TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Grundwasserökologie

Systematic investigation of antibiotic producers in groundwater

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. R. F. Vogel

Prüfer der Dissertation:

1. Univ.-Prof. Dr. R. Meckenstock
2. Univ.-Prof. Dr. S. Scherer

Die Dissertation wurde am 27.06.2008 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 17.10.2008 angenommen.

Danksagung

Ohne die Unterstützung zahlreicher Personen wäre diese Arbeit nicht entstanden.

Herrn Prof. Dr. Rainer Meckenstock danke ich für das interessante Thema und die Betreuung als Doktorvater am Institut für Grundwasserökologie, Helmholtz Zentrum München.

Herrn Prof. Dr. Siegfried Scherer gilt mein Dank für seine Bereitschaft die Aufgabe des 2. Prüfers zu übernehmen und Herrn Prof. Dr. Rudi Vogel für den Vorsitz während des Prüfungsverfahrens.

Dr. Agnes Fekete danke ich für ihre unermüdliche Hilfe und Betreuung bei allen Fragestellungen zur analytischen Chemie. Die Arbeit an HPLC, FT-ICR-MS und UPLC Geräten am Institut für Ökologische Chemie wäre außerdem nicht möglich und zudem erfolgreich gewesen ohne die Unterstützung von PD Dr. Philipe Schmitt-Kopplin, Dr. Georg Matuschek, Dr. Bassem Kanawati und Dr. Jutta Lintelmann.

Meine Kolleginnen und Kollegen sorgten für ein sehr angenehmes Arbeitsklima am Institut, herauszuheben sind Brigitta Friesleben als Unterstützung in allen Bürokratie- und Lebensfragen, Dietmar Jurrat für stets gefüllte Materialschränke und Gerätebetreuung und Michael Stöckl als Pannenhelfer bei Computerproblemen. Draženka Selesi, Muna Mangalo und Christine Stumpp lasen Teile dieser Arbeit, korrigierten den einen oder andern Fehler oder halfen bei der Formatierung. Zum Glück wurden viele Mitdoktoranden mehr als nur Kollegen und so sorgten vor allen Dingen Christine Stumpp, Muna Mangalo, Bettina Anneser, Rita Kleemann, Marc Schwientek und Robert Bauer dafür, dass ich mich während der gesamten Zeit in München sehr wohlgefühlt habe. Heike Brielmann war die beste Bürokollegin und darüber hinaus weit mehr, obwohl sie es in einem Fall absolut nicht beabsichtigt hat, vielen Dank dafür.

Stefan Rieger war zur richtigen Zeit am richtigen Ort, dafür kann ich nicht danken, aber für alles was er bis jetzt für mich gemacht hat und was zum Glück weit entfernt von der Mikrobiologie war.

Meinen Eltern danke ich dafür, dass sie nie an meinen Plänen und Entscheidungen gezweifelt haben und mich ohne zögern meinen eigenen Weg gehen ließen und natürlich für meinen Bruder, ohne ihn wäre es langweilig gewesen und viele Erfahrungen würden fehlen.

Abstract

The aim of this work was the detection of antibiotic producers in groundwater systems, the characterisation of isolated antibiotic producers, and analysis of the antibiotically active substances of the new isolated strains.

The systematic investigation of one pristine and one contaminated aquifer proofed the appearance of multiresistant antibiotic producers in both habitats. High cultivation efficiencies with 33 % in the pristine aquifer and up to 79 % in the contaminated aquifer were reached and the amount of antibiotic producers was in the range of 0.4 % - 0.6 % in the pristine and 9.5 % in the contaminated aquifer. No influence of geological or chemical parameters on the appearance of antibiotic producers was found and also cultivation efficiency was irrelevant for the amount of antibiotic producers. During the cultivation approaches 38 antibiotic producers in total were found. However, only two strains (from the pristine aquifer) carried on with antibiotic production during several cultivation steps. Due to metabolic properties and 16S rDNA gene sequence analysis these strains were related to the genus *Pseudomonas fluorescens*. With the help of HPLC, FT-ICR-MS and UPLC the produced antibiotic was identified as mupirocin. The semiquantitative analysis resulted in an amount of 1.7 – 3.5 mg/l of mupirocin in the culture supernatant.

An experimental proof for the antibiotic production *in situ* was planned and partially conducted with a column experiment and the help of TRFLP analysis. Sediment material from the sampled pristine aquifer and one isolated antibiotic producer was used. Pre-experiments showed that this proof was unrealisable this way. The dominant appearance of one restriction fragment at 490 bp, which was totally the same as for the antibiotic producer, pointed to a distinct appearance of pseudomonads in the used sediment.

Zusammenfassung

Das Ziel der Arbeit war der Nachweis von Antibiotikaproduzenten in Grundwassersystemen, die Charakterisierung isolierter Bakterienstämme und die Identifikation/Analyse der produzierten antibiotischen Substanzen.

Die systematische Untersuchung eines unbelasteten und eines belasteten Aquifers brachte den Nachweis für multiresistente Antibiotikaproduzenten in diesen Habitaten. Bei hohen Kultivierungseffizienzen von bis zu 33 % im unbelasteten und 79 % im belasteten Grundwasser konnten 0.4 % - 0.6 % Antibiotikaproduzenten im unbelasteten und 9.5 % Antibiotikaproduzenten im belasteten Grundwasser gefunden werden. Ein Einfluss von geologischen oder chemischen Eigenschaften des Aquifers auf das Vorkommen von Antibiotikaproduzenten konnte nicht festgestellt werden, auch die Kultivierungseffizienz spielte dafür keine Rolle.

Bei den Kultivierungsansätzen wurden insgesamt 38 Antibiotikaproduzenten isoliert, von denen nur zwei Stämme (aus dem unbelasteten Aquifer) auch über mehrere Kultivierungsschritte Antibiotika produzierten. Diese wurden mit Hilfe von metabolischen Eigenschaften und 16S rDNA Analyse der Art *Pseudomonas fluorescens* zugeordnet und das produzierte Antibiotikum mit Hilfe von HPLC, FT-ICR-MS und UPLC als Mupirocin identifiziert. Eine semiquantitative Analyse des produzierten Mupirocin ergab einen Gehalt von 1.7 – 3.5 mg/l Mupirocin im Kulturüberstand.

Der geplante Nachweis über den Einfluss dieser Antibiotikaproduzenten auf die mikrobielle Gemeinschaft im Sediment des ursprünglichen Aquifers, mit Hilfe eines Säulenversuchs und TRFLP Analysen, konnte nach einigen Vorversuchen nicht zu Ende geführt werden. Das dominante Auftreten eines Restriktionsfragments mit 490 bp entsprach dem des einzusetzenden Antibiotikaproduzenten und ließ auf ein ausgeprägtes Vorkommen von *Pseudomonaden* im verwendeten Sediment schließen.

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List of Abbreviations

AFW	artificial fresh water medium
AHL	acetyl homoserine lactone
ATCC	American type culture collection
ATP	adenosine triphosphate
BHL	N-butanoyl-l-homoserine lactone
BTEX	benzene, toluene, ethylbenzene, xylene
cAMP	cyclic adenosine monophosphate
CID	collision induced dissociation
DAPI	4',6-diamidino-2-phenylindole
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
FT-ICR-MS	Fourier-transform ion cyclotron resonance mass spectrometry
HPLC	high performance liquid chromatography
MIC	minimum inhibitory concentration
MPN	most probable number
MRSA	methicillin resistant *Staphylococcus aureus*
NAG	N-acetylglucosamine
NAM	N-acetyl muramic acid
NB	nutrient broth medium
OHHL	N-(3-oxohexanoyl)-l-homoserine lactone
PAH	polycyclic aromatic hydrocarbone
PBP	penicilline binding protein
PFA	paraformaldehyde
PHB	poly-3-hydroxybutyrate
Str	*Streptomyces* medium

TRFLP	terminal restriction fragment length polymorphism
UPLC	ultra performance liquid chromatography

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1 Introduction

For over 60 years antibiotics are used for medical application and can be declared as very important and highly effective substances in medicine. Many diseases lost their scare but soon it was realised that no magic bullet was found. The possibility that pathogens do not respond to the treatment was detected three years after mass production of penicillin and resistant pathogens are wide spread. There is a constant need for new antibiotics but the last years were not very productive for new antibiotics. Fewer products were introduced in the market than from the starting in the 1930th. The last new antibiotic class were the lipopeptides with daptomycin as representative (Butler, 2005, Clardy, *et al.*, 2006, Lam, 2007). Many pharmaceutical companies quit the search for antibiotics or minimised there budget (Projan, 2003). The research on finding new antibiotics was mostly focused on soil because of the antibiotic-producing *Streptomycetes*, and bacterial biocontrol as a hint for antibiotic production from soil organisms (Mazzola, 2002, Baltz, 2006).

The other way to get new antibacterial compounds was the use of combinatorial chemistry or molecular methods to directly find new targets in the microorganisms (Freiberg & Brotz-Oesterhelt, 2005, Baltz, 2006). Also plants, insects, and higher animals were used to detect new antibacterial substances (Sheridan, 2006). However, groundwater sediment did not get into the focus of antibiotic research and also a complete systematic detection of resistant organisms in groundwater did not occur. With taking into account that antibiotics are often modifications of natural products and less than half of all antibiotics are totally synthetically produced (Champness, 2000) a new natural environment should be investigated for antibiotic-producing bacteria.

1.1 Aim of the Work

The search for antibiotics was conducted in many environments and with different approaches but groundwater sediment was neglected from this research until now. Therefore, the natural background of antibiotic concentrations or antibiotic producers in this environment is unknown so far. Important questions are if new antibacterial substances can be found in groundwater and if concentrations *in situ* are high enough to show an antibiotic effect on surrounding organisms. High interest for antibiotic producers can be found in rhizosphere research. A control of plant pathogens with natural bacterial communities could be observed here (Raaijmakers*, et al.*, 2002, Garbeva*, et al.*, 2004). It was also possible to isolate anaerobic bacterial strains from soil samples, which were tested for antibiotic production with specific test strains (Sturgen & Casida, 1962). This work was no longer followed up and no further publications on this field could be found. A direct reference from soil and groundwater can not be made because of different conditions in these habitats. But it can be presumed that everywhere we can find bacteria also antibiotic producers might be detectable, maybe especially in groundwater because the competition for nutrients in this nutrient poor environment maybe higher. It would be important to show if antibiotic-producing bacteria occur in groundwater and if the produced concentrations are high enough to show an effect.

The risk caused by drugs reaching the groundwater directly or by insufficient waste water treatment is repeated (Sacher*, et al.*, 2001, Kreuzinger*, et al.*, 2004). However, if there would be an evidence that bacteria itself bring a certain concentration of antibiotics into the groundwater the whole discussion should be reconsidered.

The cultivation of bacteria from special habitats emerged as very difficult and only about 1 % of all bacteria could be cultivated under lab conditions (Amann*, et al.*, 1995). To find unknown bacteria in groundwater and if and how much antibiotic producers occur, and maybe to get new antibiotics we have to follow another direction. Today's cultivation methods under aerobic conditions and with complex cultivation media bring along the same bacterial classes or organisms every time. An encouraging way is the high throughput cultivation. With this method it is possible to

get many bacterial cultures with low costs. A special cultivation medium (low nutrient conditions) can help to turn groundwater bacteria into laboratory cultures. Selected test organisms should help to identify produced antibiotics. These investigations then state the natural frequency and the influence on the surrounding bacterial community of antibiotic producers in groundwater. Possible differences between groundwater bodies will be detectable more easily and new antibiotic producers can be characterised in detail. To complete the work antibiotically active substances of antibiotic producers from groundwater or sediment samples may be analysed with analytical techniques like high performance liquid chromatography (HPLC), Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and ultra performance liquid chromatography (UPLC).

2 State of the Art

2.1 Cultivation of Bacteria

Many investigations deal with the cultivability of bacteria, especially for bacteria from characteristic habitats cultivation is complicated because adaptation to given conditions is huge. The phenomenon of “plate count anomaly” is well known and the fact that direct microscopic cell counts are much higher than viable cell counts shows the great problem with cultivation of bacteria (Amann*, et al.*, 1995). To get more information about uncultured organisms molecular techniques were helpful and the uncultivated diversity was visualised (Green & Keller, 2006). The knowledge alone on uncultivated bacteria is not sufficient we need to know if the uncultivated bacteria have the opportunity to produce antibiotics in laboratory cultures. Furthermore, detection of the DNA of an organism found in a habitat is surely no evidence for present activity or if only a resting form was found. Modifications at cultivation media were made and effects of growth inducers and signalling molecules were tested to get the preferably highest amount of cultivable organisms (Bussmann*, et al.*, 2001, Bruns*, et al.*, 2002). Cultivation media are either defined media with specific salts, sugar, amino acids, or other ingredients, these are minimal media. Complex media are from not defined components like meat- or yeast extract. But it is also possible to get complex media with low nutrient conditions. Fundamentally necessary for every bacterium and therefore for every medium are energy-, carbon-, nitrogen-, and sulphur sources and alike some ions (salts). Also an addition of buffer components is important. Amino acids, basis, vitamins, or signalling molecules can boost growth of bacteria and are used as growth substance. Media with low substrate concentrations are applicable for oligotrophic habitats, but there are no media developed especially for groundwater habitats. One possibility to get a medium for cultivation is to take water from the initially settled habitat. Groundwater is then filtered, sterilized and added with buffer components and growth substances. This method was used for sea water habitats and new bacteria could be cultivated (Zengler*, et al.*, 2002). Also diluted and sieved sediment was successfully used as cultivation basis (Vester &

Ingvorsen, 1998). To get defined conditions for cultivation it would be helpful to use a defined medium for groundwater bacteria. Therefore, an artificial freshwater medium from Bartsch & Overmann (Bartscht*, et al.*, 1999) was tested. Important for successful cultivation and antibiotic production are special signal molecules or defined substrate concentrations. An addition of acetyl homoserine lactones (AHLs), cyclic adenosine monophosphate (cAMP) and adenosine tri phosphate (ATP) raises cultivability (Bruns*, et al.*, 2003). Also oxygen concentration seems to affect cultivability (Bussmann*, et al.*, 2001), however, in this work also anaerobe conditions were investigated.

2.2 Antibiotics

Antibiotics are from the original definition low molecular products of the metabolism of microorganisms. The molecular mass is M < 2000 Da, and small concentrations of < 200 µg/ml inhibit the growth of microorganisms (Waksman & Lechevalier, 1949). The term antibiotic is derived from the Greek (anti= against, biotikos= belonging to life) and means “against live”. This definition is no longer precise because antibiotics with higher molecular masses were isolated and chemical produced antibiotics (chemotherapeutics) are on the market.

Antibiotics are products of the secondary metabolism of bacteria and do not play a role for vegetative development of antibiotic producers. This raises the question why some organisms have these metabolic pathways and the relevant genes. Maybe the molecules used now as antibiotics had another implication and were in former times displaced by more effective molecules. But the genes for these precursor molecules are not lost. These could be approved that some molecules against protein synthesis show effects on gene transfer, cell growth, translation, transcription, and mutagenesis (Davies, 1990).

Further discussions on the value of secondary metabolites include the following points:

- secondary metabolites are build to eliminate toxic depletion products
- secondary metabolites could be storage molecules with posterior benefit
- secondary metabolites are produced as special inter or intra cellular signalling molecules (Gräfe, 1992)

To isolate antibiotic producers from aquifer sediment signalling molecules and substrates should stimulate antibiotic production (Demain, 1998) and fulfil the special conditions to start the secondary metabolite production. The antibiotic biosynthesis pathways are complicated and a big number of genes need to be expressed. Different signalling molecules are known to stimulate secondary metabolite synthesis. Butanolides, found in *Actinomycetes*, acetyl homoserin lactones (AHLs) in Gram-negative bacteria (Whitehead*, et al.*, 2001), Oligopeptides in Gram-positive organisms and B-factor (3-(1-butylphosphoryl)-adenosine) in *Amycolatopsis mediterranii* (Kawaguchi*, et al.*, 1988, Demain, 1998) belong to this molecules. For example antibiotic production in *Erwinia carotovora* is regulated by OHHL (N-(3-Oxohexanoyl)-L-homoserinlacton, an AHL), temperature, and carbon source (Coulthurst*, et al.*, 2005).

Primers with the known sequences can be used to detect genes for antibiotic production. Many antibiotic synthesis genes are coded in clusters and the corresponding resistance gene is often also in this gene cluster (Martin & Liras, 1989). The diversity is high and it is not possible to find the sequences for all antibiotic classes and it would be also not possible to find something unknown with detecting genes for antibiotic production.

Depending on their structure and effect antibiotics are divided into several classes. A short overview about naturally and synthetically synthesised antibiotics is given in Table 1. Further information can be required from the following literature (Demain, 1983, Piepersberg, 1995, Brickner, 1996, Kleinkauf & VonDohren, 1996, Drlica, 1999, Scholar, 2000, Hoffmann*, et al.*, 2002, Walsh, 2003, Süssmuth & Wohlleben, 2004, Brogden, 2005, Chatterjee*, et al.*, 2005, Coulthurst*, et al.*, 2005, Franklin &

Snow, 2005, Katz & Ashley, 2005) and a short overview of the most important antibiotic classes should be given in this work also.

Table 1 Antibiotic classes and mechanism of action.

Antibiotic classes **Subclasses**	**Important substances**	**Mechanism of action**
ß-Lactam antibiotics		Peptidoglycane transpeptidase is inhibited by binding of the ß-lactam ring, cell wall synthesis is averted.
Penicillin	Penicillin G Ampicillin, Methicillin	
Cephalosporins	Cephalotin	
Cephamycins	Cephamycin C	
Carbapenems	Meropenem	
Monobactams		
Polyketides		
	Mupirocin	Inhibition of isoleucyl t RNA synthesis
Macrolides	Erythromycin	Inhibition of protein synthesis by binding through 50S subunit of ribosomes. Elongation of proteins impossible.
Tetracyclines	Tetracycline, Doxycycline	Inhibition of protein synthesis by binding through 30S subunit of ribosomes
Lincosamide	Clindamycin, Lincomycin	Similar to macrolides
Aminoglycosides	Neomycin, Kanamycin Amikacin, Gentamicin Tobramycin, Rifampicin Streptomycin	Binding through 30S-subunit of ribosomes, no more binding of N-Formylmethionyl-t-RNA possible, start of protein synthesis stopped. Additionally aminoacyl-t-RNA can not be bind to ribosomes and the proteins can not be elongated.
Gyrase inhibitor	Nalidixic acid, Cibrofloxacin	Binding through Topoisomerases II; No gyrase effect.
Chloramphenicols	Chloramphenicol, Thiamphenicol	Inhibition of protein synthesis by binding through 50S subunit of ribosomes.
Peptide antibiotics		Cytoplasmic membrane is attacked and gets porous. Vancomycin: Inhibition of peptidoglycane cross linking
Lantibiotics	Nisin, Subtilin	
Glykopeptides	Vancomycin	
Polypepetids	Bacitracin, Colistin Polymyxin	
Nitroimidazoles	Metronidazol, Ornidazol Tinidazol	Impairment and death of bacteria by reaction with DNA bases
Oxazolidinones	Linezolid	Formation of initiation complex of protein synthesis is blocked.
Sulfonamides	Trimethoprim	Folic acid synthesis is disturbed and growth of bacteria not possible

Another classification of antibiotics is the mode of action in the target organisms. The targets in bacteria can be absent or modified in the host to minimize the danger of side effects. Important mechanisms are:

- inhibition of cell wall biosynthesis (ß-lactames, vancomycin)
- inhibition of protein synthesis by blocking of 30S or 50S subunit of the ribosomes (aminoglycosides, tetracyclines, macrolides)
- inhibition of DNA Replication (gyrase inhibitor, ciprofloxacin)
- inhibition of folic acid biosynthesis (sulfonamides, trimethopim)
- destruction of the bacterial cell wall by peptide antibiotics.

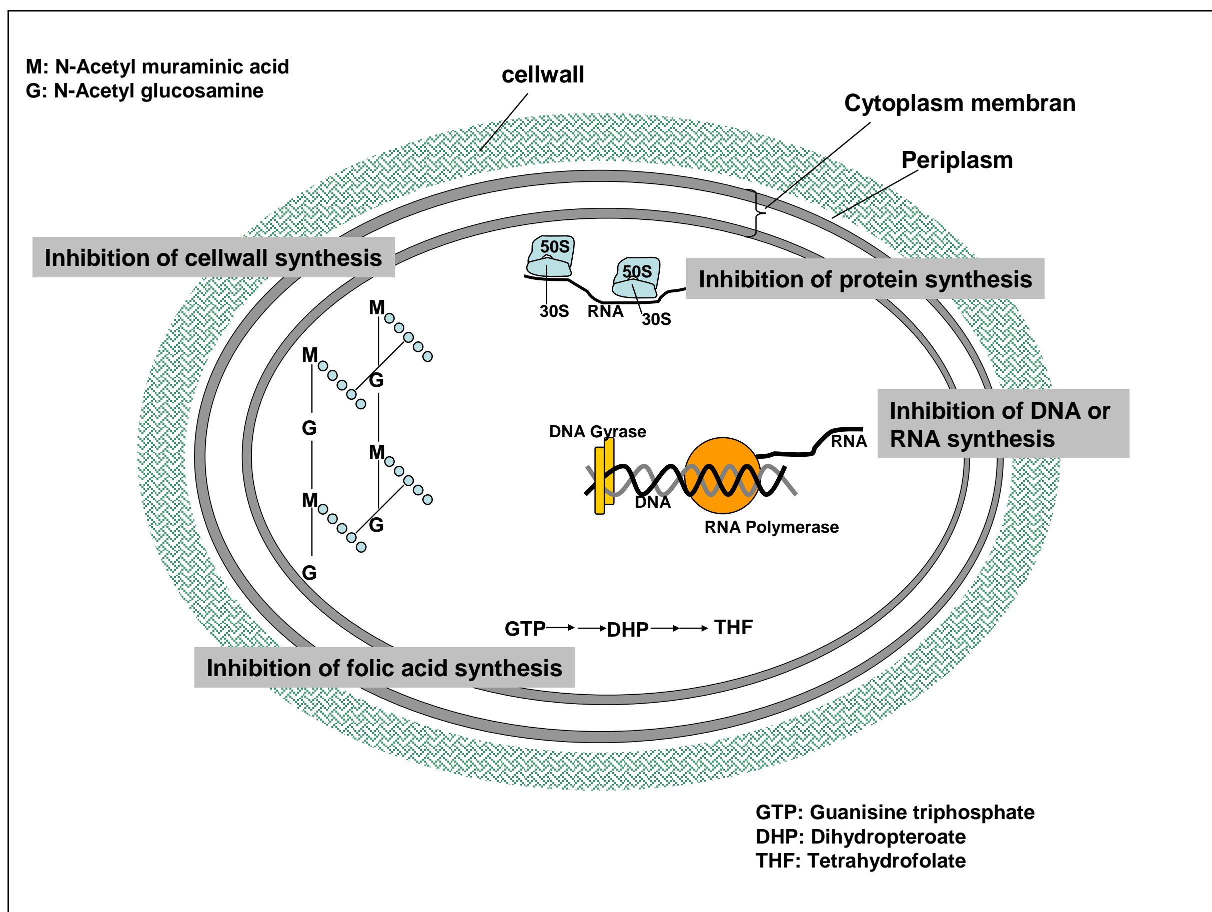

Figure 1 Mode of action of antibiotics (Modified from Walsh (2003)).

Depending on the mode of action an antibiotic has a bacteriostatic or bactericidal effect. Bacteriostatic substances inhibit cell division but do not kill the bacteria directly like in the case of bactericidal substances. Bactericidal effects often occur only during cell division like in ß-lactam antibiotics.

2.2.1 Antibiotic classes

2.2.1.1 ß-lactam antibiotics

β-lactam antibiotics, as the most widely-used group of antibiotics available, include penicillin derivatives, cephalosporins, cephamycins, carbapenems, monobactams, and β-lactamase inhibitors. All members of this broad antibiotic class contain a β-lactam nucleus in its molecular structure.

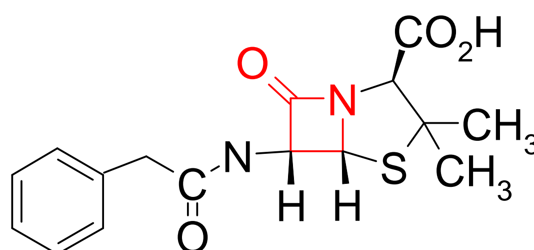

Figure 2 Penicillin G as representative of the ß-lactam antibiotics. The red part marks the typical ß-lactam structure.

The ß-lactams also belong to the nonribosomally derived peptide antibiotics (see below). Mainly active against Gram-positive bacteria, the recently developed substances of broad-spectrum β-lactam antibiotics are also active against various Gram-negative organisms. The bactericidal ß-lactam antibiotics inhibit the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for a stable cell wall, especially in Gram-positive organisms. The final transpeptidation step in the synthesis of the peptidoglycan is catalysed by transpeptidases, also known as penicillin-binding proteins (PBPs). D-alanyl-D-alanine are the terminal amino acid residues on the precursor N-acetyl muraminic acid (NAM)-/N-acetyl glucosamine (NAG)-peptide subunits of the developing

peptidoglycan layer. The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine allows their binding to the active site of penicillin-binding proteins (PBPs). The β-lactam ring binds irreversibly to the Ser_{403} residue of the PBP active site and prevents the final crosslinking of the growing peptidoglycan layer resulting in disrupting cell wall synthesis. Inhibition of cross-linkage by β-lactams causes also an accumulation of peptidoglycan precursors, which stimulates autolytic hydrolases to digest existing peptidoglycan without the production of new peptidoglycan. The bactericidal action of β-lactam antibiotics is additionally enhanced.

2.2.1.2 Macrolide antibiotics

The macrolides, belonging to the polyketide class of natural products are a group of antibiotics whose activity stems from the presence of a macrolide ring (usually 14, 15 or 16-membered), a large macrocyclic lactone ring to which one or more deoxy-sugars can be attached.

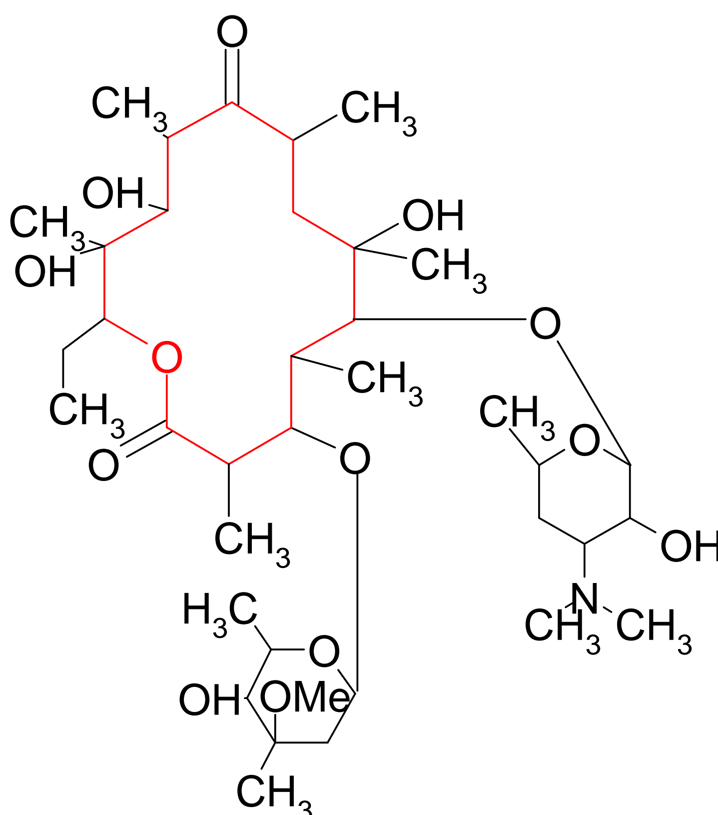

Figure 3 Erythromycin as representative of the macrolide antibiotics. The red part marks the typical structure of macrolides.

The mechanism of action of the macrolides is inhibition of bacterial protein biosynthesis during elongation. The molecules bind irreversibly to the 50S subunit of the bacterial ribosome and inhibit the translocation of peptidyl tRNA. Only high concentrations can cause a bactericidal effect but the action is mainly bacteriostatic. The macrolides are broad spectrum antibiotics and can be used to treat several infections.

2.2.1.3 Tetracycline antibiotics

Tetracyclines are a group of broad-spectrum antibiotics but the intensive use caused high occurrence of bacterial resistance and therefore the application is reduced nowadays. The name is derived from the four ("tetra-") hydrocarbon rings and they are a subclass of the polyketides.

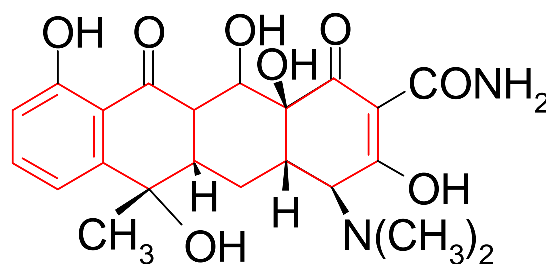

Figure 4 Tetracyclin as a representative of tetracycline antibiotics. The red part marks the typical tetracycline structure.

Tetracyclines inhibit protein synthesis by inhibiting translation. The molecules bind to the 16S part of the 30S ribosomal subunit and interfere with the amino-acyl tRNA for the binding site at the ribosome.

2.2.1.4 Aminoglycoside antibiotics

Aminoglycosides also inhibit protein synthesis. By binding to the bacterial 30S ribosomal subunit, the translocation of the peptidyl-tRNA from the A-site to the P-site of the ribosome is inhibited. Additionally misreading of mRNA leads to nonsense proteins. However, as for several antibiotics the exact mechanism of action is not fully understood. Also for aminoglycosides the concentration is crucial to the effect caused in the bacterial cell; bactericidal effect can be initiated by high concentrations

of the antibiotic. The nonsense proteins (from misreading of the mRNA) cause wrong activities in the cell and also in the cytoplasmic membrane. This changes the cellular metabolism and osmotic stability of the cell. Aminoglycosides that are derived from bacteria of the *Streptomyces* genus are named with the suffix –mycin. Those which are derived from *Micromonospora* have the suffix -micin. They are effective against a wide spectrum of bacteria but mainly used to treat Gram-negative organisms.

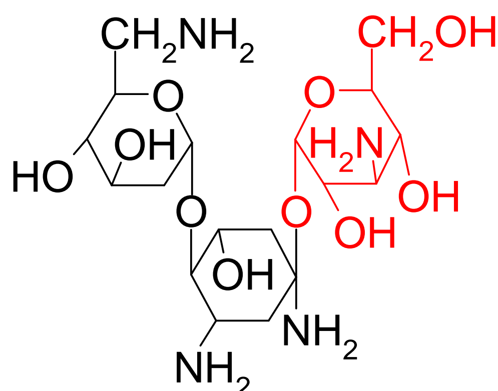

Figure 5 Kanamycin as a representative of the aminoglycoside antibiotics. The red part marks the typical aminoglycoside structure.

2.2.1.5 Gyrase inhibitors (Quinolones)

The quinolones are a family of broad-spectrum, bactericidal antibiotics with nalidixic acid as parent compound.

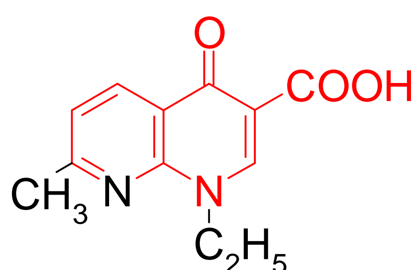

Figure 6 Nalidixic acid as the parent compound of quinolone antibiotics. The red part marks the typical structure of the quinolones.

For clinical application mostly fluorochinolones (synthetic class of molecules), which have a fluoro group attached to the central ring system are used. When bacterial DNA gyrase (coiling of the DNA molecule) or the topoisomerase IV enzyme

(decoiling of the DNA molecule) is inhibited by quinolones an inhibition of DNA replication and transcription occurs in the bacterial cell. The good flux of quinolones through porins in cell membranes makes them effective against intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*. In Gram-negative bacteria often DNA gyrase is targeted by quinolones, whereas topoisomerase IV is the target for many Gram-positive bacteria.

2.2.1.6 Chloramphenicol

Chloramphenicol is a bacteriostatic antibiotic. It was the first antibiotic to be manufactured synthetically on a large scale. Chloramphenicol is effective against a wide variety of microorganisms and is still commonly used in low income countries because it is very cheap. Though it can cause a very serious side effect: aplastic anemia where bone marrow does not produce sufficient new cells to replenish blood cells. The bacterial protein synthesis is inhibited by binding to the 23S rRNA of the 50S ribosomal subunit. Therefore peptidyl transferase activity of the bacterial ribosome is stopped and an elongation with new amino acids of the peptides is not possible.

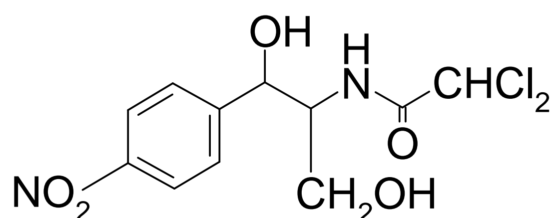

Figure 7 Chemical structure of the antibiotic chloramphenicol.

2.2.1.7 Peptide antibiotics

Peptide antibiotics are divided into two classes depending on their biosynthesis pathway. On the one hand glycopeptide antibiotics (vancomycin), lipoglycopeptides, ß-lactam antibiotics, and polypeptides, which are nonribosomally derived. On the other hand the lantibiotics, which are ribosomally derived and posttranslationally modified. Often these molecules act as cationic membrane inserting molecules and disrupt the bacterial cell membrane or act as depolarising agent. Daptomycin is also known to block the biosynthesis of the lipoteichoic acid as a component of the outer

membrane of Gram-positive bacteria. Vancomycin prevents incorporation of N-acetylmuramic acid (NAM) - and N-acetylglucosamine (NAG)-peptide subunits into the peptidoglycan matrix, which forms the major structural component of Gram-positive cell walls.

2.2.2 Mupirocin

As mupirocin was found as the active substance of the two new isolated strains during this work a short overview about this substance is given below. The antibacterial activity of *Pseudomonas fluorescens* isolated from a soil sample was first described in 1887 (Baader & Garre, 1887) and in 1971 the substance with antibiotic effect was identified as pseudomonic acid (Fuller*, et al.*, 1971). Later it could be shown that derivatives of pseudomonic acid are produced simultaneously by the same organism. (Chain & Mellows, 1977, Chain & Mellows, 1977, Feline*, et al.*, 1977). The most important and major form is pseudomonic acid A also known as mupirocin and used as a topical antibiotic for skin infections (Sutherland*, et al.*, 1985). Only topical use is possible because the pseudomonic acid molecule (Figure 8) is quickly metabolised to monic acid in human serum.

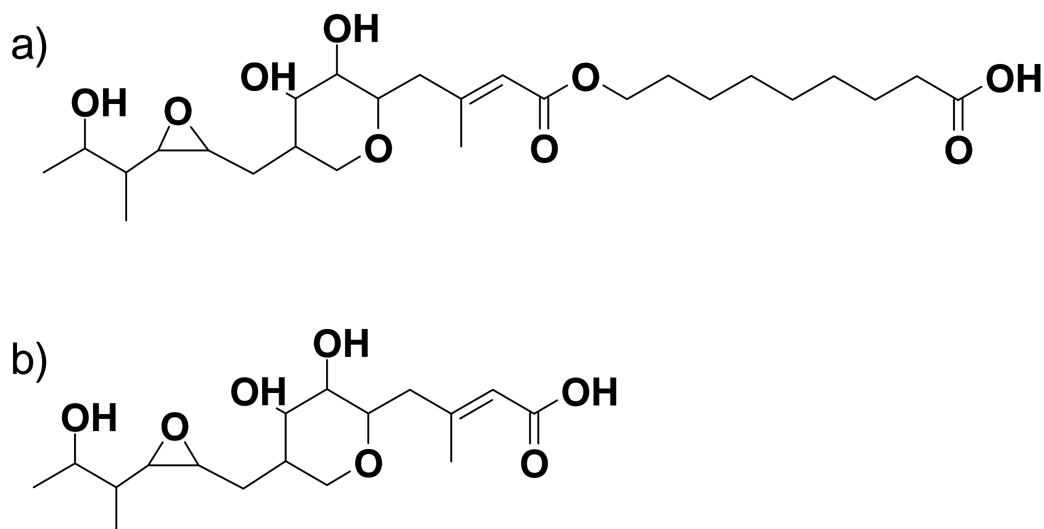

Figure 8 Structure of a) mupirocin and b) inactive monic acid.

Mupirocin is also not stable over longer periods in solution or in solid state because of a rupture of the epoxide ring (Figure 24a) (Crowley, 1999). Maybe this is one reason why studies about mupirocin as contamination in the environment are not available to my knowledge. The polyketide antibiotic mupirocin targets isoleucyl-tRNA synthase at the isoleucine binding site, this prevents further incorporation of isoleucine. The inhibition of bacterial protein and RNA synthesis of sensitive bacterial strains occurs by depleted cellular levels of isoleucin-charged tRNAs (Ward & Campoli-Richards, 1986, Parenti*, et al.*, 1987). The main activity of mupirocin is against Gram-positive bacteria especially *Staphylococcus* and *Streptococcus* species. Lower or no activity can be found against Gram-negative strains and anaerobic organisms. It is also useful against methicillin-reistant *Staphylococcus aureus* (MRSA). For all antibiotics known so far, bacteria developed a resistance and also mupirocin resistant strains of MRSA were detected (Cookson, 1998). The resistance mechanism is given by a mutation of the isoleycyl-tRNA synthetase or by an additional new isoleycyl-tRNA synthetase (Cookson, 1998). The producer strain *Pseudomonas fluorescens* NCIMB10586 has two different isoleucyl-tRNA synthases and one enzyme is highly resistant against the produced mupirocin (Yanagisawa & Kawakami, 2003). Up to now, no cross resistance to other antibiotics could be detected (Conly & Johnston, 2002). The biosynthesis cluster of *Pseudomonas fluorescens* NCIMB10586 was identified and the closest relationship could be found to the polyketide biosynthesis genes in *Bacillus subtilis* or the erythromycin biosynthesis genes (a macrolide antibiotic and also a polyketide) in *Saccharopolyspora erythraea* (El-Sayed*, et al.*, 2001, El-Sayed*, et al.*, 2003). There were also be hints found for a quorum-sensing-dependent regulation of mupirocin production (El-Sayed*, et al.*, 2001).

2.3 Antibiotic producers and there appearance in the environment

Antibiotics are mainly produced by soil fungi or bacteria. For example the first useful natural antibiotic penicillin was found by Flemming in 1929. Penicillin is produced by the fungi *Penicillium notatum* and belongs to the ß-lactam class of the antibiotics. The first antibiotic produced from a bacterium was streptomycin from *Streptomyces griseum*. It was detected in 1944 by the group of Selman Waksman (Jones*, et al.*, 1944) and belongs to the aminogycoside class of antibiotics. Also the last new discovered antibiotic class with its representative platensimycin is produced by a *Streptomyces* species (*Streptomyces platensis,* Wang, *et al.*, (2006)). *Actinobacteria* are typical members of the microbial community in soils and oxic sediments and are often found as the most prominent antibiotic producers. 50 % of the *Streptomycetes*, which belong to the *Actinobacteria*, produce antibiotics (Baltz*, et al.*, 2005, Baltz, 2006). In fact 55 % of the clinically used antibiotics are from *Streptomycetal* origin (Champness, 2000, Newman & Cragg, 2007) and the number of antimicrobial compounds of this genus was estimated to 100,000 which may be discovered in future (Watve*, et al.*, 2001). *Streptomycetes* are easily cultivated, which is reflected by the large number of cultured members in this genus (Hugenholtz*, et al.*, 1998, Clardy*, et al.*, 2006). *Streptomycetes* are strict aerobes and only one exception of antibiotic production under anaerobic conditions has been found yet (Sturgen & Casida, 1962), although these results have not been reproduced so far. Substances belonging to the antibiotic classes tetracycline, macrolide, lincosamide, aminoglycoside, chloramphenicol, and gyrase inhibitors are produced by *Streptomyces* species. But also other bacteria like *Bacillus* strains are known as antibiotic producers as example for bacilysin a peptide antibiotic (Walker, 1971, Stein, 2005). Even if many soil bacteria were found to be antibiotic producers there are only few studies on the ecological role of antibiotic production and the influence of antibiotic producers on microbial communities in an environmental habitat (Wiener, 1996, Wiener, 2000, Davelos*, et al.*, 2004), in contrast to rhizosphere research. Strains from the genus *Pseudomonas* play an important role in case of bacterial biocontrol in rhizosphere of plants (Thomashow, 1996, Raaijmakers*, et al.*, 1997).

Their ability to inhibit other bacteria was well known already at the end of the 19th century (Baader & Garre, 1887). Due to the fact that *Pseudomonads* are ubiquitous (Megha*, et al.*, 2007) the research about the antagonistic capacity in nature should be enhanced.

2.4 Resistances and occurrence of resistant organism in the environment

The ability of bacteria to get along with an antibiotic (or other harmful substances like heavy metals) is called resistance. In this work resistance is always used in the context of antibiotic resistance. Antibiotic resistance evolves naturally via natural selection through random mutation, but it can also be engineered by applying an evolutionary stress on a population. Resistances can be present on a plasmid or developed by changes on the chromosome. Placed on a plasmid, resistances can be transferred fast from one bacterial strain to another and transformation also occurs in the environment (Kruse & Sorum, 1994). The fitness of bacteria can be depleted by carrying a resistance gene, which can be balanced by adoption. A good growth is also possible without the selection pressure antibiotic (Modi & Adams, 1991, Lenski, 1998). Especially resistances on chromosomes do not get lost easily (Gillespie, 2001). If a bacterium carries several resistance genes, it is called multiresistant. The test for antibiotic susceptibility takes place with the disc diffusion assay or Kirby-Bauer Test (Bauer*, et al.*, 1966). Small filter discs are saturated with a selected antibiotic concentration and applied through the test organisms on an agar plate. The susceptibility can be detected by measuring of inhibition zones around the filter disc. This method gives the possibility also to measure the efficiency from culture supernatant of unknown bacteria.

Bacteria developed different resistance mechanisms against applied antibiotics (Walsh, 2000). Inactivation of drug substances (lactamases against penicillin), efflux pumps to minimize intracellular concentrations, or modification of target molecules can eliminate efficiency of the antibiotics (Figure 9). But resistances can also be developed naturally by Gram-negative cell wall, or absence of special transport

proteins in anaerobe bacteria. Resistances occur naturally in the environment in the antibiotic-producing bacteria. All antibiotic producers carry resistance genes to ensure their own survival (Hopwood, 2007, Nodwell, 2007) and have a selection benefit in environments with antibiotics. Therefore, the occurrence of natural antibiotic producers implies that one will always find a certain background of antibiotic resistances. The origin of resistances seems to be the antibiotic producers. The pool of resistance genes is therefore very old but did not spread in the past because of the local effects of antibiotics. With the wide application of antibacterial substances resistances became more and more important and therefore also finding the origin of resistances. One hint that an important pool for resistances are the antibiotic producers was found when it was realised that antibiotic preparations contain an amount of DNA. These can be transformed into the antibiotic treated organism resulting in resistant strains (Aminov & Mackie, 2007).

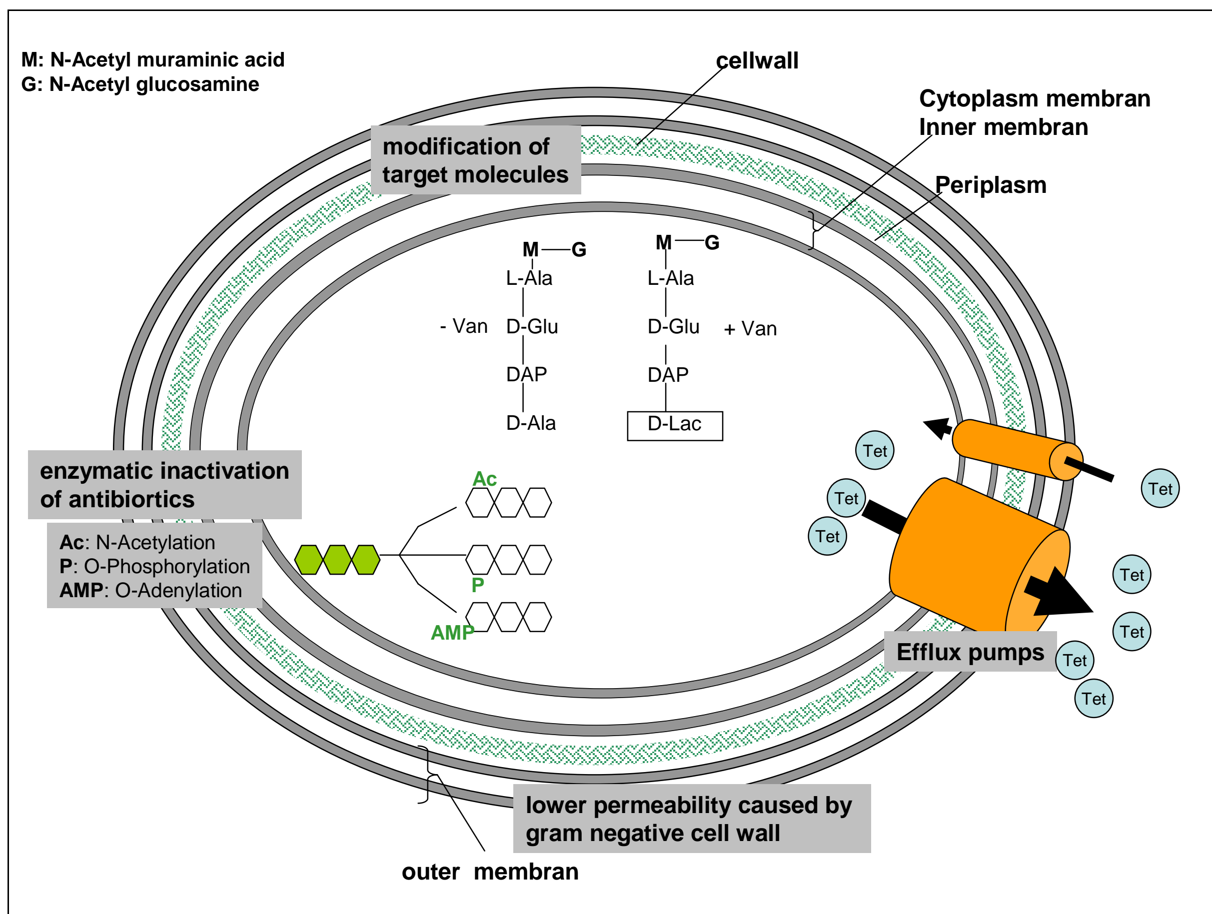

Figure 9 Resistance mechanisms of bacteria (modified from Walsh (2003)).

The appearance of resistances can occur a short time after clinical introduction or can be absent for many years. Vancomycin resistance just occurred 30 years after application and a long time there was hope that no resistance would occur at all. The abundance of drug application is decisive for resistance formation. Especially in hospitals a high selection pressure exists for resistance formation. Bacteria with a MIC (minimum inhibitory concentration) higher than 32 µg/l are seen as resistant (Walsh, 2003). Cross resistances against several antibiotics with the same target are common. Resistances occur for many antibiotics even during medication.
In the US each year 90.000 patients die because of hospital germs where no effective antibiotic could be found (Smith, 2005). There are strains of *Staphylococcus aureus* that can not be treated with Vancomycin (Kacica & McDonald, 2004) which was seen as last chance antibiotic for Gram-positive pathogens, that are resistant against many other antibiotics.

The risk of antibiotic resistant organism in the environment or only the genes coding for the resistances was realised in the last years and discussed in literature. Entry of manure or not sufficiently treated waste water into the environment can cause resistances in the bacterial community (McKeon*, et al.*, 1995, Guillemot, 1999, Teuber, 2001, Seveno*, et al.*, 2002, Wegener, 2003). Especially in these habitats small but not lethal dose rates can be expected. The continuous selection pressure leads to reproduction and accumulation of resistant organisms. Manure can additionally contain a certain amount of resistant organisms that can directly get into the environment.

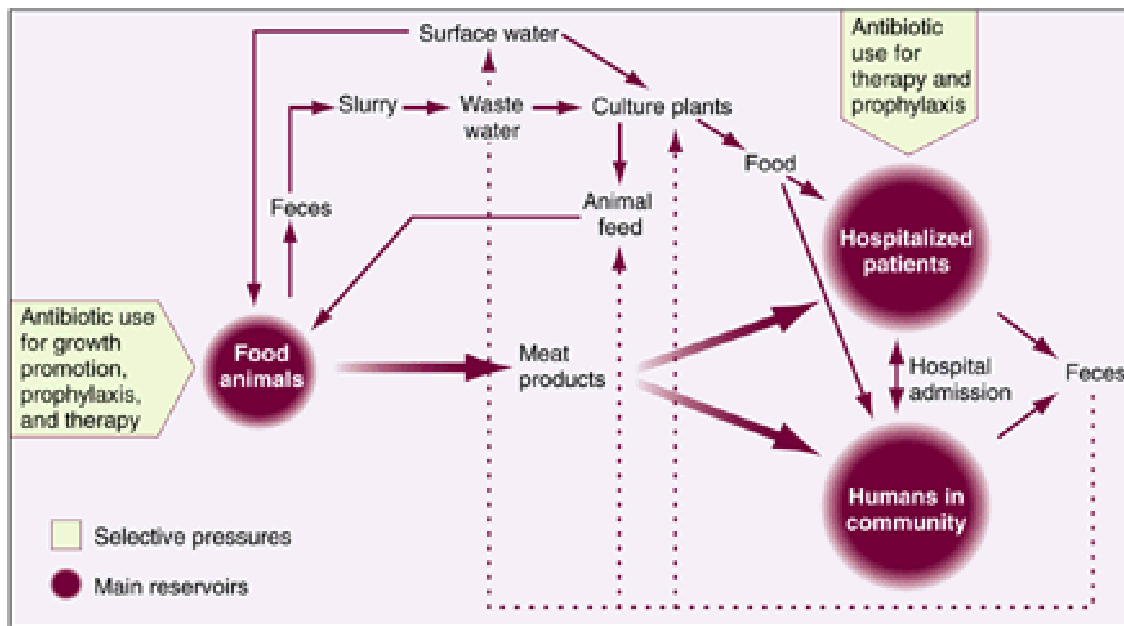

Figure 10 Network of resistances. Ecological relationships between antibiotic-resistant bacteria and resistance genes: selective pressures, main reservoirs, and routes of transmission from Witte, (1998)).

But not only development of resistances is recognised. Especially microorganisms in waste water treatment plants are exposed to the antibiotics and a shift in the bacterial community induced by the antibiotics can inhibit the function of the treatment system. Accumulated resistant bacteria then get to the environment by water or sewage sludge as fertiliser. In Germany there are no strict, precise regulations about pharmaceuticals in groundwater and therefore also not for antibiotic components. According to the drinking water directive § 6 it is not allowed that water for human use contains chemicals in a concentration that health would be endangered. Concentrations of chemicals must be kept as low as it is possible with tenable efforts (http://www.dvgw.de/wasser/recht-trinkwasserverordnung/trinkwasserverordnung/) but it is not really known at which concentrations antibiotics can cause resistances in nature and maybe concentrations reached by tenable efforts or by natural antibiotic producers are high enough.

Mostly resistant bacteria and resistance genes are described from clinical or veterinary isolates but also other environments were investigated, especially soil habitats. This might correlate with the problems explained before and even if humans

enhanced the evolution and distribution of resistant genes they can not be the only cause (Josephson, 2006). Resistant organisms found in natural, pristine environments, like Antarctica showed that the development and transfer of resistant genes occurred on a global scale (Kobori*, et al.*, 1984, Seveno*, et al.*, 2002). Molecular methods allowed to detect resistant genes explained by the term resistome also in uncultivated organisms and visualised the dimension of the resistance pool in the environment (Alonso*, et al.*, 2001, Riesenfeld*, et al.*, 2004, D'Costa*, et al.*, 2006, D'Costa*, et al.*, 2007, Wright, 2007). The isolation of resistant organisms or the verification of resistant genes was possible from diverse locations like urban or agricultural areas, soil, forest, and water. All soil derived isolates belonging to the genus *Streptomycetes,* where over half of all antibiotic producers stem from, were found to be multi resistant. Resistances against 2 to 15 of the tested antibiotics were found, with most of the strains being resistant against 7 antibiotics (D'Costa*, et al.*, 2006). The distribution of streptomycin resistance can not be correlated to the treated or untreated sampling sites when *Streptomycetes* from different sampling sites were compared for the occurrence of resistance (Tolba*, et al.*, 2002). This can also emphasise the wide spread occurrence of antibiotic resistance. It also hints for a special resistance pattern or an environmental selection for resistance genes, which could be detected (Kelch & Lee, 1978, Alonso*, et al.*, 2001, Singer*, et al.*, 2006) and bacteria sharing a certain environment seem to share a common mode for developing antibiotic resistance.

Also aquatic habitats were investigated for the occurrence of resistant organisms and the verification was possible in all tested environments, ranging from rivers and lakes to groundwater and drinking water. In bottled spring water where 70 % of the grown cultures were resistant to 2 to 4 antibiotics whereas the remaining strains showed resistance against 6 to 9 antibiotics (Mary*, et al.*, 2000). In drinking water, sampled directly at the water supplies 33 % in average were found to carry multiple resistance (Armstrong*, et al.*, 1981). In contrast, 63 % of the isolates found in groundwater, which was used to provide waterworks, showed multiple resistance against two or more compounds (El-Zanfaly*, et al.*, 1988). The amount of resistant organisms seems to be very equal to raw and biologically treated sewage or in groundwater below leaking sewers, where up to 80 % of the organisms were found to

be resistant against two antibiotics. The amount of bacteria with more resistances was obviously lower (Gallert*, et al.*, 2005). These data could only be compared carefully because not always the same concentrations or even antibiotic substances were used in the studies to test for resistance. One important point is also the organism carrying the resistance. Non pathogenic bacteria would not directly affect human health and the studies investigated totally differ from the amounts of bacteria of different bacterial families. However, all information about resistant bacteria in the environment point out that resistance and multi resistance also occurs under natural conditions and humans only enhance proliferation.

3 Material and Methods

3.1 Sampling sites

The first sampling site was an aquifer with homogenous sandy sediment located in the River Rhine Valley in Düsseldorf, Western Germany. The sampled aquifer is tar oil contaminated while situated in a former gasworks site. More information about the area can be found in Wisotzky, (1997). Sediment was taken from core drilling under argon atmosphere. The sediment was filled into glass bottles covered with associated groundwater and stored at 4 °C. A first drilling took place in June 2005 but the first complete cultivation approach of these samples was performed in February 2006. A second drilling was done in February 06 and these samples were cultivated within the next 3 days after sampling. While this aquifer is investigated regularly for natural attenuation purposes it was possible to get water samples from March 2007 which were treated nearly like the sediment samples within 3 days after sampling.

The second sampling site is located in Scheyern, which is 50 km north of Munich, on an experimental farm area. The sampling area is located on fallow land whereas the groundwater recharges in a forest area before it reaches the sampling site. Sediment was taken by core drilling and stored under argon. Sediment samples were filled into sterile 50 ml plastic tubes and cooled at 4 °C. All samples were analysed within three days after sampling. The stratigraphy of the aquifer is shown in Figure 11. From a first drilling in May 2006; samples from 2.8 m, 5.5 m, 7 m and 10 m were used for cultivation experiments. A second drilling in October 2006 gave the opportunity to use samples from 1.0 m to 30 m depth. The two drilling points were located in a distance of two meters to each other.

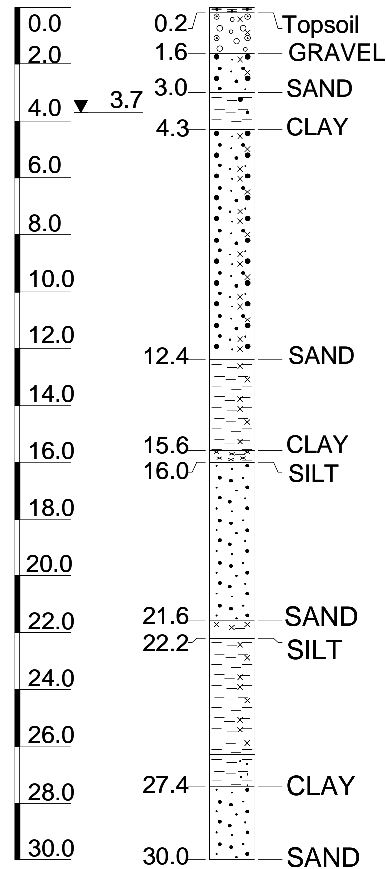

Figure 11 Stratigraphy of the aquifer on the sampling site in Scheyern.

3.2 Cultivation of bacteria from sediment samples

The isolation and cultivation of microorganisms from sediment samples was performed in artificial fresh water medium (AFW) (Bartscht, *et al.*, 1999) supplemented with *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), N-butanoyl-l-homoserine (BHL), and cyclic adenosine monophosphate (cAMP) (10 µm each) as growth inducers. This medium was prepared under aerobic or anaerobic conditions depending on the later cultivation conditions. For anaerobic cultivation 60 ml of AFW were added to 100 ml serum flasks, flushed with N_2/CO_2 (80/20, v/v) and closed with butyl stoppers.

In order to detach living bacterial cells from the sediment particles, 4 g of wet sediment were added to 60 ml of AFW in a 100 ml serum bottle and shaken with 600

rpm for one hour. Then the sediment was allowed to settle down and 0.8 ml of the supernatant were taken for 4',6-Diamidino-2-phenylindole (DAPI) staining and cell counting (see below). Two parallel 60 ml samples per depth were prepared; one was kept under anoxic conditions and flushed with nitrogen gas, the other sample was kept under oxic conditions. The cell number was adjusted to a density of five cells in 200 µl AFW and 200 µl aliquots were distributed into 96 well microtiter plates (Nunc, Wiesbaden, Germany). Three parallel plates were filled for every sample depth. The plates for anaerobic cultivation were prepared and stored in a glove box under N_2/H_2 atmosphere (95/5, v/v). Cell growth was determined by measuring the optical density at 595 nm ($OD_{595\ nm}$) after one month (aerobic cultivations) with a multilable counter Victor 3 (Perkin Elmer, Fremont, USA) or after three month (anaerobic cultivations) with the multilable counter ELx800UV (Bad Friedrichshall, Germany). All grown cultures were tested for antibiotic production. Most probable number (MPN) cultivations were performed in triplicates for every depth to determine the cultivation efficiency on AFW and a special *Streptomyces* medium (Fluka, Buchs, Switzerland).

Cell number in the water samples was directly determined by DAPI staining and the same cultivations were set up.

3.2.1 DAPI staining

For determination of cell numbers, 0.8 ml 6 % paraformaldehyde (PFA) (end concentration 3 %) were added to 0.8 ml of cells and incubated at room temperature for 15 minutes. After this fixation step, 0.4 ml of 4',6-Diamidino-2-phenylindole (DAPI) solution (10 µg/ml) were added and incubated for another 15 minutes in the dark. Samples were filtered with a vacuum pump on a 0.2 µm polycarbonate filter (Whatman, Dassel, Germany) and cells were counted under a fluorescence microscope (Axioscope Zeiss, Jena, Germany) at an excitation wavelength of 290 nm.

3.3 Cultivation of test organisms

Nine bacterial strains and one yeast strain were used to test the environmental cultures for antibiotic production and to determine the produced antibiotic (Table 2). The strains were grown at 30 ℃ and kept on agar pl ates at 4 ℃ until they were needed for experiments. The cultures were streaked on new agar plates every week. *Saccharomyces cerevisiae* (DSMZ 70449) was grown on universal medium for yeast (Yeast extract 3 g/l, Malt extract 3 g/l, Peptone from soybean 5 g/l, Glucose 10 g/l, Medium 186 DSMZ). *Bacillus clausii* (DSMZ 8716) was grown in alkaline Nutrient broth where Nutrient broth (described below) is adjusted to pH of 9.7 with a 1 M Na-bicarbonate solution ($NaHCO_3$ 4.2 g/100ml, Na_2CO_3 5.3 g/100ml, Medium 31 DSMZ). All other strains were grown in Nutrient broth medium (NB) (Peptone 5 g/l, Meat extract 5 g/l, Medium 1 DSMZ).

3.4 Antibiotic susceptibility test

Grown cultures from sediment samples were tested for antibiotic production. Supernatants (50 µl) of grown cultures were transferred from the 96 well cultivation plate to a new plate and dried at 50 ℃ until all l iquid was evaporated. This was repeated three times. Then, the precipitate was covered with 80 % ethanol to sterilize the wells and dried again at 50 ℃. Antibiotic susc eptibility test organisms were grown over night in 5 ml cultures and transferred into a 250 ml Erlenmeyer flask with 50 ml Mueller Hinton broth (meat infusion 2.0 g/l; casein hydrolysate 17.5 g/l; starch 1.5 g/l, composition like ready available broth from Sigma, Hamburg, Germany). The cultures were shaken with 200 rpm for 5 hours. To prepare the testing solution the $OD_{595\ nm}$ of the cultures was detected and a sample of bacterial cell culture was mixed with Mueller Hinton Agar (0.8 % agar) to an optical density of 0.01. 50 µl/well of this mixture were added with a multichannel pipette to cover the dried culture supernatant and allowed to solidify. Before and after growth of 18 hours the $OD_{595\ nm}$ was measured with the Victor3 microtiterplate reader. The difference in optical densities was very low when an antibiotic inhibited growth of the test organism.

Standard antibiotics (Table 4) were tested in the same manner as controls. Stock solutions of antibiotics were prepared and pipetted into the 96 well plates and treated similar to the culture supernatants. The stock solutions for streptomycin, penicillin, vancomycin, cephalothin, nalidixic acid, lincomycin were prepared in ultrapure water, for chloramphenicol, erythromycin, and tetracyclin 95 % ethanol was used, and nisin was dissolved in 0.02 M HCl. The first screening for antibiotic producers from sediment samples was conducted with *Bacillus subtilis*. If the test was positive for antibiotic production and *B. subtilis* was not able to grow, a bigger culture of the original sediment culture was grown and tested with the other test organisms (Table 2).

Table 2 Test organisms used in the susceptibility test for strains cultivated from sediment samples.

Testorganism (Bacterial collection no.)	Resistance
Bacillus subtilis subsp subtilis (DSMZ 10)	-
Escherichia.coli (DSMZ 498)	-
Saccharomyces cerevisiae (DSMZ 70449)	-
Escherichia coli (Institut Pasteur 103983)	ß-lactame antibiotics
*Escherichia coli (*Institut Pasteur 75.17)	Gyrase inhibitors
Ochrobactrum grignonense (Institut Pasteur 107373)	Polypeptide antibiotics
Bacillus subtilis subsp spizizenii (DSMZ 347)	Glycopeptide antibiotics
Bacillus clausii (DSMZ 8716)	Makrolides
Bacillus cereus (Institut Pasteur 69.12)	Tetracyclines
Streptococcus agalactiae (Institut Pasteur 108247)	Chloramphenicoles

3.5 Identification of antibiotic-producing bacterial strains

3.5.1 DNA Extraction

DNA Extraction for sequencing of the unknown bacterial strains was done with the Fast DNA® Spin for Soil Kit (MP Biomedicals, Eschwege, Germany). As inoculum a pellet of an 18 hours grown culture (5 ml) of the bacteria was used. DNA concentration was detected afterwards with NanoDrop ND-1000 spectrometer (Thermo Scientific, Schwerte, Germany).

3.5.2 PCR amplification of 16S rDNA genes and sequencing

As master mix was prepared:

H_2O add to 50 µl

10x PCR-Buffer 5.0 µl

25 mM $MgCl_2$ 3.0µl

20 µg/µl BSA 0.5 µl

10 mM dNTPs 0.5 µl

50 µM f-primer 0.5 µl (Ba27f)

50 µM r-primer 0.5 µl (907r)

5 U/µl MBI Taq 0.25 µl

(DNA-template 1 µl) not included in Master Mix

Table 3 Sequences of used primers for 16S rDNA gene amplicons.

Primer	Sequence
Ba 27f	AGA GTT TGA TCM TGG CTC AG
Ba 519f	CAG CMG CCG CCG TAA TA
Ba 907r	CCG TCA ATT CMT TTR AGT TT
Ba 1492r	CGG YTA CCT TGT TAC GAC TT

The profile for the Thermocycler Mastercycler ® Ep Gradient (Eppendorf, Hamburg, Germany) was:

94 ℃ 5 min

94 ℃ 30 sec }

52 ℃ 30 sec } 20 - 25 cycles (depending on DNA con centration)

70 ℃ 60 sec }

70 ℃ 5 min

4 ℃ hold

By gel electrophoresis the amplicons were checked and purified on MinElute columns (Qiagen, Hamburg, Germany) and re-eluted in 25 µl of elution buffer. Again a quantification of the DNA content was done with the NanoDrop spectrometer.

The amplicons (50 ng) were used for cycle sequencing reaction. The master mix contains:

BigDye terminator v3.1 premix	1 µl
5x BigDye sequencing buffer	1 µl
10 µM sequencing primer	1 µl (Ba27f, Ba519r, Ba1492r)
Template	up to 2 µl
H_2O	add to 5 µl final volume

Thermal protocol:

96 ℃ 1 min

96 ℃ 10 sec

50 ℃ 5 sec } 25 (- 30) cycles

60 ℃ 4 min

4 ℃ hold

The cycle sequencing product was desalted with DyeEx Sephadex G50 spin columns (Qiagen, Hamburg, Germany) and sequenced by capillary electrophoresis on an ABI 3730 analyzer (AME Biosience, Toroed, Norway).

The 16S gene sequences of the two new antibiotic-producing strains were submitted in GeneBank with the accession numbers D7: EU680857 and G11: EU680856.

3.5.3 Gram staining of bacterial cell wall

The Gram staining was performed with the Gram staining set from (Sigma, Hamburg, Germany). Furthermore a Gram typing with 3% KOH solution was tested. A drop of potassium hydroxide solution was brought onto a slide and mixed 5 to 10 seconds with cell material from a pellet. When getting sticky filaments the bacterium is Gram-negative.

3.5.4 Test for catalase-, oxidase- activity, and metabolic properties

The test for catalase activity was done with 3% aqueous solution of H_2O_2. A grown colony was mixed with the solution. Frothing indicated catalase activity.

A solution of 0.1 g of citric acid and 1.0 g 4-amino-N, N-dimethylanilin-dihydrochlorid was prepared and a soaked filter spread with a grown colony. A blue colouring showed the existence of cytochrom c-oxidase.

To test for metabolic properties of D7 and G11 the standard test kit api® 20NE from Biomérieux (Nürtingen, Germany) was used.

3.5.5 Determination of growth temperature

The optimal growth temperature for the strains was detected for agar cultures and for liquid cultures. Nutrient broth medium was used and temperatures between 4 ℃ and 60 ℃ adjusted.

For growth in liquid cultures 5 ml of medium was inoculated with cells of an overnight culture to an $OD_{595\ nm}$ of 0.01. For growth on solidified medium the strains were streaked out on the agar plates and incubated at the given temperature.

3.5.6 Preparation of bacterial cells for electron microscopy

The electron microscopy was carried out at the Institute of Pathology at the Helmholtz Center Munich by Mrs. Jennen. Up to the first fixation step the protocol was carried out at our institute with the fixation buffer offered from the Institut of Pathology.

2 ml of an overnight culture of D7 and G11 in NB medium (28 °C, 200 rpm) were centrifuged and the pellet was transferred to a BEEM capsule and the remaining medium eliminated by pipetting. The pellet was covered with 2.5 % glutaraldehyde in cacodylat buffer (Science Services, Munich, Germany). Three washing steps with cacodylat buffer (each 20 minutes) followed. In a second fixation step chrome osmium acid mixture was used for 1 – 2 hours. The mixture contained one part buffer solution (100 ml 5 % aqueous $K_2Cr_20_7$ solution set to pH 7.2 with 12 ml 2.5n KOH), one part salt solution (3.4 % Na Cl), and two parts 2 % OsO_4 solution. Afterwards (minimal) three times rinsing with bidest water followed. After that five dehydration steps with 30 % ethanol, 50 % ethanol, 70 % ethanol, 90 % ethanol, 96 % ethanol each 15 to 20 minutes were carried out. For embedding first a 1:1 mixture of EPON 812 solution and propylenoxyde for one hour was applied with a following step of pure EPON solution over night. The next day the EPON was replaced by fresh EPON solution and the polymerisation step at 60 °C for 24 – 48 hours followed. In an ultramicrotome (Reichert-Jung, Leica, Wetzlar, Germany) sections of 60 - 70 nm thickness were cut and rinsed to cooper grids. The contrastation of the sections occurred with 0.5 % uranylacetate and 3 % leadcitrate. The sections were investigated at a transmission electron microscope EM 10 CR (Zeiss, Jena, Germany)

3.6 Identification of the antibiotically active substance

3.6.1 Treatment of bacterial supernatant

For identification of the antibacterial active substance the strains D7 and G11 were grown in 50 ml of NB medium or 50 ml of artificial freshwater medium (AFM, (Bartscht*, et al.*, 1999). AFM as a poor mineral medium was chosen to have less disturbing components in the chromatographic analysis, but both media were compared because a better bacterial growth was detected in the rich nutrient broth medium. After 18 hours of growth the cells were divided from the supernatant by centrifugation at 4800 rpm in a Megafuge 1.0R centrifuge (Fisher Scientific, Schwerte, Germany). The supernatant was filtered through a 0.22 µm filter, frozen at 80 ℃ and lyophilised in a freeze drier (VirTis Com pany, New York, USA). After freeze drying the residues were dissolved in 2 ml of ultra pure water and used for further analysis.

3.6.2 Semi preparative HPLC analysis

The resolved supernatants were fractionated on a Perkin-Elmer Series HPLC (Überlingen, Germany) equipped with a diode array detector 235C set to 215 nm. The separation was performed on a LiChrospher 100 RP-18 column (150×4.6 mm id, 5 µm, Macherey-Nagel, Düren, Germany). For linear solvent strength gradient 0.1 % formic acid (A) and 100% acetontirile containing 0.06 % formic acid (B) was used. The following gradient profile was used: 0 - 15 min from 0 % to 100 % B, 15 - 25 min 100 % B, 25 - 30 min from 100 % to 0 % B, and 30 - 45 min 0 % B. The flow rate was 1 ml/min causing pressure drop on the column between 95 - 160 bar. The injection volume was 20 µl. The eluent was fractioned from 0 until 30 min at a rate of 0.5 ml/tube (Gilson FC203B fraction collector, Limburg, Germany).

3.6.3 Antibiotic susceptibility test

In order to identify antibiotically active HPLC fractions samples were transferred into a 96 well microtiter plate and dried at 50 °C until all liquid was evaporated. The test for antibiotic activity was conducted with *B.subtilis* subsp. *subtilis* (DSMZ 10) as a very sensitive control strain. *B. subtilis* was grown in 5 ml over night cultures (medium like described for cultivation) and transferred into 50 ml Mueller Hinton Broth (Meat infusion 2.0 g/l; casein hydrolysate 17.5 g/l; starch 1.5 g/l), and shaken for 5 hours at 200 rpm. The $OD_{595\ nm}$ of the culture was measured and a sample was mixed with Mueller Hinton Agar (0.8 % agar) to a final $OD_{595\ nm}$ of 0.01. 50 µl of this mixture were added with a multichannel pipette to each well to cover the dried culture supernatants and allowed to solidify. Before and after growth of 18 hours the $OD_{595\ nm}$ was measured with a Victor3 microtiterplate reader (Perkin Elmer, Überlingen, Germany). The difference in optical densities was very low when an active fraction inhibited growth of the test organism. Tetracycline dissolved in ethanol (30 µg/well) served as a control.

3.6.4 FT-ICR-MS analysis

High-resolution mass spectra were acquired on a Fourier transform ion cyclotron resonance mass Spectrometer (Bruker, Bremen, Germany), equipped with a 12 Tesla superconducting magnet and an Apollo II ESI source. Samples containing 75 % methanol were infused with the micro-electrospray source at a flow rate of 120 µl/h with a nebuliser gas pressure of 20 psi, and a drying gas pressure of 15 psi at 250 °C. Negative and positive electrospray ionisation was used. Spectra were externally calibrated on clusters of arginine (10 mg/l in methanol); calibration errors in the relevant mass range were always below 0.1 ppm. In the mass range of 150 – 2000 m/z 1 MW time domain was applied. The ion accumulation time in the ion source was set to 2 s and 128 scans were accumulated per sample. Before Fourier transformation of the time-domain transient, a sine apodisation was performed. For fragmentation experiments, collision-induced dissociation (CID) was carried out. The mass window of 1 Da of the parent ions was selected between the ion source and the analyzer cell with the quadrupole and were fragmented by varying the excitation

power (15, 20, 25 eV). Argon as a collision gas was pulsed into the chamber at a pressure of 1 x 10^{-8} Torr, with 50 ms gas pulse duration. On resonance CID provided abundant structural information. The ion accumulation time in the ion source was set to 0.2 s and 64 scans were accumulated for the samples.

3.6.5 UPLC analysis

A UPLC Acquity System (Waters, Darmstadt, Germany) equipped with a 2996 PDA detector was applied for analysis. A reversed phase column with dimensions of 2.1 x 100 mm, filled with BEH C18 packing material with 1.7 µm particle sizes (Waters, Darmstadt, Germany, Milford, MA, USA) was used for the separation. The column was thermostated at 40 °C, and the autosampl er at 27 °C. 5 µl of sample were injected via a partial loop with needle overfill injection. The optimised system was run with a linear gradient starting with water containing 20 % to 100 % acetonitrile in 2 minutes. The flow rate was set to be 0.6 ml/min which results in a maximum system pressure of 750 bars. Detection was performed at 220 nm at a scan rate of 40 Hz.

3.7 Column experiment as proof of antibiotic production under close to *in situ* conditions

A column experiment was planned to find out if antibiotic producers have an influence on the surrounding bacterial community. The plan was to show if differences in terminal restriction fragment length polymorphism (TRFLP) and the resistance profile of the bacterial community occur.

For this experiment sterile glass columns (length 10 cm, diameter 16 mm) were filled with sediment from a pristine aquifer and flow through with AFW medium 50 times diluted to prevent anoxic zones in the columns. The columns were closed with 16 mm viton stoppers (Saint Gobain, Paris, France) and the medium was pumped through viton tubes with 1.02 mm in diameter (ISMATEC, Glattbrugg, Switzerland) or

steel capillaries (Harry Rieck Edelstahl GmbH, Hilden, Germany). To prevent bacterial growth from the columns into the tubes or the medium reservoir 0.2 µm filters (Millipore, Boston, USA) were switched ahead the column inlet. Tubes and stoppers or filters were connected by syringe needles (TYCO, Neustadt, Germany). The flow of 0.3 ml/min was caused by a syringe pump (ISMATEC, Glattbrug, Switzerland). The columns and the medium reservoir were covered with aluminium foil to prevent destruction of medium compounds by light. Flow through samples were taken every week and sediment samples at the starting and end point of the experiment. The first four weeks all columns were treated the same and only medium was pumped to regenerate the columns. After this time four different treatments were planned: one sediment control untreated, one column where an antibiotic producer should be added, one column treated with a standard antibiotic, and one column where a non antibiotic-producing bacterium should be added to check if addition of biomass changed the conditions. This step of the experiment and conclusive results were not possible because of problems in the bacterial community composition (see results).

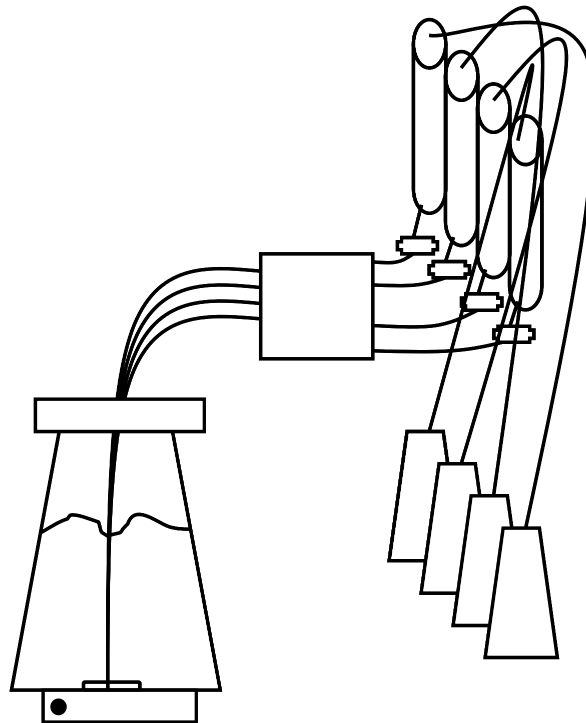

Figure 12 Overview of the column experiment.

3.7.1 TRFLP community analysis of column flow through and sediment samples

The water samples were filtered on 0.2 µm Filter which were cut in small pieces and treated like the sediment samples in the Fast DNA ® Spin for Soil Kit from MP Biomedicals (Eschwege, Germany). The PCR for 5-6-carboxyfluorescein (FAM-) labeled rDNA amplicons was performed with Ba27f (-FAM) and 907r primer. The master mix was prepared like explained above but the Ba27f primer was used with FAM labelling. Also the thermal profile for the cycler was the same as above. By gel electrophoresis the amplicons were checked and purified on MinElute columns (Quiagen, Hamburg, Gemany) and re-eluted in 25 µl of elution buffer. Again a quantification of the DNA content was done with the NanoDrop spectrometer. Amplicons were restricted for 2 hours at 37 °C with enzyme Msp1 under the following conditions:

PCR product 20 ng (NanoDropquantified)

Restriction enzyme 0.3 µl (*Msp*I)

10x buffer (incl. BSA) 1 µl

H_2O add to 10 µl in total

The restricted PCR products were desalted by DyeEx Spin columns; the following was loaded onto a 96-well sequencing plate:

Desalted Amplicons 1 µl

HiDi Formamide 13 µl

(Formamide containing a 1/300 dilution of MapMarker-1000 ROX Size Standard)

3.7.2 Resistance test for column flow through

Artificial fresh water agar plates were prepared without or with addition of one antibiotic (Penicillin 15 µg/ml, Chloramphenicol 30 µg/ml, Vancomycin 30 µg/ml, or Tetracycline 10 µg/ml). 10 µl of column flow through were diluted with 990 µl of sterile medium and 100 µl of the dilution plated onto the agar plates. The plates were stored

at room temperature and cell counts made after two days and after four days of incubation.

4 Results

4.1 Development of a high throughput antibiotic susceptibility test

The routine method for antibiotic susceptibility testing is the disc diffusion or Kirby Bauer test. To obtain a comparably sensitive high throughput screen for antibiotic-producing microbes the test was transferred to 96 well plates as described. The results for the new test with standard antibiotics and the supernatants of two antibiotic producers isolated in the study were compared to the disc diffusion test (Table 4). At similar antibiotic concentrations the two tests showed comparable inhibition of growth. For example tetracyclin, cephalothin, streptomycin and chloramphenicol showed an inhibition at all concentrations between 5 µg and 150 µg/well and with *Escherichia coli* or *B.subtilis* in both test systems (Table 4). With nalidixic acid a slightly lower inhibition was detected in the microtiter plate test for the concentrations 75 µg and 30 µg but with all other antibiotics and all concentrations the same sensitivity of the test organisms towards the antibiotics could be detected.

The test was also performed in liquid culture in a 96 well microtiter plate (Data not shown). This test could not be utilised because the susceptibility was very low and the test was not reproducible.

Table 4 Applied amounts of antibiotics used for the susceptibility test. The four concentrations are correlated to the four results given for microtiter plates or disc diffusion test. + total inhibition of growth, - no inhibition of growth, (+) a slight but visible inhibition. Numbers give the diameter of the inhibition area by the antibiotic on the disc.

Antibiotic	Antibiotic class	Amount (µg/well) or (µg/disc)	Limit of inhibition *B.subtilis*								Limit of inhibition *E.coli*							
			microtiterplates				disc diffusion test				microtiterplates				disc diffusion test			
Tetracyclin	Tetracyclines	150, 75, 30, 15	+	+	+	+	4.6	4.6	4.6	4.6	+	+	+	+	2.4	2.3	2.3	2.3
Cephalothin	ß-lactames	150, 75, 30, 15	+	+	+	+	2.8	2.8	1.6	1.4	+	+	+	+	2.1	2.0	1.6	1.5
Nisin	Lantibiotics	50, 25, 10, 5	-	-	-	-	0.8	0.8	(+)	(+)	-	-	-	-	0.9	0.9	-	-
Vancomycin	Glycopeptide antibiotics	50, 25, 10, 5	+	+	+	-	2.0	1.7	1.6	1.3	+	+	+	-	1.3	0.8	0.8	-
Streptomycin	Aminoglycosidse	50, 25, 10, 5	+	+	+	+	2.0	1.2	1.1	0.6	+	+	+	+	1.2	1.0	1.0	-
Nalidixic Acid	Gyrase inhibitor	150, 75, 30, 15	+	(+)	(+)	-	1.6	1.2	1.0	-	+	-	-	-	1.2	0.9	-	-
Chloramphenicole	Chloramphenicoles	150, 75, 30, 15	+	+	+	+	2.3	2.0	1.8	1.8	+	+	+	+	3.2	2.7	2.0	1.8
Erythromycin	Macrolides	50, 25, 10, 5	+	+	+	+	1.9	1.9	1.7	1.7	+	(+)	(+)	(+)	(+)	(+)	-	-
		Amount (U/well) or (U/disc)																
Lincomycin	Linconsamide antibiotics	65, 32.5, 15, 7.5	+	+	(+)	(+)	1.5	1.2	0.8	0.7	+	(+)	(+)	(+)	(+)	(+)	-	-
Penicillin	ß-lactames	50, 25, 10, 5	+	+	(+)	(+)	1.8	1.7	1.3	1.2	+	+	+	-	1.7	1.4	-	-
		Amount (µl/well)																
Supernatant D7	unknown	150	+				2.5				-				-			
Supernatant G11	unknown	150	+				2.3				-				-			
Medium alone	-	150	-				-				-				-			
Water control	-	150	-				-				-				-			

4.2 Cultivation of bacteria

4.2.1 Distribution of antibiotic producers in sediment samples from a contaminated aquifer

Cultivation of microorganisms from samples of the sediment core taken in June 2005 was performed with two different media and for oxic and anoxic conditions. The comparison between MPN cultivations and total microscopic cell counts of the microbes from the sediment extracts before cultivation shows cultivation efficiency up to 79 %, for aerobic cultivated samples (Table 5).

Table 5 Cultivation efficiency (Düsseldorf 2005) in %, reverenced to total cell counts with DAPI staining before cultivation for the different cultivation settings and two different media. AFW: artificial fresh water medium, Str: *Streptomyces* medium.

	Düsseldorf 2005			
Depth (m)	**Cultivation efficiency (%) oxic conditions**		**Cultivation efficiency (%) anoxic conditions**	
	AFW	**Str**	**AFW**	**Str**
6.85	47.5	0.01	1.15	0.01
7.6	0.33	0.00	6.67	0.01
9.5	79.2	0.00	0.00	0.00
11	79.2	0.00	0.08	0.00

The highest cell number was detected at 7.6 m but this is not very different from the cell numbers in 9.5 and 11 m depth (Figure 13). In general the MPN cultivations followed the profile for total cell counts with the exceptions in 6.85 m where the total cell count was lower than the MPN with AFW medium. However, the cultivation efficiency for anoxic conditions was lower than for oxic cultivation (Table 5). No samples in the anoxic cultivation could be found with cultivation efficiency higher

than 0.01 % (Table 5). Antibiotic producers could be detected in two depths of the aerobic cultivations (Figure 13) 6.85 m (10), and 9.5 m (8) (number of antibiotic producers in brackets), but no antibiotic producers could be detected in anoxic cultivations.

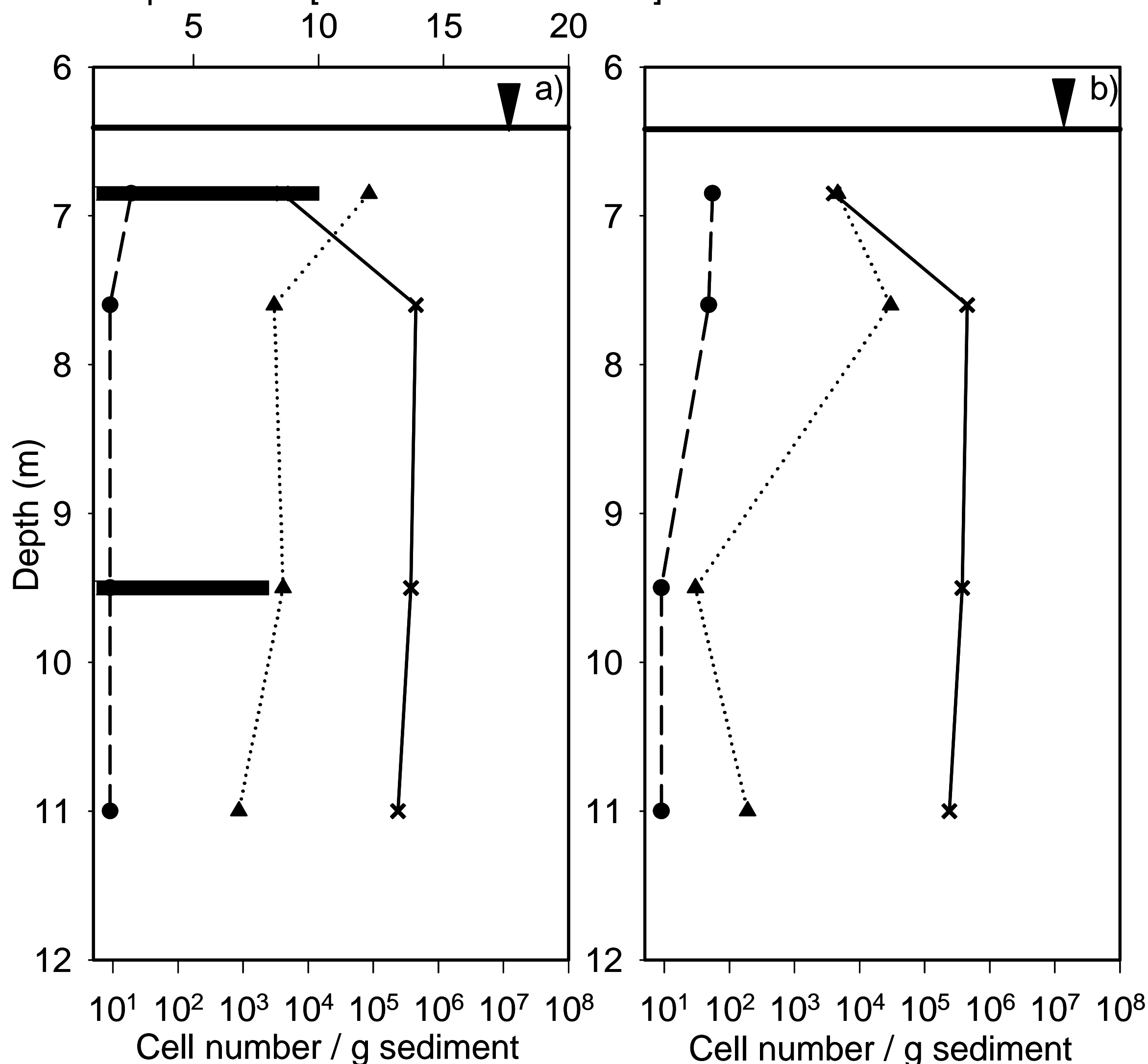

Figure 13 Cultivation of total microorganisms from sediment samples of the drilling in Düsseldorf 2005 for a) oxic and b) anoxic conditions; solid line: total cell counts obtained by DAPI staining; dotted lines: most probable number (MPN) counts cultivated with artificial fresh water (AFW) medium; dashed lines: MPN counts cultivated with *Streptomyces* medium.

All antibiotic-producing strains identified in the first screening were subcultured and purified. However, all strains lost the ability to inhibit test organisms after a few further cultivation steps. It was not possible to reproduce the antibiotic effect, even if the former antibiotic producers were cultivated in smaller volumes of medium or in sterile sediment. The occurrence of antibiotic producers did not correlate with the cultivation efficiency, because antibiotic producers could be found also in 6.85 m depths where the lowest cultivation efficiency could be detected (Table 5).

Table 6 Resistance of isolated antibiotic producers against standard antibiotics, + strain is resistant, - strain is sensitive. Strains of the Düsseldorf 2005 cultivation.

Antibiotic (µg)	Strain																	
	Depth - 6.85 m										Depth – 9.5 m							
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8
Tetracyclin (30)	-	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-
Cephalothin (30)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nisin (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vancomycin (10)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin (10)	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-
Nalidixic acid (30)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Chloramphenicol (30)	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+
Erythromycin (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lincomycin (15 Units)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillin (10 Units)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

In total 18 antibiotic producers out of 189 grown cultures could be detected for the cultivation in a contaminated aquifer. This means that approximately 9.5 % of the cultured bacterial community were antibiotic producers. All antibiotic producers were found to be multi resistant against the standard antibiotics tested (Table 6).

Streptomycin and chloramphenicol were effectively in killing most but not all of the isolated strains. Only strain 8 and 10 (-6.85 m) and strain 8 (-9.5 m) were resistant against chloramphenicol and strain 2, 3, and 6 (-9.5 m) against streptomycin. There was no strain that could not be killed by one of the applied antibiotics.

Cultivation of microorganisms from samples of the sediment core was performed with AFW medium and *Streptomyces* medium to compare for growth on a rich medium. Cultivation efficiency was in average 26.7 times higher with the AFW medium than with *Streptomyces* medium indicating the sustainability of the AFW medium to cultivate also organisms from contaminated environments.

Cultivation of microorganisms from samples of the sediment core taken in February 2006 was performed with two different media and for oxic and anoxic conditions. Cultivation efficiency should be also detected by MPN counts but the growth of this cultivation approach was very poor and no data for results could be obtained. It was only possible to detect the cell numbers before cultivation by DAPI staining (Figure 14).

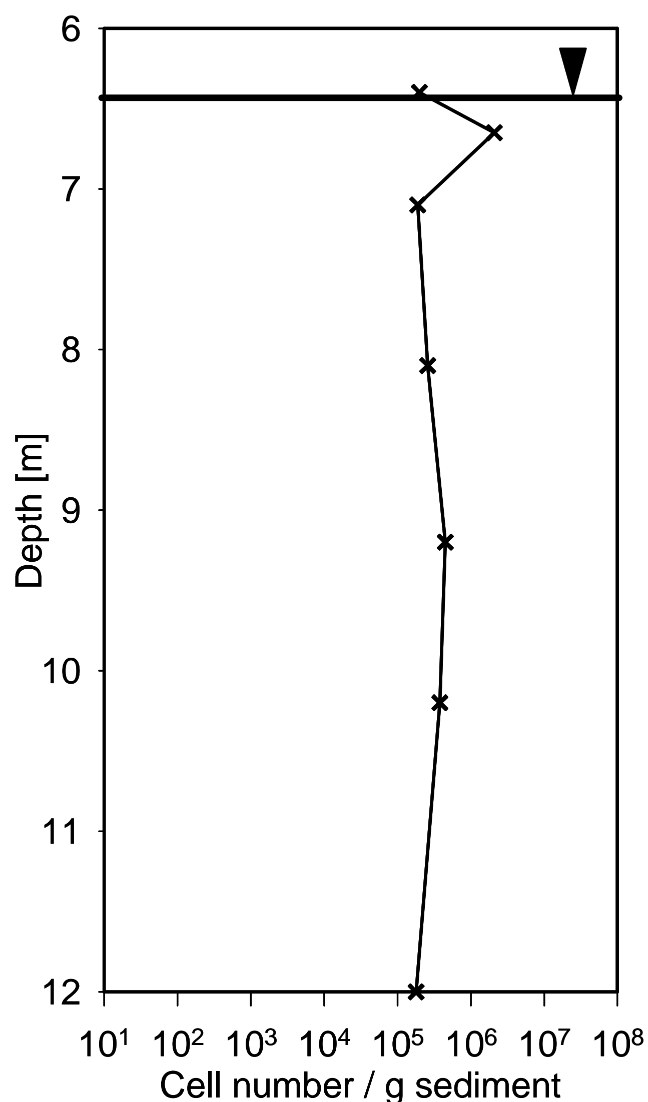

Figure 14 Total cell counts obtained by DAPI staining for sediment samples from the drilling in Düsseldorf February 2006.

The cultivation of bacteria from water samples from March 2007 was more successful. The cultivation was performed with two different media and for oxic and anoxic conditions. The comparison between MPN cultivations and total microscopic cell counts of the microbes from the sediment extracts before cultivation shows cultivation efficiency up to 47.7 %, for aerobic and 55.45 % for anaerobic cultivated samples (Table 7). The highest cell number was detected at 6.7 m (Figure 15). The MPN cultivations followed the profile for total cell counts especially with the AFW medium under aerobic conditions. One exception could be found in 11.19 m depth where a small peak in the cultivation efficiency could be detected. The cultivation efficiency for anoxic conditions was at the lower depth higher than for the oxic conditions (Table 7). From 6.85 m to 12.6 m all sampled depth showed higher cultivation efficiency under oxic conditions. No antibiotic producers could be detected in the whole cultivation approach.

Table 7 Cultivation efficiency (Düsseldorf 2007) in %, reverenced to total cell counts with DAPI staining before cultivation for the different cultivation settings and two different media. AFW: artificial fresh water medium; Str: *Streptomyces* medium.

Depth (m)	Düsseldorf March 2007			
	Cultivation efficiency (%) oxic conditions		Cultivation efficiency (%) anoxic conditions	
	AFW	Str	AFW	Str
6.56	0.96	0.06	43.2	0.65
6.64	1.64	0.00	55.4	0.55
6.70	3.69	0.45	43.8	0.35
6.83	15.2	0.00	5.41	-
7.11	4.10	1.23	0.97	0.54
7.46	0.06	0.00	-	0.00
8.65	0.41	0.02	0.23	-
8.75	5.26	0.08	-	-
11.19	47.7	0.36	-	0.00
12.52	0.95	0.43	-	-

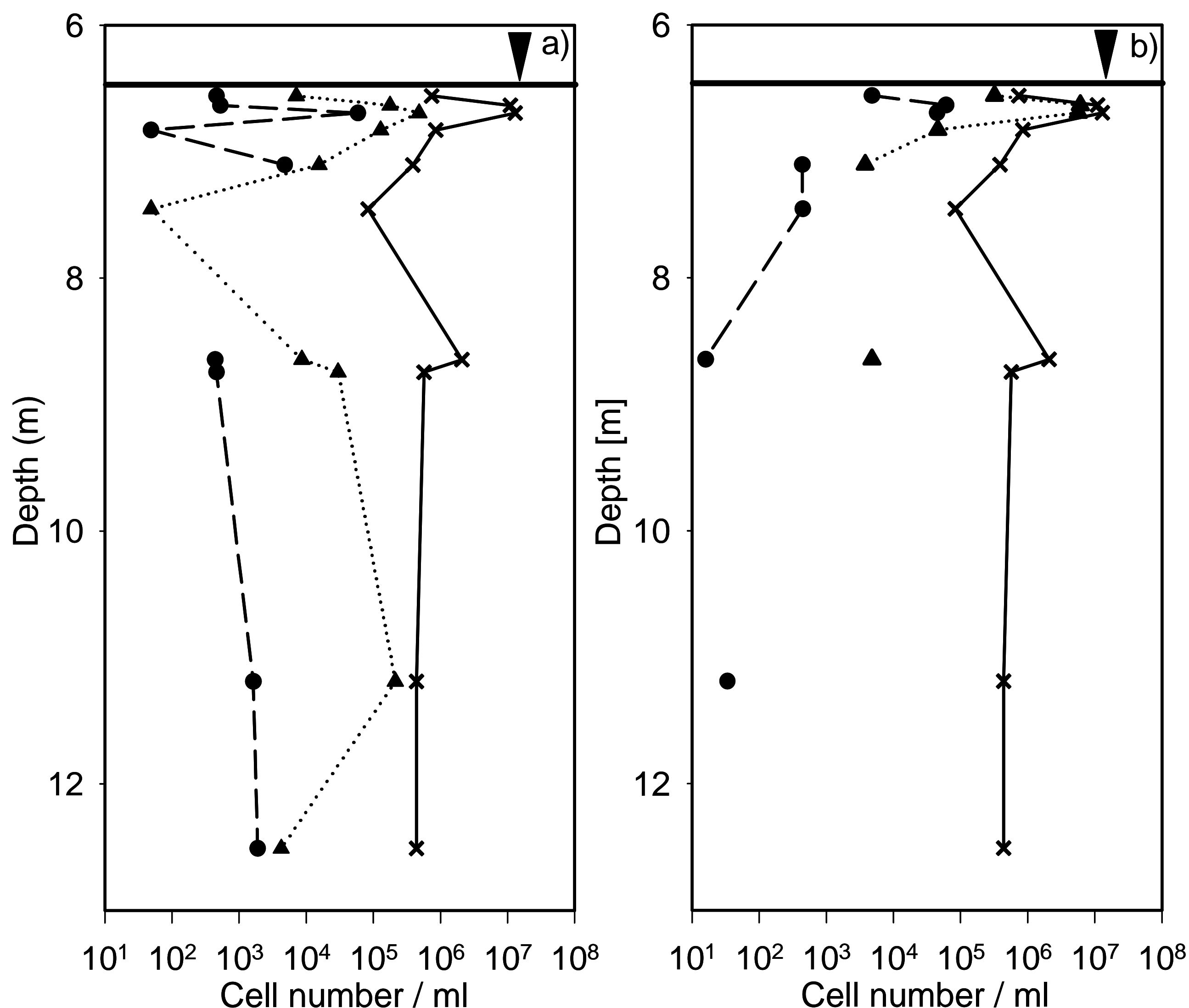

Figure 15 Cultivation of microorganisms from water samples in Düsseldorf 2007 for a) oxic and b) anoxic conditions; solid line: total cell counts obtained by DAPI staining; dotted lines: most probable number (MPN) counts cultivated with artificial fresh water (AFW) medium; dashed lines: MPN counts cultivated with *Streptomyces* medium.

4.2.2 Distribution of antibiotic producers in sediment samples from a pristine aquifer

Cultivation of microorganisms from samples of the sediment core was performed with two different media and for oxic and anoxic conditions. The comparison between MPN cultivations and total microscopic cell counts of the microbes from the sediment extracts before cultivation showed cultivation efficiency up to 33 %, for aerobic cultivated samples (Table 8). Especially at the transition zones from sand to clay or close to these areas high cell numbers and high cultivation rates could be found. In the sampled aquifer the clay zones were between 2 – 3 m, between 12.40 – 15.60 m and between 22.20 – 27.40 m. The highest cell numbers were detected at 2.3 m, 4.3 m, 11 m, and 21.5 m (Figure 16) with slight decreases in depth. At 20 m and 26 m peaks with similar bacterial counts as in upper parts of the aquifer could be found.

In general the MPN cultivations followed the profile for total cell counts with some exceptions in 5.3 m and 15.9 m where a decrease in total cell counts and a peak in MPN counts could be detected. This systematic held for both aerobic and anaerobic cultivations. However, the cultivation efficiency for anoxic conditions was lower than for oxic cultivation (Table 8). Only in some samples at 5.9 m, 15.9 m, 18 m and 18.9 m cultivation is higher than 1 % of total cell counts (Table 8).

Cultivation of microorganisms from samples of the sediment core was performed with AFW medium and *Streptomyces* medium to compare for growth on a rich medium. Cultivation efficiency was in average 13.7 times higher with the AFW medium than with *Streptomyces* medium indicating the sustainability of the AFW medium to cultivate organisms from oligotrophic environments. Only at 17 m depth the cultivation was better with *Streptomyces* medium and for both media the same cultivation efficiency could be found in the depth of 18.9 m, 19.5 m, 21.5 m, and 22 m (Table 8). In the anaerobic cultivation the cultivation efficiency was higher for *Streptomyces* medium than for AFW in the depth of 9.0 m, 25.0 m and 26.0 m.

Table 8 Cultivation efficiency in %, reverenced to total cell counts with DAPI staining before cultivation for the different cultivation settings and two different media. AFW: Artificial Fresh Water Medium; Str: *Streptomycetes* Medium; for 29 m and 30 m it was not possible to get the cultivation efficiency.

Depth (m)	Drilling October 06				Depth (m)	Drilling May 06	
	Cultivation efficiency (%) oxic conditions		Cultivation efficiency (%) anoxic conditions			Cultivation efficiency (%) oxic conditions	
	AFW	Str	AFW	Str		AFW	Str
1.0	11.70	2.01	0.15	0.07	2.8	0.84	0.02
2.3	0.86	0.09	0.01	0.01	5.5	11.40	0.14
3.0	2.29	0.19	0.15	0.00	7.0	0.05	0.01
4.3	0.24	0.05	0.01	0.00	10.0	0.12	0.04
5.3	16.88	2.56	0.00	0.00			
5.9	0.11	0.01	2.71	0.12			
6.9	0.23	0.04	0.00	0.00			
7.9	1.07	0.23	0.04	0.02			
9.0	25.95	6.52	0.93	4.53			
10.0	19.78	1.14	0.11	0.02			
11.0	9.15	0.49	0.13	0.01			
12.0	17.16	8.27	0.06	0.02			
12.6	16.97	6.62	0.27	0.03			
13.7	0.22	0.05	0.00	0.00			
14.7	0.28	0.22	0.01	0.01			
15.9	33.07	9.65	3.84	3.71			
17.0	0.22	0.29	0.70	0.21			
18.0	19.11	2.92	17.67	0.05			
18.9	22.19	22.19	1.13	0.00			
19.5	3.96	3.96	0.03	0.04			
20.5	2.53	2.53	0.06	0.22			
21.5	2.38	2.38	0.06	0.01			
22.0	7.60	7.60	0.00	0.00			
23.0	2.22	0.26	0.00	0.00			
24.0	2.81	0.18	0.00	0.00			
25.0	4.53	0.51	0.05	215.87			
26.0	4.39	0.02	0.01	4.70			
27.0	4.78	4.22	0.00	0.00			
28.0	12.81	5.11	0.01	2.00			

Antibiotic producers could be detected in five depths of the aerobic cultivations (Figure 16) 2.3 m (1), 4.3 m (2), 7 m (2), 21.5 m (12), 30.0 m (1) (number of antibiotic producers in brackets), but no antibiotic producers could be detected in anoxic cultivations. The occurrence of antibiotic producers did not correlate with the cultivation efficiency. Some areas with high cell counts and good cultivation efficiency could be detected around 5.0 m, 10.0 m and 20.0 m. At around 5.0 m and 10.0 m the highest cell numbers could be found at the transition from clay to sand layers of the sediment but the antibiotic producers were found in 2.3 m, 4.3 m, 7.0 m, 21.5 m, and 30.0 m depths.

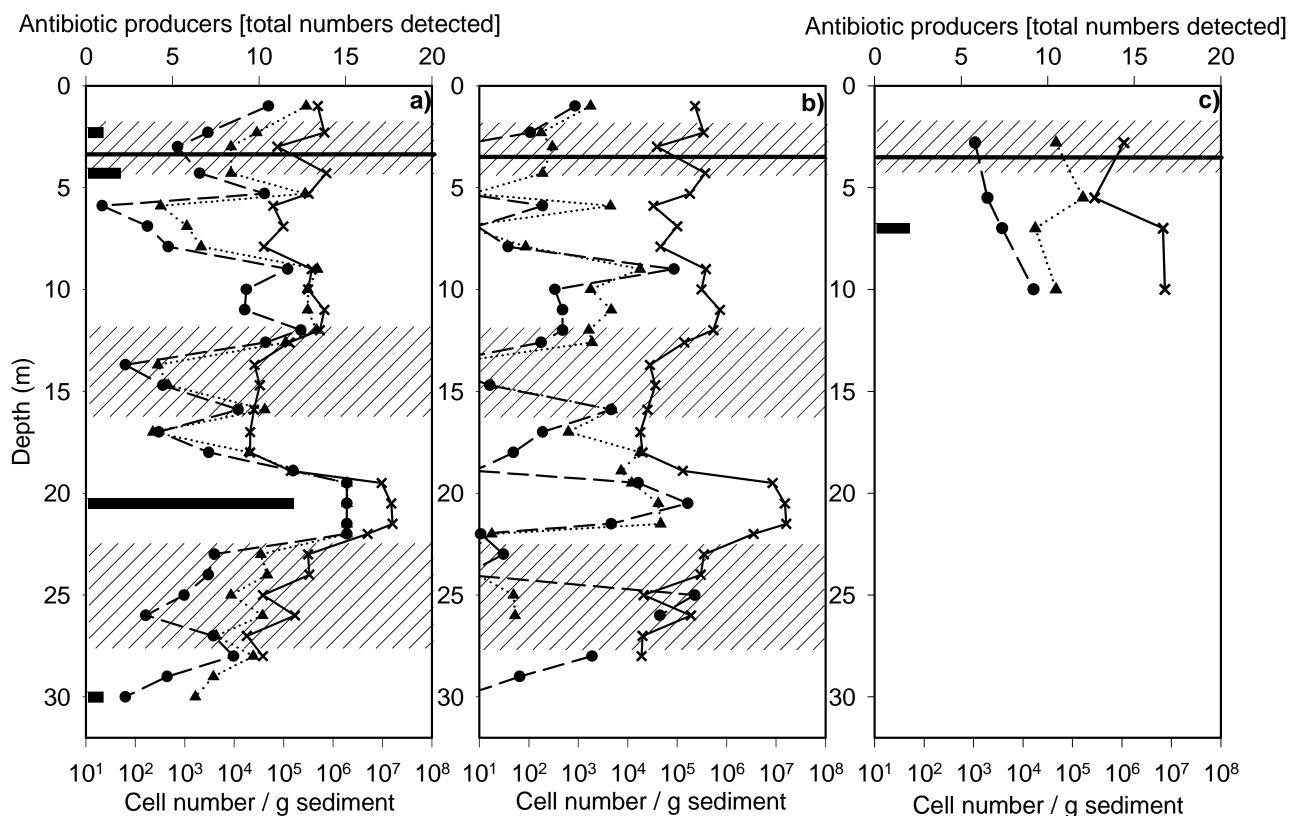

Figure 16 Cultivation of microorganisms from sediment samples of the drilling in Scheyern for a) October 06 oxic, b) October 06 anoxic conditions, and c) May06 oxic conditions; solid line: total cell counts obtained by DAPI staining; dotted lines: most probable number (MPN) counts cultivated with AFW medium; dashed lines: MPN counts cultivated with Streptomyces medium. The striped areas mark clay zones in the aquifer.

The two antibiotic producers from 7 m depth (D7 and G11) are from the sampling May 06 and displayed in Figure 16c). All antibiotic-producing strains identified in the first screening were subcultured and purified. However, all but these two strains lost the ability to inhibit test organisms after a few further cultivation steps. It was not possible to reproduce the antibiotic effect, also when the former antibiotic producers were cultivated in smaller volumes of medium or in sterile sediment.

In the first drilling (May 06), two antibiotic producers out of 484 grown cultures could be detected. This means hat approximately 0.4 % of the cultured bacterial community were antibiotic producers. For the second drilling (October 06), in total 16 antibiotic producers were detected out of 2608 cultures which accounts for 0.6 % antibiotic producers. All antibiotic producers were found to be multi resistant against the standard antibiotics tested (Table 9). Streptomycin and chloramphenicol were effectively in killing most but not all of the isolated strains. Strain 8 and 10 (6.85 m) and strain 8 (9.5 m) were resistant against chloramphenicol and strain 12, 15, and 16 against streptomycin. There was no strain that could not be killed by one of the applied antibiotics.

Table 9 Resistance of isolated antibiotic producers against standard antibiotics, + means strain is resistant, - means strain is sensitive. Strains of the Scheyern cultivation October 06 were named 1 - 16 starting with the strain found in the lowest depth.

Antibiotic	**Strain**																	
(µg) (Units U)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**D7**	**G11**
Tetracyclin (30)	-	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+
Cephalothin (30)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nisin (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vancomycin (10)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin (10)	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-
Nalidixic acid (30)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chloramphenicol(30)	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
Erythromycin (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lincomycin (15 U)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillin (10 U)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

D7 and G11 produced antibiotics constantly and inhibited growth of Gram-positive test organisms (Figure 17). All Gram-positive test strains and one Gram-negative test strain (*Ochrobactrum grignonense*) were more or less inhibited by the culture supernatants of D7 and G11.

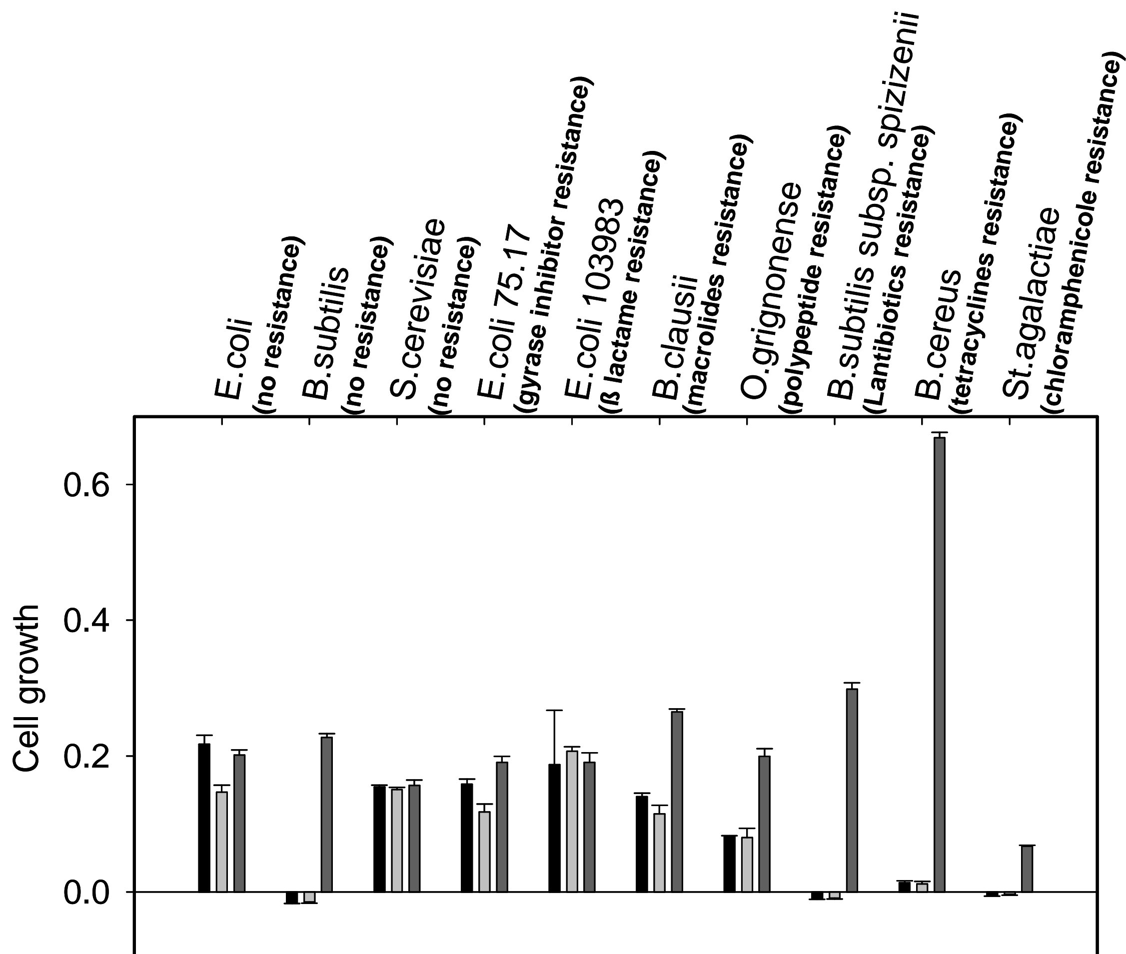

Figure 17 Influence of supernatant of antibiotic producers on growth of test organisms Black bar: supernatant from strain D7, light grey bar: supernatant of strain G11, dark grey bar: growth without antibiotic.

The strains with resistances against lantibiotics/glycopeptide antibiotics (*Bacillus subtilis* subspecies *spizizenii*), tetracyclines (*Bacillus cereus*), and chloramphenicole. antibiotics (*Streptococcus agalactiae*) did not grow at all. The test strain *Bacillus clausii* with macrolide resistance was able to grow in the presence of the supernatants indicating that our two isolates might produce a macrolide antibiotic.

4.3 Characterisation of isolated antibiotic producers

Two novel antibiotic-producing strains, D7 and G11, were isolated from aquifer sediment. Both were Gram-negative and catalase and oxidase positive and did not grow in the absence of molecular oxygen. Growth was successfully tested with AFW, nutrient broth and LB medium in liquid media and on agar plates. The optimal growth temperature of both strains was 28 ℃ with a wide r anging from 15 – 37 ℃ (Figure 18a). Growth of D7 and G11 was detected within a broad pH range from nearly 4.0 to 10.0 (Figure 18b).

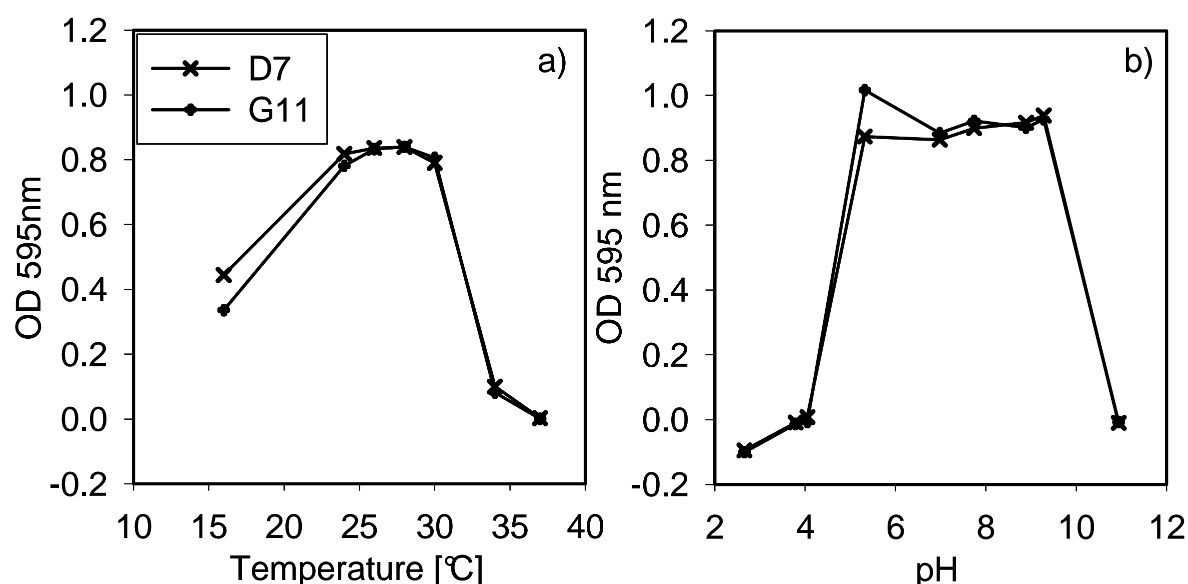

Figure 18 Growth conditions for the strains D7 and G11 under different a) temperature and b) pH conditions.

The metabolic abilities of both strains were tested with api® 20NE kits and indicated a broad substrate spectrum with the ability to assimilate glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, potassium gluconate, capric acid, malic acid, and citrate (Table 10). Usage of the api® 20NE test indicates a typical metabolic profile for the genus *Pseudomonas.* There could be found no difference in metabolic properties compared to the *P. fluorescens* type strain ATCC 13525 (Table 10). Both strains were motile and showed fluorescence at the typical wavelength of fluorescein (excitation 485 nm emission 535 nm) in small amounts but increasing with increasing

cell numbers (data not shown). The supernatant of the two isolated strains also showed haemolysis activity on blood agar plates (Merck, Darmstadt, Germany).

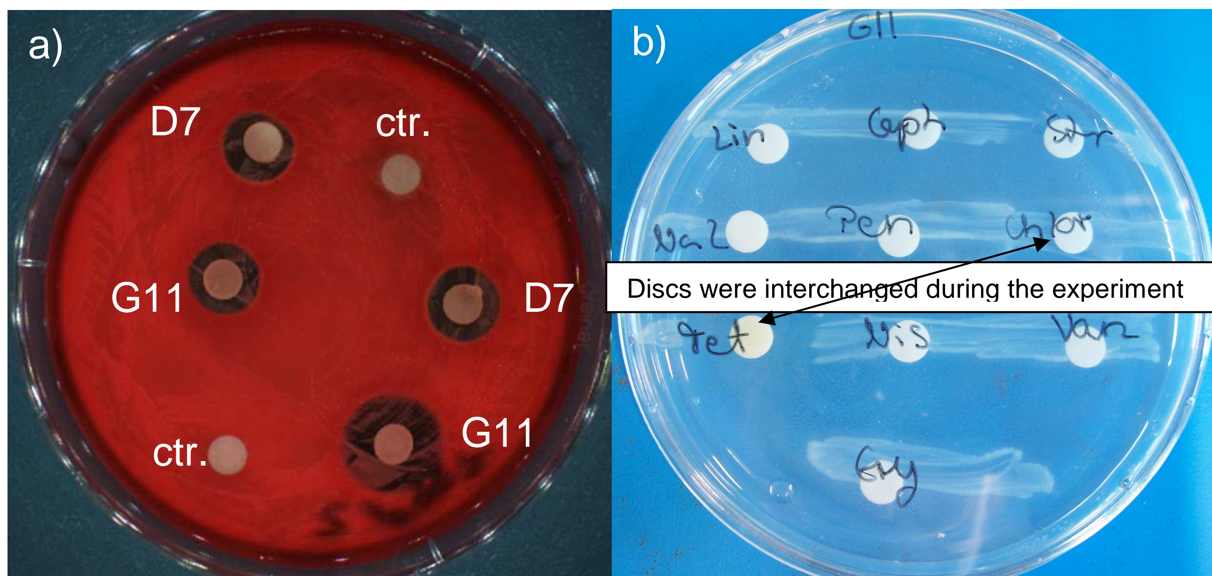

Figure 19 a) Haemolysis caused by supernatants of strain D7 and G11 grown in artificial fresh water medium (left) and nutrient broth (right) on filter discs. Controls did not cause haemolysis. b) Resistance test with strain G11 on Mueller Hinton agar. Concentrations on filter discs: Lincomycin (Lin) 15 Units/disc, Cephalothin (Ceph) 30 µg/disc, Streptomycin (Str) 10 µg/disc, Nalidixic acid (Nal) 30 µg/disc, Penicillin (Pen) 10 Units/disc, Chloramphenicol 30 µg/disc, Tetracyclin 30 µg/disc, Nisin 10 µg/disc, Vancomycin 10 µg/disc, Erythromycin 10 µg/disc.

The producers were resistant against many commercially available antibiotics (tetracycline, cephalothin, nisin, vancomycin, nalidixic acid, erythromycin, lincomycin, and penicillin) (Figure 19b) and were only sensitive to streptomycin and chloramphenicol.

16S rDNA analysis revealed as closest relatives the genus *Pseudomonas* (Figure 20). The closest relation with 99 % of the strains D7 and G11 was given to *Pseudomonas* sp. EP25, *Pseudomonadaceae bacterium* KVD-1700-26, *Pseudomonas* sp. MPU101, and *Pseudomonas* sp. IC038 (Figure 20).

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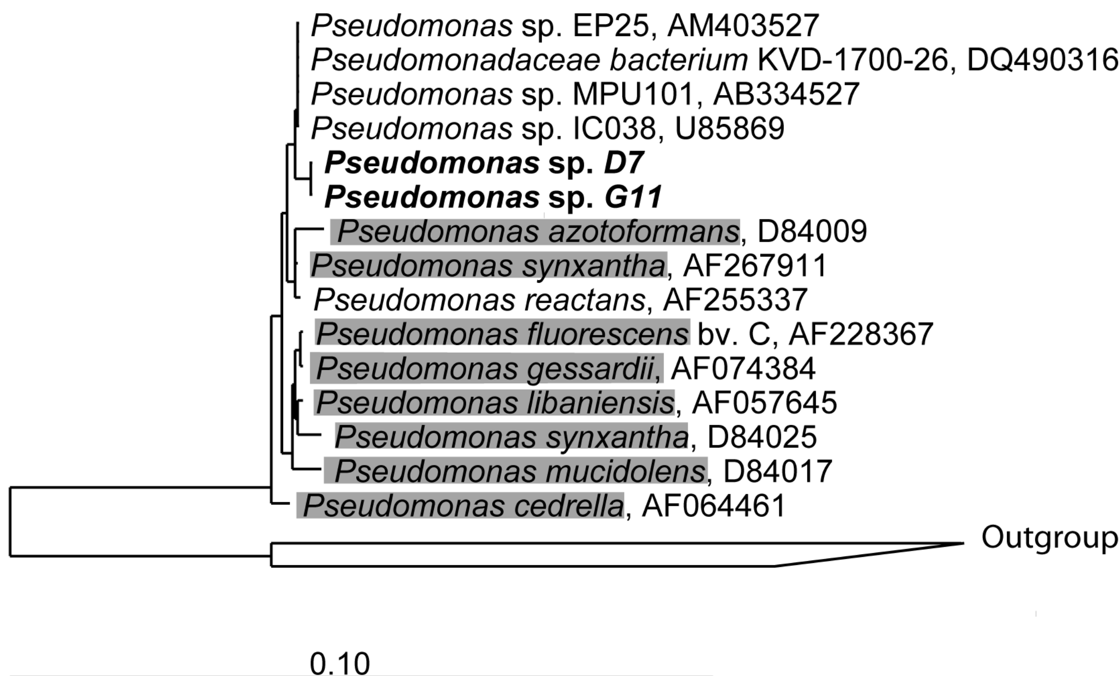

Figure 20 Phylogenetic tree of *Pseudomonas* species related to D7 and G11. The grey highlighted strains belong to the *Pseudomonas fluorescens* subcluster of the genus *P. fluorescens* (Anzai*, et al.*, 2000). For tree calculation the outgroup consisted of a broad collection of organisms that belong to the kingdoms Archea and Bacteria. The bar indicates 10 % estimated sequence divergence.

The electron microscopic pictures show the Gram-negative cell wall (Figure 21c), poly-hydroxyalkanoate inclusions in the cell (Figure 21a), and the bacterial chromosome (Figure 21c). Figure 21b) gives an overview of the bacterial culture. The photographs of D7 and G11 looked totally the same and therefore a selection was made and only pictures of D7 were presented.

Table 10 Properties of the new bacterial isolates, + enzyme or (metabolic) reaction available, - enzyme or (metabolic) reaction not available.

Property	Strain D7	Strain G11	*P. fluorescens* type strain ATCC13525
cell morphology	rod	rod	rod
growth optimum	28 ℃	28 ℃	28 - 30 ℃
motility	+	+	+
fluorescent	+	+	+
spore formation	--	--	--
oxidase	+	+	+
catalase	+	+	+
Gram stain	negative	negative	negative
GC content	59.9	59.9	59 – 61%
pH range	4.5 - 10	4.5 - 10	
nitrate reduction	--	--	--
indole production	--	--	--
glucose fermentation	--	--	--
arginine dihydrolase	--	+	+
urease	--	--	--
ß-glucosidase	--	--	--
protease	+	+	+
ß-galactosidase	--	--	--
Assimilation of:			
D-glucose	+	+	+
L-arabinose	+	+	+
D-mannose	+	+	+
D-mannitol	+	+	+
N-acetylglucosamin	+	+	+
D-maltose	--	--	--
potassium gluconate	+	+	+
capric acid	+	+	+
apidic acid	--	--	--
malic acid	+	+	+
citrate	+	+	+
phenylacetic acid	--	--	--

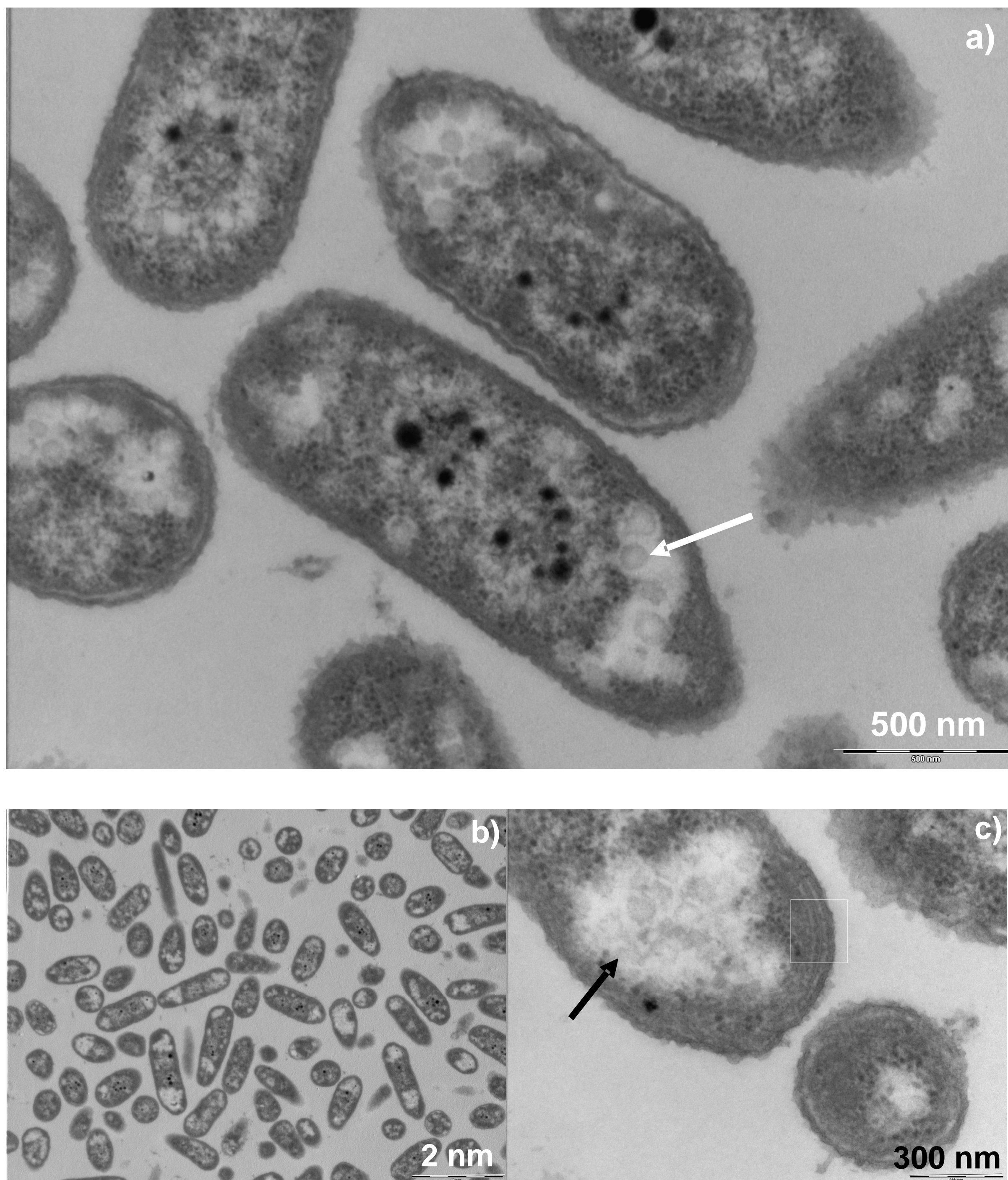

Figure 21 Transmission electron microscopic picture of strain D7; a) white arrow: poly-hydroxyalkanoate inclusion in the bacterial cell; b) overview of the bacterial culture; c) white frame: Gram-negative cell wall, black arrow: bacterial chromosome.

4.4 Identification of the active substance

Since cell-free supernatants of strains G11 and D7 showed antibacterial activity (Figure 17), a strategy for the identification of bioactive component(s) was designed based on liquid chromatographic separation and mass spectrometric detection.

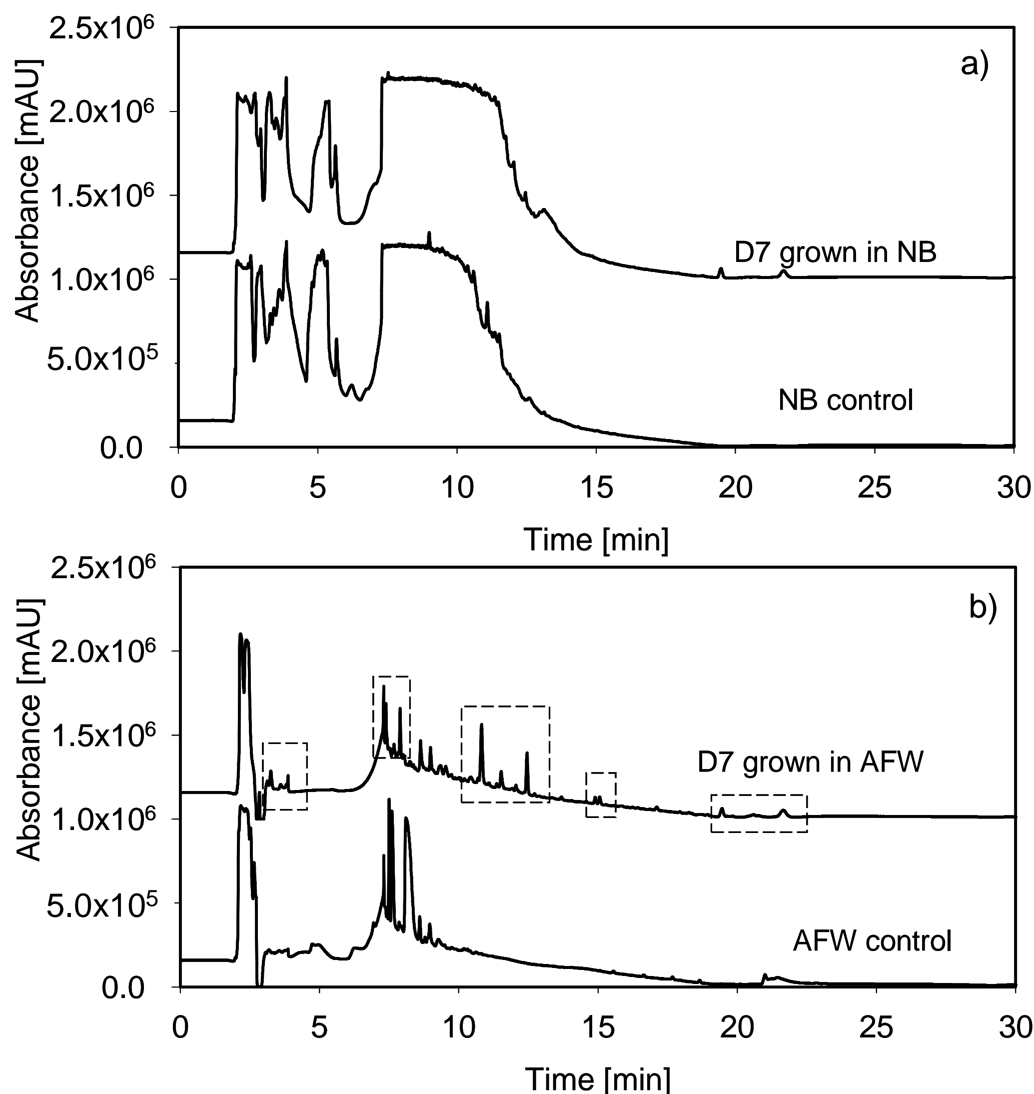

Figure 22 HPLC chromatogram of bacterial supernatant of strain D7 grown in two different media a) nutrient broth medium (NB), b) artificial fresh water (AFW), the boxes mark peaks that only occurred in the chromatogram of bacterial supernatant measurements.

Several HPLC peaks were identified in supernatants of AFW grown cultures (Figure 22b). Fractions containing the peaks were tested for antimicrobial activity and compared with the same fractions from the pure medium control. An antibacterial activity against the test strain *B. subtilis* was detected in fraction 25 of the HPLC-chromatogram (Figure 22) with a retention time of 12.5 minutes.

Fractions exhibiting antimicrobial activity (fraction 25) were subjected to FT-ICR-MS analysis. The samples were measured in both, positive and negative, ionisation mode. In positive mode, several m/z peaks over the whole mass range were observed (data not shown). The mass spectra of the same sample measured at negative mode were less complex with a dominant peak at 499.2905 m/z (Figure 23). The elemental composition considering C, H, N, and O was determined as $C_{26}H_{44}O_9$ with a relative error of 0.3 ppm that indicated mupirocin (Figure 23) as a possible structure (http://chem.sis.nlm.nih.gov/chemidplus/) which is known to have antibacterial activity.

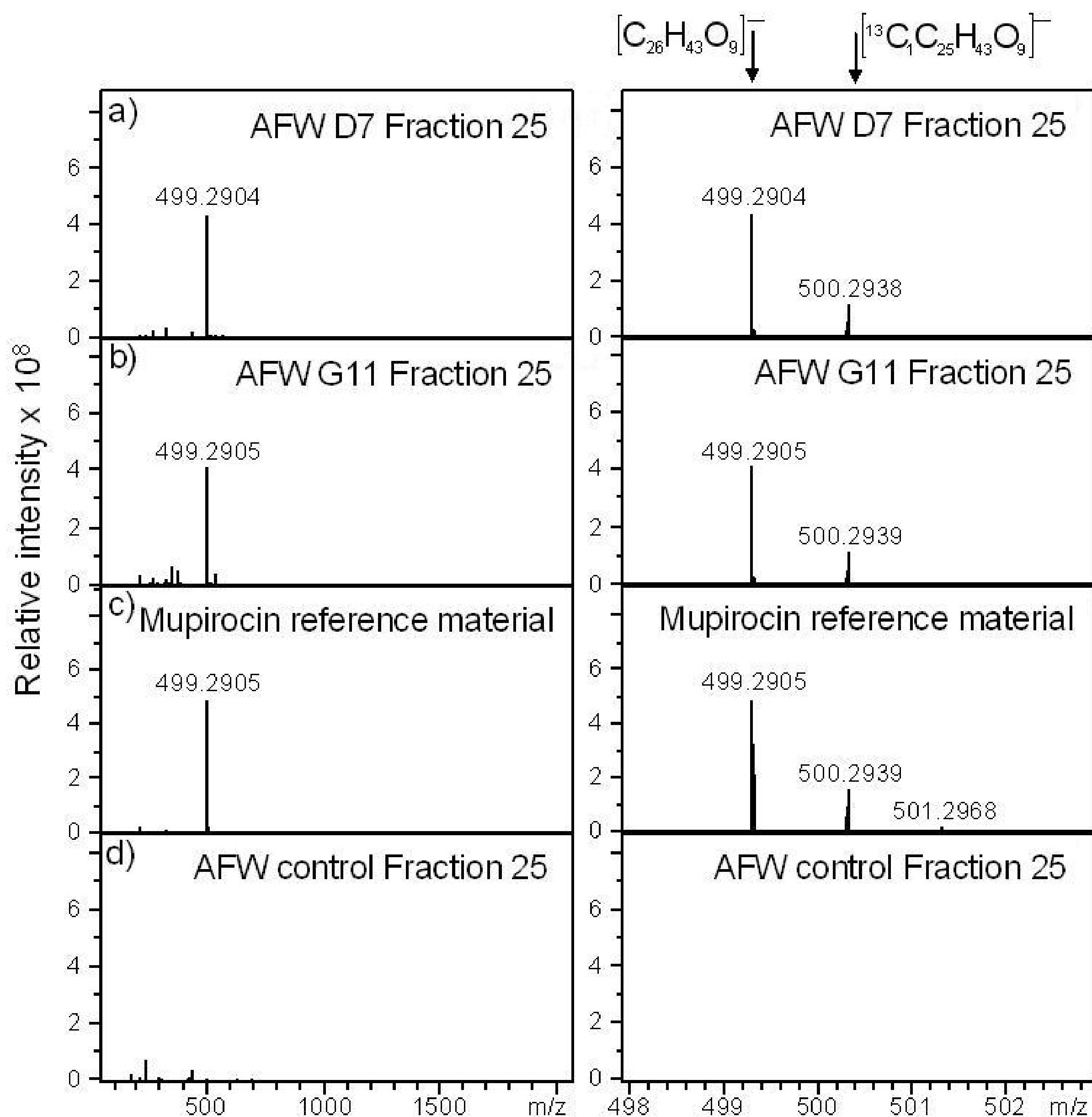

Figure 23 Dominant peaks of the FT-ICR mass spectrum at a range of 150 - 2000 m/z (left column) and 498 - 503 m/z (right column) from HPLC Fraction 25 of: a) culture supernatant of strain D7, b) culture supernatant of strain G11, c) standard mupirocin as reference material, d) pure artificial fresh water medium control.

To get more information of mupirocin structure by FT-ICR-MS, fragmentation with different collision-induced dissociations (CID) (15, 20, and 25 eV) was applied and mass spectra of the fraction 25 was compared to the ones purchased of mupirocin

reference material (Figure 24a). Mupirocin dissociated into two fragments with m/z of 299.1081 and 255.1600 and the second one fragmented further at the ester group into m/z of 173.1182. These fragments were also observed in the supernatant fractions of D7 and G11 (Figure 24a) when CID was applied.

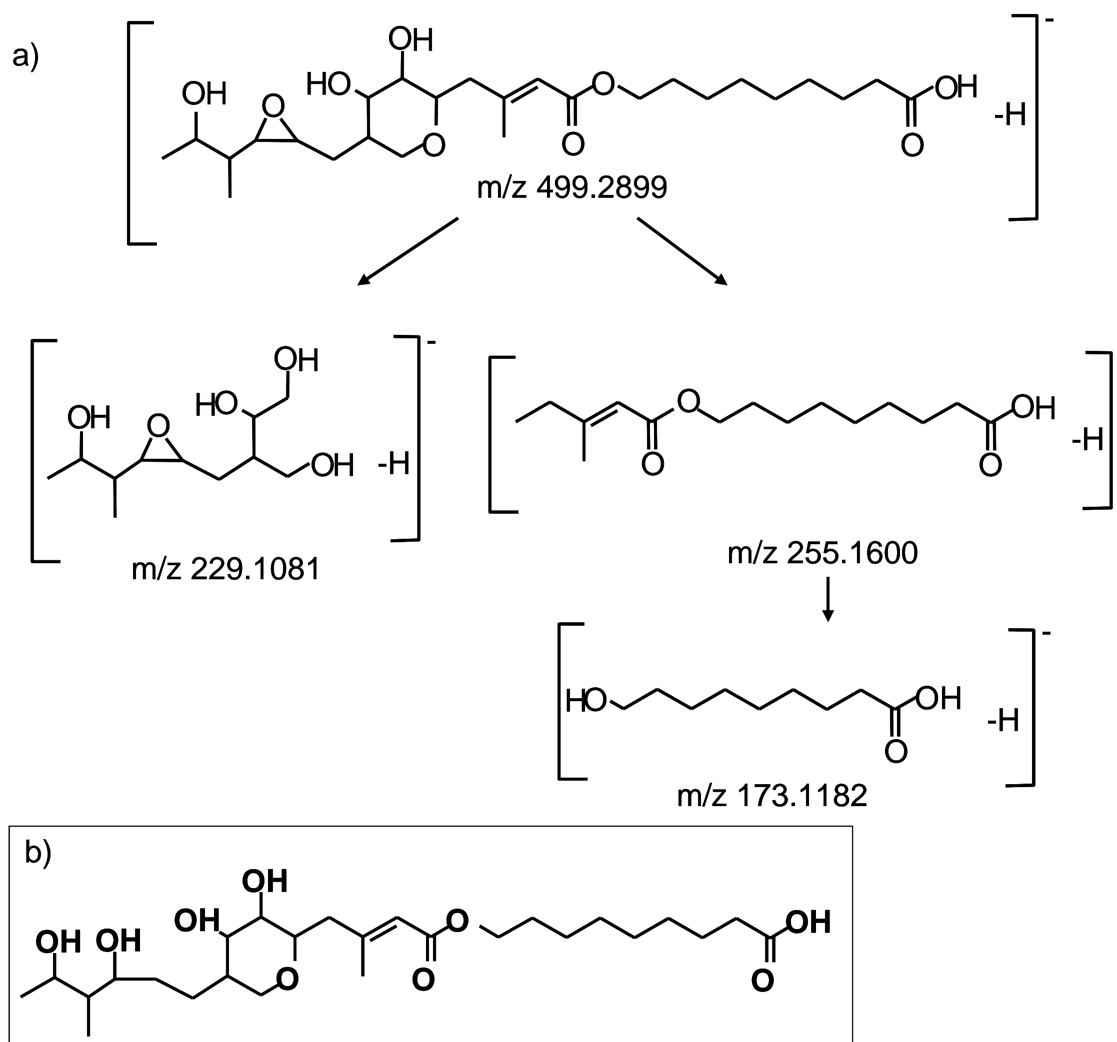

Figure 24 a) Mupirocin fragmentation pattern, b) Mupirocin with opened epoxy ring.

The same supernatant fractions of D7 and G11 were separated by UPLC after optimisation of the method for the determination of mupirocin. Injecting the standard solution, two peaks were observed near to each other (Figure 25) and their resolution increased with decrease of the pH of the mobil phase. On the other hand, low pH of the separation solvent had to be used to get the mupirocin at neutral form since its

predicted pKa was 4,88 (Pallas, Software predicting pKa,logP, logD values and metabolites based on structural formulae of compounds) and thus a pH less then 3.0 was required. Presumably, during the separation and/or storage the epoxy ring of mupirocin (Figure 24) might open resulting in a hydroxyl group. The new metabolite has different retention behaviour that could be visualised by UPLC due to the higher resolution efficiency (Murphy, 2006, Novakova*, et al.*, 2006, Englmann*, et al.*, 2007).

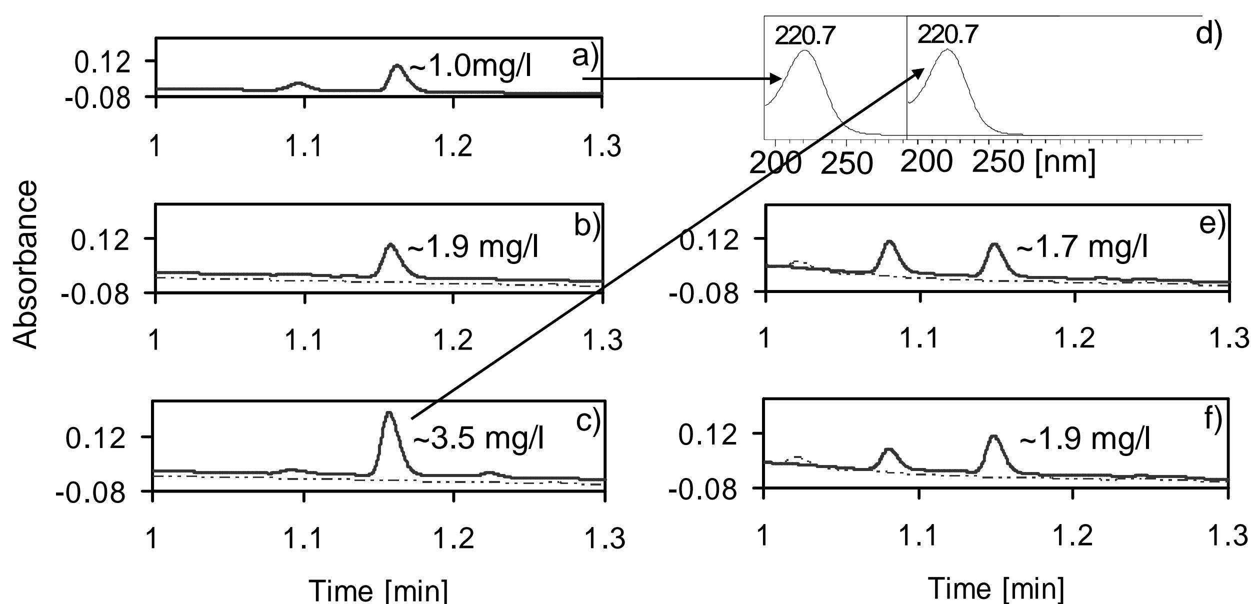

Figure 25 Chromatogram of UPLC analysis with semiquantitive information of the mupirocin concentration of a) mupirocin standard, b) bacterial supernatant from strain D7 in AFW medium, c) bacterial supernatant from strain G11 in AFW medium, d) Absorbance wavelength of mupirocin standard and bacterial supernatant from strain G11 in AFW medium, e) bacterial supernatant from strain D7 in NB medium, f) bacterial supernatant from strain G11 in NB medium. The dashed line represents the pure medium as control.

At the retention time of mupirocin a peak was observed in the fractionated samples with an identical UV-Vis spectrum to the reference (Figure 25). Thus the results obtained from the FT/ICR-MS, MS/MS and UPLC reflects the high reliability of mupirocin identification. Since two peaks of the reference material were observed,

semi-quantification on the amount of the mupirocin was possible. Figure 25 shows the chromatogram of the concentrated supernatants. The produced amount was at low mg/l range (around 1.8 - 3.5 mg/l) and independency on the type of the supernatant was observed, although the amount of the opened form of the mupirocin was higher in NB medium.

4.5 Influence of antibiotic producers on bacterial community composition

The TRFLP analysis showed a small number of community members in the samples. A high peak occurred at 490 base pairs and the problem occurring during the experiment was that the TRFLP peak of the pure culture for the isolated strain, which should be used as antibiotic producers in the columns, was also at 490 base pairs. The prominent peak at 490 established from the beginning of the experiment and staid present. Some peaks appeared after an incubation time of 2 weeks but not with a higher intensity then the peak at 490 base pairs. The community seems to be not very diverse in the columns because of less peaks occurring. The picture does not changed when the incubation time was elongated. When adding the antibiotic producer D7 to the columns the result, after a few days looks like the result for the pure culture (data not shown).

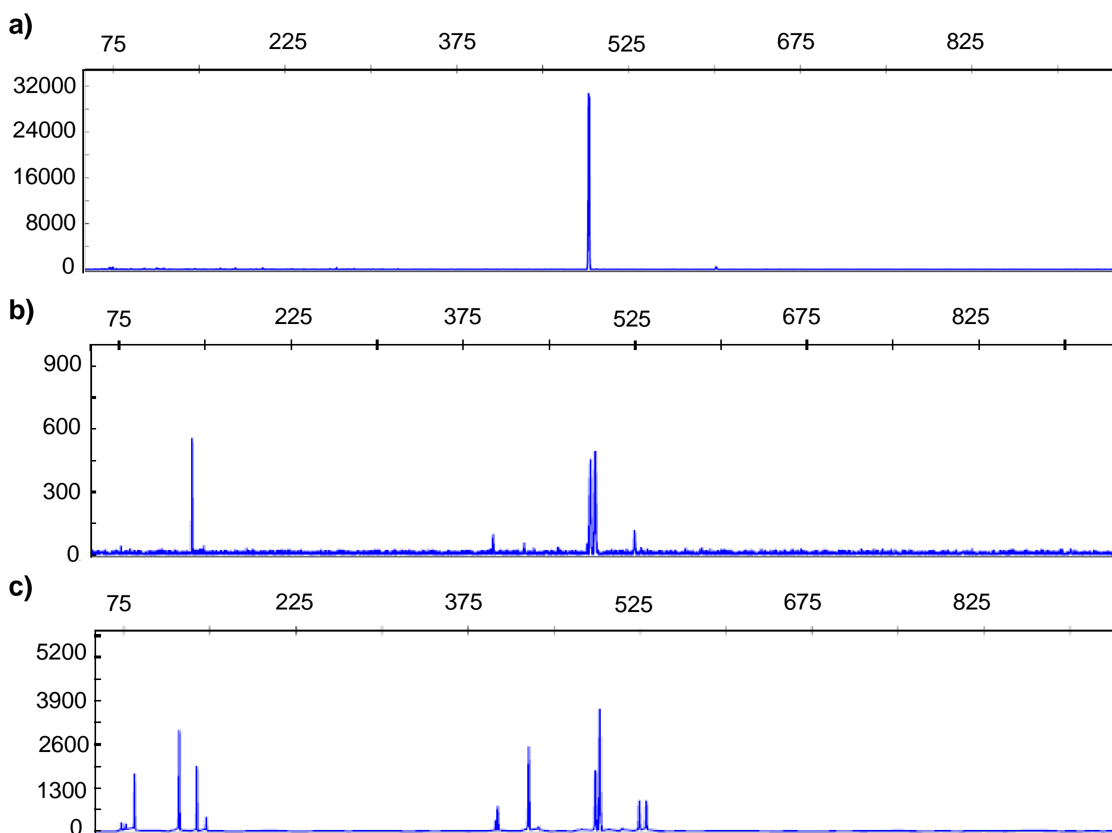

Figure 26 TRFLP fingerprint of the bacterial community in flow through of sediment columns a) pure culture of the antibiotic-producing strain D7, b) flow through after 1 week of incubation without antibiotic producer, c) flow through after 2 weeks of incubation without antibiotic producers.

5 Discussion

5.1 Cultivation of bacteria from sediment and water samples

In this study, the abundance of antibiotic-producing strains in a pristine and a contaminated aquifer is presented. Although the pristine sampling site was located on an experimental farmland, it was not impacted by antibiotics so far. No declaration about antibiotics can be made for the contaminated aquifer because the main interest was lying on the tar oil contamination (Wisotzky, 1997).

5.1.1 Cultivation of bacteria from the pristine aquifer in Scheyern

Pristine aquifers, which are usually used for drinking water production are specified by very oligotrophic conditions (low organic carbon content) and, thus, by an extremely low density of microbial populations. In general, the number in these systems is in the range of 10^5 – 10^7 cells/g dry sediment but dependent on flow, dissolved oxygen, and hydrocarbon concentration cell numbers can vary from 10^2 – 10^8 cells/g dry sediment (Ghiorse & Balkwill, 1983, Wilson*, et al.*, 1983, Ghiorse & Wilson, 1988). The distribution of microbial cells in aquifers is most abundant at the capillary fringe with decreasing tendency towards depth (Wilson*, et al.*, 1983). Usually, cell numbers do not fall below 10^5 cells/g dry sediment in shallow aquifers. In the investigated pristine sediment, which penetrated several aquifer layers, a very fluctuating cell density was observed. Here, from the capillary fringe (four meters below ground) down to 30 m depth, total cell numbers were about 10^6 cells/g. Interestingly, a slightly decreasing cell density towards depth was observable with higher cell numbers at the fractions between clay and sand layers. This effect is due to microsites, where substrates diffuse from organic rich layers (clay) into sandy layers, which offer enough porosity for organisms to live (Ghiorse & Wilson, 1988). The number of cultivated bacteria more or less followed the trends of the microscopic cell counts. Nevertheless, the cultivation efficiency under oxic conditions varied from 0.11 % (5.9 m depth) to 33.07 % (15.9 m depth) of total cell counts over depth.

30 % cultivation efficiency is an astonishing high number compared to the general statement that only up to 1 % of the total cells can get cultivated with regular media (Amann*, et al.*, 1995, Bernard*, et al.*, 2000). Recently, Bruns *et al.* (2002) developed a medium for high throughput cultivation in microtiter plates, which produced unusual high cultivation efficiencies. Key parameters in the medium were the low nutrient concentrations and the presence of microbial messenger molecules. In the present work, this cultivation approach was adapted to sediment samples. The obtained cultivation efficiencies of up to 33 % depicted that a representative portion of the microbial community has been studied. With high resolution of sampling of approximately 1 m it was investigated if the occurrence of antibiotic-producing microbes or the cultivation efficiency might depend on the geochemical conditions in different sediment layers. The cultivation efficiency was not reproducible for the same depth (10 m, Table 8) at different drillings. However, we have to take into account that there was a time span of six month between both sampling dates and the boreholes were 2 m apart.

The amount of antibiotic producers cultivated from the aquifer ranged between 0.4 % to 4.8 % of the local community. The majority of the detected antibiotic producers stemmed from one sampling depth (20.5 m, Figure 16) but due to overall low number of positives no clear statistic correlation to cultivation efficiency, depth or stratigraphy of the aquifer could be made. Only one antibiotic producer stemmed from a clayey zone of the aquifer (depth 2.3 m, Figure 16a). In the depth where antibiotic producers can be found (2.3 m, 4.3 m, 20.5 m, and 30.0 m) cultivation efficiencies ranged from 0.24 % at 4.3 m to 2.53 % in 20.5 m and in depths with high cultivation efficiency like 9 m, 15.9 m, or 18.9 m no antibiotic producer could be detected. The highest cell numbers and 12 antibiotic producers could be identified at 20.5 m depth, where a higher but still comparably low amount of dissolved organic carbon was present (Schwientek, 2008). High cell numbers for the anaerobic cultivation can be found in oxic parts of the aquifer (around 20 m depth). However this was not surprising because it has been shown that anaerobic processes occur within micro sites of oxygenated environments (Martino*, et al.*, 1998, Beeman & Bleckmann, 2002).

Anaerobic antibiotic producers might be detectable in the aquifer sediments and therefore an anaerobic cultivation approach was established. A putative anaerobic

microorganism might produce a totally new antibiotic, which is not related to known ones, and there is only one publication on anaerobic antibiotic production (Sturgen & Casida, 1962). Furthermore, oxygen is needed many antibiotic biosynthesis pathways known so far (Flickinger & Perlman, 1979, Elibol & Mavituna, 1999). On the one hand, the fact that no antibiotic producers were detected supports the limited knowledge of this topic. On the other hand, it is not surprising that aerobic antibiotic producers were isolated in anoxic parts of the aquifer as aerobic organisms are frequently found in anoxic aquifers (Bakermans*, et al.*, 2002).

Comparing the efficiencies of the anoxic and oxic cultivations the latter revealed approximately 10-fold higher counts.

5.1.2 Cultivation of bacteria from the contaminated aquifer in Düsseldorf

The investigated aquifer is mainly contaminated by benzene, toluene, ethylbenzene, and xylenes (BTEX), the highest contaminant can be found directly below the unsaturated zone in 6.8 m depth. But these contaminants were only detected in groundwater and this were not adsorbed to the sediment samples. Another less important contamination is by polycyclic aromatic hydrocarbons (PAHs), which can be found adsorbed to the sediment in 8.8 m depth (Anneser*, et al.*, 2007).

Higher cell numbers were found in the groundwater samples compared to sediment, normally higher cell numbers can be found in sediment samples (Griebler & Lueders, 2008). This can be explained by the not 100 % effective detachment of bacterial cells during cell preparation from the sediment especially when living cells should be detached. Cell numbers in sediment samples are nearly in the same range (2×10^5 - 4.5×10^5) for the sampling in June 2005 (Figure 13) and February 2006 (Figure 14) although the samples of June 2005 were stored few months before detecting the cell numbers. Not all samples were from the same depth during the drillings and it is not possible to tell if the low cell number of 4×10^3 in 6.85 m could be found again. The PAH load in the sediment at 8.8 m depth seems not to influence the bacterial cell number because no significant increase or decrease was detected at this depth in both samplings (Figure 13 and Figure 14). The highest BTEX load for

the groundwater samples could be found in 6.8 m depth (Anneser*, et al.*, 2007) and the highest cell counts can be detected slightly above this zone at the fringe of the contaminant plume. This behaviour can be explained by the plume fringe concept: mixing processes that take place at fringes ensure a contact of electron donors and acceptors (Bauer*, et al.*, 2008), which allows a high occurrence of bacteria in this zone. Also the highest enzymatic activity was detected for this aquifer layer (Anneser*, et al.*, 2007). A special bacterial community, which can cope with the contaminants, seemed to be established in this layer. This also explains why the highest cultivation efficiency under anoxic conditions was in the first three sampling points for the groundwater samples (Table 7). Because of the high carbon load due to the contaminants, oxygen is used up quickly, anaerobic bacteria established, and good cultivation efficiencies under anoxic conditions were detected.

The small peak in bacterial cell numbers at 8.65 m depth (Figure 15) can be explained by the PAH contamination in 8.8 m depth. Also the PAHs are adsorbed to the sediment, bacteria in groundwater metabolize the components and increase the cell number.

The cultivations of bacteria from contaminated sediment revealed the identification of 18 antibiotic producers they were detected from the samples in June 2005. 10 of these strains came from 6.85 m depth, the zone with high BTEX contamination. 8 strains were from 9.5 m depth a layer where no high contamination was detected but a contamination of PAHs is present. It is wrong to draw the conclusion that antibiotic producers were isolated mainly from contaminated aquifers as they were also found in pristine ones. Additionally, the cultivation of bacteria from water samples from the contaminated aquifer revealed no antibiotic producers. Again no correlation between cultivation efficiency and antibiotic producers can be found in these samples because cultivation efficiency was lower for sediment samples in 6.85 m (Table 5) then in 9.5 m, however more antibiotic producers could be detected.

5.2 Detection and characterisation of antibiotic producers

For detection of antibiotic producers from the high throughput cultivations the microtiter cultivation set up was also further developed into a new antibiotic susceptibility test to enable easy and fast testing of cultivated organisms. The test turned out to be useful as a fast screening test system to analyse the effect of antibiotics on microorganisms. The same amount of antibiotics supplied to the test organisms in liquid cultures often did not inhibit growth of the test organisms (data not shown). In comparison to the typical antibiotic susceptibility test with agar plates and filter discs, the test with agar cultures in microtiter plates offers a fast and material saving method to check the susceptibility of bacteria. The test system was very reproducible; standard deviations from three parallel measurements were always below 10 % of the signals. The test in microtiter plates is a good alternative to the antibiotic disc test because it is easily performed in high throughput. The result is much easier detectable by optical density measurements in plate readers, and as sensitive as the well established Kirby-Bauer Test (Bauer, *et al.*, 1966)(Table 4).

The actual number of antibiotic producers in the samples might have been larger than identified due to the limited sensitivity of the screening test. Because all cultures from the sediment samples were unknown at the time point of testing for antibiotic production some might have produced concentrations of antibiotics lower than needed for detection which might have prohibited finding of antibiotic producers.

As an indicator for classification of the produced antibiotics a test system with resistant test strains was established. The test organisms carried resistances against different antibiotic classes (Table 2). This allowed an exclusion of certain antibiotic classes for the substances produced by the two isolated strains D7 and G11. Resistances against chloramphenicol, lantibiotics, polypeptide antibiotics, and tetracycline were not effective against the used supernatants. No resistant Gram-positive strain for every antibiotic class could be received and consequently a more precise classification of the compounds produced by the strains D7 and G11 was not possible. The only Gram-positive strain that was able to grow in the test with supernatants of the two isolated strains was *B. clausii* (Figure 17). This bacterium carries a macrolide resistance indicating that the produced antibiotic from D7 and

G11 might belong to the macrolides. The supernatants also inhibited *O. grignonense* as the only Gram-negative tested. The identification of the active substance in the supernatant of D7 and G11 as mupirocin explains the resistance of *B. clausii* to the substance. Mupirocin is a polyketide antibiotic where also the macrolides belong to. Maybe a cross resistance is the reason for *B. clausii* growing under the influence of the active supernatant fraction. The problem of cross resistance between mupirocin and macrolides is unimportant because the effective mechanism of mupirocin can not be found in any other antibiotics up to now (Cookson, 1998). A resistance to mupirocin is not described for this *B. clausii* strain. Only a few Gram-negative bacteria were found to be sensitive to mupirocin (Sutherland, *et al.*, 1985) and only one of our Gram-negative test strains (*O. grignonense*) was sensitive to the bacterial supernatant of D7 and G11 (Figure 17) although the sensitivity seemed not to be as high as in the sensitive Gram-positive strains. *O. grignonense* was until now not investigated especially for resistance purposes and is used due to its resistances to peptide antibiotics (Lebuhn, *et al.*, 2000) in this test system.

The fact that most of the antibiotic producers, which were isolated lost there antibiotic production after 2 - 3 transfers indicates that the cultivation conditions were not ideal to sustain antibiotic production. Antibiotics are secondary metabolites and many are only produced under special conditions (Rokem, *et al.*, 2007), which are unknown for these strains. In nature, the task of secondary metabolites, which we commonly use as antibiotics, is not always the fight against other bacteria. Often the impact of an antibiotic is dependent on the concentration, where lower concentrations are stimulating protein synthesis, modulate metabolic functions or work as intermicrobial signalling agents (Davies, *et al.*, 2006, Linares, *et al.*, 2006, Yim, *et al.*, 2006). However, the strains D7 and G11 produce antibiotics under all tested conditions and were used for further characterisation.

Two new mupirocin-producing *Pseudomonas* strains were isolated from the pristine Scheyern aquifer. The occurrence of *Pseudomonads* is ubiquitous in the environment (Megha, *et al.*, 2007). Special habitats of *Pseudomonads* are for example soil and rhizosphere (Lugtenberg, 2004). *Pseudomonads* are also often found in contaminated aquifers because they are able to use a high number of substances as energy or carbon source and can often resist toxic substances (Fahy, *et al.*, 2006,

Hendrickx, *et al.*, 2006, Iribar, *et al.*, 2008). The bacterial genus *Pseudomonas* is classified by negative Gram stain, the absence of gas formation from glucose, no photosynthetic pigments, a positive oxidase and catalase test, no spore formation, a negative indole test, and motility (Krieg & Holt, 1984). Most of the *Pseudomonads* can reduce nitrate what distinguishes the species *P. fluorescens* from the other species of the genus (Madigan, 2006). These characteristics were all found for the isolated strains. The 16S rDNA gene sequence of D7 and G11 showed 99 % similarity to several *Pseudomonas* strains and also to the *P. fluorescens* type strain (Table 10). The closest relatives of D7 and G11 are represented by *Pseudomonas* sp. EP25, *Pseudomonadaceae bacterium* KVD-1700-26, *Pseudomonas* sp. MPU101, and *Pseudomonas* sp. IC038 (Figure 20). These strains were isolated from deep sea sediments (*Pseudomonas* sp. EP25), a Hawaiian volcanic deposit (*Pseudomonadaceae bacterium* KVD-1700-26), and antarctic sea ice (*Pseudomonas* sp. IC038). A direct comparison of the 16S rDNA gene sequences to the known mupirocin producer *Pseudomonas fluorescens* NCIMB 1010586 is not possible because the sequence is not available in public databases for this producer strain.

Because a huge number of strains have been accommodated in the genus *Pseudomonas* seven subclusters in the genus were formed (Anzai, *et al.*, 2000). The closely (99 %) related strains *P. azotoformans*, *P. synxantha*, *P. fluorescens*, *P. gessardii*, *P. libaniensis*, *P. synxantha*, *P. mucodolens*, and *P. cedrella* (Figure 20) are all members of this *P. fluorescens* subcluster. The strains of this subcluster are mostly known as bacterial bio control organisms or plant pathogens (Mavrodi, *et al.*, 2001, Wechter, *et al.*, 2002, Garbeva, *et al.*, 2004, Raaijmakers, *et al.*, 2006, Megha, *et al.*, 2007) and often polyketides (like mupirocin) are the effective substances (Bender, *et al.*, 1999). The pictures from transmission electron microscopy (Figure 21) also support the metabolic and phylogenetic results of D7 and G11. The Gram-negative cell wall was clearly detectable and also the size and cell morphology indicate that a *Pseudomonas* strain was isolated. The whitish inclusion bodies might be poly-hydroxyalkanoate (PHA), which is the typical storage substance for *P. fluorescens,* but poly-hydroxybutyrate like in many other bacteria is not produced by *P. fluorescens* (Krieg & Holt, 1984).

The interesting fact that mupirocin producers were found in groundwater samples demonstrated that even in pristine aquifers we have to face a certain abundance of antibiotic producers. This also implies the natural abundance of antibiotic resistant organisms in such habitats. In fact our isolates were multiresistant against tetracycline, cephalothin, nisin, vancomycin, nalidixic acid, erythromycin, lincomycin, and penicillin. This further indicates that multiresistant microorganisms have to be expected also independent from anthropogenic influences.

Naturally *Pseudomonads* are often highly resistant against antibiotics, pesticides or protection substances of plants (Shafiani & Malik, 2003). The resistance against antibiotics was also shown for the isolated strains. Many *Pseudomonads* are pathogens and contain virulence factors that are mobile on plasmids and that can be transferred to other strains (Chakrabarty, 1976). This is also the case for resistance genes often located on a R-plasmid (Chakrabarty, 1976). The ability to spread these resistance genes into an environmental bacterial community is given and can become dangerous for human health.

5.3 Detection of the active substance

An identification strategy for the determination of active components applying different analytical techniques has been built up. At first, semi-preparative HPLC have been used for the collection of the separated components. This step was applied as clean-up process that allowed decreasing the number of the presented components in the sample. HPLC has been applied earlier for the determination of antibiotics in liquid samples such as surface water and groundwater. Additionally several HPLC (Marzo & Dal Bo, 1998) or LC-MS protocols exist (Niessen, 1998) for the detection of macrolide antibiotics belonging to the polyketides like mupirocin (Kanfer*, et al.*, 1998, Lingerfelt & Champney, 1999, Schlüsener*, et al.*, 2003, de la Huebra & Vincent, 2005). The fractions were checked for antibiotic activity and the ones showing positive results were then studied by FT-ICR-MS. FT-ICR-MS especially with high magnetic field (12 Tesla) provides ultrahigh resolution of the masses as their m/z and thus allows the accurate determination of the elemental

composition even for complex samples (Hertkorn*, et al.*, 2007). Nevertheless one elemental composition may represent more than one structure. Additional methods like MS/MS, chromatography or capillary electrophoresis have to be applied (Li*, et al.*, 2007, Li, 2007) in combination with reference material for comparison purpose. In this case mupirocin was assumed from the m/z data from FT-ICR-MS after a first database search. UPLC has been used for confirmation study that differs in the analysis base compared to MS and provides faster separation as HPLC (within 1 - 5 minutes instead of 15 - 45 minutes) and higher resolution of the peaks. The two possible forms of mupirocin (Figure 25) were baseline separated. Since the reference material provides two peaks, the method enables only semi-quantification but the UPLC method has an advantage of differentiation of the two forms of the mupirocin. Mupirocin was determined by UPLC from the supernatant without further clean up procedures with concentrations in the low mg/l range (1.7 - 3.5 mg/l). The ratio of the two mupirocin forms was dependant on the type of the medium. In AFW medium the mupirocin with epoxy ring was dominant, until the ratio of the two forms in medium was between 0.3 - 0.5.

5.4 Antibiotic producers and resistances in the environment

The fact that multi resistant antibiotic-producing microbes were cultivable from aquifers, which had no exposure to manure from pig farming, shed a new light on the discussion on the release of antibiotics into the environment. The results indicate that antibiotic-producing organisms have to be expected even at very pristine environments. Thus, also antibiotic resistant microbes should be present in the same habitats. Indeed, the isolated antibiotic-producing strains were all multiresistant against commercially available antibiotic classes. The concentrations used for the resistance test of the strains were chosen by taking in account antibiotic concentrations used in other studies testing bacterial resistance (Jogersen *et al.*, 1994). Pathogenic bacteria or especially hospital strains that developed a antibiotic resistance may need much higher concentrations of antibiotic substances but for this study resistances in the environment were important and not the situation in clinic.

Infact, antibiotic-producing organisms have developed resistance mechanisms against the own antibiotic (Nodwell, 2007) and might also spread this competence within the microbial community (Trevors, 1987, Kruse & Sorum, 1994). The occurrence of multiresistant microbes is therefore not restricted to human environments such as hospitals. The findings of this study indicate that there is a certain background of antibiotic resistances in pristine aquifers (D'Costa*, et al.*, 2006). In the past, mostly soil was considered as a habitat for antibiotic resistant microorganisms, but resistant microorganisms were also detected in drinking water (Armstrong*, et al.*, 1981), mineral water (Messi*, et al.*, 2005), and groundwater from rural areas (McKeon*, et al.*, 1995). It is not clear at present if such resistant microbes constitute a threat to human health or if they might exchange resistance genes with pathogenic microorganisms. Nevertheless, the present study indicates that antibiotic producers and resistant microbes are far more spread in the environment than anticipated so far.

Especially, the finding of mupirocin producers were found in groundwater habitats offers the possibility to screen in so far not investigated ecosystems for putative new antibiotic producers.

The influence of antibiotics on the composition of the bacterial community in contaminated soils could be already demonstrated and resulted in reduced numbers of soil bacteria and shifts in the fungal:bacterial ratio (Thiele-Bruhn, 2005, Thiele-Bruhn & Beck, 2005). In order to monitor the effect of *in situ* produced antibiotics on inherent microbial populations in groundwater sediment a column experiment with sediment material from the investigated site in Scheyern, which was spiked with the isolated antibiotic producer D7, was performed. Control experiments without antibiotic producers contained 16S rDNA gene sequences that formed the dominant restriction fragment (T-RF) of 490 bp in length. However, this T-RF was also obtained from the 16S rDNA sequence of both isolated antibiotic producers (D7 and G11). Because of the very close relationship of *Pseudomonas* strains based on the 16S rRNA gene differentiation between inherent and introduced *Pseudomonas* strains was not possible. The sediment was stored at 4 °C before it was used for the columns and an important problem may be that all other bacteria were more or less totally suppressed at low temperatures. However, *Pseudomonas* strains were able to

establish faster when the conditions improved. When the growth of all organisms was successfully suppressed during storage also the conditions in the column set up could have enhanced growth of *Pseudomonas* strains. Some plans arose to solve the occurring problems but all of them brought the experiment away from close to *in situ* conditions. The idea to introduce a typical antibiotic-producing strain with another 16S rRNA gene sequence into the column experiment was neglected because the ideal conditions for the strains might be too far away from natural conditions. For example a nutrient rich medium might be necessary for the growth of the producer strain. Also the idea to sterilize the sediment before running the experiment was neglected. Depending on the conditions used for sterilizing spore formers might accumulate and the composition of the bacterial community shifted from the original one. Under harsher sterilizing conditions also the sediment characteristics might change. At the time of planning and setting up the column experiment no fresh sediment was available but maybe it is possible to plan such an experiment with the next drilling campaign.

6 Conclusion and Outlook

The results of this work answer the question if antibiotic producers occur in groundwater habitats. The isolation of two new mupirocin-producing *Pseudomonas fluorescens* strains from a pristine aquifer demonstrates that also under-investigated environments are promising for the research of new antibiotically active substances. The isolation of these mupirocin producers supports the assumption that much more interesting and previously unidentified compounds might be present. The mode of action of mupirocin is until now unique for an antibiotic and maybe groundwater bacteria offer much more unique possibilities.

The distribution of antibiotic producers was randomly and not distributed to geological or chemical parameters. However, this fact requires a high number of cultivation efforts discovering novel interesting bacterial strains. The applied high throughput cultivation approach and the related high throughput susceptibility test were useful in solving this problem and especially the susceptibility test could be a fast and material saving detection method for future applications. It would be challenging to investigate further aquifers to approve or also disprove this random distribution.

All isolated antibiotic producers, also from the pristine aquifer, showed multiresistances, which confirms that not only the influence of antibiotics causes resistances. The natural abundance of resistances should be taken into account when the bacterial community in habitats contaminated with wastewater or manure is investigated, maybe like a blank value or standard condition. To show if the new isolated bacterial strains have the ability to transfer their resistance genes to other bacteria especially pathogenic strains would be very interesting. This would shed light on the risk of natural occurring resistances to human health.

The most important field for future investigations should elucidate the influence of antibiotic producers on the bacterial community. The performed experiment with sediment columns can be used for this purpose but freshly drilled sediment should be filled in to prevent the problems that occurred during this study. Additionally some pre-experiments under more artificial conditions may be promising. For example

sterile material in the column equipped with a defined bacterial community can be used in combination with one of the isolated antibiotic producers.

In the environment, the concentration of antibiotics released by bacterial producer strains could be comparably low. But even these low concentrations could probably exhibit an antibiotically effect on the bacterial community. However, the natural bacterial community could adapt to the antibiotics and consequently develop resistances. This should be taken into account when discussing about artificial contamination with antibiotics or resistant strains from manure that can sometimes found in groundwater.

7 Literature

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