and potato fields

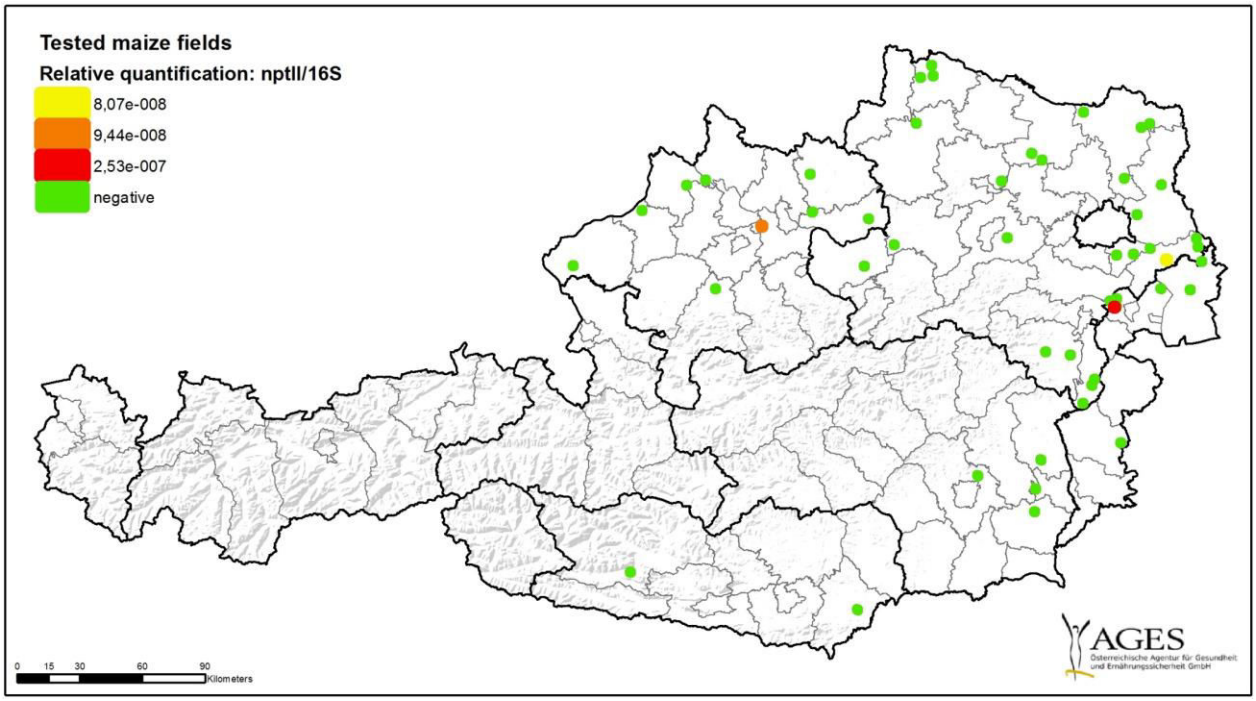

Figure 12: Relative quantification: comparison nptII/maize fields

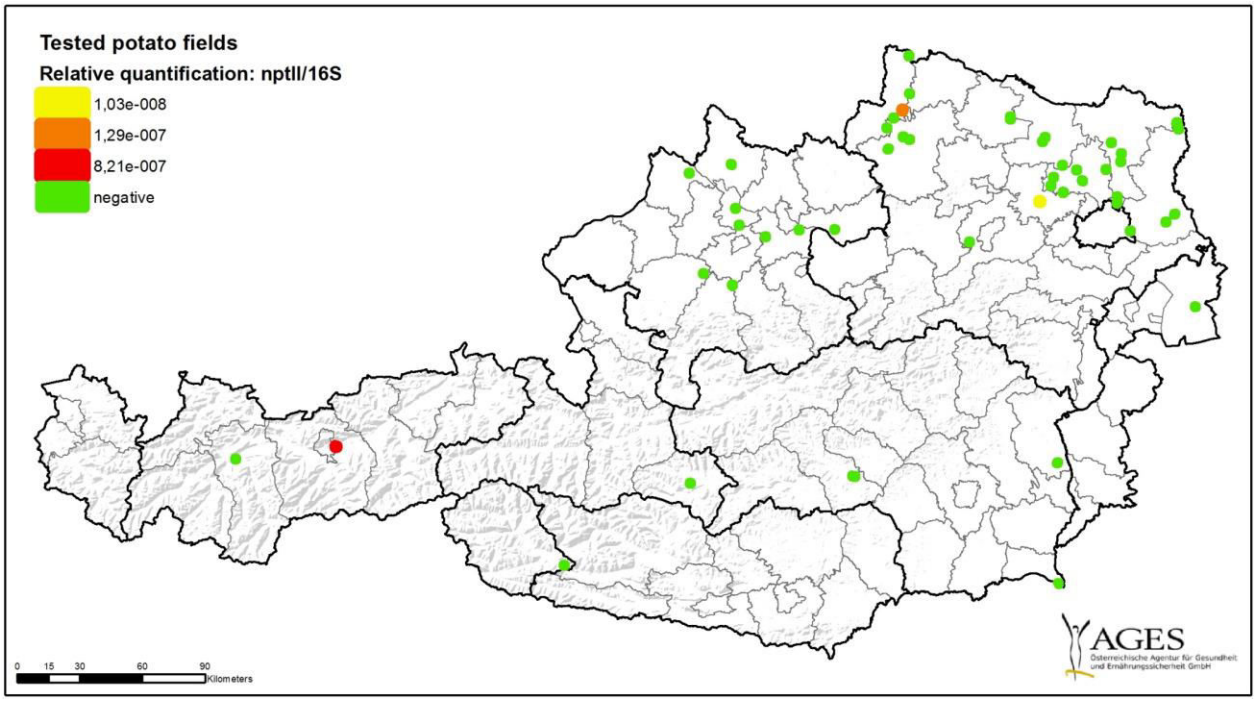

Figure 13: Relative quantification: comparison nptII/potato fields

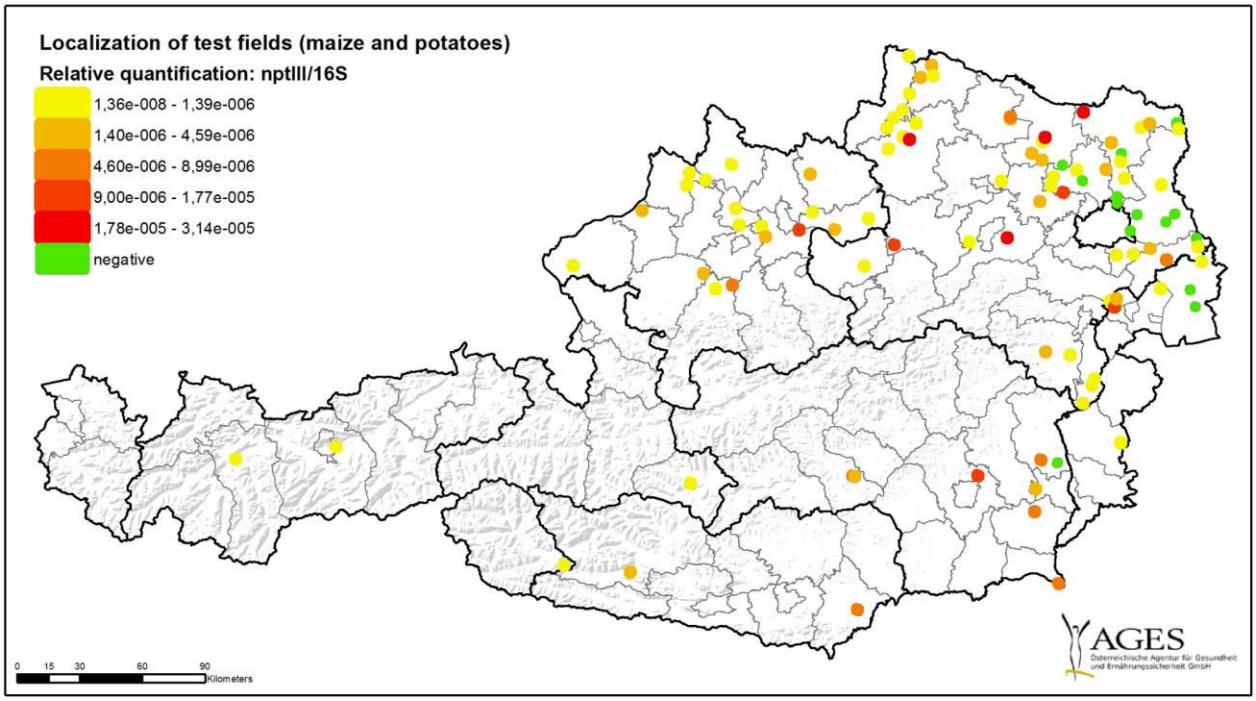

Figure 14: Relative quantification: comparison nptIII/maize and potato fields

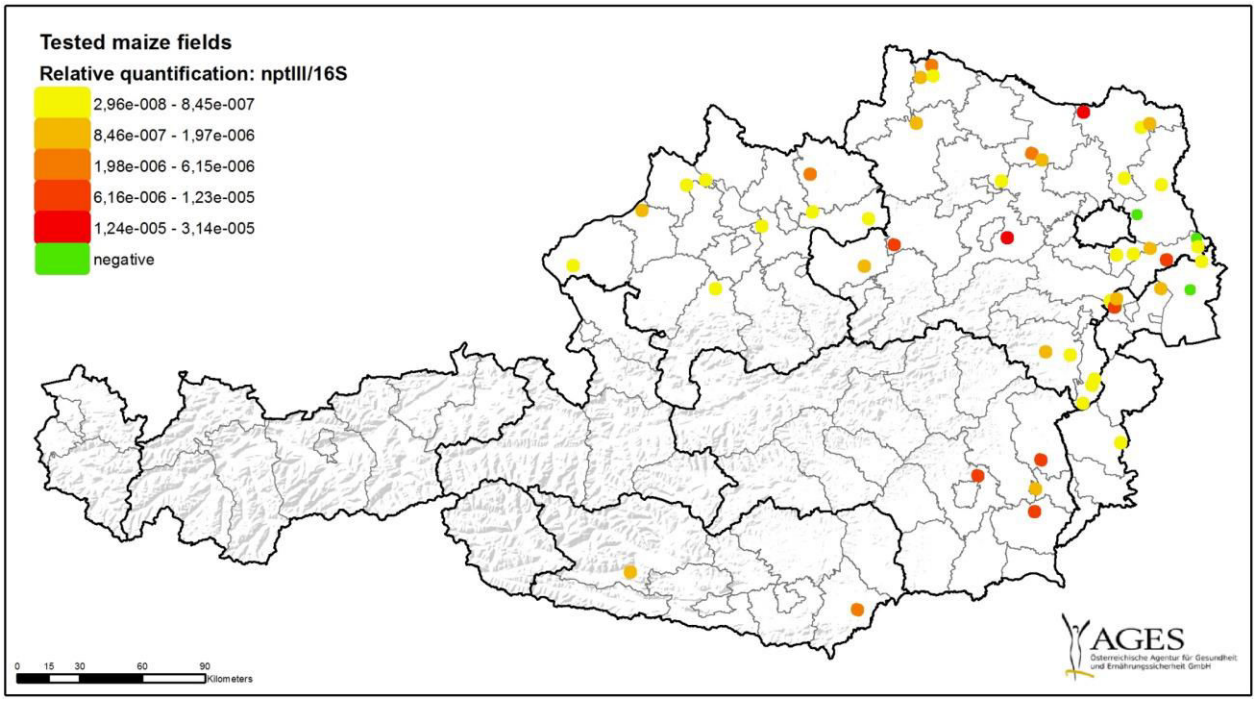

Figure 15: Relative quantification: comparison nptIII/maize fields

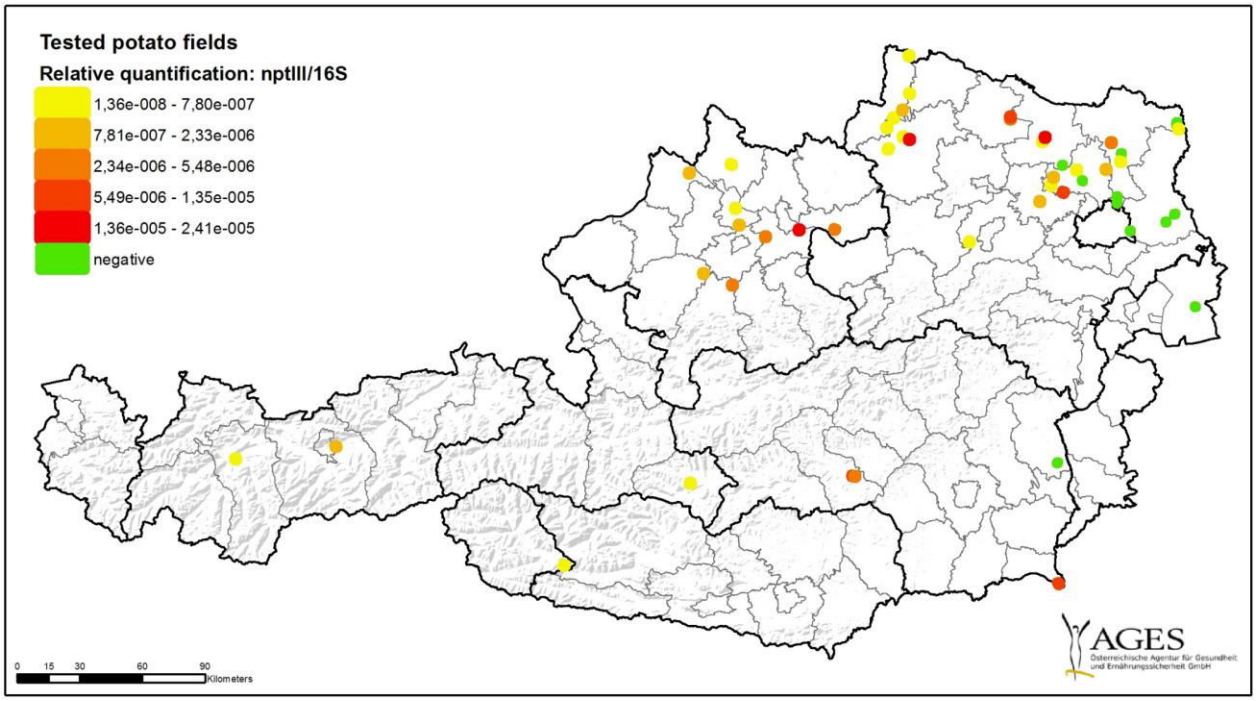

Figure 16: Relative quantification: comparison nptIII/potato fields

1.10 Statistical interpretation of the PCR results

1.10.1 Material/Methods

NptII and nptIII was tested in 100 soil samples. NptII was detected in six and nptIII in 85 soil samples, as can be seen in Figure 17.

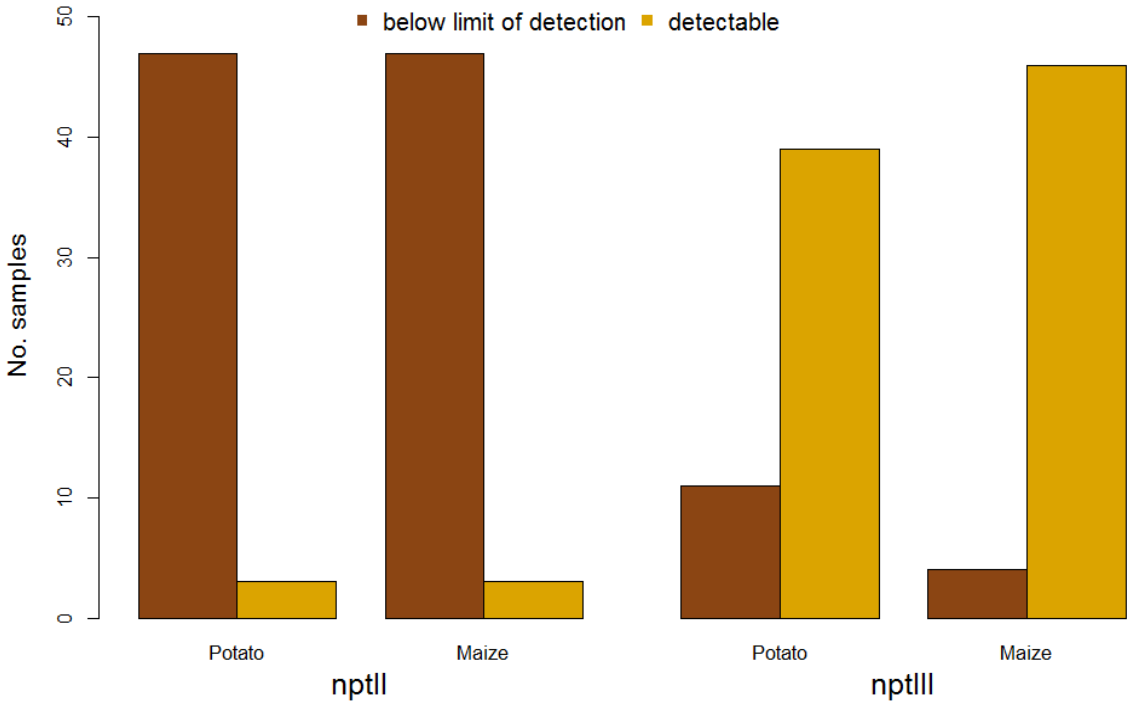

Figure 17: Sample results for nptII and nptIII.

Aside from nptII and nptIII, many additional soil parameters were collected within each soil sample. These parameters were recorded during the sampling procedure, on laboratory analysis and from GIS based data. The essential parameters are listed in Table 27.

Some parameters were excluded from the statistical analyses because they were either not suitable or had high correlation with other parameters: the weather data and air temperature correlated with soil moisture, the parameter "silt" correlated with "sand" and "clay". Furthermore, the parameter “pH: CA-acetate” was removed from the statistical analysis due to an insufficient number of observations. The parameter “inorganic fertilizer” was excluded, because it was divided into a large number of subcategories and not enough observations were available in each category.

	Parameter
During sampling	Crop (Frucht)
	Preceding crop (Vorfrucht)
	Organic fertilizer of animal origin (organischer Dünger tierischen Ursprungs)
	Inorganic fertilizer (anorganischer Dünger)
	Soil humidity (Bodenfeuchtigkeit)
	Weather data (Witterungsdaten)
	Air temperature (Lufttemperatur in °C)
GIS based data	Soil type (Typengruppe)
	Value of humus (Humuswert)
	Water conditions (Wasserverhältnis)
Laboratory analysis	pH: $CaCl_2$
	pH: Ca-acetate
	Phosphorus (P): CAL
	Potassium (Kalium (K): CAL
	Humus (Humusgehalt)
	Sand (Sand)
	Silt (Schluff)
	Clay (Ton)

Table 27: Data source for the soil parameters

The laboratory analysis of one soil sample was missing and for two soil samples the “organic fertilizer of animal origin” was not indicated.

Prior to the statistical analysis, the values of the soil parameters were standardized in order to allow better comparability. The values were centered around zero by subtracting the mean value and scaled by dividing by the standard deviation.

1.10.2 Statistical analyses

The metric soil parameters are illustrated using Box-Whisker-plots (Figure 18, Figure 22 and Figure 26). These plots show the median, upper and lower quartiles, as well as extreme values and outliers. Outliers (displayed as points) are depicted if their distance from the corresponding quartile exceeds 1.5 times the interquartile range. In addition to the Box-Whisker-plots, the metric parameters are furthermore illustrated using the mean values and the corresponding 95% confidence intervals; see Figure 19 and Figure 23 (65).

The non-metric (categorical) soil parameters are illustrated in mosaic plots (Figure 20, Figure 21, Figure 24 and Figure 25). The distribution of the different values of the categorical parameters (x-axis), classified according to the detectability of nptII or nptIII respectively (y-axis), is illustrated using stacked rectangles. The sizes of the rectangles represent the number of cases corresponding to the particular combination of the attributes.

The probability of detecting nptII or nptIII was modeled using logistic regression with a logit link. The probability π of a positive result, or rather the proportion $\pi/(1-\pi)$ of the probability and the complementary probability (the so-called odds), is modeled in relation to relevant (significant) risk factors x_i as follows:

$$\pi_i = \frac{\exp(\boldsymbol{x_i}^T \boldsymbol{\beta})}{1 + \exp(\boldsymbol{x_i}^T \boldsymbol{\beta})}$$

$$\log\left(\frac{\pi_i}{1-\pi_i}\right) = \beta_1 \boldsymbol{x_{1,i}} + \cdots + \beta_p \boldsymbol{x_{p,i}}$$

In practice, the transformed regression coefficients $\exp(\beta_i)$ are used for the interpretation of the relationship between the explanatory variables β_i and the odds, as the transformed values are directly proportional to the odds. A value of $\exp(\beta_i) > 1$ indicates a positive relationship between the corresponding covariate and the odds of a positive result, i.e., the probability of detection is increased in the presence of the risk factor x_i. For categorical data, the influence of a certain covariate is to be interpreted in relation to a pre-defined reference category.

All statistical analyses in this section were done using the open source software R (51).

1.10.3 nptII prevalence in soils: qualitative results

Figure 18 shows a Box-Whisker-plot of the standardized metric soil parameters grouped by the detectability of nptII. Figure 19 shows the same data, illustrated by their mean values and corresponding 95% confidence intervals. The medians of the detectable and the non-detectable cases differ for all parameters with the exception of “Silt”, as can be seen in Figure 18. A clear separation is, however, not visible. Figure 19 shows overlapping confidence intervals for the detectable and the non-detectable cases for all parameters. The 95% confidence intervals of the mean values of the nptII-positive cases are rather wide due to the small number of “positive” observations.

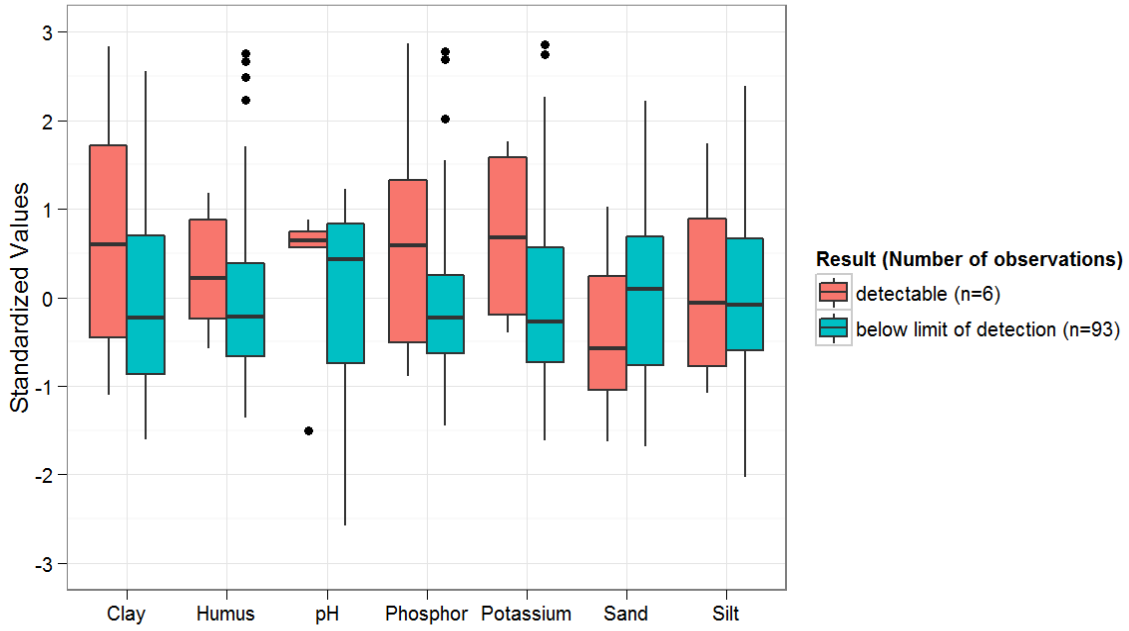

Figure 18: Box-Whisker-Plot of the metric soil parameters grouped by nptII-detectability.

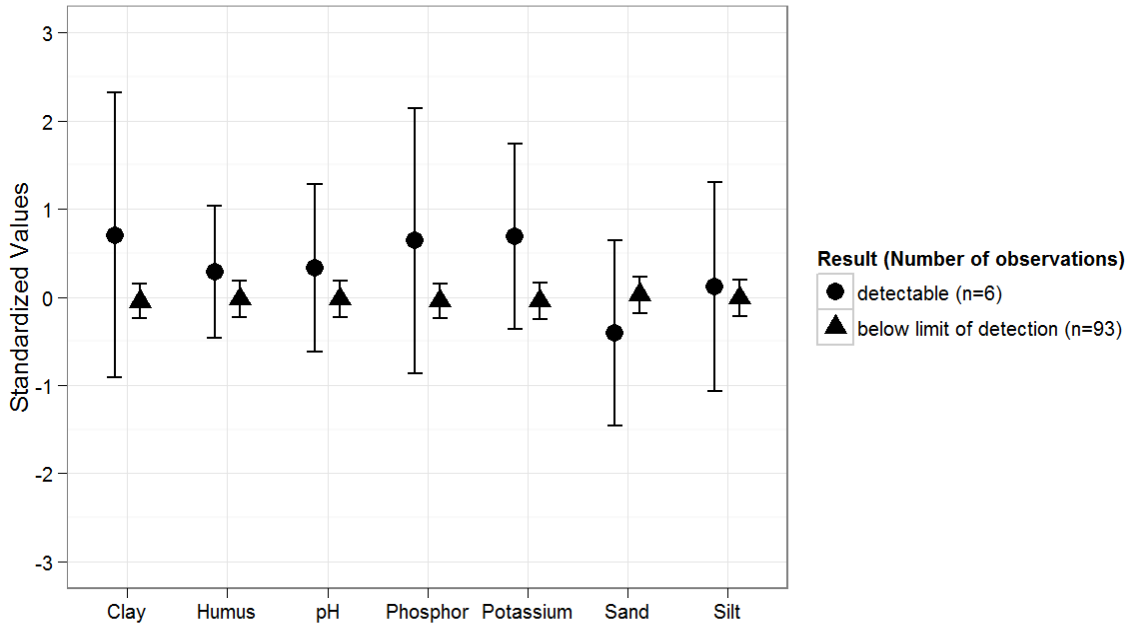

Figure 19: Mean values and related 95% confidence intervals of the metric soil parameters grouped by nptII-detectability.

The non-metric soil parameters are displayed in mosaic plots in Figure 20 and Figure 21. Whether nptII was detected or not is shown on the y-axis. “0” indicates that nptII could not be detected, “1” indicates that nptII was detected in the sample. No nptII was detected for the soils with preceding crop “maize” and for the soil humidity “wet”. The number of nptII positive cases is a bit higher for the

soil type “chernozem” than for the others. In general, the low number of nptII positive soil samples is reflected in the lower areas within the mosaic plots.

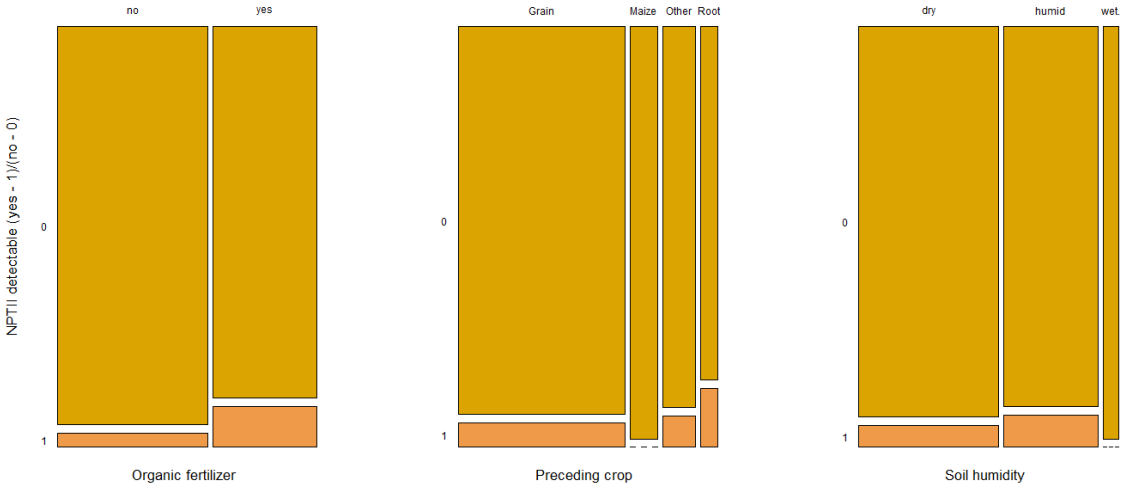

Figure 20: Mosaic plot of nptII-detectability (yes 1/ no 0) for the parameters determined during sampling.

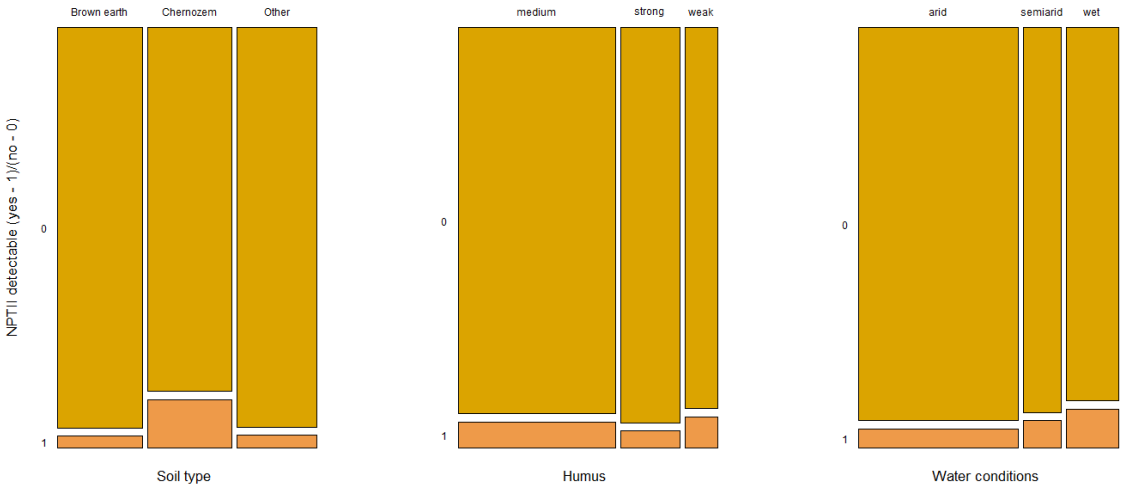

Figure 21: Mosaic plot of nptII-detectability (yes 1/ no 0) for the parameters based on GIS data.

In the logistic regression model, none of the soil parameters showed a statistically significant influence on nptII detectability. For the metric variables, this was already hinted by the overlap of the confidence intervals in Figure 19. The lack of significant parameters may, however, be partly due to the low number of nptII positive cases.

1.10.4 nptIII prevalence in soils: qualitative results

The standardized metric soil parameters for nptIII, grouped by detectability, are shown in Figure 22 and Figure 23. For all soil parameters, with the exception of pH, the Box-Whisker-plots show similar median values and overlapping data. The 95% confidence intervals in Figure 23 also overlap for the different soil parameters with the exception of pH.

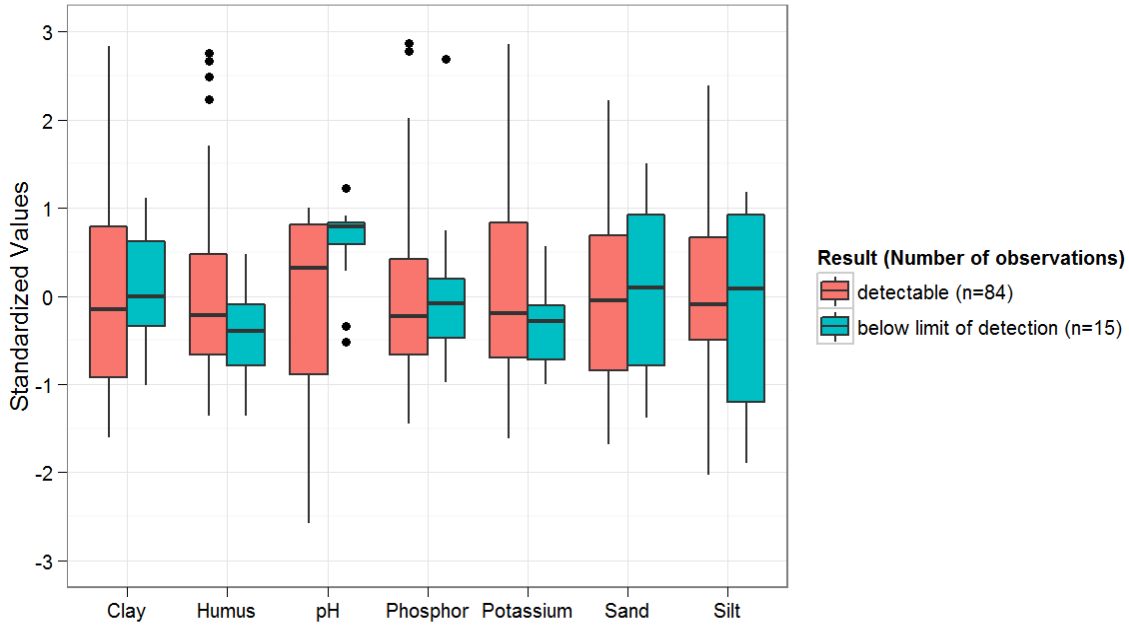

Figure 22: Box-Whisker-plot of the metric soil parameters grouped by nptIII-detectability.

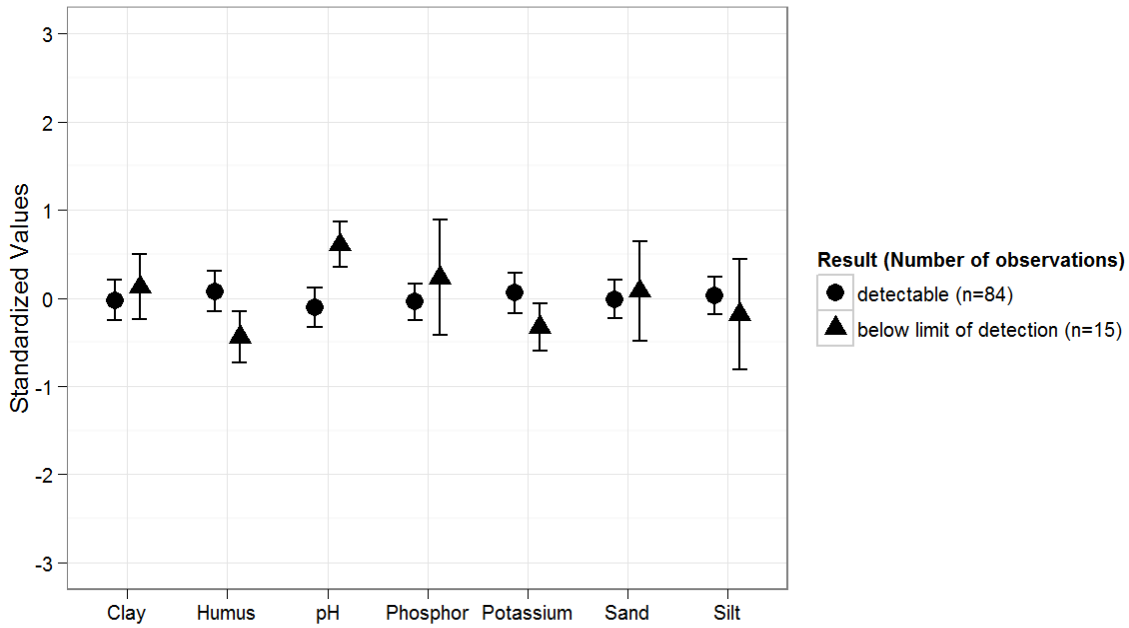

Figure 23: Mean values and related 95% confidence intervals of the metric soil parameters grouped by nptIII-detectability.

The mosaic plot for the non-metric soil parameters are given in Figure 24 and Figure 25. In contrast to nptII, the detection frequency was higher for nptIII. This is reflected in the size of the red areas in the mosaic plots. For some categories (preceding crop: maize, soil humidity: wet, humus: strong), nptIII was detected in all samples. The left mosaic plot in Figure 24 indicates that the probability of detecting nptIII is larger if "organic fertilizer of animal origin" was used. In Figure 25, the mosaic plot for soil type shows that the probability of detecting nptIII is larger for "brown earth" and "other" than for "chernozem".

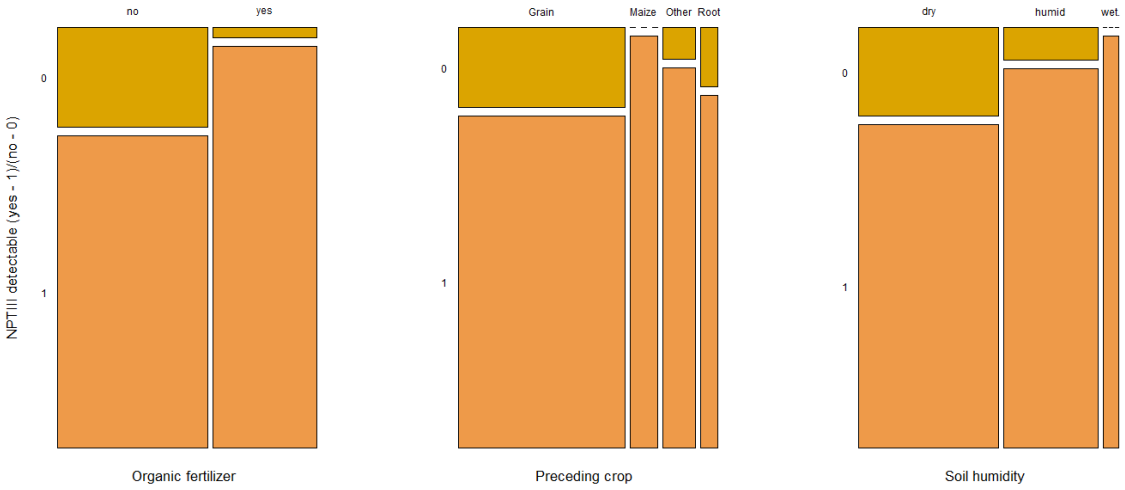

Figure 24: Mosaic plot of nptIII-detectability (yes 1/ no 0) for the parameters determined during sampling.

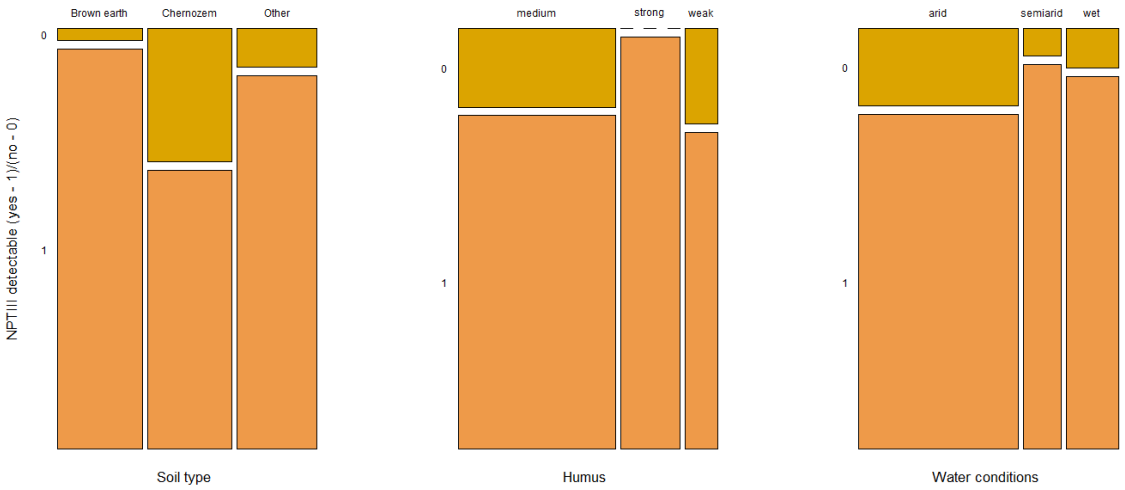

Figure 25: Mosaic plot of nptIII-detectability (yes 1/ no 0) for the parameters based on GIS data.

For nptIII, the results of the logistic regression model are shown in Table 28. Only the variable “organic fertilizer of animal origin” showed a statistically significant relationship with nptIII. According to the model, the odds of detecting nptIII increase by a factor of 12 (estimated coefficient: 2.492, p-value: < 0.001) if "organic fertilizers of animal origin" are used.

	Parameter	p-value
Intercept	3.638	<0.001
Organic fertilizer of animal origin	2.492	0.02

Table 28: Estimated model parameter for the probability of the nptIII-detection.

The univariate analysis in Figure 23 suggests a significant influence of pH on the detectability of nptIII. This could, however, not be verified in the multivariate approach. One reason for this might be that the variable pH correlates with other predictors, particularly with the significant parameter “organic fertilizer of animal origin”. Figure 26 displays the pH values grouped by the bivariate variable “organic fertilizer of animal origin”. The Box-Whisker-plot shows a separation of the data which indicates such a correlation.

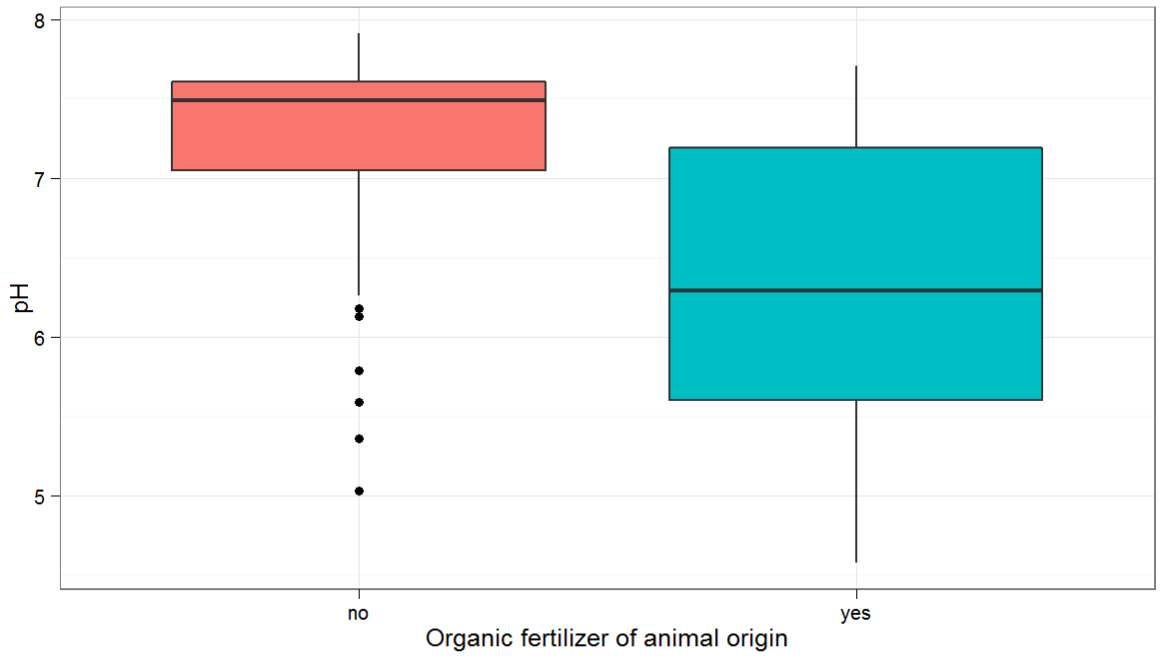

Figure 26: Box-Whisker-plot: pH grouped by organic fertilizer of animal origin (red box: no / blue box: yes).

1.10.5 nptIII prevalence in soils: quantitative results

In addition to the qualitative results (detectable/below limit of detection), the absolute amount of nptII and nptIII, respectively, was determined for the different soil samples. In order to enable comparability of the results, these quantitative values were normalized by relating them to the number of 16S copies in the respective samples.

Figure 27 displays the results of the proportion of nptIII to 16S grouped by the non-metric parameters. The categories of the parameters are shown in the legend. For example, the median of the nptIII to 16S-ratio is higher in samples where organic fertilizer of animal origin was used compared to samples without organic fertilizer of animal origin.

The correlation between the proportion of nptIII to 16S and the metric soil parameters is displayed in a correlation diagram (Figure 28). The variable names are displayed on the diagonal. In the lower half the relationships between two variables are displayed in scatter plots. The upper half shows the correlation coefficient as a pie chart. Blue colours represent positive correlation coefficients and red colours denote negative correlation coefficients. For example, a full circle stands for a correlation coefficient of 1 (blue) or -1 (red) (67).

No significant correlation between the proportion of nptIII to 16S and the metric soil variables could be found. This is visible in the scatter plot (first column) as well as in the pie charts (first row).

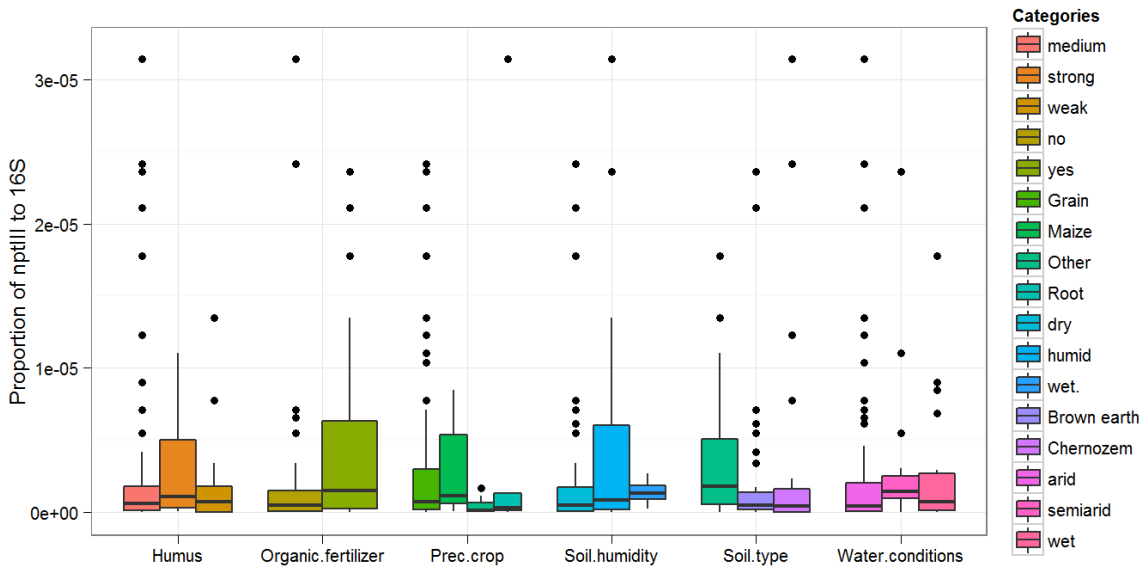

Figure 27: Box-Whisker-plot: Proportion of nptIII to 16S for the non-metric soil parameters.

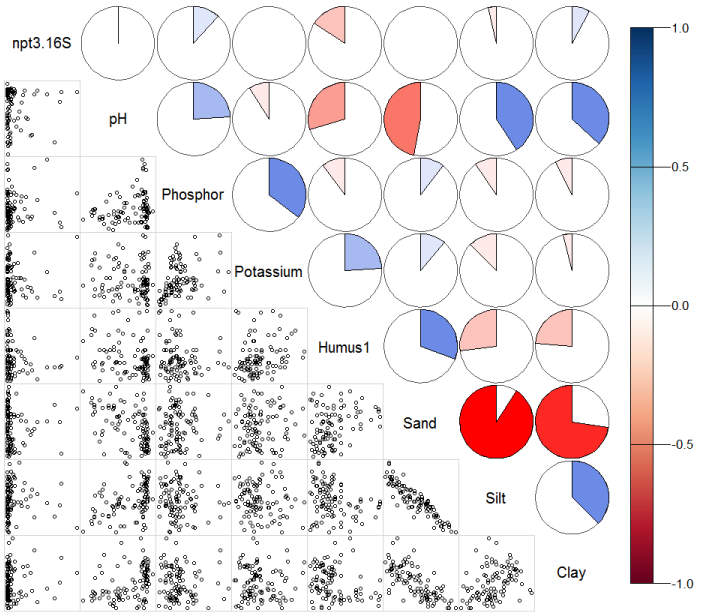

Figure 28: Scatter- and Correlation-plot for nptIII/16S and the metric soil parameters.

1.11 Variability of nptII and nptIII sequences in selected maize and potato fields: pyrosequencing

Soils of three maize and three potato fields established to contain nptII gene copies were analysed for sequence variability by pyrosequencing. The same approach was chosen for thirty potato fields identified as nptIII positive. DNA was isolated from 1 g of soil using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions. The resulting DNA eluate was concentrated in a vacuum centrifuge. The pellet was resuspended in 150 µl of H_2O (Sigma, Austria; molecular biology grade) and repurified with the PowerClean Kit (MoBio) according to the manufacturer's instructions. Standard thermoblock PCRs were performed on an Eppendorf thermocycler using the HotStarTaq Plus Polymerase (Qiagen, Hilden, Germany) and primers which generated nptII or nptIII full length amplicons. These amplicons were purified with the QIAquick PCR Purification Kit (Qiagen).

The purified nptII amplicons from the different fields were pooled into a single tube and the nptIII amplicons were pooled in a different tube. Both tubes were sent for FLX 454 long range sequencing to LGC Genomics GmbH, Berlin, Germany. The obtained data were analysed with the Clustal Omega and JalView tools at the EBI (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalo) and blasted via MegaBLAST at NBCI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

A total of 67221 reads were obtained from nptII pyrosequencing. NptIII 454 sequencing retrieved 39640 reads. 172 sequences were labelled "unsorted".

For nptII the minimum length of the obtained sequences was 84 nucleotides, the maximum fragment length was 1412 nucleotides. The most abundant fragment length was between 500 – 600 nts, followed by fragments with a length of 600 – 700 nts (Figure 29).

For nptIII the minimum length of the obtained sequences was 29 nucleotides, the maximum fragment length was 839 nucleotides. The most abundant fragment length was between 500 – 600 nts, followed by fragments with a length of 400 – 500 nts (Figure 30).

After clustering of the retrieved sequences according to their length (nptII: 831 -700 bp; nptIII: 839 – 500 bp) and to a characteristic signature sequence (20 nucleotides following 3' of the ATG start codon) a randomly compiled subsample containing approx. 2% of the retrieved nptII and 4% of the obtained nptIII sequences were analysed. Sequence alignment with the wild type nptII sequence (GenBank Acc.No. V00618) showed up to 98% homology but also a substantial variability in the form of point mutations and deletions became obvious. The same was true for the sequence alignment of nptIII fragments with the aph(3')-IIIa reference sequence (Genbank Acc.No. V01547).

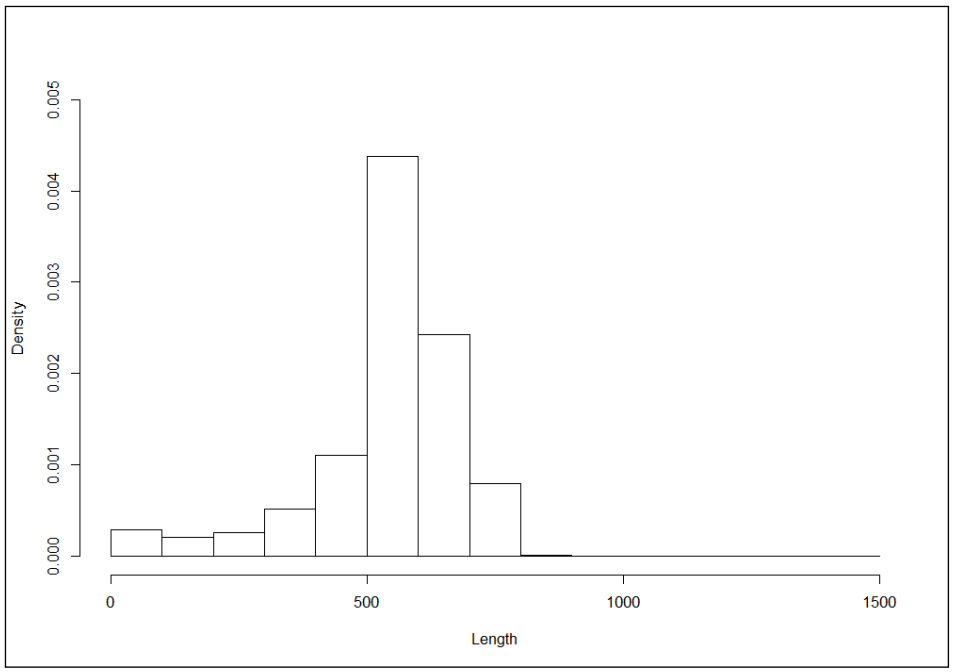

Figure 29: Lengths and relative abundance of retrieved nptII sequences from six fields

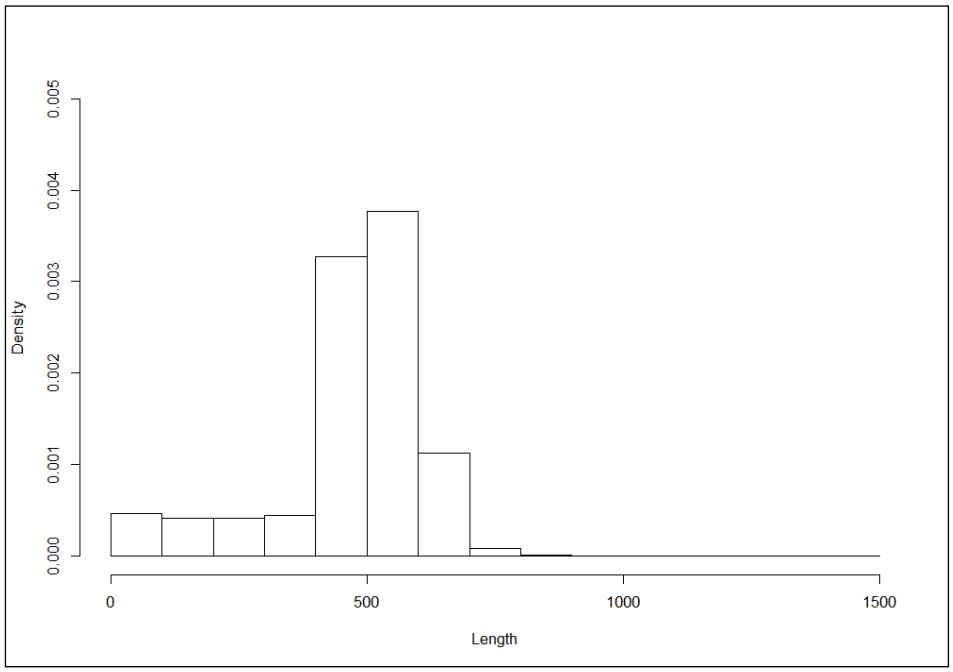

Figure 30: Lengths and relative abundance of retrieved nptIII sequences from 30 fields

1.12 NptII/nptIII prevalence in kanamycin resistant bacterial strains from agricultural soils

Bacterial strains resistant to kanamycin were obtained from the Institute of Plant Health, AGES, Vienna (for a description of the bacterial strain isolation procedures from soil samples see sections 1.8.2.1 and 1.8.2.3). Strains for PCR testing were recovered from glycerol stocks on nutrient agar plates.

Three to six colonies of each bacterial strain were collected from the nutrient agar plate with an inoculation loop and transferred into 100 µl Quickextract^{tm} DNA Extraction Solution 1.0 (Epicentre Biotechnologies). The resulting bacterial suspension was vortexed, incubated at 65°C for 7 min and for an additional 5 min at 98°C on a standard thermoblock thermocycler. Two µl of a 10^{-1} dilution of the resulting solution served as template for the nptII/nptIII TaqMan Double Screening as well as for the 16S rRNA TaqMan real time PCR assay.

1.12.1 16S TaqMan real time PCR assay - Check for the presence of amplifiable DNA

16S TaqMan real time PCR was performed as described above (see 1.9.2). For PCR conditions please see section 3.3; Table 62 and Table 63.

1.12.2 NptII/nptIII TaqMan Double Screening Assay

The nptII/nptIII TaqMan double screening assay was performed as described above (see 1.9.3). For PCR conditions please see Table 58 and Table 59.

1.12.3 Results: Prevalence of nptII/nptIII in the collected bacterial strains resistant to kanamycin (from reference soil samples)

From 396 validly tested kanamycin resistant strains (2 drop outs, 2 negative 16S PCR results) isolated from all references soil samples none of them tested positive for nptII (Table 29). Seven isolates appeared to carry nptIII.

A statistical evaluation of these results retrieved the following (Table 29): The prevalence of nptII in the tested sample collection was between 0% and 0.8% (95% confidence interval). The prevalence of nptIII was between 0% and 3.3%. The prevalence of nptIII (1.8%) is higher than that of nptII (0%) according to the cultivable bacterial strain collection but the frequency of both genes appear to be low in Austrian agricultural soils used for potato and maize cultivation.

These results indicate that the resistance phenotype of the cultivable fraction of soil bacteria insensitive to kanamycin does not rely predominantly on the presence of nptII and nptIII resistance genes.

	Number of isolates from soil	Number of positive isolates	Prevalence estimator	Confidence interval (95%)[1)]
nptII	396	0	0%	[0%; 0,8%]
nptIII	396	7	1,8%	[0%; 3,3%]

Table 29: Prevalence of nptII/nptIII positive strains from maize and potato fields in Austria.

1) Single-sided confidence interval according to Clopper-Pearson.

	Number of isolates from soil	Number of positive isolates	Prevalence estimator	Confidence interval (95%)[1)]
nptII	199	0	0%	[0%; 1,5%]
nptIII	199	3	1,5%	[0%; 3,9%]

Table 30: Prevalence of nptII/nptIII positive strains from maize fields in Austria.

1) Single-sided confidence interval according to Clopper-Pearson.

	Number of isolates from soil	Number of positive isolates	Prevalence estimator	Confidence interval (95%)[1)]
nptII	197	0	0%	[0%; 1,5%]
nptIII	197	4	2.0%	[0%; 4.6%]

Table 31: Prevalence of nptII/nptIII positive strains from potato fields in Austria.

1) Single-sided confidence interval according to Clopper-Pearson.

A nptII prevalence of 0% - 1.5% within a 95% confidence interval was obtained for bacterial isolates from maize fields (Table 30). The same prevalence was observed for nptII in potato fields (Table 31). A nptIII prevalence within a 95% confidence interval of 0% - 3.9% for bacterial isolates obtained from maize fields (see Table 30). In potato fields the nptIII prevalence is between 0% - 4.6% (Table 31).

This statistical subsample analysis was also indicative for a low prevalence of nptII and nptIII in the tested sample collection.

In the tested maize fields three isolates carrying the nptIII gene could be detected, however only two could unambiguously be identified as *Mucilaginibacter* sp. and *Pedobacter cryoconitis*. When

compared with the results from the bacterial biodiversity study, these two taxa represented 0.33% and 0.49% of the bacteria which could be resolved down to genus level, respectively. In the case of tested potato fields, *Stenotrophomonas* sp., *Wautersiella* sp. and *Pedobacter* spp. could be identified carrying the nptIII gene. These taxa represented 0.1%, < 0.01% and 0.27% of the bacterial taxa which could be resolved down to genus level in the corresponding soil sample, respectively.

Detailed PCR results can be found in section 2.3, Table 36.

1.13 Determination of the bacterial biodiversity in selected soil samples: pyrosequencing

1.13.1 Experimental design

For accessing the bacterial diversity in selected soil samples a 454 Sequencing of the bacteria 16S rDNA of the total soil DNA was performed. For that 16S primer specific for bacteria (described in Weisburg et al. 1991 (64) and Muyzer et al. 1995 (42)) were designed as amplicon fusion primer according to the Roche 454 Sequencing Technical Bulletin No. 013-2009 (F. Hoffmann-La Roche Ltd). The PCR reaction was performed using KAPA2G Robust DNA Polymerase (Kapa Biosystems, Inc., Woburn, MA, USA) with concentrations according to the manufacturer. Primers were used in 0.5 µM concentration and template was 1 µl (1:10 dilution of the total soil DNA) in 20 µl total volume. The annealing temperature was 56°C. The thermocycler used for amplification was a Biometra T3000 (Biometra GmbH, Göttingen, Germany). The different tagged PCR amplicons were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and equimolar aliquots of all samples were pooled and sent for 454 sequencing (LGC Genomics GmbH, Berlin, Germany). The obtained data have been analysed with the Ribosomal Database Project Classifier (7, 61) for assignment of rRNA sequences into the bacterial taxonomy.

1.13.2 Interpretation of the microbial biodiversity: pyrosequencing analysis

1.13.2.1 Data

Additional analyses were carried out for the ten reference test fields (Table 1). Apart from soil parameters (boron, cadmium, copper, zinc, iron, manganese, see Figure 31), the biodiversity of the soil bacteria was examined. In Table 32 an overview of the ten reference soils is given. The “Lab-No.” indicates the crop (first letter: K for potato and M for maize), the soil type (third letter: A for Fluvisol (=“Auboden”), B for brown earth, S for chernozem, R for relict soil) and the pH characterization (a for alkaline, n for neutral and s for acidic) of the soils. The soil types “Fluvisol”, Cambisol and Chernozem were each sampled three times, while the relict soil was sampled once.

Lab-No.	Id	Soil type	pH characterization	Crop
K1Aa	E4648N2800	Fluvisol (Auboden)	alkaline	Potato
K2Ba	E4800N2840	Cambisol (Brown earth)	alkaline	Potato
K3Sn	E4834N2816	Chernozem	neutral	Potato
K4Bs	E4594N2825	Cambisol (Brown earth)	acidic	Potato
K5Sa	E4824N2812	Chernozem	alkaline	Potato
M1As	M34_0009	Fluvisol (Auboden)	acidic	Maize
M2Bs	M34_0020	Cambisol (Brown earth)	acidic	Maize
M3Sa	M34_0055	Chernozem	alkaline	Maize
M4Rs	M34_0124	Relict soil	acidic	Maize
M5An	E4574N2632	Fluvisol (Auboden)	neutral	Maize

Table 32: Overview of the ten soil samples.

The sequences were classified according to their taxa phylum, order, family and genus where possible. Table 33 contains the number of sequences for which a particular taxon could be identified and the number of different groups of each taxon in parenthesis for each soil sample.

For the soil sample “K1Aa” (potato, Auboden, alkaline) the phylum could be determined for 48 989 sequences, in total bacteria of 11 different phyla were classified. For 25 781 sequences, the order could be determined (42 different orders). For 17 811 sequences the taxon family (105 different families) was identified and for 11 378 sequences, the genus could be specified, yielding 250 different bacterial genera. The results of the other soil samples are to be interpreted analogously. The difference in the numbers of classified bacteria between the hierarchical levels is due to the fact that the taxa become increasingly more difficult to appoint as the classification becomes more specific.

For the statistical analyses, the relative frequencies of the different taxa are used. In order to allow for better comparability, the soil parameters are standardized. The values are centered around zero by subtracting the mean value and scaled by dividing by the standard deviation.

Lab-No.	Phylum	Order	Family	Genus
K1Aa	48989 (11)	25781 (42)	17811 (105)	11378 (250)
K2Ba	35254 (11)	20673 (31)	12468 (82)	7157 (194)
K3Sn	40489 (12)	24864 (38)	16957 (95)	10245 (244)
K4Bs	39370 (12)	21794 (36)	14259 (88)	8563 (228)
K5Sa	42063 (12)	23635 (40)	16549 (97)	10429 (218)
M1As	31082 (11)	18594 (41)	12886 (92)	7922 (210)
M2Bs	41671 (13)	26354 (43)	15946 (113)	8105 (263)
M3Sa	32436 (11)	19445 (37)	12231 (93)	7153 (220)
M4Rs	45658 (13)	33167 (43)	20058 (109)	10210 (268)
M5An	35722 (13)	15500 (43)	9858 (102)	5332 (263)

Table 33: Number of the specified bacteria and number of different groups in parenthesis within each taxon.

1.13.2.2 Methods

The correlation between different metric soil parameters is displayed in a correlation diagram (see Figure 32 and Figure 35). The variable names (soil samples) are displayed on the diagonal. In the lower half the relationships between two variables are displayed as scatter plots. The upper half shows the correlation coefficient as a pie chart. Blue colors represent positive correlation coefficients and red colors denote negative correlation coefficients. A full circle stands for a correlation coefficient of 1 (blue) or -1 (red), an empty circle denotes a correlation coefficient of 0 (Wright, 2012).

Agglomerative hierarchical clustering is used to identify similar groups with respect to certain metric parameters in the data. The difference between two soil samples is measured using the n-dimensional Euclidean distance between the parameter vectors associated with the soil samples. For soil samples X and Y and associated parameter vectors x and y, the Euclidean distance is defined as follows:

$$d(x,y) = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2}$$

At initialization, each soil sample is considered as a separate cluster. The soil samples are then iteratively grouped according to the Ward method. The clusters are formed subject to a minimization of the sum of the squared errors. The visualization of hierarchical agglomerative cluster analysis is done by dendrograms (see Figure 33, Figure 37, Figure 38 and Figure 39)(3).

Three different indices are used as diversity measures for bacterial communities (26). The richness index S is defined as the total number of different groups in a taxon.
The Shannon index considers the number of different groups and the number of elements in each group. It is calculated as follows:

$$H' = -\sum_i p_i \ln(p_i), \text{where } p_i = \left({}^{n_i}/_{N}\right).$$

The Shannon index therefore increases if the number of groups increases and if the number of individuals per group is balanced.
The third diversity index is the so-called Shannon evenness index, which is defined as the Shannon index H' divided by the logarithm of the richness S:

$$E = \frac{H'}{\ln S}$$

If the number of "individuals" is equal in all groups, the Shannon evenness index is 1, which is the maximal attainable value.

All statistical analyses in this section were done using the open source software R (51).

1.13.2.3 ***Results***

Soil parameters

Figure 31 shows the standardized values of the soil parameters for the 10 soil samples. Figure 32 illustrates the correlations between the soil samples for the soil parameters. The soil samples K5Sa and M3Sa correlate strongly. This correlation is also visible in Figure 31. The other soil samples show a weaker correlation with respect to their soil parameters. The results of the cluster analysis are shown in Figure 33, which illustrates the degree of similarity between the different soil samples. At first, the soil samples K5Sa and M3Sa are joined together. Then the soil sample K2Ba is added to the same cluster. The other soil samples build clusters at larger Euclidean distances from each other. The soil parameters of M1As, K4Bs and M4Rs are more similar than the soil samples of the first cluster, and the soil samples K3Sn, M2Bs, K1Aa and M5An, which are also combined in a cluster.

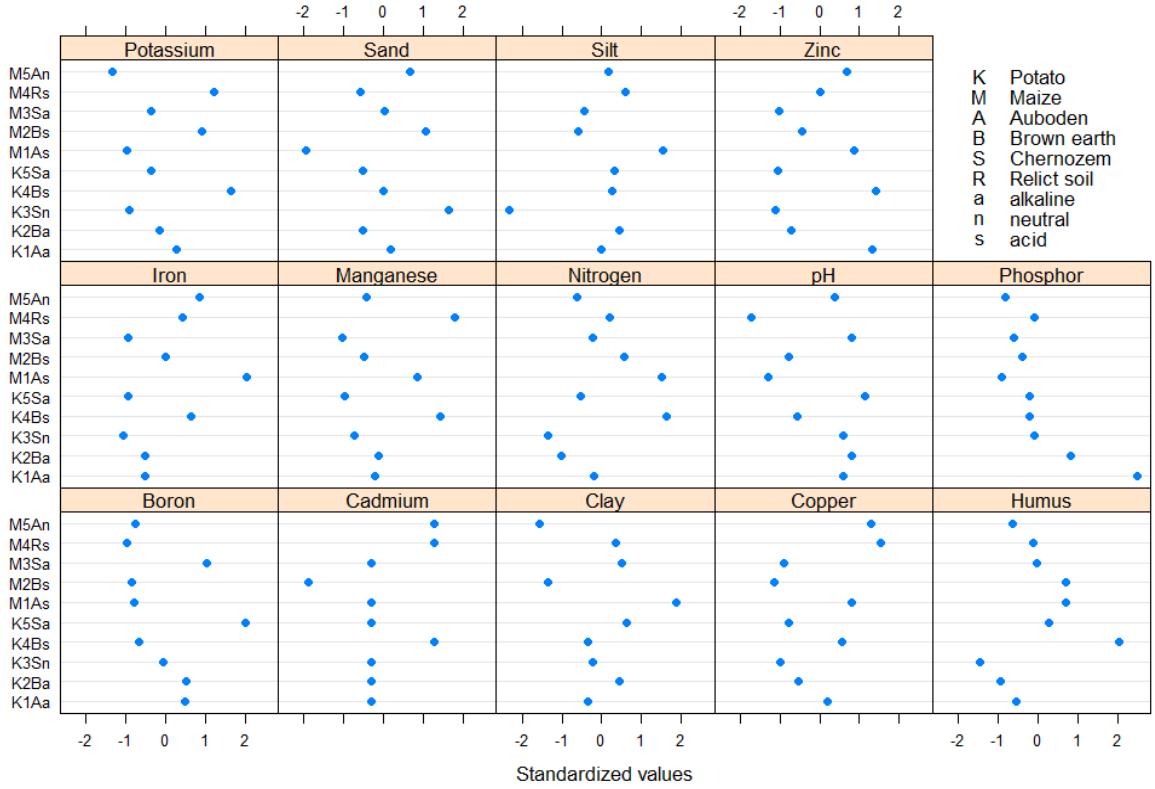

Figure 31: Scatter plot of the different metric soil parameters for the ten soil samples.

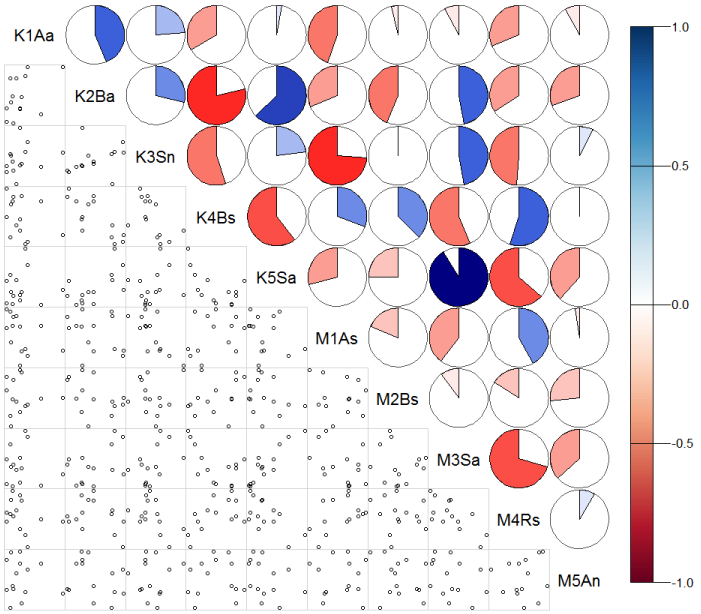

Figure 32: Scatter plot and correlation diagram of the ten soil samples relating to the soil parameters.

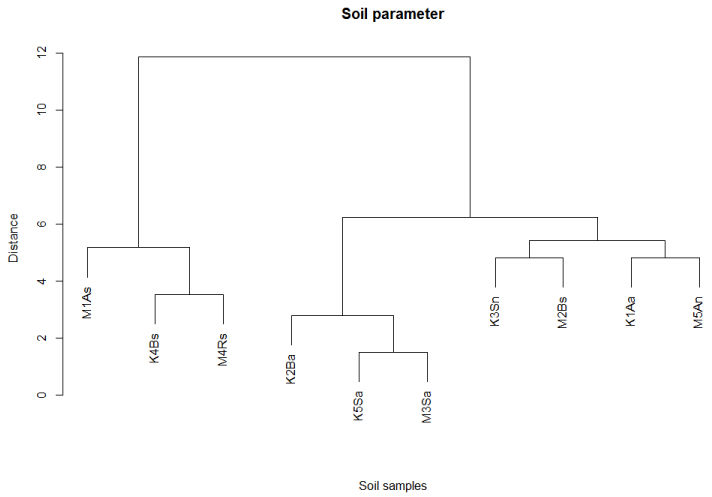

Figure 33: Dendrogram: agglomerative hierarchical clustering of the ten reference soil samples relating to the soil parameters.

Biodiversity: Phylum

Figure 34 shows the relative frequency of different bacteria with respect to the phylum for the ten soil samples. The most common phyla are Proteobacteria, Actinobacteria, Acidobacteria and Gemmatimonadetes. Approximately 30 % of the soil bacteria could not be classified at phylum level. These bacteria are combined in the group “unclassified” in the graphic. The ten soil samples show a high correlation with respect to the frequency of bacteria at phylum level. This is illustrated in the correlation diagram in Figure 35 and in the dendrogram in Figure 36. All correlation coefficients are higher than 0.75. Clusters are formed by the soil samples K3Sn, M2Bs, K5Sa, the soil samples K2Ba, M3Sa, M4Rs, and the soil samples K4Bs, M1As, K1Aa, M5An.

The unclassified phyla were not taken into account for the calculated correlations and cluster analysis.

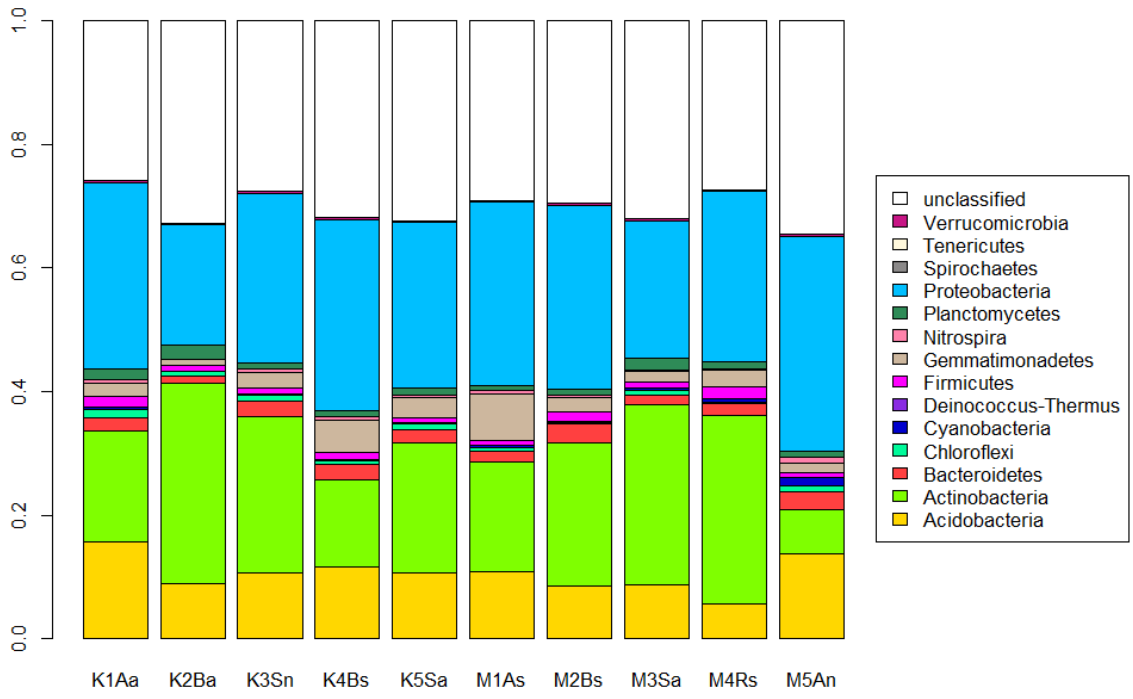

Figure 34: Relative frequency of the bacteria within each soil at the taxon level phylum.

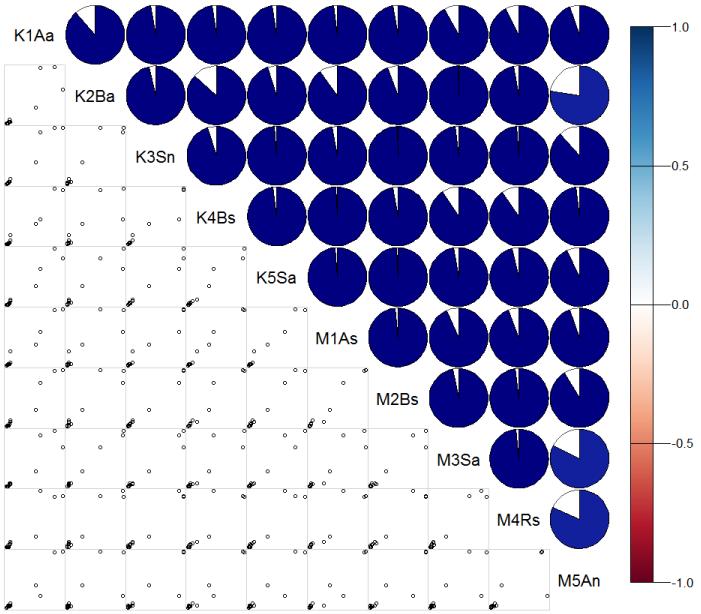

Figure 35: Scatter plot and correlation diagram of the ten soil samples relating to the biodiversity at the taxon level phylum.

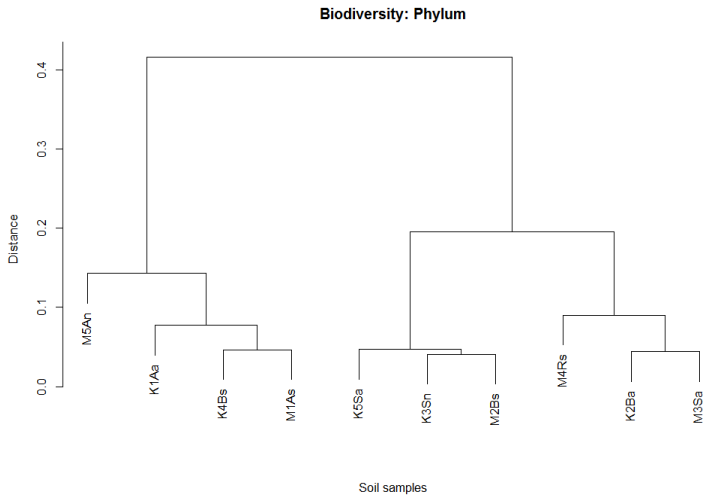

Figure 36: Dendrogram: agglomerative hierarchical clustering of the ten reference soil samples relating to the biodiversity at the taxon level phylum.

Biodiversity: Order

The taxon level "order" could be specified for approximately 60 % of the bacteria with phylum classification (see Table 33). The unclassified bacteria are not included in the analyses. The number of different orders in the ten soil samples varied between 31 and 43.

Two different main clusters show up in the dendrogram, as can be seen in Figure 37. The two clusters consist of the soil samples M5An, K1Aa, K4Bs, K5Sa, M1As, and M4Rs, K2Ba, M3Sa, K3Sn, M2Bs.

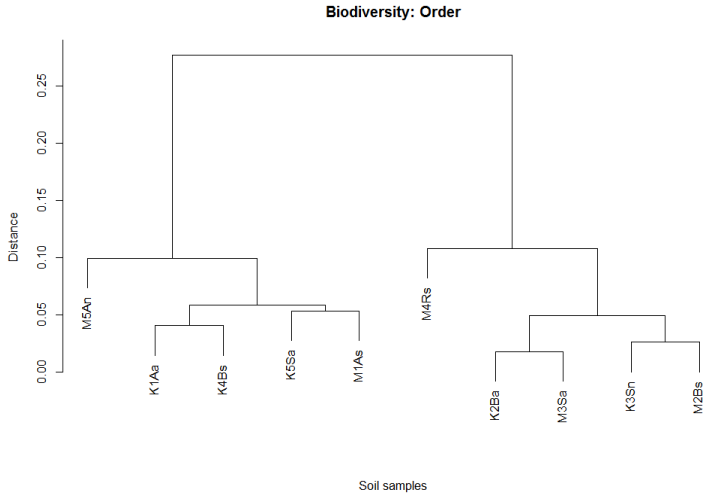

Figure 37: Dendrogram: agglomerative hierarchical clustering of the ten reference soil samples relating to the biodiversity at the taxon level order.

Biodiversity: Family

The taxon "family" could be determined for approximately 65 % of the bacteria with specified order (see Table 33). Between 82 and 113 different families were identified in the ten soil samples. The dendrogram in Figure 38 shows multiple clusters. The samples can be divided onto two rough

clusters: M5An, K2Ba, M3Sa, K1Aa K3Sn and K5Sa make up the first cluster while K4Bs, M1As,M2Bs and M4Rs make up the second cluster.

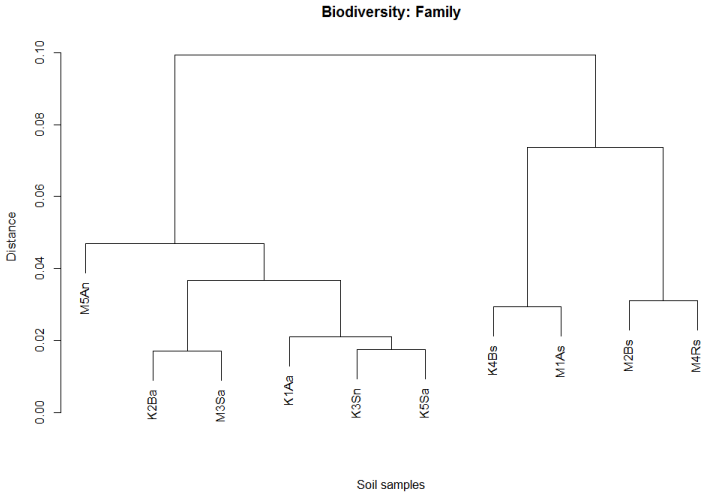

Figure 38: Dendrogram: agglomerative hierarchical clustering of the 10 reference soil samples relating to the biodiversity at the taxon level family.

Biodiversity: Genus

For about 60 % of the bacteria with classified families, the genus could be identified (see Table 33). The number of different genera varies between 194 and 268 in the 10 soil samples. The relative frequencies of the most common genera per soil sample are shown in the tables in the Annex. Throughout all the soil samples, with the exception of K2Ba, the dominant genus was “Gemmatimonas”. In soil sample K2Ba, the genus “Blastococcus” was the most frequent genus. Figure 39 shows the results of the cluster analysis. Two main clusters can be identified in the dendrogram. The soil samples K4Bs and M1As make up one cluster, while all the other soil samples are combined into the second cluster.

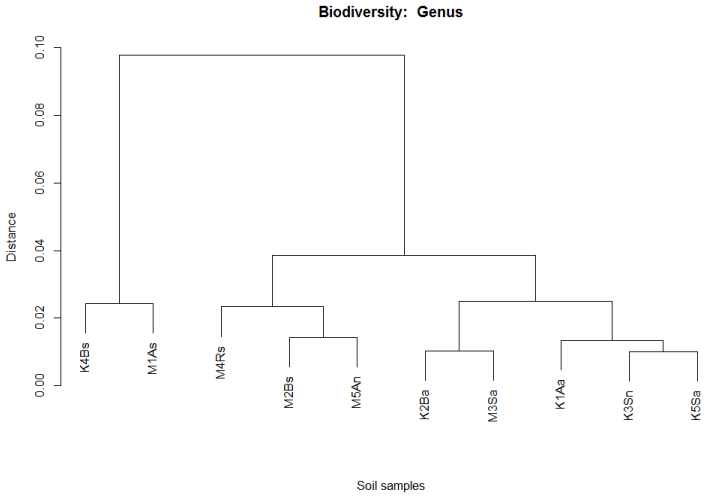

Figure 39: Dendrogram: agglomerative hierarchical clustering of the 10 reference soil samples relating to the biodiversity at the taxon level genus.

Diversity indices

For each of the taxa, three different diversity indices were calculated; see chapter “methods”. The richness of each taxon is shown in Figure 40. The richness of the 10 soil samples lies between 11 and 13 for phylum, between 31 and 43 for order, between 82 and 113 for family and between 194 and 268 for genus. The lowest richness index was found for soil sample K2Ba.

The results of the Shannon index are illustrated in Figure 41 for the 10 soil samples and the different taxa. It not only considers the number of different communities but also the abundance in each community. The Shannon index increases for increased number of groups and a more balanced number of group individuals between the groups. At the taxon phylum the differences between the soil samples are minimal. The Shannon index varies a bit more for the other taxa. In particular, the low index for the soil sample M1As at taxon genus is noticeable. By comparing this value with Table 47 in the annex, it is visible that one bacterial genus is dominant.

The results of the Shannon evenness index are shown in Figure 42. The evenness index lies between 0 and 1, attaining its maximal value if all communities are evenly distributed within a sample. The dominant bacteria at genus taxon for the soil sample M1As is also visible in this graph.

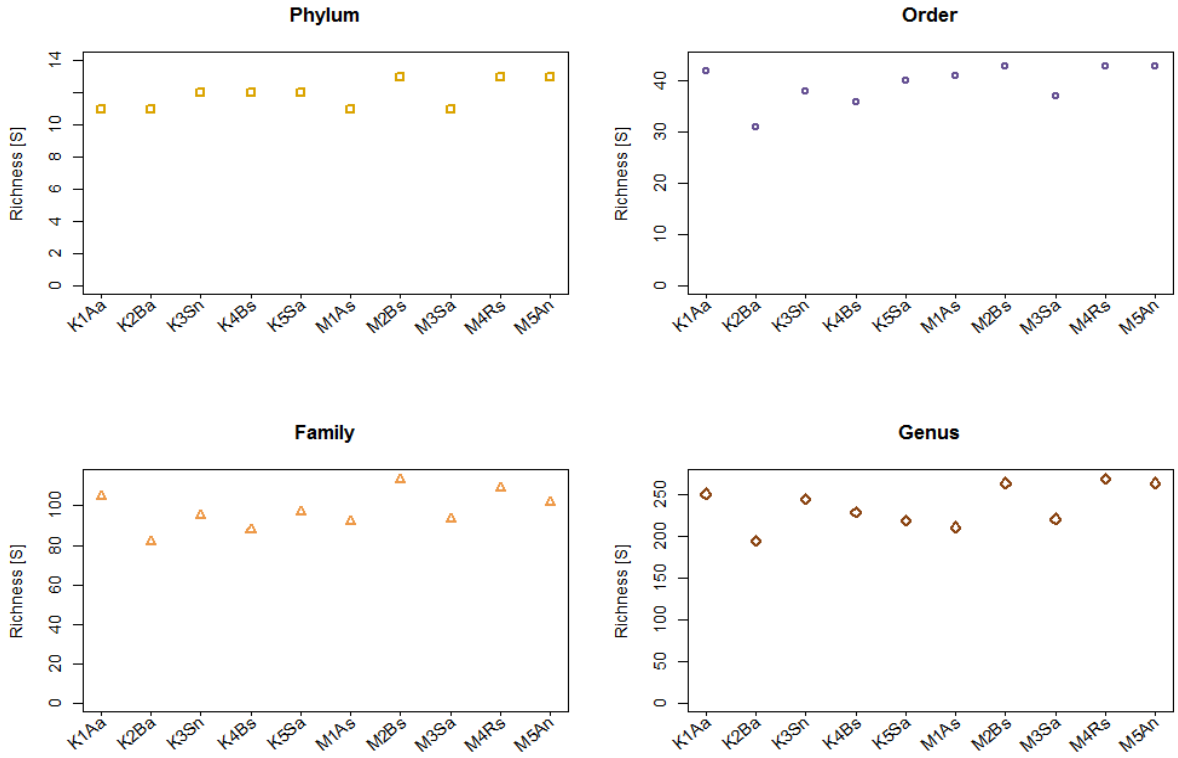

Figure 40: Richness at taxa phylum, order, family and genus for the 10 soil samples.

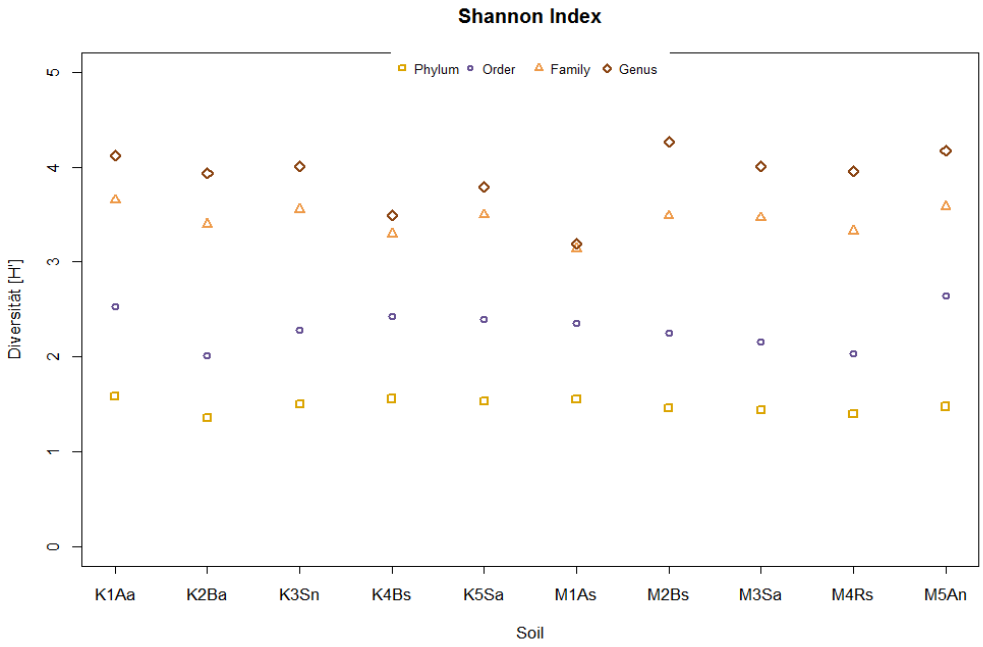

Figure 41: Shannon index for the taxa phylum, order, family and genus for the 10 soil samples.

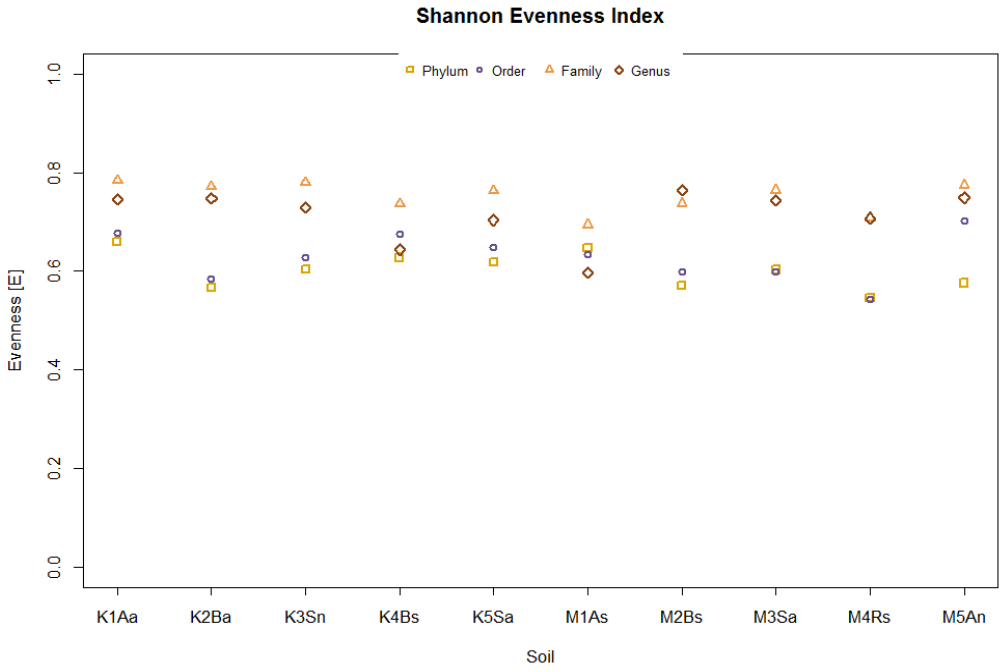

Figure 42: Shannon Evenness index for the taxa phylum, order, family and genus for the 10 soil samples.

1.13.3 Selected results: summary and interpretation

1.13.3.1 Isolation on kanamycin

- All Isolated strains could also be found on genus level in pyrosequencing. This is a good cross check of the suitability of the methods used and indicated the high resolution of the pyrosequencing.
- Some of the isolated bacteria could be identified as possibly of clinical relevance, however it is not known if the strains specifically isolated in this study would bear a risk of having an influence on human health in any case.

1.13.3.2 Clustering on soil parameters:

- Correlation in soil parameters of K5 and M3 (both from Chernozem soils with similar high pH) clustering also with K2 (brown earth soil with similar pH). All 3 soils did not receive organic fertilizer.

1.13.3.3 Pyrosequencing

General observations (trends) on phylum level:

- The higher the number of Actinobacteria, the lower the number of Gemmatimonadetes (for description of the taxon see (68)) and Proteobacteria (soil K2, M3, M4)

Relevant representatives in the Actinobacteria are in the Streptomyces group, and these are able to synthetize antibacterial, antifungal, and antiparasitic drugs, and also a wide range of other bioactive compounds (63) which could provide an advantage while competing for nutrients with soil bacteria from other groups (38).

- Low content on Acidobacteria in acidic relict soil which had the highest content in boron of the soils tested (soil M4).
- High number of Acidobacteria in Auboden, with low potassium concentration and predominatly low nitrogen concentration in the soil (soil K1, M5).
- In acidic Auboden soil with high content in humus, TOC and nitrogen, lower number of Acidobacteria/Actinobacteria/Proteobacteria but a high amount of Gemmatimonadetes could be detected (soil M1).
- In soils with low content in humus, TOC and nitrogen, high number of Proteobacteria could be registered (soil M5, K4):

The more prominent abundance of Proteobacteria in samples with lower soil nitrogen content could be explained by the competition advantage due to n-fixing capability of several representatives in this taxon (chemoautotrophic Proteobacteria, (38)).

- Clay soil with high content in nitrogen and iron but low content in phosphorus contain a high number of Actinobacteria and a low number of Proteobacteria (soil K2):

Proteobacteria possibly being outcompeted by Actinobacteria under these conditions (63).

On genus level:

- Soil K2: Blastococcus is the most abundant genus in the heavy granulitic alkaline soil with high iron and nitrogen, but low phosphorus content (nota bene from the 10 soils analysed here, this was the only one with sugar beet as preceding crop)
- Clustering of K4 and M1 on genus level, due to high Gemmatimonas prevalence:

Possibly linked to relatively high content of soil nitrogen which decreases bacterial biodiversity (54). Another possible effect on a decreased bacterial biodiversity could be the influence of a relatively low pH (19) in K4 and M1 soils. A cumulation of these two effects would also be plausible. This fact is also visible in the lower Shannon Index on genus level, indicating a lower bacterial biodiversity on this taxonomic level in these two soils.

For more detailed results on the frequency of different genera in the tested soil samples see Table 42, Table 43, Table 44, Table 45, Table 46, Table 47, Table 48, Table 49, Table 50, and Table 51. An overview over the most frequently observed genera and their potential to cause diseases in humans can be found in Table 52 in Appendix A.

1.14 Conclusions: Prevalence of nptII and nptIII resistance genes in soils of maize and potato fields in Austria

The aim of the project was to establish the baseline frequency of neomycin phosphotransferase II and III genes in maize and potato growing regions in Austria. To achieve this goal bacterial strains resistant to kanamycin were isolated from soils of ten reference fields and analysed for the presence of nptII and nptIII resistance genes with TaqMan real time PCR. Additionally, total DNA was extracted from soils of 50 maize and 50 potato fields and the gene copy number of these two aminoglycoside phosphotransferases in these habitats was established by TaqMan real time PCR.

The naturally occurring background load of nptII resistance genes in agricultural habitats used for the cultivation of maize and potatoes in Austria appears to be low. The obtained quantitative results indicate that the resistance gene pools of the maize and potato fields under investigation are not saturated with nptII gene copies. The baseline frequency of nptII in these natural habitats is distinctly lower compared to the prevalence of nptIII in the same habitats. NptII could not be detected in bacterial strains isolated from ten reference soils supporting also the observation of a low prevalence of nptII in agricultural environments used for the cultivation of the crops mentioned above. In summary the abundance of nptII appears to be low in the resistance gene pools under investigation. It is likely that an anthropogenic input of exogenous DNA carrying aminoglycoside phosphotransferase (3')-IIa gene homologues is of relevance: A long-term and constant exposure of these ecosystems with exogenous nptII carrying DNA via root exsudates or plant cell decay might be capable of elevating the abundance of this resistance determinant in the relevant environments.

The naturally occurring background load of nptIII in maize and potato growing fields is usually higher compared to the situation with nptII reaching peak concentrations in some maize fields of up to 62 000 copies per gram soil. But the nptIII copy number is still several orders magnitude lower compared to the abundance of e.g. tetracycline resistance determinants in pig manure (10^7 – 10^9 copies per g) used for soil fertilization (27). In the present setting statistical evaluation showed a significant correlation between nptIII prevalence and the application of organic fertilizer of animal origin in Austrian maize and potato fields. Out of 396 bacterial isolates cultivated from the soils of ten reference fields 7 isolates appeared to be carriers of nptIII. This observation also supports the conclusion that nptIII is more prevalent in naturally occurring soil bacterial populations of agricultural origin compared to the situation with nptII. An additional input of exogenous DNA carrying aph(3')-IIIa genes into the analysed ecosystems would be more likely of less significance compared to an additional input of nptII into the same habitats.

2 Appendix A: Individual Test Results

2.1 Pilot study

2.1.1 Sample collection

In order to test the methodology for taking soil samples in maize and potato fields, a basic approach was developed and tested in May 2011 on a fodder maize field (Figure 43) in the township of Söchau located in the south-eastern part of Styria. The field size was 1.13 hectares. The soil was loamy. Harvest was estimated to take place in September. In this experiment the feasibility of the method and the time effort of soil sampling as well as the following analytics in the laboratory were tested.

Figure 43: **Test field in the pilot study in the township of Söchau in Styria.**

A composite soil sample - consisting of ten single samples - of the test field was taken following a predefined sampling scheme as shown in section 1.6.4.1, Figure 9. The plants were removed from the soil and the soil adhering on the roots of the ten selected small maize plants was collected in a bucket. The sampling plan followed the shown scheme and did not respect different soil qualities of the field which were optically visible. The developed sampling plan of the ten plants tested in the pilot study proved to be appropriate. After removal of a plant from the soil and the extraction of soil material from the rooting zone, the maize plant was planted again in the pilot study. The working time for sampling a small field was estimated and proved to be around one hour. In this working time the estimated time for field identification, soil sampling, homogenising, sieving through a sieve with a mesh size of 2 mm and storing the sample in a plastic bag and a small proportion of soil in a 50 ml sterile tube as well as the sample labelling were included. With larger fields in comparison to the pilot test field (1.13 ha) a higher time effort would be expected due to larger distances on the field which were to be covered. No detailed protocol about field conditions was created in the pilot study. The methodology tested in that pilot study proved to be feasible.

2.1.2 DNA Recovery from soil samples: PowerSoil™ DNA Isolation Kit (MO BIO Laboratories)

To establish the bacterial DNA recovery rate from soil samples with the PowerSoil DNA Isolation Kit 4 aliquots of soil from the pilot study test field were spiked with a defined number of *Erwinia amylovora* (10^8 cells per gram soil). DNA preparation was performed according to the manufacturer's

instructions. The total soil DNA was diluted according to Table 34 and quantified in 4 replicates using an *Erwinia amylovora* specific TaqMan assay to determine the DNA recovery.

The mean DNA recovery from soil using this procedure was approx. 60%. No inhibition was detectable at a 10^{-1} dilution of the Powersoil DNA eluate (Table 34).

Sample	Dilution 1:x	Bacterial spike/µl	Cfu/µl TaqMan results	Recovery (%)	Mean DNA recovery (%)	SD	Comment
FEa1	1	250000	14	0,01	0,04	0,07	inhibited
FEa2	1	250000	1	0,00			
FEa3	1	250000	2,72	0,00			
FEa4	1	250000	339	0,14			
FEa1	10	25000	15292	61,17	63,23	5,32	ok
FEa2	10	25000	16176	64,70			
FEa3	10	25000	17443	69,77			
FEa4	10	25000	14315	57,26			
FEa1	100	2500	1608	64,32	68,72	6,16	ok
FEa2	100	2500	1904	76,16			
FEa3	100	2500	1784	71,36			
FEa4	100	2500	1576	63,04			
FEa1	1000	250	152	60,80	61,48	16,88	ok
FEa2	1000	250	163	65,20			
FEa3	1000	250	201	80,40			
FEa4	1000	250	98,8	39,52			

Table 34: Comparative results of the quantitative DNA extraction recovery with the PowerSoil Kit

2.2 Qualitative screening for nptII/nptIII in total DNA from soil

Isolate	16S	nptII	nptIII	Isolate	16S	nptII	nptIII
K1	+	-	+	M1	+	-	+
K2	+	-	+	M2	+	-	+
K3	+	-	-	M3	+	-	+
K4	+	-	+	M4	+	-	+
K5	+	-	-	M5	+	-	+
FK1	+	-	+	FM1	+	-	+
FK2	+	-	+	FM2	+	-	+
FK3	+	+	+	FM3	+	-	+
FK4	+	-	-	FM4	+	-	+
FK5	+	-	-	FM5	+	+	+
FK6	+	+	+	FM6	+	-	+
FK7	+	-	+	FM7	+	-	+
FK8	+	-	+	FM8	+	-	+
FK9	+	-	-	FM9	+	-	+
FK10	+	-	+	FM10	+	-	+
FK11	+	-	+	FM11	+	-	+
FK12	+	-	+	FM12	+	-	+
FK13	+	-	-	FM13	+	-	+
FK14	+	-	+	FM14	+	-	-
FK15	+	-	+	FM15	+	+	+
FK16	+	-	+	FM16	+	-	+
FK17	+	-	+	FM17	+	-	+
FK18	+	-	+	FM18	+	-	+
FK19	+	-	+	FM19	+	-	-
FK20	+	-	-	FM20	+	+	+
FK21	+	-	+	FM21	+	-	+
FK22	+	-	-	FM22	+	-	+
FK23	+	-	+	FM23	+	-	+
FK24	+	-	+	FM24	+	-	+
FK25	+	-	+	FM25	+	-	+
FK26	+	-	+	FM26	+	-	-
FK27	+	-	+	FM27	+	-	-
FK28	+	-	+	FM28	+	-	+
FK29	+	-	+	FM29	+	-	+
FK30	+	-	+	FM30	+	-	+
FK31	+	-	-	FM31	+	-	+
FK32	+	-	-	FM32	+	-	+
FK33	+	-	-	FM33	+	-	+
FK34	+	-	+	FM34	+	-	+
FK35	+	-	+	FM35	+	-	+
FK36	+	-	+	FM36	+	-	+
FK37	+	-	+	FM37	+	-	+
FK38	+	+	+	FM38	+	-	+
FK39	+	-	+	FM39	+	-	+
FK40	+	-	+	FM40	+	-	+
FK41	+	-	+	FM41	+	-	+
FK42	+	-	+	FM42	+	-	+
FK43	+	-	+	FM43	+	-	+
FK44	+	-	+	FM44	+	-	+
FK45	+	-	+	FM45	+	-	+

Table 35: Results of nptII/nptIII double screening and 16S TaqMan real time PCR assays in soil.

2.3 Qualitative screening for the prevalence of nptII/nptIII in kanamycin resistant soil bacteria

Isolate	16S	nptII	nptIII	Isolate	16S	nptII	nptIII
M1/1	+	-	-	K1/1	+	-	-
M1/2	+	-	-	K1/2	+	-	-
M1/3	+	-	-	K1/3	+	-	-
M1/4	+	-	-	K1/4	+	-	-
M1/5	+	-	-	K1/5	+	-	-
M1/6	+	-	-	K1/6	+	-	-
M1/7	+	-	-	K1/7	-		
M1/8	+	-	-	K1/8	+	-	-
M1/9	+	-	-	K1/9	+	-	-
M1/10	+	-	-	K1/10	+	-	-
M1/11	+	-	-	K1/11	+	-	-
M1/12	+	-	-	K1/12	+	-	-
M1/13	+	-	-	K1/13	+	-	-
M1/14	+	-	-	K1/14	+	-	-
M1/15	+	-	-	K1/15	+	-	-
M1/16	+	-	-	K1/16	+	-	-
M1/17	+	-	-	K1/17	+	-	-
M1/18	+	-	-	K1/18	+	-	-
M1/19	+	-	-	K1/19	+	-	-
M1/20	+	-	-	K1/20	+	-	-
M1/21	+	-	-	K1/21	+	-	-
M1/22	+	-	-	K1/22	+	-	-
M1/23	+	-	-	K1/23	+	-	-
M1/24	+	-	-	K1/24	+	-	-
M1/25	+	-	-	K1/25	+	-	-
M1/26	+	-	-	K1/26	+	-	-
M1/27	+	-	-	K1/27	+	-	-
M1/28	+	-	-	K1/28	+	-	-
M1/29	+	-	-	K1/29	+	-	-
M1/30	+	-	-	K1/30	+	-	-
M1/31	+	-	-	K1/31	+	-	-
M1/32	+	-	-	K1/32	+	-	-
M1/33	+	-	-	K1/33	+	-	-
M1/34	+	-	-	K1/34	+	-	-
M1/35	+	-	-	K1/35	+	-	-
M1/36	+	-	-	K1/36	+	-	-
M1/37	+	-	-	K1/37	+	-	-
M1/38	+	-	+	K1/38	+	-	-
M1/39	+	-	-	K1/39	+	-	-
M1/40	+	-	-	K1/40	+	-	-
M2/1	+	-	-	K2/1	+	-	-
M2/2	+	-	-	K2/2	+	-	-
M2/3	+	-	-	K2/3	+	-	-
M2/4	+	-	-	K2/4	+	-	-
M2/5	+	-	-	K2/5	+	-	-
M2/6	+	-	-	K2/6	+	-	-
M2/7	+	-	-	K2/7	+	-	-
M2/8	+	-	-	K2/8	+	-	-
M2/9	+	-	-	K2/9	+	-	-
M2/10	drop out			K2/10	+	-	-
M2/11	+	-	-	K2/11	+	-	-

Isolate	16S	nptII	nptIII	Isolate	16S	nptII	nptIII
M2/12	+	-	-	K2/12	+	-	-
M2/13	+	-	-	K2/13	+	-	-
M2/14	+	-	-	K2/14	+	-	-
M2/15	+	-	-	K2/15	+	-	-
M2/16	+	-	-	K2/16	+	-	-
M2/17	+	-	-	K2/17	+	-	-
M2/18	+	-	-	K2/18	+	-	-
M2/19	+	-	-	K2/19	+	-	-
M2/20	+	-	-	K2/20	+	-	-
M2/21	+	-	-	K2/21	+	-	-
M2/22	+	-	-	K2/22	+	-	-
M2/23	+	-	-	K2/23	+	-	-
M2/24	+	-	-	K2/24	+	-	-
M2/25	+	-	-	K2/25	+	-	-
M2/26	+	-	-	K2/26	+	-	-
M2/27	+	-	-	K2/27	+	-	-
M2/28	+	-	-	K2/28	+	-	-
M2/29	+	-	-	K2/29	+	-	-
M2/30	+	-	-	K2/30	+	-	-
M2/31	+	-	+	K2/31	+	-	-
M2/32	+	-	-	K2/32	+	-	-
M2/33	+	-	-	K2/33	+	-	-
M2/34	+	-	-	K2/34	+	-	-
M2/35	+	-	-	K2/35	+	-	-
M2/36	+	-	-	K2/36	+	-	-
M2/37	+	-	-	K2/37	+	-	-
M2/38	+	-	-	K2/38	+	-	-
M2/39	+	-	-	K2/39	+	-	-
M2/40	+	-	-	K2/40	+	-	-
M3/1	+	-	-	K3/1	+	-	-
M3/2	+	-	-	K3/2	+	-	-
M3/3	+	-	-	K3/3	+	-	-
M3/4	+	-	-	K3/4	+	-	-
M3/5	+	-	-	K3/5	+	-	-
M3/6	+	-	-	K3/6	+	-	-
M3/7	+	-	-	K3/7	+	-	-
M3/8	+	-	-	K3/8	+	-	-
M3/9	+	-	-	K3/9	+	-	-
M3/10	+	-	-	K3/10	+	-	-
M3/11	+	-	-	K3/11	+	-	-
M3/12	+	-	-	K3/12	+	-	-
M3/13	+	-	-	K3/13	+	-	-
M3/14	+	-	-	K3/14	+	-	-
M3/15	+	-	-	K3/15	+	-	-
M3/16	+	-	-	K3/16	+	-	-
M3/17	+	-	-	K3/17	+	-	-
M3/18	+	-	-	K3/18	+	-	-
M3/19	+	-	-	K3/19	+	-	-
M3/20	+	-	-	K3/20	+	-	-
M3/21	+	-	-	K3/21	+	-	-
M3/22	+	-	-	K3/22	+	-	-
M3/23	+	-	-	K3/23	+	-	-
M3/24	+	-	-	K3/24	+	-	-
M3/25	+	-	-	K3/25	+	-	-
M3/26	+	-	-	K3/26	+	-	-

Isolate	16S	nptII	nptIII	Isolate	16S	nptII	nptIII
M3/27	+	-	-	K3/27	+	-	-
M3/28	+	-	-	K3/28	+	-	-
M3/29	+	-	-	K3/29	+	-	-
M3/30	+	-	-	K3/30	+	-	-
M3/31	+	-	-	K3/31	+	-	-
M3/32	+	-	-	K3/32	+	-	-
M3/33	+	-	-	K3/33	+	-	-
M3/34	+	-	-	K3/34	+	-	-
M3/35	+	-	-	K3/35	+	-	-
M3/36	+	-	-	K3/36	+	-	-
M3/37	+	-	-	K3/37	+	-	-
M3/38	+	-	-	K3/38	+	-	-
M3/39	+	-	-	K3/39	-		
M3/40	+	-	-	K3/40	+	-	-
M4/1	+	-	-	K4/1	+	-	-
M4/2	+	-	-	K4/2	+	-	-
M4/3	+	-	-	K4/3	+	-	-
M4/4	+	-	-	K4/4	+	-	-
M4/5	+	-	-	K4/5	+	-	+
M4/6	+	-	-	K4/6	+	-	-
M4/7	+	-	-	K4/7	+	-	-
M4/8	+	-	-	K4/8	+	-	-
M4/9	+	-	+	K4/9	+	-	-
M4/10	+	-	-	K4/10	+	-	-
M4/11	+	-	-	K4/11	+	-	-
M4/12	+	-	-	K4/12	+	-	-
M4/13	+	-	-	K4/13	+	-	-
M4/14	+	-	-	K4/14	+	-	-
M4/15	+	-	-	K4/15	+	-	-
M4/16	+	-	-	K4/16	+	-	-
M4/17	+	-	-	K4/17	+	-	-
M4/18	+	-	-	K4/18	+	-	-
M4/19	+	-	-	K4/19	+	-	-
M4/20	+	-	-	K4/20	+	-	-
M4/21	+	-	-	K4/21	+	-	-
M4/22	+	-	-	K4/22	+	-	-
M4/23	+	-	-	K4/23	+	-	-
M4/24	+	-	-	K4/24	+	-	-
M4/25	+	-	-	K4/25	+	-	+
M4/26	+	-	-	K4/26	+	-	+
M4/27	+	-	-	K4/27	+	-	-
M4/28	+	-	-	K4/28	+	-	-
M4/29	+	-	-	K4/29	+	-	-
M4/30	+	-	-	K4/30	+	-	-
M4/31	+	-	-	K4/31	+	-	-
M4/32	+	-	-	K4/32	+	-	-
M4/33	+	-	-	K4/33	+	-	-
M4/34	+	-	-	K4/34	+	-	-
M4/35	+	-	-	K4/35	+	-	-
M4/36	+	-	-	K4/36	+	-	+
M4/37	+	-	-	K4/37	+	-	-
M4/38	+	-	-	K4/38	+	-	-
M4/39	+	-	-	K4/39	+	-	-
M4/40	+	-	-	K4/40	+	-	-
M5/1	+	-	-	K5/1	+	-	-

Isolate	16S	nptII	nptIII	Isolate	16S	nptII	nptIII
M5/2	+	-	-	K5/2	+	-	-
M5/3	+	-	-	K5/3	+	-	-
M5/4	+	-	-	K5/4	+	-	-
M5/5	+	-	-	K5/5	+	-	-
M5/6	+	-	-	K5/6	+	-	-
M5/7	+	-	-	K5/7	+	-	-
M5/8	+	-	-	K5/8	drop out		
M5/9	+	-	-	K5/9	+	-	-
M5/10	+	-	-	K5/10	+	-	-
M5/11	+	-	-	K5/11	+	-	-
M5/12	+	-	-	K5/12	+	-	-
M5/13	+	-	-	K5/13	+	-	-
M5/14	+	-	-	K5/14	+	-	-
M5/15	+	-	-	K5/15	+	-	-
M5/16	+	-	-	K5/16	+	-	-
M5/17	+	-	-	K5/17	+	-	-
M5/18	+	-	-	K5/18	+	-	-
M5/19	+	-	-	K5/19	+	-	-
M5/20	+	-	-	K5/20	+	-	-
M5/21	+	-	-	K5/21	+	-	-
M5/22	+	-	-	K5/22	+	-	-
M5/23	+	-	-	K5/23	+	-	-
M5/24	+	-	-	K5/24	+	-	-
M5/25	+	-	-	K5/25	+	-	-
M5/26	+	-	-	K5/26	+	-	-
M5/27	+	-	-	K5/27	+	-	-
M5/28	+	-	-	K5/28	+	-	-
M5/29	+	-	-	K5/29	+	-	-
M5/30	+	-	-	K5/30	+	-	-
M5/31	+	-	-	K5/31	+	-	-
M5/32	+	-	-	K5/32	+	-	-
M5/33	+	-	-	K5/33	+	-	-
M5/34	+	-	-	K5/34	+	-	-
M5/35	+	-	-	K5/35	+	-	-
M5/36	+	-	-	K5/36	+	-	-
M5/37	+	-	-	K5/37	+	-	-
M5/38	+	-	-	K5/38	+	-	-
M5/39	+	-	-	K5/39	+	-	-
M5/40	+	-	-	K5/40	+	-	-

Table 36: NptII/nptIII PCR results of kanamycin resistant strains isolated from soil samples

Drop out: no growth after re-cultivation from cryo-stock on nutrient agar.

M1-5: 40 bacterial isolates (1-40) from five soil types representative for maize growing areas in Austria

K1-5: 40 bacterial isolates (1-40) from five soil types representative for potato growing areas in Austria

2.4 Classification of bacterial strains resistant to kanamycin isolated from soil

Isolate	BLAST result	Match (max, identity)
K1_1	Pedobacter sp.	99
K1_2	Ensifer adhaerens	99
K1_4	Sphingobacterium multivorum	98
K1_5	Ensifer adhaerens	99
K1_6	Ensifer adhaerens	99
K1_7	Uncultured bacterium	99
K1_8	Pedobacter sp.	99
K1_9	Klebsiella sp.	98
K1_10	Klebsiella sp.	97
K1_11	Flavobacterium sp.	98
K1_12	Chryseobacterium sp.	99
K1_13	Pedobacter panaciterrae	99
K1_14	Paenibacillus apiarius	99
K1_15	Flavobacterium sp.	99
K1_16	Ensifer adhaerens	99
K1_17	Chryseobacterium sp.	99
K1_18	Pedobacter sp.	99
K1_19	Ensifer adhaerens	100
K1_20	Ensifer adhaerens	99
K1_21	Variovorax paradoxus	99
K1_23	Solitalea canadensis	99
K1_26	Pedobacter heparinus	98
K1_27	Flavobacterium sp.	98
K1_28	Klebsiella pneumoniae	98
K1_29	Variovorax paradoxus	99
K1_30	Ensifer adhaerens	100
K1_31	Ensifer adhaerens	99
K1_32	Sphingobacterium sp.	99
K1_33	Pedobacter agri	99
K1_34	Pedobacter steynii	99
K1_35	Flavobacterium sp.	98
K1_38	Ensifer adhaerens	99
K1_39	Dyadobacter sp.	96
K1_40	Sphingobacterium sp.	99
K2_1	Ensifer adhaerens	99
K2_2	Pedobacter piscium	98
K2_3	Paenibacillus apiarius	99
K2_4	Pedobacter sp.	99
K2_5	Ensifer adhaerens	100
K2_6	Chitinophagaceae	96
K2_7	Ensifer adhaerens	99
K2_8	Rhizobiaceae	96
K2_9	Paenibacillus apiarius	99
K2_10	Pedobacter sp.	98
K2_11	Ensifer adhaerens	99
K2_14	Paenibacillus terrigena	99
K2_15	Chitinophaga sp.	96
K2_16	Ensifer adhaerens	100

Isolate	BLAST result	Match (max, identity)
K2_18	Lysobacter gummosus	99
K2_19	Pedobacter sp.	99
K2_20	Pedobacter panaciterrae	99
K2_21	Dyadobacter fermentans	99
K2_22	Chitinophaga sp.	99
K2_23	Chitinophaga sp.	98
K2_26	Ensifer adhaerens	99
K2_27	Chitinophaga sp.	96
K2_28	Dyadobacter fermentans	99
K2_29	Dyadobacter fermentans	99
K2_31	Chitinophaga sp.	95
K2_32	Dyadobacter sp.	97
K2_33	Chitinophaga sp.	95
K2_36	Chitinophaga sp.	95
K2_37	Chitinophaga sp.	96
K2_38	Ensifer adhaerens	99
K2_40	Chitinophaga sp.	96
K3_1	Ensifer adhaerens	99
K3_2	Lysobacter sp.	100
K3_3	Dyadobacter sp.	97
K3_4	Microbacterium phyllosphaerae	99
K3_6	Ensifer adhaerens	100
K3_7	Pedobacter sp.	98
K3_8	Pedobacter sp.	98
K3_9	Flavobacterium sp.	99
K3_10	Pedobacter sp.	99
K3_11	Stenotrophomonas maltophilia	99
K3_12	Flavobacterium sp.	99
K3_14	Pedobacter panaciterrae	99
K3_15	Rhizobiaceae	96
K3_16	Pedobacter sp.	98
K3_17	Pedobacter panaciterrae	99
K3_18	Pedobacter panaciterrae	99
K3_19	Ensifer adhaerens	99
K3_20	Flavobacterium sp.	98
K3_21	Paenibacillus ehimensis	99
K3_22	Chryseobacterium joostei	99
K3_23	Mucilaginibacter sp.	98
K3_25	Paenibacillus sp.	99
K3_26	Mucilaginibacter sp.	98
K3_27	Stenotrophomonas maltophilia	99
K3_30	Stenotrophomonas rhizophila	100
K3_31	Ensifer adhaerens	99
K3_32	Mucilaginibacter sp.	97
K3_33	Dyadobacter fermentans	99
K3_34	Mucilaginibacter sp.	98
K3_35	Mucilaginibacter sp.	99
K3_36	Flavobacterium sp.	99
K3_37	Sphingoterrabacterium pocheensis	99
K3_40	Chitinophaga sp.	97
K4_1	Pedobacter sp.	99
K4_2	Ensifer adhaerens	100
K4_3	Chryseobacterium balustinum	99
K4_4	Flavobacterium sp.	99

Isolate	BLAST result	Match (max, identity)
K4_5	Stenotrophomonas sp.	98
K4_6	Stenotrophomonas rhizophila	99
K4_7	Pedobacter sp.	99
K4_8	Flavobacterium sp.	99
K4_9	Ensifer adhaerens	100
K4_10	Sphingobacterium sp.	100
K4_11	Ensifer adhaerens	100
K4_12	Flavobacterium sp.	97
K4_13	Ensifer adhaerens	99
K4_14	Pedobacter sp.	99
K4_15	Flavobacterium sp.	99
K4_16	Sanguibacter sp.	100
K4_17	Pedobacter sp.	96
K4_19	Flavobacterium sp.	99
K4_20	Ensifer adhaerens	100
K4_21	Pedobacter sp.	99
K4_25	Wautersiella sp.	97
K4_26	Pedobacter sp.	98
K4_27	Flavobacterium sp.	99
K4_28	Dyadobacter sp.	99
K4_29	Paenibacillus sp.	99
K4_30	Sphingobacterium faecium	99
K4_31	Pedobacter sp.	99
K4_32	Chryseobacterium sp.	99
K4_33	Pedobacter panaciterrae	99
K4_34	Pedobacter sp.	99
K4_35	Uncultured bacterium	94
K4_36	Pedobacter panaciterrae	98
K4_37	Pedobacter sp.	98
K4_38	Pedobacter sp.	98
K4_40	Flavobacterium sp.	99
K5_1	Ensifer adhaerens	99
K5_2	Ensifer adhaerens	99
K5_3	Sphingobacterium sp.	98
K5_4	Flavobacterium sp.	97
K5_5	Paenibacillus sp.	99
K5_6	Paenibacillus sp.	99
K5_7	Sphingobacterium sp.	96
K5_8	Chryseobacterium sp.	98
K5_9	Ensifer adhaerens	99
K5_10	Uncultured bacterium	99
K5_11	Dyadobacter sp.	99
K5_12	Ensifer adhaerens	99
K5_13	Uncultured bacterium	99
K5_15	Uncultured bacterium	97
K5_16	Flavobacterium sp.	100
K5_18	Pedobacter sp.	99
K5_19	Ensifer adhaerens	99
K5_20	Oerskovia enterophila/jenensis	99
K5_21	Sphingobacterium sp.	98
K5_22	Stenotrophomonas rhizophila	99
K5_23	Flavobacterium sp.	97
K5_25	Stenotrophomonas maltophilia	99
K5_26	Flavobacterium sp.	98

Isolate	BLAST result	Match (max, identity)
K5_27	Sphingobacterium sp.	98
K5_28	Sphingobacterium sp.	97
K5_29	Pedobacter sp.	97
K5_30	Variovorax paradoxus	99
K5_31	Stenotrophomonas rhizophila	99
K5_32	Variovorax paradoxus	99
K5_33	Chryseobacterium sp.	99
K5_34	Flavobacterium sp.	98
K5_35	Dyadobacter sp.	99
K5_36	Variovorax paradoxus	99
K5_37	Sphingobacterium sp.	98
K5_39	Cytophaga sp.	99
K5_40	Uncultured bacterium	95
M1_1	Flavobacterium sp.	99
M1_2	Uncultured bacterium	100
M1_3	Micrococcineae	94
M1_4	Pedobacter suwonensis/terrae	99
M1_5	Chryseobacterium sp.	99
M1_6	Ensifer adhaerens	100
M1_9	Pedobacter sp.	97
M1_10	Pedobacter suwonensis/terrae	99
M1_11	Chryseobacterium sp.	99
M1_12	Flavobacterium sp.	98
M1_13	Ensifer adhaerens	100
M1_14	Chryseobacterium sp.	99
M1_15	Pedobacter sp.	97
M1_16	Micrococcineae	93
M1_17	Pedobacter soli	99
M1_18	Flavobacterium sp.	99
M1_19	Oerskovia jenensis	99
M1_20	Flavobacterium johnsoniae	99
M1_21	Solitalea canadensis	99
M1_22	Pedobacter sp.	97
M1_23	Chryseobacterium sp.	99
M1_25	Luteifibra arvensicola	99
M1_26	Solitalea canadensis	100
M1_27	Flavobacterium sp.	98
M1_28	Pedobacter sp.	99
M1_29	Chryseobacterium sp.	99
M1_30	Mucilaginibacter sp.	98
M1_31	Mucilaginibacter sp.	97
M1_32	Pedobacter sp.	98
M1_33	Sphingobacteriaceae	97
M1_34	Pedobacter sp.	99
M1_35	Sphingobacterium sp.	99
M1_36	Pedobacter africanus/heparinus	99
M1_37	Solitalea canadensis	100
M1_38	Mucilaginibacter sp.	98
M1_39	Pedobacter sp.	99
M1_40	Chryseobacterium sp.	99
M2_1	Ensifer adhaerens	100
M2_2	Pedobacter panaciterrae	99
M2_3	Cellulosimicrobium cellulans	99
M2_4	Flavobacterium sp.	99

Isolate	BLAST result	Match (max, identity)
M2_5	Pedobacter sp.	98
M2_6	Pedobacter panaciterrae	100
M2_7	Microbacterium oxydans	100
M2_8	Uncultured bacterium	99
M2_9	Flavobacterium sp.	99
M2_10	Flavobacterium sp.	99
M2_11	Oerskovia enterophila	99
M2_12	Pedobacter panaciterrae	99
M2_13	Chryseobacterium sp.	99
M2_14	Ensifer adhaerens	99
M2_15	Uncultured bacterium	99
M2_16	Elizabethkingia meningoseptica	99
M2_17	Sphingobacterium sp.	99
M2_18	Pedobacter sp.	99
M2_19	Oerskovia jenensis	99
M2_20	Flavobacterium sp.	99
M2_21	Mucilaginibacter sp.	98
M2_22	Dyadobacter sp.	98
M2_23	Mucilaginibacter sp.	98
M2_25	Pedobacter soli	99
M2_26	Mucilaginibacter sp.	98
M2_27	Sphingobacteriaceae	96
M2_28	Pedobacter panaciterrae	99
M2_29	Dyadobacter fermentans/beijingensis	99
M2_30	Sphingobacteriaceae	98
M2_31	Pedobacter cryoconitis	99
M2_33	Sphingobacterium sp.	98
M2_34	Pedobacter terrae	99
M2_35	Pedobacter sp.	99
M2_36	Chryseobacterium sp.	99
M2_37	Sphingobacterium sp.	99
M2_40	Variovorax paradoxus	99
M3_1	Ensifer adhaerens	100
M3_2	Mucilaginibacter sp.	97
M3_3	Ensifer adhaerens	100
M3_4	Ensifer adhaerens	99
M3_5	Paenibacillus apiarius	99
M3_6	Chryseobacterium sp.	99
M3_7	Pedobacter sp.	99
M3_8	Chitinophaga sp.	95
M3_10	Pedobacter sp.	99
M3_11	Paenibacillus apiarius	99
M3_12	Chryseobacterium joostei	99
M3_13	Paenibacillus apiarius	99
M3_15	Ensifer adhaerens	99
M3_16	Ensifer adhaerens	100
M3_17	Flavobacterium sp.	99
M3_18	Sphingobacterium sp.	99
M3_19	Ensifer adhaerens	100
M3_22	Dyadobacter sp.	99
M3_23	Dyadobacter sp.	98
M3_24	Chitinophaga sp.	96
M3_25	Paenibacillus sp.	99
M3_26	Dyadobacter sp.	99

Isolate	BLAST result	Match (max, identity)
M3_27	Stenotrophomonas maltophilia	99
M3_28	Flavobacterium sp.	99
M3_29	Ensifer adhaerens	100
M3_30	Pedobacter sp.	98
M3_33	Pedobacter suwonensis/terrae	98
M3_34	Chitinophaga sp.	95
M3_35	Uncultured bacterium	99
M3_36	Pedobacter sp.	98
M3_37	Uncultured bacterium	98
M3_38	Stenotrophomonas maltophilia	99
M3_39	Ensifer adhaerens	100
M3_40	Chryseobacterium sp.	99
M4_1	Chitinophaga sp.	99
M4_2	Pedobacter sp.	98
M4_3	Stenotrophomonas maltophilia	99
M4_4	Chryseobacterium sp.	99
M4_5	Sphingobacteriaceae	94
M4_6	Pedobacter sp.	99
M4_7	Pedobacter sp.	99
M4_8	Pedobacter panaciterrae	98
M4_10	Pedobacter panaciterrae	99
M4_11	Sphingobacteriaceae	98
M4_12	Pedobacter sp.	99
M4_13	Pedobacter sp.	99
M4_14	Sphingobacterium sp.	97
M4_16	Sphingobacteriaceae	94
M4_17	Pedobacter panaciterrae	99
M4_18	Flavobacteriaceae	96
M4_19	Uncultured bacterium	96
M4_20	Pedobacter panaciterrae	99
M4_21	Stenotrophomonas maltophilia	99
M4_22	Stenotrophomonas maltophilia	99
M4_23	Pedobacter sp.	99
M4_24	Pedobacter panaciterrae	99
M4_26	Mucilaginibacter sp.	98
M4_27	Mucilaginibacter sp.	99
M4_28	Pedobacter sp.	98
M4_29	Pedobacter sp.	98
M4_30	Stenotrophomonas maltophilia	99
M4_31	Mucilaginibacter sp.	98
M4_33	Chitinophaga arvensicola	98
M4_34	Pedobacter soli	99
M4_37	Uncultured bacterium	94
M4_38	Uncultured bacterium	99
M4_39	Pedobacter sp.	98
M4_40	Uncultured bacterium	99
M5_1	Pedobacter suwonensis/terrae	99
M5_2	Flavobacterium sp.	96
M5_3	Chryseobacterium sp.	98
M5_4	Chryseobacterium sp.	98
M5_5	Oerskovia enterophila	99
M5_6	Ensifer adhaerens	100
M5_7	Ensifer adhaerens	100
M5_8	Stenotrophomonas maltophilia	99

Isolate	BLAST result	Match (max, identity)
M5_9	Pedobacter soli	99
M5_10	Chryseobacterium sp.	99
M5_11	Chryseobacterium sp.	99
M5_12	Pedobacter suwonensis/terrae	99
M5_13	Stenotrophomonas maltophilia	99
M5_15	Flavobacterium sp.	99
M5_16	Variovorax paradoxus	99
M5_17	Pedobacter suwonensis/terrae	99
M5_18	Chryseobacterium sp.	98
M5_19	Pedobacter sp.	99
M5_20	Flavobacterium sp.	98
M5_21	Pedobacter sp.	99
M5_22	Pedobacter sp.	98
M5_24	Chryseobacterium sp.	98
M5_25	Chryseobacterium sp.	99
M5_26	Solitalea canadensis	99
M5_27	Pedobacter suwonensis/terrae	99
M5_28	Dyadobacter fermentans	99
M5_29	Sphingobacteriaceae	95
M5_30	Stenotrophomonas maltophilia	99
M5_31	Variovorax paradoxus	99
M5_32	Dyadobacter sp.	99
M5_33	Sphingobacteriaceae	99
M5_34	Pedobacter sp.	98
M5_35	Dyadobacter sp.	97
M5_36	Bacteroidetes	99
M5_37	Uncultured bacterium	99
M5_38	Variovorax paradoxus	99
M5_39	Sphingobacteriaceae	98
M5_40	Pedobacter sp.	99

Table 37: Taxonomic characterization of isolated bacterial strains resistant to kanamycin from reference fields.

M1-5: 40 bacterial isolates (1-40) from five soil types representative for maize growing areas in Austria.

K1-5: 40 bacterial isolates (1-40) from five soil types representative for potato growing areas in Austria.

Species highlighted in yellow may be of clinical relevance (53, 62).

2.5 Quantitative PCR results in soil: 16S rRNA gene copy number

Soil Sample	Mean copy number per g soil	SD[1)]	Soil Sample	Mean copy number per g soil	SD[1)]
FM1	2,70E+09	5,54E+07	**FK1**	3,52E+09	2,10E+08
FM2	1,51E+09	1,19E+08	**FK2**	1,39E+09	6,78E+07
FM3	8,22E+08	5,08E+07	**FK3**	3,04E+09	1,18E+08
FM4	1,52E+09	4,28E+07	**FK4**	5,64E+08	8,30E+06
FM5	1,42E+09	8,34E+07	**FK5**	1,11E+09	4,54E+07
FM6	1,45E+09	4,98E+07	**FK6**	1,26E+09	6,76E+07
FM7	1,05E+09	3,48E+07	**FK7**	5,22E+08	1,26E+07
FM8	1,62E+09	6,54E+07	**FK8**	1,16E+09	4,58E+07
FM9	1,02E+09	4,14E+07	**FK9**	8,52E+08	3,04E+07
FM10	1,50E+09	7,20E+07	**FK10**	5,88E+07	4,52E+06
FM11	3,42E+09	6,94E+07	**FK11**	1,21E+09	2,36E+08
FM12	5,48E+09	2,36E+08	**FK12**	6,94E+08	1,21E+08
FM13	1,99E+09	1,32E+08	**FK13**	1,33E+08	3,68E+07
FM14	1,58E+09	2,96E+07	**FK14**	8,62E+08	1,46E+08
FM15	3,38E+09	1,61E+08	**FK15**	7,00E+08	5,52E+07
FM16	1,88E+09	8,28E+07	**FK16**	1,00E+09	1,95E+08
FM17	1,03E+09	6,62E+07	**FK17**	4,28E+08	1,23E+08
FM18	7,62E+08	1,15E+08	**FK18**	2,72E+08	3,12E+07
FM19	1,53E+09	6,78E+07	**FK19**	7,70E+08	7,16E+07
FM20	1,71E+09	8,50E+07	**FK20**	1,08E+09	2,20E+08
FM21	1,70E+09	6,62E+07	**FK21**	3,64E+08	2,14E+07
FM22	1,58E+09	1,55E+08	**FK22**	6,96E+08	1,08E+08
FM23	1,30E+09	3,66E+07	**FK23**	3,88E+08	2,46E+07
FM24	1,59E+09	1,14E+08	**FK24**	3,34E+08	3,10E+07
FM25	1,08E+09	4,96E+07	**FK25**	2,58E+09	3,38E+07
FM26	1,36E+09	2,14E+08	**FK26**	8,28E+08	2,96E+07
FM27	9,60E+08	4,58E+07	**FK27**	7,64E+08	9,44E+06
FM28	1,24E+09	1,41E+08	**FK28**	2,10E+09	6,46E+07
FM29	1,54E+09	1,89E+08	**FK29**	1,39E+09	6,40E+07
FM30	1,49E+09	3,80E+07	**FK30**	1,28E+09	5,02E+07
FM31	1,56E+09	8,14E+07	**FK31**	8,00E+08	2,94E+07
FM32	8,14E+08	1,11E+08	**FK32**	1,30E+09	3,82E+07
FM33	1,14E+09	7,26E+07	**FK33**	6,68E+08	2,52E+07
FM34	8,26E+08	4,70E+07	**FK34**	2,26E+09	4,88E+07
FM35	1,35E+09	4,24E+07	**FK35**	1,51E+09	3,48E+07
FM36	1,96E+09	6,16E+07	**FK36**	1,70E+09	5,42E+07
FM37	1,98E+09	7,64E+07	**FK37**	1,98E+09	4,32E+07
FM38	1,55E+09	4,34E+07	**FK38**	8,72E+08	1,54E+07
FM39	1,24E+09	2,34E+07	**FK39**	1,05E+09	4,62E+07
FM40	3,04E+08	1,65E+07	**FK40**	1,36E+09	4,66E+07
FM41	1,67E+09	6,66E+07	**FK41**	1,37E+09	8,06E+07
FM42	3,86E+09	1,04E+08	**FK42**	2,12E+09	1,60E+08
FM43	1,41E+09	4,24E+07	**FK43**	2,52E+09	1,86E+08
FM44	1,43E+09	5,68E+07	**FK44**	1,69E+09	6,86E+07
FM45	2,58E+09	2,56E+07	**FK45**	2,20E+09	9,28E+07
M1	2,40E+09	2,94E+07	**K1**	1,64E+09	4,92E+07
M2	3,68E+09	1,71E+08	**K2**	9,74E+08	1,48E+07
M3	1,25E+09	4,58E+07	**K3**	2,20E+09	1,25E+08
M4	1,57E+09	4,26E+07	**K4**	3,42E+09	1,77E+08
M5	3,04E+09	1,14E+08	**K5**	1,27E+09	2,38E+07

Table 38: 16S rRNA gene copy number in agricultural soils in Austria.

1) standard deviation

2.6 Quantitative PCR results in soil: nptII gene copy number

Soil Sample	Mean copy number /g soil	SD[1)]	Soil Sample	Mean copy number /g soil	SD[1)]
FM1	negative[2)]		**FK1**	negative	
FM2	negative		**FK2**	negative	
FM3	negative		**FK3**	3,12E+01	-[3)]
FM4	negative		**FK4**	negative	
FM5	1,34E+02	1,17E+02	**FK5**	negative	
FM6	negative		**FK6**	1,62E+02	-[3)]
FM7	negative		**FK7**	negative	
FM8	negative		**FK8**	negative	
FM9	negative		**FK9**	negative	
FM10	negative		**FK10**	negative	
FM11	negative		**FK11**	negative	
FM12	negative		**FK12**	negative	
FM13	negative		**FK13**	negative	
FM14	negative		**FK14**	negative	
FM15	8,56E+02	9,40E+01	**FK15**	negative	
FM16	negative		**FK16**	negative	
FM17	negative		**FK17**	negative	
FM18	negative		**FK18**	negative	
FM19	negative		**FK19**	negative	
FM20	1,38E+02	-[3)]	**FK20**	negative	
FM21	negative		**FK21**	negative	
FM22	negative		**FK22**	negative	
FM23	negative		**FK23**	negative	
FM24	negative		**FK24**	negative	
FM25	negative		**FK25**	negative	
FM26	negative		**FK26**	negative	
FM27	negative		**FK27**	negative	
FM28	negative		**FK28**	negative	
FM29	negative		**FK29**	negative	
FM30	negative		**FK30**	negative	
FM31	negative		**FK31**	negative	
FM32	negative		**FK32**	negative	
FM33	negative		**FK33**	negative	
FM34	negative		**FK34**	negative	
FM35	negative		**FK35**	negative	
FM36	negative		**FK36**	negative	
FM37	negative		**FK37**	negative	
FM38	negative		**FK38**	7,16E+02	9,00E+01
FM39	negative		**FK39**	negative	
FM40	negative		**FK40**	negative	
FM41	negative		**FK41**	negative	
FM42	negative		**FK42**	negative	
FM43	negative		**FK43**	negative	
FM44	negative		**FK44**	negative	
FM45	negative		**FK45**	negative	
M1	negative		**K1**	negative	
M2	negative		**K2**	negative	
M3	negative		**K3**	negative	
M4	negative		**K4**	negative	
M5	negative		**K5**	negative	

Table 39: NptII gene copy number in maize and potato fields in Austria

1) standard deviation 2) nptII concentration below the detection limit

3) single replicate positive only

2.7 Quantitative PCR results in soil: nptIII gene copy number

Soil Sample	Mean copy number /g soil	SD[1)]	Soil Sample	Mean copy number /g soil	SD[1)]
FM1	1,66E+04	1,33E+03	**FK1**	2,17E+02	5,44E+01
FM2	5,48E+02	6,44E+01	**FK2**	9,64E+02	1,40E+02
FM3	1,62E+03	8,52E+02	**FK3**	7,08E+03	1,34E+03
FM4	1,72E+03	4,00E+01	**FK4**	negative	
FM5	2,42E+02	-[3)]	**FK5**	negative	
FM6	4,68E+01	1,13E+01	**FK6**	1,30E+03	1,81E+02
FM7	5,56E+02	3,15E+02	**FK7**	1,26E+04	2,70E+02
FM8	2,38E+02	-[3)]	**FK8**	1,98E+03	1,79E+02
FM9	8,64E+03	1,26E+03	**FK9**	negative	
FM10	3,14E+02	3,32E+02	**FK10**	7,92E+02	1,02E+02
FM11	9,00E+02	3,33E+02	**FK11**	3,21E+03	1,11E+02
FM12	1,62E+02	[3)]	**FK12**	2,37E+03	3,95E+02
FM13	3,25E+03	8,00E+02	**FK13**	negative	
FM14	negative[2)]		**FK14**	3,99E+02	1,10E+02
FM15	4,16E+04	3,67E+03	**FK15**	3,16E+02	6,24E+01
FM16	2,51E+02	-[3)]	**FK16**	2,24E+02	1,66E+02
FM17	1,70E+03	2,93E+02	**FK17**	2,94E+03	3,41E+02
FM18	7,96E+02	2,50E+02	**FK18**	9,28E+02	3,54E+02
FM19	negative		**FK19**	5,12E+02	3,11E+01
FM20	1,32E+04	6,36E+02	**FK20**	negative	
FM21	2,78E+02	2,02E+02	**FK21**	2,84E+02	1,04E+02
FM22	4,48E+02	2,48E+02	**FK22**	negative	
FM23	2,50E+03	5,00E+01	**FK23**	2,74E+03	1,80E+02
FM24	7,56E+02	1,39E+02	**FK24**	7,04E+03	1,19E+03
FM25	2,55E+04	3,72E+03	**FK25**	8,20E+03	1,38E+03
FM26	negative		**FK26**	1,00E+03	8,16E+00
FM27	negative		**FK27**	1,06E+03	4,36E+02
FM28	1,28E+02	-[3)]	**FK28**	1,15E+03	2,96E+02
FM29	6,48E+02	2,65E+01	**FK29**	4,96E+02	7,12E+01
FM30	2,64E+03	3,65E+02	**FK30**	2,18E+03	1,85E+02
FM31	2,27E+02	1,09E+02	**FK31**	negative	
FM32	2,38E+03	5,52E+02	**FK32**	negative	
FM33	1,34E+02	1,10E+02	**FK33**	negative	
FM34	1,21E+03	8,44E+01	**FK34**	4,24E+03	1,54E+02
FM35	1,80E+03	1,66E+02	**FK35**	8,28E+03	1,06E+03
FM36	6,16E+04	2,49E+03	**FK36**	1,11E+04	7,32E+02
FM37	1,19E+03	4,44E+02	**FK37**	1,36E+03	3,77E+02
FM38	4,68E+03	6,28E+02	**FK38**	9,88E+02	4,16E+02
FM39	5,16E+03	1,93E+03	**FK39**	2,41E+02	8,20E+01
FM40	2,57E+02	-[3)]	**FK40**	9,93E+01	2,42E+01
FM41	1,84E+04	1,89E+03	**FK41**	3,82E+02	9,48E+01
FM42	3,75E+02	4,44E+02	**FK42**	1,39E+03	1,30E+02
FM43	6,04E+01	-[3)]	**FK43**	9,13E+01	4,32E+01
FM44	1,48E+04	6,16E+03	**FK44**	6,53E+02	2,09E+01
FM45	2,32E+04	8,68E+03	**FK45**	1,01E+04	1,70E+03
M1	3,44E+03	1,96E+03	**K1**	2,91E+04	2,19E+03
M2	6,45E+02	3,59E+02	**K2**	1,32E+01	-[3)]
M3	5,21E+02	2,09E+02	**K3**	negative	
M4	2,26E+03	3,13E+02	**K4**	3,39E+03	3,54E+03
M5	4,79E+03	3,96E+02	**K5**	negative	

Table 40: NptIII gene copy number in maize and potato fields in Austria

1) standard deviation 2) nptIII concentration below the detection limit

3) single replicate positive only

2.8 Relative quantification: results

Relative quantification: maize			Relative quantification: potato		
Soil sample	nptII/16S	nptIII/16S	Soil sample	nptII/16S	nptIII/16S
FM1	-	6,15E-06	FK1	-	6,17E-08
FM10	-	2,09E-07	FK10	-	1,35E-05
FM11	-	2,63E-07	FK11	-	2,65E-06
FM12	-	2,96E-08	FK12	-	3,41E-06
FM13	-	1,63E-06	FK13	-	-
FM14	-	-	FK14	-	4,63E-07
FM15	2,53E-07	1,23E-05	FK15	-	4,51E-07
FM16	-	1,34E-07	FK16	-	2,24E-07
FM17	-	1,65E-06	FK17	-	6,87E-06
FM18	-	1,04E-06	FK18	-	3,41E-06
FM19	-	-	FK19	-	6,65E-07
FM2	-	3,63E-07	FK2	-	6,94E-07
FM20	8,07E-08	7,72E-06	FK20	-	-
FM21	-	1,64E-07	FK21	-	7,80E-07
FM22	-	2,84E-07	FK22	-	-
FM23	-	1,92E-06	FK23	-	7,06E-06
FM24	-	4,75E-07	FK24	-	2,11E-05
FM25	-	2,36E-05	FK25	-	3,18E-06
FM26	-	-	FK26	-	1,21E-06
FM27	-	-	FK27	-	1,39E-06
FM28	-	1,03E-07	FK28	-	5,48E-07
FM29	-	4,21E-07	FK29	-	3,57E-07
FM3	-	1,97E-06	FK3	1,03E-08	2,33E-06
FM30	-	1,77E-06	FK30	-	1,70E-06
FM31	-	1,46E-07	FK31	-	-
FM32	-	2,92E-06	FK32	-	-
FM33	-	1,18E-07	FK33	-	-
FM34	-	1,46E-06	FK34	-	1,88E-06
FM35	-	1,33E-06	FK35	-	5,48E-06
FM36	-	3,14E-05	FK36	-	6,53E-06
FM37	-	6,01E-07	FK37	-	6,87E-07
FM38	-	3,02E-06	FK38	8,21E-07	1,13E-06
FM39	-	4,16E-06	FK39	-	2,30E-07
FM4	-	1,13E-06	FK4	-	-
FM40	-	8,45E-07	FK40	-	7,30E-08
FM41	-	1,10E-05	FK41	-	2,79E-07
FM42	-	9,72E-08	FK42	-	6,54E-07
FM43	-	4,28E-08	FK43	-	3,62E-08
FM44	-	1,03E-05	FK44	-	3,87E-07
FM45	-	8,99E-06	FK45	-	4,59E-06
FM5	9,44E-08	1,70E-07	FK5	-	-
FM6	-	3,23E-08	FK6	1,29E-07	1,03E-06
FM7	-	5,30E-07	FK7	-	2,41E-05
FM8	-	1,47E-07	FK8	-	1,71E-06
FM9	-	8,47E-06	FK9	-	-
M1	-	1,43E-06	K1	-	1,77E-05
M2	-	1,75E-07	K2	-	1,36E-08
M3	-	4,17E-07	K3	-	-
M4	-	1,44E-06	K4	-	9,91E-07
M5	-	1,58E-06	K5	-	-

Table 41: Relative quantification: quotient nptII/16S and nptIII/16S gene copy number

2.9 Microbial biodiversity – Pyrosequencing: genus frequency, pathogen status

Genus	Relative frequency (%)
Gemmatimonas	12,54
Ilumatobacter	4,75
Steroidobacter	4,74
Skermanella	4,45
Marmoricola	4,20
Nocardioides	4,03
Nitrospira	3,60
Blastococcus	2,88
Solirubrobacter	2,28
Arthrobacter	2,12
Methylibium	1,95
Streptomyces	1,94
Opitutus	1,78
Microlunatus	1,69
Amaricoccus	1,57
Caldilinea	1,56
Iamia	1,51
Lysobacter	1,47
Bacillus	1,46
Nitrosospira	1,44
Other	38,04

Table 42: Relative frequency of classified bacteria at genus level within the soil sample K1Aa.

Genus	Relative frequency (%)
Blastococcus	10,13
Gemmatimonas	6,93
Ilumatobacter	5,99
Skermanella	5,45
Solirubrobacter	5,35
Nocardioides	4,25
Microlunatus	3,97
Steroidobacter	3,94
Agromyces	3,35
Iamia	2,71
Pseudonocardia	2,65
Arthrobacter	2,26
Pirellula	1,97
Nitrosospira	1,90
Marmoricola	1,86
Nitrospira	1,29
Balneimonas	1,24

Gemmata	1,12
Streptomyces	1,03
Bacillus	1,01
Other	31,60

Table 43: Relative frequency of classified bacteria at genus level within the soil sample K2Ba.

Genus	Relative frequency (%)
Gemmatimonas	13,17
Blastococcus	6,73
Skermanella	5,45
Steroidobacter	4,61
Arthrobacter	3,80
Microlunatus	3,41
Solirubrobacter	3,38
Nocardioides	3,18
Nitrospira	3,12
Marmoricola	3,07
Ilumatobacter	2,80
Pseudonocardia	2,36
Nitrosospira	2,29
Streptomyces	1,79
Agromyces	1,43
Opitutus	1,41
Flavobacterium	1,33
Lysobacter	1,21
Tumebacillus	1,13
Balneimonas	1,12
Other	33,21

Table 44: Relative frequency of classified bacteria at genus level within the soil sample K3Sn.

Genus	Relative frequency (%)
Gemmatimonas	34,80
Nitrospira	4,34
Nocardioides	2,83
Ilumatobacter	2,73
Marmoricola	2,69
Methylibium	2,02
Phenylobacterium	2,00
Bradyrhizobium	1,87
Blastococcus	1,86
Steroidobacter	1,60
Solirubrobacter	1,56
Flavobacterium	1,51
Terrabacter	1,45
Arthrobacter	1,40

Opitutus	1,39
Pedomicrobium	1,35
Nitrosospira	1,11
Singulisphaera	1,02
Terrimonas	1,02
Massilia	0,98
Other	30,47

Table 45: Relative frequency of classified bacteria at genus level within the soil sample K4Bs.

Genus	Relative frequency (%)
Gemmatimonas	18,41
Skermanella	6,40
Blastococcus	5,92
Steroidobacter	4,83
Nocardioides	4,28
Solirubrobacter	4,15
Nitrospira	3,23
Ilumatobacter	2,97
Marmoricola	2,70
Arthrobacter	2,50
Nitrosospira	2,10
Lysobacter	1,99
Pseudonocardia	1,78
Microlunatus	1,70
Agromyces	1,59
Iamia	1,55
Lentzea	1,52
Streptomyces	1,32
Sphingobium	1,23
Amaricoccus	1,21
Other	28,62

Table 46: Relative frequency of classified bacteria at genus level within the soil sample K5Sa.

Genus	Relative frequency (%)
Gemmatimonas	41,61
Marmoricola	3,19
Nitrospira	3,19
Nocardioides	2,63
Phenylobacterium	2,37
Methylibium	2,20
Blastococcus	2,08
Arthrobacter	1,78
Solirubrobacter	1,72
Bradyrhizobium	1,63
Steroidobacter	1,62

Lysobacter	1,50
Singulisphaera	1,48
Nitrosospira	1,25
Terrabacter	1,22
Ilumatobacter	1,21
Streptomyces	1,17
Opitutus	0,96
Ktedonobacter	0,93
Anaeromyxobacter	0,86
Other	25,40

Table 47: Relative frequency of classified bacteria at genus level within the soil sample M1As.

Genus	Relative frequency (%)
Gemmatimonas	17,57
Bradyrhizobium	2,91
Arthrobacter	2,58
Nocardioides	2,38
Methylibium	2,37
Rhodanobacter	2,26
Marmoricola	2,22
Solirubrobacter	2,21
Nitrospira	2,13
Dokdonella	1,95
Opitutus	1,80
Singulisphaera	1,67
Humicoccus	1,64
Phenylobacterium	1,63
Blastococcus	1,57
Microlunatus	1,49
Nitrosospira	1,42
Terrabacter	1,41
Pseudonocardia	1,38
Bacillus	1,33
Other	46,08

Table 48: Relative frequency of classified bacteria at genus level within the soil sample M2Bs.

Genus	Relative frequency (%)
Gemmatimonas	11,66
Blastococcus	7,75
Nocardioides	5,98
Skermanella	4,99
Ilumatobacter	4,71
Solirubrobacter	4,61
Marmoricola	3,27
Microlunatus	2,87

Nitrosospira	2,70
Arthrobacter	2,29
Steroidobacter	2,24
Iamia	2,17
Agromyces	1,97
Nitrospira	1,92
Pirellula	1,72
Pseudonocardia	1,59
Streptomyces	1,26
Opitutus	1,22
Phenylobacterium	1,05
Microbacterium	1,01
Other	33,02

Table 49: Relative frequency of classified bacteria at genus level within the soil sample M3Sa.

Genus	Relative frequency (%)
Gemmatimonas	16,84
Rhodanobacter	9,37
Marmoricola	4,57
Nocardioides	4,22
Singulisphaera	3,86
Blastococcus	2,80
Arthrobacter	2,53
Streptomyces	2,40
Nitrosospira	2,27
Kribbella	2,21
Terrabacter	2,17
Phenylobacterium	1,84
Solirubrobacter	1,81
Mycobacterium	1,67
Nitrospira	1,39
Dyella	1,34
Bradyrhizobium	1,30
Caulobacter	1,30
Massilia	1,27
Mucilaginibacter	1,06
Other	33,78

Table 50: Relative frequency of classified bacteria at genus level within the soil sample M4Rs.

Genus	Relative frequency (%)
Gemmatimonas	15,51
Nitrospira	9,08
Ilumatobacter	3,53
Steroidobacter	3,24
Flavobacterium	3,11

Methylibium	2,79
Opitutus	2,68
Arthrobacter	2,61
Geobacter	2,36
Nocardioides	1,88
Lysobacter	1,69
Pirellula	1,52
Terrimonas	1,52
Marmoricola	1,50
Duganella	1,43
Massilia	1,43
Pedomicrobium	1,31
Phenylobacterium	1,26
Nitrosospira	1,22
Hyphomicrobium	1,01
Other	39,32

Table 51: Relative frequency of classified bacteria at genus level within the soil sample M5Sa.

Genus	Potential to cause disease in humans	Comment
Acetivibrio	No (41)	Plant cell wall degradation (10)
Acetonema	No (41)	Strictly anaerobic (29)
Achromobacter	Yes. A. xylosoxidans: Septicemia in noscomial settings (41)	Reduction of nitrite but not of nitrate
Acidisphaera	No (41)	
Acidothermus	No (41)	
Acidovorax	Pathogen status not established (41)	Aerobic. Environmental organism
Acrocarpospora	No. (41)	
Actinoallomurus	No. (41)	
Actinocatenispora	No. (41)	
Actinocorallia	No. (41)	
Actinomadura	No. (41)	
Actinomycetospora	No. (41)	
Actinoplanes	No?	Teicoplanin producer
Actinospica	No. (41)	
Actinosynnema	No. (41)	
Actinotalea	No. (41)	
Adhaeribacter	No. (41)	
Aeromicrobium	No. (41)	
Afifella	No. (41)	
Afipia	No. (41)	
Agreia	No. (41)	
Agrococcus	No. (41)	
Agromonas	No. (41)	
Agromyces	No. (41)	
Algoriphagus	No. (41)	
Alicyclobacillus	No. (41)	
Alkalibacter	No. (41)	
Alkaliphilus	No. (41)	
Allokutzneria	No. (41)	
Altererythrobacter	No. (41)	
Alterococcus	No. (41)	
Amaricoccus	No. (41)	
Aminobacter	No. (41)	
Ammoniphilus	No. (41)	
Amycolatopsis	No?	Vancomycin producer
Anaerobacter	No. (41)	
Anaerolinea	No. (41)	

Genus	Potential to cause disease in humans	Comment
Anaeromyxobacter	No. (41)	
Anaerosporobacter	No. (41)	
Anaerovorax	No. (41)	
Ancylobacter	No. (41)	
Aneurinibacillus	No. (41)	
Anoxybacillus	No. (41)	
Aquabacterium	No. (41)	
Aquicella	No. (41)	
Aquincola	No. (41)	
Archangium	No. (41)	
Arenibacter	No. (41)	
Arenimonas	No. (41)	
Arthrobacter	Yes. Bacteremia, urinary tract infections (41)	
Asanoa	No. (41)	
Aspromonas	No. (41)	
Asticcacaulis	No. (41)	
Aurantimonas	No. (41)	
Aureispira	No. (41)	
Azoarcus	No. (41)	
Azohydromonas	No. (41)	
Azospirillum	No. (41)	
Bacillus	Yes. Certain species. Opportunistic or obligate. Food poisoning (41)	
Bacteriovorax	No. (41)	
Balneimonas	No. (41)	
Bdellovibrio	No. (41)	
Beijerinckia	No. (41)	
Bellilinea	No. (41)	
Belnapia	No. (41)	
Blastobacter	No. (41)	
Blastococcus	No. (41)	
Blastomonas	No. (41)	
Blastopirellula	No. (41)	
Bosea	Not listed (41)	
Bradyrhizobium	No (41)	
Brevibacillus	Yes. Endophtalmitis (41)	
Brevundimonas	Yes. Bacteremia (rare cases) (41)	
Burkholderia	Yes. B. pseudomallei: Severe infections (meloidosis); B. cepacia: Nosocomial infections (41)	
Byssovorax	No (41)	
Caedibacter	No (41)	
Caenimonas	No (41)	
Caldalkalibacillus	No (41)	
Caldilinea	No (41)	
Caryophanon	No (41)	
Catellatospora	No (41)	
Catellibacterium	No (41)	
Catelliglobosispora	No (41)	
Catenulispora	No (41)	
Catenuloplanes	No (41)	
Caulobacter	No (41)	
Cellulomonas	No (41)	
Cellulosimicrobium	No (41)	
Cellvibrio	No (41)	
Chelatococcus	No (41)	
Chitinimonas	No (41)	
Chitinophaga	No (41)	
Chloroflexus	No (41)	
Chondromyces	No (41)	
Chryseobacterium	No (41)	
Clostridium	Yes. Several species pathogens (41)	
Cohnella	No (41)	
Comamonas	No (41)	
Conexibacter	No (41)	
Corallococcus	No (41)	
Corynebacterium	Yes. Several species induce severe	

Genus	Potential to cause disease in humans	Comment
	infections (41)	
Couchioplanes	No. (41)	
Craurococcus	No. (41)	
Croceicoccus	No. (41)	
Cryobacterium	No. (41)	
Cryptosporangium	No. (41)	
Cupriavidus	No. (41)	
Curtobacterium	Yes (41)	
Curvibacter	No. (41)	
Cystobacter	No. (41)	
Cytophaga	No. (41)	
Dactylosporangium	No. (41)	
Dechloromonas	No. (41)	
Defluviicoccus	No. (41)	
Dehalobacter	No. (41)	
Dehalogenimonas	No. (41)	
Deinococcus	No. (41)	
Delftia	No. (41)	
Demequina	No. (41)	
Denitratisoma	No. (41)	
Derxia	No. (41)	
Desulfitobacterium	No. (41)	
Desulfobulbus	No. (41)	
Desulfocapsa	No. (41)	
Desulforhopalus	No. (41)	
Desulfosporosinus	No. (41)	
Desulfovibrio	Yes. Opportunistic intestinal tract inhabitant (appendicitis) (41)	
Desulfuromonas	No. (41)	
Dethiobacter	No. (41)	
Devosia	No. (41)	
Dietzia	No?	
Dokdonella	No. (41)	
Duganella	No. (41)	
Dyadobacter	No. (41)	
Dyella	No. (41)	
Dysgonomonas	No. (41)	
Effluviibacter	No. (41)	
Elioraea	No. (41)	
Elizabethkingia	No. (41)	
Emticicia	No. (41)	
Enhygromyxa	No. (41)	
Ensifer	No. (41)	
Enterococcus	Yes. Urinary tract infections. Intrabdominal infections. Bacteremia, endocarditis. (41)	
Epilithonimonas	No. (41)	
Erythrobacter	No. (41)	
Erythromicrobium	No. (41)	
Ferribacterium	No. (41)	
Ferruginibacter	No. (41)	
Filibacter	No. (41)	
Filimonas	No. (41)	
Filomicrobium	No. (41)	
Flavisolibacter	No. (41)	
Flavobacterium	Pathogen status not established (41)	
Fluoribacter	No. (41)	
Fluviicola	No. (41)	
Frateuria	No. (41)	
Friedmanniella	No. (41)	
Frigoribacterium	No. (41)	
Gallicola	No. (41)	
Geminicoccus	No. (41)	
Gemmata	No. (41)	
Gemmatimonas	No. (41)	
Geobacillus	No. (41)	
Geobacter	No. (41)	
Geodermatophilus	No. (41)	

Genus	Potential to cause disease in humans	Comment
Georgenia	No. (41)	
Geothrix	No. (41)	
Glycomyces	No. (41)	
Gordonia	Yes. Cutaneous infections. Morbidity factor in immunocompromised patients (41)	
Gracilibacter	No. (41)	
Haliangium	No. (41)	
Haliscomenobacter	No. (41)	
Halobacillus	Pathogen status not established (41)	
Haloplasma	No. (41)	
Hamadaea	No. (41)	
Herbaspirillum	No. (41)	
Herbidospora	No. (41)	
Herminiimonas	No. (41)	
Herpetosiphon	No. (41)	
Humibacillus	No. (41)	
Humicoccus	No. (41)	
Hyalangium	No. (41)	
Hydrogenophaga	No. (41)	
Hymenobacter	No. (41)	
Hyphomicrobium	No. (41)	
Iamia	No. (41)	
Ideonella	No. (41)	
Ilumatobacter	No. (41)	
Inhella	No. (41)	
Inquilinus	No. (41)	
Isoptericola	No. (41)	
Isosphaera	No. (41)	
Janthinobacterium	No. (41)	
Jeotgalibacillus	No. (41)	
Jiangella	No. (41)	
Kaistella	No. (41)	
Kaistia	No. (41)	
Kibdelosporangium	No. (41)	
Kineococcus	No. (41)	
Kineosporia	No. (41)	
Kitasatospora	No. (41)	
Knoellia	No. (41)	
Kocuria	No. (41)	
Kofleria	No. (41)	
Krasilnikovia	No. (41)	
Kribbella	No. (41)	
Ktedonobacter	No. (41)	
Kutzneria	No. (41)	
Labrys	No. (41)	
Laceyella	No. (41)	
Lacibacter	No. (41)	
Lactobacillus	Yes. Orofacial infections. (41)	
Larkinella	No. (41)	
Lechevalieria	No. (41)	
Legionella	Yes. Severe infections, pneumonia. (41)	
Leifsonia	No. (41)	
Lentzea	No. (41)	
Leptonema	No. (41)	
Leucobacter	No. (41)	
Limnobacter	No. (41)	
Longilinea	No. (41)	
Luedemannella	No. (41)	
Luteibacter	No. (41)	
Luteimonas	No. (41)	
Luteolibacter	No. (41)	
Lutispora	No. (41)	
Lysinibacillus	No. (41)	
Lysobacter	No. (41)	
Magnetospirillum	No. (41)	
Marinactinospora	No. (41)	
Marinibacillus	No. (41)	

Genus	Potential to cause disease in humans	Comment
Marmoricola	No. (41)	
Massilia	No. (41)	
Mesorhizobium	No. (41)	
Methylibium	No. (41)	
Methylobacillus	No. (41)	
Methylobacterium	Yes. Skin ulcers, septicaemia etc…(41)	On vegatation but also in hospital environments
Methylocapsa	No. (41)	
Methylocystis	No. (41)	
Methylophilus	No. (41)	
Methylosinus	No. (41)	
Methylotenera	No. (41)	
Microbacterium	Yes. Foreign body infections, bacteremia. (41)	
Microbispora	No. (41)	
Microcella	No. (41)	
Microlunatus	No. (41)	
Micromonospora	No. (41)	Producer of gentamicin
Micropruina	No. (41)	
Microvirga	No. (41)	
Mitsuaria	No. (41)	
Modestobacter	No. (41)	
Mogibacterium	No. (41)	
Mucilaginibacter	No. (41)	
Muricauda	No. (41)	
Mycobacterium	Yes. Severe infections (41)	
Mycoplana	No. (41)	
Myroides	No. (41)	
Myxococcus	No. (41)	
Nakamurella	No. (41)	
Nannocystis	No. (41)	
Naxibacter	No. (41)	
Nevskia	No. (41)	
Niastella	No. (41)	
Nitrobacter	No. (41)	
Nitrosomonas	No. (41)	
Nitrosospira	No. (41)	
Nitrospira	No. (41)	
Nocardia	Yes. Rare infections, immunocompromised. (41)	
Nocardioides	No. (41)	
Nocardiopsis	Yes. Mycetomas, skin infections. Rare reports. (41)	
Nonomuraea	No. (41)	
Novosphingobium	No. (41)	
Nubsella	No. (41)	
Oceanibaculum	No. (41)	
Ochrobactrum	Yes. Catheder related infections. (41)	
Oerskovia	Yes. Foreign body infections, bacteremia. (41)	
Okibacterium	No. (41)	
Opitutus	No. (41)	
Orientia	Yes. Scrub typhus. (41)	
Ornithinicoccus	No. (41)	
Ornithinimicrobium	No. (41)	
Oryzihumus	No. (41)	
Oscillibacter	No. (41)	
Oxalicibacterium	No. (41)	
Oxobacter	No. (41)	
Paenibacillus	Yes. Meningitis, wound infections. (41)	
Paenisporosarcina	No. (41)	
Pandoraea	No. (41)	
Paracoccus	Yes. Paracoccioides. Granulomatous infection. (41)	
Paracraurococcus	No. (41)	
Parapedobacter	No. (41)	
Parasegetibacter	No. (41)	
Parvibaculum	No. (41)	

Genus	Potential to cause disease in humans	Comment
Patulibacter	No. (41)	
Paucibacter	No. (41)	
Pedobacter	Pathogen status not established (41)	Activated sludge, soil, fish
Pedomicrobium	No. (41)	
Pelomonas	No. (41)	
Pelotomaculum	No. (41)	
Phaselicystis	No. (41)	
Phenylobacterium	No. (41)	
Phycicoccus	No. (41)	
Phycicola	No. (41)	
Phyllobacterium	No. (41)	
Pigmentiphaga	No. (41)	
Pilimelia	No. (41)	
Pimelobacter	No. (41)	
Pirellula	No. (41)	
Planctomyces	No. (41)	
Planifilum	No. (41)	
Planococcus	Pathogen status not established (41)	Marine environments
Planomicrobium	No. (41)	
Planomonospora	No. (41)	
Plantibacter	No. (41)	
Polaromonas	No. (41)	
Pontibacter	No. (41)	
Porphyrobacter	No. (41)	
Procabacter	No. (41)	
Promicromonospora	No. (41)	
Propionibacterium	Yes. Skin infections, akne, foreign body infections surgical wounds. (41)	
Propionivibrio	No. (41)	
Prosthecobacter	No. (41)	
Prosthecomicrobium	No. (41)	
Proteiniborus	No. (41)	
Pseudoclavibacter	No. (41)	
Pseudogulbenkiania	No. (41)	
Pseudolabrys	No. (41)	
Pseudomonas	Yes. Severe infections (41)	
Pseudonocardia	No. (41)	
Pseudorhodobacter	No. (41)	
Pseudorhodoferax	No. (41)	
Pseudoxanthomonas	No. (41)	
Pyxidicoccus	No. (41)	
Quadrisphaera	No. (41)	
Ramlibacter	No. (41)	
Rathayibacter	No. (41)	
Renibacterium	No. (41)	
Rhizobacter	No. (41)	
Rhizobium	No. (41)	
Rhodanobacter	No. (41)	
Rhodobacter	No. (41)	
Rhodococcus	Yes. Opportunistic pathogen. Immunocompromised patients (41)	
Rhodoferax	No. (41)	
Rhodopila	No. (41)	
Rhodopirellula	No. (41)	
Rhodoplanes	No. (41)	
Rhodopseudomonas	No. (41)	
Rhodovarius	No. (41)	
Rickettsia	Yes. Rickettsialpox, typhus, murine typhus. Several species of indeterminant pathology. (41)	
Roseateles	No. (41)	
Roseococcus	No. (41)	
Roseomonas	No. (41)	
Rubellimicrobium	No. (41)	
Rubrobacter	No. (41)	
Rudaea	No. (41)	
Rummeliibacillus	No. (41)	
Runella	No. (41)	

Genus	Potential to cause disease in humans	Comment
Saccharibacillus	No. (41)	
Saccharococcus	No. (41)	
Saccharomonospora	Yes. (41)	
Saccharopolyspora	Yes. (41)	
Saccharothrix	No. (41)	
Salinibacterium	No. (41)	
Sandaracinobacter	No. (41)	
Sandarakinorhabdus	No. (41)	
Sanguibacter	No. (41)	
Schlesneria	No. (41)	
Sedimentibacter	No. (41)	
Sediminibacterium	No. (41)	
Segetibacter	No. (41)	
Serinibacter	No. (41)	
Shimazuella	No. (41)	
Shinella	No. (41)	
Silanimonas	No. (41)	
Simplicispira	No. (41)	
Singulisphaera	No. (41)	
Sinorhizobium	No. (41)	
Skermanella	No. (41)	
Smithella	No. (41)	
Solibacillus	No. (41)	
Solimonas	No. (41)	
Solirubrobacter	No. (41)	
Solitalea	No. (41)	
Sorangium	No. (41)	
Sphaerisporangium	No. (41)	
Sphaerobacter	No. (41)	
Sphingobacterium	Yes. (41)	
Sphingobium	No. (41)	
Sphingomonas	Yes. (41)	
Sphingopyxis	No. (41)	
Sphingosinicella	No. (41)	
Spirillospora	No. (41)	
Spirosoma	No. (41)	
Sporacetigenium	No. (41)	
Sporichthya	No. (41)	
Sporobacter	No. (41)	
Sporomusa	No. (41)	
Sporosarcina	Yes. (41)	
Sporotalea	No. (41)	
Sporotomaculum	No. (41)	
Stackebrandtia	No. (41)	
Staphylococcus	Yes. (41)	
Starkeya	No. (41)	
Stella	No. (41)	
Stenotrophomonas	Yes. (41)	
Steroidobacter	No. (41)	
Sterolibacterium	No. (41)	
Stigmatella	No. (41)	
Streptacidiphilus	No. (41)	
Streptomyces	Yes. ?	Antibiotic producers
Streptosporangium	No. (41)	
Symbiobacterium	No. (41)	
Syntrophobacter	No. (41)	
Syntrophomonas	No. (41)	
Tatlockia	No. (41)	
Tepidanaerobacter	No. (41)	
Tepidimicrobium	No. (41)	
Terrabacter	No. (41)	
Terrimonas	No. (41)	
Tetrasphaera	No. (41)	
Thermacetogenium	No. (41)	
Thermincola	No. (41)	
Thermoactinomyces	Yes. (41)	
Thermobacillus	No. (41)	
Thermobifida	No. (41)	

Genus	Potential to cause disease in humans	Comment
Thermobispora	No. (41)	
Thermoflavimicrobium	No. (41)	
Thermomonas	No. (41)	
Thermomonospora	No. (41)	
Thermopolyspora	No. (41)	
Thiobacillus	No. (41)	
Tissierella	No. (41)	
Trichococcus	No. (41)	
Truepera	No. (41)	
Tsukamurella	No. (41)	
Tumebacillus	No. (41)	
Turicibacter	No. (41)	
Uliginosibacterium	No. (41)	
Umezawaea	No. (41)	
Undibacterium	No. (41)	
Ureibacillus	No. (41)	
Variovorax	No. (41)	
Verrucomicrobium	No. (41)	
Virgisporangium	No. (41)	
Vogesella	No. (41)	
Wautersia	No. (41)	
Williamsia	No. (41)	
Xanthomonas	No. (41)	
Xylella	No. (41)	
Xylophilus	No. (41)	
Zavarzinella	No. (41)	

Table 52: Characterisation of bacterial taxa on genus level according to possible clinical relevance in soil samples (pyrosequencing)

Genera highlighted in yellow contain species with human pathogenic potential.

“No”: not listed in (41), thus, not recognized as major health threat at the time of printing the Clinical Manual of Microbiology (7th Edition, 1999).

3 Appendix B: Material and Methods

3.1 Random sampling plan – theoretical considerations

3.1.1 Sampling plan for areas used for the cultivation of maize

Fifty test areas with maize cultivation had been determined as part of the BINATS project and were used also for the present project(48, 49). In the case of maize squares with a side length of 625 m were used for calculations. The BINATS sampling plan was adopted for the current project. In addition, 25 substitute areas were sampled in a manner similar to the sampling of the potato areas (see section 3.1.2). This pool of substitute areas was necessary to avoid a shortage of representative test fields if the entry on the originally planned test site was prohibited by the land owner. The classification scheme is shown in Table 53 and Table 54 below.

climatic clusters	average temperature	average rainfall
c1	8.7	8.0
c2	8.0	11.0
c3	7.0	8.8

Table 53: Maize growing regions in Austria: Climatic clusters including average temperature and rainfall.

cluster	number of cells	
	population	substitute
Fluvisol – c1	353	3
Fluvisol – c2	340	3
Cambisol – c1	1388	10
Cambisol – c2	2986	18
Cambisol – c3	1985	12
Stagnosol– c1	557	4
Stagnosol – c2	852	6
Chernozem – c1	499	4
remnant	3173	15
total	**12133**	**25**

Table 54: Maize growing regions in Austria: Nine types of maize cropland and their corresponding numbers of cells.

The spatial distribution of the sampling areas and substitute sampling areas for soils from maize fields is provided in section 1.5.1, Figure 1.

3.1.1.1 Reference areas: maize

Five reference areas for an in depth analysis of soil parameters, microbiological biodiversity and cultivation and kanamycin resistance testing had to be determined. These were randomly chosen from the areas included in the sampling plan taking into account the following prerequisites (weighted from high to low importance):

1. high abundance of the soil type
2. different soil types to cover a wide array of biodiversity
3. different pH cluster

The selected soil types for the maize reference areas are shown in section 1.5.1, Table 1.

3.1.2 Sampling plan for areas used for the cultivation of potatoes

For the determination of the sampling locations, the territory of Austria was divided into regular 1km x 1km grid cells. The sampling population consisted of those cells for which at least 80% of the area lay inside the territory of Austria and where the area of cropland used for the cultivation of potatoes in 2009 and 2010 exceeded a minimum threshold. This minimum threshold was set to 0.975ha in order to guarantee that the sampling population contained at least 90% of the Austrian cropland.

Using Cluster Analysis, the grid cells were categorized into three distinct groups with respect to climatic factors such as average temperature and rainfall. Taking further factors such as pH-value and soil type into account, nine types of potato cropland were identified. The three climatic groups and the resulting nine types of potato cropland are provided in Table 55 and Table 56 below.

climatic clusters	average temperature	average rainfall
c1	7.8	10.4
c2	6.4	7.6
c3	9.1	9.3

Table 55: Potato growing regions in Austria: Climatic clusters including average temperature and rainfall.

In a first step, 50 samples were proportionally allocated to the different types of cropland according to their occurrence in Austria. In an attempt to balance the number of samples per type of potato cropland, a minimum number of four samples per cluster was enforced. The resulting distribution of the number of samples can be seen in Table 56. For each type of cropland, the specific sample areas were then chosen at random. 25 additional substitute areas were sampled in the same way.

cluster	number of cells		
	population	sampling	substitute
alkaline – Fluvisol – c1	83	4	2
alkaline – Fluvisol – c3	117	4	2
acidic – Cambisol – c1	258	5	3
acidic – Cambisol – c2	880	7	4
alkaline – Cambisol – c3	117	4	2
acidic – Cambisol – c3	169	4	2
alkaline – Chernozem – c3	1100	9	4
acidic – Chernozem – c3	116	4	2
remnant	884	9	4
total	**3724**	**50**	**25**

Table 56: Potato growing regions in Austria: Nine types of potato cropland and their corresponding numbers of cells

The spatial distribution of the areas included in the sampling plan and the locations of the substitute sampling areas are provided in section 1.5.2, Figure 2:

3.1.2.1 Reference areas: potato

Five reference areas for an in depth analysis of soil parameters, microbiological biodiversity, cultivation, characterization and kanamycin resistance testing of bacterial strains were determined. These were randomly chosen from the areas included in the sampling plan taking into account the following prerequisites (weighted from high (1) to low importance (3)):

1. high abundance of the soil type
2. different soil types to cover a wide array of biodiversity
3. different pH cluster

Almost 90% of Austrian potato cropland consists of the following soil types: Cambisol (41.2%), Chernozem (34.9%), Fluvisol (7.3%) and atypical soil (6.6%). Therefore these soil types were taken into account when selecting the five reference areas. Table 57 gives an overview of the numbers of cropland areas (= cells) in the population and in the sampling plan.

	population		sampling plan		reference areas	
	pH-value		pH-value		pH-value	
soil type	alkaline	acidic	alkaline	acidic	alkaline	acidic
Cambisol	150	1383	4	16	1	1
Chernozem	1175	123	10	4	1	1
Fluvisol	219	53	9		1	
atypical soil	221	25	2			

Table 57: Soil types of potato cropland and their corresponding numbers of cells in Austria (sampling population) and the sampling plan.

One specific reference area of each of the selected soil types was subsequently chosen at random (Table 57).

3.2 PCR conditions for nptII/nptIII real time PCR TaqMan Double and 16S TaqMan real time screening assays

All real time PCR assays were implemented on the LightCycler LC480 real time PCR platform (Roche, Austria) using 96-well microtiter plates. All pipetting steps were performed manually under strict adherence to good laboratory practices (distinct sample preparation and PCR mastermix pipetting rooms, separated PCR platform and post-PCR analysis room, separate laboratory equipment and gowns in each area, routine decontamination of the equipment with 10% sodium hypochlorite, pipetting exclusively in laminar airflow hoods and daily decontamination of these hoods with 10% sodium hypochlorite and/or UV radiation).

NptII and nptIII gene targets in bacterial isolates were detected simultaneously in a single well.

Of the 10^{-1} diluted bacterial Quickextract DNA solution 1.0 (Epicentre; Madison, USA), an amount of 2 µl was transferred into 8 µl of the real time PCR TaqMan assay mix resulting in total PCR assay volume of 10 µl. PCR TaqMan Double Assays were prepared according to the recommendations of the manufacturer (Ingenetix, Vienna). Briefly, 8 µl of the real time PCR mastermix comprising of 5 µl of the LC480 Probe Master (Roche, 2x), 0.5 µl of the nptII mix (Ingenetix), 0.5 µl of the nptIII mix (Ingenetix), and 2 µl H_2O (molecular biology grade, Sigma) were transferred into a 96 well microplate and supplemented by 2 µl of the DNA template solution. For details see Table 58.

The results were analysed using the second derivative maximum method of the LightCycler LC480 software version 1.5. with default parameters.

Each 96-well microtiter plate contained two negative controls (H_2O as template) and nptII and nptIII plasmid positive controls in duplicates. The PCR run was only valid if all negative controls were negative and all positive controls positive. Otherwise the run was repeated. The amplification conditions are depicted in Table 59. For PCR primer sequences see Table 60. Die amplicon lengths for the nptII, nptIII and 16S specific TaqMan PCRs are 129 bp, 82 bp, and 571 bp, respectively. The position of the nptII amplicon and TaqMan probe relative to the nptII reference sequence (Genbank accession No. V00618) is depicted in Figure 44. The characteristics of the nptIII amplicon (reference sequence: V01547) can be found in Figure 45.

The same DNA extraction solution from each sample was tested with the nptII/nptIII TaqMan Double Assay and with the 16S TaqMan DNA extraction/amplification control assay. Samples with a negative 16S TaqMan assay result (Cp > 27) were excluded from statistical analysis if the nptII and/or the nptIII PCR result were negative.

Reagent	Volume
LC480 Probemaster (Roche, Austria):	5 µl
nptII mix (Ingenetix, Austria): primer concentration: 0.6 µM probe concentration: 0.2 µM probe label : FAM	0.5 µl
nptIII mix (Ingenetix; Austria): primer concentration: 0.6 µM probe concentration: 0.2 µM probe label : 5'YYE	0.5 µl
H_2O (Sigma, molecular biology grade):	2 µl
Template:	2 µl
Total:	10 µl

Table 58: PCR TaqMan Double Screening Assay: Components

Item	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1 x
Amplification: Denaturation Annealing and elongation	 95°C 60°C	 10 s 20 s	45 x
Cooling	40°C	continuous	1 x

Table 59: PCR TaqMan nptII/nptIII Double Screening Assay: PCR conditions (LC480 96-well microplate format).

Primer	Sequence (5' → 3')	Length
np2-F:	GAT CTC CTG TCA TCT CAC CTT GCT	24 nts
np2-R	TCG CTC GAT GCG ATG TTT C	19 nts
nptIII-F	ACA TAT CGG ATT GTC CCT ATA CGA A	25 nts
nptIII-R	TCG GCC AGA TCG TTA TTC AGT A	22 nts
16S_F	TGG AGA GTT TGA TCM TGG CTC AG	23 nts
16S_R	CTT TAC GCC CAR TRA WTC CG	20 nts

Table 60: PCR Primer for TaqMan nptII/nptIII Double Screening and 16S Single Assay.

Probe	Sequence (5' → 3')	Length
nptII	FAM-TCATGGCTGATGCAATGCGGC-BHQ-1	21 nts
nptIII	YYE -AGACAGCCGCTTAGCCGAATTGGATT-BHQ-1	26 nts
16S	confidential business information	

Table 61: TaqMan probe sequences.

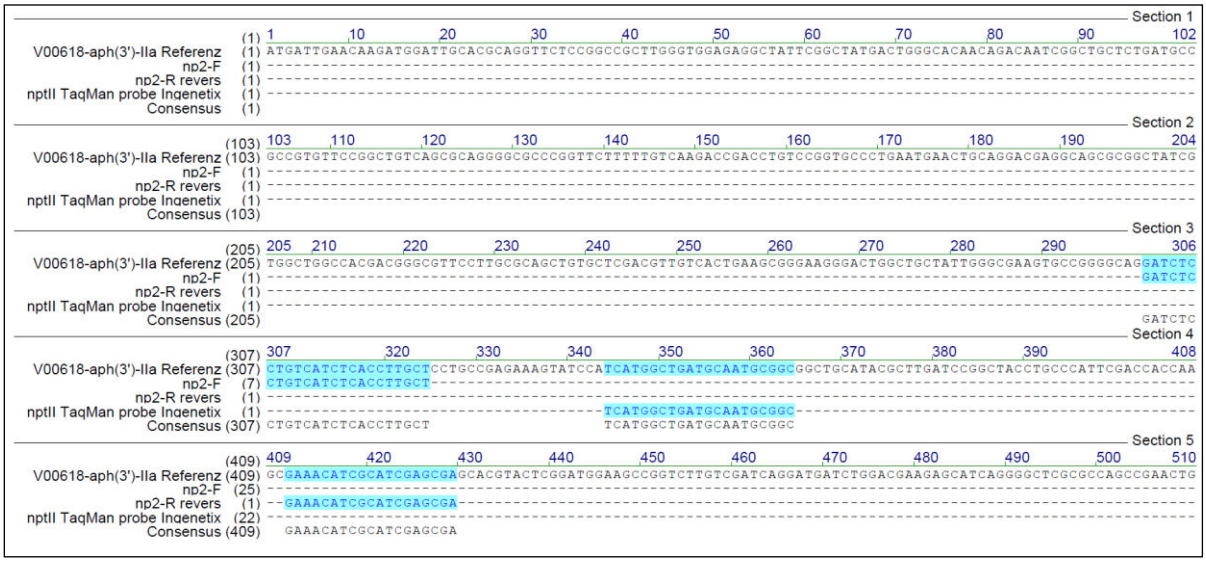

Figure 44: nptII TaqMan amplicon: primers and probe

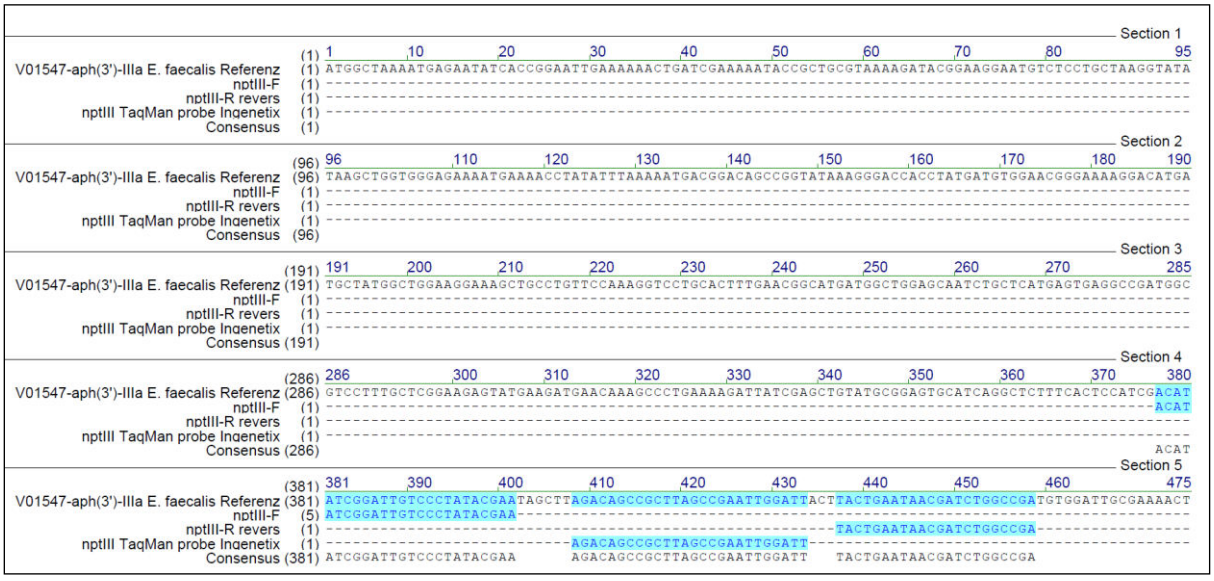

Figure 45: nptIII TaqMan amplicon: primers and probe

3.3 Amplification and DNA extraction control: 16S rRNA TaqMan Single Screening Assay

Each soil bacterial isolate was tested for amplifiable DNA and for PCR inhibition by analysing the respective Epicenter DNA extract with an "internal" control 16S TaqMan Assay.

The 16S TaqMan PCR assay conditions were as follows: 8 µl of the real time PCR mastermix comprising of 5 µl of the LC480 Probe Master (Roche, 2x), 0.5 µl of the 16S TaqMan probe (Ingenetix), 0.28 µl of the 16S primermix (Ingenetix; degenerated, 500 nM) and 2.22 µl H_2O (molecular biology grade, Sigma) were transferred into a 96 well microplate and supplemented by 2 µl of the DNA template solution. PCR was run on a LightCycler LC480 device (Roche). 16S rRNA TaqMan PCR specific details concerning assay composition and PCR conditions are depicted in Table 62 and Table 63.

The results were analysed using the second derivative maximum method of the LightCycler LC480 software version 1.5 with default parameters.

Reagent	Volume
LC480 Probemaster (Roche, Austria):	5 µl
Primer 16S (degenerated, mix) (Ingenetix, Austria): primer concentration: 0.5 µM	0.28 µl
probe concentration: 0.2 µM probe label : Cy5	0.5 µl
H_2O (Sigma, molecular biology grade):	2.22 µl
Template:	2 µl
Total:	10 µl

Table 62: PCR TaqMan 16S Single Assay: composition

Item	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1 x
Amplification: Denaturation Annealing and elongation	 95°C 60°C	 10 s 1 min	 45 x
Cooling	40°C	continuous	1 x

Table 63: PCR TaqMan 16S Single Assay: PCR conditions (LC480 96-well microplate format).

3.4 PCR conditions for quantitative nptII, nptIII and 16S single assays

	nptII	nptIII	16S
Probes			
Label:	FAM	5'YYE	Cy5
Concentration:	0.2 µM	0.2 µM	0.2 µM
Primers			
Forward	0.6 µM	0.6 µM	0.5 µM primer mix (degenerated, universal 16S)
Revers	0.6 µM	0.6 µM	
PCR conditions			
A) Initial denaturation	95°C/10 min/1x	95°C/10 min/1x	95°C/10 min/1x
B) Cycling	45 x	45 x	45 x
denaturation	95°C/10 s	95°C/10 s	95°C/10 s
annealing + elongation	60°C/20 s	60°C/20 s	60°C/1 min
C) Cool down	40°C/continuous	40°C/continuous	40°C/continuous
Assay			
Template volume	2 µl	2 µl	2 µl
PCR mix volume	8 µl	8 µl	8 µl
Total PCR assay volume	10 µl	10 µl	10 µl

Table 64: LightCycler LC480 real time TaqMan PCR parameters for quantitative single assays.

3.5 Validation of the PCR Assays

Data acquisition for the validation of the TaqMan PCR assays was performed as follows:

A semilogarithmic limited dilution of a pCR2.1 plasmid containing the full length nptII or nptIII or 16S rDNA of *E. coli* was prepared (750 000 - 0.38 copies/assay). The PCR measurement of each dilution step was performed in at least 8 replicates per run. A statistical analysis of at least 3 independent PCR runs resulting in at least 24 data points for each dilution step was performed. 95% detection limit was calculated by probit regression analysis. The linear quantification limit was established by the following formula: LOQ = 10* sigma/S (sigma = standard deviation of the response variables; S = slope of the calibration curve). Inter and Intra-assay variability was characterized by the calculation of the standard deviation and the coefficient of variation of each dilution step.

For details on the validation procedure see part E of the project.

TaqMan system	95% detection limit (copies/assay; median)	Linear quantification limit (copies/assay)
nptII	7.9	43
nptIII	11.4	318.1
16S	-[1)]	56.2

Table 65: Detection and linear quantification limits of the TaqMan PCRs.

1) Not applicable due to 16S rDNA background in PCR reagents.

nptII TaqMan Assay	Intra-assay variability	Inter-assay variability
SD min	0.05	0.13
SD max	0.7	1.07
CV (%) min	0.19	0.60
CV (%) max	1.97	2.13
Measurements	216	216

Table 66: Reproducibility of the nptII TaqMan PCR system.

Analysis range: 48 – 750,000 copies/assay.

nptIII TaqMan Assay	Intra-assay variability	Inter-assay variability
SD min	0.02	0.27
SD max	1.51	1.11
CV (%) min	0.10	1.34
CV (%) max	5.15	3.46
Measurements	253	253

Table 67: Reproducibility of the nptIII TaqMan PCR system.

Analysis range: 48 – 750,000 copies/assay

16S TaqMan Assay	Intra-assay variability	Inter-assay variability
SD min	0.04	0.17
SD max	0.24	0.54
CV (%) min	0.16	0.58
CV (%) max	0.70	2.14
Measurements	168	168

Table 68: Reproducibility of the 16S TaqMan PCR system.

Analysis range: 48 – 750,000 copies/assay

3.6 Applied procedure to avoid soil contamination from previously tested fields

To avoid problems concerning DNA cross-contamination between soil samples which may lead to false positive results in the following sensitive TaqMan real time PCR analyses, a diluted DanKlorix-solution (1:10 DanKlorix with distilled water) was used for decontamination of the instruments used for soil sampling in the field. The rakes were put into the decontamination solution for at least two to five minutes (Figure 46). The hark-stick was also cleaned using the same solution. The whole harks were then washed with distilled water and dried in the air. Special care was taken in avoiding contact of the instruments with shoes or clothing during soil sampling on the field which could have resulted in unintended sample contamination. Medical gloves were used in the field to avoid contaminations from the skin. Plastic bags for the soil samples were stored in an extra, closed box in the car for additional contamination security reason.

Figure 46: Decontamination with diluted DanKlorix-solution in the field.

3.7 Survey of field soil parameters

A protocol sample sheet was completed for each test field. The following information and parameters were recorded:

- Name of the field biologist
- Date and time of sampling
- Closest village on the Austrian map
- Code for the test area
- Field number
- Crop: maize or potato
- Usage of the standard sampling plan or method deviation
- Checklist: Ten single soil samples taken
- Confirmation of homogenisation of the composite sample
- Confirmation of sieving
- Confirmation of labelling of the plastic bag with the soil sample
- Storage (ice-box or in the car, recording of storage temperature)
- Handover of the soil samples to the AGES soil sample collection center (date)
- Labelling of the sample for special analyses

- Moisture condition of the field soil (wet – moist – dry)
- Soil type (Chernozem – Cambisol – Fluvisol)
- Comment if sampling design had to be adopted
- Weather conditions (sunny – clear – cloudy – rainy)
- Air temperature (10-15°C, 16-20°C, 21-25°C, 26-30°C, more than 30°C)
- Hygiene protocol: confirmation of usage of plastic gloves
- Decontamination of the sieve in a 10% DanKlorix-solution followed by a washing procedure with distilled water, air drying
- Decontamination of instruments in a 10% DanKlorix-solution followed by a washing procedure with distilled water, air drying
- Compliance of entering prohibitions.

3.8 Handling and sieving of composite soil samples

The fresh weight of the composite soil sample material of all ten test plants per field was approx. 750 g. After collection, the composite soil samples were cooled in a small car-refrigerator or in a cool-bag to approximately 8°C. Temperature was regularly controlled and recorded in the protocol. The soil samples were transferred to the AGES soil sample collection site in Vienna as soon as possible after collection (ideally on the same day) and were stored in a cold-store chamber (air-conditioned to a temperature below 10°C) until sieving.

Before starting the sieving process, all instruments (sieves, scissors, buckets etc…) were decontaminated in a DanKlorix-solution (1:10 with distilled water, Figure 47) and then dried. To accelerate the process, the instruments were also blow-dried.

Figure 47: Demonstration of the decontamination procedure in the laboratory.

Decontamination of all instruments was conducted with a 10% DanKlorix-solution.

Each composite soil sample was homogenized in a decontaminated bucket by hand using a medical glove. Then a small portion of the soil material was sieved through a decontaminated sieve with a mesh size of 2 mm. From this material a 50 ml sterile plastic tube was filled with this sieved material, labelled and cooled again in the cold-store chamber until further analysis. This sieved material was used for DNA-analysis and for cultivation of soil bacteria. The remaining not sieved material was filled into a twofold labelled plastic bag and also stored in the cold store chamber. This material was used for the analysis of soil parameters.

3.9 Instruction manual to avoid cross-contaminations during the soil sieving procedure

All soil samples were handled according to the following instructions:

1. **Use medical gloves throughout the whole sieving procedure!**
   The sieve is put into a 10% DanKlorix-solution on both sides for at least five minutes. Afterwards it is extensively washed with distilled water (all fringes and the net on both sides). The net is then joggled and put on a freshly laid out kitchen paper until it is dry. A hair dryer can be used for acceleration of the drying process. The decontaminated sieve is to be touched with new medical gloves only. Do not use the same gloves which have been used during the DanKlorix-handling.

2. Clean a bucket generously with a 10% DanKlorix-solution using a kitchen paper and expose it for at least five minutes. Afterwards rinse the bucket walls - handling of two persons, spin the bucket - and the fringes twice with distilled water. Additionally, rinse the bottom of the bucket with distilled water once again and pour out the remaining water. Dry the bucket. You can also use a fresh kitchen paper for drying the bucket's insides. Use medical gloves! The kitchen paper has to be stored separately. Storing should be avoided in the area where sieving is performed to avoid unintended contaminations with the soil of former samples.

3. The soil sample which is wrapped twice in plastic bags is prepared for sieving. The first plastic bag is removed. The labelled bag is saved as a reminder for labelling the new plastic bag.

4. New clean medical gloves are used for the next steps.

5. Scissors are decontaminated using a 10% DanKlorix-solution and afterwards wiped with a kitchen paper which is imbued with distilled water.

6. The plastic bag containing the soil sample is wiped along the seam with 10% DanKlorix-solution and then dried with a fresh kitchen paper.

7. Using the disinfected scissors the bag is cut along the steam for a short distance. Through this hole the soil material is then carefully dumped into the prepared decontaminated and dried bucket. Crumbly fractions of the composite soil sample are then crushed with the hand (use medical gloves) and afterwards the sample is homogenized. Be aware of contact with the soil or the bucket's insides, e. g. with the forearm. Do not touch the bucket outside with your hand protected with a glove because of contamination risks with soil of former samples.

8. **For this step two persons are recommended**: the soil is being transferred from the bucket into a new plastic bag. After that a small part of the sample is dumped into a decontaminated sieve (both persons have to use medical gloves). This portion of soil is sieved through a sieve with a mesh-wide of 2 mm into the same bucket. A prepared 50 ml plastic tube is held over a dustbin by

the first person, the second person takes the sieved soil out of the bucket with new medical gloves and trickles it without touching the tube into the plastic tube until it has reached the 50 ml mark. The other person screws down the tube without touching the inside of the tube cap.

9. The 50 ml plastic tube is labelled (project FEAR, test area number, date and location of sampling, initials for the crops: M for maize and K for potato (German: Kartoffel). Likewise the plastic bag is labelled directly on the bag's label with a water-resistant pen. Additionally, a likewise labelled tesa-stripe is stuck onto the knotted bag and on the tube, respectively. The sample for soil parameter analysis is put into a second fresh plastic bag (protection for being torn and consequently for sample mixing) and is knotted.

10. The 50 ml plastic tube for analysis of soil bacteria (living soil sample) has to be stored in a cool place of approximately 4°C and should be processed immediately. The soil sample in the plastic bag for analysing the soil parameters can also be stored at room temperature for several days without losing quality.

11. The sieves are cleaned with a brush in a separate room and should be free of soil for further usage. Be aware of soil dust which could contaminate subsequent samples. Please do not store buckets for subsequent samples close to this dust cleaning area.

12. For sieving the next soil sample, the procedure is repeated following the protocol from item 1 to item 11.

3.10Allowance for soil sampling

For compensation all collaborating farmers received the data of the soil parameter analysis of their provided test fields accompanied with recommendations on fertilizer usage to optimize crop yields.

3.11Determination of soil parameters

The samples (bulk soil, not sieved) were handed over to the Department for Sustainable Plant Production, AGES, Vienna. This Department is officially certified for soil parameter analysis and is producing this kind of data on a routine basis according to relevant national (ÖNORM) and international guidelines (http://www.ages.at/landwirtschaft/produkte-und-tarife/tarife-des-geschaeftsfeldes-ernaehrungssicherung/boden/).

All soil samples got a certificate for analysis. This certificate may be used for interactions with federal authorities.

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Frequency of Environmental Antibiotic Resistance

Part C:

NptII and nptIII Prevalence in Feed

Final Report

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1 Prevalence of nptII and nptIII in maize and potato samples used as feed in Austria

1.1 Summary

Total DNA extracts from maize and potatoes - corresponding to ARM gene carrying transgenic maize and potato varieties with approval for marketing and cultivation in the European Union - were checked for the presence of nptII and nptIII resistance gene copies using TaqMan real time PCR. Additionally the cultivable cell number from plant associated bacteria and the kanamycin resistance rates of these bacterial populations were obtained.

The prevalence of nptII and nptIII resistance genes in total DNA extracts from maize and potatoes appeared to be low in Austria during the testing period. No samples contained nptII or nptIII copy numbers above the detection limit. A statistical evaluation showed that within a confidence level of 95% less than 6.9% of the maize and potato samples would be carriers of nptII or nptIII.

Phenotypic resistance to kanamycin varied considerably within bacterial populations obtained from maize samples. A minimum of 0.01% resistant to kanamycin was observed with a bacterial population from a maize sample from VST Freistadt, Upper Austria, whereas 73.05% were resistant in a maize sample from Gleisdorf, Styria. Resistance to kanamycin was more homogenously distributed within bacterial populations obtained from potato samples showing considerably lower overall resistance rates to kanamycin (minimum: 0.002%; maximum: 6.6%) compared to maize samples.

In the kanamycin resistant fraction of plant associated bacteria nptII was only detected in a single isolate from maize (prevalence estimator: 1.1%; 95% confidence interval: [0%;4.9%]), nptIII was not present in the analysed collection of bacteria (0%; [0%;3.2%]). For bacteria isolated from potatoes it was calculated that within a confidence level of 95% less than 4% would be carriers of nptII or nptIII genes. Concerning the PCR results obtained from the kanamycin resistant bacterial isolates it should be noted that only a relatively small number of bacterial strains (maize: nptII: 94, nptIII: 93; potato: nptII/nptIII: 73) was analysed for the presence of nptII or nptIII resistance genes and contamination of agar plates with fungi during the recovery of kanamycin resistant strains from maize samples complicated the evaluation of the results from this environment.

In conclusion the obtained results support the hypothesis of a low naturally occurring background load of nptII and nptIII genes in maize and potatoes cultivated and used as feed in Austria during the testing period.

1.2 Zusammenfassung

Gesamt-DNA Extrakte aus Mais und Kartoffeln, jenen Futtermitteln, von denen gentechnisch veränderte Varianten bereits in der Europäischen Union zum Anbau und Verkauf zugelassen worden sind, wurden mittels TaqMan Real Time PCR auf die Anwesenheit von nptII und nptIII Resistenzgenkopien untersucht. Zusätzlich wurde die Gesamtzellzahl und die Kanamycinresistenzrate der aus diesen Proben zu gewinnenden Bakterienpopulationen ermittelt.

Während des Analysezeitraums war die Häufigkeit von nptII und nptIII Resistenzgenen in den getesteten Gesamt-DNA Extrakten aus Mais und Kartoffelproben niedrig. Keine einzige Probe

enthielt nptII oder nptIII Kopien über dem Detektionslimit. Die statistische Auswertung des Versuchs zeigte, dass mit einer Sicherheit von 95% davon ausgegangen werden kann, dass weniger als 6,9% der Proben aus dem Versuchsgebiet nptII oder nptIII positiv sind.

Die phänotypischen Kanamycin Resistenzraten der aus Maisproben gewonnenen Bakterienpopulationen variierten stark. Eine Probe zeigte eine Resistenzrate von 0,01% (AGES-Versuchsstation Freistadt, Oberösterreich), während eine maximale Resistenzrate von 73,05% bei einer Probe aus Gleisdorf, Steiermark, ermittelt wurde. Bei Bakterienpopulationen, die von Kartoffelproben angezüchtet wurden, sind die Resistenzraten etwas gleichmäßiger verteilt, dafür aber substantiell niedriger (Minimum: 0,002%; Maximum: 6,6%) als im Vergleich zur Situation beim Mais.

Bei den Kanamycin resistenten Einzelkolonien aus Maisproben konnte ein einziges Isolat als nptII positiv detektiert werden (Prävalenzschätzer: 1,1%; 95% Konfidenzintervall: [0%;4,9%]). NptIII konnte in keinem Isolat - weder aus Mais noch aus Kartoffelproben - nachgewiesen werden. Für Bakterien aus Kartoffelproben kann mit 95%iger Sicherheit davon ausgegangen werden, dass weniger als 4% der aus dem Testgebiet isolierten Stämme nptII oder nptIII tragen. Bezüglich der PCR Ergebnisse, die für die bakteriellen Einzelisolate ermittelt worden sind, muss darauf hingewiesen werden, dass es sich nur um eine geringe Probenanzahl gehandelt hat (Mais: nptII: 94, nptIII: 93; Kartoffel: nptII/nptIII: 73). Überdies haben Schwierigkeiten bei der Anzucht von Einzelkolonien aus Maisproben die Auswertung dieses Teils der Untersuchung erschwert.

Insgesamt unterstützen die ermittelten Ergebnisse die Hypothese einer niedrigen Hintergrundbelastung der in Österreich angebauten und als Futtermittel verwendeten Mais- und Kartoffelpflanzen mit nptII und nptIII Resistenzgenen während des Untersuchungszeitraumes.

1.3 Aims

The objectives of the present project were:

1. Determination of the frequency of nptII and nptIII gene copies in bacterial populations associated with plants used as feed. This is typically performed by analysis of total DNA extracted from these sources. The data are acquired from habitats before exposure to exogenous plant derived ARM gene containing DNA. The naturally occurring background load with nptII and nptIII resistance genes in theses environments should be established.

2. The obtained data should serve as reference baseline for comparison if the impact of cultivation of ARM gene containing transgenic plants on resistance gene frequencies in these natural habitats will have to be evaluated in the future.

3. Samples should be collected from relevant plants used as feed – in the present case from maize and potatoes corresponding to ARM gene carrying transgenic maize and potato varieties with approval for marketing and cultivation in the European Union – and should be collected in a representative fashion.

4. The samples should be analysed by classical cultivation methods for characterization of residing bacterial species. Their total cultivable cell number and the phenotypic kanamycin resistance ratios should be determined, to get an overview about the abundance of resistance to this kind of aminoglycoside antibiotic.

1.4 Introduction

The neomycin phosphotransferase II gene is the most abundantly used ARM gene in plant gene technology and also the transgenic maize line MON863 and the GM potato variety EH92-527-1 – both approved for cultivation in the European Union - are carriers of nptII (6, 15, 16). Cultivation of transgenic crops has rapidly increased over the past 15 years (13), which increases the potential for contacts between transgenic DNA and bacteria capable for uptake of free DNA (17).

Facing a global crisis in the therapy of infectious diseases caused by antibiotic resistant bacteria (9, 11) the superfluous presence of the antibiotic resistance gene nptII in each plant cell of these transgenic crops has led to concerns (14, 19) because a horizontal transfer of the plant derived ARM genes to soil or gut bacteria cannot be excluded a priori (5). A potential uptake of plant derived resistance genes either directly by human pathogens or via a cascade of several other intermittent host bacterial species which subsequently may interfere with antimicrobial therapy has to be evaluated carefully (10). The overall impact of horizontal gene transfer from transgenic plants to soil or gut bacteria should be taken carefully into consideration for risk assessment of antibiotic resistance marker genes (12, 18). As plant surfaces usually are densely populated with bacteria, these microbial populations are in proximal location of ARM gene sources and potentially the first prokaryotic candidates which are primarily exposed to transgenic plant derived resistance genes.

The EFSA GMO Panel released an Opinion on the use of antibiotic resistance genes in GM plants in 2004 providing a 3-tiered classification scheme for antibiotic resistance genes (category 1: no risk for human and animal health and the environment; category 2: should only be used for authorized field trials; category 3: should not be used in transgenic crops in general). The Panel concluded that the use of nptII as a selection marker did not pose a risk to the environment or to human and animal health (7). EFSA provided two additional statements including a “Joint Scientific Opinion of the GMO and BIOHAZ Panels“ on the use of ARM genes in GM plants in 2009, which resulted in the same conclusions (5, 8). Two Members of the BIOHAZ Panel expressed a critical minority opinion. Additionally this Joint Opinion pointed to limitations related among others to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source and stressed the importance of taking those and other uncertainties described in that Opinion into account (5). Two reviews on the risk assessment of antibiotic resistance marker genes also concluded on the innocuousness of nptII (2, 10). None of these evaluations considered it necessary to back up their conclusions with quantitative information on the prevalence of nptII in natural habitats. However, data on the prevalence, diversity and the ecology of antibiotic resistance genes in naturally occurring bacterial populations in soils or on plant surfaces are a prerequisite for evaluation of the consequences of a possible transfer of plant derived antibiotic resistance marker genes to bacterial communities (4).

To our knowledge there is no information available about the prevalence of nptII and nptIII in bacteria associated with plant surface habitats.

In the present project we analysed the resistance gene pools of plant associated bacteria and checked total DNA extracts of dried maize kernels and potato juice – two plant lines used as examples for feed applications - for the presence of nptII and nptIII resistance genes. Phenotypic kanamycin resistance rates were calculated and resistant bacterial isolates were checked for their nptII and nptIII carrier-status. The obtained results should provide information about the nptII and nptIII background load in the habitats under investigation before exposure to exogenously presented ARM genes.

1.5 Materials and Methods

1.5.1 Sampling plan 2011 for varietal analysis of table potatoes

The risk-based test- and sampling-plan for the varietal analysis in table potatoes is assessed annually on the ground of previous year's results of analytical tests and specific statistical methods, respectively. On the basis of analysed data of the previous year and specific statistical methods, for 2011 the plan was recommended as follows: 186 samples risk-based, 16 samples of follow-up inspections (1)(3).

The calculation of sample size for 2011 is based on risk assessment, targeting protection against fraud, and a safety of 95%, and was done in consideration of an objection rate in 2010 of 7% with a precision requirement of +/- 4%. Also on basis of the model of market observation without underlying data from inspections, but considering a presumed objection rate, arithmetically a comparable sample size would result.

In the following, the division of the 186 samples over the Federal States for 2011 is given, based on consumption data with a 9-weeks observation period, and divided into 4 sampling dates (Table 1). These 186 samples were rounded up to 189 to allow a division by 9 (= 9 Federal States). Of the 189 routine samples mentioned, in total 50 samples from the third sampling date (September) of 2011 were planned to be integrated into the actual research project.

	Consumers 2008		**Sampling plan 2011, calculated**	**Basis 189 dividable by 9**	**Sampling plan 2011 Recommendation (Basis 189)**
Federal States	**in 1000**	**%**	**Consummation (Basis 186)**	**Consummation (Basis 189)**	**Samples**
Burgenland	282	3.4	6.29	6.4	9
Kärnten	560	6.7	12.50	12.7	9
Niederösterreich	1,601	19.2	35.73	36.3	36
Oberösterreich	1,409	16.9	31.45	32.0	36
Salzburg	528	6.3	11.78	12.0	9
Steiermark	1,206	14.5	26.92	27.3	27
Tirol	702	8.4	15.67	15.9	18
Vorarlberg	366	4.4	8.17	8.3	9
Wien	1,680	20.2	37.49	38.1	36
Total	**8,334**	**100**	**186**	**189**	**189**

Table 1: Potato sampling plan for 2011 (3).

1.5.2 Maize samples from AGES testing sites (1)

Test basis are corn cob patterns of maize varieties which - as standard and comparative varieties - serve as reference values for crop yield in the most important production areas within the framework of the official technical variety examination in Austria. The selection of habitats monitored is done very targeted and multi-staged; therefore, a production output related distribution can be assumed.

1.5.3 Selection and number of tested maize and potato samples

Forty nine maize and 51 potato samples were randomly selected from the incoming routine samples at AGES, Linz, (see 1.5.1 and 1.5.2) and subject for the bacterial cultivation approach. 42 total DNA extracts, each, were submitted to the PCR testing facility at AGES-IMED, Vienna. This reduction in the number of total DNA extracts was taken into consideration during the statistical evaluation and was compensated by larger confidence intervals.

1.5.4 Procedure to enumerate and differentiate bacteria

The method served to determine microbial numbers of aerobic bacteria. Additionally, colonies detected were diagnostically differentiated as indicator micro-organisms (1).

Culture medium and solutions:

Triphenyltetrazolium chloride (TTC) solution:

1% (w / v): 1 g of 2.3.5-Triphenyltetrazolium chloride was dissolved in 100 ml of distilled water and sterilized by membrane filtration.

Kanamycin solution:

200 mg kanamycin were dissolved with 2 ml distilled water and sterilized by membrane filtration.

Tryptic soy agar (TSA) with (TTC):

Tryptone soy agar was weight and suspended with distilled water according to manufacturer's instructions. Heat and cook the medium briefly up to the full-permanent resolution of the agar-agar. After autoclaving the medium for 15 minutes at 121 °C and cooling down to ≤ 50°C 1 ml TTC solution was added per liter. 15 - 20 ml portions of the agar were aliquoted into sterile petri dishes (94 x 16mm). After solidification the plates were pre-dried at room temperature in the dark. The prepared medium was clear and colorless to pale yellow and was stored at 2-10 ° C up to two months.

Tryptic soy agar (TSA) with TTC and kanamycin:

TSA with TTC was prepared as described above. Additionally, after cooling down the medium to 50 °C 1 ml kanamycin solution per liter was added. The prepared agar plates were stored at 2-10 °C up to two weeks.

Suspension solution:

Sodiumchloride-Peptone solution:

20 g sodiumchloride, 5 g peptone (tryptic), 1.5 ml Tween 80 were dissolved with 4800 ml distilled water and mixed on the magnetic stirrer until complete homogenization.

Buffer solution:

2,9 g sodiumhydrogenphosphate and 12,5 g di-sodiumhydrogenphosphate were dissolved in 200 ml distilled water, mixed and heated until complete homogenization on the magnetic stirrer.

Buffer solution and Sodiumchloride-Peptone solution were mixed and autoclaved 15 minutes at 121 °C (pH 7.0 ± 0.2). Additionally a few aliquots of 9 ml were prepared into glass tubes before autoclaving.

Preparation of colony count plates with potato juice:

1.2 ± 0.2 ml potato juices was extracted from 1 potato with a glass pistil and collected into a sterile tube. The first dilution step was made with 1 ml potato juice and 9 ml sterile suspension solution (10^{-1}). Further decimal dilution steps, 10^{-2} to 10^{-4}, were produced with suspension solution. 0.1 ml of dilution 10^{-2}, 10^{-3} and 10^{-4} were pipetted onto two TSA plates and distributed evenly using a sterile spreader (e.g. DRIGALSKI spatula). 0.1 ml direct potato juice and 0.1 ml of dilutions 10^{-1} and 10^{-2} were pipetted onto two TSA plates with kanamycin and distributed with a DRIGALSKI spatula.

Preparation of colony count plates with maize suspension:

20 g milled maize was weight into a sterile glass bottle. The initial suspension was produced by adding 180 ml sterile suspension solution. The initial suspension thus prepared was treated on a horizontal shaking apparatus for 20 minutes. To produce the first dilution 5 ml of the initial solution are pipetted into 45 ml suspension solution. Further decimal dilutions are produced from the first dilution by pipetting 1 ml into 9 ml suspension solution. 0.1 ml of dilution 10^{-2}, 10^{-3} and 10^{-4} are pipetted onto two TSA plates and 0.1 ml of dilution 10^{-1}, 10^{-2} and 10^{-3} are pipetted onto TSA plus kanamycin plates and distributed evenly with a DRIGALSKI spatula.

Incubation:

The bacteria count plates were incubated for 2 days at 30 °C. The plates were stored at room temperature, strictly avoiding direct sunlight. For analysis and evaluation of colonies for determining micro-organism numbers the plates were counted on day 3. All colonies which showed recognizable reduction of TTC were counted. For differentiation of micro-organisms the plates were counted on day 4. If insufficient differentiation was observed plates were left on room temperature for over 5 days.

Calculation of micro-organism numbers:

Analysis and evaluation had to be performed with at least two of the count plates produced out of one or several dilutions. Colonies of evaluable plates were counted as colony forming units per gram (cfu/g), respectively as colony forming unit per ml (cfu/ml) according to the following formula:

Calculation with two dilutions:

$$N = \frac{\Sigma c}{(n_1 + [n_2 * 0{,}1])} * 10^d$$

N = number of colony forming units per gram or ml

ΣC = summary of colonies on count plates of two consecutive dilutions

n_1 = number of count plates of lower dilution

n_2 = number of count plates of higher dilution

d = dilution factor of lowest dilution

Calculation with one dilution:

$$N = \frac{\Sigma c}{n} * 10^{d}$$

N = number of colony forming units per gram or ml

ΣC = summary of colonies on count plates

n = number of count plates

d = dilution factor

Differentiation of micro-organisms:

Identification of micro-organisms (species and morphological groups) suited to criteria of the "Standard operating procedure for identifying bacteria, yeasts, moulds and *Dematiaceae* as product typical and spoilage indicating micro-organisms" (20). All detectable indicator micro-organisms were counted on count plates as summarized in Table 2:

No.	*Indicator organism*	*Morphology*
1	Yellow colored bacteria	Yellow to orange, flat and round colonies
2	Pseudomonas / Enterobacteriaceae	Flat, whitish beige colonies, reddish in center and partially clear and narrow to expanded borders
3	Mucous Pseudomonas	Rosy with clear border, round to amoeboid shape, extremely slimy
4	Bacillus spp.	Big morphological diversity, partially irregular wrinkled, notched, vesicular or folded colonies, slimy to dry, smooth or rough borders, center mostly reddish
5	Coccus	Mostly rosy and smooth or on surface emarginated, convex and round colony
6	Streptomycetes	Strong, leathery, small, convex colonies, strong adherence on medium, emarginated borders, after 7 days whitish mycelium and typical earthy smell

Table 2: Differentiation of indicator strains.

1.5.5 DNA extraction of maize and potato juice

200 µl potato juice and 200 mg milled maize were transferred into 2 ml DNAase free tubes. DNA extraction was performed with the DNeasy Plant Mini Kit from Qiagen (Hildesheim, Germany; Article Number: 69106*)* according the manufacturer's instructions. The final eluation volume was 100 µl.

DNA recovery was checked by spiking 1 ml of potato juice with 2.7 x 10^{8} *E. coli* cells / ml. Logarithmic dilutions down to 10^{-9} were prepared and DNA from 1 ml of the 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} step were extracted with the DNeasy Plant Mini Kit as decribed above. 2 µl of the eluate were applied as template for single copy gene real time PCR. The 10^{-6} dilution representing 5.4 gene copies / assay could be reproducibly detected. This equals 270 copies / ml matrix indicating that PCR inhibition was

no issue. Based on this observation the detection limit was approx. 2000 copies/ml for nptII and 2750 copies/ml for nptIII.

1.5.6 DNA extraction of bacterial isolates from maize and potato samples

A single colony from agar plates supplemented with 100 µg/ml kanamycin was removed with an inoculation loop and transferred in 100 µl Prepman Ultra reagent (ABI, Austria). The colony was resuspended thoroughly and the suspension was homogenised by vortexing. The tube was incubated at 95°C for 10 min and after cooling centrifuged at maximum speed for 3 min. A 10^{-1} dilution of the clear supernatant was used as template for the TaqMan real time PCRs.

1.5.7 16S TaqMan real time PCR assay - Check for the presence of amplifiable DNA

Each of the DNA isolates from potato and maize samples was checked for inhibition and for the presence of amplifiable DNA by adding 2 µl of the original DNeasy DNA eluate to the 16S TaqMan real time PCR assay. For PCR assay and amplification conditions see section 2.2. All samples were analysed at least in duplicates. Both replicates had to be positive to achieve a positive 16S TaqMan assessment.

16S TaqMan PCR results were classified as positive, if the crossing point (Cp) value was <27. This crossing point equals a single copy gene number of approx. 8400 copies/assay. If the amplification curve showed a Cp ≥27, the assay was classified as negative. A sample retrieving a single negative 16S PCR result was re-analysed and – if again negative – excluded from statistical analysis if nptII and/or nptIII PCRs were also negative. PCR amplification inhibition was checked by testing 1:5 and 1:25 serial dilutions.

1.5.8 NptII/nptIII TaqMan double screening assay for total DNA from maize and potato samples

Each of the DNA isolates from potato and maize samples were checked for the presence of nptII and/or nptIII genes by adding 2 µl of the original DNeasy DNA eluate to the nptII/nptIII TaqMan real time PCR double assay. For PCR conditions please see Appendix section 2.1. All of the samples were tested at least in duplicates, some of them four times. A single positive replicate was sufficient to classify the sample as positive.

The same DNA extraction solution from each sample was tested with the nptII/nptIII TaqMan Double Assay and with the 16S TaqMan DNA extraction/amplification control assay. Samples with a negative 16S TaqMan assay result (Cp > 27) were excluded from statistical analysis if the nptII and/or the nptIII PCR result were negative.

A sample was classified as nptII and/or nptIII positive if PCR amplification occurred before Cp 40. Single replicates producing an amplification curve with a Cp ≥40 were re-analysed. A sample was classified as nptII and/or nptIII negative only, if the 16S rRNA TaqMan assay was positive and all nptII/nptIII replicates showed no amplification in the respective PCR assay. The detection limit was approx. 2000 copies/ml for nptII and 2750 copies/ml for nptIII for total DNA extractions.

1.6 Results

1.6.1 Results: PCR Screening for nptII/nptIII in total DNA isolated from maize samples

DNA preparation from dried maize kernels (flour) was performed as described in section 1.5.5.

42 DNA eluates were sent to the PCR testing facility at AGES-IMED, Vienna, for real time TaqMan PCR analysis. None of the maize samples was nptII or nptIII positive (Table 3). Detailed results concerning 16S and nptII/nptIII qualitative PCR results as well as source of the maize sample and analysed variety can be found in Table 4. Samples were obtained from AGES experimental fields.

Data retrieved in the present experimental setting indicated that within a confidence level of 95% less than 6.9% of the maize samples obtained from the AGES test areas would be expected to be carriers for nptII as well as for nptIII (Table 3).

	Number of maize samples	Number of positive maize samples	Prevalence estimator	Confidence interval (95%)[1)]
nptII	42	0	0%	[0%;6.9%]
nptIII	42	0	0%	[0%;6.9%]

Table 3: NptII/nptIII prevalences in total DNA extracts from maize: summary

1) Confidence interval was calculated according to Clopper-Pearson

Feed/Maize Results: total DNA isolation					PCR results		
Serial No.	Internal Number	District	Variety	Sample	16S	nptII	nptIII
1	PCR/061011/001	NÖ/VST Fuchsenbigl	PR37Y12	Mais1	+	-	-
2	PCR/061011/002	NÖ/VST Fuchsenbigl	DKC4964	Mais2	+	-	-
3	PCR/061011/003	NÖ/VST Fuchsenbigl	DKC4408	Mais3	+	-	-
4	PCR/061011/004	KT/VST Hörzendorf	P8400	Mais4	+	-	-
5	PCR/061011/005	KT/VST Hörzendorf	LG3258	Mais5	+	-	-
6	PCR/061011/006	KT/VST Hörzendorf	ESGaranl	Mais6	+	-	-
7	PCR/061011/007	OÖ/VST Linz	ES Palazzo	Mais7	+	-	-
8	PCR/061011/008	OÖ/VST Linz	NK Falkone	Mais8	+	-	-
9	PCR/061011/009	OÖ/VST Linz	Ambrosini	Mais9	+	-	-
10	PCR/061011/010	OÖ/VST Linz	ES Garant	Mais10	+	-	-
11	PCR/061011/011	OÖ/VST Linz	LG 3258	Mais11	+	-	-
12	PCR/061011/012	OÖ/VST Linz	P8400	Mais12	+	-	-
13	PCR/061011/013	NÖ/VST Grabenegg	NK Octet	Mais13	+	-	-
14	PCR/061011/014	NÖ/VST Grabenegg	DKC3511	Mais14	+	-	-
15	PCR/061011/015	NÖ/VST Grabenegg	PR38A79	Mais15	+	-	-
16	PCR/061011/016	NÖ/VST Grabenegg	ES Garant	Mais16	+	-	-
17	PCR/061011/017	NÖ/VST Grabenegg	P8400	Mais17	+	-	-
18	PCR/061011/018	NÖ/VST Grabenegg	LG3258	Mais18	+	-	-
19	PCR/061011/019	ST/VST Gleisdorf	DKC4961	Mais19	+	-	-
20	PCR/061011/020	ST/VST Gleisdorf	PR37Y12	Mais20	+	-	-
21	PCR/061011/021	ST/VST Gleisdorf	DKC4408	Mais21	+	-	-
22	PCR/061011/022	ST/VST Gleisdorf	PR3Y12	Mais22	+	-	-
23	PCR/061011/023	ST/VST Gleisdorf	DKC4408	Mais23	+	-	-
24	PCR/061011/024	ST/VST Gleisdorf	DK4964	Mais24	+	-	-
25	PCR/061011/025	KSTLK 01	PR37Y12	Mais25	+	-	-
26	PCR/061011/026	KSTLK 01	DKC4408	Mais26	+	-	-
27	PCR/061011/027	ST/VST Gleisdorf	NK Octet	Mais27	+	-	-
28	PCR/061011/028	ST/VST Gleisdorf	DKC3511	Mais28	+	-	-
29	PCR/061011/029	ST/VST Gleisdorf	PR38A79	Mais29	+	-	-
30	PCR/061011/030	OÖ/VST Linz	LG3258	Mais30	+	-	-
31	PCR/061011/031	OÖ/VST Linz	ES Garant	Mais31	+	-	-
32	PCR/061011/032	OÖ/VST Linz	P8400	Mais32	+	-	-
33	PCR/061011/033	OÖ/VST Linz	NC Octet	Mais33	+	-	-
34	PCR/061011/034	OÖ/VST Linz	DKC3511	Mais34	+	-	-
35	PCR/061011/035	OÖ/VST Linz	PR38A79	Mais35	+	-	-
36	PCR/061011/036	OÖ/VST Freistadt	Ambrosini	Mais36	+	-	-
37	PCR/061011/037	OÖ/VST Freistadt	ES Palazzo	Mais37	+	-	-
38	PCR/061011/038	OÖ/VST Freistadt	NK Falkone	Mais38	+	-	-
39	PCR/061011/039	ST/VST Gleisdorf	DKC4964	Mais39	+	-	-
40	PCR/061011/040	ST/VST Gleisdorf	PR37Y12	Mais40	+	-	-
41	PCR/061011/041	ST/VST Gleisdorf	DKC4408	Mais41	+	-	-
42	PCR/061011/042	ST/VST Gleisdorf	DKC3511	Mais42	+	-	-

Table 4: NptII/nptIII prevalences in total DNA extracts from maize: detailed results

1.6.2 Results: PCR Screening for nptII/nptIII in total DNA isolated from potato samples

DNA preparation from potato juice was performed as described in section 1.5.5.

42 DNA eluates were available for real time TaqMan PCR testing. None of them were nptII or nptIII positive (Table 5). Detailed results concerning 16S and nptII/nptIII qualitative PCR results as well as source of the poato sample and analysed variety can be found in Table 6. Samples were obtained from individual farmers.

Data retrieved in the present experimental setting indicated that within a confidence level of 95% less than 6.9% of the potato samples obtained from the routine sampling program would be carriers for nptII as well as for nptIII (Table 5).

	Number of potato samples	Number of positive potato samples	Prevalence estimator	Confidence interval (95%)[1)]
nptII	42	0	0%	[0%;6.9%]
nptIII	42	0	0%	[0%;6.9%]

Table 5: NptII/nptIII prevalences in total DNA extracts from potatoes: summary

1) Confidence interval was calculated according to Clopper-Pearson

Feed/Potato results: total DNA Potato extract				PCR results		
Serial No.	Sample Number	District	Variety	16S	nptII	nptIII
1	**10**	NÖ/Waschbach	Ditta	+	-	-
2	**11**	NÖ/Waschbach	Tosca	+	-	-
3	**14**	NÖ/Stockerau	Agata	+	-	-
4	**15**	NÖ/Hof	Okama	+	-	-
5	**16**	W/Wien	Ditta	+	-	-
6	**17**	W/Wien	Ditta	+	-	-
7	**19**	NÖ/Niederfellabrunn	Agata	+	-	-
8	**20**	NÖ/Niederfellabrunn	Marabel	+	-	-
9	**21**	NÖ/Flandorf	Impala	+	-	-
10	**22**	S/St. Andrä	Jaerla	+	-	-
11	**23**	S/Tamsweg	Ditta	+	-	-
12	**24**	NÖ/Höbersdorf	Agata	+	-	-
13	**27**	NÖ/Niederrußbach	Ditta	+	-	-
14	**28**	NÖ/Ladendorf	Ditta	+	-	-
15	**31**	NÖ/Wittau	Ditta	+	-	-
16	**34**	NÖ/Gerasdorf	Ditta	+	-	-
17	**35**	NÖ/Gerasdorf	Desiree	+	-	-
18	**43**	NÖ/Zaina	Ditta	+	-	-
19	**44**	NÖ/Gemeinlebarn	Ditta	+	-	-
20	**48**	OÖ/Eferding	Annabelle	+	-	-
21	**49**	OÖ/Eferding	Frühkartoffel	+	-	-
22	**50**	OÖ/St. Aegidi	Freya	+	-	-
23	**51**	OÖ/St. Aegidi	Red Lady	+	-	-
24	**52**	OÖ/St. Aegidi	Mirage	+	-	-
25	**53**	OÖ/Eferding	Tosca	+	-	-
26	**55**	OÖ/Eferding	Erika	+	-	-
27	**56**	NÖ/Gaubitsch	Ditta	+	-	-
28	**57**	NÖ/Gaubitsch	Ditta	+	-	-
29	**60**	NÖ/Unterrohrbach	Ditta	+	-	-
30	**66**	NÖ/Rückersdorf-Harmannsdorf	Prinzess	+	-	-
31	**67**	NÖ/Zwerndorf	Anuschka	+	-	-
32	**68**	NÖ/Rückersdorf-Harmannsdorf	Marabel	+	-	-
33	**70**	B/Frauenkirchen	Prinzess	+	-	-
34	**71**	NÖ/Großau bei Raabs	Nicola	+	-	-
35	**76**	NÖ/Schweigens	Ditta	+	-	-
36	**77**	OÖ/Mitterkirchen im Machland	Ditta	+	-	-
37	**78**	OÖ/Mitterkirchen im Machland	Agria	+	-	-
38	**83**	OÖ/Ohlsdorf	Ditta	+	-	-
39	**84**	OÖ/Ohlsdorf	Ukama	+	-	-
40	**85**	ST/Laßnitzhöhe	Annabelle	+	-	-
41	**86**	ST/Graz	Frieslander	+	-	-
42	**87**	ST/Hausmannstätten	Nicola	+	-	-

Table 6: NptII/nptIII prevalences in total DNA extracts from potatoes: detailed results

1.6.3 Cultivation of bacterial strains and kanamycin resistance rates

The total colony count plates for potato juice and maize samples were performed with tryptone soy agar (TSA) containing triphenyltetrazoliumchloride (TTC). For testing kanamycin resistance the enumeration of bacteria were performed with TSA/TTC plates additionally containing 100 mg / L kanamycin.

Colony count plates were performed to enumerate bacteria of 49 maize samples. The average number of cfu was 7.05 x 10^5 (Table 7) and the main bacteria groups were differentiated into enterobacteriaceae, spore-producing bacteria, pseudomonads, yellow-colored bacteria (Table 8). On plates of two samples spherical and rod-shaped bacteria were determined as well. The reason for lower enumeration numbers compared to potato samples could be argued with the heating and drying process of the maize samples after harvesting. Because of that it would be possible that bacteria were damaged within that procedure. On the other hand bacteria which overcame the drying procedure became more robust which might be the cause for less reduction on kanamycin plates. The average number of cfu on kanamycin plates was just four fold reduced and was represented with the value 1.57 x 10^5. The bacteria groups represented on these plates were enterobacteriaceae, spore-producing bacteria and pseudomonads (Table 9). Interestingly, just 4 samples showed growth of yellow-colored bacteria on those plates. It seemed that these bacteria were generally more sensitive to kanamycin. A minimum of 0.01% resistant to kanamycin was observed with a bacterial population from a maize sample from VST Freistadt, Upper Austria, whereas 73.05% were resistant in a maize sample from Gleisdorf, Styria (Table 7).

In contrast to the potato samples the rate of resistance of maize is differently divided. 33% (16 samples) of the maize samples showed a rate of resistance less than 10 percent (Figure 1) and further 33% showed a rate of resistance between 10 and 75%. Moreover, kanamycin plates of 30% of maize samples (15 samples) were overgrown with *Fusarium spp.* and completely lacked bacterial growth. The according average enumeration number of these samples resulted from count plates without kanamycin was 2.26 x 10^4 cfu. As mentioned previously, the reason for that general lower enumeration number could be the damaging effect of the heating and drying treatment of the maize samples. The effect of the antibiotic might additionally stress bacteria which results in a complete lack of growth on the kanamycin plates.

51 potato juice samples were tested. The resulting average number of colony forming units (cfu) per ml potato juice was 8.08 x 10^5 (Table 10). The main groups of bacteria represented on these count plates were enterobacteriaceae, spore-producing bacteria, pseudomonads, yellow-colored bacteria, spherical-shaped bacteria and mucous pseudomonads (Table 11). The same samples plated on kanamycin plates showed an average number of cfu of 2.3 x 10^3 (Table 10). Generally, a reduction of two log steps on kanamycin count plates could be observed. Thus, the rate of resistance (cfu on TSA+TTC+kanamycin/cfu on TSA+TTC in %) was calculated. Resistance to kanamycin was more homogenously distributed within bacterial populations obtained from potato samples showing considerably lower overall resistance rates to kanamycin (minimum: 0.002%; maximum: 6.6%) compared to maize samples (Table 10). Moreover, the results indicated that 94% of the samples (48 samples out of 51) showed less than 10% kanamycin resistance (Figure 1). 6% (three samples) were overgrown with moulds. Additionally it was noticeable that the main bacteria group grown on the count plate with kanamycin was mucous pseudomonads (Table 12). Approximately 80% of the potato plates nearly showed a homogenous population with mucous pseudomonades of more than 50%. The reason for that remains unclear.

Summarized it was observed that the bacterial cell numbers from potato sources were higher than those of maize samples. Moreover potato samples showed in 94% of cases a rate of resistance less than 10%. In contrast to maize samples the enumeration numbers were generally lower. The rate of resistance is differently divided. 33% represented less than 10% resistance, another 33% showed

resistance between 10 and 75 % and 30% of the samples were overgrown with *Fusarium spp.* The reason for that might be the previous heating and drying process of the maize samples.

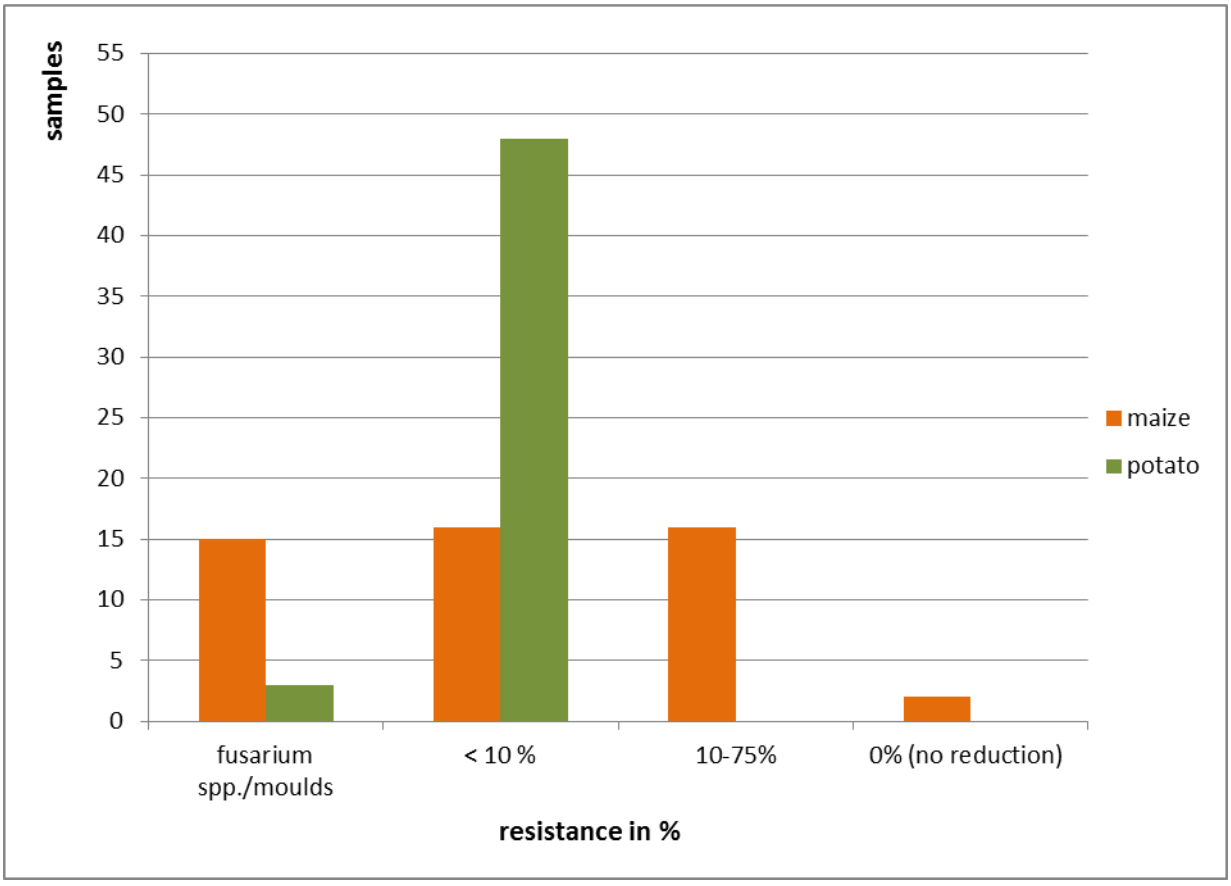

Figure 1: Abundance of kanamycin resistant isolates from maize and potato samples

		Complete medium	Complete + kanamycin		
Location of sampling	**Maize sample (laboratory code)**	**cfu / g maize**	**cfu / g maize**	**Kanamycin resistant bacteria**	**Reduction in log steps**
VST FUCHSENBIGL	PCR/061011/001	3,36E+05	4,32E+04	12,86%	1
	PCR/061011/002	1,78E+05	4,41E+04	24,78%	1
	PCR/061011/003	3,09E+04	1,50E+03	4,85%	1
VST HÖRZENDORF	PCR/061011/004	2,15E+06	7,90E+05	36,74%	1
	PCR/061011/005	4,65E+06	2,01E+06	43,23%	0
	PCR/061011/006	1,99E+06	3,01E+05	15,13%	1
VST LINZ	PCR/061011/007	1,16E+06	3,14E+04	2,71%	2
	PCR/061011/008	4,14E+05	1,00E+04	2,42%	1
	PCR/061011/009	1,92E+05	2,36E+03	1,23%	2
VST LINZ	PCR/061011/010	1,14E+05	1,00E+03	0,88%	2
	PCR/061011/011	8,27E+04	*Fusarium spp.*	-	-
	PCR/061011/012	6,36E+03	3,50E+03	55,03%	0
VST GRABENEGG	PCR/061011/013	4,40E+06	1,15E+06	26,14%	0
	PCR/061011/014	5,97E+04	6,14E+04	no reduction	0
	PCR/061011/015	1,50E+06	1,09E+05	7,27%	1
VST GRABENEGG	PCR/061011/016	4,91E+04	*Fusarium spp.*	-	-
	PCR/061011/017	7,62E+04	1,67E+04	21,92%	0
	PCR/061011/018	1,90E+04	1,14E+04	60,00%	0
VST GLEISDORF	PCR/061011/019	3,32E+04	*Fusarium spp.*	-	-
	PCR/061011/020	4,68E+04	1,09E+04	23,29%	0
	PCR/061011/021	2,32E+04	4,68E+03	20,17%	1
VST GLEISDORF	PCR/061011/022	2,41E+04	*Fusarium spp.* spp.	-	-
	PCR/061011/023	1,77E+04	7,14E+02	4,03%	2
	PCR/061011/024	6,82E+04	3,41E+04	50,00%	0
K STLK 01	PCR/061011/025	8,00E+03	*Fusarium spp.*	-	-
	PCR/061011/026	*Fusarium spp.*	*Fusarium spp.*	-	-
VST GLEISDORF	PCR/061011/027	4,50E+03	6,50E+03	no reduction	0
	PCR/061011/028	*Fusarium spp.*	*Fusarium spp.*	-	-
	PCR/061011/029	4,07E+04	4,75E+03	11,67%	1
VST LINZ	PCR/061011/030	4,82E+04	1,36E+03	2,82%	1
	PCR/061011/031	3,50E+04	*Fusarium spp.*	-	-
	PCR/061011/032	1,57E+04	1,23E+02	0,78%	2
VST LINZ	PCR/061011/033	1,22E+05	3,08E+03	2,52%	2
	PCR/061011/034	2,00E+03	*Fusarium spp.*	-	-
	PCR/061011/035	2,44E+04	*Fusarium spp.*	-	-
VST FREISTADT	PCR/061011/036	2,45E+06	2,00E+02	0,01%	4
	PCR/061011/037	8,00E+03	1,90E+03	23,75%	0
	PCR/061011/038	2,27E+04	*Fusarium spp.*	-	-
VST GLEISDORF	PCR/061011/039	1,00E+03	*Fusarium spp.*	-	-
	PCR/061011/040	2,50E+03	*Fusarium spp.*	-	-

		Complete medium	Complete + kanamycin		
Location of sampling	**Maize sample (laboratory code)**	**cfu / g maize**	**cfu / g maize**	**Kanamycin resistant bacteria**	**Reduction in log steps**
	PCR/061011/041	5,00E+02	*Fusarium spp.*	-	-
VST GLEISDORF	PCR/061011/042	9,09E+03	*Fusarium spp.*	-	-
	PCR/061011/043	1,41E+05	1,03E+05	73,05%	0
	PCR/061011/044	3,00E+03	1,00E+02	3,33%	1
VST GLEISDORF	PCR/061011/045	7,64E+05	4,50E+03	0,59%	2
	PCR/061011/046	6,00E+05	4,10E+05	68,33%	0
	PCR/061011/047	8,00E+05	1,32E+03	0,17%	2
VST LINZ	PCR/061011/048	1,99E+06	1,73E+05	8,69%	1
	PCR/061011/049	4,73E+06	1,00E+03	0,02%	3

Table 7: Kanamycin resistance in bacteria recovered from dried maize kernels

	Trypton soy agar (TSA) with triphenyltetrazoliumchloride (TTC) (%)						
Maize sample (laboratory code)	**Entero-bacteriaceae ***	**Spore producing bacteria***	**Pseudo-monads***	**Yellow colored bacteria***	**Spherical-shaped bacteria***	**Rod-shaped bacteria***	**Other bacteria***
PCR/061011/001	80			20			0
PCR/061011/002	40		30	25			5
PCR/061011/003	85		3	8			4
PCR/061011/004	13	16	10	3		50	8
PCR/061011/005	30		65				5
PCR/061011/006	13		80				7
PCR/061011/007			28	72			0
PCR/061011/008	27	5	12	56			0
PCR/061011/009			2	90			8
PCR/061011/010				100			0
PCR/061011/011		19	16	65			0
PCR/061011/012			50	50			0
PCR/061011/013	32		33	16	16		3
PCR/061011/014	31		45	24			0
PCR/061011/015			22	78			0
PCR/061011/016	49		38	13			0
PCR/061011/017			90	10			0
PCR/061011/018			23	77			0
PCR/061011/019				100			0
PCR/061011/020			40	53			7
PCR/061011/021	16	7	7	65			5
PCR/061011/022	40		50	6			4
PCR/061011/023	32	8	20	40			0
PCR/061011/024	39	7	12	42			0
PCR/061011/025	75			25			0
PCR/061011/026							*Fusarium spp.*
PCR/061011/027	50			50			0
PCR/061011/028							*Fusarium spp.*
PCR/061011/029				100			0
PCR/061011/030	48		7	45			0
PCR/061011/031		3	8	89			0
PCR/061011/032	10	40	17	33			0
PCR/061011/033	37			63			0
PCR/061011/034			100				0
PCR/061011/035		10	15	75			0
PCR/061011/036			10	90			0
PCR/061011/037				100			0
PCR/061011/038			17	83			0

	Trypton soy agar (TSA) with triphenyltetrazoliumchloride (TTC) (%)						
Maize sample (laboratory code)	Entero-bacteriaceae *	Spore producing bacteria*	Pseudo-monads*	Yellow colored bacteria*	Spherical-shaped bacteria*	Rod-shaped bacteria*	Other bacteria*
PCR/061011/039			100				0
PCR/061011/040	100						0
PCR/061011/041			100				0
PCR/061011/042	52		24	24			0
PCR/061011/043	89	2	7	2			0
PCR/061011/044				100			0
PCR/061011/045	20		75	5			0
PCR/061011/046			100				0
PCR/061011/047	49		17	34			0
PCR/061011/048	20		66	14			0
PCR/061011/049				100			0

Table 8: Differentiation of strains retrieved from dried maize kernels on complete medium.

* approximate percental recovery of bacterial groups

	TSA+TTC + Kanamycin (100 mg/L) (%)					
Maize sample (laboratory code)	Entero-bacteriaceae*	Spore producing bacteria*	Pseudo-monads*	Yellow-colored bacteria*	Rod-shaped bacteria*	Other bacteria*
PCR/061011/001	80			20		0
PCR/061011/002	90		5			5
PCR/061011/003	95					5
PCR/061011/004		16	45		30	9
PCR/061011/005			95			5
PCR/061011/006		20	75			5
PCR/061011/007	86		14			0
PCR/061011/008	overgrown with moulds					100
PCR/061011/009			95			5
PCR/061011/010			100			0
PCR/061011/011						*Fusarium spp.*
PCR/061011/012			100			0
PCR/061011/013			95			5
PCR/061011/014			100			0
PCR/061011/015			100			0
PCR/061011/016						*Fusarium spp.*
PCR/061011/017			100			0
PCR/061011/018	50		50			0
PCR/061011/019						*Fusarium spp.*
PCR/061011/020			95			5

	TSA+TTC + Kanamycin (100 mg/L) (%)					
Maize sample (laboratory code)	**Entero-bacteriaceae***	**Spore producing bacteria***	**Pseudo-monads***	**Yellow-colored bacteria***	**Rod-shaped bacteria***	**Other bacteria***
PCR/061011/021	100					0
PCR/061011/022						*Fusarium spp.*
PCR/061011/023	90					10
PCR/061011/024				99		1
PCR/061011/025						*Fusarium spp.*
PCR/061011/026						*Fusarium spp.*
PCR/061011/027	25	50	25			0
PCR/061011/028						*Fusarium spp.*
PCR/061011/029			90			10
PCR/061011/030	100					0
PCR/061011/031						*Fusarium spp.*
PCR/061011/032		50	8	42		0
PCR/061011/033		7	93			0
PCR/061011/034						*Fusarium spp.*
PCR/061011/035						*Fusarium spp.*
PCR/061011/036		60		40		0
PCR/061011/037	100					0
PCR/061011/038						*Fusarium spp.*
PCR/061011/039						*Fusarium spp.*
PCR/061011/040						*Fusarium spp.*
PCR/061011/041						*Fusarium spp.*
PCR/061011/042						*Fusarium spp.*
PCR/061011/043	100					0
PCR/061011/044	100					0
PCR/061011/045	67		33			0
PCR/061011/046			100			0
PCR/061011/047	29	8	42	21		0
PCR/061011/048	15			85		0
PCR/061011/049	15		85			0

Table 9: Differentiation of strains retrieved from dried maize kernels on complete medium supplemented with kanamycin.

* approximate percental recovery of bacterial groups

	Complete medium	Complete medium + Kanamycin		
Laboratory code (PCK)	Cfu / ml potato juice	Cfu / ml potato juice	Kanamycin resistant bacteria	Reduction in log steps
11*0010	1,30E+06	2,30E+03	0,177%	3
11*0011	5,40E+05	2,30E+03	0,426%	2
11*0014	5,00E+05	moulds	-	5
11*0015	2,20E+06	5,00E+04	2,273%	2
11*0016	5,00E+05	1,10E+03	0,220%	2
11*0017	1,10E+06	2,00E+03	0,182%	3
11*0019	7,00E+05	7,00E+03	1,000%	2
11*0020	2,00E+04	2,50E+02	1,250%	2
11*0021	3,10E+04	2,00E+02	0,645%	2
11*0022	2,00E+04	1,00E+02	0,500%	2
11*0023	2,10E+05	2,00E+02	0,095%	3
11*0024	2,80E+05	1,50E+02	0,054%	3
11*0027	3,40E+04	2,00E+02	0,588%	2
11*0028	2,40E+06	2,00E+03	0,083%	3
11*0031	1,10E+06	3,60E+03	0,327%	3
11*0034	1,50E+05	4,00E+02	0,267%	3
11*0035	1,30E+06	1,40E+04	1,077%	2
11*0043	1,20E+06	4,10E+03	0,342%	3
11*0044	9,60E+05	4,60E+03	0,479%	2
11*0048	3,40E+06	5,40E+03	0,159%	3
11*0049	2,00E+04	1,00E+01	0,050%	3
11*0050	8,20E+06	3,20E+04	0,390%	2
11*0051	2,40E+05	4,50E+01	0,019%	4
11*0052	3,20E+05	8,50E+02	0,266%	3
11*0053	3,70E+06	1,20E+05	3,243%	1
11*0055	1,10E+05	4,40E+03	4,000%	2
11*0056	1,80E+06	4,00E+04	2,222%	2
11*0057	7,00E+04	6,60E+02	0,943%	2
11*0060	1,20E+05	2,30E+02	0,192%	3
11*0066	1,60E+06	3,30E+04	2,063%	2
11*0067	1,90E+04	moulds	-	4
11*0068	2,00E+04	5,50E+02	2,750%	2
11*0070	1,10E+06	3,90E+04	3,545%	2
11*0071	4,10E+05	1,00E+01	0,002%	4
11*0076	1,00E+06	8,50E+02	0,085%	4
11*0077	1,20E+05	5,50E+03	4,583%	2
11*0078	4,60E+05	1,50E+01	0,003%	4
11*0083	4,50E+05	1,80E+03	0,400%	2
11*0084	3,30E+04	5,50E+01	0,167%	3
11*0085	3,50E+04	1,80E+02	0,514%	2

	Complete medium	Complete medium + Kanamycin		
Laboratory code (PCK)	Cfu / ml potato juice	Cfu / ml potato juice	Kanamycin resistant bacteria	Reduction in log steps
11*0086	8,50E+05	4,10E+03	0,482%	2
11*0087	4,20E+05	3,20E+03	0,762%	2
11*0089	3,20E+04	5,50E+01	0,172%	3
11*0094	1,50E+03	moulds	-	3
11*0112	2,09E+05	2,05E+03	0,981%	2
11*0113	9,64E+04	3,73E+03	3,869%	1
11*0121	2,36E+04	4,50E+01	0,191%	3
11*0122	4,09E+04	3,68E+02	0,900%	2
11*0123	4,77E+05	3,15E+04	6,604%	1
11*0124	7,64E+05	3,68E+03	0,482%	2
11*0125	5,23E+05	5,15E+03	0,985%	2

Table 10: Kanamycin resistance in bacteria recovered from potato juice

	Trypton soy agar (TSA) with triphenyltetrazoliumchloride (TTC) (%)							
Lab code (PCK)	Entero-bacteriaceae *	Spore producing bacteria*	Pseudo-monads*	Yellow colored bacteria*	Strepto-mycetes*	Mucous pseudomonads*	Spherical-shaped bacteria*	Other bacteria*
11*0010	5	1	2			90		2
11*0011	10	20	4			60		6
11*0014		30	2	7			55	6
11*0015	2	2	6	10		70	4	6
11*0016		45				50		5
11*0017		20				75		5
11*0019		15	2			75	3	5
11*0020						95		5
11*0021	10	20	30	3		30		7
11*0022	5	20				75		0
11*0023	10	65				20		5
11*0024		10	85					5
11*0027	10	80		5				5
11*0028		5	30	10		50		5
11*0031	15	20				55	5	5
11*0034		20	1				70	9
11*0035		40				55		5
11*0043		15				10	70	5
11*0044		45				30	20	5
11*0048		15				70		15
11*0049		95						5

	Trypton soy agar (TSA) with triphenyltetrazoliumchloride (TTC) (%)							
Lab code (PCK)	Entero-bacteriaceae *	Spore producing bacteria*	Pseudo-monads*	Yellow colored bacteria*	Strepto-mycetes*	Mucous pseudomonads*	Spherical-shaped bacteria*	Other bacteria*
11*0050	10	1	80			3		6
11*0051		5	60			5	25	5
11*0052		5	85			5		5
11*0053			85			10		5
11*0055			65			30		5
11*0056						95		5
11*0057		2		4		85		9
11*0060		2				90		8
11*0066	7			15		70		8
11*0067							100	0
11*0068	70	10		5		10		5
11*0070			75				20	5
11*0071		80	5				12	3
11*0076		10			25		60	5
11*0077		50	20	8		10	12	0
11*0078							95	5
11*0083	9	40	25			20		6
11*0084		20	5			55	14	6
11*0085		85					10	5
11*0086	1	2	70	13			7	7
11*0087			5	5			85	5
11*0089	80	15						5
11*0094	100							0
11*0112	21	21	10		16	20	5	7
11*0113	18	18	36		27			1
11*0121		50	20		2	4	7	17
11*0122		14	67				10	9
11*0123	14	9	65		12			0
11*0124		21			79			0
11*0125	4	14	8		74			0

Table 11. Differentiation of strains retrieved from potatoes on complete medium.

* approximate percental recovery of bacterial groups

	TSA+TTC + kanamycin (100 mg/L) (%)						
Lab code (PCK)	Entero-bacteriaceae *	Spore producing bacteria*	Pseudo-monads*	Yellow colored bacteria*	Strepto-mycetes*	Mucous pseudo-monads*	Other bacteria*
11*0010						95	5
11*0011						95	5
11*0014							moulds
11*0015						95	5
11*0016						95	5
11*0017			10			85	5
11*0019			10			85	5
11*0020						95	5
11*0021						95	5
11*0022						95	5
11*0023						95	5
11*0024			65			30	5
11*0027						95	5
11*0028			50			50	0
11*0031						95	5
11*0034						95	5
11*0035						95	5
11*0043						95	5
11*0044						95	5
11*0048						95	5
11*0049						100	0
11*0050				10		85	5
11*0051	1					90	9
11*0052			1			90	9
11*0053			4	35		55	6
11*0055						95	5
11*0056						95	5
11*0057	1					90	9
11*0060						95	5
11*0066			45			50	5
11*0067							moulds
11*0068			50			45	5
11*0070			30			65	5
11*0071		100					0
11*0076	5		80			10	5
11*0077						95	5
11*0078			100				0
11*0083			45			50	5
11*0084			45			50	5

	TSA+TTC + kanamycin (100 mg/L) (%)						
Lab code (PCK)	Entero-bacteriaceae *	Spore producing bacteria*	Pseudo-monads*	Yellow colored bacteria*	Strepto-mycetes*	Mucous pseudo-monads*	Other bacteria*
11*0085	9		26			60	5
11*0086			43			50	7
11*0087			22			70	8
11*0089	95						5
11*0094							moulds
11*0112						100	0
11*0113	26		24			40	10
11*0121						90	10
11*0122			25		63		12
11*0123	40		50		3	3	4
11*0124					92	8	0
11*0125					14	86	0

Table 12: Differentiation of strains retrieved from potatoes on complete medium supplemented with kanamycin.

* approximate percental recovery of bacterial groups

1.6.4 Prevalence of nptII and nptIII in kanamycin resistant bacteria from maize samples

DNA was isolated from the isolated strains as described in section 1.5.6. Using an altered evaluation scheme (see below) 94 PCR results were egligible for statistical evaluation for nptII, 93 were valid for nptIII prevalence evaluation.

One bacterial isolate (48/K4) was positive for nptII resulting in a prevalence estimator of 1.1% (95% confidence interval: [0%;4.9%]) (Table 13). No isolate carried copies of nptIII (confidence interval: [0%;3.2%]).

Suppression of fungal growth was insufficient on some of the kanamycin containing agar plates used for the isolation of kanamycin resistant strains resulting in rapid overgrowth of bacterial colonies. To ensure a pure isolate single colonies were already obtained after short overnight incubations. The colonies selected for further processing, consequently, were only small and contained low amounts of DNA. This procedure resulted in only 65 isolates being 16S TaqMan PCR positive if Cp 27 was used as cut off (Table 14; for detailed PCR results see Table 16). Using this cut off value only 57.5% of the isolates were statistically evaluable (Table 15). To obtain some meaningful data from this experiment an altered evaluation scheme was checked for applicability (for details see below).

	Number of bacterial isolates	Number of positive bacterial isolates	Prevalence estimator	Confidence interval (95%)[1)]
nptII	94	1	1.1%	[0%;4.9%]
nptIII	93	0	0%	[0%;3.2%]

Table 13: NptII/nptIII prevalence in kanamycin resistant strains from maize

1) according to Clopper-Pearson

Altered evaluation scheme

Due to an insufficient repression of fungal growth during the isolation of the kanamycin resistant strains many isolates were negative in the 16S TaqMan assay. The main reason for the low DNA contents of these isolates was the small size of the colony which was removed by an inoculation loop from the surface of the agar plate. The resulting DNA amounts were, thus, low resulting in Cp-values >27 in the subsequent PCR analyses. However, the 16S cut off value was chosen very high by intention to provide a large margin of safety. Therefore crossing points above Cp 27 in fact do not indicate the complete absence of 16S target molecules. As shown in Table 14 Cp 27 corresponds to approx. 8400 16S rRNA gene target copies per 16S TaqMan assay. The subsequent – higher - Cp-values are correlated to the corresponding copy numbers. Assuming a maximum rRNA gene copy number per bacterial cell of 20, then the 95% detection limit for the single copy gene nptIII (approx. 11 copies/assay) is reached in the present 16S TaqMan assay at a Cp of 32 (Table 14). Using Cp 32 as cut off value for the 16S TaqMan assay still guarantees an input of single copy gene templates above the 95% detection limit of the assay. Under these circumstances a nptII and/or nptIII positive colony

would still provide enough nptII and nptIII template molecules to be detected in the respective nptII/nptIII screening assay.

Table 15 shows the ascending number of 16S TaqMan positive samples if the respective Cp-blocks displayed in Table 14 were integrated into the evaluation until the 95% detection limit for single copy nptIII genes is reached.

Block:	1	2	3	4	5	7	8	9
Cp:	**27**	**28 < x <= 29**	**29 < x <= 30**	**30 < x <= 31**	**31 < x <= 32**	**32 < x <= 33**	**33 < x <= 34**	**x> 34**
cumulative 16S positive samples:	65	70	76	78	94	100	106	113
16S copy number/assay	8400	4200 - 2100	2100 - 1050	1050 - 525	525 - 260	260 - 130	130 - 65	< 65
Single copy gene number/assay[1)]	420	210 - 105	105 – 52.5	52.5 – 26.25	26.25 – 13.125			

Table 14: 16S TaqMan PCR results: correlation between Cp value and gene copy number

1) Assuming a maximum 16S rRNA gene copy number of 20 per bacterial cell and nptII/nptIII as single copy genes.

16S	**number of samples**	**%**
positive	65	57,52
negative	48	42,48
total	113	100
incl. block[1)]	2	
16S	**number of samples**	**%**
positive	70	61,95
negative	43	38,05
total	113	100
incl. block[1)]	3	
16S	**number of samples**	**%**
positive	76	67,26
negative	37	32,74
total	113	100
incl. block[1)]	4	
16S	**number of samples**	**%**
positive	78	69,03
negative	35	30,97
total	113	100
incl. block[1)]	5	
16S	**number of samples**	**%**
positive	94	83,19
negative	19	16,81
total	113	100

Table 15: Modified evaluation of the 16S PCR results

1) for corresponding block number and Cp values see Table 14.

Maize: kanamycin resistant bacterial isolates					PCR results		
Serial No.	Internal Number	Variety	Sample number	Bacterial strain	16S	nptII	nptIII
1	PCR/061011/004	P8400	4/k1	rod-shaped bacteria	-	-	-
2	PCR/061011/004	P8400	4/K2	Pseudomonas	+	-	-
3	PCR/061011/005	LG3258	5/K1	Pseudomonas	+	-	-
4	PCR/061011/005	LG3258	5/K2	Pseudomonas	+	-	-
5	PCR/061011/006	ESGaranl	6/K1	Pseudomonas	+	-	-
6	PCR/061011/006	ESGaranl	6/K2	Pseudomonas	+	-	-
7	PCR/061011/007	ES Palazzo	7/K1	Enterobacteriaceae	-	-	-
8	PCR/061011/007	ES Palazzo	7/K2	Pseudomonas	-	-	-
9	PCR/061011/010	ES Garant	10/k1	Mucous Pseudomonas	+	-	-
10	PCR/061011/012	P8400	12/K1	Pseudomonas	+	-	-
11	PCR/061011/012	P8400	12/K2	Pseudomonas	+	-	-
12	PCR/061011/013	NK Octet	13/k1	Pseudomonas	-	-	-
13	PCR/061011/013	NK Octet	13/K2	Pseudomonas	+	-	-
14	PCR/061011/014	DKC3511	14/K1	Pseudomonas	+	-	-
15	PCR/061011/014	DKC3511	14/K2	Pseudomonas	+	-	-
16	PCR/061011/015	PR38A79	15/K1	Pseudomonas	+	-	-
17	PCR/061011/015	PR38A79	15/K2	Pseudomonas	+	-	-
18	PCR/061011/017	P8400	17/K1	Pseudomonas	-	-	-
19	PCR/061011/017	P8400	17/K2	Pseudomonas	-	-	-
20	PCR/061011/018	LG3258	18/K1	Pseudomonas	+	-	-
21	PCR/061011/018	LG3258	18/K2	Pseudomonas	-	-	-
22	PCR/061011/018	LG3258	18/K3	Enterobacteriaceae	+	-	-
23	PCR/061011/020	PR37Y12	20/K1	Pseudomonas	+	-	-
24	PCR/061011/020	PR37Y12	20/K2	Pseudomonas	+	-	-
25	PCR/061011/021	DKC4408	21/K1	Enterobacteriaceae	-	-	-
26	PCR/061011/021	DKC4408	21/K2	Enterobacteriaceae	-	-	-
27	PCR/061011/024	DK4964	24/K1	Yellow colored bacteria	-	-	-
28	PCR/061011/024	DK4964	24/K2	Yellow colored bacteria	-	-	-
29	PCR/061011/027	NK Octet	27/K1	Pseudomonas	+	-	-
30	PCR/061011/030	LG3258	30/K1	Enterobacteriaceae	+	-	-
31	PCR/061011/030	LG3258	30/K2	Enterobacteriaceae	+	-	-
32	PCR/061011/032	P8400	32/K1	Spore forming bacteria	+	-	-
33	PCR/061011/032	P8400	32/K2	Yellow colored bacteria	-	-	-
34	PCR/061011/032	P8400	32/K3	Pseudomonas	+	-	-
35	PCR/061011/033	NC Octet	33/K1	Pseudomonas	+	-	-
36	PCR/061011/033	NC Octet	33/K2	Pseudomonas	+	-	-
37	PCR/061011/037	ES Palazzo	37/K1	Enterobacteriaceae	+	-	-
38	PCR/061011/037	ES Palazzo	37/K2	Enterobacteriaceae	+	-	-
39	PCR/061011/043	NK Octet	43/K1	Enterobacteriaceae	+	-	-
40	PCR/061011/043	NK Octet	43/K2	Enterobacteriaceae	+	-	-
41	PCR/061011/043	NK Octet	43/K3	Enterobacteriaceae	+	-	-
42	PCR/061011/043	NK Octet	43/K4	Enterobacteriaceae	+	-	-
43	PCR/061011/043	NK Octet	43/K5	Enterobacteriaceae	+	-	-
44	PCR/061011/043	NK Octet	43/K6	Enterobacteriaceae	+	-	-
45	PCR/061011/043	NK Octet	43/K7	Enterobacteriaceae	+	-	-
46	PCR/061011/043	NK Octet	43/K8	Enterobacteriaceae	+	-	-
47	PCR/061011/043	NK Octet	43/K9	Enterobacteriaceae	+	-	-
48	PCR/061011/043	NK Octet	43/K10	Enterobacteriaceae	+	-	-
49	PCR/061011/045	P8400	45/K1	Enterobacteriaceae	-	-	-

Maize: kanamycin resistant bacterial isolates					PCR results		
Serial No.	Internal Number	Variety	Sample number	Bacterial strain	16S	nptII	nptIII
50	PCR/061011/045	P8400	45/k2	Enterobacteriaceae	-	-	-
51	PCR/061011/045	P8400	45/K3	Enterobacteriaceae	-	-	-
52	PCR/061011/045	P8400	45/K4	Enterobacteriaceae	+	-	-
53	PCR/061011/045	P8400	45/K5	Pseudomonas	-	-	-
54	PCR/061011/045	P8400	45/k6	Pseudomonas	-	-	-
55	PCR/061011/045	P8400	45/K7	Pseudomonas	-	-	-
56	PCR/061011/045	P8400	45/K8	Pseudomonas	-	-	-
57	PCR/061011/045	P8400	45/K9	Pseudomonas	-	-	-
58	PCR/061011/045	P8400	45/k10	Pseudomonas	-	-	-
59	PCR/061011/045	P8400	45/k11	Pseudomonas	-	-	-
60	PCR/061011/045	P8400	45/k12	Pseudomonas	-	-	-
61	PCR/061011/045	P8400	45/k13	Pseudomonas	-	-	-
62	PCR/061011/045	P8400	45/k14	Pseudomonas	-	-	-
63	PCR/061011/045	P8400	45/k15	Pseudomonas	-	-	-
64	PCR/061011/046	LG3258	46/K1	Pseudomonas	+	-	-
65	PCR/061011/046	LG3258	46/K2	Pseudomonas	+	-	-
66	PCR/061011/046	LG3258	46/K3	Pseudomonas	+	-	-
67	PCR/061011/046	LG3258	46/K4	Pseudomonas	-	-	-
68	PCR/061011/046	LG3258	46/K5	Pseudomonas	+	-	-
69	PCR/061011/046	LG3258	46/K6	Pseudomonas	+	-	-
70	PCR/061011/046	LG3258	46/K7	Pseudomonas	+	-	-
71	PCR/061011/046	LG3258	46/K8	Pseudomonas	+	-	-
72	PCR/061011/046	LG3258	46/k10	Pseudomonas	+	-	-
73	PCR/061011/047	ES Garant	47/K1	Pseudomonas	+	-	-
74	PCR/061011/047	ES Garant	47/K2	Pseudomonas	+	-	-
75	PCR/061011/047	ES Garant	47/K3	Pseudomonas	+	-	-
76	PCR/061011/047	ES Garant	47/K4	Pseudomonas	+	-	-
77	PCR/061011/047	ES Garant	47/K5	Yellow colored bacteria	-	-	-
78	PCR/061011/047	ES Garant	47/K6	Pseudomonas	+	-	-
79	PCR/061011/047	ES Garant	47/K7	Pseudomonas	+	-	-
80	PCR/061011/047	ES Garant	47/K8	Pseudomonas	+	-	-
81	PCR/061011/047	ES Garant	47/K9	Yellow colored bacteria	-	-	-
82	PCR/061011/047	ES Garant	47/K10	Yellow colored bacteria	-	-	-
83	PCR/061011/047	ES Garant	47/K11	Yellow colored bacteria	-	-	-
84	PCR/061011/047	ES Garant	47/K12	Yellow colored bacteria	-	-	-
85	PCR/061011/047	ES Garant	47/K13	Enterobacteriaceae	-	-	-
86	PCR/061011/047	ES Garant	47/K14	Enterobacteriaceae	-	-	-
87	PCR/061011/047	ES Garant	47/K15	Enterobacteriaceae	+	-	-
88	PCR/061011/047	ES Garant	47/K16	Enterobacteriaceae	-	-	-
89	PCR/061011/047	ES Garant	47/K17	Enterobacteriaceae	-	-	-
90	PCR/061011/047	ES Garant	47/K18	Enterobacteriaceae	+	-	-
91	PCR/061011/047	ES Garant	47/K19	Enterobacteriaceae	+	-	-
92	PCR/061011/047	ES Garant	47/K20	Enterobacteriaceae	+	-	-
93	PCR/061011/048	ES Fortress	48/K1	Yellow colored bacteria	-	-	-
94	PCR/061011/048	ES Fortress	48/K2	Yellow colored bacteria	-	-	-
95	PCR/061011/048	ES Fortress	48/K3	Yellow colored bacteria	-	-	-
96	PCR/061011/048	ES Fortress	48/K4	Yellow colored bacteria	-	+	-
97	PCR/061011/048	ES Fortress	48/K5	Yellow colored bacteria	-	-	-
98	PCR/061011/048	ES Fortress	48/K6	Yellow colored bacteria	-	-	-
99	PCR/061011/048	ES Fortress	48/K7	Yellow colored bacteria	-	-	-
100	PCR/061011/048	ES Fortress	48/K8	Yellow colored bacteria	-	-	-

Maize: kanamycin resistant bacterial isolates					PCR results		
Serial No.	Internal Number	Variety	Sample number	Bacterial strain	16S	nptII	nptIII
101	PCR/061011/048	ES Fortress	48/K9	Yellow colored bacteria	-	-	-
102	PCR/061011/048	ES Fortress	48/K10	Yellow colored bacteria	-	-	-
103	PCR/061011/048	ES Fortress	48/K11	Enterobacteriaceae	-	-	-
104	PCR/061011/048	ES Fortress	48/K12	Enterobacteriaceae	-	-	-
105	PCR/061011/049	DK315 Waxy	49/K1	Mucous Pseudomonas	+	-	-
106	PCR/061011/049	DK315 Waxy	49/K2	Mucous Pseudomonas	+	-	-
107	PCR/061011/049	DK315 Waxy	49/K3	Mucous Pseudomonas	+	-	-
108	PCR/061011/049	DK315 Waxy	49/K4	Mucous Pseudomonas	+	-	-
109	PCR/061011/049	DK315 Waxy	49/K5	Mucous Pseudomonas	+	-	-
110	PCR/061011/049	DK315 Waxy	49/K6	Mucous Pseudomonas	+	-	-
111	PCR/061011/049	DK315 Waxy	49/K7	Mucous Pseudomonas	+	-	-
112	PCR/061011/049	DK315 Waxy	49/K8	Mucous Pseudomonas	+	-	-
113	PCR/061011/049	DK315 Waxy	49/K10	Mucous Pseudomonas	+	-	-

Table 16: NptII/nptIII prevalence in kanamycin resistant bacteria from maize: detailed results

16S TaqMan PCR results were classified as positive, if the crossing point (Cp) value was ≤27.

1.6.5 Prevalence of nptII and nptIII in kanamycin resistant bacteria from potato samples

DNA was isolated from the isolated strains as described in section 1.5.6. The results were evaluated using Cp 27 as cut off value for 16S TaqMan PCR positivity (= standard procedure).

From 84 PCR assays 73 were egligible for statistical evaluation for nptII and for nptIII prevalence evaluation (i.e. they were positive for the 16S TaqMan PCR).

None of the bacterial isolates were positive for nptII or nptIII (95% confidence interval: [0%;4.0%]).

	Number of bacterial isolates	Number of positive bacterial isolates	Prevalence estimator	Confidence interval (95%)[1)]
nptII	73	0	0%	[0%;4.0%]
nptIII	73	0	0%	[0%;4.0%]

Table 17: NptII/nptIII prevalence in kanamycin resistant strains from potatoes

1) according to Clopper-Pearson

For detailed PCR results, potato varieties investigated, and taxonomic characterisation of the isolated bacteria as routinely performed in the laboratory see Table 18.

Feed/Potato results: bacterial isolates (Kanamycin resistant)					PCR results		
Serial number	Internal number	Variety	Sample Code	Bacterial Strain	16S	nptII	nptIII
1	11*0010	Ditta	10/K1	Mucous Pseudomonas	+	-	-
2	11*0010	Ditta	10/k2	Mucous Pseudomonas	+	-	-
3	11*0011	Tosca	11/K1	Mucous Pseudomonas	-	-	-
4	11*0011	Tosca	11/k2	Mucous Pseudomonas	-	-	-
5	11*0015	Okama	15/K1	Mucous Pseudomonas	+	-	-
6	11*0015	Okama	15/K2	Mucous Pseudomonas	+	-	-
7	11*0016	Ditta	16/K1	Mucous Pseudomonas	+	-	-
8	11*0016	Ditta	16/K2	Mucous Pseudomonas	+	-	-
9	11*0017	Ditta	17/K1	Mucous Pseudomonas	+	-	-
10	11*0017	Ditta	17/K2	Pseudomonas	-	-	-
11	11*0019	Agata	19/K1	Pseudomonas	-	-	-
12	11*0019	Agata	19/K2	Pseudomonas	+	-	-
13	11*0020	Marabel	20/1	Mucous Pseudomonas	-	-	-
14	11*0020	Marabel	20/2	Mucous Pseudomonas	+	-	-
15	11*0021	Impala	21/1	Mucous Pseudomonas	+	-	-
16	11*0021	Impala	21/2	Mucous Pseudomonas	+	-	-
17	11*0022	Jaerla	22/1	Mucous Pseudomonas	+	-	-
18	11*0023	Ditta	23/1	Mucous Pseudomonas	+	-	-
19	11*0023	Ditta	23/2	Pseudomonas	+	-	-
20	11*0024	Agata	24/1	Pseudomonas	+	-	-
21	11*0024	Agata	24/2	Pseudomonas	+	-	-
22	11*0024	Agata	24/3	Mucous Pseudomonas	+	-	-
23	11*0027	Ditta	27/1	Mucous Pseudomonas	+	-	-
24	11*0027	Ditta	27/2	Mucous Pseudomonas	+	-	-
25	11*0028	Ditta	28/1	Pseudomonas	+	-	-
26	11*0028	Ditta	28/2	Pseudomonas	+	-	-
27	11*0031	Ditta	31/K1	Mucous Pseudomonas	+	-	-
28	11*0031	Ditta	31/K2	Mucous Pseudomonas	+	-	-
29	11*0035	Desiree	35/1	Mucous Pseudomonas	+	-	-
30	11*0035	Desiree	35/2	Mucous Pseudomonas	+	-	-
31	11*0043	Ditta	43/1	Mucous Pseudomonas	+	-	-
32	11*0043	Ditta	43/2	Mucous Pseudomonas	+	-	-
33	11*0044	Ditta	44/1	Mucous Pseudomonas	+	-	-
34	11*0044	Ditta	44/2	Mucous Pseudomonas	+	-	-
35	11*0048	Anabell	48/1	Mucous Pseudomonas	+	-	-
36	11*0048	Anabell	48/2	Mucous Pseudomonas	+	-	-
37	11*0049	Frühkartoffel	49/1	Mucous Pseudomonas	+	-	-
38	11*0050	Freya	50/1	Mucous Pseudomonas	+	-	-
39	11*0050	Freya	50/2	Mucous Pseudomonas	+	-	-
40	11*0051	Red Lady	51/1	Mucous Pseudomonas	+	-	-
41	11*0051	Red Lady	51/2	Enterobacteriaceae	+	-	-
42	11*0052	Mirage	52/1	Pseudomonas	+	-	-
43	11*0053	Tosca	53/1	Yellow colored bacteria	-	-	-
44	11*0053	Tosca	53/2	Yellow colored bacteria	-	-	-

Feed/Potato results: bacterial isolates (Kanamycin resistant)					PCR results		
Serial number	Internal number	Variety	Sample Code	Bacterial Strain	16S	nptII	nptIII
45	11*0053	Tosca	53/3	Pseudomonas	+	-	-
46	11*0053	Tosca	53/4	Pseudomonas	+	-	-
47	11*0055	Erika	55/1	Mucous Pseudomonas	+	-	-
48	11*0055	Erika	55/2	Mucous Pseudomonas	+	-	-
49	11*0056	Ditta	56/1	Mucous Pseudomonas	+	-	-
50	11*0056	Ditta	56/2	Mucous Pseudomonas	+	-	-
51	11*0057	Ditta	57/1	Enterobacteriaceae	+	-	-
52	11*0057	Ditta	57/2	Mucous Pseudomonas	+	-	-
53	11*0057	Ditta	57/3	Yellow colored bacteria	-	-	-
54	11*0060	Ditta	60/K2	Mucous Pseudomonas	+	-	-
55	11*0060	Ditta	60/K1	Mucous Pseudomonas	+	-	-
56	11*0066	Prinzess	66/K1	Mucous Pseudomonas	+	-	-
57	11*0066	Prinzess	66/K2	Pseudomonas	+	-	-
58	11*0066	Prinzess	66/K3	Pseudomonas	-	-	-
59	11*0068	Marabel	68/K1	Mucous Pseudomonas	+	-	-
60	11*0068	Marabel	68/K2	Mucous Pseudomonas	+	-	-
61	11*0070	Prinzess	70/K1	Mucous Pseudomonas	+	-	-
62	11*0070	Prinzess	70/k2	Pseudomonas	+	-	-
63	11*0076	Ditta	76/k1	Mucous Pseudomonas	+	-	-
64	11*0076	Ditta	76/K2	Pseudomonas	-	-	-
65	11*0076	Ditta	76/K3	Enterobacteriaceae	+	-	-
66	11*0077	Ditta	77/K1	Mucous Pseudomonas	+	-	-
67	11*0077	Ditta	77/K2	Mucous Pseudomonas	+	-	-
68	11*0078	Agria	78/K1	Pseudomonas	+	-	-
69	11*0078	Agria	78/K2	Pseudomonas	+	-	-
70	11*0083	Ditta	83/K1	Pseudomonas	+	-	-
71	11*0083	Ditta	83/K2	Pseudomonas	+	-	-
72	11*0083	Ditta	83/K3	Pseudomonas	+	-	-
73	11*0083	Ditta	83/K4	Mucous Pseudomonas	+	-	-
74	11*0084	Ukama	84/K1	Mucous Pseudomonas	+	-	-
75	11*0084	Ukama	84/K2	Pseudomonas	+	-	-
76	11*0084	Ukama	84/K3	Pseudomonas	+	-	-
77	11*0085	Anabell	85/K1	Mucous Pseudomonas	+	-	-
78	11*0085	Anabell	85/K2	Enterobacteriaceae	+	-	-
79	11*0085	Anabell	85/K3	Pseudomonas	+	-	-
80	11*0086	Frieslander	86/K1	Mucous Pseudomonas	+	-	-
81	11*0086	Frieslander	86/K2	Pseudomonas	+	-	-
82	11*0087	Nicola	87/K1	Pseudomonas	+	-	-
83	11*0087	Nicola	87/K2	Pseudomonas	-	-	-
84	11*0087	Nicola	87/K3	Enterobacteriaceae	-	-	-

Table 18: NptII/nptIII prevalence in kanamycin resistant bacteria from potatoes: detailed results

1.7 Conclusions

The prevalence of nptII and nptIII resistance genes in total DNA extracts from maize and potatoes appeared to be low in Austria during the testing period. No samples contained nptII or nptIII copy numbers above the detection limit. A statistical evaluation showed that within a confidence level of 95% less than 6.9% of maize and potato samples would be carriers of nptII or nptIII.

Phenotypic resistance to kanamycin varied considerably within bacterial populations obtained from maize samples. A minimum of 0.01% resistant to kanamycin was observed with a bacterial population from a maize sample from VST Freistadt, Upper Austria, whereas also 73.05% were resistant in a maize sample from Gleisdorf, Styria. Resistance to kanamycin was more homogenously distributed within bacterial populations obtained from potato samples showing considerably lower overall resistance rates to kanamycin (minimum: 0.002%; maximum: 6.6%) compared to maize samples.

In the kanamycin resistant fraction of plant associated bacteria nptII was only detected in a single isolate from maize (prevalence estimator: 1.1%; [0%;4.9%]), nptIII was not present in the analysed collection of bacteria (0%; [0%;3.2%]). For bacteria isolated from potatoes none contained nptII or nptIII, but it was calculated that within a confidence level of 95% less than 4% would be carriers for these resistance genes. Concerning the PCR results obtained from the kanamycin resistant bacterial isolates it should be noted that only a relatively small number of bacterial strains (maize: nptII: 94, nptIII: 93; potato: nptII/nptIII: 73) has been analysed for the presence of nptII or nptIII resistance genes. Low DNA contents due to an insufficient size of the isolated single colony during the recovery of kanamycin resistant strains from maize samples complicated the evaluation of the results from the maize environment. The small number of evaluable bacterial isolates is reflected and compensated by larger confidence intervals in the statistical evaluation. If more samples would have been available for statistical evaluation the 95% confidence interval would have been narrower.

In conclusion the obtained results support the hypothesis of a low naturally occurring background load of nptII and nptIII genes in maize and potato samples used as feed in Austria during the testing period.

2 Appendix

2.1 PCR conditions for nptII/nptIII real time PCR TaqMan Double and 16S TaqMan real time screening assays

All real time PCR assays were implemented on the LightCycler LC480 real time PCR platform (Roche, Austria) using 96-well microtiter plates. All pipetting steps were performed manually under strict adherence to good laboratory practices (distinct sample preparation and PCR mastermix pipetting rooms, separated PCR platform and post-PCR analysis room, separate laboratory equipment and gowns in each area, routine decontamination of the equipment with 10% sodium hypochlorite, pipetting exclusively in laminar airflow hoods and daily decontamination of these hoods with 10% sodium hypochlorite and/or UV radiation).

NptII and nptIII gene targets were detected simultaneously in a single well.

A volume of 2 µl of the DNeasy Plant Mini kit (Qiagen, Germany) eluate was transferred into 8 µl of the real time PCR TaqMan assay mix resulting in a total PCR assay volume of 10 µl. PCR TaqMan Double Assays were prepared according to the recommendations of the manufacturer (Ingenetix, Vienna). Briefly, 8 µl of the real time PCR mastermix comprising of 5 µl of the LC480 Probe Master (Roche), 0.5 µl of the nptII mix (Ingenetix), 0.5 µl of the nptIII mix (Ingenetix), and 2 µl H_2O (molecular biology grade, Sigma) were transferred into a 96 well microplate and supplemented by 2 µl of the DNA template solution. For details see Table 19.

The results were analysed using the second derivative maximum method of the LightCycler LC480 software version 1.5. with default parameters.

Each 96-well microtiter plate contained two negative controls (H_2O as template) and nptII and nptIII plasmid positive controls in duplicates. The PCR run was only valid if all negative controls were negative and all positive controls positive. Otherwise the run was repeated. The amplification conditions are depicted in Table 20. For PCR primer sequences see Table 21. The amplicon lengths for the nptII, nptIII and 16S specific TaqMan PCRs are 129 bp, 82 bp, and 571 bp, respectively. The position of the nptII amplicon and TaqMan probe relative to the nptII reference sequence (GenBank accession No. V00618) is depicted in Figure 2. The characteristics of the nptIII amplicon (reference sequence: V01547) can be found in Figure 3.

The same DNA eluate from each sample was tested with the nptII/nptIII TaqMan Double Assay and with the 16S TaqMan DNA extraction/amplification control assay. Samples with a negative 16S TaqMan assay result (Cp > 27) were excluded from statistical analysis if the nptII and/or the nptIII PCR result were negative.

Reagent	Volume
LC480 Probemaster (Roche, Austria):	5 µl
nptII mix (Ingenetix, Austria): primer concentration: 0.6 µM probe concentration: 0.2 µM probe label : FAM	0.5 µl
nptIII mix (Ingenetix; Austria): primer concentration: 0.6 µM probe concentration: 0.2 µM probe label : 5'YYE	0.5 µl
H_2O (Sigma, molecular biology grade):	2 µl
Template:	2 µl
Total:	10 µl

Table 19: PCR TaqMan Double Screening Assay: components

Item	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1 x
Amplification: Denaturation Annealing and elongation	 95°C 60°C	 10 s 20 s	 45 x
Cooling	40°C	continuous	1 x

Table 20: PCR TaqMan nptII/nptIII Double Screening Assay: PCR conditions (LC480 96-well microplate format).

Primer	Sequence (5' → 3')	Length
np2-F:	GAT CTC CTG TCA TCT CAC CTT GCT	24 nts
np2-R	TCG CTC GAT GCG ATG TTT C	19 nts
nptIII-F	ACA TAT CGG ATT GTC CCT ATA CGA A	25 nts
nptIII-R	TCG GCC AGA TCG TTA TTC AGT A	22 nts
16S_F	TGG AGA GTT TGA TCM TGG CTC AG	23 nts
16S_R	CTT TAC GCC CAR TRA WTC CG	20 nts

Table 21: PCR Primer for TaqMan nptII/nptIII Double Screening and 16S Single Assay.

Probe	Sequence (5' → 3')	Length
nptII	FAM-TCATGGCTGATGCAATGCGGC-BHQ-1	21 nts
nptIII	YYE -AGACAGCCGCTTAGCCGAATTGGATT-BHQ-1	26 nts
16S	confidential business information	

Table 22: TaqMan probe sequences.

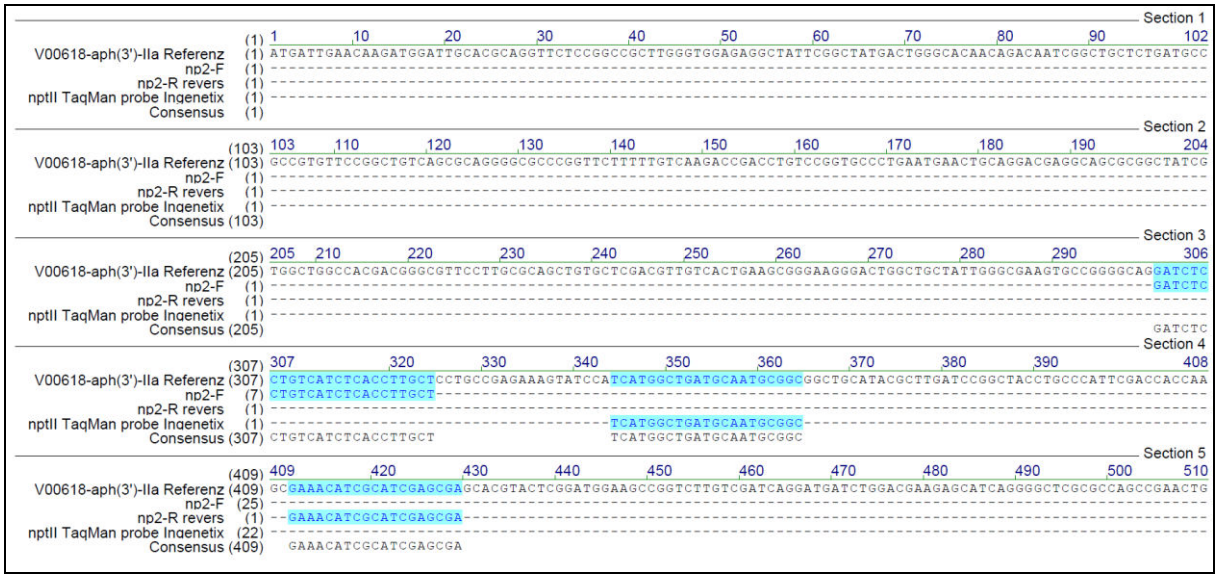

Figure 2: nptII TaqMan amplicon: primers and probe

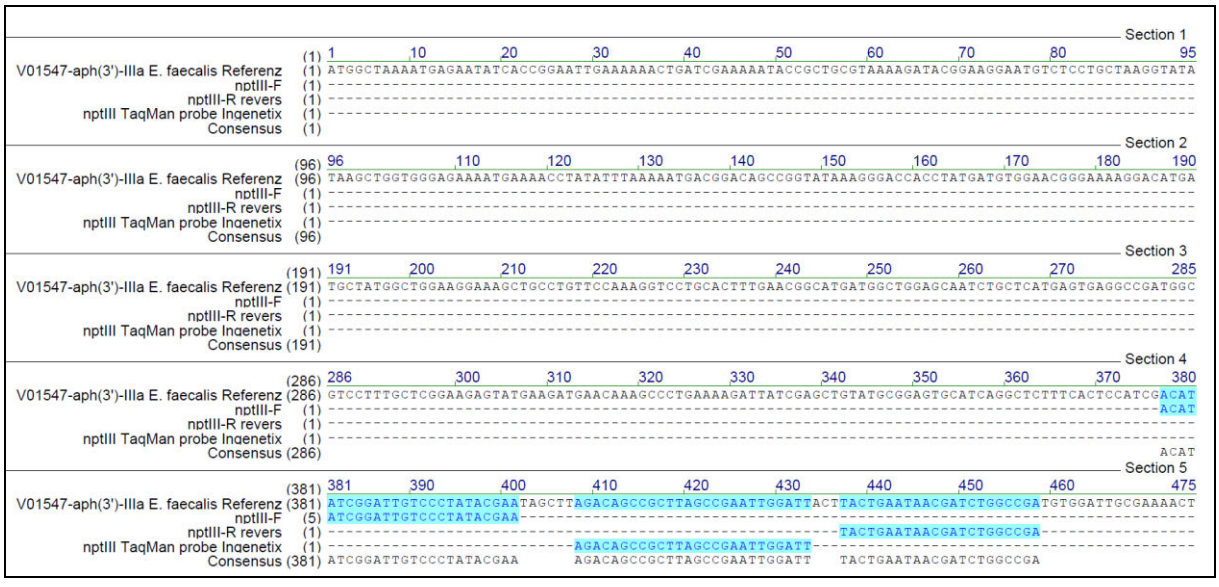

Figure 3: nptIII TaqMan amplicon: primers and probe

2.2 Amplification and DNA extraction control: 16S rRNA TaqMan Single Screening Assay

Each maize and potato sample DNA extract was tested for amplifiable DNA and for PCR inhibition by analysing the respective Plant DNA Mini kit (Qiagen, Germany) extract with an "internal" control 16S TaqMan Assay.

The 16S TaqMan PCR assay conditions were as follows: 8 µl of the real time PCR mastermix comprising of 5 µl of the LC480 Probe Master (Roche, Austria), 0.5 µl of the 16S TaqMan probe (Ingenetix), 0.28 µl of the 16S primermix (Ingenetix; degenerated, 500 nM) and 2.22 µl H_2O (Sigma, molecular biology grade) were transferred into a 96 well microplate and supplemented by 2 µl of the DNA eluate solution. PCR was run on a LightCycler LC480 device (Roche). 16S rRNA TaqMan PCR specific details concerning assay composition and PCR conditions are depicted in Table 23 and Table 24.

The results were analysed using the second derivative maximum method of the LightCycler LC480 software version 1.5 with default parameters.

Reagent	Volume
LC480 Probemaster (Roche, Austria):	5 µl
Primer 16S (degenerated, mix) (Ingenetix, Austria): primer concentration: 0.5 µM	0.28 µl
probe concentration: 0.2 µM probe label : Cy5	0.5 µl
H_2O (Sigma, molecular biology grade):	2.22 µl
Template:	2 µl
Total:	10 µl

Table 23: PCR TaqMan 16S Single Assay: composition

Item	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1 x
Amplification: Denaturation Annealing and elongation	 95°C 60°C	 10 s 1 min	 45 x
Cooling	40°C	continuous	1 x

Table 24: PCR TaqMan 16S Single Assay: PCR conditions (LC480 96-well microplate format).

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Frequency of Environmental Antibiotic Resistance

Part D:

Mosaic Genes;

Selection Pressure;

Modelling Horizontal Gene Transfer in Soil Habitats

Final Report

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1 Potential of nptII to form mosaic genes

1.1 Summary

In silico analysis of nptII gene sequences shows no extended regions of DNA similarity with other aminoglycoside phosphotransferase genes. The identified contiguous stretches of identical sequence are extremely short. This observation indicates that homologous recombination between nptII and the other aminoglycoside phosphotransferase genes under investigation will not be the primary route for the exchange of DNA fragments and sequence evolution. However, a remaining possibility for exchange of DNA sequences between aminoglycoside phosphotransferase genes is illegitimate recombination due to the identification of microhomologies. Sequence alignments using standard parameters of the BLAST algorithm did not reveal the presence of nptII mosaic genes of natural origin in GenBank sequence entries. Data retrieved from the *in silico* analysis available at the time of query did not provide substantial support to the hypothesis of an involvement of the nptII gene in the formation of mosaic genes with altered resistance patterns within the family of aminoglycoside phosphotransferases. However, there are indications from experiments performed in the course of the project that nptII containing DNA might recombine with certain aminoglycoside phosphotransferase genes cloned into *Acinetobacter baylyi* during natural genetic transformation.

A computer model simulating the horizontal transfer of plant derived antibiotic resistance genes into soil bacterial communities expanding pivotal work from Townsend et al. indicates that fixation of the new trait is a long term process extremely dependent on the selection pressure prevailing in the respective habitat. Taking plant degradation processes into account the frequency of horizontal gene transfers plays also a role to a certain extent concerning the fixation of a new phenotype in the population.

The effects of selection pressure mediated by antibiotics in soils are comprehensively discussed. Analysis of recent literature reveals that antibiotics at drug concentrations several hundred-fold below the minimum inhibitory concentrations of susceptible bacteria may select effectively for resistant bacteria and that low antibiotic concentrations found in natural environments are supposed to be important for the enrichment and maintenance of resistance in bacterial populations.

1.2 Zusammenfassung

Eine *in silico* Analyse der nptII Gensequenz mit anderen Aminoglycosidphosphotransferasegenen zeigt keine ausgeprägten Bereiche mit DNA Ähnlichkeit. Die identifizierten zusammenhängenden DNA Abschnitte mit totaler Sequenzidentität sind extrem kurz. Das deutet darauf hin, dass homologe Rekombination zwischen nptII und den anderen untersuchten Aminoglycosidphosphotransferasegenen nicht der primäre Weg zum Austausch von DNA Fragmenten und zur Sequenzevolution ist. Es verbleibt jedoch die Möglichkeit eines DNA Austausches zwischen Aminoglycosidphosphotransferasegenen durch illegitime Rekombination, da Bereiche mit Microhomologien auftreten. BLAST Sequenzalignments mit Standardparametern zeigen, dass sich keine nptII Mosaikgene natürlichen Ursprungs in GenBank befinden. Die aus der *in silico* Analyse gewonnenen Daten unterstützen kaum die Hypothese einer Beteiligung von nptII an der Bildung von Mosaikgenen mit anderen Aminoglycosidphosphotransferasegenen, die zur Veränderung des Resistenzmusters führen können. Es gibt jedoch Hinweise aus im Rahmen des Projekts durchgeführten Experimenten, dass nptII codierende DNA mit ausgewählten Aminoglycosidphosphotransferasegenen, die zuvor in *Acinetobacter baylyi* kloniert worden waren, nach natürlicher genetischer Transformation rekombinieren.

Ein Computer-Modell zur Simulation des horizontalen Gentransfers von aus Pflanzen freigesetzten Antibiotikaresistenzgenen auf die Bakteriengemeinschaften im Boden erweitert das Schlüsselwerk von Townsend et al. und bestätigt, dass die Fixierung eines neu eingebrachten Gens ein langfristiger Prozess ist, welcher extrem vom Selektionsdruck im jeweiligen Habitat abhängig ist. Bezieht man die Abbauprozesse von pflanzlichem Material ins Model mit ein, zeigt sich dass auch die Häufigkeit des horizontalen Gentransfers bei der Fixierung eines neuen Genotyps in einem gewissen Ausmaß eine Rolle spielt.

Die Wirkung des durch Antibiotika in Böden erzeugten Selektionsdrucks wird umfangreich diskutiert. Die Analyse aktueller Literatur zeigt, dass Antibiotikamengen, die mehr als das Hundertfache unter den tatsächlichen minimalen Hemmkonzentrationen von empfindlichen Bakterien liegen, effektiv resistente Bakterien selektieren und dass diese niedrigen, in natürlicher Umgebung zu findenden Antibiotikakonzentrationen wichtig für Anreicherung und Erhalt von Resistenzen in Bakterienpopulationen sind.

1.3 Background

The formation of mosaic genes was initially observed with penicillin binding proteins (pbp) in *Streptococcus pneumoniae* and was found to constitute a threat for public health due to treatment failure of infectious diseases caused by this pathogen (45, 86). This observation is of relevance for the risk assessment of aph(3')-IIa due to the following reasons:

1. The incriminated pbp gene fragments are transferable via natural genetic transformation (40, 41) - equivalent to the expected transfer mode of free aph(3')-IIa encoding plant DNA fragments to soil or gut bacteria.

2. The formation of mosaic genes has been observed in natural environments (suggesting favourable conditions for transfer also of ARM genes) (45).

3. The formation of pbp mosaics in *S. pneumoniae* is an extremely rare event – orders of magnitude lower than the already low putative frequency of horizontal ARM gene transfer from plants to bacteria. Nevertheless pbp mosaics appeared in pathogenic strains after rare HGT events and turned into a problem for public health (45).

4. According to the EFSA GMO Panel there is already a large reservoir of aph(3')-IIa genes present in natural environments (27, 28). Given that this assumption is true there would be ample opportunities for frequent recombinations between incoming aph(3')-IIa fragments and chromosomally or extrachromosomally encoded bacterial nptII genes.

5. Plant-derived aph(3')-IIa fragments may carry mutations or suffer lesions when present as free DNA in the environment which may, after homologous recombination, lead to an altered substrate specificity of the newly generated aminoglycoside phosphotransferase. An altered substrate specificity could result in the inactivation of additional and/or different aminoglycoside antibiotics compared to the inactivation spectrum of the original (i.e. "wild type") nptII gene product. Bacteria carrying a mosaic derivative of the wild type aph(3')-IIa sequence may therefore differ in their resistance phenotype and may not respond to standard treatment schemes, if these bacteria cause infectious diseases.

6. Incoming aph(3')-IIa fragments may also be expected to recombine with other endogenously present aminoglycoside phosphotransferase genes which show sufficient sequence similarity. The result of these homologous recombination events would be aminoglycoside phosphotransferases with a different antibiotic inactivation spectrum compared to the

corresponding “wild type” phosphotransferase causing the same consequences as indicated above.

Mosaic patterns have been detected in addition to pbp genes in tetracycline resistance determinants (tetO/tetW/tet32 genes), autolysin genes (lytA), neuraminidase genes (nanA), surface proteins (pspA), immune response modulation M serotypes (emm gene family), streptokinase genes (ska), shikimate metabolism genes (aroE), glutamine synthetase genes (glnA) etc. in various bacterial species (7, 24, 51, 77, 81, 95, 100).

The formation of mosaic genes is, thus, a common theme in bacterial evolution covering a large array of different gene families coding for various functions (e.g. antibiotic resistance, virulence, metabolic “housekeeping” genes)(6).

Currently, there is no experimental information available whether aph(3’)-IIa has the potential to form mosaic patterns which may become clinically relevant. This workpackage is dealing with the potential of aph(3’)-IIa to form genetic mosaics.

1.4 Strategy for the detection of mosaic nptII gene variants: *In silico* approach

1.4.1 NptII nucleotide sequence alignment studies: NCBI/BLAST

In a first *in silico* approach, a bioinformatics based analysis was conducted to determine whether there were mosaic nptII sequences already deposited in the GenBank database:

For this purpose the aph(3’)-IIa reference sequence (V00618) was aligned with the deposited database sequences in GenBank using the nucleotide BLAST tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome; last access: 15.2.2012). To retrieve a manageable amount of hits, artificial sequences and genes, vector sequences (including cloning vectors, expression vectors, shuttle vectors, transformation vectors, eukaryotic vectors, eukaryotic transformation vectors), and plasmids (broad host range, promiscuous, wide host range, eukaryotic) were excluded from the search. Standard BLAST parameters using the megablast algorithm were applied. The retrieved hits all corresponded to the wild type nptII sequences - in several cases containing a few mutations spread over the whole gene (795 bp) compared to the reference sequence V00618. However, no larger aberrant but contiguous sequence fragments indicative for the formation of gene mosaics were identified. A few hits with a sequence coverage between 5 and 77% were obtained, but these results were either marked as partial sequence or were obtained from mammalian, plant or fungal sources.

There were only two results of some interest: an artificial viral construct showing a part of the nptII gene fused to a fluorescence protein encoding gene. However, this gene combination was designed in the laboratory and had passed unexpectedly through the sequence filter of the BLAST algorithm. A second sequence contained an nptII fragment of 330 bp (5’ part of the nptII gene) which was fused to a 16S rRNA gene fragment. This rearrangement was most likely a sequencing or PCR artefact maybe due to an aberrant recombination event during PCR since the submitter provided a large amount of 16S rRNA sequences within a single upload which all contained native 16S rRNA sequences but did not discuss this aberrant sequence in the corresponding publication. Both hits were excluded from further analysis due to lack of relevance.

Using standard BLAST parameters it was not possible to detect nptII sequences that obviously contained DNA fragments of different phylogenetic origins. Thus, it appears that at least at the time of query the GenBank database did not contain entries of nptII sequences with mosaic patterns.

In a second *in silico* approach the potential of nptII to build mosaic gene patterns with other aminoglycoside phosphotransferase genes was analysed. The most prominent aminoglycoside phosphotransferase (3') genes including gene variants, different nomenclature, GenBank accession number as well as the corresponding aminoglycoside resistance profiles are presented in Table 1. The available corresponding nucleotide and amino acid sequences of aph(3') genes used for subsequent analyses are collected in Table 2.

The data for the remaining aminoglycoside 3''-phosphotransferases, aminoglycoside 4-phosphotransferases, aminoglycoside 6-phosphotransferases, aminoglycoside 2'' phosphotransferases, aminoglycoside 7''-phosphotransferase, and aminoglycoside 9-phosphotransferases can be found in Table 3, Table 4, Table 5, Table 6, Table 7, and Table 8, respectively. Corresponding resistance profiles - if available - are summarized in Table 9. The information was retrieved from Shaw et al. (85) unless otherwise stated. It is noted that this reference is from 1993 and does therefore not provide a full overview of all phosphotransferases present in the environment today. However, data were crosschecked with Ramirez et al. (80) - who provided an update on this issue in 2010 - and the tables were extended if relevant information was available.

1.4.2 Aminoglycoside phosphotransferases: collected data

Information concerning gene variants, different nomenclature, GenBank accession number, resistance profiles of the most important aminoglycoside (3') phosphotransferases are displayed below (Table 1). Data were retrieved from Shaw et al. (85) if not otherwise stated. Corresponding sequence information can be found in Table 2.

APH(3')												
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Resistance**									**Source**
aph(3')-I:	aph(3')-Ia	aphA-1	J01839 V00359	Km	Neo	Prm	Rsm	Lvdm	GmB				*E. coli* (98) Tn903 monomer: 31 kDa kcat/Km: 10E6 – 10E8 M^{-1} s^{-1}
	aph(3')-Ib	aphA-like	M20305	Km	Neo	Prm	Rsm	Lvdm	GmB				*E. coli* (98)
	aph(3')-Ic	aphal-IAB apha7	M37910	Km	Neo	Prm	Rsm	Lvdm	GmB				*Klebsiella pneumoniae* (98)
aph(3')-II:	aph(3')-IIa	aphA-2	V00618 X57709	Km	Neo	Prm	Rsm		GmB	But	(Amk)		*E. coli* (98) Tn5
	aph(3')-IIb		X90856										*P. aeruginosa*
aph(3')-III:	aph(3')-IIIa		V01547	Km	Neo	Prm	Rsm	Lvdm	GmB	But	Amk	Isp	Enterococcus/ Staphylococcus (98)
aph(3')-IV:	aph(3')-IVa	aphA4	X01986	Km	Neo	Prm	Rsm			But			*Bacillus circulans* (98)
aph(3')-V:	aph(3')-Va	aphA-5a	K00432	Km (98)	Neo	Prm	Rsm						*S. fradiae* (98)
	aph(3')-Vb	aphA-5b rph	M22126	Km (98)	Neo	Prm	Rsm						*S. ribosidificus* (98)
	aph(3')-Vc	aphA-5c	S81599(80)	Km (98)	Neo	Prm	Rsm						*Micromonospora chalcea* (98)
aph(3')-VI:	aph(3')-VIa	aphA6	X07753	Km	Neo	Prm	Rsm		GmB	But	Amk	Isp	*A. baumannii* (98)
	aph(3')-VIb			Km	Neo	Prm	Rsm		GmB	But	Amk	Isp	
aph(3')-VII:	aph(3')-VIIa	aphA7	M29953	Km	Neo	Prm (98)	Rsm (98)	Lvdm (98)	GmB (98)	But (98)	(Amk)	Isp (98)	*C. jejuni* (98)

Table 1: Aminoglycoside 3'-phosphotransferases: resistance profiles

Km: kanamycin; Neo: neomycin; Prm: paromomycin; Rsm: ribostamycin; Lvdm: lividomycin; GmB: gentamicin B; But: butirosin; Amk: amikacin; Isp: isepamicin

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(3')-I:	aph(3')-Ia	aphA-1	J01839	obsolete version; replaced by V00359
			V00359	>gi|43025:1162-1977 **aph(3')-Ia** Transposon Tn903, aphA-1 like ATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTAT ATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCC CGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTC AGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATG CATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGG TGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCT TTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGA GTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCC ATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAA TTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGA ACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGA TATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAA
			CAA23656	>gi|43027|emb|CAA23656.1| **APH(3')-Ia** unnamed protein product [Escherichia coli], aphA-1 like MSHIQRETSCSRPRLNSNMDADLYGYKWARDNVGQSGATIYRLYGKPDAPELFLKHGKGSVANDVTDEMV RLNWLTEFMPLPTIKHFIRTPDDAWLLTTAIPGKTAFQVLEEYPDSGENIVDALAVFLRRLHSIPVCNCP FNSDRVFRLAQAQSRMNNGLVDASDFDDERNGWPVEQVWKEMHKLLPFSPDSVVTHGDFSLDNLIFDEGK LIGCIDVGRVGIADRYQDLAILWNCLGEFSPSLQKRLFQKYGIDNPDMNKLQFHLMLDEFF
	aph(3')-Ib	aphA-like	M20305	>gb|M20305.1|RP43APHA: **aph(3')-Ib** 779-1594 Plasmid RP4 aphA gene encoding aminoglycoside 3'-phosphotransferase, complete cds GTGAACGATATTGATCGAGAAGAGCCCTGCGCAGCCGCTGCCGTGCCCGAGAGCATGGCGGCTCACGTGA TGGGATACAAATGGGCGCGTGATAAGGTTGGTCAGTCCGGCTGCGCGGTCTATCGGCTGCATAGCAAGTC AGGCGGCTCCGACTTGTTTCTGAAGCACGGCAAAGATGCTTTTGCCGACGACGTGACTGATGAAATGGTG AGATTGCGTTGGCTGGCGGGGCACATTTCTGTGCCCTCCGTTGTAAGCTTCGTTCGCACGCCCAATCAGG CATGGCTCCTGACAACAGCAATACATGGAAAAACGGCATATCAAGTGCTGAAATCGGATTTCGGAGCCCG TCTCGTTGTTGTTGACGCATTGGCGGCGTTCATGCGCCGACTGCATGCGATCCCAGTGAGCGAATGCTCC GTTCAACAGTGGACCACGCATGCAGGCTTGCCCGAGCGCGGGAGTATCGAGGCGGGGGTTGTTGATGTCG ATGACTTCGATAAGGAGCGCGAAGGGTGGACGGCCGAACAGGTTTGGGAGGCGATGCATCGCCTCCTACC GCTCGCGCCGGACCCAGTCGTGACGCACGGCGATTTTTCACTCGATAATCTACTTATCGTCGAAGGTAAG GTAGTCGGCTGCATCGACGTTGGGCGGGCTGGTATTGCTGATCGATACCAAGACCTTGCCGTGTTATGGA ACTGTCTTGAGGAGTTCGAACCTTCGCTTCAGGAGAGGCTTGTTGCGCAATATGGCATTGCCGATCCGGA TAGGCGCAAGCTGCAATTTCATCTCCTGCTGGACGAACTTTTCTAA

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
			AAA26412	>gi|294757|gb|AAA26412.1| **APH(3')-Ib** aminoglycoside 3'-phosphotransferase like peptide [Plasmid RP4] MNDIDREEPCAAAAVPESMAAHVMGYKWARDKVGQSGCAVYRLHSKSGGSDLFLKHGKDAFADDVTDEMV RLRWLAGHISVPSVVSFVRTPNQAWLLTTAIHGKTAYQVLKSDFGARLVVVDALAAFMRRLHAIPVSECS VQQWTTHAGLPERGSIEAGVVDVDDFDKEREGWTAEQVWEAMHRLLPLAPDPVVTHGDFSLDNLLIVEGK VVGCIDVGRAGIADRYQDLAVLWNCLEEFEPSLQERLVAQYGIADPDRRKLQFHLLLDELF
	aph(3')-Ic	aphal-IAB apha7	M37910 useless entry	>gi|150542|gb|M37910.1| **aph(3')-Ic** PBWIABA Plasmid pBWH77 (from K. pneumoniae) aminoglycoside-3', 5''-phosphotransferase-I, 5' end CCCGGTACAGATACGCCCAGCGGCCATTGACCTTCACGTAGGTTTCATCCATGTGCCACGGGCAAAGATC GGAAGGGTTACGCCAGTACCAGCGCAGCCGTTTTTCCATTTCAGGCGCATAACGCTGAACCCAGCGGTAT ATCGTGGAGTGATCGACATTCACTCCGCGTTCAGCCAGCATCTCCTGCAGCTCACGGTAACTGATGCCGT ATTTGCAGTACCAGCGTACGGCCCACAGAATGATGTCACGCTGAAAATGCCGGCCTTTGAATGGGTTCAT GTGCAGCTCCATCAGCAAAAGGGGATGATAAGTTTATCACCACCGACTATTTGCAACAGTGCCGATAAAA ATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAAATACAAGGGGTGTT**ATG**
			X62115.1	>gi|48988:410-1225 **aph(3')-Ic** K. pneumoniae plasmid pBWH77 aphA7 ATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACCTGGATGCTGATTTAT ATGGGTATAGATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCC CAATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTC AGACTAAACTGGCTGACGGCATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATG CATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGG TGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCT TTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCTA GTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCC ATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAA TTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGA ACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGA TATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTGA
			CAA44024	>gi|48989|emb|CAA44024.1| **APH(3')-Ic** neomycin phosphotransferase [Klebsiella pneumoniae] MSHIQRETSCSRPRLNSNLDADLYGYRWARDNVGQSGATIYRLYGKPNAPELFLKHGKGSVANDVTDEMV RLNWLTAFMPLPTIKHFIRTPDDAWLLTTAIPGKTAFQVLEEYPDSGENIVDALAVFLRRLHSIPVCNCP FNSDRVFRLAQAQSRMNNGLVDASDFDDERNGWPVEQVWKEMHKLLPFSPDSVVTHGDFSLDNLIFDEGK LIGCIDVGRVGIADRYQDLAILWNCLGEFSPSLQKRLFQKYGIDNPDMNKLQFHLMLDEFF

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(3')-II:	aph(3')-IIa	aphA-2	V00618	>gi|43748:151-945 **aph(3')-IIa** Transposon Tn5 fragment encoding neomycin and kanamycin resistance (neo) and a fragment of the reading frame of a further protein ATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACT GGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCT TTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTG GCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTAT TGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGC TGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGC ATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGAC CCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGC CGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCG GCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTA TCGCCTTCTTGACGAGTTCTTCTGA
			CAA23892.1	>gi|43749|emb|CAA23892.1| **APH(3')-IIa** neomycin phosphotransferase [Escherichia coli] MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQDEAARLSWL ATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADAMRRLHTLDPATCPFDHQAKHR IERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARMPDGEDLVVTHGDACLPNIMVENGRFSGFIDCG RLGVADRYQDIALATRDIAEELGGEWADRFLVLYGIAAPDSQRIAFYRLLDEFF
			X57709	>gi|40924|emb|X57709.1| **aph(3')-IIa** E. coli transposon Tn5 DNA for aminoglycoside-phosphotransferase gene aphA-2 ATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACT GGGCACAACAGACAATCGGCTGCTCTGATGGCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCT TTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTG GCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTAACTGAAGCGGGAAGGGACTGGCTGCTAT TGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGC TGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGC ATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGAC CCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGC CGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCG GCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTA TCGCCTTCTTGACGAGTTCTTCTGA
			Translation of X57709 by Expasy Tool	>aminoglycoside-phosphotransferase **APH(3')-IIa** (aphA-2) E. coli transposon Tn5 X57709 MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDGAVFRLSAQGRPVLFVKTDLSGALNEL QDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADAM RRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARMPD GEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADRF LVLYGIAAPDSQRIAFYRLLDEFF

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
	aph(3')-IIb	aph(3")IIps	X90856	>gi|953190:1305-1874 **aph (3')-IIb** Pseudomonas aeruginosa aph(3")IIps gene TCAGTTCAGCCGGTACTGCGCCAGTTCCTTCGCCGAAAGCACGCGCATCCGTTTGGGATCGGTGCTTTGC ATGGCCTGTACCAAGGTGCTGGGGATATCCATTTCGCGGAAGTAGGCGGCGGAGTCGTAGCGCATGCCGT TGGCCGAGCGCGAGTGCAGCGAGTCGCCGTGGGCGTAGTAGGGGCGGTGCAGGCCGACGTAGCCGCGTAC GGTCTTGCGCTTGCCGGCTGCCAGCAGGTAGACGCAGGTGCCCTGGCAGACGCTGCTCGACGGCACCAGC GCGTCGAAGCCGGATTCGCGCAGCAGGCGGCCCATGCGGATCGCCTCCGGCACGCTGCCGCCGATGCTGT TCAGCAGGAGCAGCTTGCGCGAGTAGGTTCCCGGATTGTTGCGCAAACCCTTGAGCAGCGTTTCGTAGTC GCCCGGCGCGATCTCCTCCGAGACGCGGACCGTCAGCACCCGCCCCATGCTCTTGTGTTGCAGCGAATGC ACTTCGACCCTGGCCAGTGCGGGAGTCGCGCAGAACACGGCGAGGCAGGCGAGGACGGTCCGGAGGCGGA GGCGGGGCAT
			CAA62365	>gi|953191|emb|CAA62365.1| **APH (3')-IIb** aminoglycoside phosphotransferase (3")IIps [Pseudomonas aeruginosa] MHDAATSMPPQAPSTWADYLAGYRWRGQGEGCSAATVHRLEAARRPTLFVKQEVLSAHAELPAEIARLRW LHGAGIDCPQVLNETQSDGRQWLLMSAMPGDTLSALAQRDELEPERLVRLVAAALRRLHDLDPAACPFDH RLERRLDTVRQRVEAGLVDEADFDDDHRGRSATELYRLLLDRRPAVEDLVVAHGDACLPNLLAEGRRFSG FIDCGRLGVADRHQDLALAARDIEAELGAAWAEAFLVEYGGDIDGERLAYFRLLDEFF
	aph(3')-IIc		Not available (74, 80)	
aph(3')-III:	aph(3")-IIIa	aphA3	**V01547**	>gi|47033:535-1329 **aph(3')-IIIa** Streptococcus faecalis kanamycin resistance gene encoding a 3'5''-aminoglycoside phosphotransferase of type III. The gene resides on plasmid pJH1 ATGGCTAAAATGAGAATATCACCGGAATTGAAAAAACTGATCGAAAAATACCGCTGCGTAAAAGATACGG AAGGAATGTCTCCTGCTAAGGTATATAAGCTGGTGGGAGAAAATGAAAACCTATATTTAAAAATGACGGA CAGCCGGTATAAAGGGACCACCTATGATGTGGAACGGGAAAAGGACATGATGCTATGGCTGGAAGGAAAG CTGCCTGTTCCAAAGGTCCTGCACTTTGAACGGCATGATGGCTGGAGCAATCTGCTCATGAGTGAGGCCG ATGGCGTCCTTTGCTCGGAAGAGTATGAAGATGAACAAAGCCCTGAAAAGATTATCGAGCTGTATGCGGA GTGCATCAGGCTCTTTCACTCCATCGACATATCGGATTGTCCCTATACGAATAGCTTAGACAGCCGCTTA GCCGAATTGGATTACTTACTGAATAACGATCTGGCCGATGTGGATTGCGAAAACTGGGAAGAAGACACTC CATTTAAAGATCCGCGCGAGCTGTATGATTTTTTAAAGACGGAAAAGCCCGAAGAGGAACTTGTCTTTTC CCACGGCGACCTGGGAGACAGCAACATCTTTGTGAAAGATGGCAAAGTAAGTGGCTTTATTGATCTTGGG AGAAGCGGCAGGGCGGACAAGTGGTATGACATTGCCTTCTGCGTCCGGTCGATCAGGGAGGATATCGGGG AAGAACAGTATGTCGAGCTATTTTTTGACTTACTGGGGATCAAGCCTGATTGGGAGAAAATAAAATATTA TATTTTACTGGATGAATTGTTTTAG
			CAA24789.1	>gi|47034|emb|CAA24789.1| **APH(3')-IIIa** unnamed protein product [Enterococcus faecalis] MAKMRISPELKKLIEKYRCVKDTEGMSPAKVYKLVGENENLYLKMTDSRYKGTTYDVEREKDMMLWLEGK LPVPKVLHFERHDGWSNLLMSEADGVLCSEEYEDEQSPEKIIELYAECIRLFHSIDISDCPYTNSLDSRL AELDYLLNNDLADVDCENWEEDTPFKDPRELYDFLKTEKPEEELVFSHGDLGDSNIFVKDGKVSGFIDLG RSGRADKWYDIAFCVRSIREDIGEEQYVELFFDLLGIKPDWEKIKYYILLDELF

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(3')-IV:	aph(3')-IVa	aphA4	X01986	>gi|39413:277-1065 **aph(3')-IVa** Bacillus circulans aph gene for amino-glycoside phosphotransferase ATGAACGAAAGTACGCGTAATTGGCCGGAGGAACTTCTTGAGCTTCTCGGGCAGACGGAACTAACCGTCA ACAAAATCGGATATTCCGGAGATCACGTCTATCACGTGAAAGAGTACAGGGGCACCCCCGCATTTCTGAA AATCGCCCCCAGTGTATGGTGGAGAACGCTCCGGCCCGAAATTGAAGCGCTCGCTTGGCTGGACGGGAAG CTCCCGGTTCCCAAAATTTTGTACACGGCCGAACACGGCGGGATGGACTACTTGCTGATGGAGGCGCTAG GCGGAAAAGACGGCTCCCACGAAACGATCCAGGCGAAGCGGAAACTGTTTGTGAAGCTGTACGCGGAAGG GCTCCGAAGCGTGCATGGCCTCGATATCCGCGAATGTCCGCTGTCGAACGGGCTGGAGAAGAAGCTCCGG GATGCGAAAAGAATAGTCGATGAGAGCCTGGTGGACCCGGCCGATATAAAAGAGGAGTACGATTGCACGC CGGAGGAATTGTACGGGCTATTGCTTGAGAGTAAGCCGGTAACCGAAGATCTGGTTTTTGCGCACGGAGA TTACTGTGCTCCGAATCTGATTATCGACGGTGAGAAGCTGTCGGGATTTATCGATCTCGGACGTGCGGGC GTGGCGGACCGTTATCAGGACATCAGCCTGGCGATCCGCAGCCTCCGGCACGATTACGGCGACGACCGCT ACAAAGCGCTCTTCCTGGAACTTTACGGGCTGGACGGGCTTGACGAGGACAAGGTCCGGTATTACATCCG GCTGGATGAATTTTTTTGA
			CAA27061.1	>gi|39414|emb|CAA27061.1| **APH(3')-IVa** unnamed protein product [Bacillus circulans] MNESTRNWPEELLELLGQTELTVNKIGYSGDHVYHVKEYRGTPAFLKIAPSVWWRTLRPEIEALAWLDGK LPVPKILYTAEHGGMDYLLMEALGGKDGSHETIQAKRKLFVKLYAEGLRSVHGLDIRECPLSNGLEKKLR DAKRIVDESLVDPADIKEEYDCTPEELYGLLLESKPVTEDLVFAHGDYCAPNLIIDGEKLSGFIDLGRAG VADRYQDISLAIRSLRHDYGDDRYKALFLELYGLDGLDEDKVRYYIRLDEFF
aph(3')-V:	aph(3')-Va	aphA-5a	K00432	>gb|K00432.1| **aph(3')-Va** STMAPH:307-1113 S.fradiae aminoglycoside phosphotransferase (aph) gene ATGGACGACAGCACGTTGCGCCGGAAGTACCCGCACCACGAGTGGCACGCAGTGAACGAAGGAGACTCGG GCGCCTTCGTCTACCAGCTCACCGGCGGCCCCGAGCCCCAGCCCGAGCTCTACGCGAAGATCGCCCCCCG CGCCCCCGAGAACTCCGCCTTCGACCTGTCCGGCGAGGCCGACCGGCTGGAGTGGCTCCACCGCCACGGG ATCCCCGTCCCCCGCGTCGTCGAGCGCGGTGCCGACGACACCGCCGCGTGGCTCGTCACGGAGGCCGTCC CCGGCGTCGCGGCGGCCGAGGAGTGGCCCGAGCACCAGCGGTTCGCCGTGGTCGAGGCGATGGCGGAGCT GGCCCGCGCCCTCCACGAGCTGCCCGTGGAGGACTGCCCCTCCGACCGGCGCCTCGACGCGGCGGTCGCC GAGGCCCGGCGGAACGTCGCCGAGGGCTTGGTGGACCTCGACGACCTGCAGGAGGAGCGGGCCGGGTGGA CCGGCGACCAGCTCCTGGCGGAGCTCGACCGCACCCGTCCCGAGAAGGAGGACCTGGTCGTCTGCCATGG CGACCTGTGCCCCAACAACGTCCTGCTCGACCCCGGGACCTGCCGGGTCACCGGCGTGATCGACGTCGGC CGCCTCGGGGTCGCCGACCGCCACGCCGACATCGCCTTGGCCGCCCGCGAGCTGGAGATCGACGAGGACC CCTGGTTCGGCCCCGCCTACGCCGAGCGGTTCCTGGAGCGGTACGGCGCCCACCGCGTCGACAAGGAGAA GCTGGCCTTCTACCAGCTTCTCGACGAGTTCTTCTAG
			AAA26699.1	>gi|153161|gb|AAA26699.1| **APH(3')-Va** aminoglycoside phosphotransferase [Streptomyces fradiae] MDDSTLRRKYPHHEWHAVNEGDSGAFVYQLTGGPEPQPELYAKIAPRAPENSAFDLSGEADRLEWLHRHG IPVPRVVERGADDTAAWLVTEAVPGVAAAEEWPEHQRFAVVEAMAELARALHELPVEDCPSDRRLDAAVA EARRNVAEGLVDLDDLQEERAGWTGDQLLAELDRTRPEKEDLVVCHGDLCPNNVLLDPGTCRVTGVIDVG RLGVADRHADIALAARELEIDEDPWFGPAYAERFLERYGAHRVDKEKLAFYQLLDEFF

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
	aph(3')-Vb	aphA-5b rph	M22126	>gb|M22126.1| **aph(3')-Vb** STMRPHA:373-1164 Streptomyces ribosidificus aminoglycoside phosphotransferase (rph) gene, complete cds ATGGAAAGCACGTTGCGCCGGACATACCCGCACCACACTTGGCACCTCGTGAACGAAGGAGACTCGGGCG CCTTCGTCTACCGCCTCACCGGACACGGGCCCGAGCTCTACGCGAAGATCGCCCCCCGCACCCCCGAGAA CTCCGCCTTCCACCTCGACGGCGAGGCCGACCGCCTCGACTGGCTCGCCCGCCATGGCATCTCGGTCCCC CGTGTCGTCGAGCGCGGTGCCGACGACACCACCGCCTGGCTCGTCACCGAGGCCGTGCCCGGCGCCGCGG CCTCCGAGGAGTGGCCCGAGGACGAGCGGGCGGCCGTTGTCGACGCGATCGCCGAAATGGCCCGCACCCT CCATGAACTCCCCGTGTCCGAGTGCCCCTTCGACCGCCGCCTCGACGTCACCGGCGAGGCCCGGCACAAC GTCCGCGAGGGCCTGGTCGACCTCGACGACCTCCAGGAGGAGCCGGCCGGCTGGACCGGCGACCAACTCC TGGCCGAACTCGACCTGACGCGGCCCGAGAAGGAGGACTTGGTCGTCTGCCATGGCGACCTGTGCCCCAA CAACGTGCTGCTCGACCCCGAGACCCACCGGATCACCGGGCTGATCGACGTCGGCCGCCTCCGGCTCGCC ACCTGCCACGCCGACCTCGCCCTCGCCGCCCGCGAACTGGCGATCGACGAGGACCCGTGGTTCGGCCCCG CATACGCCGAACGGTTCCTCGAACGGTACGGGGCCCACCACGTCGACCAGGAGAAGATGGCCTTCTACCA GCTGCTCGACGAGTTCTTCTAG
			AAC32025.1	>gi|153435|gb|AAC32025.1| **APH(3')-Vb** aminoglycoside phosphotransferase [Streptomyces ribosidificus] MESTLRRTYPHHTWHLVNEGDSGAFVYRLTGHGPELYAKIAPRTPENSAFHLDGEADRLDWLARHGISVP RVVERGADDTTAWLVTEAVPGAAASEEWPEDERAAVVDAIAEMARTLHELPVSECPFDRRLDVTGEARHN VREGLVDLDDLQEEPAGWTGDQLLAELDLTRPEKEDLVVCHGDLCPNNVLLDPETHRITGLIDVGRLRLA TCHADLALAARELAIDEDPWFGPAYAERFLERYGAHHVDQEKMAFYQLLDEFF
	aph(3')-Vc	aphA-5c	S81599.1	>gi|244648|gb|S81599.1| **aph(3')-Vc** aphA-5c=aminoglycoside-O-phosphoryl-transferase Type Vc [Micromonospora chalcea, 69-683, genomic, 1220 nt] GCCGGACCGGTAGCGGGTCCGCTCGTGGTCGGCGACGCGGAGTTCGCGCCGAGACCGACACCGCCGAGGC CGACGTCACCCAGGACGCGGAGGCCGCCGTGAGCACCGGCGCGCCGGGCGTCATCGGGCCGACCGCCGAC GGGAGCACCGCGGCCGAGGCGGACGAGCAGTCGGGCACCCGGGCGAGATCCTCAGTGAGCCGAACCGCTC GTGTCCGAAATTGTTCAAGCAGTATCAACATTCCCCGAGAAAGCCGCGTGACCTCTGCCATGATTCCGAC CATGTACGCCATGTTGCGCCGGAAATACCAGCACTACGAATGGACCTCCGTGAACGAAGGAGATTCGGGC GCCTCCGTTTACCGCCTCGCCGGACAGCAGCCCGAGCTCTATGTGAAATTCGCTCCGCGCGAACCGGAAA ATTCCGCGTTCGACCTGGCGGGCGAGGCCGACCGGCTCACCTGGCTCACCCGCCACGGCATCCCGGTTCC GTGCATTGTCGAGTGCGGCGGCGACGACACCTCGGTTTTCCTCGTCACCGAGGCCGTCACCGGCGTATCG GCCGCCGAGGAGTGGCCGGAGCACCAGCGCTTCGCCGTCGTCGAGGCGATGGCCGACCTCGCCCGCACCC TGCACGAACTGCCCGTTGGTGGCTGCCCCTTCGATCGCAGCCTGGCGGTGACGGTTGCCGAAGCCCGCCA CAACCTACGCGAGGGCCTCGTGGACCTGGACGACCTCCAAGAGGAGCACGCCAACTGGTCCGGTGACCAG CTTCTCGCCGAGCTCGACCGAACGCGGCCCGAGAAAGAGGATCTGGTCCTCTGCCACGGGGACCTGTGCC CCAACAACGTGCTGCTCGATCCCGAGACATGCCGAGTCACCGGAATGATCGATGTGGGCCGCCTCGGCCG CGCCGATCGCCACGCCGACCTGGCCCTCGCCGCCCGCGAGCTGGAGATCGACGAGGATCCCTGGTTTGGC CCCGAGTACGCCCAGCGGTTCCTCGAACGCTACGGCGCGCACCACGTCGACGAGAACAAGATGGCCTTTT ACCAGCTGCTCGACGAGTTTTTCTAGAAAGGCTCCGTTAGGTCTTCGGGTGCGGTCTGTTCCTGGACATG GTGGATGGATGGACGCACATGAAGTGAACCGTGTCCGGGCGAAGTTGACGTTGTACGTGGCTGACGTGTT CGCATCGGCGCCGCGTAAGGACCAGCGGGC

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
			AAB21326.1	>gi|244649|gb|AAB21326.1| **APH (3')-Vc** aminoglycoside-O-phosphoryl-transferase Type Vc [Micromonospora chalcea] MYAMLRRKYQHYEWTSVNEGDSGASVYRLAGQQPELYVKFAPREPENSAFDLAGEADRLTWLTRHGIPVP CIVECGGDDTSVFLVTEAVTGVSAAEEWPEHQRFAVVEAMADLARTLHELPVGGCPFDRSLAVTVAEARH NLREGLVDLDDLQEEHANWSGDQLLAELDRTRPEKEDLVLCHGDLCPNNVLLDPETCRVTGMIDVGRLGR ADRHADLALAARELEIDEDPWFGPEYAQRFLERYGAHHVDENKMAFYQLLDEFF
aph(3')-VI:	aph(3')-VIa	aphA6	X07753	>gi|38662:103-882 **aph(3')-VIa** Acinetobacter baumannii aphA-6 gene ATGGAATTGCCCAATATTATTCAACAATTTATCGGAAACAGCGTTTTAGAGCCAAATAAAATTGGTCAGT CGCCATCGGATGTTTATTCTTTTAATCGAAATAATGAAACTTTTTTTCTTAAGCGATCTAGCACTTTATA TACAGAGACCACATACAGTGTCTCTCGTGAAGCGAAAATGTTGAGTTGGCTCTCTGAGAAATTAAAGGTG CCTGAACTCATCATGACTTTTCAGGATGAGCAGTTTGAATTCATGATCACTAAAGCGATCAATGCAAAAC CAATTTCAGCGCTTTTTTTAACAGACCAAGAATTGCTTGCTATCTATAAGGAGGCACTCAATCTGTTAAA TTCAATTGCTATTATTGATTGTCCATTTATTTCAAACATTGATCATCGGTTAAAAGAGTCAAAATTTTTT ATTGATAACCAACTCCTTGACGATATAGATCAAGATGATTTTGACACTGAATTATGGGGAGACCATAAAA CTTACCTAAGTCTATGGAATGAGTTAACCGAGACTCGTGTTGAAGAAAGATTGGTTTTTTCTCATGGCGA TATCACGGATAGTAATATTTTTATAGATAAATTCAATGAAATTTATTTTTTAGATCTTGGTCGTGCTGGG TTAGCAGATGAATTTGTAGATATATCCTTTGTTGAACGTTGCCTAAGAGAGGATGCATCGGAGGAAACTG CGAAAATATTTTTAAAGCATTTAAAAAATGATAGACCTGACAAAAGGAATTATTTTTTAAAACTTGATGA ATTGAATTGA
			CAA30578.1	>gi|38663|emb|CAA30578.1| **APH(3')-VIa** unnamed protein product [Acinetobacter baumannii] MELPNIIQQFIGNSVLEPNKIGQSPSDVYSFNRNNETFFLKRSSTLYTETTYSVSREAKMLSWLSEKLKV PELIMTFQDEQFEFMITKAINAKPISALFLTDQELLAIYKEALNLLNSIAIIDCPFISNIDHRLKESKFF IDNQLLDDIDQDDFDTELWGDHKTYLSLWNELTETRVEERLVFSHGDITDSNIFIDKFNEIYFLDLGRAG LADEFVDISFVERCLREDASEETAKIFLKHLKNDRPDKRNYFLKLDELN
	aph(3')-VIb		Not available (34, 80)	
aph(3')-VII:	aph(3')-VIIa	aphA7	M29953	>gb|M29953.1|CAJAPHA7A:296-1048 **aph(3')-VIIa** Campylobacter jejuni kanamycin phosphotransferase (aphA-7) gene, complete cds ATGAAATATATCGATGAAATTCAAATTCTGGGAAAATGTTCAGAGGGTATGTCTCCAGCAGAAGTATATA AATGCCAGCTTAAAAATACTGTATGCTATCTGAAAAAAATTGACGATATATTTTCAAAAACCACATACAG CGTGAAAAGAGAAGCTGAGATGATGATGTGGTTATCCGATAAACTGAAAGTACCAGATGTAATCGAATAC GGAGTACGAGAACATTCAGAATATTTGATCATGAGTGAGTTAAGGGGGAAACACATAGATTGCTTTATTG ATCATCCAATAAAATATATTGAGTGCTTGGTAAACGCACTTCATCAGCTACAAGCAATAGATATAAGAAA CTGCCCATTTTCATCCAAAATAGATGTTCGATTAAAAGAACTAAAATATCTTTTGGATAACAGAATTGCC GATATTGATGTATCGAATTGGGAAGATACAACAGAATTTGATGATCCAATGACGTTATATCAGTGGCTTT GCGAAAATCAACCTCAAGAAGAACTGTGTCTCTCTCATGGAGATATGAGCGCTAATTTTTTTGTATCTCA TGATGGAATATATTTTTATGATTTGGCAAGATGTGGAGTTGCAGACAAATGGTTGGATATAGCATTTTGT GTCAGAGAGATTCGAGAATATTATCCTGATTCTGATTATGAAAAATTCTTTTTTAACATGTTGGGACTTG AACCGGATTATAAAAAAATTAACTATTACATTTTATTAGATGAGATGTTTTAG

APH(3')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
			AAA76822.1	>gi|144187|gb|AAA76822.1| **APH (3')-VIIa** kanamycin phosphotransferase [Campylobacter jejuni] MKYIDEIQILGKCSEGMSPAEVYKCQLKNTVCYLKKIDDIFSKTTYSVKREAEMMMWLSDKLKVPDVIEY GVREHSEYLIMSELRGKHIDCFIDHPIKYIECLVNALHQLQAIDIRNCPFSSKIDVRLKELKYLLDNRIA DIDVSNWEDTTEFDDPMTLYQWLCENQPQEELCLSHGDMSANFFVSHDGIYFYDLARCGVADKWLDIAFC VREIREYYPDSDYEKFFFNMLGLEPDYKKINYYILLDEMF
	aph(3')-VIIb	aphA	Not available	
			Not available	
aph(3')-VIII:			M20305.1 (76)	>gb|M20305.1|RP43APHA:779-1594 **aph(3')-VIII** Plasmid RP4 aphA gene encoding aminoglycoside 3'-phosphotransferase, complete cds GTGAACGATATTGATCGAGAAGAGCCCTGCGCAGCCGCTGCCGTGCCCGAGAGCATGGCGGCTCACGTGA TGGGATACAAATGGGCGCGTGATAAGGTTGGTCAGTCCGGCTGCGCGGTCTATCGGCTGCATAGCAAGTC AGGCGGCTCCGACTTGTTTCTGAAGCACGGCAAAGATGCTTTTGCCGACGACGTGACTGATGAAATGGTG AGATTGCGTTGGCTGGCGGGGCACATTTCTGTGCCCTCCGTTGTAAGCTTCGTTCGCACGCCCAATCAGG CATGGCTCCTGACAACAGCAATACATGGAAAAACGGCATATCAAGTGCTGAAATCGGATTTCGGAGCCCG TCTCGTTGTTGTTGACGCATTGGCGGCGTTCATGCGCCGACTGCATGCGATCCCAGTGAGCGAATGCTCC GTTCAACAGTGGACCACGCATGCAGGCTTGCCCGAGCGCGGGAGTATCGAGGCGGGGGTTGTTGATGTCG ATGACTTCGATAAGGAGCGCGAAGGGTGGACGGCCGAACAGGTTTGGGAGGCGATGCATCGCCTCCTACC GCTCGCGCCGGACCCAGTCGTGACGCACGGCGATTTTTCACTCGATAATCTACTTATCGTCGAAGGTAAG GTAGTCGGCTGCATCGACGTTGGGCGGGCTGGTATTGCTGATCGATACCAAGACCTTGCCGTGTTATGGA ACTGTCTTGAGGAGTTCGAACCTTCGCTTCAGGAGAGGCTTGTTGCGCAATATGGCATTGCCGATCCGGA TAGGCGCAAGCTGCAATTTCATCTCCTGCTGGACGAACTTTTCTAA
			P14509 (76)	>gi|125469|sp|P14509.1| **APH(3')-VIII** KKA8_ECOLX RecName: Full=Aminoglycoside 3'-phosphotransferase; AltName: Full=APH(3')VIII; AltName: Full=Kanamycin kinase, type VIII; AltName: Full=Neomycin-kanamycin phosphotransferase type VIII MNDIDREEPCAAAAVPESMAAHVMGYKWARDKVGQSGCAVYRLHSKSGGSDLFLKHGKDAFADDVTDEMV RLRWLAGHISVPSVVSFVRTPNQAWLLTTAIHGKTAYQVLKSDFGARLVVVDALAAFMRRLHAIPVSECS VQQWTTHAGLPERGSIEAGVVDVDDFDKEREGWTAEQVWEAMHRLLPLAPDPVVTHGDFSLDNLLIVEGK VVGCIDVGRAGIADRYQDLAVLWNCLEEFEPSLQERLVAQYGIADPDRRKLQFHLLLDELF

Table 2: Aminoglycoside 3'-phosphoptransferases: nomenclature, accession numbers and sequences.

Data were retrieved from Shaw et al. (85) if not otherwise stated. Sequence information was obtained from NCBI nucleotide and protein databases (GenBank, PDB, Swissprot etc…)

APH(3'')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(3'')-I:	aph(3'')-Ia	aphE, aphD2	X53527	>gi|47077|emb|X53527.1| **aph(3'')-Ia** Streptomyces griseus aphE gene for streptomycin phosphotransferase ATGAGTGATCACCCCGGGCCGGGGGCCGTCACGCCGGAGCTGTTCGGCGTGGGCGGCGACTGGCTGGCCG TCACCGCGGGCGAATCGGGCGCCTCCGTCTTTCGCGCCGCGGACGCCACCCGGTACGCCAAGTGCGTGCC CGCCGCGGACGCGGCCGGTCTTGAGGCGGAACGCGACCGGATCGCCTGGCTGAGCGGGCAGGGCGTACCG GGCCCCCGCGTCCTCGACTGGTACGCCGGTGACGCGGGCGCCTGCCTGGTCACCCGTGCCGTCCCCGGCG TACCCGCTGATCGGGTGGGCGCCGATGACCTTCGCACTGCCTGGGGGGCCGTCGCGGACGCGGTCCGTCG GCTGCACGAGGTGCCCGTGGCCTCGTGTCCGTTCCGCCGGGGGCTGGACTCCGTGGTCGACGCCGCCCGT GACGTGGTGGCCCGTGGCGCGGTGCATCCGGAGTTCCTGCCGGTGGAGCAGCGGCTCGTTCCCCCGGCGG AGCTGCTGGCCCGGCTCACCGGGGAGCTCGCCCGTCGGCGCGATCAGGAGGCCGCCGACACGGTCGTCTG CCACGGTGATCTCTGCCTGCCCAACATCGTCCTCCATCCGGAGACCCTGGAGGTGTCGGGCTTCATCGAC CTGGGACGGCTCGGGGCGGCCGACCGCCACGCCGACCTGGCGCTGCTGCTGGCCAACGCGCGCGAGACCT GGGTGGACGAGGAGCGGGCGCGGTTCGCCGACGCGGCGTTCGCCGAGCGTTACGGCATCGCCCCGGACCC GGAACGGCTGCGCTTCTACCTCCATCTCGATCCGCTCACCTGGGGCTAG
			CAA37605.1	>gi|47078|emb|CAA37605.1| **APH(3'')-Ia** unnamed protein product [Streptomyces griseus] MSDHPGPGAVTPELFGVGGDWLAVTAGESGASVFRAADATRYAKCVPAADAAGLEAERDRIAWLSGQGVP GPRVLDWYAGDAGACLVTRAVPGVPADRVGADDLRTAWGAVADAVRRLHEVPVASCPFRRGLDSVVDAAR DVVARGAVHPEFLPVEQRLVPPAELLARLTGELARRRDQEAADTVVCHGDLCLPNIVLHPETLEVSGFID LGRLGAADRHADLALLLANARETWVDEERARFADAAFAERYGIAPDPERLRFYLHLDPLTWG
	aph(3'')-Ib	strA, orfH	M28829	>gb|M28829.1|RSFRMRA: strA 63-866 Plasmid RSF1010, complete sequence TTGAATCGAACTAATATTTTTTTTGGTGAATCGCATTCTGACTGGTTGCCTGTCAGAGGCGGAGAATCTG GTGATTTTGTTTTTCGACGTGGTGACGGGCATGCCTTCGCGAAAATCGCACCTGCTTCCCGCCGCGGTGA GCTCGCTGGAGAGCGTGACCGCCTCATTTGGCTCAAAGGTCGAGGTGTGGCTTGCCCCGAGGTCATCAAC TGGCAGGAGGAACAGGAGGGTGCATGCTTGGTGATAACGGCAATTCCGGGAGTACCGGCGGCTGATCTGT CTGGAGCGGATTTGCTCAAAGCGTGGCCGTCAATGGGGCAGCAACTTGGCGCTGTTCACAGCCTATCGGT TGATCAATGTCCGTTTGAGCGCAGGCTGTCGCGAATGTTCGGACGCGCCGTTGATGTGGTGTCCCGCAAT GCCGTCAATCCCGACTTCTTACCGGACGAGGACAAGAGTACGCCGCTGCACGATCTTTTGGCTCGTGTCG AACGAGAGCTACCGGTGCGGCTCGACCAAGAGCGCACCGATATGGTTGTTTGCCATGGTGATCCCTGCAT GCCGAACTTCATGGTGGACCCTAAAACTCTTCAATGCACGGGTCTGATCGACCTTGGGCGGCTCGGAACA GCAGATCGCTATGCCGATTTGGCACTCATGATTGCTAACGCCGAAGAGAACTGGGCAGCGCCAGATGAAG CAGAGCGCGCCTTCGCTGTCCTATTCAATGTATTGGGGATCGAAGCCCCCGACCGCGAACGCCTTGCCTT CTATCTGCGATTGGACCCTCTGACTTGGGGTTGA

			AAA26442.1	>gi|551976|gb|AAA26442.1| STRA Sm resistance protein A [Plasmid RSF1010] MNRTNIFFGESHSDWLPVRGGESGDFVFRRGDGHAFAKIAPASRRGELAGERDRLIWLKGRGVACPEVIN WQEEQEGACLVITAIPGVPAADLSGADLLKAWPSMGQQLGAVHSLSVDQCPFERRLSRMFGRAVDVVSRN AVNPDFLPDEDKSTPLHDLLARVERELPVRLDQERTDMVVCHGDPCMPNFMVDPKTLQCTGLIDLGRLGT ADRYADLALMIANAEENWAAPDEAERAFAVLFNVLGIEAPDRERLAFYLRLDPLTWG
			strB NC_001740.1	>gb|M28829.1|str B RSFRMRA:866-1702 Plasmid RSF1010, complete sequence ATGTTCATGCCGCCTGTTTTTCCTGCTCATTGGCACGTTTCGCAACCTGTTCTCATTGCGGACACCTTTT CCAGCCTCGTTTGGAAAGTTTCATTGCCAGACGGGACTCCTGCAATCGTCAAGGGATTGAAACCTATAGA AGACATTGCTGATGAACTGCGCGGGGCCGACTATCTGGTATGGCGCAATGGGAGGGGAGCAGTCCGGTTG CTCGGTCGTGAGAACAATCTGATGTTGCTCGAATATGCCGGGGAGCGAATGCTCTCTCACATCGTTGCCG AGCACGGCGACTACCAGGCGACCGAAATTGCAGCGGAACTAATGGCGAAGCTGTATGCCGCATCTGAGGA ACCCCTGCCTTCTGCCCTTCTCCCGATCCGGGATCGCTTTGCAGCTTTGTTTCAGCGGGCGCGCGATGAT CAAAACGCAGGTTGTCAAACTGACTACGTCCACGCGGCGATTATAGCCGATCAAATGATGAGCAATGCCT CGGAACTGCGTGGGCTACATGGCGATCTGCATCATGAAAACATCATGTTCTCCAGTCGCGGCTGGCTGGT GATAGATCCCGTCGGTCTGGTCGGTGAAGTGGGCTTTGGCGCCGCCAATATGTTCTACGATCCGGCTGAC AGAGACGACCTTTGTCTCGATCCTAGACGCATTGCACAGATGGCGGACGCATTCTCTCGTGCGCTGGACG TCGATCCGCGTCGCCTGCTCGACCAGGCGTACGCTTATGGGTGCCTTTCCGCAGCTTGGAACGCGGATGG AGAAGAGGAGCAACGCGATCTAGCTATCGCGGCCGCGATCAAGCAGGTGCGACAGACGTCATACTAG
			AAA26443.1 strB	>gi|152579|gb|AAA26443.1| strB Sm resistance protein B [Plasmid RSF1010] MFMPPVFPAHWHVSQPVLIADTFSSLVWKVSLPDGTPAIVKGLKPIEDIADELRGADYLVWRNGRGAVRL LGRENNLMLLEYAGERMLSHIVAEHGDYQATEIAAELMAKLYAASEEPLPSALLPIRDRFAALFQRARDD QNAGCQTDYVHAAIIADQMMSNASELRGLHGDLHHENIMFSSRGWLVIDPVGLVGEVGFGAANMFYDPAD RDDLCLDPRRIAQMADAFSRALDVDPRRLLDQAYAYGCLSAAWNADGEEEQRDLAIAAAIKQVRQTSY

Table 3: Aminoglycoside 3''-phosphotransferases: nomenclature, accession numbers and sequences.

Gene variants, other nomenclature and accession numbers were retrieved from Ramirez et al. (80) if not otherwise stated.

APH(4)				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(4)-I:	aph(4)-Ia	hph	V01499	>gi|40922:231-1256 E. coli gene aph(4) coding for hygromycin B resistance. (encodes aminocyclitol phoshotransferase that inactivates by covalent addition of a phosphate to the 4-position of hygromycin) ATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCG ACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGT CCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCC GCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCC GTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGA GGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGA ATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAA CTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGA CTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGC ATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCT TCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGC AGGATCGCCGCGGCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGAC GGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTG TCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGA TAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAG
			CAA24743.1	>gi|40923|emb|CAA24743.1| unnamed protein product [Escherichia coli K-12] MKKPELTATSVEKFLIEKFDSVSDLMQLSEGEESRAFSFDVGGRGYVLRVNSCADGFYKDRYVYRHFASA ALPIPEVLDIGEFSESLTYCISRRAQGVTLQDLPETELPAVLQPVAEAMDAIAAADLSQTSGFGPFGPQG IGQYTTWRDFICAIADPHVYHWQTVMDDTVSASVAQALDELMLWAEDCPEVRHLVHADFGSNNVLTDNGR ITAVIDWSEAMFGDSQYEVANIFFWRPWLACMEQQTRYFERRHPELAGSPRLRAYMLRIGLDQLYQSLVD GNFDDAAWAQGRCDAIVRSGAGTVGRTQIARRSAAVWTDGCVEVLADSGNRRPSTRPRAKE

APH(4)				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
	aph(4)-Ib	hyg	X03615	>gi|47133:232-1230 Streptomyces hygroscopicus hyg gene for hygromycin B phosphotransferase GTGACACAAGAATCCCTGTTACTTCTCGACCGTATTGATTCGGATGATTCCTACGCGAGCCTGCGGAACG ACCAGGAATTCTGGGAGCCGCTGGCCCGCCGAGCCCTGGAGGAGCTCGGGCTGCCGGTGCCGCCGGTGCT GCGGGTGCCCGGCGAGAGCACCAACCCCGTACTGGTCGGCGAGCCCGACCCGGTGATCAAGCTGTTCGGC GAGCACTGGTGCGGTCCGGAGAGCCTCGCGTCGGAGTCGGAGGCGTACGCGGTCCTGGCGGACGCCCCGG TGCCGGTGCCCCGCCTCCTCGGCCGCGGCGAGCTGCGGCCCGGCACCGGAGCCTGGCCGTGGCCCTACCT GGTGATGAGCCGGATGACCGGCACCACCTGGCGGTCCGCGATGGACGGCACGACCGACCGGAACGCGCTG CTCGCCCTGGCCCGCGAACTCGGCCGGGTGCTCGGCCGGCTGCACAGGGTGCCGCTGACCGGGAACACCG TGCTCACCCCCCATTCCGAGGTCTTCCCGGAACTGCTGCGGGAACGCCGCGCGGCGACCGTCGAGGACCA CCGCGGGTGGGGCTACCTCTCGCCCCGGCTGCTGGACCGCCTGGAGGACTGGCTGCCGGACGTGGACACG CTGCTGGCCGGCCGCGAACCCCGGTTCGTCCACGGCGACCTGCACGGGACCAACATCTTCGTGGACCTGG CCGCGACCGAGGTCACCGGGATCGTCGACTTCACCGACGTCTATGCGGGAGACTCCCGCTACAGCCTGGT GCAACTGCATCTCAACGCCTTCCGGGGCGACCGCGAGATCCTGGCCGCGCTGCTCGACGGGGCGCAGTGG AAGCGGACCGAGGACTTCGCCCGCGAACTGCTCGCCTTCACCTTCCTGCACGACTTCGAGGTGTTCGAGG AGACCCCGCTGGATCTCTCCGGCTTCACCGATCCGGAGGAACTGGCGCAGTTCCTCTGGGGGCCGCCGGA CACCGCCCCCGGCGCCTGA
			CAA27276.1	>gi|581682|emb|CAA27276.1| unnamed protein product [Streptomyces hygroscopicus] MTQESLLLLDRIDSDDSYASLRNDQEFWEPLARRALEELGLPVPPVLRVPGESTNPVLVGEPDPVIKLFG EHWCGPESLASESEAYAVLADAPVPVPRLLGRGELRPGTGAWPWPYLVMSRMTGTTWRSAMDGTTDRNAL LALARELGRVLGRLHRVPLTGNTVLTPHSEVFPELLRERRAATVEDHRGWGYLSPRLLDRLEDWLPDVDT LLAGREPRFVHGDLHGTNIFVDLAATEVTGIVDFTDVYAGDSRYSLVQLHLNAFRGDREILAALLDGAQW KRTEDFARELLAFTFLHDFEVFEETPLDLSGFTDPEELAQFLWGPPDTAPGA

Table 4: Aminoglycoside 4-phosphotransferases: nomenclature, accession numbers and sequences.

Gene variants, other nomenclature and accession numbers were retrieved from Ramirez et al. (80) if not otherwise stated.

APH(6)				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(6)-I:	aph(6)-Ia	aphD, strA	Y00459	>gi|1621271:2825-3748 Streptomyces griseus genes strA (aphD), strB1, strD, strF, strG, strH, strI, strK, strR, strS and strT ATGAGTTCGTCGGACCACATCCACGTCCCGGACGGCCTGGCCGAGTCGTACAGCAGAAGCGGTGGCGAGG AAGGGCGCGCCTGGATCGCCGGACTTCCCGCTCTCGTCGCGCGATGCGTCGACCGCTGGGAGCTGAAGAG GGACGGCGGCGTCCGCTCCGGTGAGGCCTCCCTCGTGGTGCCGGTGCTGCGTGCTGACGGCACCCGGGCG GCGCTCAAACTCCAGATGCCCCGGGAAGAGACGACGGCCGCGCTGATCGGCCTGCGAGCCTGGGGCGGGG ACGGCATGGTGCGGCTGCTCGACCACGACGAGGAGAGCAGCACGATGCTGCTGGAACGCCTGGACGGTTC GCGGACGCTGGCGTCGGTCGAGGACGACGACGAGGCCATGGGCGTCCTCGCCGGGCTGCTGAACCGGCTG CACTCCGTTCCGGCACCTCCGGGGCTGCGGGGTCTGGGAGAGATCGCCGGCGCCATGGTGGAGGAAGTTC CCTCCGCTGTCGACTCGTTGGCGGATCCGGAGGACCGTAGCCGGTTGCGCGGCTGGGCGTCGGCCGTGGC CGAGCTGGTGGGCGAGCCCGGTGACCGCGTCCTGCACTGGGACCTGCACTACGAGAACGTGCTGGCCGCC GAGCGCGAACCGTGGCTGGCCATCGACCCCGAGCCGCTGGTCGGCGACCCGGGGTTCGACCTGTGGCCGG CCCTGGACACCGGTTGGGAGCGGATCGAGGCCACCGGTGACGCGCGGCGGGTGGTCCGGCGGCGCTTCGA CCTGCTGACGGAATCGCTGGAGCTGGACCGCGGGAGGGCGGCCGGGTGGACCCTGGCCCGGCTCCTGCAG AACACCCTGTGGGACATCGAGGACGGGCTGACGGCGATCGCCCCCTCCCAGATCGCCGTGGCCGAAGCGC TGGCGAAGCCCTGA
			CAA68516.1	>gi|49263|emb|CAA68516.1| streptomycin-6-phosphotransferase [Streptomyces griseus] MSSSDHIHVPDGLAESYSRSGGEEGRAWIAGLPALVARCVDRWELKRDGGVRSGEASLVVPVLRADGTRA ALKLQMPREETTAALIGLRAWGGDGMVRLLDHDEESSTMLLERLDGSRTLASVEDDDEAMGVLAGLLNRL HSVPAPPGLRGLGEIAGAMVEEVPSAVDSLADPEDRSRLRGWASAVAELVGEPGDRVLHWDLHYENVLAA EREPWLAIDPEPLVGDPGFDLWPALDTGWERIEATGDARRVVRRRFDLLTESLELDRGRAAGWTLARLLQ NTLWDIEDGLTAIAPSQIAVAEALAKP

	aph(6)-Ib	sph	X05648 X05648.1	>gi|47104:382-1305 Streptomyces glaucescens gene sph for hydroxystreptomycin phosphostransferase ATGAGCACGTCAAAACTGGTGGAGATCCCGGAACCCCTGGCGGCGTCGTACGCCCGCGCCTTCGGCGAGG AGGGACAGGCATGGATCGCCGCCCTGCCCGCGCTGGTCGAGGAATTACTGGACCGCTGGGAGCTGACGGC GGACGGCGCCTCCGCGTCGGGCGAGGCCTCCCTCGTGCTGCCGGTGCTGCGCACCGACGGCACCCGCGCC GTCCTCAAGCTCCAGCTGCCCAGGGAGGAGACCTCCGCCGCCATCACCGGACTGCGCACCTGGAACGGGC ACGGCGTCGTGCGGCTGCTCGACCACGACCCGCGCAGCAGCACCATGCTCCTGGAGCGGCTGGACGCGTC CCGCACGCTGGCCTCGGTCGAGGACGACGACGCCGCCATGGGCGTACTCGCCGGGCTGCTGGCCCGGCTG GTGTCCGTCCCCGCGCCGCGGGGGCTGCGCGGCCTCGGCGACATCGCCGGCGCCATGCTGGAGGAGGTGC CGCGGGCGGTCGCGGCGCTGGCCGACCCGGCCGACCGGCGGCTGCTGAACGACTGGGCGTCGGCGGTGGC CGAACTGGTCGGCGAACCCGGCGACCGGATGCTGCACTGGGACCTGCACTACGGCAACGTCCTCGCCGCC GAGCGCGAACCCTGGCTCGCCATCGACCCCGAACCGCTCGCCGGTGACCCCGGCTTCGACCTGTGGCCCG CCCTGGACAGCCGGTGGGACGACATCGTCGCACAGCGGGACGTCGTACGCGTCGTGCGACGCCGCTTCGA CCTGCTGACCGAGGTCCTCGGCCTGGACCGGGCACGGGCGGCCGGCTGGACGTACGGCAGGCTGCTGCAG AACGCCCTGTGGGACATCGAGGACGGCAGTGCCGCCCTCGACCCCGCCGCCGTCACGCTCGCACAGGCGC TGCGGGGCCACTGA
			CAA29136.1	>gi|47106|emb|CAA29136.1| unnamed protein product [Streptomyces glaucescens] MSTSKLVEIPEPLAASYARAFGEEGQAWIAALPALVEELLDRWELTADGASASGEASLVLPVLRTDGTRA VLKLQLPREETSAAITGLRTWNGHGVVRLLDHDPRSSTMLLERLDASRTLASVEDDDAAMGVLAGLLARL VSVPAPRGLRGLGDIAGAMLEEVPRAVAALADPADRRLLNDWASAVAELVGEPGDRMLHWDLHYGNVLAA EREPWLAIDPEPLAGDPGFDLWPALDSRWDDIVAQRDVVRVVRRRFDLLTEVLGLDRARAAGWTYGRLLQ NALWDIEDGSAALDPAAVTLAQALRGH
	aph(6)-Ic	str	X01702 X01702.1	>gi|43744:485-1285 Transposon Tn5 central region with bleomycin and streptomycin resistance genes ATGGAGCGCTGGCGCCTGCTGCGCGACGGCGAGCTGCTCACCACCCACTCGAGCTGGATACTTCCCGTCC GCCAGGGGGACATGCCGGCGATGCTGAAGGTCGCGCGCATTCCCGATGAAGAGGCCGGTTACCGCCTGTT GACCTGGTGGGACGGGCAGGGCGCCGCCCGAGTCTTCGCCTCGGCGGCGGGCGCTCTGCTCATGGAGCGC GCGTCCGGGGCCGGGGACCTTGCACAGATAGCGTGGTCCGGCCAGGACGACGAGGCTTGCAGGATCCTCT GCGACACCGCCGCTCGTCTGCACGCGCCGCGGTCCGGACCGCCGCCCGATCTCCATCCGCTACAGGAATG GTTCCAGCCGCTTTTCCGGTTGGCCGCTGAGCACGCGGCACTTGCGCCCGCCGCCAGCGTAGCGCGCCAA CTTCTGGCGGCGCCGCGCGAGGTGTGCCCGCTCCACGGCGACCTGCACCACGAGAACGTGCTCGACTTCG GCGACCGCGGCTGGCTGGCCATCGACCCGCACGGACTGCTCGGCGAGCGCACCTTCGACTATGCCAACAT CTTCACGAATCCCGATCTCAGCGACCCCGGTCGCCCGCTTGCGATCCTGCCGGGCAGGCTGGAGGCTCGA CTCAGCATTGTGGTCGCGACGACCGGGTTTGAGCCCGAACGGCTTCTTCGCTGGATCATTGCATGGACGG GCTTGTCGGCAGCCTGGTTCATCGGCGACGGCGACGGCGAGGGCGAGGGCGCTGCGATTGATCTGGCCGT AAACGCCATGGCACGCCGGTTGCTTGACTAG
			CAA25854.1	>gi|43747|emb|CAA25854.1| unnamed protein product [Escherichia coli] MERWRLLRDGELLTTHSSWILPVRQGDMPAMLKVARIPDEEAGYRLLTWWDGQGAARVFASAAGALLMER ASGAGDLAQIAWSGQDDEACRILCDTAARLHAPRSGPPPDLHPLQEWFQPLFRLAAEHAALAPAASVARQ LLAAPREVCPLHGDLHHENVLDFGDRGWLAIDPHGLLGERTFDYANIFTNPDLSDPGRPLAILPGRLEAR LSIVVATTGFEPERLLRWIIAWTGLSAAWFIGDGDGEGEGAAIDLAVNAMARRLLD

	aph(6)-Id	strB, orfi	M28829 M28829.1	>gb|M28829.1|RSFRMRA:866-1702 Plasmid RSF1010, complete sequence ATGTTCATGCCGCCTGTTTTTCCTGCTCATTGGCACGTTTCGCAACCTGTTCTCATTGCGGACACCTTTT CCAGCCTCGTTTGGAAAGTTTCATTGCCAGACGGGACTCCTGCAATCGTCAAGGGATTGAAACCTATAGA AGACATTGCTGATGAACTGCGCGGGGCCGACTATCTGGTATGGCGCAATGGGAGGGGAGCAGTCCGGTTG CTCGGTCGTGAGAACAATCTGATGTTGCTCGAATATGCCGGGGAGCGAATGCTCTCTCACATCGTTGCCG AGCACGGCGACTACCAGGCGACCGAAATTGCAGCGGAACTAATGGCGAAGCTGTATGCCGCATCTGAGGA ACCCCTGCCTTCTGCCCTTCTCCCGATCCGGGATCGCTTTGCAGCTTTGTTTCAGCGGGCGCGCGATGAT CAAAACGCAGGTTGTCAAACTGACTACGTCCACGCGGCGATTATAGCCGATCAAATGATGAGCAATGCCT CGGAACTGCGTGGGCTACATGGCGATCTGCATCATGAAAACATCATGTTCTCCAGTCGCGGCTGGCTGGT GATAGATCCCGTCGGTCTGGTCGGTGAAGTGGGCTTTGGCGCCGCCAATATGTTCTACGATCCGGCTGAC AGAGACGACCTTTGTCTCGATCCTAGACGCATTGCACAGATGGCGGACGCATTCTCTCGTGCGCTGGACG TCGATCCGCGTCGCCTGCTCGACCAGGCGTACGCTTATGGGTGCCTTTCCGCAGCTTGGAACGCGGATGG AGAAGAGGAGCAACGCGATCTAGCTATCGCGGCCGCGATCAAGCAGGTGCGACAGACGTCATACTAG
			AAA26443.1	>gi|152579|gb|AAA26443.1| Sm resistance protein B [Plasmid RSF1010] MFMPPVFPAHWHVSQPVLIADTFSSLVWKVSLPDGTPAIVKGLKPIEDIADELRGADYLVWRNGRGAVRL LGRENNLMLLEYAGERMLSHIVAEHGDYQATEIAAELMAKLYAASEEPLPSALLPIRDRFAALFQRARDD QNAGCQTDYVHAAIIADQMMSNASELRGLHGDLHHENIMFSSRGWLVIDPVGLVGEVGFGAANMFYDPAD RDDLCLDPRRIAQMADAFSRALDVDPRRLLDQAYAYGCLSAAWNADGEEEQRDLAIAAAIKQVRQTSY

Table 5: Aminoglycoside 6-phosphotransferases: nomenclature, accession numbers and sequences.

Gene variants, other nomenclature and accession numbers were retrieved from Ramirez et al. (80) if not otherwise stated.

APH(2'')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(2''):	aph(2'')-Ia	aph(2'')-bifunctional	AP003367 AP003367.1	>gi|14020884:17224-18663 Staphylococcus aureus subsp. aureus Mu50 plasmid VRSAp DNA, complete sequence TCAATCTTTATAAGTCCTTTTATAAATTTCTTTTCTACCATTTTCGATAAATTCCTGTTTAATATTTTTA ATTCCATAAACAATAGTTTCAATAGGATAATATTCTTCAACTATATCTTGATATTCTTTTGCTTTCTCAA TATCTATATTTCCATACATTCTTAATATATCTTCTCCAAAATTTGTTCCTATTTCTTCTTCACTATCTTC AAGTAAGTATATAAAATCACAATATTCATCTATAATTCCAGAATCTCCAAAATCAATTATTCCAGTTAAT CTATTATTGCCATCTAACAATAGATGATTACAACTAAAATCATTATGGCATAAACACTTTTTACCCTCAA AAACTGTTGTTGCATTTAGTCTTTCCATAAAACTTTCTATATAATCTTTTTCTATATCAGTTAAATCATT ATAAATAGTTTCACGCAACAATATATACTCTTCTAATACATTTTGTTTATTATCAATAGTACATTCACTA ATATCTGTATAATCTAAACCGTGCATTTGTCTTAAAAAACTGGCAATATCTCGTTTTAACAAATTTTGTT CTTCTTCTGACATAGTAGAATAAATTTCTGGTGTTAAAAAAGTTCCTTTAATTTCTTTATAACCTAGTAT AGATAATTCATCACTAATATACGAATATTCAATATTAGGAATTTTTACATTAGTTTCTAAATTTGTATTT AAAAAATTATATATTGCTTTTTCTTTTGCATAACCTTTTTTCTTATTAGTACTAAATTTTGTTTTAAAAA TGTATTCATTATTAACTAAATATGCCACACTATCATAACCACTACCGATTATTTCAATACTATCTACTTT GAAATTATCAAAGTAATGCTCAATTAAATATTTCATTGCCTTAACATTTGTGGCATTATCATCATATCTA TATTCCATTAAATAACAATCTTCTTTTTTGCCCTCGTGTAATTCATGTTCTGGCAAATCTTCAATAATTC TAAAACCAGATTTTTGGTATGCCCTTATTGCTCTTGGATTATTTTTATGAGGGTCTAAAATAACTGCATT AGCATTTCTTTCTTTTTTCAAAAATTCAAAAATCAATTTAATATATCTTGTACCAATTCCTTTACTCCAA TAATTTGGCTCTCCTATAAATTGATCCATACCATAGACTATCTCATCAGTTTTTGGATAATGATAATCAG TATATAACTCATCATACATTTTATATATTTGTCCATATCCAATAGGAACATTGTTATATTCAATAATTAC TCTAAAAACTTCATCTTCCCAAGGCTCTGTATAATGTTTTTTTAATGATTCTAATGTATATTTTTTATCT CTACCACCATAAAATTCTAATACTCTTTCATCAGTTAACCATTTTAACATCAAAGGAAAATCATCATCTA TTAAAGTTCTTATACATATTTCATTTTCAACTATATTCAT
			BAB47534.1	>gi|14020910|dbj|BAB47534.1| N-acetyltransferase [Staphylococcus aureus subsp. aureus Mu50] MNIVENEICIRTLIDDDFPLMLKWLTDERVLEFYGGRDKKYTLESLKKHYTEPWEDEVFRVIIEYNNVPI GYGQIYKMYDELYTDYHYPKTDEIVYGMDQFIGEPNYWSKGIGTRYIKLIFEFLKKERNANAVILDPHKN NPRAIRAYQKSGFRIIEDLPEHELHEGKKEDCYLMEYRYDDNATNVKAMKYLIEHYFDNFKVDSIEIIGS GYDSVAYLVNNEYIFKTKFSTNKKKGYAKEKAIYNFLNTNLETNVKIPNIEYSYISDELSILGYKEIKGT FLTPEIYSTMSEEEQNLLKRDIASFLRQMHGLDYTDISECTIDNKQNVLEEYILLRETIYNDLTDIEKDY IESFMERLNATTVFEGKKCLCHNDFSCNHLLLDGNNRLTGIIDFGDSGIIDEYCDFIYLLEDSEEEIGTN FGEDILRMYGNIDIEKAKEYQDIVEEYYPIETIVYGIKNIKQEFIENGRKEIYKRTYKD

APH(2'')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
	aph(2'')-IIa		AF207840	>gb|AF207840.1|:122-1021 Enterococcus faecium aminoglycoside phosphotransferase (aph(2')-Ib) gene, complete cds ATGGTTAACTTGGACGCTGAGATATATGAGCACTTAAATAAACAGATAAAAATAAATGAACTCCGTTATT TATCGTCCGGCGATGATAGTGATACTTTTTTGTGTAATGAACAATATGTTGTGAAAGTTCCTAAACGAGA TTCTGTTAGAATTTCTCAGAAACGAGAGTTTGAATTGTATCGTTTTTTAGAAAACTGTAAGCTATCTTAT CAAATCCCTGCGGTAGTGTATCAAAGTGACCGATTTAATATTATGAAATATATTAAAGGGGAACGTATTA CTTATGAGCAGTATCATAAGTTGAGTGAAAAGGAAAAGGATGCCCTTGCATATGATGAAGCGACGTTTTT GAAAGAGTTACATTCCATAGAGATTGATTGTTCTGTCAGTTTGTTTTCAGATGCTCTGGTGAATAAGAAA GATAAGTTTTTGCAAGATAAAAAATTACTTATAAGTATTCTGGAAAAGGAGCAGCTGTTAACTGATGAGA TGTTGGAACATATCGAAACAATATATGAAAACATATTAAACAATGCTGTTTTATTTAAATATACCCCTTG TTTGGTACATAATGATTTCAGTGCAAATAACATGATTTTTAGAAATAATAGACTGTTTGGAGTTATTGAT TTTGGCGATTTTAATGTAGGTGACCCGGATAATGATTTTTTGTGCTTGCTGGATTGTAGTACAGATGATT TCGGGAAAGAATTTGGCAGGAAGGTATTAAAATACTATCAGCATAAGGCGCCGGAAGTAGCAGAAAGAAA AGCAGAGCTTAATGATGTATATTGGTCGATAGACCAAATTATTTATGGTTATGAAAGAAAAGATAGGGAA ATGTTGATTAAGGGTGTTTCTGAATTGCTACAAACACAAGCAGAGATGTTTATATTTTAG
			AAG13458.1	>gi|10130002|gb|AAG13458.1|AF207840_1 aminoglycoside phosphotransferase [Enterococcus faecium] MVNLDAEIYEHLNKQIKINELRYLSSGDDSDTFLCNEQYVVKVPKRDSVRISQKREFELYRFLENCKLSY QIPAVVYQSDRFNIMKYIKGERITYEQYHKLSEKEKDALAYDEATFLKELHSIEIDCSVSLFSDALVNKK DKFLQDKKLLISILEKEQLLTDEMLEHIETIYENILNNAVLFKYTPCLVHNDFSANNMIFRNNRLFGVID FGDFNVGDPDNDFLCLLDCSTDDFGKEFGRKVLKYYQHKAPEVAERKAELNDVYWSIDQIIYGYERKDRE MLIKGVSELLQTQAEMFIF
			AF337947	>gb|AF337947.1|:272-1171 Escherichia coli aminoglycoside phosphotransferase (aph(2')-Ib) and aminoglycoside acetyltransferase (aac(6')-Im) genes, complete cds ATGGTTAACTTGGACGCTGAGATATATGAGCACTTAAATAAACAGATAAAAATAAATGAACTCCGTTATT TATCGTCCGGCGATGATAGTGATACTTTTTTGTGTAATGAACAATATGTTGTGAAAGTTCCTAAACGAGA TTCTGTTAGAATTTCTCAGAAACGAGAGCTTGAATTGTATCGTTTTTTAGAAAACTGTAAGCTATCTTAT CAAATCCCTGCGGTAGTGTATCAAAGTGACCGATTTAATATTATGAAATATATTAAAGGGGAACGTATTA CTTATGAGCAGTATCATAAGTTGAGTGAAAAGGAAAAGGATGCCCTTGCATATGATGAAGCGACGTTTTT GAAAGAGTTACATTCCATAGAGATTGATTGTTCTGTCAGTTTGTTTTCAGATGCTCTGGTGAATAAGAAA GATAAGTTTTTGCAAGATAAAAAATTACTTATAAGTATTCTGGAAAAGGAGCAGCTGTTAACTGATGAGA TGTTGGAACATATCGAAACAATATATGAAAACATATTAAGCAATGCTGTTTTATTTAAATATACCCCTTG TTTGGTACATAATGATTTCAGTGCAAATAACATGATTTTTAGAAATAATAGACTGTTTGGAGTTATTGAT TTTGGCGATTTTAATGTAGGTGACCCGGATAATGATTTTTTGTGCTTGCTGGATTGTAGTACAGATGATT TCGGGAAAGAATTTGGCAGGAAGGTATTAAAATACTATCAGCATAAGGCGCCGGAAGTAGCAGAAAGAAA AGCAGAGCTTAATGATGTATATTGGTCGATAGACCAAATCATTTATGGTTATGAAAGAAAAGATAGGGAA ATGTTGATTAAGGATGTTTCTGAATTGCTACAAACACAAGCAGAGATGTTTATATTTTAG

APH(2'')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
			AAK63040.1	>gi|14485642|gb|AAK63040.1|AF337947_1 aminoglycoside phosphotransferase [Escherichia coli] MVNLDAEIYEHLNKQIKINELRYLSSGDDSDTFLCNEQYVVKVPKRDSVRISQKRELELYRFLENCKLSY QIPAVVYQSDRFNIMKYIKGERITYEQYHKLSEKEKDALAYDEATFLKELHSIEIDCSVSLFSDALVNKK DKFLQDKKLLISILEKEQLLTDEMLEHIETIYENILSNAVLFKYTPCLVHNDFSANNMIFRNNRLFGVID FGDFNVGDPDNDFLCLLDCSTDDFGKEFGRKVLKYYQHKAPEVAERKAELNDVYWSIDQIIYGYERKDRE MLIKDVSELLQTQAEMFIF
	aph(2'')-IIIa		U51479 U51479.1	>gb|U51479.1|EGU51479:196-1116 Enterococcus gallinarum gentamicin resistance protein gene, complete cds ATGAAACAAAATAAACTTCACTATACCACAATGATAATGACTCAGTTCCCAGATATAAGCATACAATCCG TCGAGTCGCTTGGTGAGGGCTTTAGGAATTACGCGATCCTCGTCAATGGAGATTGGGTTTTTCGTTTTCC CAAGAGTCAACAAGGTGCAGACGAATTGAACAAAGAAATCCAATTGCTACCTCTGTTGGTCGGTTGTGTT AAGGTGAATATTCCACAGTATGTATATATCGGAAAGCGAAGTGATGGAAATCCCTTCGTGGGCTACCGTA AAGTCCAAGGCCAAATCTTGGGTGAAGACGGGATGGCCGTTTTTCCCGATGATGCAAAAGATCGACTGGC GCTGCAACTTGCTGAGTTCATGAATGAGCTAAGCGCATTTCCTGTTGAAACTGCCATATCAGCCGGGGTT CCTGTTACAAACCTGAAAAATAAAATTCTCTTGCTATCGGAAGCTGTGGAGGATCAGGTGTTCCCTCTTC TTGATGAGTCTTTAAGGGACTATCTCACGCTGCGCTTCCAATCCTATATGACTCATCCGGTATATACACG ATATACGCCGAGACTAATTCACGGCGATTTGTCACCTGATCATTTTTTGACGAATTTGAATTCACGTCAG ACCCCATTAACAGGCATTATCGATTTTGGTGATGCCGCAATAAGTGATCCCGATTATGATTATGTATACC TTTTGGAAGATTGCGGCGAGCTGTTTACTCGGCAAGTGATGGCTTATAGAGGCGAGGTTGACTTGGATAC TCACATCAGAAAAGTCTCCTTGTTCGTAACGTTCGATCAAGTCAGTTACCTGTTAGAAGGCTTAAGGGCA AGGGATCAGGACTGGATTTCTGAAGGGTTAGAGCTTTTGGAAGAGGATAAGGCCAACAATTTTGGTGCGA ACAGTGCTTAA
			AAB49832.1	>gi|1854639|gb|AAB49832.1| gentamicin resistance protein [Enterococcus gallinarum] MKQNKLHYTTMIMTQFPDISIQSVESLGEGFRNYAILVNGDWVFRFPKSQQGADELNKEIQLLPLLVGCV KVNIPQYVYIGKRSDGNPFVGYRKVQGQILGEDGMAVFPDDAKDRLALQLAEFMNELSAFPVETAISAGV PVTNLKNKILLLSEAVEDQVFPLLDESLRDYLTLRFQSYMTHPVYTRYTPRLIHGDLSPDHFLTNLNSRQ TPLTGIIDFGDAAISDPDYDYVYLLEDCGELFTRQVMAYRGEVDLDTHIRKVSLFVTFDQVSYLLEGLRA RDQDWISEGLELLEEDKANNFGANSA

APH(2'')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
	aph(2'')-IVa		AF016483	>gi|3080754|gb|AF016483.1| Enterococcus casseliflavus aminoglycoside modifying enzyme (aph(2'')-Id) gene, complete cds AAAATGCTGCAGAGATAGGTTATGCAAGATTTTTATATGGAAATAGCATGAAACAAAAATGAATTAACTA TAATTACTAAATTCTTATTTACAGTCAATCAGATTTTTGATTAAAAGGAGTAGAGAAGTCATGAGAACTT ATACTTTCGACCAGGTAGAAAAGGCAATAGAGCAGTTATATCCTGATTTTACTATCAATACAATAGAGAT TTCAGGAGAAGGCAATGACTGTATTGCATATGAAATAAACAGGGATTTCATTTTTAAATTTCCAAAGCAT TCAAGAGGATCTACTAATCTTTTTAATGAAGTAAATATACTCAAAAGAATCCACAATAAATTACCCCTCC CCATTCCGGAGGTGGTTTTTACAGGAATGCCATCAGAAACGTACCAAATGTCTTTCGCAGGTTTTACAAA AATTAAAGGAGTACCATTGACACCTCTTCTACTCAATAATCTGCCGAAGCAATCTCAAAATCAGGCAGCT AAGGACCTGGCCCGATTTCTAAGTGAACTTCACAGCATAAACATCTCTGGATTCAAAAGTAATCTGGTAT TAGATTTTCGAGAGAAGATAAATGAAGATAATAAAAAAATCAAAAAGTTACTATCCAGGGAATTAAAGGG TCCCCAGATGAAGAAAGTGGATGATTTTTACAGGGATATTCTAGAGAACGAAATCTACTTCAAATACTAT CCTTGTCTTATTCATAACGATTTTAGCAGTGATCATATTTTATTTGATACCGAAAAAAATACTATTTGTG GAATAATCGATTTTGGAGATGCAGCTATTTCTGATCCCGACAATGATTTTATAAGTTTGATGGAAGATGA TGAAGAATACGGCATGGAATTTGTATCAAAAATATTGAACCATTACAAACATAAGGATATACCGACAGTT TTGGAAAAATATAGGATGAAAGAAAAATACTGGTCGTTCGAAAAGATTATCTATGGAAAGGAATATGGTT ATATGGATTGGTATGAAGAGGGATTAAATGAAATCAGAAGCATTAAAATTAAATAGATATTTAATATAAA GGACTGCTAATGTACTCATCAATAGCAATCCTTTTTTAGAATCCGGAATC
			AAC14693.1	>gi|3080755|gb|AAC14693.1| APH(2'')-Id [Enterococcus casseliflavus] MRTYTFDQVEKAIEQLYPDFTINTIEISGEGNDCIAYEINRDFIFKFPKHSRGSTNLFNEVNILKRIHNK LPLPIPEVVFTGMPSETYQMSFAGFTKIKGVPLTPLLLNNLPKQSQNQAAKDLARFLSELHSINISGFKS NLVLDFREKINEDNKKIKKLLSRELKGPQMKKVDDFYRDILENEIYFKYYPCLIHNDFSSDHILFDTEKN TICGIIDFGDAAISDPDNDFISLMEDDEEYGMEFVSKILNHYKHKDIPTVLEKYRMKEKYWSFEKIIYGK EYGYMDWYEEGLNEIRSIKIK

APH(2'')				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
	aph(2'')-Ie		AY939911 AY939911.1	>gb|AY939911.1|:3536-4441 Enterococcus casseliflavus plasmid TnpA (tnpA), aph(2'')-Ie, and RepD (repD) genes, complete cds; and Str (str) gene, partial cds ATGACAACTTATACTTTCGACCAGGTAGAAGAGGCAATAGAGCAGTTATATCCTGATTTTACTATCAATA CAATAGAGATTTCAGGAGAAGGCAATGACTGTATTGCATATGAAATAAACGGGAATTTTATTTTTAAATT TCCAAAGCATTCAAGAGCTTCGATTAATCTCTTGAATGAAGTAACCGTACTCAAAACAATCCACAATGAA TTATCACTACCCATTCCCGAGGTGGTTTTTACAGGAATGCCATCAGAAATGTGCCAAATGTCTTTCGCAG GTTTTACAAAAATTAAAGGAGTACCTTTGACACCTCTTCTACTCAAAAATCTGCCGAAGCAATCTCAAGA TCAGGCAGCTAAGGACCTGGCCCGATTTTTAAGTGAACTTCACAGCATAAATATCTCTGGATTCAAAAGT AATCTGGTATTAGATTTTCGAGAGAAGATAAATGAAGATAATAAAAAAATCAAAAAGTTACTATCCAGGG AATTAAAGGGTCACCAGATGAAGAAAGTGGATGATTTTTACAGGGATATTCTAGACAACGAAATCTACTT CAAATACTATCCTTGTCTTATTCATAACGATTTCAGCAGCGATCATATTTTATTTGATACCGAAAAAAAT ACCATTTGTGGAATAATCGATTTTGGAGATGCAGCTATTTCTGATCCCGACAATGATTTTATAAGTTTGA TGGAAGATGATGAAGAGTACGGCATGGAATTCGTATCAAAAATATTGAACCATTACAAACATAAGGATAT ACCGACAGTTTTGGAAAAATATATGATGAAAGAAAAATACTGGTCGTTCGAAAAGATTATCTATGGAAAG GAATATGGTTATATGGATTGGTATGAAGAGGGATTAAATGAAATCAGAAGCATTAAAATTAAATAG
			AAX38178.1	>gi|61104771|gb|AAX38178.1| aph(2'')-Ie [Enterococcus casseliflavus] MTTYTFDQVEEAIEQLYPDFTINTIEISGEGNDCIAYEINGNFIFKFPKHSRASINLLNEVTVLKTIHNE LSLPIPEVVFTGMPSEMCQMSFAGFTKIKGVPLTPLLLKNLPKQSQDQAAKDLARFLSELHSINISGFKS NLVLDFREKINEDNKKIKKLLSRELKGHQMKKVDDFYRDILDNEIYFKYYPCLIHNDFSSDHILFDTEKN TICGIIDFGDAAISDPDNDFISLMEDDEEYGMEFVSKILNHYKHKDIPTVLEKYMMKEKYWSFEKIIYGK EYGYMDWYEEGLNEIRSIKIK

Table 6: Aminoglycoside 2''-phosphotransferases: nomenclature, accession numbers and sequences.

Gene variants, other nomenclature and accession numbers were retrieved from Ramirez et al. (80) if not otherwise stated.

APH(7'')(98)				
Gene	**Gene variants**	**Other nomenclature**	**Accession No.**	**Sequence**
aph(7'')(98)	aph(7'')-Ia		Not available (80)	

Table 7: Aminoglycoside 7''-phosphotransferase: nomenclature, accession numbers and sequences.

Gene variants, other nomenclature and accession numbers were retrieved from Ramirez et al. (80) if not otherwise stated.

APH(9)(98)				
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Sequence**
aph(9)(98)	Aph(9)-Ia		U94857	>gb|U94857.1|LPU94857:151-1146 Legionella pneumophila spectinomycin phosphotransferase (aph) gene, complete cds ATGCTAAAACAACCAATTCAAGCTCAACAACTTATCGAACTTTTGAAAGTGCATTATGGAATTGATATTC ATACAGCACAATTCATCCAGGGTGGTGCTGATACGAATGCATTTGCATATCAAGCAGATTCAGAATCCAA GTCTTATTTCATAAAGCTAAAATACGGCTATCATGATGAAATTAATTTATCGATAATCCGTCTTTTACAT GATTCTGGAATAAAAGAAATTATTTTTCCTATCCATACACTTGAAGCAAAATTATTCCAGCAACTAAAGC ATTTTAAAATAATTGCGTATCCATTTATTCATGCGCCCAATGGTTTCACCCAAAATTTAACAGGAAAACA GTGGAAACAGCTTGGAAAAGTATTAAGACAAATTCATGAAACATCAGTTCCCATCTCGATTCAACAACAA TTAAGAAAAGAAATATACTCCCCTAAATGGCGTGAAATAGTCAGATCCTTTTATAATCAAATTGAATTTG ATAATTCAGATGATAAGCTCACGGCTGCCTTTAAATCTTTTTTTAACCAAAATAGTGCTGCAATTCATCG ATTAGTTGATACTTCAGAAAAACTATCTAAAAAAATTCAACCTGATTTAGATAAATACGTACTATGTCAT TCTGATATACATGCGGGCAATGTGTTAGTCGGTAATGAAGAGTCGATTTACATTATTGATTGGGATGAGC CTATGTTAGCTCCAAAAGAACGTGATTTGATGTTCATAGGTGGTGGCGTTGGTAATGTATGGAATAAACC CCATGAAATCCAATATTTTTATGAAGGTTATGGTGAAATAAATGTCGATAAAACAATTTTGTCTTATTAC AGGCATGAACGAATTGTCGAAGATATCGCAGTATACGGGCAAGACTTGCTTTCACGTAATCAAAACAATC AGTCCAGACTTGAAAGTTTTAAATATTTTAAAGAAATGTTTGATCCAAACAACGTTGTTGAAATAGCTTT TGCTACAGAGCAGTAA
			AAB58447.1	>gi|2145190|gb|AAB58447.1| spectinomycin phosphotransferase [Legionella pneumophila 130b] MLKQPIQAQQLIELLKVHYGIDIHTAQFIQGGADTNAFAYQADSESKSYFIKLKYGYHDEINLSIIRLLH DSGIKEIIFPIHTLEAKLFQQLKHFKIIAYPFIHAPNGFTQNLTGKQWKQLGKVLRQIHETSVPISIQQQ LRKEIYSPKWREIVRSFYNQIEFDNSDDKLTAAFKSFFNQNSAAIHRLVDTSEKLSKKIQPDLDKYVLCH SDIHAGNVLVGNEESIYIIDWDEPMLAPKERDLMFIGGGVGNVWNKPHEIQYFYEGYGEINVDKTILSYY RHERIVEDIAVYGQDLLSRNQNNQSRLESFKYFKEMFDPNNVVEIAFATEQ

APH(9)(98)				
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Sequence**
			CR628337	>gi|53752796:1710246-1711238 Legionella pneumophila str. Lens complete genome ATGCTAAGAAATAACATTCCGGATCAACACCTCATTGCGCTTTTGAAAGTCTATTATGGAATTGATATTC ATACTGTACAACTCATCGTGGGTGGTGCTGATATGAATGCCTTTGGATATAAAGCGGATTCAGAATCCAA TTCTTATTTCGTAAAGCTGAAATACGGCCATCATGATGAAATTAATTTATCAATCATTCGCCTCTTGCAT GATTCTGGAATAAAAGAAATTATTTTTCCTATCTACACACGTGATACAAAATTATTTCAGCAAATAGATC ATTTTAAAATAATAGTGTATCCATTTATTAATGCGCCCAATGGCTTCACTCAAAATTTAACAGAAAAACA GTGGCACCAGCTTGGAAAAGTATTAAGACAAATTCATGAAACTTCTGTTCCCACCGCTATTCAGCAACGA TTAAGGAAAGAAACTTATTCGCCTAAATGGCGTGAAATGGTTAGATCTTTTTATAACAAAATTGGATTTG ATGATTCAGATGATCAAATTACTACTGACTTCAAAACTTTTTTTAATCAAAATATCGATTCAATTCATCG ATTAGTCGATTCTTCAGAAGAACTATCTAAAAAAATTCAACCCGATTTAGATAAATATGTACTGTGTCAT TCTGATGTACATGCGGGCAATGTATTGGTCGTTAATGAGGAATCTATTTACATTATTGATTGGGATGAGC CCATGTTAGCGCCAAAAGAGTGTGATTTGATGTTCATTGGCGGTGGCATTGGTAATGTATGGAATAAACA CCATGAAATCAATTATTTTTATGAAGGTTATGGTAAAACAAACGTTGATAAAATAATTTTATCTTATTAC AGACATGAACGAATTGTCGAAGATATAGCTGTATATGGACAAGACTTACTTTCACGTGATCAAAACGACG AGTCCAGACTTGAAAGTTTTAAACATTTTAAATCGATGTTTACTCCAAATGATGTTGTTGAAATAGCTTT TTCTTCGGATTAA
			Expasy Translation	>aph(9')-I? MLRNNIPDQHLIALLKVYYGIDIHTVQLIVGGADMNAFGYKADSESNSYFVKLKYGHHDE INLSIIRLLHDSGIKEIIFPIYTRDTKLFQQIDHFKIIVYPFINAPNGFTQNLTEKQWHQ LGKVLRQIHETSVPTAIQQRLRKETYSPKWREMVRSFYNKIGFDDSDDQITTDFKTFFNQ NIDSIHRLVDSSEELSKKIQPDLDKYVLCHSDVHAGNVLVVNEESIYIIDWDEPMLAPKE CDLMFIGGGIGNVWNKHHEINYFYEGYGKTNVDKIILSYYRHERIVEDIAVYGQDLLSRD QNDESRLESFKHFKSMFTPNDVVEIAFSSD

APH(9)(98)				
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Sequence**
			U70376	>gb|U70376.2|:2696-3466 Streptomyces flavopersicus spectinomycin biosynthesis gene cluster, complete sequence TCAGTTACGGCGCGTGGCCCAACGGTCGTACGTGCACAGCCCGTGCGGGCTGCGCTCGAACAGCGTGCGC CTCCTGCATGACGAGCACCTGCTCACTTGCAACGAGGCCCCCGAGCACCCGCCCCGCTCCGGTCCTGCCG GCGTCTGCTCCGCGATGGACCCCCTTCCACCGCGTGCCCAGGTGTCCGCCGGGCATGCCGCCATGTCGGC CGGGCCCTTGCAGGAGCGGTCGGTGACATGGGCCGTGAGCGCCGCGGGTGTCGCGTCGGTCAGCTGTGCC GTGGTGACGTTTCCGGTGGCGTACCCCTCCCGGCGGGCCAGTTCGAGCAGGGTGGGCAGCGGCTCGTTGG TGTCCGGTGTCCGGGAGATGCGCCCGTTGACCGTCTTGCGCCCGGTGGCCCACGCCGTGGCGCCGGCTGC GGAGTCGGTGACGTAGTCGGGGTGGCCCCGCTCGTCCACCGCGGAGGTCAGGGACGATCCGGTCATCGGC AGCCGGTCCATCGTGAGCCGACCCTCGGCGGCGACGGCGTAGTTGCGCGCGGCGGTGATCTCCGAGTCAC CCATGCCGTCGCCTATGAGGAGGATTACGTTCCGCGCCGGGGAGGGCCTGAAATCTCCGGAAACTGGTCC GGACGGAGGCGCTGCGGAGGACACGGTCACCGATGTCAGCAGGACACCGGTGACCGCGCCCCACCGCCGC ACCCGTCGCTTTCCCTGCCGCACCGCATGGACCGGCCCTCCGCCTCGTCGGTCCGGAAAAGCTGCCGTCA T
	Aph(9)-Ib	spcN	U70376.2	>gb|U70376.2|:7526-8518 Streptomyces flavopersicus spectinomycin biosynthesis gene cluster, complete sequence TCAGCTCGGTACTTCGGAGTTCAGATGGTCGAGAGTCTCGGCGAAGCTCTGCCAAGCGGCTTCGGTGTCG GAGGTGCGCTGGTGTTCCCCGCGGAACCACTCGACGAACTCGGCGACGTCCAGCAGGCTCCACCGCAGCC GGTAGAGCGCCAGCGCGGCCGGGTCGGGCGTGTGCCCGGTCAGTTCGGTGTAGCGGGCGAGAGCTGCCGG GTCGTCCGAGATCAGGGAGAGGTCCCGTTCGGGTATCGCGAGGCCCACCGTGTCCCAGTCCACCAGCACA TAGCCGTCCTCACCAAGGATCAGGTTCCCCGGGTGCGGCTCGCCGTGTGTGACGACCAGCGGGGCGCCGC GGCCCCGTACCCGCGCCACCAGTTCGCCGAACTCCGCCATCCGCCCGCGGAGCGTCGCCTCGTGCTCGGC CAGCAACTCGCGGGCCGGCTCGGAGAACGGCCCGCCCGTCCAGGTTCCGGACGGCTCGGCCAGCGCGGTG TGCACGCCATCCAGTCCGGTCGGCACCATGTCGGTGGTCGGGGTGCACTTCGGCGGTGCCTGGCCGTGCA ATTCTGCGAGCAGCACCAGCACCTGGTCCCGCTCCCGCTCCGTCAGCTTCTGGCCGAACTCCCCGGGTCG GGCCGAGACATGGGGAAATACGGTCAACGCGTACCGCGAGTCCAGCGGGACCAGTGAAGCGCCGTCACTC GTGGTCCGGGGTGCCACCACGAACGGCAGGCCGCCCTGCTCACGCAAGTGCACCGCGGTGTCCATGGCTC TCCGCAGACCTCGCAGTGCCGCCGGGGCACCGTGCCCGCAGTGCTCCTTGTGTTCGAGGTCGGAGACGGT GGCGAACCACCGCTGCCCGTCGTCACCGGTGATGTGCCAGTGATAGTCGCCGAAGCCGAGCGGCGCGTAC GACGCACTGGTCGTGGAGATACCGAATTCTCGTAGTCCCTGAAATAGGCTTTCCTGGTCCAGGTTCTCAG GAAGATCTTCCAT

APH(9)(98)				
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Sequence**
			AAD45540.1	>gi|5579419|gb|AAD45540.1|U70376_2 streptomycin-6-phosphate phosphatase [Streptomyces netropsis] MTAAFPDRRGGGPVHAVRQGKRRVRRWGAVTGVLLTSVTVSSAAPPSGPVSGDFRPSPARNVILLIGDGM GDSEITAARNYAVAAEGRLTMDRLPMTGSSLTSAVDERGHPDYVTDSAAGATAWATGRKTVNGRISRTPD TNEPLPTLLELARREGYATGNVTTAQLTDATPAALTAHVTDRSCKGPADMAACPADTWARGGRGSIAEQT PAGPERGGCSGASLQVSRCSSCRRRTLFERSPHGLCTYDRWATRRN
			AAB66655.1	>gi|1575682|gb|AAB66655.1| SpcN [Streptomyces netropsis] MEDLPENLDQESLFQGLREFGISTTSASYAPLGFGDYHWHITGDDGQRWFATVSDLEHKEHCGHGAPAAL RGLRRAMDTAVHLREQGGLPFVVAPRTTSDGASLVPLDSRYALTVFPHVSARPGEFGQKLTERERDQVLV LLAELHGQAPPKCTPTTDMVPTGLDGVHTALAEPSGTWTGGPFSEPARELLAEHEATLRGRMAEFGELVA RVRGRGAPLVVTHGEPHPGNLILGEDGYVLVDWDTVGLAIPERDLSLISDDPAALARYTELTGHTPDPAA LALYRLRWSLLDVAEFVEWFRGEHQRTSDTEAAWQSFAETLDHLNSEVPS

Table 8: Aminoglycoside 9-phosphotransferases: nomenclature, accession numbers and sequences.

Gene variants, other nomenclature and accession numbers were retrieved from Ramirez et al. (80) if not otherwise stated.

APH(3'')																
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Resistance**												
aph(3'')-I:	aph(3'')-Ia	aphE, aphD2	X53527										Sm		
	aph(3'')-Ib	strA, orfH	M28829										Sm		
APH(4)																
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Resistance**												
aph(4)-I:	aph(4)-Ia	hph	V01499											HygB		
	aph(4)-Ib	hyg	X03615											HygB		
APH(6)																
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Resistance**												
aph(6)-I:	aph(6)-Ia	aphD, strA	Y00459										Sm		
	aph(6)-Ib	sph	X05648										Sm		
	aph(6)-Ic	str	X01702										Sm		
	aph(6)-Id	strB, orfi	M28829										Sm		
APH(2'')																
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Resistance**												
aph(2'')	aph(2'')-Ia	aph(2'')-bifunctional	AP003367										Gm		in combination with aac(6')

APH(7'')(98)															
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Resistance**											
aph(7'')(98)			Not available											HygB	
APH(9)(98)															
Gene	**Gene variants**	**other nomenclature**	**Accession No.**	**Resistance**											
aph(9)(98)		spcN	U94857	Spc											

Table 9: Resistance profiles of other than 3'-aminoglycoside phosphotransferases.

Information was retrieved from Shaw et al. (85) if not otherwise stated.

1.4.3 Phylogenetic analyses

A phylogenetic analysis of the aminoglycoside phosphotransferase gene sequences identified in the database searches (NCBI nucleotide/GenBank database; last access: 16.2.2012) was conducted in order to determine the potential of aph(3')-IIa – fragments to induce the formation of mosaic gene structures after recombination with sequence-similar phosphotransferase genes.

For the results of the phylogenetic analysis of the most prominent aminoglycoside phosphotransferase genes see Figure 1. Aph(3')-IIa does not show close phylogenetic relationship to other aminoglycoside phosphotransferase genes. This observation reflects the absence of extensive stretches of sequence homology between the tested aminoglycoside phosphotransferase genes and aph(3')-IIa.

Aminoglycoside phosphotransferase genes which show sufficient DNA similarity for homologous recombination with nptII genes were determined by dual sequence alignments with the aph(3')-IIa reference sequence V00618. This approach was used to identify contiguous stretches of high DNA similarity between nptII and various other aminoglycoside phosphotransferase genes and to determine the length of identified blocks of homologous regions. As example a dual alignment between aph(3')-IIa and aph(3')-IVa is presented in Figure 2 and Figure 3. Sequence fragments highlighted in yellow indicate homologous regions.

The results of the dual alignments are depicted in Table 10. Potential sequence regions prone for homologous recombination with parts of the aph(3')-IIa sequence are presented. Extensive stretches of exact DNA sequence homology to the aph(3')-IIa were not detected for the analysed aminoglycoside phosphotransferase genes. The identified contiguous stretches of 100% similarity usually showed a maximum length of 7 – 9 bp. There were several locations detectable showing extended microhomologies. These regions were also indicated in Table 10 because they may serve as targets for illegitimate recombination but that process will only take place at a substantial lower frequency compared to homologous recombination (79).

The results of the dual alignments were used as basis for determining the aminoglycoside phosphotransferase gene to be used as the target for nptII recombination in *Acinetobacter baylyi* natural transformation experiments (see section 1.5)

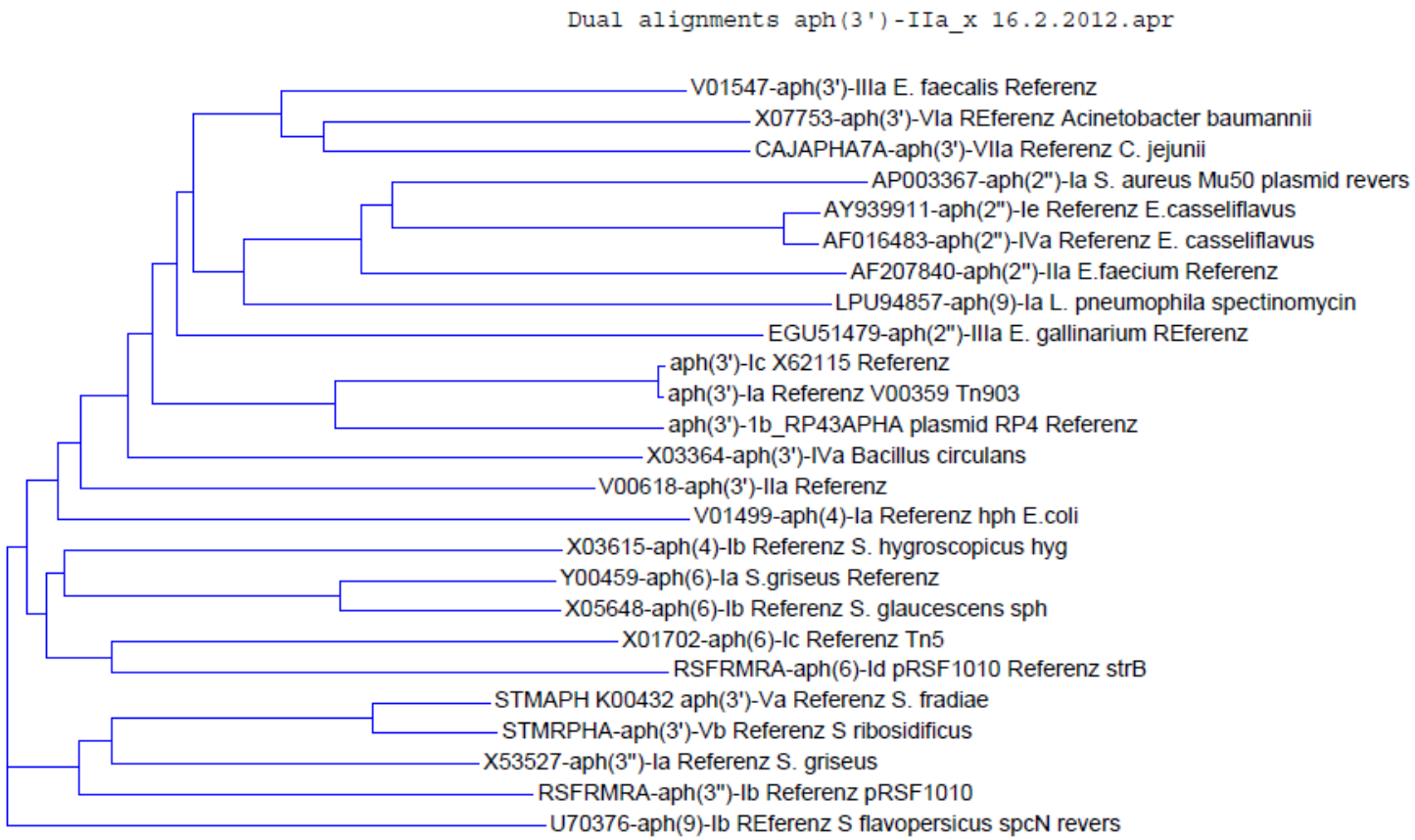

Figure 1: Aminoglycoside phosphotransferase genes: phylogenetic tree.

(ClustalW analysis; VectorNTI 7.1)

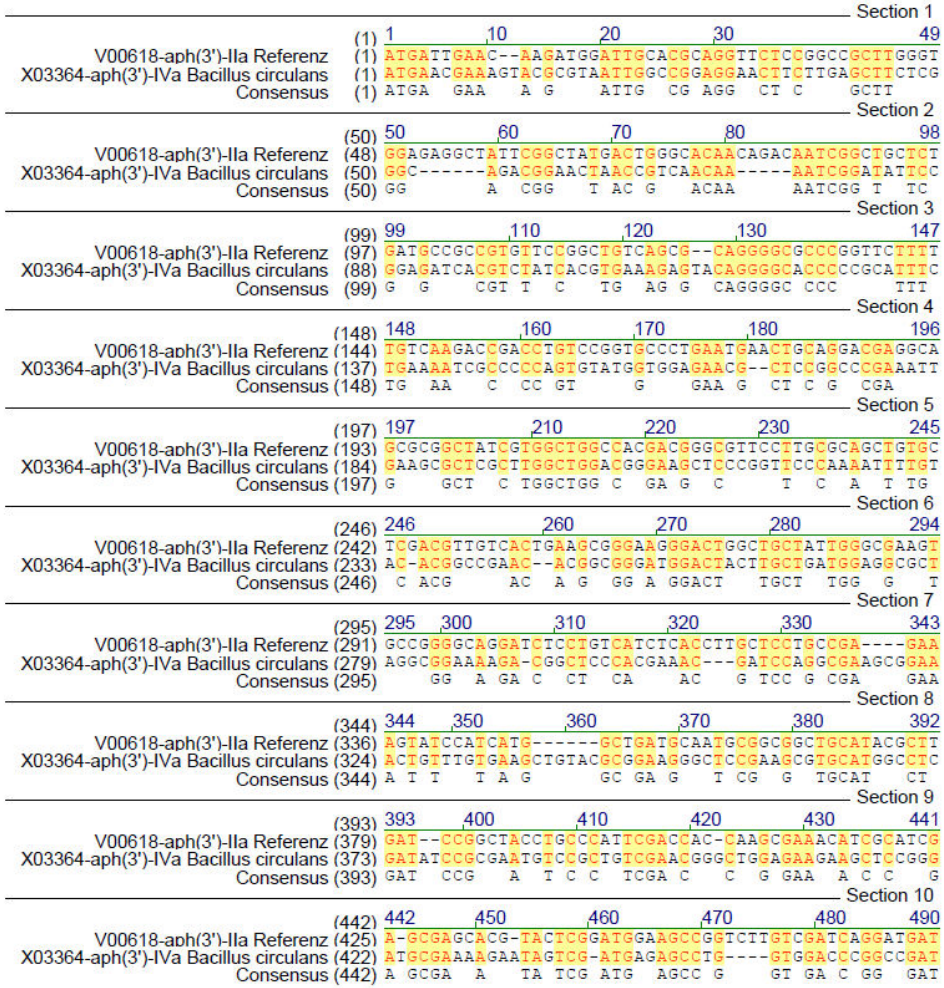

Figure 2: Dual alignment of aph(3')-IIa and aph(3')-IVa.

(ClustalW analysis; VectorNTI 7.1)

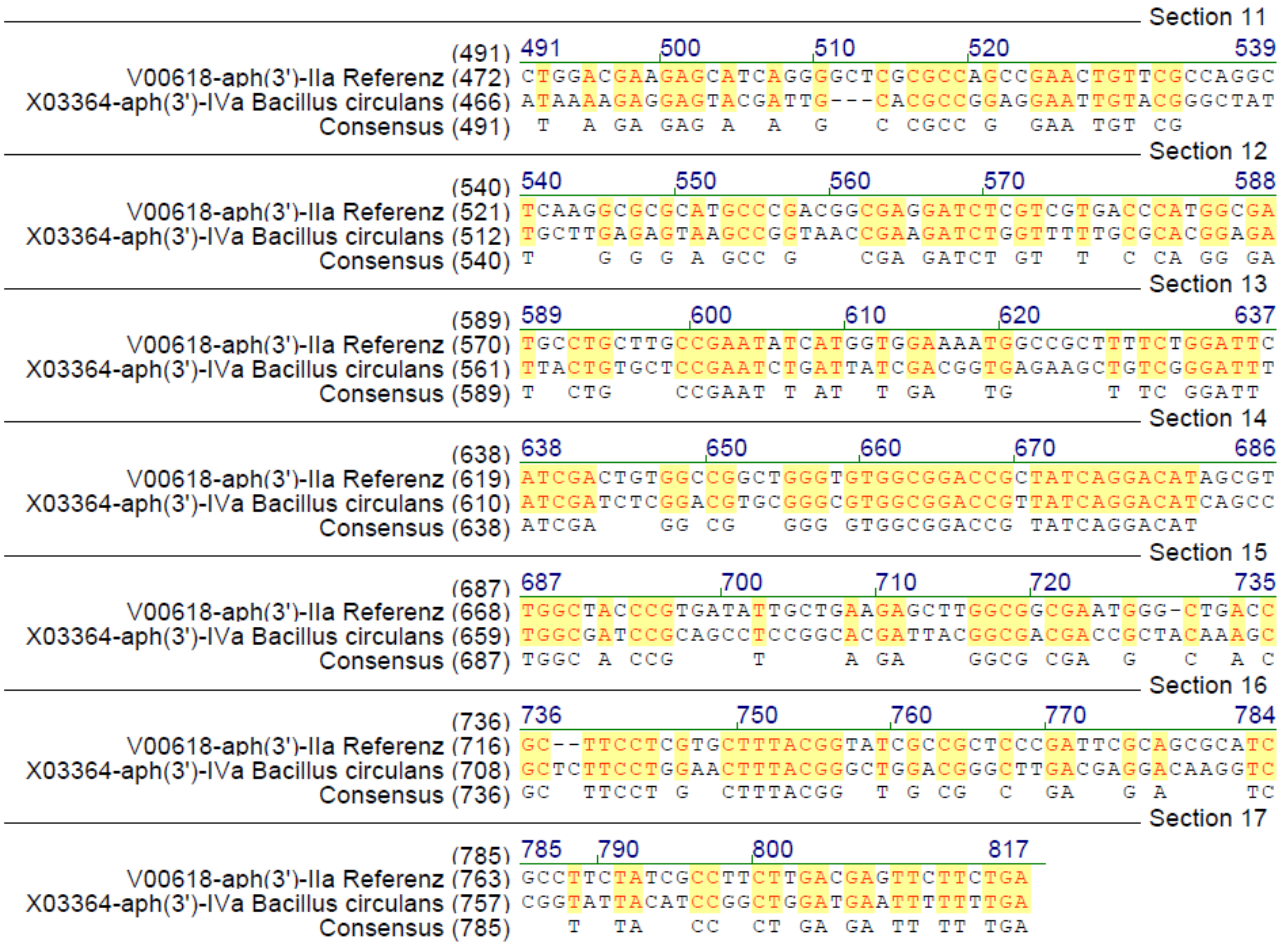

Figure 3: Dual alignment of aph(3')-IIa and aph(3')-IVa (continued).

(ClustalW analysis; VectorNTI 7.1)

1.4.4 Results of aph(3')-IIa dual alignments with other aminoglycoside phosphotransferases

The analysis results of the dual alignments are shown in Table 10. DNA regions with high DNA similarity that could potentially enable homologous recombination with parts of the aph(3')-IIa sequence are presented. No extensive stretches of 100% DNA sequence similarity to aph(3')-IIa were detected for the analysed aminoglycoside phosphotransferase genes. The detected contiguous stretches of similarity usually showed a maximum continual length of 7 – 9 bp. There were several locations showing extended microhomologies. These regions are also indicated in Table 10 because they may serve as targets for illegitimate recombination; a process taking place with substantial lower frequency compared to homologous recombination (79).

Aminoglycoside phosphotransferase	Homologous region with aph(3')-IIa 5' → 3'[1)]	Position according to pdf-file[2)]	Comment
aph(2'')-Ia	GACTGGGCACAACAGACAAT	334 - 354	microhomology: 20 bp; identity:13 bp interesting region
aph(2'')-Ie	GCTATGACTG	93 - 103	microhomology: 10 bp; identity: 9 bp 3' block of homology appears with several other aph's (positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa homologous 3' block: 7 bp
	GGATGATCT	531 - 540	microhomology: 9 bp; identity: 8 bp homologous 5' block: 7 bp
aph(2'')-IIa	GAAGAGCTT	785 - 794	microhomology: 9 bp; identity: 8 bp homologous 3' block: 7 bp
aph(2'')-IIIa	GCCGCTTGG	77 - 84	microhomology: 9 bp; identity: 8 bp homologous 3' block: 7 bp
	TCCTCGTGCTTTACGGT--ATC	798 - 830	microhomology: 20 bp; identity: 15 bp interesting region
aph(2'')-IVa	GCTATGACTGG---GCACA	92 - 108	microhomology: 16 bp; identity: 13 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa; see aph(2'')-Ie
aph(3')-Ia	GGCTATGACTGGGCAC	75 - 88	microhomology: 16 bp; identity: 12 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa; see aph(2'')-Ie
	TTCTTGACGAGTTCTTCTGA	814 - 833	microhomology: 20 bp; identity: 15 bp 3' end of gene

aph(3’)-Ib	TATTCGGCTATGACTGGGCAC	69 - 89	microhomology: 21 bp; identity: 13 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3’)-IIa; see aph(2’’)-Ie
	TGGCTGGC	222 - 229	homologous block: 8 bp
	ATGCGGCGGCTGCATACGCT	387 - 406	microhomology: 20 bp; identity: 16 bp
	TTCGACCACCAAGCGAAACATCGCAT	426 - 452	microhomology: 26 bp; identity: 17 bp
	GCCGAAC	536 - 543	homologous block: 7 bp
	GTCGTGACCCATGGCGAT	592 - 608	microhomology: 18 bp; identity: 16 bp
	GGTGTGGCGGACCGCTATCAGGACATAGC	675 - 704	microhomology: 29 bp; identity: 20 bp
aph(3’)-Ic	TTCTTGACGAGTTCTTCTGA	814 - 833	microhomology: 20 bp; identity: 16 bp 3’ end of gene
aph(3’)-IIa	100%		
aph(3’)-IIb	GGGACTGGCTGCT	271-283	microhomology: 13 bp; identity: 11 bp
	TGGGCGAAGTGCCGGGG	286-303	microhomology: 17 bp; identity: 14 bp
	TGCGGCGGCTGCA	376-388	microhomology: 13 bp; identity: 12 bp
	CTTGATCCGGC	393-403	microhomology: 11 bp; identity: 10 bp
	GGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCG	631-668	microhomology: 38 bp; identity: 30 bp
	CTTCCTCGT	735-744	homologous block: 9 bp
	TTGACGAGTTCTTCT	797-811	microhomology: 15 bp; identity: 14 bp
aph(3’)-IIIa	AATATCATGGTGGAAAATGGC	611 – 832	microhomology: 21 bp; identity: 16 bp
	GGCGGAC	670 – 677	homologous block: 7 bp
aph(3’)-IVa	CAGGGGC	128 – 135	homologous block: 7 bp
	TGGCTGG	199 – 216	homologous block: 7 bp
	GGGTGTGGCGGACCGCTATCAGGACAT	655 – 682	microhomology: 27 bp; identity: 25 bp interesting region
	CTTTACGG	749 – 757	homologous block: 8 bp
	CTTGACGAGTTCTTCTGA	800 – 817	microhomology: 18 bp; identity: 13 bp 3’ end of gene
aph(3’)-Va	CTGCCCATTCGACC	415 – 429	microhomology: 14 bp; identity: 12 bp
	CCATGGCGA	591 – 599	homologous block: 9 bp
	TCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA	809 – 843	microhomology: 35 bp; identity: 30 bp 3’ end of gene interesting region
aph(3’)-Vb	TGCCCATTCGACCACC	406 – 423	microhomology: 16 bp; identity: 14 bp
	CCATGGCGA	477 – 586	homologous block: 9 bp
	GCTGACCGCTTCCTCG	746 –762	microhomology: 16 bp; identity: 14 bp
	ATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA		microhomology: 36 bp; identity: 28 bp 3’ end of gene interesting region
aph(3’)-Vc	GAGGATCTCGTCGT	562-575	microhomology: 14 bp; identity: 12 bp

	ACCGCTTCCTCG	753-764	microhomology: 12 bp; identity: 10 bp
	CTTCTTGACGAGTTCTTCT	815-834	microhomology: 19 bp; identity: 16 bp 3' end of gene interesting region
aph(3')-VIa	CCCATGGCGAT	581 - 590	microhomology: 11 bp; identity: 10 bp
	TGGCCG-GCTGGGTGTGGCGGA	646 - 667	microhomology: 22 bp; identity: 18 bp
aph(3')-VIIa	ACCTGCCCATT	393 - 404	microhomology: 11 bp; identity: 10 bp
aph(3')-VIIIa	TGGCTGGC	222-230	homologous block: 8 bp
	ATGCGGCGGCTGCATACGCT	387-407	microhomology: 20 bp; identity: 16 bp
	GCCGAACTG-TTCG	536-549	microhomology: 14 bp; identity: 11 bp
	GTCGTGACCCATGGCGAT	591-608	microhomology: 18 bp; identity: 16 bp
	GGATTCATCGACTGTGGCCGG	651-662	microhomology: 21 bp; identity: 16 bp
aph(3'')-Ia	CGGCTA-TGACTGGGC	60 - 75	microhomology: 16 bp; identity: 12 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa; see aph(2'')-Ie
	GCTGATGCAATGCGGCGGCTGCA	369 - 381	microhomology: 23 bp; identity: 17 bp
	GCCCGACGGCG-----AGGA	545 - 565	microhomology: 20 bp; identity: 15 bp
	TTTCTGGATTCATCGAC	640 - 656	microhomology: 17 bp; identity: 14 bp
	CGTGCTTTACGGTATCGCC	770 - 789	microhomology: 19 bp; identity: 16 bp
aph(3'')-Ib	TGACTGG	66 - 72	homologous block: 7 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa; see aph(2'')-Ie
	CCATGGCGATGCCTGCTTGCCGAATATCATGGTGGA	585 - 620	microhomology: 36 bp; identity: 31 bp interesting region
	AGCGCATCGCCTTCTATC	804 - 820	microhomology: 18 bp; identity: 15 bp
aph(4)-Ia	GCCCATTCG	421 - 429	homologous block: 9 bp
	GGAAAATGGCCGCTT	647 - 661	microhomology: 15 bp; identity: 13 bp
aph(4)-Ib	AGGCTATTCGGCTATGACTGG	199 - 219	microhomology: 21 bp; identity: 16 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa; see aph(2'')-Ie
	GCGAAGT--GCCGGG	441 - 455	microhomology: 15 bp; identity: 12 bp
aph(6)-Ia	CGGCTGCA	433 - 440	homologous block: 8 bp
aph(6)-Ib	TTCGGCTATGACTG---GGCA	61 - 81	microhomology: 21 bp; identity: 14 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa; see aph(2'')-Ie
	GCCGAACTGTTCGCC	561 -575	microhomology: 15 bp; identity: 13 bp
	GCTGACCG	776 - 783	homologous block: 8 bp
aph(6)-Ic	?		no obvious region of microhomology
aph(6)-Id	?		no obvious regions of microhomology
aph(9)-Ia	TGTGCTCGACGTTGTCAC	313 - 335	microhomology: 18 bp; identity: 12 bp

aph(9)-Ib	CGGCTATGACTGGGCACAACA	108 - 128	microhomology: 21 bp; identity: 17 bp region positioned around nucleotide 70 according to the numbering scheme of V00618 on aph(3')-IIa; see aph(2'')-Ie

Table 10: Results: dual alignments of aph(3')-IIa with other aminoglycoside phosphotransferases.

1) Only identical DNA stretches of a length of 7 bp (or larger) or otherwise noticeable sequences are reported. Identical nucleotides between query and subject sequence are depicted in red.

2) See specific aph designation (which was aligned to aph(3')-IIa) in title of the corresponding pdf-files. Files are available upon request.

1.4.5 Structure – activity conservation in the gene sequence of nptII

A detailed analysis of the nptII gene sequence reveals that important regions (i. e. sequences coding for the ATP binding, the antibiotic binding, and the active catalytic site of the enzyme) are broadly dispersed over the 795 bp of the whole gene (Figure 4). Random replacement of sequences by DNA fragments of 50 to 100 bp (and longer) most certainly lead to severe distortions in secondary and tertiary structure of the amino acid chain resulting in a non-functional enzyme. NptII genes with random indels will therefore most probably be non-functional and eliminated from the population over time.

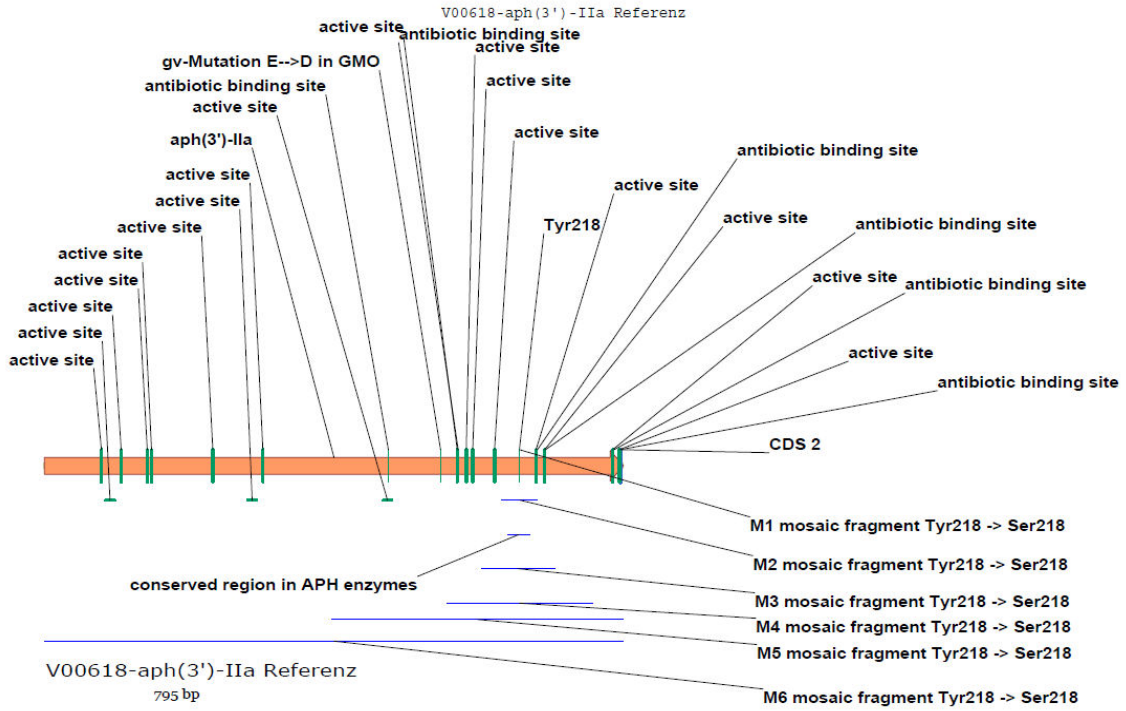

Figure 4: Important DNA sequence regions in aph(3')-IIa.

M1 – M6 are artificially designed DNA fragments containing a mutation which leads to an alteration of the substrate specificity of NptII (image generated by VectorNTI 7.1).

1.4.6 Conclusions of the *in silico* analyses

NptII gene sequences show no extended regions of DNA similarity with other aminoglycoside phosphotransferase genes. The identified contiguous stretches of identical sequence are extremely short. This observation indicates that homologous recombination between nptII and the other aminoglycoside phosphotransferase genes under investigation will not be the primary route for the exchange of DNA fragments and sequence evolution. However, a remaining possibility for exchange of DNA sequences between aminoglycoside phosphotransferase genes is illegitimate recombination. This is because short sequence stretches of microhomology have been identified which may serve as anchors for recombining DNA fragments. Nevertheless, sequence alignments using standard parameters of the BLAST algorithm did not reveal the presence of nptII mosaic genes of natural origin in GenBank sequence entries. Functional nucleotide segments crucial for the formation of the active catalytic site were present over the entire nptII gene. The opportunities for exchange of gene fragments will therefore be limited to sequence conserved variants of the resistance gene for enzymatic activity to be retained after the formation of mosaic structures.

In conclusion, the data retrieved from the *in silico* analysis available at the time of query do not provide substantial support to the hypothesis of an involvement of the nptII gene in the formation of mosaic genes with altered resistance patterns within the family of aminoglycoside phosphotransferases.

1.5 Experimental design

1.5.1 Examination of the interactions between aph(3')-IIa fragments with other aminoglycoside phosphotransferase genes encoded in potential bacterial acceptor strains

An experimental approach for testing the potential of aph(3')-IIa to generate mosaic gene formation in related phosphotransferase genes was developed. The aph(3')-IIa gene was used as donor DNA exposed to naturally transformable *Acinetobacter baylyi* cells harbouring different aminoglycoside phosphotransferase genes.

The hypothesis that aminoglycoside phosphotransferase genes may serve as aph(3')-IIa fragment acceptors was further investigated as follows: Two aminoglycoside phosphotransferase genes containing long stretches of sequence similarity with aph(3')-IIa were transformed into *A. baylyi* generating two different *A. baylyi* strains with specific aminoglycoside resistance profiles. A donor DNA vector containing the nptII wild type sequence plus an *A. baylyi* homologous anchor region was subsequently constructed. The donor DNA vector was used in natural transformation assays. If DNA uptake and recombination occurred, the transformed bacteria were expected to be identifiable due to significant changes in the minimum inhibitory concentration (MIC) or an altered resistance profile to aminoglycosides. Any transformants (i.e. growing colonies on selective agar showing MIC changes or an altered resistance profile) would then be subject to PCR of relevant genome regions for further characterization of mosaic formations.

This experimental outline was proposed by Prof. Dr. Kaare Nielsen and Sara Domingues. The practical execution of the experiments was performed under his supervision by a PhD student (Sara Domingues) in Norway and Portugal. Some theoretical background information for the design of the experiments was supplied by the Austrian collaborators (see section 1.4.3; Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, Table 7, Table 8, Table 9, and Table 10). A typical alignment between aph(3')-IIa and a related aminoglycoside phosphotransferase (e.g. aph(3')-IVa) is shown in Figure 2 and Figure 3). This dual alignment check with nptII was performed with all aminoglycoside phosphotransferase genes represented in Table 10. The results of the alignments are presented in section 1.4.4.

The aph(3')-IIa, or nptII, aminoglycoside-modifying enzyme has a similarity <61 % to the other aph enzymes at the amino acid level (85) and an identity <57,7% in terms of nucleotide sequence as compared by VectorNTI 7.1 analysis. Only microhomologies were detected between the nptII (GenBank # V00618) and the other aph genes. Based on the microhomologies, four aph genes were chosen: the aph(3')-Va (GenBank # K00432), the aph(3'')-Ib (GenBank # M28829), the aph(4)-Ib (GenBank # X03615) and the aph(6)-Ia (GenBank # Y00459). The aph(3'')-Ib has 49.4% of overall nucleotide identity with nptII, one homologous block with 7 bp and two microhomology blocks with 36 bp (with 31 bp identical) and 18 bp (with 15 bp identical); the aph(3')-Va has 50.2% of overall nucleotide identity with nptII, one homologous block with 9 bp and two microhomology blocks of 35 bp (with 30 bp identical) and 14 bp (with 12 bp identical); the aph(4)-Ib has 40.9% of overall nucleotide identity with nptII, two microhomology blocks with 21 bp (with 16 bp identical) and 15 bp (with 12 bp identical); the aph(6)-Ia has 42.2% of overall nucleotide identity with nptII and one homologous block of 8 bp.

Two of the genes were ordered from a commercial supplier with their natural promoter, the aph(3')-Va and the aph(3'')-Ib (called aph variants below). As these genes are not naturally isolated from *A. baylyi*, it was considered that a stronger promoter could be needed to detect expression of the genes in *A. baylyi*.

Details on the practical experimentation with these genes can be found in section 1.5.2.

1.5.2 Constructs for capturing hybrid aminoglycoside resistance genes by natural transformation

According to de Vries and Wackernagel, short stretches of sequence identity (3–8 bp) between donor and recipient DNA is enough for illegitimate recombination events in *Acinetobacter baylyi* BD413, if there is one homologous region that can serve as anchor (21). The experimental plan was to insert the two different aph type genes into a chromosomal location in the *A. baylyi* strain BD413 genome, and then transform this bacterium with a nptII gene also containing a homologous region (anchor) of the gene adjacent to where the aph was inserted. A recombination between the aph of the recipient bacterium and the nptII gene containing donor DNA was expected to lead to changes in the antibiotic resistance profile and/or minimum inhibitory concentrations (MIC) that could be revealed on antibiotic-containing agar-plates.

The two genes *aph(3')-Va* (no resistance to any of the available antibiotics) and *aph(3'')-Ib* (streptomycin resistant) with natural promoters were cloned into pTM4 and the resulting vectors were named pTM4-AR1 and pTM4-AR2, respectively.

a) DNA constructs to be inserted in the recipient A. baylyi genome:

1. The aph gene variants were each inserted in the vector pTM4 (which has two homologous regions with a *A. baylyi* BD413 gene):

- vector: pTM4, Ecl136II-linearized (Figure 5)

- insert: de novo-synthesized aph variant genes, recovered from a vector as EcoRV- fragments

The aph variants (aph(3')-Va and aph(3'')-Ib) with natural promoters were ordered as DNA sequences from a commercial supplier.

Vector pTM4-AR1 was introduced into *E. coli* EC100 and pTM4-AR2 into *E. coli* DH5α by chemical transformation. Vectors were then extracted with a Qiaprep Spin Miniprep kit (Qiagen, Germany).

2. The aph variants were subsequently inserted by natural transformation of *A. baylyi* BD413 by the plasmids constructed in step 1. Double homologous recombination occurring at the flanking regions ensured additive integration of the resistance gene.

A. baylyi BD413 (ACIAD3309::nptII-sacB) was naturally transformed (liquid transformation) with pTM4-AR1 and pTM4-AR2 and selection was done in LB with sucrose (50 g/L) and streptomycin (20 µg/mL), respectively.

Although the *aph(3')-Va* gene does not confer resistance to any of the available antibiotics in the performing laboratory, the acquisition of the fragment into *A. baylyi* BD413 (containing a nptII-sacB segment into the ACIAD3309 gene) can be selected by resistance to sucrose (50 g/L) and loss of kanamycin resistance (10 µg/mL) (the gene *aph(3')-Va* substitutes the nptII-sacB).

Obtained transformants from transformation of *A. baylyi* BD413 (ACIAD3309::nptII-sacB) by pTM4-AR1 and pTM4-AR2 were tested to determine the MIC for kanamycin by Etest (Biomerieux, France).

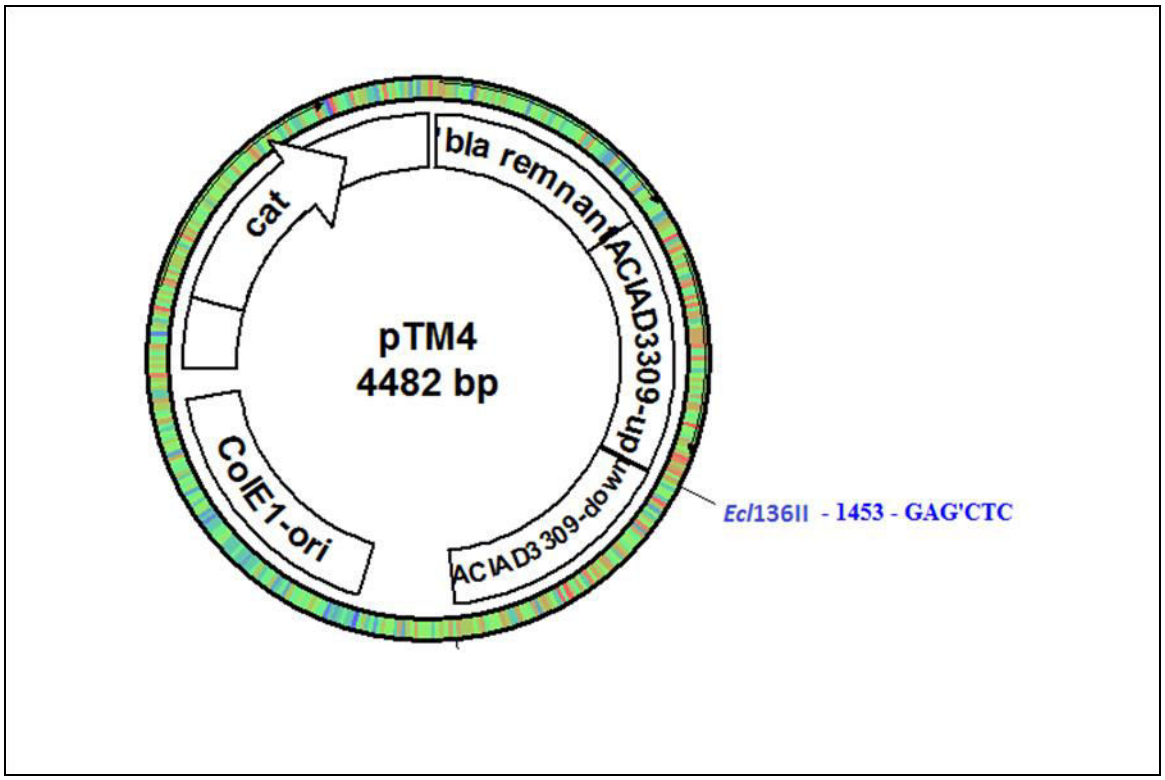

Figure 5: Cloning vector pTM4 containing *A. baylyi* homologous regions (=ACIAD)

b) DNA construct to be used as Donor DNA

1. The nptII gene was inserted in a plasmid vector downstream of an anchor region with sequence homology to the genomic DNA flanking the site where the aph variants were inserted in the *A. baylyi* genome (Figure 6).

- vector: pTM4, digested with Ecl136II and KspAI (HpaI) (excision of a homologous flank with the *A. baylyi* BD413 genome; only one side with homology).

- insert: pBlue-Km1, 1.5-kbp HincII-fragment (containing nptII) (Figure 7)

The resulting construct, pSDKH1, was introduced into *E. coli* EC100 by chemical transformation and selected with kanamycin (25 µg/mL). pSDKH1 was extracted with a Qiaprep Spin Miniprep kit (Qiagen, Germany).

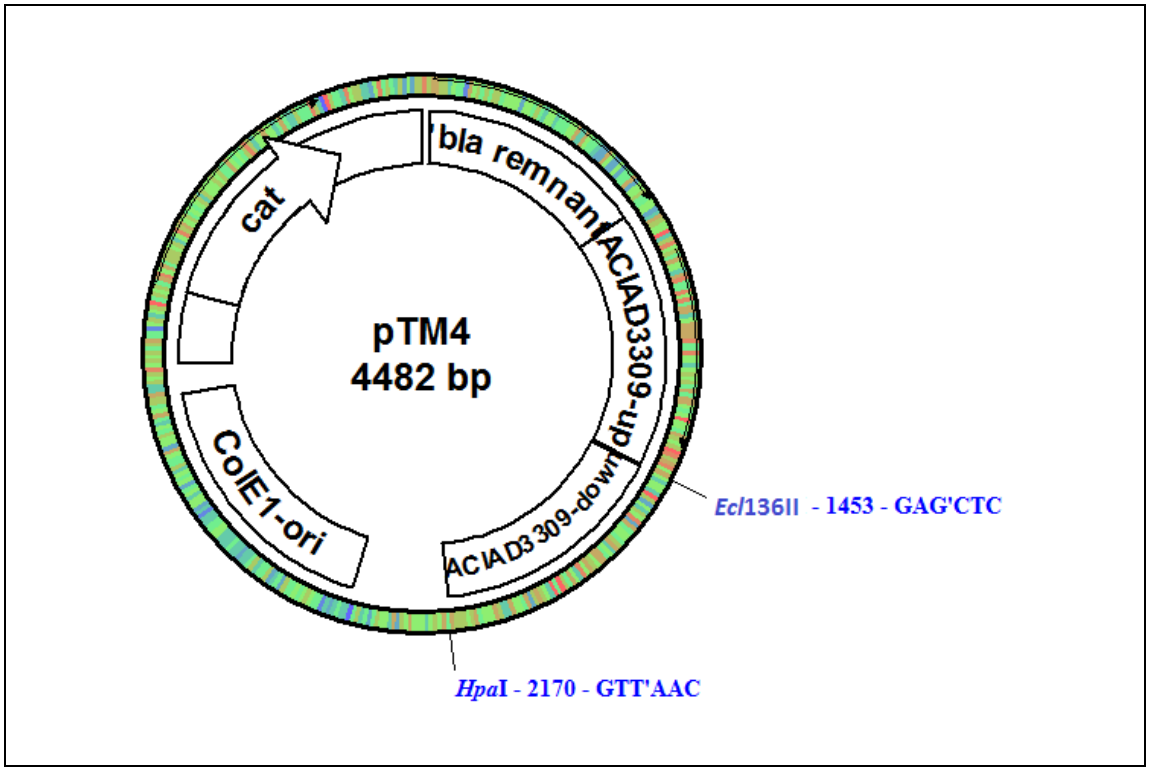

Figure 6: Excision of downstream *A. baylyi* ACIAD fragment from pTM4

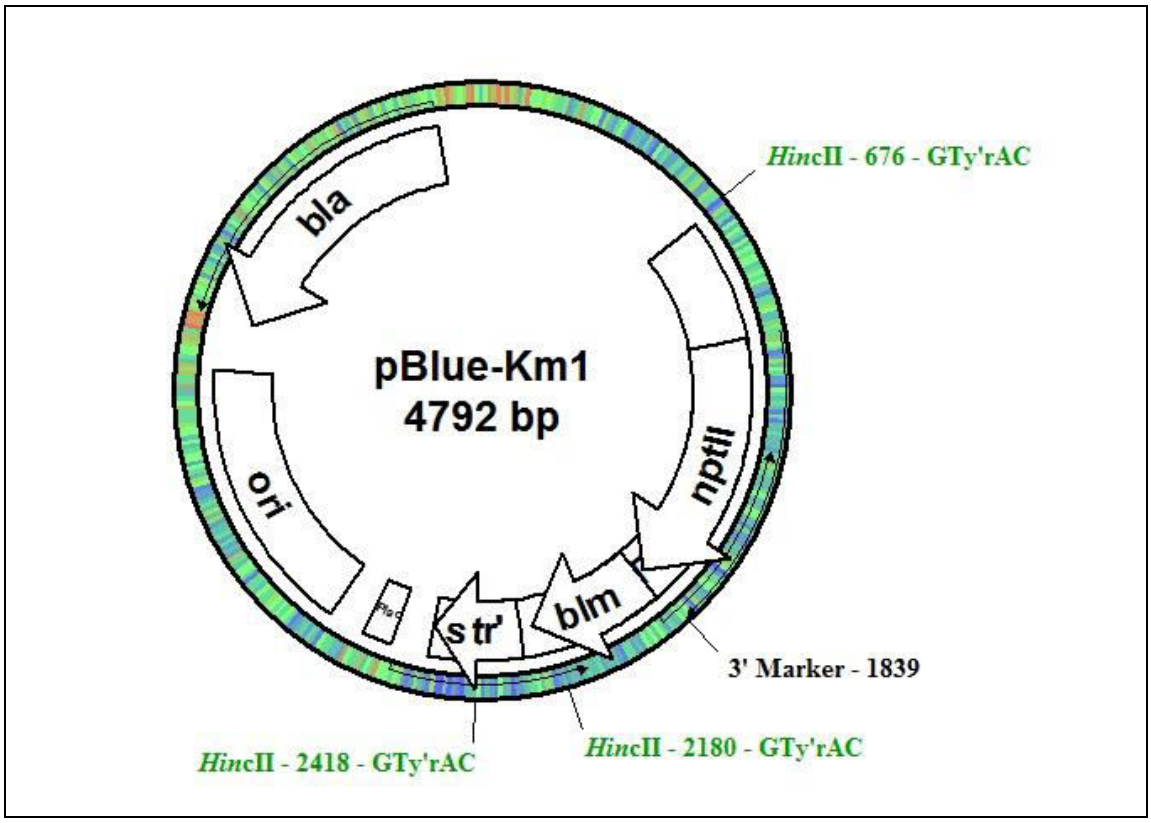

Figure 7: Donor vector for nptII donor DNA construction

Resulting construct, used as donor DNA in natural transformation experiments (Figure 8):

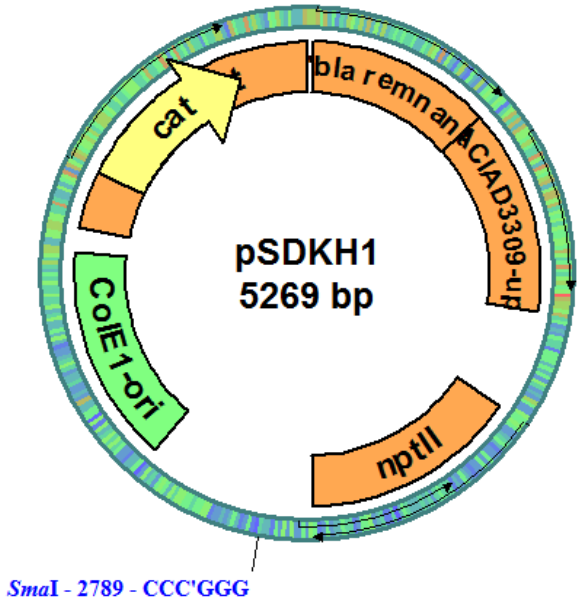

Figure 8: Resulting insertion of nptII into plasmid pTM4 (replacement of downstream ACIAD sequence)

c) Natural transformation of *Acinetobacter baylyi* BD413 strains with genomic inserted aph variants and nptII donor DNA

pSDKH1 was digested with *Sma*I and used as donor DNA in (liquid) transformation of *A. baylyi* containing the *aph* gene variant. Selection of the transformants was done in LB with kanamycin 5, 10 and 25 µg/mL. A negative control, where water replaced the DNA in the transformation assay, was also done and plated in the three different concentrations of antibiotic.

The transformation frequency was calculated and is given as the number of transformants divided by the number of viable recipient cells:

Transformation frequency = no. of transformants / no. of recipients

Antimicrobial susceptibility testing

Transformants obtained from transformation with pSDKH1 as donor were tested by the disc diffusion method to determine the susceptibility to kanamycin (30 µg per disc) (AB Biodisk, Sweden). The donor and the recipient bacteria were also tested, so changes in the diameter of the zone of growth inhibition could be detected.

1.6 Execution of Mosaic Gene Experiments

1.6.1 Results

a) Construction of *Acinetobacter baylyi* containing *aph* genes

Transformation with pTM4-AR1 (containing the *aph(3')-Va* gene) produced several transformants; with pTM4-AR2 (containing the *aph(3'')-Ib* gene) there were no transformants, which might be due to the relatively high concentration of streptomycin used (20 μg/mL) or due to the (weak) natural promoter.

The MIC for kanamycin of the transformants obtained with pTM4-AR1 were obtained by E-test. All of them showed a MIC of 1.5 μg/mL. One of the transformants containing the *aph(3')-Va* gene was chosen to be used as recipient strain in further transformation assays and was named *A. baylyi* AR1.

b) Natural transformation of *Acinetobacter baylyi* AR1

Natural transformation of the recipient *A. baylyi* AR1 (ACIAD3309::*aph(3')-Va*) with the linearized pSDKH1 produced transformants selected in LB with kanamycin 5 (n=2) and 10 μg/mL (n=4), but no transformants were acquired in LB with kanamycin 25 μg/mL (n=2). There were no CFUs in the negative controls.

Selection	Mean no. of transformants(CFU)	Mean no. of recipients (CFU)	Transformants per recipients
kanamycin 5 μg/mL	1×10^{2}	4.4×10^{7}	2.3×10^{-6}
kanamycin 10 μg/mL	8.8×10^{0}	1.8×10^{7}	4.9×10^{-7}

Table 11. Selection of kanamycin resistant transformants

c) Antimicrobial susceptibility of the transformants

The antimicrobial susceptibility profile of the donor bacterium, containing the plasmid pSDKH1, the recipient *A. baylyi* AR1 and selected transformants are shown in the following table:

Isolate	Kanamycin (mm)
EC100 pSDKH1	0
AR1	30
Transformant 1	15
Transformant 2	15
Transformant 3	15
Transformant 4	16
Transformant 5	14
Transformant 6	14
Transformant 7	15
Transformant 8	14
Transformant 9	16
Transformant 10	15
Transformant 11	14
Transformant 12	14
Transformant 13	15
Transformant 14	14
Transformant 15	16
Transformant 16	15
Transformant 17	15
Transformant 18	14

Table 12. Kanamycin suceptibility of transformants: disk diffusion test

Transformants 1-12 were selected in LB with kanamycin 10 μg/mL and 13-18 in LB with kanamycin 5 μg/mL.

1.6.2 Discussion and summary

In the natural transformation of *A. baylyi* containing the *aph(3')-Va* gene by DNA containing the *ntpII* gene, transformants with altered susceptibility to kanamycin were obtained. This means that recombination has likely occurred between the two genes, as it would be the case with the formation of mosaic genes. Selection with lower antibiotic concentrations produced a higher transformation frequency; this higher frequency did not affect the antimicrobial susceptibility of the transformants. Diameters of the inhibition zone around kanamycin containing discs retrieved from the transformants were close to the clinical breakpoint set by CLSI for resistant Salmonella strains (≤ 13 mm). Compared to the sensitive *A. baylyi* AR1 strain (= 30 mm) the inhibition zone diameters of the transformants show a significant reduction (14 – 16 mm).

The obtained phenotypical data indicate the induction of an increase in resistance to kanamycin after uptake of nptII containing DNA molecules, implicating mosaic structures to form in the involved genes subject to the recommendations suggested in section 1.6.3. However, sequencing of the putative target regions could not confirm the formation of gene mosaics.

1.6.3 Recommendations for further research

The nature and the location of the recombination events leading to reduced susceptibility to kanamycin in the obtained *A. baylyi* transformants should be clarified. Furthermore, the region located inside the ACIAD3309 gene of *A. baylyi* should be amplified and sequenced to elucidate the sequence changes and possible sites of recombination between the *aph(3')-Va* and the *ntpII* genes. The involved regions should be cloned to understand if the changes in kanamycin susceptibility were exclusively due to the recombination between the two genes, and more detailed information could be gained from retransformation of *A. baylyi* with a DNA fragment containing the *aph(3'')-Ib* gene under the control of a strong promoter or - alternatively – using lower concentrations of streptomycin for selection to increase the expression of the gene in *A. baylyi*.

2 Antibiotic selection in soil habitats

In this chapter the effects of antimicrobial compounds on soil bacterial populations are discussed. The focus is put on the behaviour, persistence, transport and detectability of antibiotics in soil environments. The basic framework of and the data presented below are retrieved from Boxall et al. (8) and Janzon et al. (55) if not otherwise stated.

2.1 Exposure of soil to antibiotics: routes of input

After administration of antibiotics to humans or animals for the treatment or prophylaxis of infectious diseases the compounds may be either absorbed or metabolized. In many instances antibiotics and their metabolites are excreted again in their active state. Soil environments may be exposed to antibiotics either directly via the excretion of faeces and urine by grazing animals or by dispersion of animal manure or sewage sludge used as fertilizer to fields (9, 11, 13, 42, 43, 83). Contaminated manure is especially a problem with intensely reared farm animals producing large amounts of farmyard manure, slurry and litter which is usually disposed subsequently in large quantities onto surrounding farmland (11). Typical exposure routes of antibiotics used in human and veterinary medicine are displayed in Figure 9. It is important to note that disposal of sludge from sewage treatment plants onto fields is restricted in Austria (prohibited: Salzburg, Tyrol; other federal states: allowed if amount of certain contaminants does not exceed a certain threshold; http://www.ris.bka.gv.at/Ergebnis.wxe?Abfrage=Lgbl&Lgblnummer=80/2001&Bundesland=Salzburg http://www.ris.bka.gv.at/GeltendeFassung.wxe?Abfrage=LrT&Gesetzesnummer=20000058 http://www.ris.bka.gv.at/GeltendeFassung.wxe?Abfrage=LROO&Gesetzesnummer=20000418) although sludge is used as fertilizer in other countries (8).

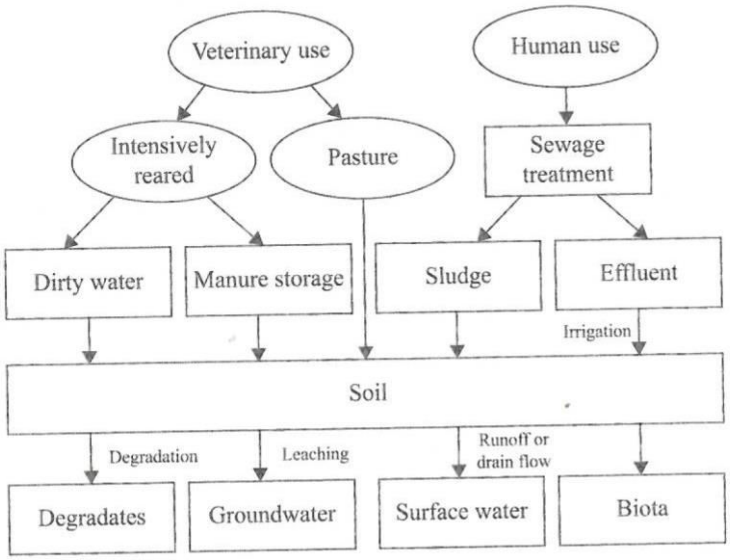

Figure 9: Exposure routes of antibiotics for soil environments.

Data from Boxall et al. (8).

Veterinary antibiotics may be degraded during storage of manure but many antimicrobial compounds are stable in these environments. Antibiotics used for human therapy are released into the public wastewater treatment systems. The resulting sewage is treated before disposal to surface waters. During the activated sludge treatment process some antibiotics may be adsorbed to particulate matter, which is separated from the liquid phase and subsequently used as fertilizer (69). Water soluble antibiotics not adsorbed to particles may reach the soil environment if treated waste water is used for irrigation (Figure 9) (90).

2.2 Detection of antibiotics in soil environments

There is plenty of information available about the occurrence of antibiotics in aquatic systems especially in surface waters (71). Considerably less studies have been performed concerning the amounts of antimicrobials is soils. In Europe the following classes of antibiotics have been in focus of some studies in soils: tetracyclines, sulfonamides, fluoroquinolones, macrolides, and diaminopyrimidines (33, 53, 68, 99). The acquisition of data concerning environmental concentrations of kanamycin was not a primary objective of the research community. The range of detection for maximum amounts of the five substances mentioned above (except for kanamycin, where no quantitative data concerning naturally occurring incidence levels were reported) was usually between 10 – 100 µg/kg soil. An Austrian study detected in pig manure up to 46 mg/kg chlortetracycline, 29 mg/kg oxytetracycline and 23 mg/kg tetracycline (68). Sulfadimidine in pig manure and sulfadiazine in chicken and turkey dung were detected in significant amounts (up to 20 and 91 mg/kg, respectively). Enrofloxacin was particularly observed in chicken and turkey samples. Chlortetracycline, enrofloxacin, and ciprofloxacin were detectable in soils. The samples were not tested for the presence of aminoglycosides (i.e. kanamycin) (68). Most analyses to date have been performed with antibiotics of veterinary origin although some quantitative data are available from testing soils after sludge application or after irrigation with waste water (37, 61). The impact of human antibiotic usage on antimicrobial concentrations in soils appears to be of less significance compared to the application of antibiotics in veterinary medicine.

2.3 Behaviour of antibiotics in soils

Antimicrobials in natural soil environments may either persist, degrade, be transported by drainage into surface or ground waters, or may be incorporated by living organisms. The way of action is determined by the physical properties of the antimicrobial. The most important parameters are: water solubility, lipophilicity, volatility and sorption potential.

2.3.1 Adsorption

The adsorption capacity of antibiotics to soil particles is dependent on the soil type and varies widely over several orders of magnitude concerning the reported sorption coefficients (K_d). The lowest K_d in soil was reported for sulfamethoxazol (0.23) (25), the highest for tylosin (range: 5.4 – 66.900) (52). This large variability is caused by the ionic nature of most antibiotics which are usually ionisable compounds with pKa values in the pH range of natural soils and, thus, may occur as negative, neutral, zwitterionic and positively charged molecules (88). Each of these molecule species may interact differently with soil particles. The sorption capacity of antibiotics is strongly influenced by the pH, cationic exchange capacity, organic carbon content and the nature of the organic carbon in soil (57, 58, 84, 87, 89). Metal oxide content and ionic strength may also play a certain role in this issue. It should be noted that most studies have checked the distribution of antibiotics between soil and water phase, only. However, the sorption capacity may be substantially influenced by the presence

of manure or sludge. It was found that the addition of sewage sludge generally decreases the sorption of sulfonamides (and other pharmaceuticals) (91).

2.3.2 Persistence

Antibiotics are usually catabolized in soils via aerobic biodegradation, although photo degradation may also play a certain role (97). The most important factors are temperature, pH and residing bacteria with catabolic potential (e.g. resistant bacteria) (54). The half-life of antibiotics in soils also depends on the chemical class of the compound. The longest half-life was reported for enrofloxacin (696 days) (8), sulfachloropyrazidine was most easily eliminated from the soil environment (2.8 days) (10). The presence of sewage sludge may either increase or decrease the persistence of antimicrobials in soils (96). Degradation of antibiotics may results in degradation products possibly of more concern than the original substance because they may have a greater toxicity, are more persistent or mobile (14). Therefore the degradation product should be also in the focus of risk assessment concerning soil persistence of antibiotics. Evidence is cumulating that disappearance of antibiotics from soil environments is not mediated by the degradation of the compound but by irreversible binding of the antibiotic to soil particles (46). This may lead to persistence of several years of the compounds (29). It is not clear whether these entrapped antimicrobials are accessible to soil organisms or may be released into the ecosystem in future (46).

2.3.3 Transport

Antibiotics can be leached out from soil and transferred to aquatic systems by drain flow, surface runoff or subsurface flow. The major factors which influence the extent of transport are determined by solubility, sorption behaviour, and persistence of the antibiotic. Physical structure, pH, organic carbon content, and cation exchange capacity of the soil matrix play a role as well as climatic conditions (including rainfall and temperature).

In semifield lysimeter experiments sulfachloropyridazine was found to leach from the soil matrix, rainfall and manure did not show a substantial effect on leaching (5). Oxytetracycline and tylosin tested during the same experiment appeared to be immobile. It was observed that the currently applied simulations for risk assessment based upon leaching models may greatly underestimate the actual transport of antibiotics to groundwater resources. However, during a large monitoring study in Germany where an extensive number of groundwater samples from areas with intense animal husbandry have been taken, no antibiotics were present above the limit of detection (50).

Tetracyclines, sulfonamides, ionophores and macrolides have been observed to be transported by water in overland flow (20, 59, 63). Runoff of highly sorptive substances like tetracyclines is lower compared to the situation with sulfonamides (20, 60). Particle selected transport on surfaces may also be of an issue for tetracyclines and macrolides (20). The presence of manure and slurry is again of importance and has been shown to increase the transport of sulfonamides (15). Snow, agricultural practices and the nature of the field influence the transport of antibiotics via runoff.

The movement of oxytetracycline and sulfachloropyridazine by drain flow to surface waters has been studied in tile drained clay soils (26, 59). The concentration of the sulfonamide in the drain flow was again higher compared to the situation of tetracycline, but it was found that tillage could reduce the transport of both compounds in the drain flow in subsequent experiments. A monitoring study of surface waters from rivers which received drainage inputs from farmlands treated with antibiotic containing manure showed a minimum concentration of 0.02 µg/L and a maximum of 21 µg/L. Both concentrations are well below any clinically relevant minimum inhibitory concentration. However, the load of stream sediments was higher reaching levels up to 813 µg/kg (12).

2.3.4 Uptake into Biota

A number of plant species take up antibiotics (e.g. tetracyclines, sulfonamides, makrolides fluoroquinolones) from contaminated soils (12, 64). However the concentrations detected in plants are very low and possibly do not pose any risk to consumers (12). Data for other biota are scare. Earthworms do take up antibiotics from soils (46). Wildlife and the food chain may be also affected by antibiotics from soil because fluoroquinolones have been found in the eggs of vultures and red kites, although the exact way of uptake is not quite clear (67).

2.4 Antibiotics in soils: State - of - Play 2012

There has been a tremendous amount of data collected over the past decade concerning the fate of antibiotics in soil environments leading to a much better understanding concerning interactions, persistence and transport of antibiotics in soils. However several knowledge gaps remain which should be closed in future research projects:

1. Information on other antibiotics for human use and for veterinary applications besides tetracyclines, macrolides, fluoroquinolones and sulfonamides is lacking
2. Routine monitoring of antibiotics should be intensified
3. Better model systems for assessing the environmental exposure to antibiotic at field and scales
4. Focusing on the environmental risks of non-extractable residues of antibiotics
5. Better assessment of the uptake of soil borne antibiotics by terrestrial organisms and into the food chain

2.5 Effects of antibiotics on soil organisms - selection

There is not much data available concerning exposure of bacterial soils populations with kanamycin residues. In an attempt to test the effect of kanamycin on the population dynamics of a Tn5 carrying *Pseudomonas fluorescens* strain two kanamycin concentrations (18 µg/g and 180 µg/g dry soil) were applied in soil (75). No effect was observed on the non-resistant parent or on the transformed Tn5 carrier strain.

A general analysis of the effects of antibiotics on soil bacteria is presented below:

The soil microbial community structure is suspected to be influenced by veterinary antibiotics used in animal husbandry. Manure was shown to contain antibiotic resistant bacteria and antibiotic residues (47, 65). These residues may influence soil bacterial communities if manure is spread on to agricultural soils (42, 47). Selection pressure may be induced and promoted by manure application (47). In soil microcosm studies it could be shown that a single application of manure containing sulfadiazine significantly increased the absolute and relative amounts of the corresponding resistance genes. The effect of manure on the abundance of the resistance genes was detectable for at least two months after the application indicating a long term bioavailability of sulfadiazine (48, 49). A selective effect on the sul2 resistance gene became evident already at an antibiotic concentration of 150 ng/g sulfadiazine (47). Approximately 10% of the soil bacteria became resistant after repeated application of antibiotic containing manure (49). For a long time it was assumed that soil will rapidly degrade or dilute introduced antibiotics, but recent studies showed that already

subinhibitory concentrations of antibiotics impose alterations in the resistance status of the soil populations under exposure by fostering horizontal gene transfer and resistance gene expression. Selection of resistance genes is facilitated already at low antibiotic levels (62).

Recent studies have described that populations of soil bacteria contain a diversity of resistance determinants forming a “soil resistome”. This soil resistome contains a vast amount of transferable resistance genes (18, 19). This environmental resistome is hypothesized to constitute a reservoir of resistance genes that may spread to human pathogenic bacteria (3, 16). Application of antibiotic selection pressure supports the rapid development of multiresistant animal and human pathogens. Interestingly, acquired resistance genes appear to be remaining in the bacterial population even after withdrawal of the antibiotic compound and, consequently, the selection pressure (4, 82). There are indications that antibiotics at drug concentrations several hundred-fold below the minimum inhibitory concentrations of susceptible bacteria may select effectively for resistant bacteria. Low antibiotic concentrations found in natural environments are supposed to be important for the enrichment and maintenance of resistance in bacterial populations (39).

Changes in the taxonomic composition are to be expected according to the antibiotic prevailing in the environment. Changes in bacterial density and community structure could be shown in a model system of activated sludge (1, 2). However there are indications that the overall taxonomic composition remains quite robust even under high antibiotic concentrations as studies in the bacterial gut populations of humans have shown (22). Closely connected with antibiotic resistance genes are corresponding genetic mobility factors which mediate horizontal gene transfer and build their own “mobilome” (70). The type of mobility factor connected to the respective resistance element and its response to antibiotic selection is of crucial importance concerning their potential to be transferred to other bacteria. However, extensive data concerning mobility factors and their behaviour under antibiotic exposure are lacking.

In addition to the selection of antibiotic resistance genes antibiotic selection pressure may serve several different other functions. It may have profound effects on crucial ecosystem services like nitrification and degradation processes in general, but also anabolic mechanisms like nitrogen fixation may be negatively affected (23, 38, 66). Community level changes have been observed including the enrichment of sulphate reducers after ciprofloxacin exposure of a salt marsh environment (17). Density and diversity of local bacterial communities are of importance when assessing potential risks for the ecosystem or for human health. A high bacterial density may increase the number of potential contacts for horizontal gene transfer. On the other hand a high bacterial density may also result in a more rapid degradation of antibiotics reducing the selection pressure again. A greater diversity may serve as a larger reservoir of potentially different resistance genes (19). Environmental bacterial communities contaminated by animal or human bacteria from sewage may share resistance factors from their gene pool with human or animal commensals or even pathogens. Such exchanges with environmental bacteria may also be conceived if ingested and coming into contact with the intestinal microflora of humans and animals. If this humans or animals are under selection pressure (= antibiotic therapy) recruitment of resistance factors from the environmental resistome is likely to be increased. Time is also an important factor to be taken into consideration: different systems and locations are exposed to antibiotics for different periods of time. Antimicrobials have significantly different acute and chronic toxicity profiles (30, 66, 92). Individual human antibiotic therapy usually last only for a few days or weeks. There are seasonal concentration peaks of antibiotics in community wastewater systems corresponding to human antibiotic treatment (35, 36). Another important parameter to consider the effects of antibiotic selection pressure is the kind of mixture of antibiotics prevailing in the habitat. Only one or a few different antimicrobials are administered simultaneously during an individual human therapy. The situation is completely different in natural environments or wastewater treatment systems where a multitude of different antimicrobials and other stressors are present.

2.6 Analysis of Austrian sales data on antimicrobial agents in animal husbandry (31, 32)

To try to get an idea about the quantity of antibiotic compounds which may reach soil environments in Austria a current study dealing with the sales of antimicrobial agents for veterinary applications ("Antibiotikamengenströme in Österreich") was analysed. This was for the first time in Austria that such kind of data were collected and evaluated. The collected data do not provide information about individual exposure of certain animals or habitats with the compounds under investigation but gives an overview about the overall amounts of antimicrobials which have been circulated during the year 2010 for veterinary purposes in Austria.

Results:

The data on overall sales of the active ingredient from veterinary antimicrobials were collected according to the recommendations of ESVAC (European surveillance of veterinary antimicrobial consumption) a consortium founded by EMA (European Medicine Agency). Twelve pharmaceutical companies producing or importing veterinary medical products and 6 wholesalers which only distribute the product provided their sales data for 2010. The sales figure was divided by the estimated weight of livestock and of slaughter animals in the corresponding year to normalize the sales data over time by animal production, generating a normalization factor: the PCU (population correction unit). However, this procedure implies that no information about the real quantitative exposure of individual animals is possible. 319 different antimicrobials for veterinary use were on the market in 2010 and included into the study protocol. 121 of these preparations were for parenteral application, 121 were orally used, 30 were applied for intramammary and 9 for intrauterine use and 38 were feed supplements. As receiving animal's cattle, pigs, poultry and sheep and goats were included but no horses or pets. At total of 62.83 tons were sold in 2010: 60.5 tons (96%) were systemically used. Tetracyclines were most frequently applied (57.6%), followed by penicillins (10.4%) and macrolides (10.3%). Only 240 kg (0.4%) of aminoglycosides were supplied in 2010. This was the antimicrobial category with the lowest application rate in Austria. There was no differentiation in distinct aminoglycosides provided. It is not possible to deduce the application rate of kanamycin from these data. The results of this study will be published on the homepage of ESVAC (http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/document_listing/document_listing_000302.jsp&mid=WC0b01ac0580153a00) by the end of 2012.

3 Modelling horizontal gene transfer in soil habitats – Effects of selection on fixation of newly acquired genes in bacterial populations

3.1 Background

If novel genes are introduced into a new environment, horizontal gene transfer (HGT) by natural genetic transformation is an important mechanism by which bacteria acquire and recombine this new genetic material. The acquisition of a genetic trait can occur immediately from the onset of exposure to new genetic material. It may, however, subsequently take many thousand generations until the trait is widespread and get fixed in the bacterial population. The dynamics of the process depend on many parameters, such as the amount of genetic material being introduced into the system, the size of the bacterial population, the proportion of the population being exposed to the DNA, the transfer rate and the selective pressure. The potential benefit of a plant derived ARM gene uptake by a bacterial cell is survival after aminoglycoside containing manure application to soils or resistance to antimicrobial chemotherapy in the mammalian gut.

Based on results of Townsend et al. (93), we construct a cohesive probabilistic framework, modelling the transfer of genetic material from the plant matter to the soil, from the soil into the bacterial population and – once the persistence in the population is established – the development of the gene in the bacterial population using stochastic simulation models. Using these models, we determine the amount of time it takes until 50% of the bacterial population under consideration have acquired the new gene as a measure of the fitness of the genetic trait.

3.2 Material and methods

For the modelling, we consider a closed bacterial population into which novel DNA is introduced via plant matter. The model is made up of three compartments:

1. the entry of the DNA into the habitat (soil),
2. the gene transfer,
3. the fixation and spread of the new gene in the bacterial population.

We use Monte-Carlo-models to determine the point of time of the gene transfer, taking into account the probability of fixation. The impact of the gene in the host population is subsequently computed using a deterministic model from population dynamics. Our aim is to characterize the necessary conditions and to identify limiting factors for the fixation of a trait. We furthermore aim at characterizing the spread of acquired genes in the host population and to convey an impression of the time frame of a possible impact.

3.2.1 Introduction of the novel DNA

In our model, we consider two distinct scenarios. In the first scenario we consider a constant presence of the resistance genes at a low concentration, which would for instance be the case for a field where the rootstock of genetically modified plants is constantly emitting small amounts of genetic material by rhizodeposition in the rhizosphere. In the second scenario, the novel genetic

material is introduced into the system only once, where it is subject to decomposition. The genetic material of decomposing plant material subsequently decreases until all of it has been destroyed by decay. This would for instance be the case if a single batch of genetically modified maize was planted and some plants remained in the soil after the harvest.

3.2.1.1 Constant exposure to the DNA

In the first scenario, we consider a constant presence of the resistance genes at a low level, which would for instance be the case in a field where the rootstock of genetically modified plants remained in the ground, constantly emitting genetic material. We consider an agricultural field of one hectare (100 m by 100 m), in which a proportion of the soil bacteria are exposed to some novel DNA. We assume a bacterial density of 4,91E+28 bacteria per 1,4E+13 square meters of land (Whitman, Coleman, & Wiebe, 1998), which amounts to a bacteria population of N = 3,51E+19 bacteria for one hectare. We furthermore assume that 0,1 % of the bacteria are exposed to the novel DNA, i.e., the number of exposed bacteria is n_{BEXP} = 3,51E+16.

3.2.1.2 Singular DNA entry

In the second scenario, the introduction of the novel genes is considered a singular event. A single maize plant (1300 g dry matter) containing novel genetic material is cut down during the harvest. The entire plant (300 cm) is worked into the soil and comes in contact with soil bacteria, giving rise to the possibility of gene transfer. Once in the soil, the plant material undergoes a decomposition process, during which the plant is degraded and the DNA is destroyed. The decomposition of the plant mass is modelled using a double exponential function (78):

$$M_{DRY}(t) = 0{,}4953e^{-1{,}09t} + 0{,}5047e^{-0{,}0039t},$$

where *t* is the time **in days** and $M_{DRY}(t)$ is the normalized dry matter at time *t*. The double exponential function considers a faster initial decomposition of the labile parts followed by slower decay of the more resistant parts of the plant. The plant matter is hence broken up into two compartments – a fast and a slowly decomposing compartment respectively – each of which decays exponentially. The model in Pote et al. (78) was fitted using measurements of the decay of the leaves of a tomato variety (Admiro). All numerical values used in the decomposition formulas (except for the estimated resistance gene copy numbers; see below) are based upon data acquired for the Admiro tomato variety by Pote et al. (78). For the fast decaying compartments the model above yields a half-decomposition time of 0,636 days, whereas the slowly decaying compartment has a half-decomposition time of 20,45 days. The decrease of the dry matter over time is illustrated in Figure 10. DNA degradation was shown to follow similar kinetics (78).

The DNA mass concentration was assumed to be 6,08E+08 copies per g dry matter. This number of potentially available resistance genes from a transgenic maize plant was calculated from data presented in Jonas et al. (56) for a typical recombinant insert in a transgenic maize variety. Hence the approximate number of available resistance gene copies from an adult maize plant at time *t* is given by

$$n_{GEN}(t) = M_{DRY}(t) \cdot 1300 \cdot 6{,}08 \cdot 10^{8}.$$

We assume that there is exactly one host bacterium for each available copy of the resistance gene, i.e., the number of exposed bacteria $n_{BEXP}(t)$ is determined by

$$n_{BEXP}(t) = n_{GEN}(t).$$

3.2.2 Horizontal gene transfer

Gene transfer events via natural transformation are relatively rare and presumably independent. The number of gene transfer events for a given time interval can therefore be modelled using a Poisson distribution parameterized by the transfer rate *r* per exposed bacterium, the number of exposed bacteria n_{BEXP} and the length of the considered time interval relative to the generation time.

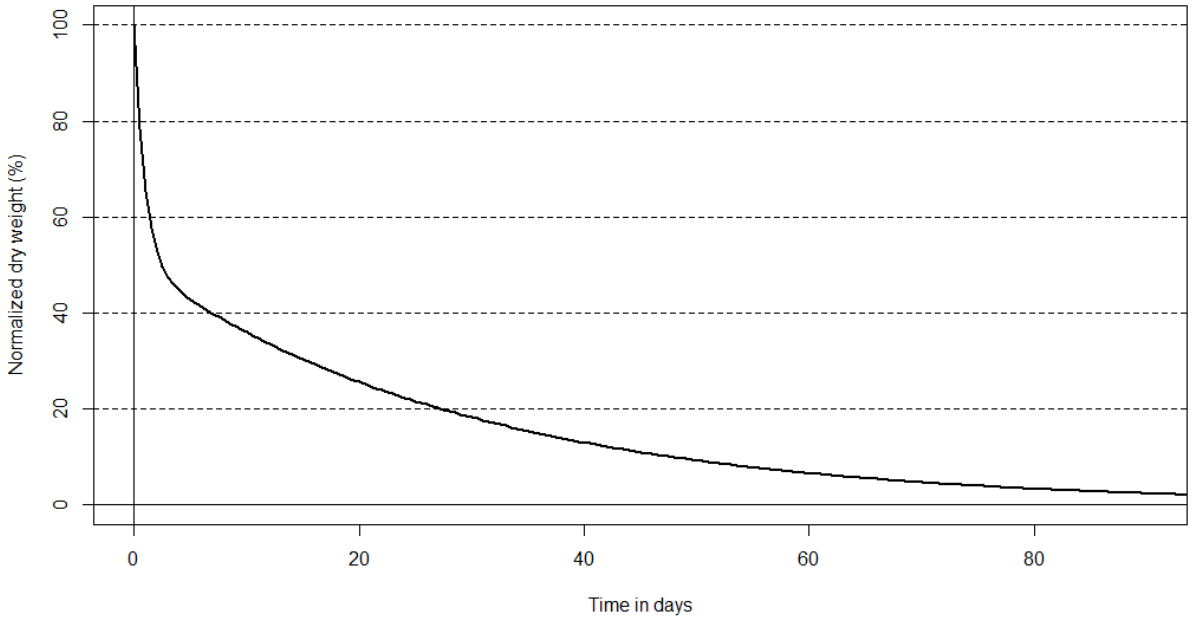

Figure 10: Decrease of normalized dry matter over time

3.2.2.1 Constant exposure to the DNA

In the first scenario, the number of exposed bacteria is constant over time. We can therefore make use of a very convenient relationship between the Poisson distribution and the exponential distribution. For a Poisson process, the length of the time intervals between consecutive events is exponentially distributed. The time T_{HGT} to the next gene transfer can hence be modelled using an exponential distribution with probability density function:

$$f_{T_{HGT}}(t) = \frac{1}{\beta \cdot e^{-t/\beta}}$$

for $t \geq 0$, where $\beta = \frac{1}{r \cdot n_{BEXP}}$.

3.2.2.2 Singular DNA entry

In the second scenario, the number of exposed bacteria decreases over time, because the amount of exogenous genes decreases due to DNA degradation. The frequency of gene transfers is therefore highest immediately after the DNA entry and it gradually decreases over time until all the available resistance gene copies have decomposed. As n_{BEXP} varies over time, the exponential distribution cannot be used to model the time intervals between HGT events. We therefore sample the number of gene transfer events for each time step and compute the time intervals between HGT events based on the results. We model the number of HGT events in the time between t_1 and t_2, using a Poisson distribution with parameter

$$\lambda = r \cdot n_{BEXP}(t_1) \cdot (t_2 - t_1).$$

3.2.3 Probability of fixation

The stability and impact of a horizontally transferred gene in the bacterial population is determined by the effects of natural selection. If the acquired gene has a neutral or a negative effect on the fitness of the reproductive potential of the bacterium, the trait is likely to vanish from the population after a few generations. Fixation, i.e., the eventual presence of the trait in all individuals of the recipient population, is only possible if the selection in the new host is positive or, for neutral or negative selection, if the transfer rate is sufficiently high. For traits with positive selection, the probability of fixation can be characterized by

$$\pi = \frac{1 - e^{-2m}}{1 - e^{-2Nm}},$$

where *N* is the size of the bacterial population and *m* is the relative Malthusian fitness coefficient; see, e.g. Nielsen et al. (72).

3.2.4 Modelling bacterial growth

According to Nielsen et al. (73), the frequency p_t of a mutant at time *t*, starting at frequency p_0, can be modelled deterministically as

$$\frac{p_t}{1 - p_t} = \frac{p_0}{1 - p_0} \cdot e^{mt},$$

where *m* again denotes the relative Malthusian fitness; see also Townsend et al. (93).

3.3 Results

In this section we present and discuss the results of the simulation models. All calculations were done using the open source software R (R Core Team, 2012).

3.3.1 Scenario 1: Constant exposure to the DNA

The first scenario represents a DNA source with a continuous release of genetic material. The level of present genetic material is constant; the effects of DNA entry into the habitat and natural decay are in an equilibrium state. Of the total population, only a fraction is actually exposed to the DNA. The timing of the gene transfer is a stochastic process, which is largely determined by the transfer rate *r*. The probability of fixation depends on the effects of natural selection which are accounted for via the relative Malthusian fitness coefficient *m*. Hence, the time at which a gene transfer event, leading to fixation within the population, takes place is largely governed by the transfer rate and the selection pressure. Once the transfer has occurred, the spread of the gene within the host population is modelled as a deterministic process governed by the fitness parameter *m* only.

3.3.1.1 The first successful HGT-event

Figure 11 shows the median time until the first HGT-event that leads to fixation in the host population occurs (note that all three axes are log-scaled in the figure). The colored areas in the figure correspond to combinations of the transfer rate and the selection coefficient for which a successful HGT-event occurred within 100.000 bacteria generations. Areas where the surface plot is not drawn in the figure, as well as missing values in the table, indicate value combinations for which no successful HGT event occurred within 100.000 bacteria generations (in the median).

Table 13: Median time (in bacteria generations) to first HGT-event that leads to fixation for varying values of the transfer rate and the selection coefficient.

		Malthusian selection coefficient m									
		1,E-10	**1,E-09**	**1,E-08**	**1,E-07**	**1,E-06**	**1,E-05**	**1,E-04**	**0.001**	**0.01**	**0.1**
Transfer rate r	**1,E-17**	-	-	-	-	-	-	8922	1010	109	14
	1,E-16	-	-	-	-	-	8991	1013	115	13	1
	1,E-15	-	-	-	-	13.385	867	81	12	2	1
	1,E-14	-	-	91.477	8243	877	87	11	2	1	1
	1,E-13	-	93.148	8957	846	114	10	1	1	1	1
	1,E-12	85.631	9479	1155	93	11	2	1	1	1	1
	1,E-11	10.703	1239	119	10	2	1	1	1	1	1
	1,E-10	965	104	13	1	1	1	1	1	1	1
	1,E-09	101	11	2	1	1	1	1	1	1	1
	1,E-08	9	1	1	1	1	1	1	1	1	1
	1,E-07	2	1	1	1	1	1	1	1	1	1
	1,E-06	1	1	1	1	1	1	1	1	1	1
	1,E-05	1	1	1	1	1	1	1	1	1	1

It can be seen that the timing of the HGT-event depends on both, the transfer rate and the selection coefficient. Even for very low transfer rates, a successful HGT-event is likely if the selection pressure is sufficiently high. For *r* = 1E-17, for instance, a successful HGT-event is likely to occur roughly within 10.000 generations for *m* = 1E-4, within 1000 generations for *m* = 1E-3, within 100 generations for *m* = 1E-2 and within 10 generations for *m* = 1E-1 (see Table 13).

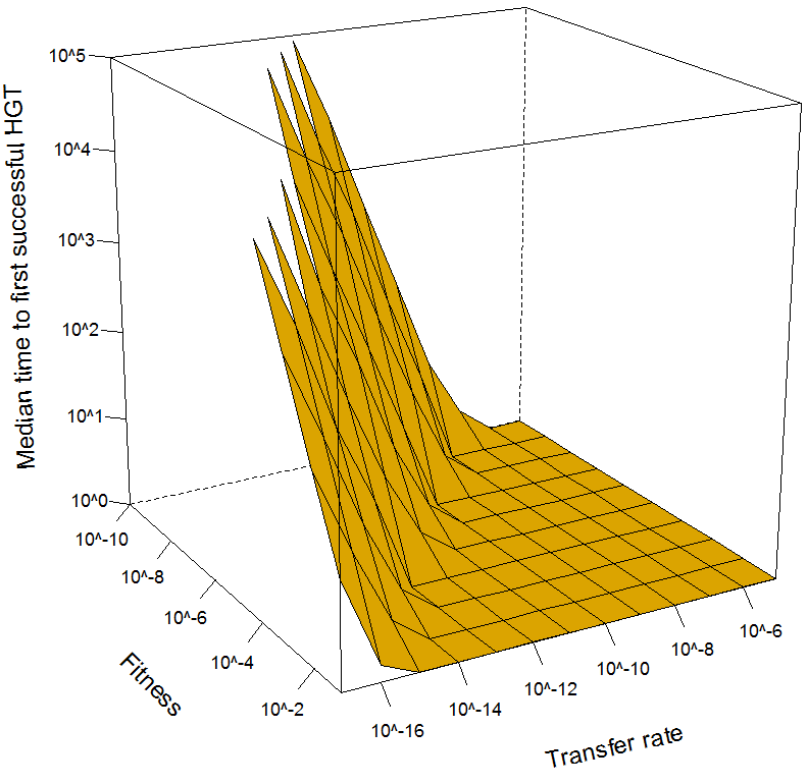

Figure 11: Median time (in bacteria generations) to first HGT-event that leads to fixation for varying values of the transfer rate and the selection coefficient.

3.3.1.2 Time until 50 % of the population have acquired the gene

Once a successful HGT-event has occurred, the gene spreads within the host population. The speed of the spread largely depends on the selection pressure in the host environment. In our simulation, we characterize the spread velocity using the parameter t_{POP50}, which is defined as the median time in bacteria generations until the novel genetic material is present in 50 % of the host population. Table 14 shows t_{POP50} for different values of m and r. Missing values indicate combinations of the selection coefficient and the transfer rate for which no successful HGT event occurred within 100.000 bacteria generations (in the median).

3.3.1.3 Univariate variation of the transfer rate r

Figure 12 and Figure 13 illustrate the influence of the transfer rate on the propagation of the gene within the bacterial host population. The figures show the median proportion of the bacterial population with the new gene over time for Malthusian selection coefficients *m* = 0,1 (Figure 12) and *m* = 0,01 (Figure 13) and for varying values for the transfer rate r (note the differing x-axes in the figures).

The figures show that a change in the transfer rate results in a shift of the curve along the x-axis, i.e., if the transfer rate decreases the time of the first successful HGT-event (and subsequently the spread of the novel gene in the population) is delayed. The shape of the curve is, however, independent of the transfer rate. A comparison of the two figures suggests that a reduction of the selection coefficient by a factor 0,1 roughly leads to a transformation of the time scale by a factor 10, as can also be seen in Table 14. The figures once more show that a HGT-event with subsequent spread

within the population is possible within a reasonable time frame even for very low values of r if the selection pressure is sufficiently high.

Table 14: Median time (in bacteria generations) until 50 % of the population have acquired the gene for varying values of the transfer rate and the selection coefficient.

		Malthusian selection coefficient m			
		0,0001	0,001	0,01	0,1
Transfer rate r	1,E-17	-	45.617	4572	461
	1,E-16	-	43.043	4309	433
	1,E-15	-	40.748	4076	410
	1,E-14	-	38.457	3849	387
	1,E-13	-	36.150	3618	364
	1,E-12	-	33.848	3388	341
	1,E-11	-	31.546	3157	318
	1,E-10	-	29.244	2927	295
	1,E-09	-	26.941	2697	272
	1,E-08	-	24.638	2467	249
	1,E-07	-	22.336	2236	226
	1,E-06	-	20.033	2006	203
	1,E-05	-	17.731	1776	180

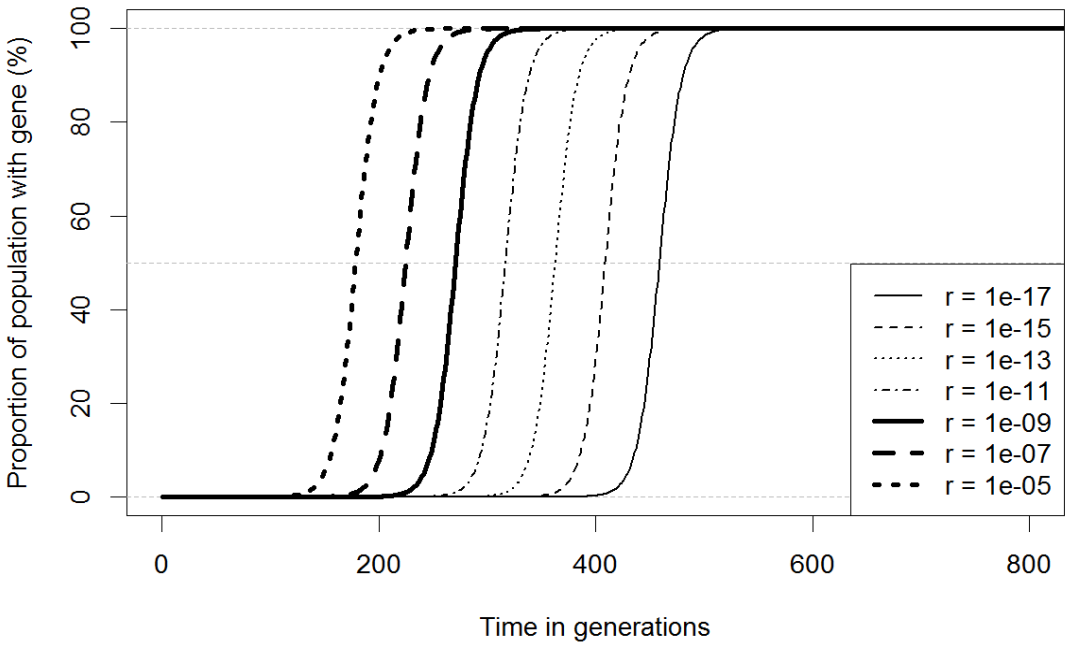

Figure 12: Median proportion of population with new gene over time (in bacteria generations) with m = 0,1 and varying values for the transfer rate r.

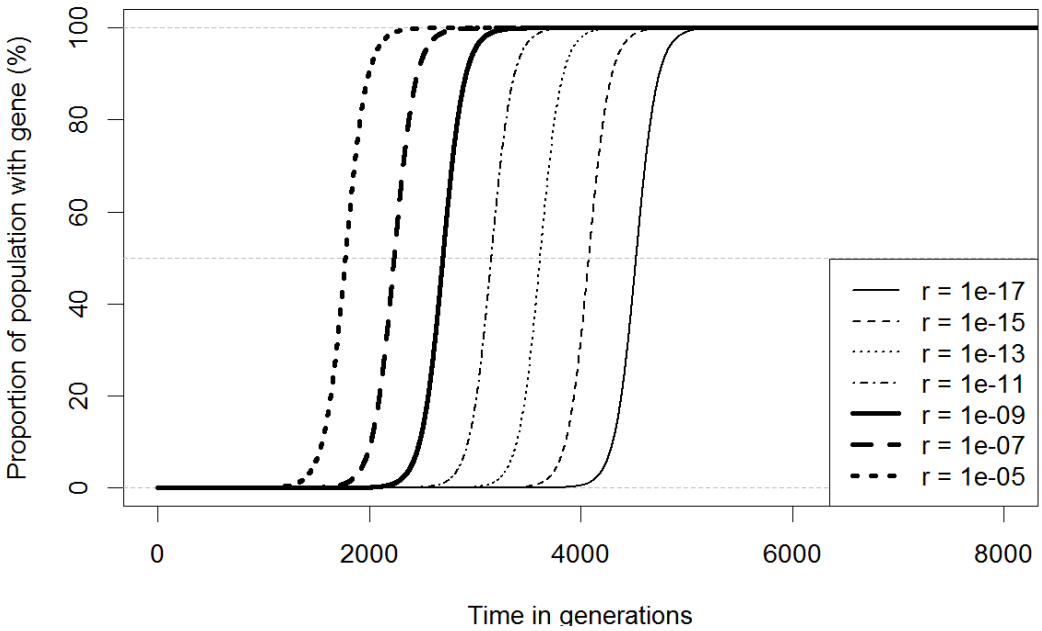

Figure 13: Median proportion of population with new gene over time (in bacteria generations) with m = 0,01 and varying values for the transfer rate r.

3.3.1.4 Univariate variation of the selection coefficient m

Figure 14 and Figure 15 illustrate the influence of selection pressure on the propagation of the gene within the bacterial host population. The figures show the median proportion of the bacterial population with the new gene over time for transfer rates *r* = 1E-13 (Figure 14) and *r* = 1E-17 (Figure 15) for selection coefficients *m* = 0,1, 0,05, 0,01, 0,005, 0,001 and 0,0005. For *m* = 1E-4 and lower, the simulation showed no significant spread within 100.000 bacteria generations. The figures once more show that the selection coefficient has an influence on, both, the timing of the first successful HGT-event and the speed of the propagation of the gene within the population. Smaller values of the selection coefficient result in a delayed first HGT-event and in a slower spread within the population.

3.3.1.5 Sensitivity analysis

One aim of the modelling was to determine which parameters have the biggest influence on the outcome. Figure 14 and Figure 15 already suggest that the system is rather sensitive with respect to the fitness coefficient. Figure 16 shows a tornado plot, illustrating the relative influence of the transfer rate, selection pressure (Malthusian fitness) and population size. In order to compare the parameters, the median time (measured in bacterial generation times) until 50% of the bacterial population carry the gene was chosen as a target value. For each of the three parameters, the values were varied by ±10% around central values of m = 1E-03, r = 1E-08 and N = 1E+09. Figure 16 shows the resulting absolute deviation of t_{POP50} from the base value of 24.137 generations.

Figure 16 clearly shows that the selection pressure (relative Malthusian fitness coefficient) has the largest influence on the speed of the propagation. The transfer rate and the population size play a smaller role. The influence of the two parameters, however, is of a comparable order.

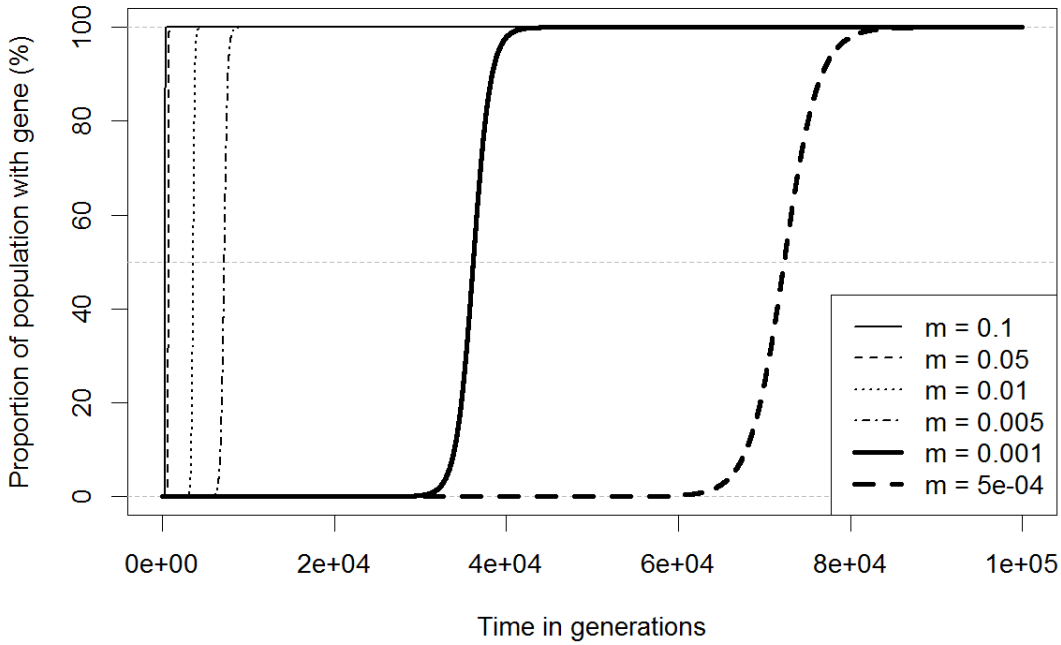

Figure 14: Median proportion of population with new gene over time (in bacteria generations) with r = 1E-13 and varying values for the selection coefficient m.

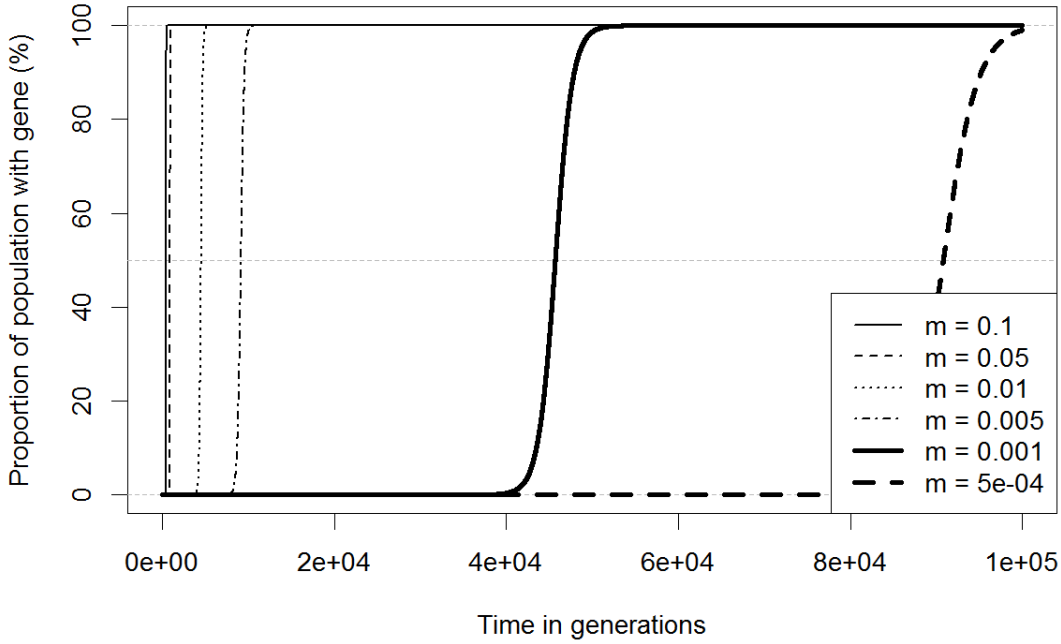

Figure 15: Median proportion of population with new gene over time (in bacteria generations) with r = 1E-17 and varying values for the selection coefficient m.

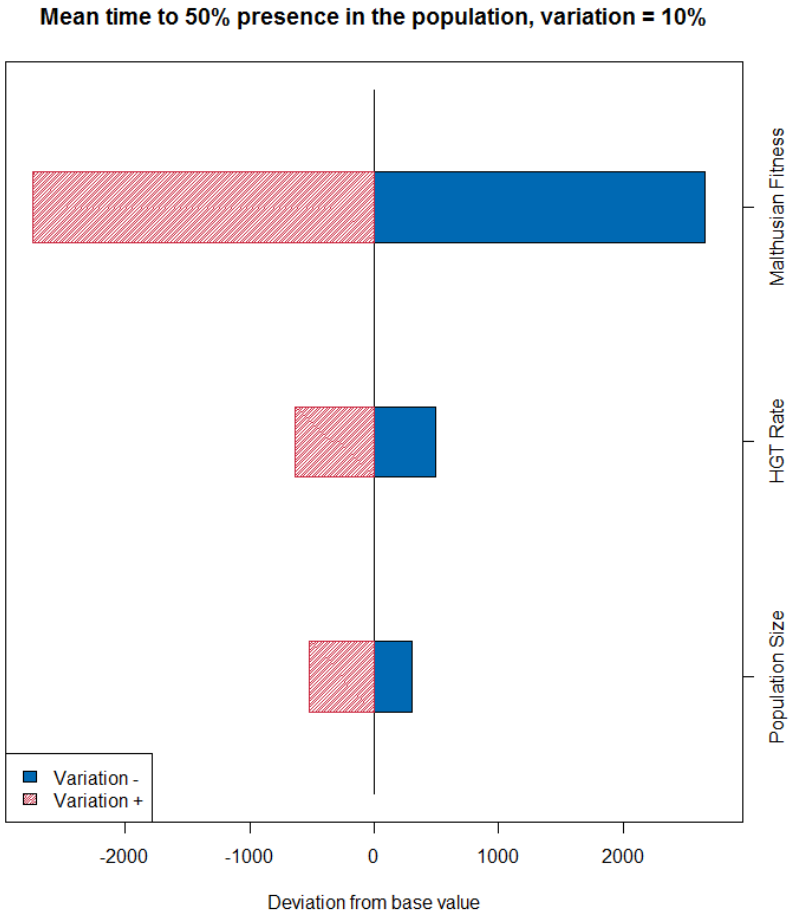

Figure 16: Tornado plot of the mean time until 50% presence of the gene in the population.

The parameters m = 1E-03, r = 1E-08 and N = 1E+09 were varied by ±10% around their base values.

3.3.2 Scenario 2: Singular DNA entry

The second scenario describes a singular event of DNA entry into the habitat. The scenario was modelled to represent a single plant of maize carrying a new resistance gene being cut down and worked into the soil. For the size of the bacterial host population, we assumed a bacterial density of 1E+09 bacteria per cubic centimetre of soil and considered the case where the plant DNA comes in contact with 10.000 cm^3 soil resulting in a bacterial population of 1E+13.

At the beginning, the exposure of the host population to the novel gene is very large. Due to decomposition, however, the number of gene copies in the soil decreases exponentially; see Figure 10. Due to the additional temporal element of the DNA decay in the model, the duration of the bacterial generations needs to be considered.

3.3.2.1 Propagation of the gene in the host population

Scenario 2 differs from the first scenario in the timing of the gene transfers. In the first scenario, the exposure is constant over time. Hence, the chance of a gene transfer is the same for every point in time. In the second scenario, the gene transfers will mainly occur at the beginning of the exposure, as the concentration of the genetic material is largest at that time. Therefore, the random element is kept minimal, as can be seen in Figure 17 below, which shows the proportion of the bacterial population carrying the new gene over time for N = 1E+13, r = 1E-08, m = 1E-03 and a generation time of 8 hours. In Figure 17, the different percentiles virtually overlap. In the median it took 23.419 bacterial generations from the start of the exposure until 50% of the population carried the new gene (2,5th percentile = 23.344; 97,5th percentile = 23.486). After a median of 28.014 generations, 99% of the population carried the new gene (2,5th percentile = 27.939; 97,5th percentile = 28.081).

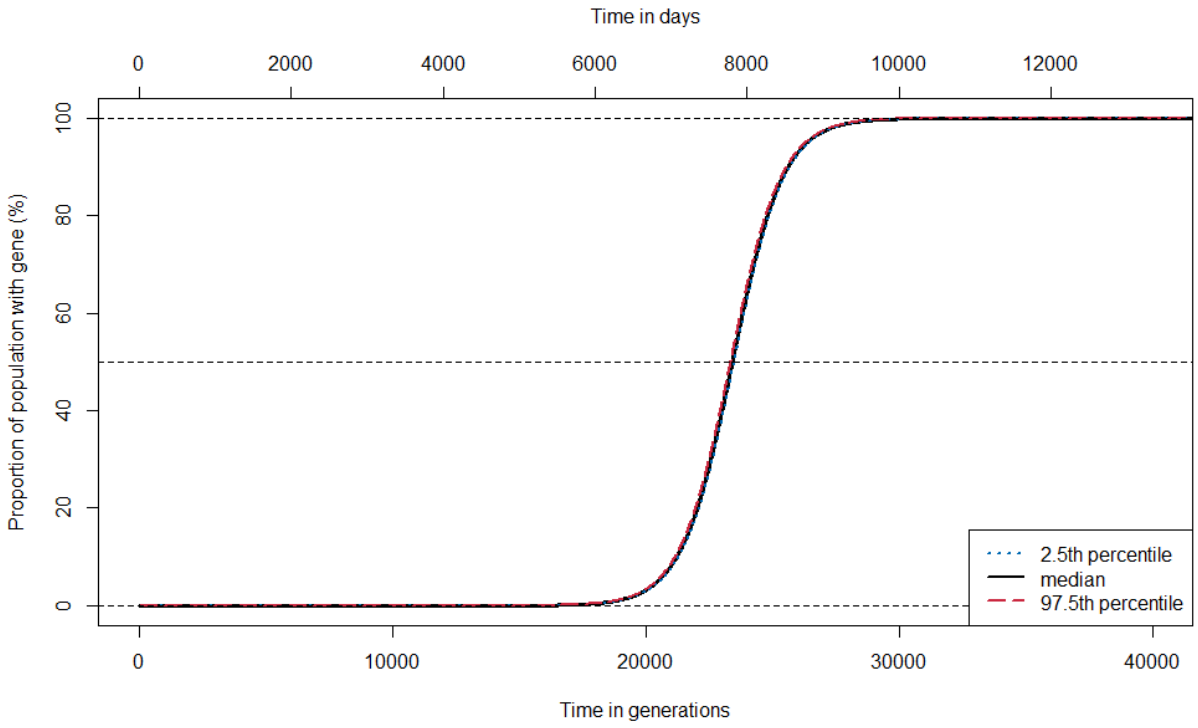

Figure 17: Proportion of bacterial population with the new gene over time for a singular DNA entry into the habitat.

Parameters used: N = 1E+13, r = 1E-08, m = 1E-03 and a generation time of 8h.

The results are very similar for an increased population size and different bacterial generation times as can be seen in Table 15. The table shows the median and the 2,5th and 97,5th percentiles for the time (in generations) until 50% and 99% of the population carry the new gene. The calculations were done for *r* = 1E-08, *m* = 1E-03 and different values for the generation time and the population size. It can be seen that for all parameter combinations, the percentiles lie very close together. The velocity of the propagation within the population seems to decrease with increasing generation time and population size. The effects are, however, not very prominent.

Figure 18 shows a tornado plot of the mean time until 50% of the population carry the new gene for a generation time of 8 hours. For each of the parameters m = 1E-03, r = 1E-08 and N = 1E+13, the values were varied by ±10%. The figure below shows the resulting deviation from the base value of

t_{POP50} = 23.419 generations. Figure 18 again confirms that the relative Malthusian fitness coefficient has the largest influence on the speed of the propagation. The transfer rate and the population size play a much smaller role.

		Time until 50% presence (in generations)			Time until 99% presence (in generations)		
Generation time	**N**	**2,5th percentile**	**median**	**97,5th percentile**	**2,5th percentile**	**median**	**97,5th percentile**
8 hours	1E+13	23.344	23.486	23.419	27.939	28.081	28.014
8 hours	1E+14	25.641	25.788	25.718	30.236	30.383	30.313
1 day	1E+13	24.319	24.569	24.436	28.914	29.164	29.031
1 day	1E+14	26.624	26.876	26.746	31.219	31.471	31.341
7 days	1E+13	25.860	26.413	26.131	30.456	31.008	30.726
7 days	1E+14	28.180	28.745	28.413	32.775	33.340	33.008

Table 15: Percentiles of the time until 50% and 99% of the population carry the new gene for r = 1E-08, m = 1E-03 and different values for the generation time and the population size.

3.3.2.2 Probability of fixation

Contrary to the first scenario where in our model the gene is always fixed in the population eventually, this scenario holds the possibility of the DNA decaying before it can transfer to the host population. This can happen if the transfer rate is too small, the Malthusian fitness is too small or if the population size is too large. Figure 19 shows a tornado plot of the probability of any gene transfer eventually becoming fixed in the population. The computations were done for a generation time of 8 hours, *m* = 1E-05, *r* = 1E-09 and *N* = 1e+13. The values for the fitness, the transfer rate and the population size were varied by ±10%. The figure below shows the resulting deviation in percent from the base value of a 51,4% probability of fixation.

As one would expect, the transfer rate seems to be the most influential factor in the model. The Malthusian fitness, however, also has an important impact on the probability of fixation. Both parameters show a direct relationship with the fixation probability, i.e., an increase of the parameters also increases the chances of fixation. The population size shows an inverse relationship to the fixation probability, the larger the population the smaller the chances of eventual fixation. The effect of the population size on the fixation probability is, however, very small.

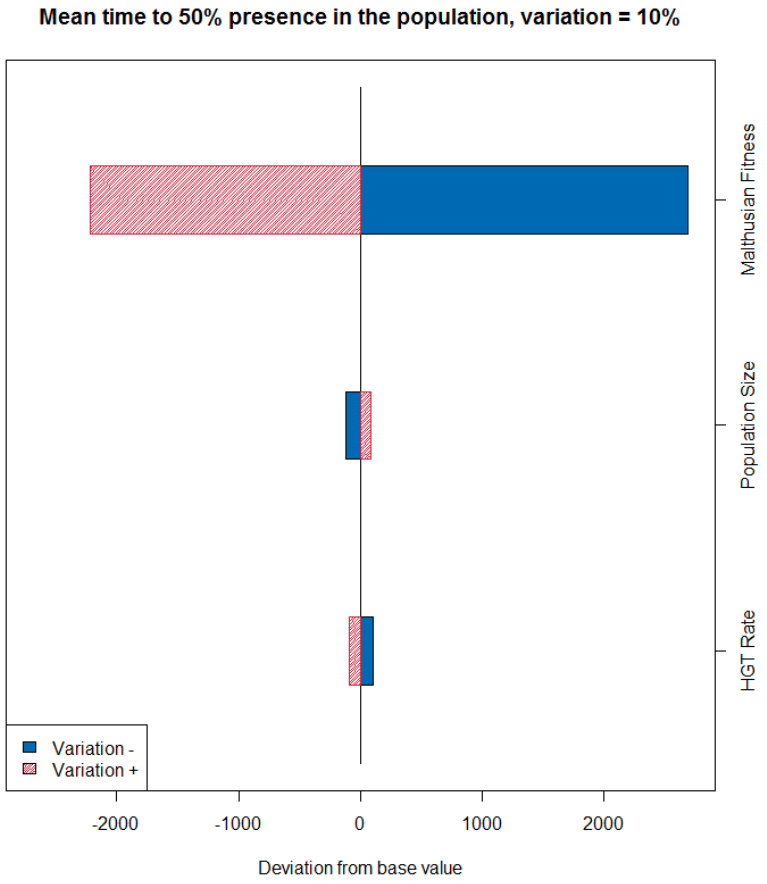

Figure 18: Tornado plot of the mean time to 50% presence of the gene in the population for a generation time of 8h.

The parameters m = 1E-03, r = 1E-08 and N = 1E+13 were varied by ±10% around their base values.

3.4 Discussion

Based on results in the literature (44, 72, 73, 78, 94), we have implemented a probabilistic framework in order to model the transfer of a novel gene (a) from the plant to the soil, (b) from the soil into the bacterial host population via horizontal gene transfer and furthermore (c) the propagation of the gene within the bacterial population through reproduction and natural selection. As horizontal gene transfers are rare events and therefore are very difficult to observe, the need for mathematical modelling arises in order to characterize the spread of a novel gene in a population subject to certain defining parameters. In our model, these parameters were the size of the bacterial population, the exposure of the bacteria to the DNA, the transfer rate and the relative Malthusian fitness parameter.

We considered two different scenarios for the entry of the novel DNA into the habitat. The first scenario dealt with a constant exposure of the population to the DNA. The DNA entry into the habitat and the natural decay are assumed to be in an equilibrium state. In this scenario the entry into the bacterial population is intrinsic, i.e., the gene is always fixed in the bacterial population. For our models it turned out that the fitness parameter plays the most important role, having the strongest influence on the time it takes for the gene to spread within the bacterial population. The population size and the transfer rate determine the time of the gene transfer. The system, however, seems to be more robust with respect to changes in these two parameters. It was further shown that even for low transfer rates successful HGT-events are possible if the selection pressure is sufficiently high.

In the second scenario, the entry of the DNA into the habitat was considered a singular event. The genetic material enters the system yielding an initially high exposure and subsequently decreases exponentially due to decomposition. The gene transfer either occurs near the beginning of the exposure or the gene is not fixed in the population at all. The probability of fixation depends on the transfer rate and the fitness coefficient, decreasing as these parameters decrease. Sensitivity analysis showed that the transfer rate is the most crucial factor for the probability of an eventual fixation, while the Malthusian fitness also plays an important role.

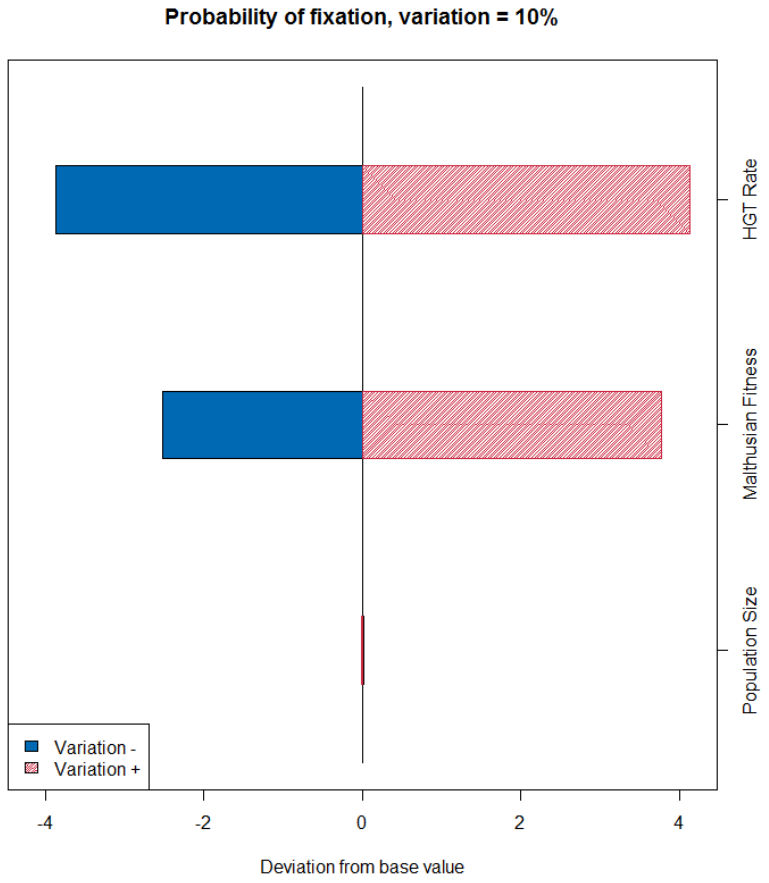

Figure 19: Tornado plot of the probability of fixation for a generation time of 8h.

The parameters m = 1E-05, r = 1E-09 and N = 1E+13 were varied by ±10% around their base values.

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Frequency of Environmental Antibiotic Resistance

Part E:

PCR Detection Methods

Final Report

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1 Development of nptII/nptIII/16S Real Time PCR detection systems

1.1 NptII and nptIII TaqMan systems: Ingenetix – company internal validation data

Below basic evaluation data concerning the nptII and nptIII TaqMan real time PCR detection systems developed by Ingenetix (Vienna, Austria) are presented. These data show the applicability of the nptII and nptIII TaqMan assays meeting the basic requirements of the present project. The TaqMan PCRs were performed on an ABI Prism 7500 device (Applied Biosystems, Austria). Based upon these results the offered nptII and nptIII TaqMan systems were adapted to and validated on the Lightcycler LC480 device (Roche, Austria).

1.1.1 NptII-TaqMan Assay

Preparation and testing of a logarithmic plasmid (containing nptII) serial dilution (1:10):

	Ct:
0.1 pg/µl	25.7
0.01 pg/µl	28.8
0.001 pg/µl	32.3
0.0001 pg/µl	35.9

Amplification curves

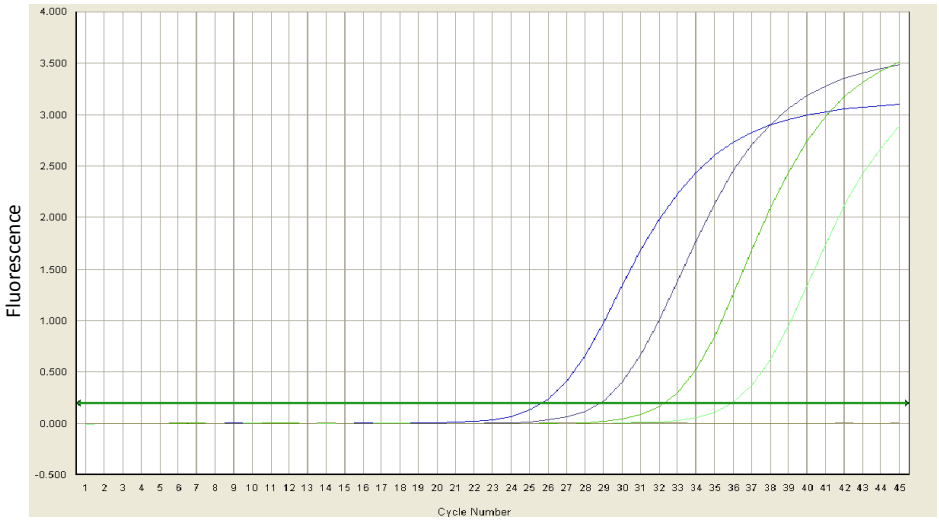

Figure 1: Amplification curve for nptII target

The amplification curves of the individual dilutions show even distances (Figure 1), the standard curve has an optimal slope (Figure 2).

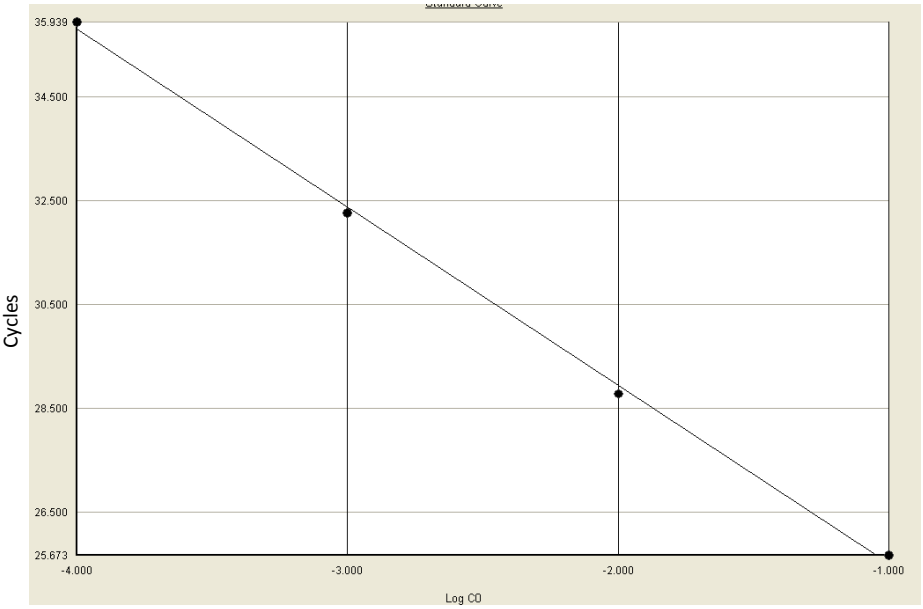

Figure 2: Standard curve for plasmid serial dilution with nptII target as shown in Figure 1.

1.1.2 NptIII-TaqMan Assay

Preparation and testing of a logarithmic plasmid (containing nptIII) serial dilution (1:10):

	Ct:
0.1 pg/µl	25.1
0.01 pg/µl	28.4
0.001 pg/µl	31.8
0.0001 pg/µl	34.9

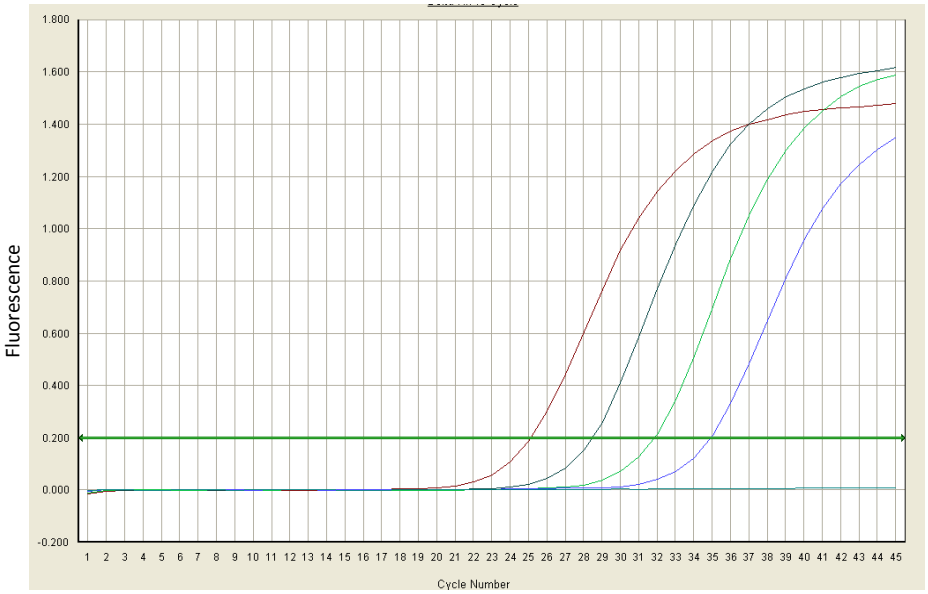

Figure 3: Amplification curve for nptIII target.

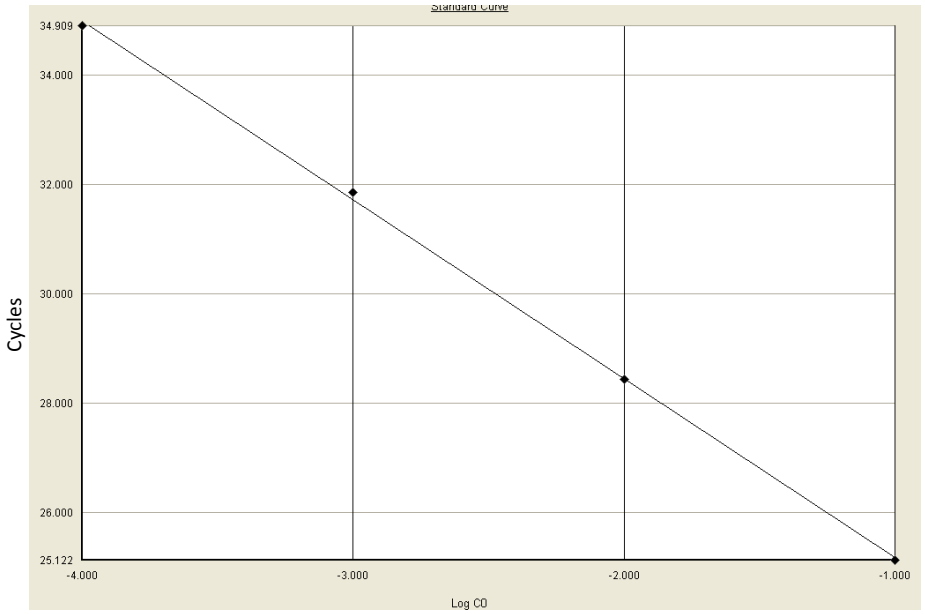

Figure 4: Standard curve for plasmid serial dilution with nptIII target as shown in Figure 3.

The amplification curves of the individual dilutions show even distances (Figure 3), the standard curve has an optimal slope (Figure 4)**.**

1.1.3 Plasmid combinations

Since nptII and nptIII genes can occur simultaneously in a sample, a duplex PCR assay in which both nptII and nptIII TaqMan systems are used simultaneously was checked. In three different situations the amplification signals were tested:

a) nptII and nptIII targets were in the same concentration in the assay (Figure 5)
b) The nptII concentration was 100 times higher than in nptIII assay (Figure 6).
c) The nptII concentration was a hundredth the concentration of nptIII in the assay (Figure 7).

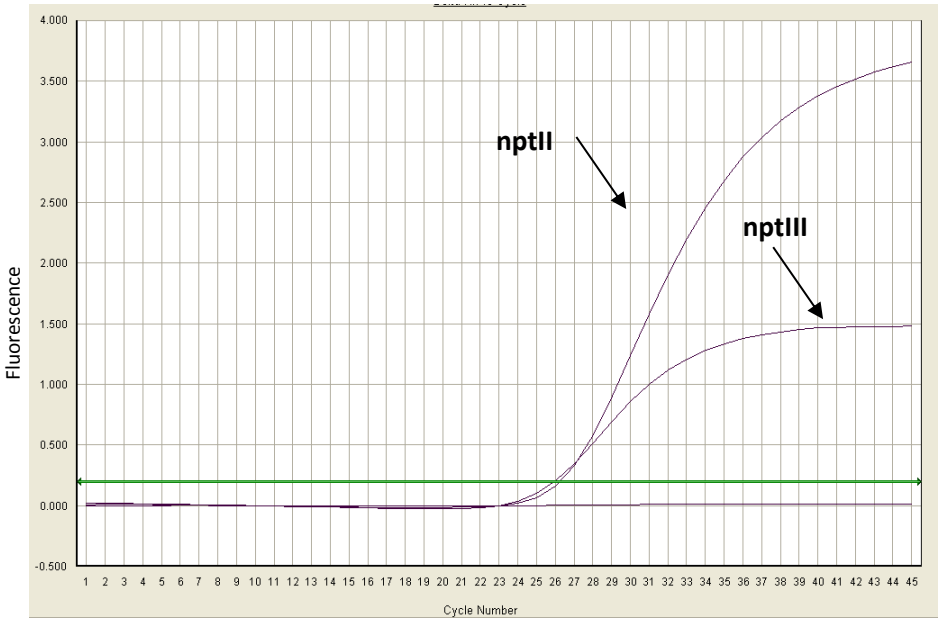

Figure 5: nptII (0.1 pg/µL), nptIII (0.1 pg/µL) plasmid in the presence of 300 nM 16S primers and 200 nM 16S primers 16S probe (can not be measured with ABI 7500 devices) and bacterial DNA (as template for 16S assay).

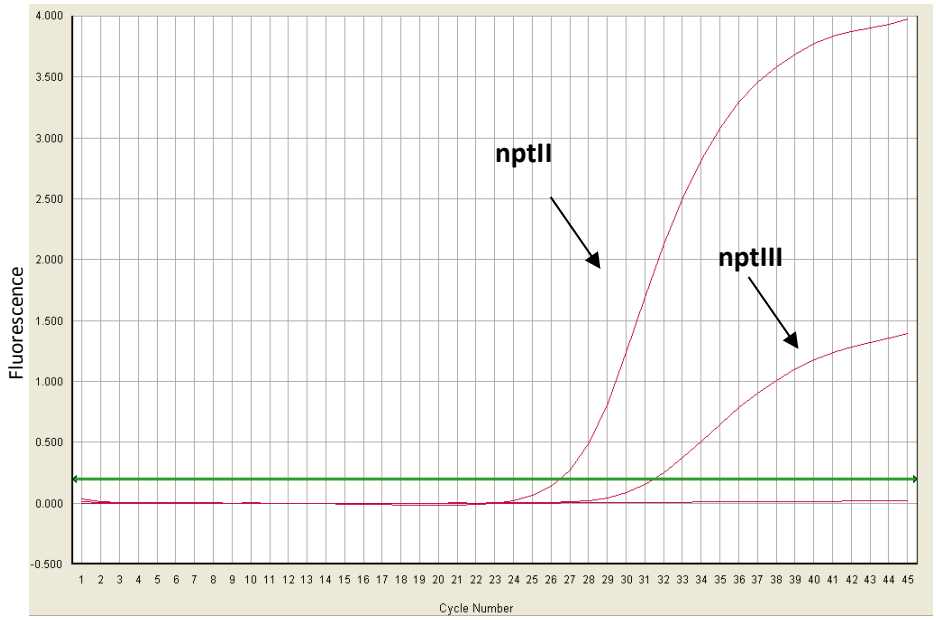

Figure 6: nptII (0.1 pg/µl), nptIII (0.001 pg/µl) in presence of 300 nM 16S primers and 200 nM 16S probe and bacterial DNA.

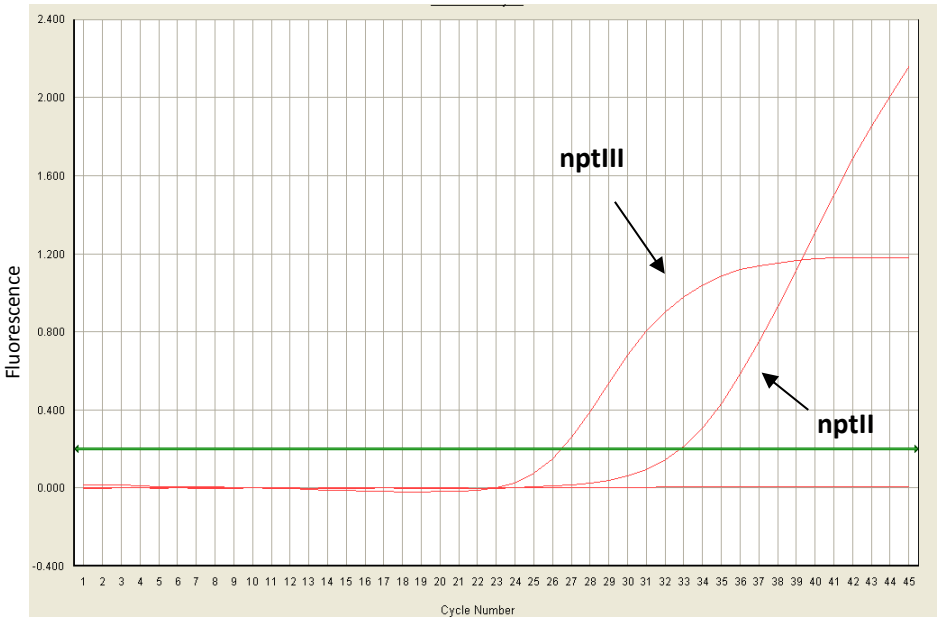

Figure 7: nptII (0.001 pg/µl), nptIII (0.1 pg/µl) in presence of 300 nM 16S primers and 200 nM 16S probe and bacterial DNA.

Results:

a) Identical target concentrations did not lead to mutual inhibition, the amplification curves appeared almost simultaneously from the amplification fluorescence emission background (purple line, Figure 5).
b) A 100 fold higher concentration of nptII target molecules in the assay resulted – as expected - in an earlier crossing point (this is equal to the "Ct-" value for ABI real time PCR thermocyclers) than nptIII. But even in this situation nptIII produces a qualitatively high-classed and clearly interpretable result (i.e. the tested isolate is clearly nptIII positive) (Figure 6).
c) NptIII present in a 100 fold higher concentration in the assay compared to nptII led - as expected - to an earlier crossing point (="Ct" value for ABI thermocyclers) for nptII. But even in this situation a qualitatively exactly interpretable result for NptII is obtained (i.e. the tested isolate is clearly nptII positive) (Figure 7).

Conclusion:

All available amplification systems were in principle well suited to meet the requirements set by the project.

1.2 Validation of the PCR systems

The following PCR systems were used during the project:

1. Duplex screening assay for the simultaneous detection of nptII and nptIII in one PCR approach (qualitative PCR) in a single well
2. Screening assay for the detection of 16S rRNA target (qualitative PCR)
3. Single assay for quantification of nptII targets (quantitative PCR)
4. Single assay for quantification of nptIII targets (quantitative PCR)
5. Single assay for quantification of 16S rRNA targets (quantitative PCR)

As key quality parameter of a real-time PCR in addition to a satisfactory specificity of the reaction, the 95% detection limit (= sensitivity) and the linear range of the used quantification PCR systems were determined.

The specificity of the nptII / nptIII PCR detection systems was tested using BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome; optimized for short input sequences, last access: 18.1.2011) and is ensured via primers and TaqMan probes.

A random sample sequencing of positive PCR results had confirmed in all cases a 100% homology to the corresponding nptII or nptIII amplicon. The primer-probe combinations were therefore specific.

The 95% detection limit and the linear range of quantification were tested in the aqueous system (PCR components plus template DNA diluted in H_2O) to determine the basic performance of the system. In addition, the inter- and intra-assay variability was determined.

1.2.1 Reference Standards

As reference standards for the validation plasmid isolations of the vector pUC19 with nptII and another pUC19 plasmid with the nptIII insert were used.

The vector pcDNA3.1/CT-GFP-TOPO (Cat. No. 46-1126, Invitrogen) which contains the neomycin resistance gene, served as a template for the amplification of nptII. The vector pCP60 was used as template for the amplification of nptIII. This vector was a gift from Dr. Günter Brader (Austrian Institute of Technology, Seibersdorf). Both amplicons were each separately cloned into pUC19 vectors which themselves carry no endogenous aminoglycoside resistances. The recombinant plasmids were used to transform *E. coli* XL1.

The reference standard for the validation of 16S rRNA was purchased (Ingenetix). It was a plasmid into which the complete 16S rRNA gene of *E. coli* had been inserted.

Amplification, transformation, cloning and plasmid isolations were carried out according to standard procedures. The identity and integrity of the inserts were confirmed via sequencing.

1.2.2 Calibration curve

The reference standards were used to prepare semilogarithmic (1:5) dilutions and to create a calibration (or standard) curve for the LightCycler 480 assays. The following concentrations of the particular plasmids were used per PCR assay and validation run:

1. 750 000 copies/assay
2. 150 000 copies/assay
3. 30 000 copies/assay
4. 6 000 copies/assay
5. 1200 copies/assay
6. 240 copies/assay
7. 48 copies/assay
8. 9,6 copies/assay
9. 1,92 copies/assay
10. 0,38 copies/assay

A H_2O negative control was included into each PCR run.

The copy number of the reference plasmid isolations was calculated with the aid of the Loschmidt constant (6.022×10^{23} particles / mol).

The above-presented semi-logarithmic dilution series was prepared for nptII, nptIII and 16S targets, each. For validation of the systems the data of at least 24 replicates per dilution step were acquired and used for statistical evaluation. These numbers were derived from a recommendation of the Paul Ehrlich Institute (PEI) for the validation of in-house PCR systems for the testing of blood products for HBV, HCV and HIV contamination that requires the testing of five dilution steps in eight replicates in three PCR runs. The PEI guideline requests a total of 120 measurement points for the establishment of the 95% detection limit. In this project 10 dilution steps (see above) were analysed to determine the 95% detection limit, each with eight replicates in more than three PCR runs.

1.2.3 PCR parameters

The LightCycler LC480 (Roche, Austria) was used with the 96 well block. The test method was based on the TaqMan assay technology (real-time PCR with hydrolysis probes). The TaqMan probes were labeled as follows:

nptII:	FAM (Filter: 533 nm)
nptIII:	5'YYE (Filter: 568 nm)
16S:	Cy5 (Filter: 670 nm)

The PCR conditions are shown in Table 1.

	nptII	nptIII	16S
Probes			
Label:	FAM	5'YYE	Cy5
Concentration:	0.2 µM	0.2 µM	0.2 µM
Primers			
Forward	0.6 µM	0.6 µM	0.5 µM primer mix (degenerated, universal 16S)
Reverse	0.6 µM	0.6 µM	
PCR conditions			
A) Initial denaturation	95°C/10 min/1x	95°C/10 min/1x	95°C/10 min/1x
B) Cycling	45 x	45 x	45 x
denaturation	95°C/10 s	95°C/10 s	95°C/10 s
annealing + elongation	60°C/20 s	60°C/20 s	60°C/1 min
C) Cool down	40°C/continuous	40°C/continuous	40°C/continuous
Assay			
Template volume	2 µl	2 µl	2 µl
PCR mix volume	8 µl	8 µl	8 µl
Total PCR assay volume	10 µl	10 µl	10 µl

Table 1: LC480 PCR parameter.

1.2.4 PCR controls

As positive controls were used:

1. Plasmid pUC19/nptII
2. Plasmid pUC19/nptIII
3. Plasmid + 16S rRNA-Gen Insert (Ingenetix; 2,11 x 10^{10} Kopien/µl)
4. E. coli XL1 + pUC19/nptII
5. E. coli XL1 + pUC19/nptIII

As negative controls were used:

1. H_2O
2. E. coli XL1

1.2.5 Evaluation of the validation runs of LC480 - applied rules

For evaluation of the validation runs the Second Derivative Maximum algorithm of the LC480 software version 1.5.39 was used. The standard-/validation curve was determined according to the manufacturer's instructions (Roche), setting the buttons determined "in run" and "high confidence".

Quality criteria for evaluation:

1. For a valid standard curve the "error" value determined by the software must be less than 0.2.
2. The efficiency of amplification should be above 1.8.
3. The "slope" of the standard curve should be between 3.2 and 3.8.
4. Negative or empty amplifications that are defined as "standard" must be excluded from the calibration.

Moreover, the following issues were observed:

1. Obvious outliers or false amplifications were excluded from the analysis (i.e. crossing point (Cp value) differs critically from the mean, calculated from all replicates of the dilution).

2. The quality of the dilution series was checked by calculating the difference between Cp values of the different dilution steps: A semilogarithmic (1:5) dilution series leads to an interval between the Cp values ideally of 2.3 cycles. Since the dilution series was designed as "limiting dilution" (the last step of dilution achieved less than zero copies/assay), the accuracy of the initial spectroscopic quantification could be roughly assessed.

1.2.6 Determination of the 95% detection limit, the quantification limit and other parameters in the aqueous system: overview and practical implementation

For the validation of the singleplex PCR a semi-logarithmic dilution series was prepared as described above (see section 1.2.2) and evaluated with the three reference standard plasmids (nptII, nptIII, 16S) starting with 750 000 copies/assay and ending with 0.38 copies/assay (see Table 2). Each dilution step was applied in at least 24 replicates. Table 2 shows a typical sample application scheme for the validation in a 96 well microtiter plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T
B	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T
C	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T
D	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T
E	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T
F	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T
G	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T
H	H2O		0,38	1,92	9,6	48	240	1200	6000	30000	150T	750T

Table 2: Sample application scheme of the semilogarithmic dilution series of plasmids (nptII, nptIII, 16S)

The numbers in each well correspond to the number of copies / assay.

150T ..... 150 000 copies / assay

Figure 8 shows the result of a semilogarithmic dilution series (nptII) in the LC480 TaqMan real-time PCR format.

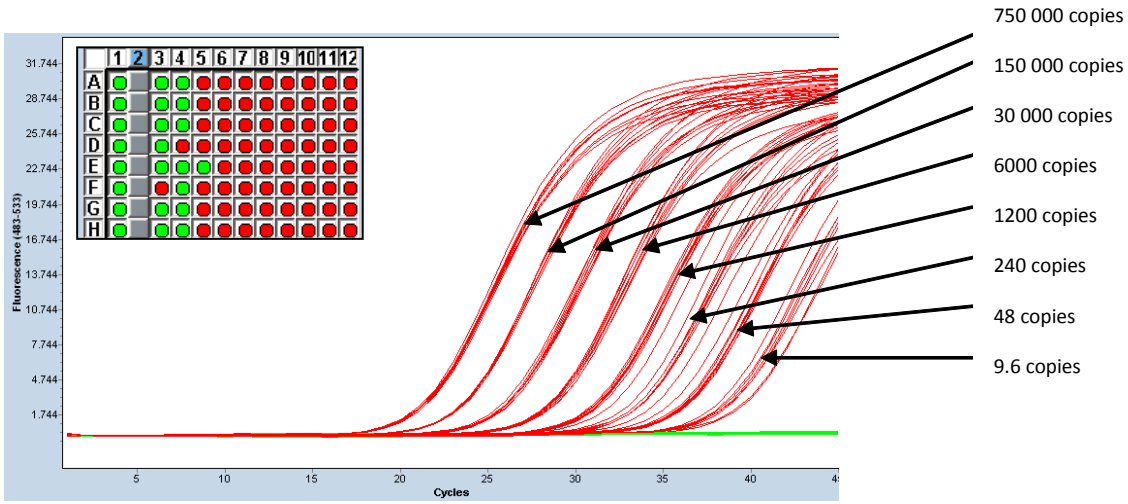

Figure 8: Semilogarithmic nptII dilution.

For designation of the dilution steps see Table 2. Column 2 was empty. All negative controls were negative (column 1).

1.2.7 95% limit of detection: nptII – TaqMan Single Assay

Data

In order to determine the limit of detection (LOD), three different runs with eight dilutions and a negative control (H_2O) each were analyzed. For each run and dilution, eigth replications were performed (see Table 3).

Copies/assay	Number of observations
0	24
0.38	24
1.92	24
9.6	24
48	24
240	24
1200	24
6000	24
30000	24

Table 3: Number of observations for the determination of the limit of detection for nptII.

Method

Based on the test design, it was assumed that observations within one block are more similar than observations of different blocks. In order to account for these dependencies, the data was modeled using a Generalized Linear Mixed Model (GLMM). As the response is coded as a dichotomous variable, the data was modeled using a binomial distribution and estimated using a probit link function. The dilution (copies/assay) is considered as a fixed effect and the block as a random effect.

The data was furthermore resampled using a bootstrapping approach with 1000 replications. For each replication, the limit of detection (LOD_m) was determined as the value, at which a positive dilution is detected by the estimated model with a probability of at least 95%. Furthermore, the 0.95 quantile of the limits of detection corresponding to the different replications ($LOD_{0.95}$) was calculated.

Results

In the first run, one value was questionably negative and therefore set as “missing value” in the analysis. The estimated models of the 1000 bootstrapping replications, as well as the resulting median and 0.95 quantile of the limit of detection are shown in Figure 9. The median 95% LOD for the nptII Real Time TaqMan Assay lies at 7.9 Copies/PCR assay with a 0.95 quantile of 10.1 copies/assay. This means that the PCR system delivers a positive signal in 95 out of 100 cases if the number of copies in the assay exceeds 7.9.

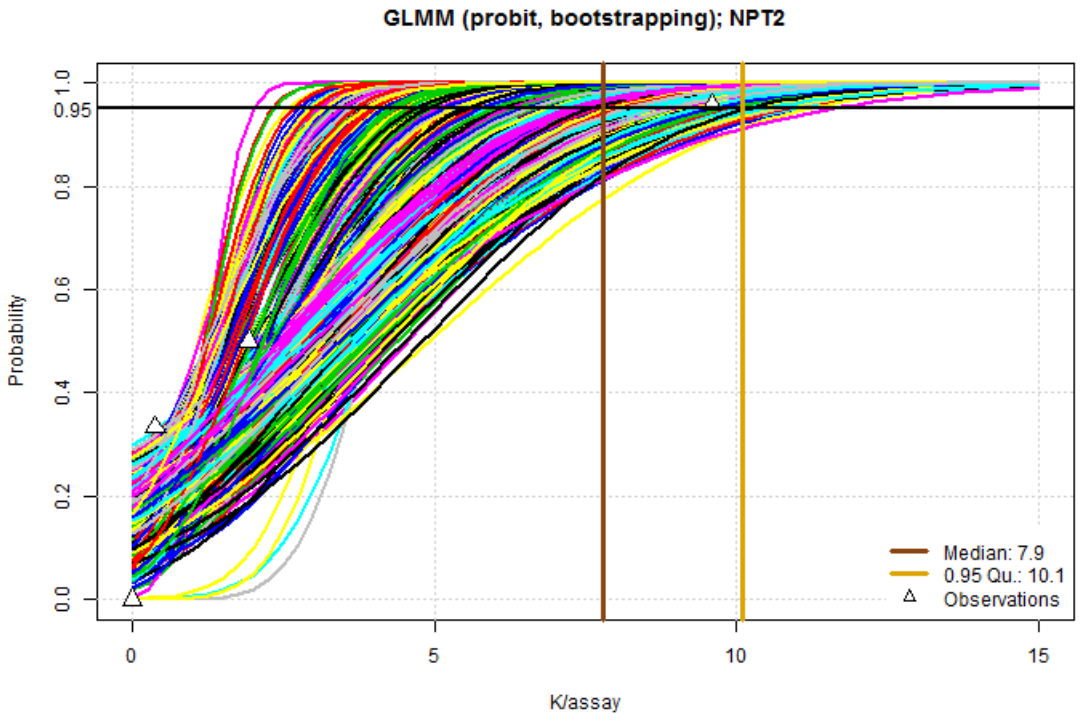

Figure 9: 95% LOD for nptII, estimated using a probit GLMM with 1000 bootstrapping replications. The resulting median limit of detection is LOD_m = 7.9 (brown) with a 0.95 quantile of $LOD_{0.95}$ = 10.1 (gold).

1.2.8 95% limit of detection: nptIII – TaqMan Single Assay

Data

For the analysis of the 95% LOD for nptIII, the results of six PCR runs were considered. One replicate (replicate 7) had to be removed in run 1, because the zero-solution delivered a positive signal. Two runs performed only three and four replicates, respectively, instead of the usual eight replicates. A total of 418 observations were available for the analysis. The numbers of observations per dilution level are shown in Table 4.

Copies/assay	Number of observations
0	38
0.38	38
1.92	38
9.6	38
48	38
240	38
1200	38
6000	38
30000	38
150000	38
750000	38

Table 4: Number of observations for the determination of the limit of detection for nptIII.

Method

See section 1.2.7.

Results

The analysis of the available data yielded a median 95% limit of detection (LOD) of 11.4 copies/assay and a 0.95 quantile of 13.3 copies/assay for the nptIII Real Time TaqMan Assay (see Figure 10).

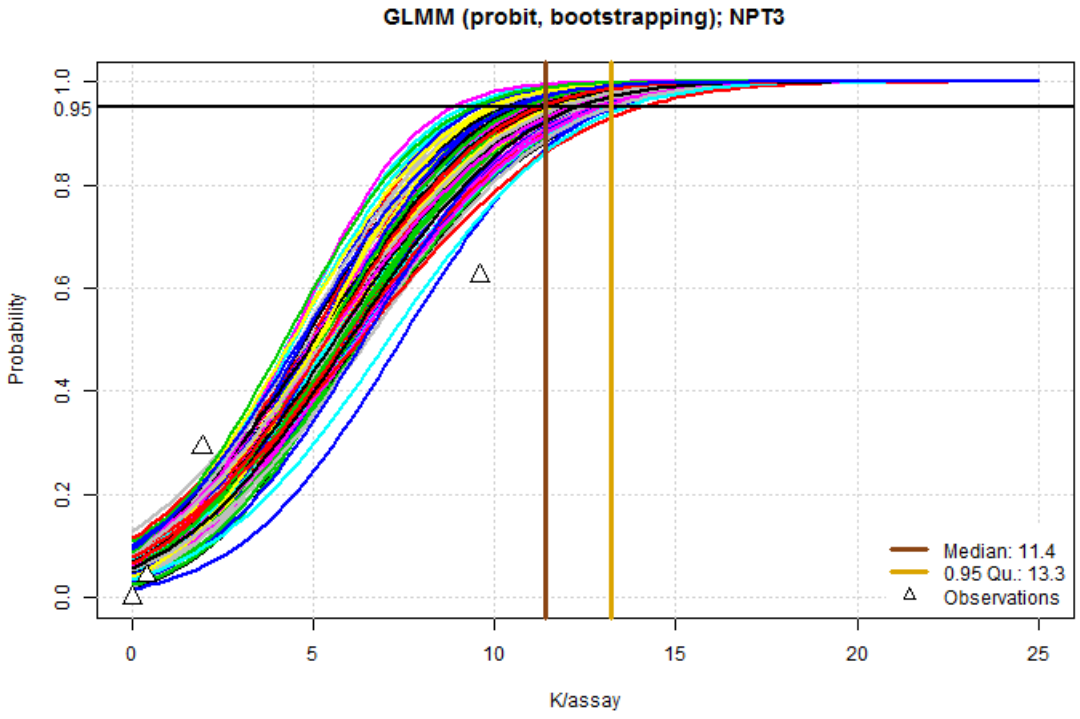

Figure 10: 95% LOD for nptIII, estimated using a probit GLMM with 1000 bootstrapping replications. The resulting median detection limit is LODm = 11.4 (brown) with a 0.95 quantile of LOD0.95 = 13.3 (gold).

1.2.9 Limit of detection: 16S rRNA – TaqMan Single Assay

The determination of the 95% LOD was not practical for the 16S rRNA TaqMan assay, as the ubiquitous occurrence of 16S rDNA (it can, e.g., be introduced via a contaminated Taq polymerase in the assay) leads to the generation of a constant background signal. For the negative control (H_2O), the background signal lies at a Cp-value of approximately 34. Values above this level, can however be quantified and the quality parameters of the PCR of a dilution series can be identified. Hence, the limit of quantification (LOQ) is rather determined for the 16S rRNA TagMan system.

1.2.10 Limit of quantification: nptII – TaqMan Single Assay

Data

Five different runs with eight replications each and a number of different dilution levels were analyzed in order to determine the limit of quantification (LOQ). Table 5 shows the number of observations, for which the real-time PCR was able to detect the gene. For run 1 and 2, the observations were only done up to a dilution of 30000 copies/assay. A total number of 233 observations were available for the determination of the limit of quantification. Questionable observations were not included in the analysis.

Copies/assay	Number of observations
0	0
0.38	0
1.92	0
9.6	17
48	24
240	24
1200	40
6000	40
30000	40
150000	24
750000	24

Table 5: Limit of Quantification: Numbers of observations for the determination of the LOQ for nptII.

Method

A common approach for the calculation of the LOQ is the use of a calibration curve. For this method, each logarithmised dilution is plotted against the corresponding Ct-value. A regression curve is subsequently estimated using linear regression. The LOQ is then calculated as follows:

LOQ = 10*sigma/S,

where S denotes the slope of the calibration curve and sigma is the standard deviation of the response variable.

Results

For nptII, the computations yielded a limit of quantification (LOQ) of 43 copies/assays, see Figure 11.

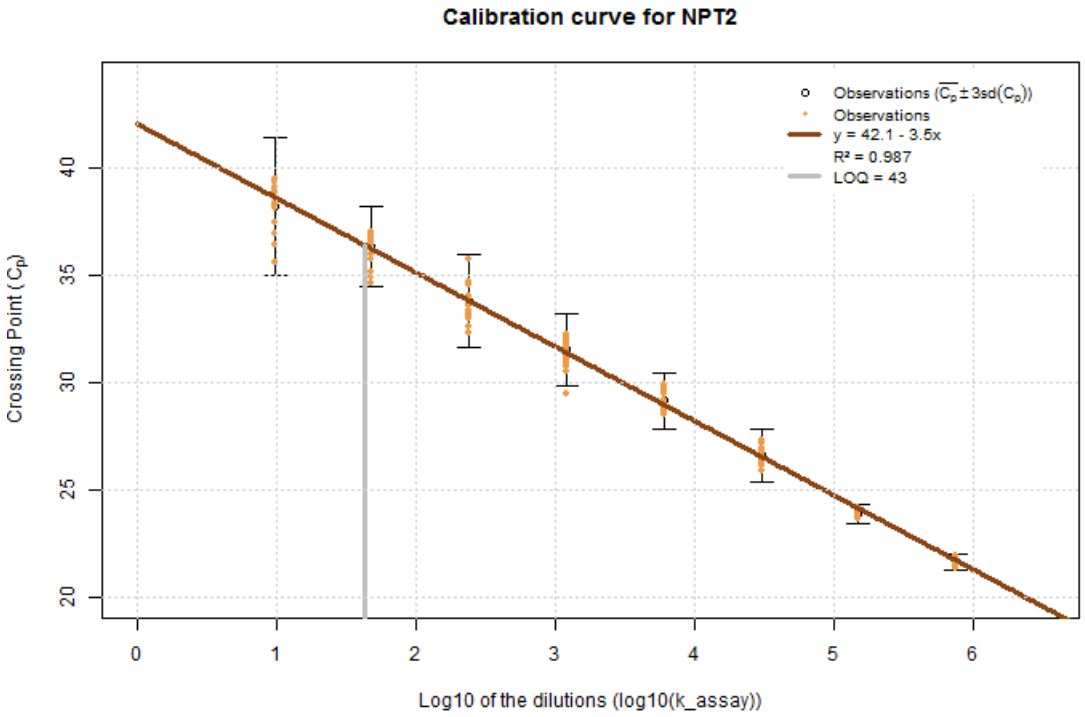

Figure 11: Limit of quantification: Calibration curve for nptII.

1.2.11 Limit of quantification: nptIII – TaqMan Single Assay

Data

The six PCR runs (in total 292 observations) were used for the calculation of the LOQ for nptIII. The numbers of observations for each dilution are given in Table 6.

Copies/Assay	Number of observations
0	0
0.38	1
1.92	10
9.6	28
48	33
240	37
1200	39
6000	39
30000	39
150000	39
750000	27

Table 6: Limit of Quantification: Numbers of observations for the determination of the LOQ for nptIII.

Method

The same method as in section 1.2.10 “Limit of quantification: nptII – TaqMan Single Assay” was used.

Results

The analysis for nptIII resulted in a limit of quantification (LOQ) of 318.1 copies/assay (see Figure 12).

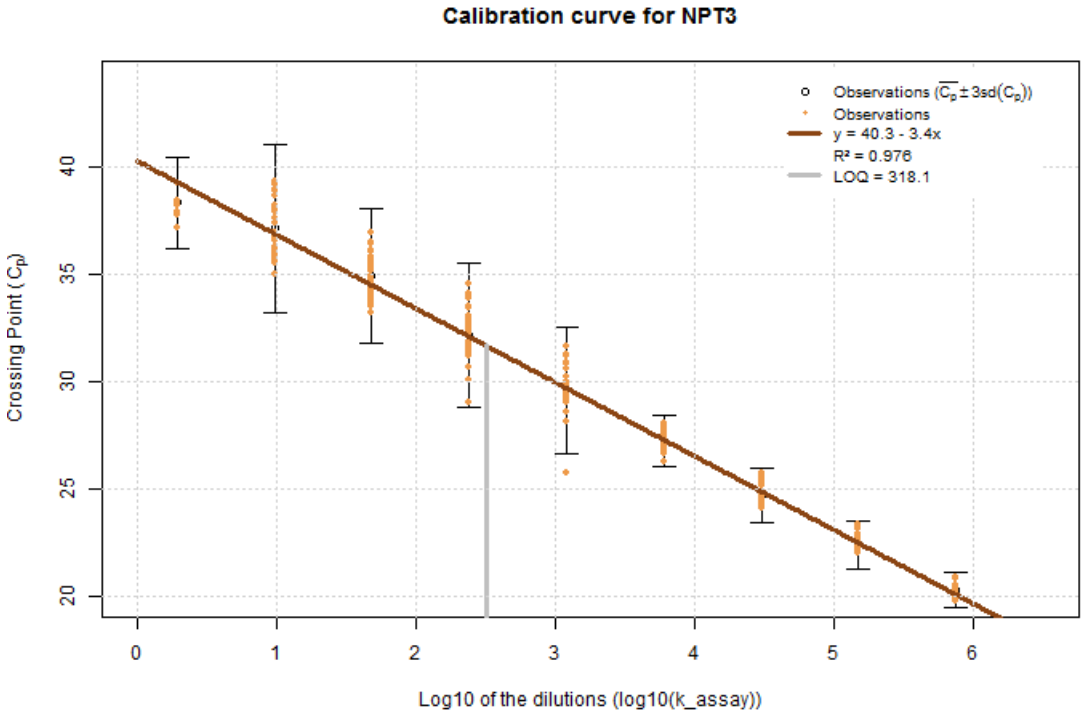

Figure 12: Limit of quantification: Calibration curve for nptIII.

1.2.12 Limit of quantification: 16S – TaqMan Single Assay

Data

The data of three PCR runs were used to calculate the LOQ for 16S. Due to the particularly high Ct-values of the highly diluted solutions (low number of copies/assay; 16S rDNA background signal), only dilutions of 48 copies/assay and higher were considered for the linear regression. In total, 168 observations were available for the analysis.

Method

The same method as in section 1.2.10 “Limit of quantification: nptII – TaqMan Single Assay” was used.

Results

For 16S, a limit of quantification (LOQ) of 56.2 copies/assay was determined (see Figure 13).

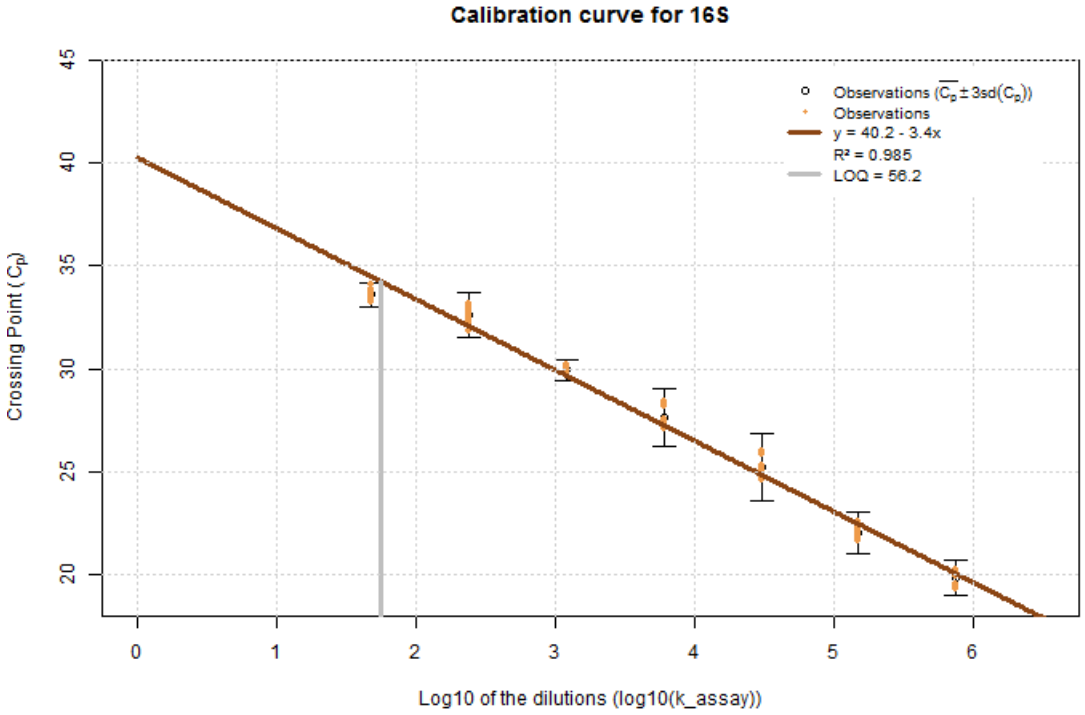

Figure 13: Limit of quantification: Calibration curve for 16S.

1.2.13 Inter- and Intra-Assay variability: nptII TaqMan Single Assay

For inter- and intra-assay variability values were analyzed above a concentration of 48 copies / assay.

The coefficient of variation (CV) for the **intra-assay** variability is between 0.19 to 1.97 percent (Table 7).

The CV for the **inter-assay** variability is between 0.6 to 2.13% (Table 7).

Therefore the system was stable.

nptII TaqMan Assay	Intra-assay variability	Inter-assay variability
SD min	0.05	0.13
SD max	0.7	1.07
CV (%) min	0.19	0.60
CV (%) max	1.97	2.13
Measurements	216	216

Table 7: Evaluation intra- and inter-assay variability (reproducibility): nptII TaqMan assay.

The standard curve parameters were determined via LightCycler software using the Second Derivative Maximum algorithm. The standard curve parameters show a low error, good amplification efficiencies and a good increase (slope) of the curves (Table 8). The generated standard curves were reliable.

Error	0.00252	0.00466	0.0239	0.0261	0.0987
Efficiency	1.82	1.957	1.955	1.88	1.922
Slope	-3,82	-3.43	-3.436	-3.649	-3.524
Y-intercept	44,2	40.81	41.42	42,03	40.53
Link:	0	244.9	216	7.831	243.3
PCR run No.:	1	2	3	4	5
Error minimum:		0.00252			
Error maximum:		0.0987			
Efficiency minimum:		1.82			
Efficiency maximum:		1.957			
Slope minimum:		-3.43			
Slope maximum:		-3,82			

Table 8: Evaluation standard / calibration curve: LC480 nptII TaqMan assay.

1.2.14 Inter- and Intra-Assay Variability: nptIII TaqMan Single Assay

For inter- and intra-assay variability values were analyzed above a concentration of 48 copies / assay.

The coefficient of variation (CV) for the **intra-assay** variability was between 0.1 to 5.15 percent (Table 9).

The CV for the **inter-assay** variability was between 1.34 and 3.46 percent (Table 9).

Therefore the system was stable.

nptIII TaqMan Assay	Intra-assay variability	Inter-assay variability
SD min	0.02	0.27
SD max	1.51	1.11
CV (%) min	0.10	1.34
CV (%) max	5.15	3.46
Measurements	253	253

Table 9: Evaluating intra-and inter-assay variability: nptIII TaqMan assay.

The standard curve parameters were determined via LightCycler LC480 software using the Second Derivative Maximum algorithm. The standard curve parameters showed a low error, good amplification efficiencies and a good increase (slope) of the curves. The generated standard curves were reliable.

Error	0.0261	0.0615	0.0241	0.0779	0.012	0.0303
Efficiency	1.927	1.976	1.876	1.923	1.972	1.954
Slope	-3.509	-3.381	-3.66	-3.522	-3.392	-3.46
Y-intercept	42.19	41.11	41.56	40.72	39.12	39.47
Link:	0	0	0	2.858	223.7	0
PCR run No.:	1	2	3	4	5	6

Error minimum:	0.012
Error maximum:	0.0779
Efficiency minimum:	1.876
Efficiency maximum:	1.976
Slope minimum:	-3.381
Slope maximum:	-3.66

Table 10: Evaluation standard / calibration curve: LC480 nptIII rRNA TaqMan Assay.

1.2.15 Inter- and Intra-Assay Variability: 16S rRNA TaqMan Single Assay

For inter-and intra-assay variability values above a concentration of 48 copies / assay were used for statistical evaluation.

The CV for the intra-assay variability for 16S rRNA Single Assay was between 0.16 and 0.70 percent (Table 11).

The CV for the inter-assay variability was between 0.58 and 2.14 percent (Table 11).

Therefore the system was stable.

16S TaqMan Assay	**Intra-assay variability**	**Inter-assay variability**
SD min	0.04	0.17
SD max	0.24	0.54
CV (%) min	0.16	0.58
CV (%) max	0.70	2.14
Measurements	168	168

Table 11: Evaluating intra-and inter-assay variability: 16S rRNA TaqMan Assay.

The standard curve parameters were determined via LightCycler software using the Second Derivative Maximum algorithm. The standard curve parameters show a low error, good amplification efficiencies and a good increase (slope) of the curves (Table 12). The generated standard curves were reliable.

Error	0.0286	0.0126	0.0476
Efficiency	1.87	1.854	1.813
Slope	-3.679	-3.73	-3.872
Y-intercept	40.91	41.87	41.48
Link:	1540	0	0
PCR run No.:	1	2	3
Error minimum:		0.0126	
Error maximum:		0.0476	
Efficiency minimum:		1.813	
Efficiency maximum:		1.87	
Slope minimum:		-3.679	
Slope maximum:		-3.872	

Table 12: Evaluation standard / calibration curve: LC480 16S rRNA TaqMan Assay

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Die vorliegende Studie beschäftigt sich mit der Häufigkeit von nptII und nptIII Antibiotikaresistenzgenen in natürlich vorkommenden Bakterienpopulationen in Österreich. [illegible]

The current study presents data concerning the prevalence of nptII and nptIII antibiotic resistance genes in naturally occurring bacterial populations in Austria. The aim of the project was to provide a comprehensive presentation of the status quo concerning the natural background load of relevant pristine Austrian ecosystems with nptII and nptIII genes ahead of an eventual cultivation of transgenic plants carrying antibiotic resistance marker genes.