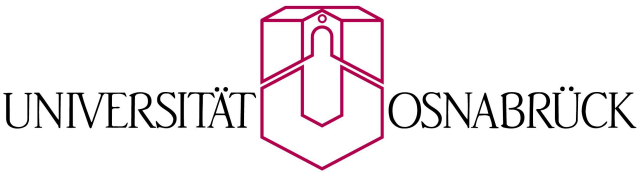

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Modeling the transfer of antibiotic drug resistance genes between *E.coli* strains

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Dieser Beitrag enthält die Diplomarbeit von Andreas Focks zum Thema "Transfer von Antibiotikaresistenzgenen". Geringfügige Korrekturen am Originaltext wurden vorgenommen.

Abstract

Horizontal gene transfer is responsible for the fast adaption of microorganisms to the application of antimicrobial drugs. Antibiotic drug resistance genes are spread mainly by means of conjugational transfer, mediated by conjugational plasmids. Modeling of the transfer of antibiotic drug resistance genes can improve the knowledge about the importance of different influence factors and provides methods for an exact evaluation of experimental data. Within this diplom thesis, a model for the description of the growth of plasmid-bearing and plasmidless cells was developed. Growth of microbial populations was described by a nutrient dependent Monod kinetic, including negative influences on microbial growth such as maintenance costs and microbial competition. Plasmid transfer was included into the model by application of a simple mass action principle. Methods for the estimation of growth-related parameters were developed, which are based on the transformation of model equations under consideration of nutrient dependence of growth and transfer processes. The estimation of plasmid transfer rates was executed by the application of the end-point method of Simonsen *et al.*. The proposed model and methods of parameter estimations were applied to data obtained from microcosm experiments performed formerly at the Federal Biological Research Center for Agriculture and Forestry (BBA). Using estimated parameter values, several scenarios were simulated and evaluated.

The results show, that a more meaningful evaluation of experimental data is possible by the application of modeling methods. Main results with respect to modeling methods are, that a homogeneous model can be suitable for the description of antibiotic gene transfer in soil. This type of model, which has been used earlier to describe liquid culture and plate experiments in the field of microbiological modeling has been validated to map growth and transfer dynamics even in the case of a heterogeneous medium such as soil. The results of model based evaluation of experimental data display, that the rhizosphere of plants has an immense positive influence on the transfer of antibiotic drug resistance genes. Assumptions concerning the stimulation of plasmid transfer by rhizospheral influence were proven to be right. Selective pressure exerted by soil amendment with antibiotics was investigated detailed. The results display, that the influence of antibiotic pressure on the transfer of antibiotic resistance is not as high as expected in the case of nourseothricin resistance.

These results show that the possibilities of prognoses concerning the spread of antibiotic drug resistance genes in soil could be improved by an extension of the proposed model. The inclusion of an activity state of cells, of more detailed nutrient turnover processes, and of plasmid transfer independent of microbial growth are proposals, which enable more exact valuations of the fate of antibiotic drug resistance genes in natural habitats.

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Chapter 1

Introduction

The transfer of antibiotic drug resistance genes is responsible for the fast adaptation of microorganisms to the usage of antibiotic compounds as pharmaceuticals. The treatment of bacterial infectious diseases by means of antibiotics is constricted by the rapid spread of antibiotic resistances. Nowadays, an infection caused by methicillin-resistant *Staphylococcus aureus* or vancomycin-resistant enterococci poses a threat to anyone, since a medication of these infections with antibiotic drugs does not succeed in any case. At the same time large amounts of antibiotic effectual compounds reach the environment from the application of antibiotic agents in human or veterinary medicine, but also as a consequence of the utilization of antibiotics as growth promoter in livestock husbandry. A selective pressure is exerted, which enhances the development and dissemination of antibiotic drug resistances.

In this context it is of outstanding interest, how the transfer of antibiotic drug resistance genes is mediated, and which environmental influence factors affect this process critically. It is helpful to apply methods of modeling for these purposes, because they provide exact means for the evaluation of experimental results.

The approach of this thesis on modeling the transfer of antibiotic resistance genes consists of the application of a simple mathematical model to data from experiments, which have formerly been performed at the Federal Biological Research Center for Agriculture and Forestry (BBA) in Braunschweig by Rüdiger Pukall. These experiments aimed at the investigation of the transfer of antibiotic resistance genes between *E. coli* strains in soil. Therefore, within this thesis a model was developed, which maps the growth dynamics of involved bacterial populations and the process of gene transfer. The model contains a parameter quantifying the rate, at which resistance genes are transmitted. For all model parameters values have been estimated from the experimental data, so that simulations can be performed. Here, the values for the transfer parameter are of particular interest.

The experiments at the BBA have been performed under variation of some environmental factors. The effect of several factors on the growth of bacterial popula-

tions and primarily onto the transfer of resistance genes was investigated by means of the mathematical model. In particular, a connection between growth of the cells and resistance gene transfer has been observed. Since some of the experiments included soils treated with antibiotics, the possibility of interaction between selective antibiotic pressure in the environment of bacteria containing resistance genes and the transfer of those genes was investigated.

This diplom thesis aims at the understanding and the mathematical description of the process of gene transfer in soil, which takes place at a very small spatial scale under heterogeneous conditions. An estimation of efficiency of genetic transfer in soil is difficult, but within this thesis methods are developed which enable the calculation of a certain plasmid's transfer efficiency, if information is available in form of cell densities of plasmid-bearing and plasmidless strains.

In the second chapter, the biological background of the transfer of drug resistance genes is elucidated. The microcosm experiments are described shortly in the third chapter, while the fourth chapter deals with the development of model equations and the parameter estimation. Results of parameter estimation methods and simulation results are presented in the fifth chapter. The influence of microbial competition, rhizospheral soil and antibiotic pressure onto genetic transfer is investigated in this chapter as well. Chapter six at last subsumes the results obtained from this diplom thesis.

Chapter 2

Biological basics

2.1 Antibiotic agents

The classical definition of antibiotics following S.A. Waksman (1941) describes antibiotics as "low molecular metabolites of microorganisms, inhibiting or even killing other microorganisms at low concentrations". Antibiotics are in fact secondary metabolites of microorganisms, whose function as agents to combat bacterial infectious diseases have only been detected in the 20th century. They are in principle usable for microbial growth control *in vivo*, that means applied to a macroscopic host organism, because they are effective even at low concentrations. This is due to their effect on substructures or metabolic pathways specifically for procaryotic cells. Only toxic side effects on the host organism prevent the most naturally occurring antibiotics of being used as antibiotic drugs (Madigan *et al.*, 2003, p. 712). Nevertheless, naturally occurring antibiotics are, besides synthetic antimicrobial drugs such as growth factor analogs[1], the most important antibiotic drugs used in human or veterinary medicine today. The effectiveness and the spectrum of activity of naturally occurring antibiotics has in many cases been improved by modifying basic structures of natural antibiotics. The resulting derivatives are the semi-synthetic antimicrobial drugs.

Antibiotics are produced naturally by fungi and bacteria. In bacteria, production of natural antibiotics as secondary metabolites is not essential for growth and reproduction of cells, it takes place during the (secondary) stationary growth phase. Due to this it is not absolutely clear, why antibiotics and other secondary metabolites are produced at all. It seems that the production of antibiotics is an optional way for bacterial cells to crowd out susceptible bacteria in competition for resources, particularly because the formation of secondary metabolites in general is dependent

[1] A growth factor is a chemical substance, which is essential in the medium, because it can not be self-synthesized by the cell. A growth factor analog is similar to a certain growth factor, but does not fulfill its function in cell growth.

on the growth conditions and will be repressed under starvation.

Table 2.1: Selection of antibiotics ordered by cellular target.

Target	Antibiotic	Chemical class	Producer
protein synthesis	erythromycin	macrocyclic lactones	*Streptomyces erythreus*
	chloramphenicol	aromatic antibiotics	*Streptomyces venezuelae*
	tetracycline	tetracyclines	*Streptomyces rimosus*
	streptomycin	aminoglycoside	*Streptomyces griseus*
	kanamycine	aminoglycoside	*Streptomyces kanamyceticus*
cytoplasm membrane	polymyxin	lipopeptide	*Bacillus polymyxa*
	bacitracin	peptid	*Bacillus licheniformis*
cell wall synthesis	cycloserine	amino acid derivatives	*Streptomyces orchidateus*
	penicillin	β-lactam	*Penicillium chrysogenum*
	cephalosporine	β-lactam	*Cephalosporium* spp.
	vancomycin	c-glycoside	*Streptomyces orientalis*
	bacitracin	peptid	*Bacillus licheniformis*
	fosfomycin	aliphatic compounds containing phosphorous	*Streptomyces fradiae*
RNA polymerase	rifamycin	macrocyclic lactones	*Nocardia mediterranei*
RNA elongation	actinomycin	chromopeptides	*Streptomyces* spp.

Antibiotics are a heterogeneous group of compounds. At present, more than 7000 natural antibiotic agents are known (Gräfe, 1992, p.15). The aim to structure this conglomeration of different compounds can be approached by grouping the antibiotic agents according to their origin, chemical structure, or mode of action. Table 2.1 lists selected antibiotics ordered by the cellular target. It shows a few outstanding aspects of antibiotics: first, the chemical structure is not always correlated with the target of the antibiotic effect, second, some antibiotics affect more than one target (e.g. bacitracin), and third, a large part of the producers seems to consist of soil microorganisms, particularly *Streptomyces* strains. More basically the effect of natural, semi-synthetic and synthetic antimicrobial agents on bacterial cells can be distinguished into three modes of biological action. While the bacteriostatic mode of action inhibits both viable cells and total cell count[2], bacteriocidal mode of action

[2]living and dead cells

reduces viable cells to zero, the dead cells remaining unaffected. The strongest mode of action is the bacteriolytic, by which both viable and total cell count are turned to zero due to the lysis of cell structures. The differences between these modes of action can be attributed to the specific ways antibiotic agents act on bacterial cells. For example bacteriostatic antibiotics such as aminoglycosides or erythromycin are often inhibitors of protein synthesis and act by binding to the ribosomes. β-lactam antibiotics such as penicillins act bacteriocidal or bacteriolytic on living cells by inhibiting cell wall synthesis and stimulating the production of autolysins.

Determined by the selective effect of antibiotics, only those cells are susceptible, which contain the specific substructure an agent acts on. In general, *broad-spectrum* antibiotics are effectively applied to both gram-positive and gram-negative bacteria[3], and a *narrow-spectrum* antibiotic agent acts only on a single group of microorganisms.

Like any other chemical effect, the effect of antibiotic agents is dependent on the concentration in the surrounding of target cells. The antimicrobial activity can be measured by a value called the *minimum inhibitory concentration* (MIC), defined as the lowest concentration of the compound that completely inhibits the growth of a test organism (Madigan *et al.*, 2003, p.704). The MIC depends on the nature of the test organism used, the inoculum size[4], and environmental conditions like temperature, pH, or aeration. By standardizing those conditions, it is possible to determine MIC values of antibiotic agents, that can be compared to other antibiotic agents.

2.2 Antibiotic resistances

Microorganisms are able to grow in the presence of antibiotics if they possess antibiotic resistances. Antibiotic resistances can be divided into natural and acquired resistances. The natural or intrinsic form of antibiotic resistance can be regarded as a genetically determined property of a cell, which matches a gap in the spectrum of action of an antibiotic agent. A classical example for natural resistance is the non-effectiveness of Penicillin G against gram-negative bacteria, whose cell wall is just impermeable for the antibiotic. Besides the natural form, most other forms of antibiotic resistances are mediated by the expression of special resistance genes. Resistance genes provide a more or less specialized way for a bacterial cell to avert the effect of an antibiotic agent. They encode enzymes for antibiotic inactivation (e.g. β-lactamases, various acetyltransferases), target modification, or active efflux of an antibiotic agent out of the cell (e.g. *tet*-genes) (Gräfe, 1992). Resistance factors

[3]Gram-positive and gram-negative are attributes bacteria can be structured on. These attributes are the result of a staining method.

[4]An inoculum is the start culture applied to a growth medium.

are widely disseminated about the microbial community. This has classically been examined by selective plating techniques. Since the 1980s molecular tools like PCR-based detection techniques have been developed, which enable molecular biologists to analyze the spread of resistance factors in the major part of bacteria, that is not accessible to traditional cultivating methods. Molecular methods have been applied, for instance, to screen environmental probes from different European countries for gentamicin resistance genes (Heuer *et al.*, 2002).

In principle, there are two major ways how a bacterial cell can acquire a resistance gene: mutation or genetic exchange. Mutations are relatively rare events (frequency 10^{-9} to 10^{-7}) during the reduplication of genetic material of the cell (Gräfe, 1992, p.324). So, the emergence of a new antibiotic resistance gene caused by spontaneous mutation is an event which does not take place very often. Mutations seem rather to alter existing resistances, even if there is evidence for spontaneously emerged antibiotic resistances in important pathogens like *M. tuberculosis* (Mazel and Davies, 1999). However, selective pressure of antibiotic compounds can lead to an amplification of mutational events. Caused by mutations, a few cells from a local population of microorganisms can acquire a selective advantage in the presence of antibiotics. Such a selective advantage could be e.g. the modification of a genetically determined target of an antibiotic agent like the ribosomal structure of a cell. The selective pressure leads to a rapid spread of the mutant cells. Possibly, this mutant form can be established at the local microenvironment even in the absence of the antibiotic, if the mutation causes no disadvantage in cell metabolism under this condition. This form of mutationally acquired resistance denotes a significant shift in the MIC of an antibiotic against this mutant. Other forms of mutationally acquired resistances only lead to a small shift in the MIC, e.g. if the efficiency of an efflux system or an alternative pathway is enhanced slightly by mutations in the respective repressor genes. A possible key role of the positive selection of low-level-shifts in the MIC for the emergence of high-level resistances in microbial communities has been postulated by Baquero *et al.* (1997). Here, it has been demonstrated experimentally, how antibiotic concentrations around the MIC of a certain agent could select positively just those cells, which have lifted their resistance level slightly.

The second possibility to gain an antibiotic resistance gene is by the means of genetic exchange. The possibilities of genetic exchange between bacterial cells are manifold. Mobile genetic elements are plasmids, transposons, phages, or gene cassettes which contribute to the dissemination of resistance genes over the bacterial community (Smalla and Sobecky, 2002). Intercellular mechanisms of genetic exchange can be discriminated against intracellular exchange. The latter is not really an exchange, but rather a transposition of resistance (and other) genes within the cell (see figure 2.1). Transposons play the central role in this process. Transposons are mobile genetic elements, which consist of one or more genes (e.g. for antibiotic resistances) flanked by insertion sequences. These insertion sequences enable

the embedded genes to leave their position in the genome and to be integrated at another position, including a transfer from the chromosome to a plasmid (or vice versa), from one plasmid to another or from other sources (e.g. phages or incorporated free DNA) to the chromosome. These "jumps"[5] enable antibiotic resistance genes to perform highly dynamic changes in their positions.

Originally, one of the main sources of antibiotic resistance genes are the microbial producers of antibiotics themselves. This assumption has been confirmed by biochemical and molecular studies (Mazel and Davies, 1999). All producers of antibiotics have developed mechanisms to auto-protect themselves in order to escape the effect of their antibiotic. Examples are the producers of aminoglycosides or streptothricines. They inactivate the active compound by acetylation. Occasionally, genes encoding those self-protection functions are set free and can be acquired by other microorganisms.

2.3 Transfer of genetic material

Intercellular transfer of genetic material in natural environments takes place as transformation, transduction, or conjugation. These processes are influenced by different factors, but they have in common that new genetic material is introduced into a cell.

Transformation can only be achieved by competent cells. Competence is the genetically determined ability of a cell, to take up free DNA from the cell's environment and integrate it into the bacterial genome. This ability can change during the life cycle of a cell, for example *Streptococcus* cells turn competent during few minutes of their growth phase (Madigan *et al.*, 2003, p. 279). As DNA can be stabilized by adsorption to soil particles, it may survive in soil for weeks or even months ready for transformation. So, competent cells have the possibility to incorporate genetic determinants originating from other dissolved cells.

In the process of transduction, bacterial genes are carried off by bacteriophages, parts from the bacterial genome usually replacing some virus genes. This process is influenced by the type of phage, since there are generalized or specific ways of transduction. The host range of bacteriophages is sometimes restricted to a single species. The replacement of virus DNA by bacterial genes often results in a defective virus particle. So, the contribution of transduction to antibiotic resistance gene transfer is supposed to be rather low.

Conjugation

Besides transformation and transduction as mechanisms with rather low frequencies, conjugation is the most significant process in the transfer of bacterial genes in the

[5]Transposons are also called "jumping genes".

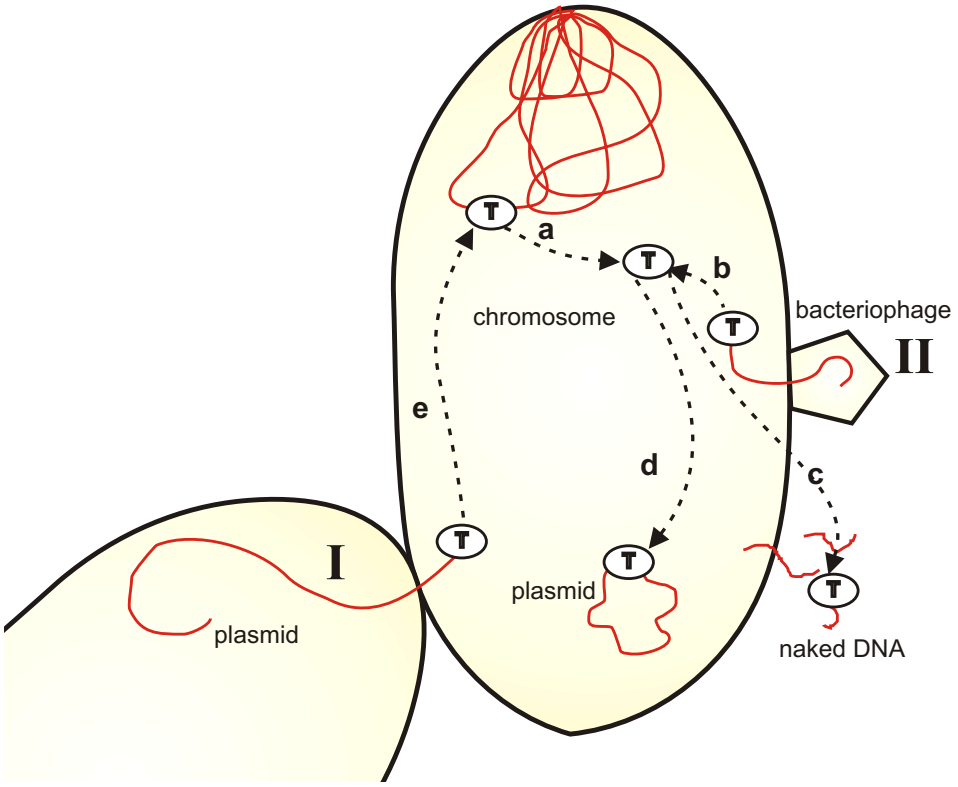


Figure 2.1: Various mechanisms of genetic exchange within bacteria. By plasmid (I) or bacteriophage (II) DNA new genes can be inserted into the cell. Inside the cell, transposons (**T**) can change their position by some of the depicted mechanisms: **a)** Excision of a transposon out of the chromosome. **b)** Excision of a transposon out of bacteriophagic DNA. **c)** Segregation of a transposon to the outside of the cell. **d)** Insertion of a transposon in plasmidic DNA. Together with the plasmid, it can now be transferred to recipient cells. **e)** Integration of a transposon into the chromosome. Graphic adapted from Levy (1997)

environment (Davison, 1999). Conjugation is a directed mechanism dependent on cell-to-cell contact. Conjugative plasmids are mostly responsible for this mechanism, as they carry the necessary genes. The responsible set of genes, called the *tra*-region within a plasmid, encodes proteins that function in DNA transfer and replication, and others that function in mating pair formation (see figure 2.2). More precisely, the latter ones encode the pilus, a structure of the cell's surface serving as the connection between the plasmid-bearing donor cell, and the target of the transfer, the plasmid-free recipient cell. During the conjugation process the pilus connects the donor and the recipient cell and stabilizes them to form a mating-pair by retracting itself. A copy of the conjugative plasmid is transferred to the recipient cell. Additional genetic material may be transferred, e.g. non self-transferable, but mobilizable[6] plasmids. The recipient cell containing the newly obtained plasmid(s) is called transconjugant cell. Transferred genetic material is not able to establish within the transconjugant cell in all cases, as it has to escape cellular DNA restriction systems, which destroy foreign DNA. Additionally, a transferred plasmid must be capable of replication and maintenance in the new host to be successful (Davison, 1999, p. 75). During the transfer process or the process of cellular division, it is possible that a plasmid gets lost from the host cell. Such a loss by *segregation* can cause the disappearance of a plasmid from a population, if it happens at a high frequency.

The accomplishment of a mating pair between cells from different strains or species is not yet understood in detail. The f-factor[7], one of the best investigated plasmids responsible for conjugation between gram-negative bacteria, was experimentally observed to be "selective" in the choice of its mating partners. As a reason for this phenomenon a surface exclusion mechanism was postulated based on the assumption, that the existence of a certain receptor at the surface of a potential mating partner decides about its ability to serve as recipient (Schumann, 1989, p.38/39). Another concept suggests, that the conjugative mating bridge is a donor-dependent structure. Hence the accomplishment of the mating pair is not the limiting factor of the observed low frequency of conjugative transfer events between divergent donor and recipient cells. Instead, the limiting element is rather postulated to be the efficiency of the actual transfer event (Waters, 1999).

However, there exists a great deal of other influence factors on conjugation. Dröge *et al.* (1998) tried to identify relevant biotic and abiotic factors influencing conjugation in the environment. Their review article includes a rating of observed both abiotic and biotic influences. For biotic factors, the physiological status of donor and recipient cells, the presence of indigenous microbes, and the presence of a rhizosphere have been considered, as well as temperature, moisture, pH, and clay minerals for abiotic factors. In spite of partially disagreeing results of different investigators it

[6]Mobilizable plasmids carry no genes for the pilus synthesis, but an origin for conjugal transfer (*oriT*).

[7]Fertility factor, this term was coined by W. Hayes (1952).

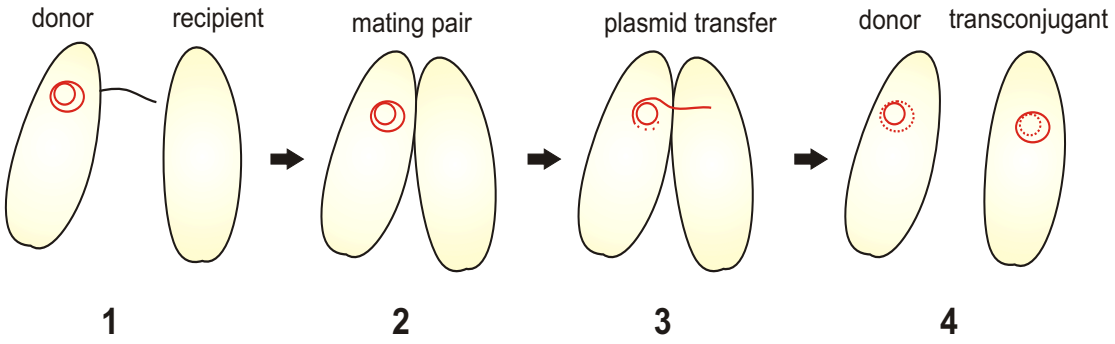


Figure 2.2: Schematic development of conjugation. Newly formed DNA is displayed dotted. From left to right: 1) The donor contacts the recipient cell via the pilus. 2) Approximation of the two cells by retraction of the pilus 3) Conjugative DNA-synthesis, plasmidic DNA is transferred single-stranded and synthesized in both donor and recipient during the transfer event. 4) Active disaggregation of the two cells. DNA in both cells is completed to double-stranded DNA. Donor cells can build up more than one pilus at the same time. Therefore, conjugation can take place in aggregates of more than 50 cells.

becomes clear, that conjugational gene transfer occurs under a wide range of environmental conditions. Influence factors can be divided into factors like nutrient availability (and so the cell's activity state) or the presence of a rhizosphere, which in general act positive on the frequency of conjugational transfer, and factors like temperature or pH, which can act either stimulating or inhibiting on conjugational events, depending on the parameter values.

Plasmids

Since plasmids play an important role in horizontal gene transfer[8], some more aspects can help to evaluate their role rightly. The discovery of conjugation, conjugative plasmids, and horizontal gene transfer since the 1950s has been boosted by the fact, that antibiotic drug resistance rose almost as fast as new antibiotic drugs were discovered and applied in the treatment of bacterial infectious diseases. Nevertheless, plasmids as an instrument for genetic exchange exist much longer than the beginning of the antibiotic era. Investigations related to genome decoding of *E. coli* show, that up to 17.6 % of the genome of an average *E. coli* cell has been acquired by

[8]Horizontal gene transfer: genetic exchange between concurrently living bacterial cells. Opposite of vertical gene transfer, which means the transfer of genes to the two daughter cells in the process of cell division.

horizontal gene transfer (Davison, 1999). Plasmids are an important evolutionary mechanism for maintaining the genetic variability of microbial populations. It becomes clear, that the selective pressure executed by the entry of antibiotic medicines into environment is only the trigger for resistance spread, because the tools for the fast genetic adaption of microbial populations to changing environmental conditions are at microbial disposal for a long time.

In general, plasmids are extra-chromosomal genetic elements, which are able to self-replicate within their hosts. Their size ranges from two to 800 kilo base pairs (kbp) (Slater *et al.*, 1988), encoding about three to 200 genes. The comparison to the size of *E. coli'*s genome with about $4 \cdot 10^6$ basepairs, coding for 900-1000 genes, yields a ratio of 0.05 % to 20 % of plasmidic to chromosomal DNA. Plasmidic genes involved in self-replication "are concerned primarily with control of the timing of the initiation process and with apportionment of the replicated plasmids between daughter cells" (Madigan *et al.*, 2003, p. 286). That means, plasmids use replication mechanisms of host cells, and only the regulation of the copy procedure is plasmid encoded. Furtheron, plasmidic genes control the number of copies in interaction with the host cell. This *copy number* of particular plasmids is related to their size: smaller plasmids can be present in a high number more than 100, larger ones only in 1-3 copies.

Plasmids exist in thousands of different types, as over 300 different naturally occurring plasmids have been isolated from *E. coli* strains alone. They can be grouped on different factors, three of which shall be mentioned here. First, there can be distinguished conjugative from non-conjugative plasmids. This distinction is based on the existence of *tra*-genes within a certain plasmid, like already mentioned above. The second way to differentiate plasmids corresponds to the range of species, the plasmids can exist and replicate in. *Narrow host range* plasmids do this only in a limited number of closely related species, while *broad host range* plasmids are able to exist in a wide range of bacterial species. The third possibility to categorize plasmids is based upon their ability to coexist within the same cell. If two different plasmids are able to exist simultaneously within one cell, they are said to be *compatible*. If coexistence is not possible, they are called *incompatible*. Therefore, plasmids can be divided into different incompatibility groups (*Inc-groups*) by sorting all plasmids into one Inc-group, which are not able to coexist within one cell. The phenomenon of incompatibility is based upon the regulation of replication. Plasmids of one incompatibility group share a similar way of replication regulation. Here, incompatibility is not alone caused by displacement, as known plasmidic genes encode inhibitor mechanisms. Due to this, maintenance of a transferred plasmid is not possible, if another plasmid of the same Inc-group occupies the host cell already. Usage of Inc-groups to categorize plasmids can help to get an overview, as the 300 different plasmids known within *E.coli* can be assigned to about 25 Inc-groups (Slater *et al.*, 1988, p.45).

Even in the case of large plasmids, there are several phenotypic functions encoded by plasmidic DNA. The spectrum reaches from resistance functions against antibiotics, heavy metals, and toxins over catabolic functions like xenobiotic or aromatic compound catabolism up to pathogenicity factors. Principally, encoded functions could be another possibility to structure plasmids, but this is inhibited by the fact, that a plasmid can carry several different functions. It is possible, that some genes are selected without antibiotic pressure, if they are present together with other selectable marker genes. One example for such a linkage between genes is the one between antibiotic resistance and heavy metal resistance genes (Alonso *et al.*, 2001).

Beneath well-known phenotypic functions encoded by plasmidic DNA, there are also so called *cryptic* plasmids, whose function is not known to date. Their discovery has brought up the question if plasmid transfer and persistence occurs by the offer of advantageous functions of their genes to the host, or by infectious transfer.

Chapter 3

Data from microcosm experiments

The probability of contact between plasmid-bearing and plasmid-free cells and therefore the possibility of plasmid transfer varies in different habitats. It is not only dependent on the average densities of the cells, but also on local heterogenous spatial conditions. While a homogenous mixing of plasmid-bearing and plasmid-free cells in well defined liquid cultures in a laboratory can be assumed, conditions can be diverse in natural habitats like soil. In order to gather information about the transfer of antibiotic drug resistance genes in near-natural terrestrial environments, R. Pukall performed a series of microcosm experiments[1] to observe the transfer of resistance plasmids between *E. coli* donor and recipient strains (Pukall *et al.*, 1996; Pukall, 1996) in his PhD thesis. With these experiments, he investigated the influence of the type of plasmid as well as the dependence of plasmid transfer on environmental conditions like microbial competitors, nutrient availability and antibiotic pressure.

In detail, several scenarios were examined during the experimental work to take into account the influence factors mentioned above. The different experimental scenarios can be classified by four factors:

1. Type of plasmid
2. Pretreatment of soil
3. Nutrient availability
4. Antibiotic pressure

The experiments have been performed to check out the ability of plasmids to transfer themselves under environmental conditions between donor and recipient cells from similar strains, but also between donor cells and autochthonous members[2]

[1] Microcosm experiments provide an experimental method to study natural processes at a small scale under well-defined conditions.

[2] Autochthonous member: natural inhabitant of a habitat, opposite of allochthon.

of the soil microbial community. *E. coli* have been chosen as donor strains, because their time of survival is limited in soil. Evidence for the transfer of resistance plasmids to members of the soil community can be analyzed by PCR based screenings of probes after the inoculated donor strains have vanished. Transfer events can be detected, even if they take place to non-cultivable strains. In different scenarios there was evidence for the transfer of the IncP-plasmid pTH16 into autochthonous cells, which have been identified as members of *Pseudomonas*, *Rhizobium*, or *Alcaligenes*. Quantitative data gained by plate countings could be applied only for the densities of the introduced *E. coli*-cells. At the beginning of each experiment, donor and recipient cells have been counted to determine initial densities per gram of soil. During the course of the experiments, densities of donor, recipient and transconjugant cells have been tracked with a time span of one to seven days between the measurements. The unit for these countings is the number of colony forming units (CFU) per gram of soil. CFU number is an usual microbiological measure for the number of viable cells in a sample. The decade logarithm is applied to the determined values, so data points are expressed in $log\, CFU\, g^{-1}$ values.

3.1 Bacterial strains and plasmids

For a survey of strains and plasmids, which used R. Pukall in the microcosm experiments see table 3.1. In all experiments, auxotrophic[3] *E.coli* K-12 (CV601) have been used as recipient strains. The donor strains were either wild-type *E.coli* or prototrophic *E.coli* K-12 (J53). The choice of an auxotrophic recipient strain allows the characterization of transconjugant cells by selective plating. The auxothrophic recipient cells may show a slow growth in comparison to the prototroph donor strains, because they lack of some essential metabolic pathways.

All plasmids, except the IncP1-plasmids, have been formerly isolated from different environmental habitats. IncP1-plasmid pTH16 has been used in the experiments as an artificial constructed plasmid, which originates from pTH10, but carries nourseothricin instead of kanamycin resistance. Attribution of the plasmids to different Inc-groups allows to group these according to their host range: the IncI1 and IncFII plasmids have a narrow host range, while the IncN, IncW3, IncP1, and IncQ plasmids have a broad host range. Not only the host range, but also the type of pilus is a property of a distinct Inc-group. The plasmids of IncI1 and IncFII groups are known to encode flexible pili, while the plasmids of IncN, IncW3, and IncP1 group encode rather short, rigid pili (Pukall, 1996, p.41). The tra^- IncQ group plasmid encodes no pilus at all. Within the broad-host-range group, the single plasmids differ in their exact configuration. That means, reproduction rates within the cell or conju-

[3]Auxotrophy: type of bacterial nutrition characterized by the dependence of certain growth factors in the medium, often provoked by mutations, in opposite to prototrophy.

Table 3.1: Bacterial strains and plasmids used in the microcosm experiments and their relevant properties. Modified from Pukall (1996), p. 8.

Strain[a]	**Plasmid**[b]	**Properties**[c]	**Resistance**[d]
Donor			
E.Coli (wild-type)			
	pIE1040	IncI1, 75 kbp, tra^+	*Nt,Sm*
	pIE1055	Inc FII, 60 kbp, tra^+	*Nt,Sm*
	pIE639	IncQ, 6.5 kbp, tra^-, mob^+	*Nt,Sm,Km*
	pIE1037	IncN, 30 kbp, tra^+	*Nt,Sm*
E.Coli K-12(J53)			
F^-, pro, met	pIE1056	IncW3, 35 kbp, tra^+	*Nt,Sm*
	pTH10	IncP1, 38 kbp, tra^+	*Tc,Km,Ap*
	pTH16	IncP1, tra^+, mod. pTH10	*Ap,Tc,Nt*
Rezipient			
E.Coli K-12(CV601)			
F^-, thr^-, leu^-, thi^-			

[a] F^-: strain is not able to initiate conjugation. pro, met: additional metabolic pathways the strain is enabled to use. thr^-, leu^-, thi^-: strain can not synthesize these amino acids.

[b] Plasmid identification number.

[c] Relevant properties of the plasmids including incompatibility group, size in kilobasepairs (kbp), and if a plasmid is self-transferable (tra^+) or not (tra^-). mob^+ means, that the plasmid is mobilizable because it contains an origin for conjugal transfer.

[d] Abbreviations: Nourseothricin *(Nt)*, streptomycin(*Sm*), kanamycin(*Km*), tetracycline(*Tc*), ampicillin(*Ap*).

gation rates are not the same for the IncP1, IncN, and IncW3 plasmids. In contrast, the nourseothricin resistance genes on the IncN, IncW3, and IncP1 plasmids are located at identical transposon Tn1826. Thus, no genetically determined differences between these three plasmids can occur regarding nourseothricin resistance.

3.2 Scenarios

The number of plasmids used, together with four basic experimental set-ups, determines a lot of different scenarios. The soil used for all microcosm-experiments has been chosen in order to offer optimal conditions for plasmid transfer. The chosen soil provides a neutral pH, a relatively high humus content, and a middle sized pore size. For every scenario, soil-filled vessels have been incubated at 28℃ under a 16/8 h light cycle. Sterile air has been added five times a day. As well, evaporated water has been regularly substituted. Donor and recipient cells have been harvested from

Table 3.2: Basic experimental set-ups and its implications.

Set-up		Implication[a]
1.	sterilized, homogenized soil	no natural microbial competitors, no predation by protozoa
2.	non-sterilized, homogenized soil	microbial competitors, predation by protozoa
3.	non-sterilized, homogenized, antibiotic amended soil 100 μg/g soil 750 μg/g soil 2500 μg/g soil	microbial competitors, predation by protozoa, selective pressure
4.	soil grown by grass	microbial competitors, predation by protozoa, presence of a rhizosphere: structuring and root exsudates

[a]Implications of the different set-ups related to microbial population dynamics. The stepwise development of the scenarios allows for a comparison of several scenarios with the aim of determining the influence of single factors.

a late exponential growth phase culture for inoculation of the microcosms (Pukall, 1996, p.32). For the experiments with nutrient amendment, yeast bouillon has been applied to the *E. coli* donor and recipient cells.

Pretreatment of the soil was different for the scenarios. In the first set-up soil has been sterilized in the autoclave three times, while the soil for the other set-ups has not been. The pretreatment of the fourth set-up included sowing of grass seeds and incubation of the microcosms, until a dense sod providing a rhizosphere has been developed. Special implications are related to the basic set-ups, as listed in table 3.2.

Nourseothricin

In the third set-up, non-sterile soil has been amended with different concentrations of nourseothricin (*Nt*). This antibiotic agent, which is a mixture of streptothricin D and F, interacts with procaryotic ribosomes and inhibits protein synthesis. In this way, it acts bacteriostatic onto both gram negative and gram positive cells. The producer of nourseothricin, *Streptomyces noursei* is naturally resistant against it mediated by acetylation of the antibiotic. The enzyme responsible for the conversion, the streptothricine-acetyltransferase is encoded by the *nat*-gene in *S. noursei*. Additionally, several more genes encoding streptothricine-acetyltransferase are known, which

show no homology to the *nat*-gene and are assumed to have developed independent of this autoprotecting gene. Some of these genes are located on the plasmids used in the experiments.

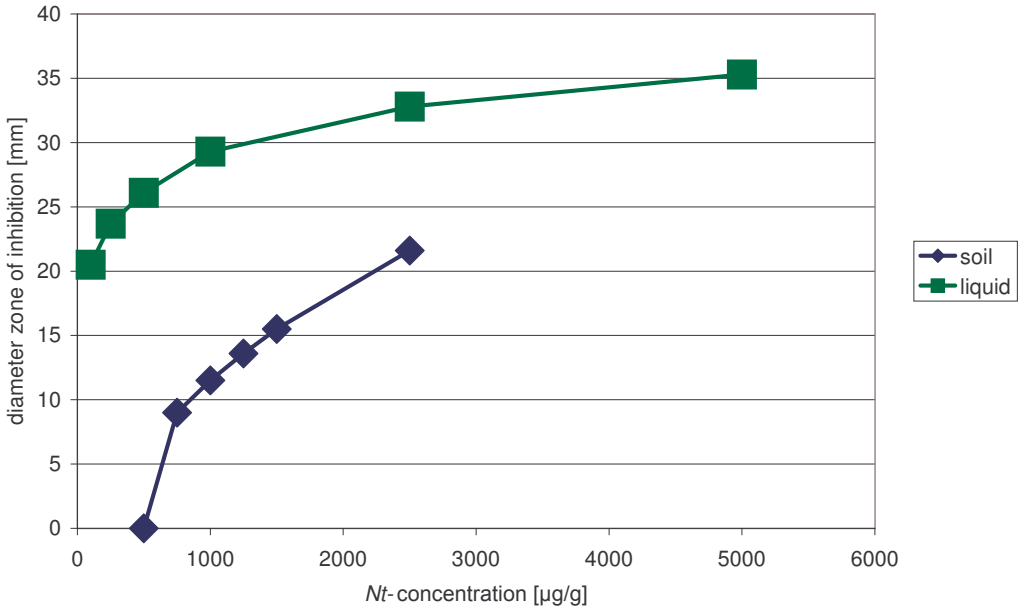


Figure 3.1: Results of agar diffusion test for nourseothricin dissolved in liquid (□) and adsorbed to soil (◇) (cf. Pukall (1996), sec. 3.4). It becomes apparent, that the effectiveness of the antibiotic in soil has been reduced.

Inclusion of antibiotic-amended soil into the experimental design offers the chance, to investigate the influence of antibiotic pressure onto the transfer of plasmids that encode resistance for this antibiotic. In the preparation of the experiments, the effect of nourseothricin on the growth of the used microorganisms has been tested by agar diffusion tests, with both liquid solved nourseothricin and nourseothricin mixed with soil. This differentiation has been made in order to measure the effect of adsorption of the antibiotic to organic matter. *Nt*-sensitive *E. coli* K-12 (CV601) has been used for testing. This sensitivity test indicated that the antimicrobial potential of nourseothricin in soil is reduced possibly due to the adsorption of the antibiotic to soil particles (cf. figure 3.1). This reduction can not be due to nourseothricin degradation by resistant microorganisms, because in the experiments only nourseothricin-sensitive *E. coli* K-12 (CV601) have been used.

Chapter 4

Modeling

Mathematical modeling is a common method to investigate complex environmental systems. A theoretical formulation of system dynamics in order to explain phenomena observed in natural systems remains always a simplification of reality. The justification for reduction and simplification of a part of the environment within a model can be found in the model's objective. The main goal of many models in the field of environmental sciences is an enhanced understanding of natural phenomena. In order to reach this aim, mathematical models try to incorporate the most important aspects of a natural system, while aspects that are not related to the underlying questions are ignored.

Application of a modeling approach can provide several advantages for a researcher. Besides the attempt to include the complexity of real systems and therefore to make quantitative predictions, models can be useful in other ways. One advantage is, that an insight into inner, otherwise not accessible dynamics of a process can be gained via the model's parameter values. Another possibility to exploit the advantages of modeling can consist in the examination of hypotheses *in silico*. This means, once a model is able to reproduce a system's dynamic approximately, model elements can be appended or removed, or parameter values can be varied, in order to evaluate the observed model reactions to these changes.

Modeling approaches in the field of microbiology are manifold. The spectrum of methods of modeling applied to problems of microbial dynamics ranges from continuous to discrete models. While continuous models are usually applied to macroscopic or large scale systems, discrete models are more often used for the examination of micro-scale phenomena. Within those two groups, the most important subgroups are characterized by the choice of their objectives, e.g the application of reaction-diffusion models to explain microbial spatial growth patterns or the usage of homogenous differential equations to model macroscopic global cycles within the continuous models group. Discrete models consider in particular microbial activities in spatial heterogeneity, e.g. for the description of biofilms or of colony morphology.

A short overview about different modeling approaches in the field of microbiology has been given by Wimpenny (1999).

Models for plasmid transfer in literature

Quite a lot of mathematical models for the investigation of phenomena in the domain of gene transfer and antibiotic resistances have been developed and applied. Mainly, two groups of scientific publications related to mathematical modeling can be distinguished: the first group consists of model developments describing plasmid transfer based on a mass action principle. One of the first models in this sense has been developed by Stewart and Levin (1977). It provides a mathematical analysis of a base model including segregation and fitness cost influence of a plasmid. Theoretical existence conditions for plasmids have been calculated from the model equations, but the model's outcome has not been compared with experimental results. Transfer events have been assumed to be proportional to the densities of donor and recipient cells. By doing so, the proportional coefficient γ can be understood as a measure of plasmid fertility, similar to a parameter for infectious transmission in models of infectious disease. Combined experimental and mathematical methods have been applied by Freter *et al.* (1982) to examine plasmid transfer in the gut of gnotobiotic mice[1]. Here, several assumptions have been conversed into mathematical relationships without resulting in progressions in modeling methods. Clewlow *et al.* (1990) developed a mathematical model, in which they combined a mass-action transfer with a logistic growth term and applied it to explain experimental data obtained from long-term nutrient limited soil microcosm experiments with *Streptomyces lividans*. They calculated parameter values from data by transforming their model equations and obtained a feasible agreement between data and simulation results.

Several contributions to the development of mathematical models in the field of plasmid transfer have been done by Lone Simonsen. Besides the investigation of the dynamics of plasmid transfer on surfaces (Simonsen, 1990), and the comparison of theoretical and real aspects of existence conditions for plasmids (Simonsen, 1991), the perhaps most important contribution was the development of an estimation method for the rate of plasmid transfer, which is based on only two measurements of cell densities (Simonsen *et al.*, 1990). Unlike, Smets *et al.* (1993) developed a model based on logistic growth and mass-action plasmid transfer, together with a parameter estimation method supposing a linear relationship between transfer frequency and evolution of donor cell numbers. For the application of this parameter estimation method, there are several measurements of cellular densities necessary.

The tradeoff between horizontal and vertical modes of plasmid transmission[2] has

[1] Mice, whose gut contains only a few controlled kinds of microorganisms

[2] Horizontal mode of transmission: conjugative plasmid transfer. Vertical mode of transmission:

been investigated both theoretically and experimentally in additional publications (Turner *et al.*, 1998; Turner, 2004). It has been stressed by them, that it is not possible for a certain plasmid to optimize both horizontal and vertical transmission at the same time. Optimizing the horizontal mode means, that a plasmid transfers itself regardless of the host's physiological state. This implicates a strong metabolic burden for the host cell, so that cell growth decelerates and the possibility for vertical plasmid transmission declines. The other way around, a plasmid optimizes its vertical mode of transmission if it burdens the host cell's metabolism only weakly. This implies low horizontal transfer rates but a better ability to compete. It has been postulated, that plasmids can switch between transmission modes by recognition of recipient densities. A broad mathematical analysis of existence conditions has been performed by Bergstrom *et al.* (2000) including a structuring of natural mechanisms that can maintain plasmids. Additionally, computer simulations have been done accounting for differences in the location of a distinct gene on the chromosome or on the plasmid.

The second group of publications deals with by applications of theoretical modeling approaches to evaluate experimental results without attempting for improvement of modeling methods, but with mostly elaborate data inquiries (e.g. Licht *et al.* (1999); Sudarshana and Knudsen (1995) and Gordon (1992)).

Other approaches of plasmid dynamic related mathematical modeling have been performed by Lagido *et al.* (2003), who assumed a stochastic spatial model for plasmid transfer focussing mainly on the establishment of contact between donor and recipient cells. The inner cellular regulation process of replication control of Plasmid C0IE1 has been investigated by Paulsson and Ehrenberg (1998), who also applied stochastic methods.

4.1 General model conditions

The usage of differential equations for the mathematical formulation of the model is indicated by the high number of microorganisms observed in the experiments. The underlying question for this work is the evaluation of the effect of different environmental conditions on the transfer of antibiotic resistance genes between *E. coli* in soil. Only the dynamics of transfer events and tightly related processes are considered. The model was supposed to be rather simple in order to keep the number of parameters as low as possible. Transfer of resistance genes and microbial growth are inextricably related processes. For this reason, the model should incorporate both growth and transfer dynamics. In this regard, the accuracy concerning this processes should be appropriate to reflect the quality of underlying experimental data.

Transfer of plasmid copies to daughter cells during the process of cell division.

The number of included or ignored processes is an important influence factor, as it determines the model's complexity. Since the resolution of the underlying experimental data remains at the scale of average densities of different microbial entities, it does not make sense to resolve the growth and transfer dynamics of modelled microorganisms more detailed. That means, intracellular processes like protein synthesis, or changes in monomeric intermediates are not considered. The unit for state variables concerning microorganisms is therefore chosen as density of cells (number of viable cells per weight unit).

The model is homogeneous in space. Already the way of taking the samples in the experiments in quantities of 1 g soil destroyed the spatial structure and caused a homogenization of the soil (Pukall, 1996, p.33). The explicit consideration of spatial dependency of cell growth and plasmid transfer seems to be not necessary. Additionally donor and recipient cells were inoculated at about equal densities in all experiments. In this case, it has been shown that the inclusion of spatial heterogeneity does not improve modeling (Simonsen, 1990). The homogeneous approach keeps the model simple, but it is necessary to evaluate its influence on the quality of simulation results.

The model must account for the fact, that the experimental design was that of a typical batch culture experiment. That means, cells grow within a closed environment, completely consuming the available initial substrate. After substrate exhaustion no more growth is possible and cell densities continuously decline until they reach zero.

4.2 Growth

Microorganisms grow mainly by cellular enlargement and binary fission. The simplest mathematical description of microbial growth therefore is based on the number of divisions n:

$$B_t = B_0 \cdot 2^n, \tag{4.1}$$

where B accounts for the number of microorganisms and the number of divisions is dependent on the length of the time period t. The measure for growth in this formulation is given by the generation time g, which is defined as the time a population needs to double its size, by

$$g = \frac{t}{n}. \tag{4.2}$$

The underlying assumption is, that the growth of microorganisms occurs at a constant rate. Formulated as time-continuous equation, this leads to the expression for infinite exponential growth of a population of microorganisms

$$\frac{dx}{dt} = \mu x \tag{4.3}$$

with the growth constant μ. To illustrate the step from discrete numbers of cells in equation 4.1 to a continuous state variable, the number of cells is now denoted as x. Unlimited growth is not likely to occur naturally. Anyhow, the growth constant μ is a basic parameter for microbial growth. More exact, it is the maximum growth rate, which can be reached under optimal conditions in the exponential growth phase of a microbial population. In reality, unlimited microbial growth can occur only for a short time period, because environmental conditions provide only limited resources. For this reason *Jaques L. Monod* introduced his concept of substrate limited growth. He established a relationship between growth rate and concentration of a growth limiting substrate very similar to the Michaelis-Menten kinetics for the effect of substrate concentration on an enzyme-catalyzed reaction:

$$\mu = \mu_{max} \frac{N}{(K_N + N)} \,. \tag{4.4}$$

Here, N denotes substrate concentration, μ_{max} is the maximum growth rate for unlimited growth, and K_N is known as saturation constant. It measures the affinity the organism has for the growth-limiting substrate. In principle, Monod's concept describes growth limitation by exhaustion of the assumed distinct limiting substrate. To take into account the consumption of substrate for cellular growth, the growth rate in equation 4.3 is replaced by 4.4, and the concentration of depleted substrate is described in a second equation:

$$\frac{dx}{dt} = \mu_{max} \frac{N}{(K_N + N)} x \tag{4.5a}$$

$$\frac{dN}{dt} = -\frac{1}{Y} \mu_{max} \frac{N}{(K_N + N)} x \,. \tag{4.5b}$$

The biomass yield Y denotes the amount of achieved biomass per unit of substrate consumed. Here, it is assumed to be a constant, although the yield in biomass has rather to be regarded as variable. The most important influence factor on the variability of Y is the ratio between the real growth yield denoting the part of the substrate used for biomass production, and the other part of substrate being used for energy requirements of the cell.

Together, μ_{max}, K_N, and Y can be regarded as a kind of "passport" data for a particular microorganism (Panikov, 1995, p. 26). By the knowledge of these parameters, which can be assessed exactly by special experimental designs, it is possible to predict the growth dynamics of a certain microorganism under defined conditions, if initial population size and substrate concentration are known. This is particularly the fact, as for equations 4.5 an analytical solution can be derived. From this basic model, a variety of models have been derived in order to clarify its disadvantages, as equations 4.5 can not describe all growth dynamics observed. For instance, growth dynamics cannot only be limited by substrate, but rather by a

specific chemical inhibitor. Particularly for soil microorganisms or microorganisms in oligotrophic habitats there are models which take into account the activity state of cells. Panikov (1995) has given an extensive overview about the possibilities to analyze specific growth dynamics far from the simple limited growth approach.

Thus far, the fact that the experiments took place in soil was not taken into account. The choice of a homogeneous modeling approach does not mean that no influence factor related to soil microbial growth can be included into the model at all. There are three factors directly belonging to soil growth dynamics, which can be incorporated: maintenance requirements, microbial biomass reutilization, and grazing by protozoa (Panikov, 1995, p.75).

For the purpose of describing batch mating experiments, Monod's concept is not sufficient, because it does not incorporate the process of cell decomposition. No negative contribution to growth dynamic has been assumed so far. Thus, energy requirements of the cell are included into the growth dynamics. The biomass required for maintenance processes like maintaining energy gradients, regulation of internal pH, or turnover of macromolecules is subtracted from the specific growth rate at a constant value m. By doing this, the value of Y can not longer be regarded as constant. This is reflected in the equations by changing the notation for Y to Y_{max} as the maximum growth yield obtained under the absence of maintenance requirements.

The reutilization of depleted microbial biomass is an important factor, if one wants to estimate the microbial production in large scale systems over a long time period. However, in microcosm experiments with a time scale of only several days the reutilization of biomass is not likely to have an influence on the observed dynamic at all. In contrast, the third process, i.e. the reduction of cells by microbial predators like protozoa, proceeds on the same time scale in the experiments took place at. Inclusion of a predatory process in cellular growth dynamics can be incorporated by assuming a constant population density F of protozoa. In proportion to this density, microbial cells are predated and therefore removed from the system. The inclusion of the described processes into equations 4.5 results in

$$\frac{dx}{dt} = (\mu_{max} \frac{N}{(K_N + N)} - m - k\ F)\ x \tag{4.6a}$$

$$\frac{dN}{dt} = -\frac{1}{Y_{max}}\ \mu_{max} \frac{N}{(K_N + N)}\ x\ , \tag{4.6b}$$

where k denotes the second order predatory constant. It should be noted, that the specific growth rate consisting of the sum of positive and negative influences can achieve negative values in comparison to the simple exponential growth model, or even to the Monod kinetic. This is a desired effect, since so it is possible to describe the dynamics of cell densities observed within a batch experiment with the models equations (cf. section 4.1). Equations 4.6 are used in the following to model the growth dynamics.

4.3 Transfer

Regarding the plasmid transfer between different bacterial cells, it is necessary to choose appropriate state variables. As mentioned before, the process of plasmid transfer includes donor, recipient and transconjugant cells. A recipient cell receives a plasmid while mating a donor cell. After the plasmid transfer, the recipient cell has turned into a transconjugant cell. Therefore, transfer can be modelled as a flow from recipient to transconjugant cell population. The transfer of a conjugative plasmid can occur only if a plasmid-bearing cell encounters a recipient cell. Without spatial heterogeneity, the chance for such an encountering between donor and recipient cells can be regarded as a random event proportional to their respective densities. Assuming, that a fraction of encounters results in the transmission of plasmid DNA, this is already a simple modeling approach for plasmid transfer. It follows a mass-action principle first proposed by Stewart and Levin (1977). In these terms, the number of newly formed transconjugant cells is proportional to the product of donor and recipient cells:

$$T = \gamma \, D \, R \ , \tag{4.7}$$

where D, R, and T are the densities of donors, recipients, and transconjugants, respectively. The constant of proportionality γ in equation 4.7 represents a plenty of influence factors on the plasmid transfer *in situ*. It should not be regarded only as the transmission of the plasmid, but rather as the successful integration of a former recipient cell into the transconjugant population. This implies the plasmid's ability to copy itself within the host cell, and to ensure that at least one plasmid copy is present in both daughter cells after binary fission. Inclusion of the transfer process into the model can be attained by the addition of newly formed transconjugants to the population of transconjugants and the simultaneously subtraction of this number from the recipient population. Unlike in Stewart and Levin (1977), here the densities of plasmid-bearing and plasmid-free cells are not distinguished, but those of donors, recipients and transconjugants.

In vivo plasmid evolution is influenced by several factors in detail. At the population level, important factors are the segregation process[3], consideration of a transitory derepression phase[4], the obtained plasmid copy number, and the influence of plasmid presence on microbial growth rates. Even though the mass action approach is quite simple, some of these factors have been taken into account in mathematical models using a mass action principle (Stewart and Levin, 1977; Simonsen *et al.*, 1990; Ganusov *et al.*, 1999). Especially Simonsen *et al.* (1990) investigated the effect of including more detailed influences on the plasmid transfer by the comparison of computer simulated transfer rates obtained by a simple mass action model vs. those

[3]Segregation means the loss of a plasmid during cell division or reproduction.

[4]Assuming that newly formed transconjugants are in a derepressed state with respect to pilus synthesis, they transfer at a faster rate than original donors.

from extended models. The results of these investigations show, that an extension of the simple modeling approach has no appreciable effect on the estimation of plasmid transfer rates using influence factors with reasonable values. Therefore, in order to keep the transfer term simple, the following assumptions are made in this model:

- a cell can contain only one plasmid,
- there is no loss of plasmids due to segregation,
- newly formed transconjugants are able to transfer their plasmid immediately,
- the transfer rate from transconjugants to recipients equals that from donors to recipients,
- bearing of a plasmid does not influence the growth rate of the bearing cell, neither positive nor negative.

Two types of dependency have been suggested to take into account that the transfer process is limited somehow. Andrup and Andersen (1999) treated the recipients as the limiting substrate analogous to Michaelis-Menten kinetics. This approach may be useful regarding a situation without nutrient limitation. It is supported by the results of Turner (2004), who observed the per-capita rate r of a plasmid-bearing population P

$$\frac{dP}{dt} = \mu\ P + \gamma\ P\ R \tag{4.8}$$

as

$$r = \frac{dP}{P\ dt} = \mu + \gamma\ R\ . \tag{4.9}$$

Therefore, he distinguished in equation 4.9 the vertical spread μ as independent of recipient concentration, while the horizontal spread γR was supposed to be proportional to the recipient concentration (cf. page 31). However, the consideration of a batch dynamic includes the depletion of nutrient and therefore the limitation of growth and transfer by the nutrient concentration. This approach has been used by Simonsen *et al.* (1990), who proposed the nutrient limitation to be of a monod type

$$\gamma(N) = \gamma_{max}\ \frac{N}{N + H_N}\ , \tag{4.10}$$

where N denotes the nutrient concentration. The transfer saturation constant H_N is equal to the saturation constant of the growth kinetics in equation 4.6, because the dependency of transfer on the nutrient availability is supposed to be very similar to the dependency of saturated growth. This approach covers the fact, that transfer events are unlikely after depletion of nutrients. Including all assumptions, the model's transfer term combines a mass action approach with nutrient limitation of the transfer:

$$\tilde{T} = \gamma_{max} \frac{N}{N + H_N} (D + T) R . \tag{4.11}$$

This term is used for the quantitative description of the transition from recipient to transconjugant population.

4.4 Complete model

The model considers densities of donors (D), recipients (R), transconjugants (T), and the nutrient concentration (N) as state variables. Cell densities are expressed as $CFU\ g^{-1}$, whereas nutrient concentration has the unit $\mu g\ g^{-1}$. Application of the derived expressions for growth and transfer to donor, recipient and transconjugant cells, respectively, yields the complete set of equations:

$$\frac{dD}{dt} = (\mu^D_{max} \frac{N}{N + H_N} - m^D - k^D F)\ D \tag{4.12a}$$

$$\frac{dR}{dt} = (\mu^R_{max} \frac{N}{N + H_N} - m^R - k^R F)\ R - \gamma_{max} \frac{N}{N + H_N} (D + T)\ R \tag{4.12b}$$

$$\frac{dT}{dt} = (\mu^R_{max} \frac{N}{N + H_N} - m^R - k^R F)\ T + \gamma_{max} \frac{N}{N + H_N} (D + T)\ R \tag{4.12c}$$

$$\frac{dN}{dt} = -\frac{1}{Y_{max}} (\mu^D_{max}\ D\ +\ \mu^R_{max}\ (R + T)) \frac{N}{N + H_N} \tag{4.12d}$$

For a summary of parameters and units see table 4.1. Maximum growth rates, maintenance costs and predation rate are assumed to be different for donor and recipient cells, respectively. As bearing of a plasmid is supposed to have no influence on growth, these factors are identical for recipient and transconjugant cells in the model.

Mathematically, this model is a system of four coupled ordinary, nonlinear differential equations. As a result of coupling the equations within the transfer terms, it is not possible to obtain an analytical solution for this system anymore. Formally, together with the initial values $D(0) = D_0,\ R(0) = R_0,\ T(0) = T_0,$ and $N(0) = N_0$ equations 4.12 form an initial value problem, which can be solved numerically, if all model parameter values are known. This has been realized in this thesis by application of the `NDSolve` method provided by `mathematica` (For details see appendix A). From the point of view of system analysis, the model is a half-open system with no continuous input. Nutrient available at start time is turned into microbial biomass, until it is depleted. Energy costs for cell maintenance and the predatory influence of protozoa form outflows transferring biomass out of the system. No non-trivial steady-state can be achieved, since for positive parameter values only the trivial

Table 4.1: Model parameters and its units.

Parameter	Meaning	Unit
μ_{max}^{D}	maximum donor growth rate	d^{-1}
μ_{max}^{R}	maximum recipient and transconjugant growth rate	d^{-1}
m^{D}	maintenance costs of donor	d^{-1}
m^{R}	maintenance costs of recipient and transconjugant	d^{-1}
$k^{D} \cdot F$	predation rate of donor	d^{-1}
$k^{R} \cdot F$	predation rate of recipient and transconjugant	d^{-1}
γ_{max}	maximum transfer rate	g CFU^{-1} d^{-1}
H_N	saturation constant for growth and transfer	$\mu g\ g^{-1}$
Y_{max}	maximum growth yield	$\mu g\ g^{-1}$
D_0	inital donor density	CFU g^{-1}
R_0	inital recipient density	CFU g^{-1}
T_0	inital transconjugant density	CFU g^{-1}
N_0	inital nutrient concentration	$\mu g\ g^{-1}$

solution exists for equations 4.12. Therefore, a stability analysis of the system is not reasonable.

The model describes the nutrient flow through the populations of donor, recipient and transconjugant cells. Caused by the assumption of a tight connection between plasmid transfer and cell growth in the model, it is a necessary pre-condition for the feasibility of a model-based analysis of an experiment, that typical batch dynamicss could be observed. That means, an initial growth phase followed by a decline of cell densities after depletion of nutrients should occur. In some scenarios, in particular those without nutrient amendment (cf. section 3.2), population growth can not be observed. In order to obtain a model suitable in these cases, equations 4.12 are simplified by omitting all terms related to nutrient concentration. The resulting set of equations 4.13 describe a simple exponential decay of donor, recipient, and

transconjugant densities. Nutrient dependency of plasmid transfer is neglected, too.

$$\frac{dD}{dt} = -(m^D + k^D F)\ D \tag{4.13a}$$

$$\frac{dR}{dt} = -(m^R + k^R F)\ R - \gamma_{max}\ \ (D+T)\ R \tag{4.13b}$$

$$\frac{dT}{dt} = -(m^R + k^R F)\ T + \gamma_{max}\ \ (D+T)\ R \tag{4.13c}$$

4.5 Parameter estimation

In principle, there are two ways to estimate model parameters from experimental data: (*i*) by means of a regression method or (*ii*) by transformations of model equations under certain conditions. The choice of one of these methods depends on quality and number of experimental data as well as on the aim of modeling. Method (*i*) is preferable in the case of a high number of data points, since under such conditions parameters can be estimated by nonlinear regression methods such as e.g. the Levenberg-Marquardt method quite exact. The choice of method (*i*) supports a model, which is dependent on parameter values being as exact as possible. Method (*ii*) can already be applied if a small number of data points is available. It does not guarantee optimal parameter values with respect to minimal deviations between data points and model results. However, a semi-quantitative evaluation of experimental data is possible by the application of method (*ii*).

R. Pukall determined the number of viable cells for donor, recipient, and transconjugant strains within the microcosm experiments. Samples were taken in parallel from all scenarios. For every scenario, the number of CFUs were determined by plate countings for both samples, and the resulting values have been transformed to the decade logarithm (Pukall (1996), sec. 2.7.1). From the parallel CFU values, R. Pukall determined mean values and standard deviations (Pukall (1996), sec. 2.9). Unfortunately, more than two values are necessary to enable the application of statistical means such as the calculation of mean values and standard deviations. So, the quality of the data values obtained from the microcosm experiments is rather low with regard to statistics.

The number of days, on which samples were taken, ranges from four to six. This results in a number of twelve to eighteen data points per scenario. In the proposed model, there are ten unknown parameter values, if the initial nutrient concentration is included. It is theoretically possible to estimate the parameter values with a nonlinear regression analysis, because the number of degrees of freedom of the system 4.12 is below the number of data points.

Nevertheless, method (*ii*) is applied in this thesis. This is mainly motivated by three facts:

- method (*ii*) is fast and safe to use, even in the case of low sample numbers,
- the objectives of this thesis can be reached well by means of method (*ii*) and results are suspected not to be improved very much by the application of method (*i*),
- method (*ii*) provides the possibility to determine the initial amount of nutrient by a kind of "backward engineering" (see equation 4.22). Initial nutrient concentration is not really a parameter value, but rather a initial condition. Using method (*i*), difficulties could arise founded on the missing initial value for the nutrient densities.

Parameter values presented in chapter 5 are shown without error values. The estimated values are verified by the comparison between simulations results and data points only. More importance is attached to the derivation of statements by comparing the results of experiments among each other.

To start the parametrization of the model, the initial population densities of donor, recipient and transconjugant cells can be taken directly from data. Values for the saturation constant H_N and the maximum growth yield Y_{max} can only be derived from data, if specially designed experiments have been performed. For example, simulations with increasing, exactly defined nutrient concentrations can be performed to determine the saturation constant. Due to the fact that such investigations are not included in the underlying experiments, these two parameter values are taken from literature (Simonsen *et al.*, 1990) as

$$H_N = 4\ \mu g\ g^{-1} \tag{4.14a}$$

$$Y_{max} = 2 \cdot 10^6\ \mu g\ g^{-1}\ . \tag{4.14b}$$

At this point, the quest for a simple model structure pays off, as it is possible to derive formulas for parameter estimation by transforming model equations for the remaining parameters. From the model equations it follows, that after depletion of nutrients only the negative term remains effective. Considering that the data point of maximum cell density constitutes the point of nutrient depletion, the following data points can be identified with the cellular decomposition process. Formally, this

can be approached by

$$\frac{dx}{dt} = -(m + k\ F)\ x \tag{4.15a}$$

$$\Leftrightarrow\ -(m + k\ F) = \int_{x(t_a)}^{x(t_b)} \frac{dx}{x}\ (\int_{t_a}^{t_b} dt)^{-1} \tag{4.15b}$$

$$\Leftrightarrow\ -(m + k\ F) = \frac{\ln x(t_b) - \ln x(t_a)}{t_b - t_a} \tag{4.15c}$$

$$\Leftrightarrow\ \delta = -\frac{\ln x(t_b) - \ln x(t_a)}{t_b - t_a}\ . \tag{4.15d}$$

That means, if t_a is the time, when maximal cell density is reached, and t_b the time of the last sample, then equation 4.15d delivers an aggregated value of the negative growth term. In the case of sterile soil, only maintenance costs have to be considered. So, the values for m of the donor and recipient population are already given by this equation. In the remaining scenarios there are two possibilities to deal with the difficulty, that values of m and $k \cdot F$ are only accessible aggregated: first, the negative term of the growth rate can be identified to

$$m + k\ F\ \equiv\ \delta\ , \tag{4.16}$$

under neglection of the differences of influences of maintenance costs and protozoal grazing, respectively. Second, under application of maintenance costs derived from the sterile soil scenarios it can be discriminated between the protozoal influences and maintenance costs by

$$k\ F\ = \delta - m\ . \tag{4.17}$$

With the knowledge about the negative term of the growth rate, the maximum growth rates can easily be calculated. Under the assumption, that $N \gg H_N$ holds initially[5], the intrinsic growth rates μ can be determined by considering a true exponential growth in the first phase

$$\frac{dx}{dt} = \mu\ x\ \Leftrightarrow\ \ \mu = \frac{\ln x(t_b) - \ln x(t_a)}{t_b - t_a}. \tag{4.18}$$

Here, t_a is the starting time point, and t_b is the time point of the maximum cell density. By application of

$$\mu_{max}\ =\ \mu + \delta\ , \tag{4.19}$$

[5]it follows, that $\frac{N}{N+H_N} \approx 1$

the maximum growth rates are accessible. The relation between the intrinsic growth rate μ and the generation time g given by the Pedersen formula

$$g = (t_a - t_b)\frac{\ln 2}{\ln x(t_b) - \ln x(t_a)} \,, \tag{4.20}$$

which allows the determination of the generation times from knowledge of μ by

$$g = \frac{\ln 2}{\mu} \,. \tag{4.21}$$

Even if the initial nutrient concentration has not been determined within the experiments, the part of nutrient consumed by the inoculated *E. coli* cells can be approximated from the model equations. Under the assumption, that the consumption results in population growth leading to the maximal observed cell density, the amount of consumed nutrient can be identified by the difference between the initial and the maximal population densities. Formally, this can be expressed as

$$N_0 = \frac{1}{Y_{max}} \left(\mu^D_{max} \left(D_{max} - D_0\right) + \mu^D_{max} \left(\left(R_{max} - R_0\right) + T_{max}\right)\right) \,, \tag{4.22}$$

if D_{max}, R_{max}, and T_{max} denote the maximal population densities of donor, recipient, and transconjugant cells, respectively. By determining the initial value of N_0 in this way, the meaning of this parameter changes from overall initial nutrient density to the amount of nutrient transformed into *E. coli* biomass during growth, given the initial and maximal densities of the different strains and the maximum yield. With other words, N_0 denotes the nutrient turnover of the system. By using the maximum growth rates μ_{max} instead of the intrinsic growth rates μ for the determination of N_0, the part of nutrients utilized for maintenance costs and removed from the system by protozoal grazing is not taken into account. Obviously, a necessary pre-condition for the feasibility of equation 4.22 is the existence of maximum values for cell densities greater than the initial values. As this pre-condition is not fulfilled in some cases, an alternative method for the determination of initial values of N_0 is given by

$$N_0 = \frac{1}{Y_{max}} \left(\mu^D \, D_{max} + \mu^R \left(R_{max} + T_{max}\right)\right) \,. \tag{4.23}$$

The underlying assumption is, that the nutrient turnover during the experiment is proportional to the initial cell density. In particular, equation 4.23 should be applied to calculate the initial value for nutrient density in connection with the simplified model 4.13.

Transfer rate

The only parameter remaining to be estimated is the plasmid transfer rate. Determination of the transfer rate directly from experimental data seems to be difficult.

Several measures have been proposed for quantifying plasmid transfer, including the ratio of transconjugants to donor cells, the ratio of transconjugants to recipient cells, and the minimum number of donors necessary to observe plasmid transfer within a fixed time period. Since these measures are dependent on cell densities, mating times, and donor/recipient ratios, it is difficult to compare results originating from different experiments. For this reason, Simonsen *et al.* (1990) have proposed a method for a general measure of plasmid transfer, which shall be applied here.

The end-point method has been derived from a model for plasmid transfer very similar to the one developed in this thesis. Alike this model, the end-point model of Simonsen considers densities of donor, recipient, and transconjugant cells as state variables, and the dynamics of growth and transfer are regarded, but it does not consider a negative term in the growth dynamics, and the growth rates are assumed to be identical for all regarded populations. Under those pre-conditions a formula for the estimation of a plasmid transfer rate was derived from the model. This aim could be achieved by obtaining a solution for the temporal change of an ad hoc measure for plasmid transfer,i.e. the transconjugant/recipient ratio, in dependence on the development of the total cell density. The equation for the transfer rate has been developed by Simonsen *et al.* to

$$\gamma \;=\; \mu \;\ln(1+\frac{T}{R}\frac{C}{D})\frac{1}{(C-C_0)}\;, \tag{4.24}$$

where C_0 denotes the complete initial cell density, C denotes the complete cell density with $C = D+R+T$ at the time of sampling, just as for the remaining variables D, R, and T. The great advantage of this end-point method is not only the possibility to compare results obtained from different experiments, but additionally the facility to determine a measure for the plasmid fertility out of only two measurements. The proposed tight relationship between growth and transfer becomes clear again, as the growth rate and the achieved cell densities are used to determine values for γ.

The main difference between Simonsen's model and the model proposed in this thesis consists of a diverse composition of growth terms. Under the assumption, that relatively low differences between the growth rates of donor and recipient cells can be neglected for an approximate determination of the transfer rate, equation 4.24 can be used to estimate the missing values for the plasmid transfer rates.

Since the determination of the plasmid transfer rate depends particularly on the observed growth rate, the end-point method cannot be applied in scenarios without growth of donor and recipient population. In order to allow an estimation of a plasmid transfer rate even if no growth occurred, a suggestion of Sudarshana and Knudsen (1995) is seized, who assumed the transfer rate to be proportional to the ratio of transconjugant cells and the product of donor and recipient cells.

This assumption can be founded by the transformation of equation 4.7 already. Neglecting nutrient dependency of the plasmid transfer and assuming that transcon-

jugant population density is small in comparison to the donor population, the plasmid transfer rate γ in the case of declining cell densities of donor and recipient cells can be determined as

$$\gamma = \frac{T}{D\,R}\,. \tag{4.25}$$

Here, D, R, and T denote non-zero population densities at an arbitrary moment of sampling.

Chapter 5

Results and discussion

In the microcosm experiments performed by R. Pukall about thirty scenarios have been sampled. It is necessary to outline shortly the outcomes of these experiments before results are presented, because it was not reasonable to estimate parameter values and simulate all experimental scenarios. The experiments aimed at the investigation of plasmid transfer in soil. Six plasmids have been used. Unexpectedly, no plasmid transfer could be detected for the IncI1 and IncFII group plasmids at all, even though donor and recipient cells have grown well. An explanation for this observation has been given by R. Pukall within his PhD-thesis, where he supposes that the structure of the pili encoded by these two plasmid groups has not been appropriate for mating pair formation in soil (Pukall, 1996, p. 76). The transfer of certain plasmid groups can also be habitat-specific within the environment. Thus, scenarios with IncI or IncF group plasmids are not considered in the simulations. Alike, scenarios with mobilizable IncQ group plasmids are not included in the following, because the transfer of these plasmids cannot be described by the proposed model.

Not only the type of plasmid, but also nutrient amendment of the soil has been a main influence factor for the occurrence of plasmid transfer in the experiments. Two of the four basic set-ups (cf. table 3.2) have been performed both with and without nutrient amendment of inoculated cells. In the scenarios with nutrient amendment, transfer was observed to occur frequently for plasmids of the IncP1, IncN, and IncW3 groups, whereas only the IncP1 plasmid could transfer itself in the scenarios without nutrient amendment, even if no growth was observed for both donor and recipient population.

5.1 Sterile and non-sterile soil

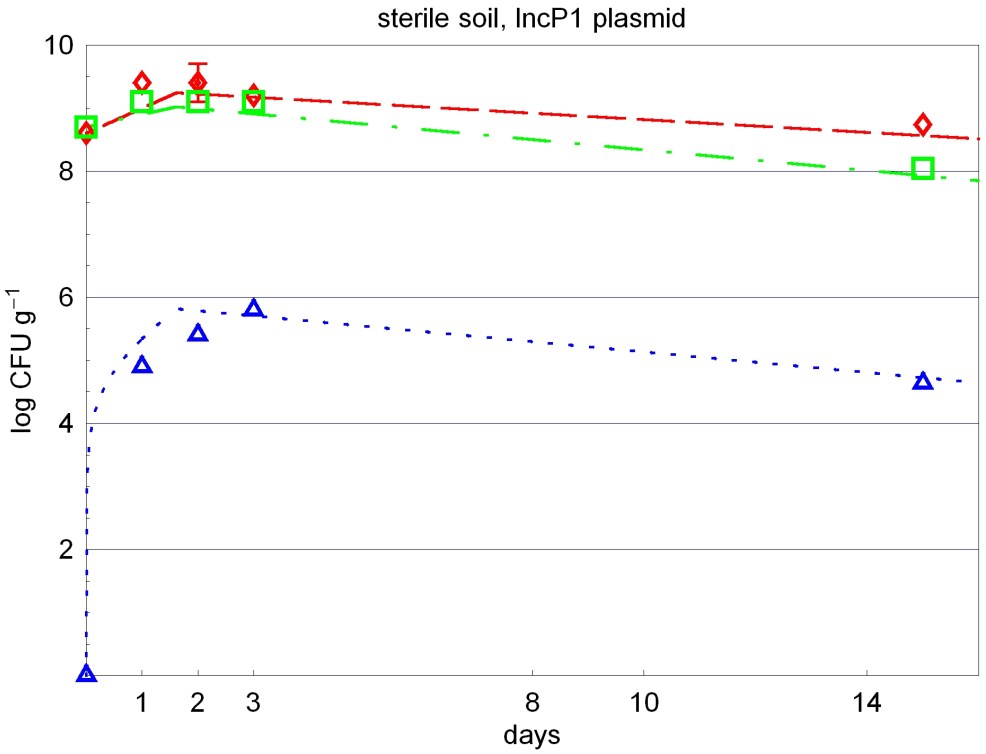
The experiments in sterile soil have been performed to examine the persistence time of *E. coli* in soil without microbial competition. Unfortunately, in two of three plas-

mid groups population densities have been measured for the first three days of the experiment only, so that the persistence in these scenarios can be valued limited only. The IncP1-group scenario has been sampled additionally after 15 days. The first scenario assayed is the IncP1-group plasmid scenario in sterile soil with nutrient amendment. Data points and simulation results are presented in figure 5.1(a). Both donor and recipient densities start at about $10^{8.5}$ viable cells per gram of soil (CFU/g). In the course of the experiment, the donor cells achieve a higher density than the recipient cells. This observation is the case for virtually all scenarios and can be explained by the auxotrophy of the recipient strain *E. coli* K-12 (CV601) (cf. section 3.1). The maximum value is obtained on the second day for both donor and recipient cells, the transconjugants achieve their maximum on the third day. The densities of donor and recipient cells do not fall below 10^8 CFU/g, those of transconjugants not below 10^4 CFU/g during the whole sampling period of fifteen days. Although they grow for the first two to three days only, cells survive very well in soil without microbial competition and protozoal grazing. Observed densities of donor and recipient strains are matched well by the simulations. The good agreement between observed and simulated cell densities displays that the model equations together with the applied parameter estimation methods are suitable for the description of the experiments. Simulation results for the transconjugants meet the order of magnitude of the data points well, even if the densities of transconjugants are slightly overestimated in the first two days. This can be caused by a lower transfer rate in the experiments caused by e.g. segregation or by a lower transconjugant growth in comparison to the recipient cells.

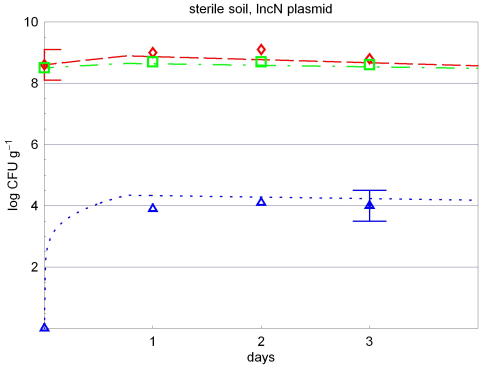
The observation of the IncN scenario shows a similar situation within the sampling time period (cf. figure 5.1(b)), but the cells do not grow as strong as in the IncP1 scenario. Regarding the IncW3 scenario, the development of the recipient cell densities does not show a batch dynamic, as the recipient cell's density first declines and rises again at the third day of sampling. This development is reflected by estimated negative values for recipient growth rate and maintenance costs (cf. table 5.1). It becomes clear that the feasibility of the parameter estimation method is given only in the case of explicit experimental batch dynamics. A simulation of this scenario using the estimated parameter values is not reasonable. The time course of experimentally observed cell densities is displayed in figure 5.1(c).

Table 5.1: Parameter values estimated for sterile nutrient amended soil.

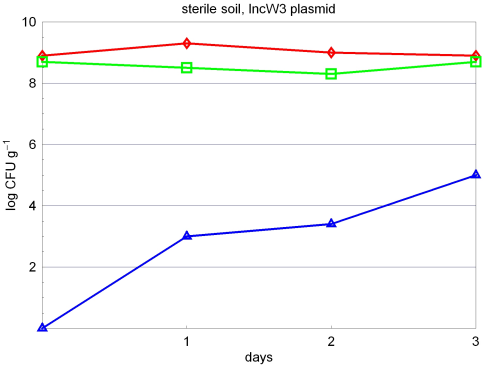
	μ^D [d^{-1}]	m^D [d^{-1}]	μ^R [d^{-1}]	m^R [d^{-1}]	γ [g CFU^{-1} d^{-1}]	N_0 [μg g^{-1}]
IncP1	0.92	0.12	0.46	0.19	$1.8 \cdot 10^{-13}$	1148
IncN	0.58	0.69	0.23	0.23	$3.7 \cdot 10^{-14}$	320
IncW3	0.92	0.46	-0.46	-0.23	$3.3 \cdot 10^{-15}$	553



(a) IncP1 plasmid



(b) IncN plasmid



(c) IncW3 plasmid

Figure 5.1: Data points and simulations (panels a) and b) only) for nutrient amended sterile soil. Panels depict the IncP1, IncN, and IncW3 plasmid scenarios. Measured values for donor, recipient and transconjugant cells are marked by diamonds (⋄), boxes (□), and triangles (△), respectively. Simulation results for donor, recipient, and transconjugant densities are displayed with dashed, dot-dashed, and dotted lines in panels a) and b), respectively. Error bars are shown, if the experimental values deviate from their mean value more than 0.2 [log CFU/g]. For parameter values, see table 5.1.

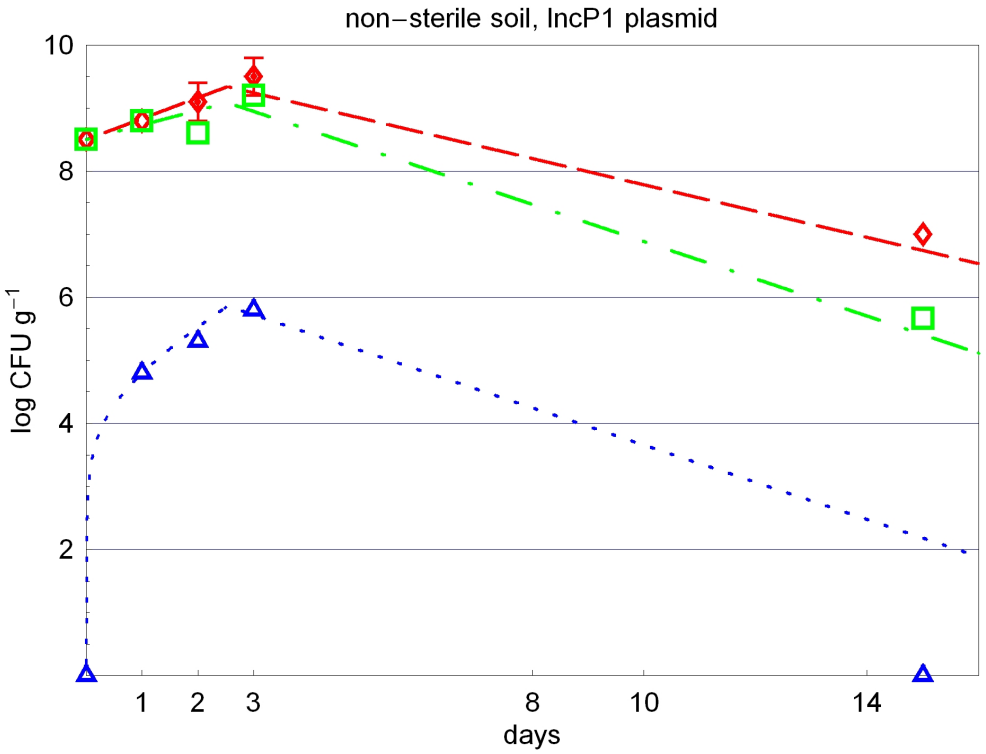
Non-sterile soil

Inoculated at similar densities compared to sterile soil, the IncP1-bearing donor strain in non-sterile, nutrient amended soil shows an increasing cell density for the first three days for both donor and recipient strain (cf. figure 5.2(a)). The maximum data values for all cell types are displayed on the third day. Growth took place similar as in the sterile IncP1 scenario. This observation is supported by the similar growth rate values estimated for the IncP1-bearing strains in sterile and non-sterile soil, respectively. Non-sterile soil influences the persistence of cells negatively. The cell density of donor cells fell below 10^8 CFU/g at the fifteenth day, in the case of recipient cells even below 10^6 CFU/g.

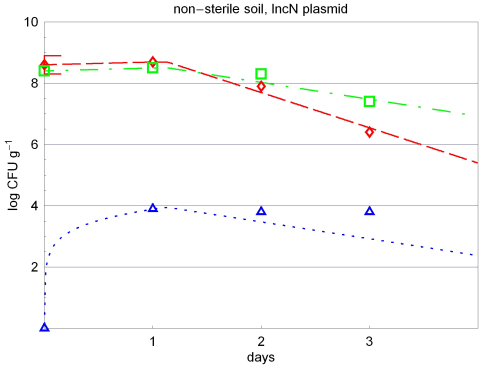
In contrast, the densities of donor and recipient cells fall below 10^8 CFU/g even at the third day in the case of IncN and IncW3 (cf. figures 5.2(b) and 5.2(c)). Nearly no growth can be observed, as it is reflected by the low growth rate estimated for the IncN scenario and the zero growth rate obtained from the IncW3 scenario. Caused by the marginal growth, the initial nutrient density cannot be calculated by equation 4.22. Instead, equation 4.23 has been used to determine N_0 for the IncN and IncW3 scenarios. The estimated parameter values are presented in table 5.2 on page 50. The plasmid transfer rate in the IncW3 scenario, which cannot be determined by equation 4.24 since the growth rate equals zero, has been estimated under the assumption, that a marginal growth occurred between the first and the second measurement.

Regarding the simulation of the IncP1 scenario, a good agreement between data points and simulation results can be detected. Overall, the data points seem to be underestimated slightly, but the data's order of magnitude and trend has been met very well. Concerning the data points, the first and the last experimental value of transconjugant density are displayed as zero. While the initial value of transconjugants is definitely zero, this cannot be stated for the last data point. The accuracy of this value depends on the detection method. Transconjugants have been sampled by laborious methods, including selective and dilution plating techniques (Pukall (1996), sec. 2.7.1). Low cell numbers are difficult to evaluate by dilution platings, since the dilution factor amplifies low cell counts stronger in comparison to higher cell numbers. The simulation overestimates the transconjugant density for a factor of 100 (cf. figure 5.2(a)), but it has to be kept in mind, that already the observation of one cell at a dilution factor of 1:100 would result in a good agreement between simulation and data values at this point.

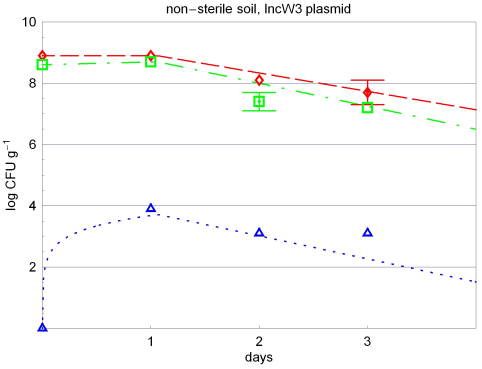
In the IncN scenario, the simulations match the experimental data well. The data points of donor and recipient cells are met nearly exactly, and the simulations hit also the dimension of transconjugants well, but they do not meet the trend of the transconjugant cells. While the data values stay at about 10^4 CFU/g, the simulation declines faster here and underestimates the transconjugant density.



(a) IncP1 plasmid



(b) IncN plasmid



(c) IncW3 plasmid

Figure 5.2: Data points and simulations for nutrient amended sterile soil. Panels depict the IncP1, IncN, and IncW3 plasmid scenarios. Measured values for donor, recipient and transconjugant cells are marked by diamonds (⋄), boxes (□), and triangles (△), respectively. Simulation results for donor, recipient, and transconjugant densities are displayed with dashed, dot-dashed, and dotted lines, respectively. Error bars are shown, if the two experimental values deviate from their mean value more than 0.2 [log CFU/g]. For parameter values, see table 5.2.

This can be traced back to the influence of e.g. transitory derepressed transconjugant cells, whose ability to synthesize pili is in a derepressed state, so that they transfer faster than other transconjugant cells.

The observation of the simulations in the IncW3 scenario obtains similar results. The data point on the second day could be caused by a systematical sampling error, because the densities of all three cell types seem to fall down in comparison to the surrounding points. A better evaluation is restricted by the low number of samples.

Overall, the evaluation of the scenarios carried out in non-sterile soil clearly indicates the influence of microbial competitors and protozoal grazing on the development of *E. coli* strains. In addition, differences in growth of the IncP1 plasmid bearing strain in comparison to the scenarios with the IncN and IncW3 plasmids are obvious.

Table 5.2: Parameter values estimated for non-sterile, nutrient amended soil.

	μ^D [d^{-1}]	δ^D [d^{-1}]	μ^R [d^{-1}]	δ^R [d^{-1}]	γ [g CFU^{-1} d^{-1}]	N_0 [μg g^{-1}]
IncP1	0.77	0.48	0.54	0.68	$2.8 \cdot 10^{-13}$	2546
IncN	0.23	2.65	0.23	1.27	$5.6 \cdot 10^{-14}$	957 [a]
IncW3	0.0	1.38	0.23	1.73	$2.5 \cdot 10^{-14}$ [b]	1039 [a]

[a]Value was determined with equation 4.23 taking account for the observed low population growth.

[b]Value was calculated considering a marginal donor growth of 0.05 [d^{-1}].

Characterizing maintenance costs and predatory influence

As proposed in section 4.5, the differences between growth in sterile and non-sterile soil can be used to quantify the influence of maintenance requirements and protozoal grazing (eq. 4.17). Assuming that maintenance costs are identical in sterile and non-sterile soil, an aggregated value for the predatory influence of protozoa can be computed. For the IncW3 scenario this is impossible due to the negative value of maintenance costs estimated in non-sterile soil. Resulting values for predatory influence in the IncP1 and IncN scenarios range from 0.36 d^{-1} to 2.42 d^{-1} (cf. table 5.3). It strikes that the maintenance costs do not vary strong, since they range from 0.12 d^{-1} to 0.23 d^{-1} only. This indicates, that the energy requirements of donor and recipient cells are relatively constant. In spite, the influence of protozoal grazing varies in comparison between the IncP1 and the IncN scenarios. Within the IncP1 scenario, the values for $k^D\ F$ are estimated to be about 0.4 d^{-1} for both donor and recipient cells, while in the IncN scenario the respective values have been calculated to 2.42 d^{-1} and 1.15 d^{-1}. Assuming, that protozoal grazing takes place unselective, it becomes likely that the influence of microbial competition has to be included into

this factor and can be supposed to be stronger than the one of protozoa. Another possibility to explain the changing values of protozoal grazing influence could be variations in protozoal densities. In any case, the IncP1-bearing strains seem to have an advantage in competition compared to the IncN-bearing strain.

Table 5.3: Estimated values for predatory influence.

	m^D [a]	δ^D [b]	m^R [a]	δ^R [b]	$k^D\ F$ [c]	$k^R\ F$ [c]	$m^D\ \delta^{D-1}$	$m^R\ \delta^{R-1}$
IncP1	0.12	0.48	0.19	0.68	0.36	0.49	0.23	0.26
IncN	0.23	2.65	0.12	1.27	2.42	1.15	0.09	0.36

[a]Maintenance costs estimated from the sterile soil scenario [d^{-1}].
[b]$\delta^D = m^D + k^D \cdot F$, estimated from the non-sterile soil scenario [d^{-1}].
[c]Calculated from equation 4.17 [d^{-1}].

Setting estimated maintenance costs and predatory or competitive influences into relation by means of comparison between sterile and non-sterile scenarios, an approximate share of maintenance costs in the common negative part of the growth rate can be determined: maintenance costs are found to contribute in a range from 9 to 36 % to the decrease of population densities (cf. last two columns in table 5.3). Since these values differ strongly, no clear conclusion can be drawn here.

Overall, it is difficult to differ between the effects of local fluctuations e.g. in protozoal densities and possible sequels of additional processes contributing to the decline of cell densities in non-sterile soil like the indirect competition for nutrients between the *E. coli* cells and autochthonous cells. The main result of the attempt to quantify single influences on the decline of cell densities is the observation, that microbial competition seems to have more influence than the predatory influence of protozoa.

5.2 Rhizosphere soil

The data obtained from the scenarios with nutrient amended rhizosphere soil display similarity to those from the previous scenarios regarding the growth differences between donor and recipient strains. Differences exist in the initial densities of donor and recipient strains and the number of samples. Initial values were about 10^6 CFU/g for both donor and recipient strains in all scenarios. Samples have been taken for the first three days, and additionally after seven and fourteen days, so that a comparison between the transfer dynamics of the different plasmids over a longer time period than in the former experiments is possible.

In the IncP1 scenario, growth occurred from the start until the second day for both donor and recipient cells (cf. figure 5.3(a)). Maximum values are $10^{8.4}$ CFU/g

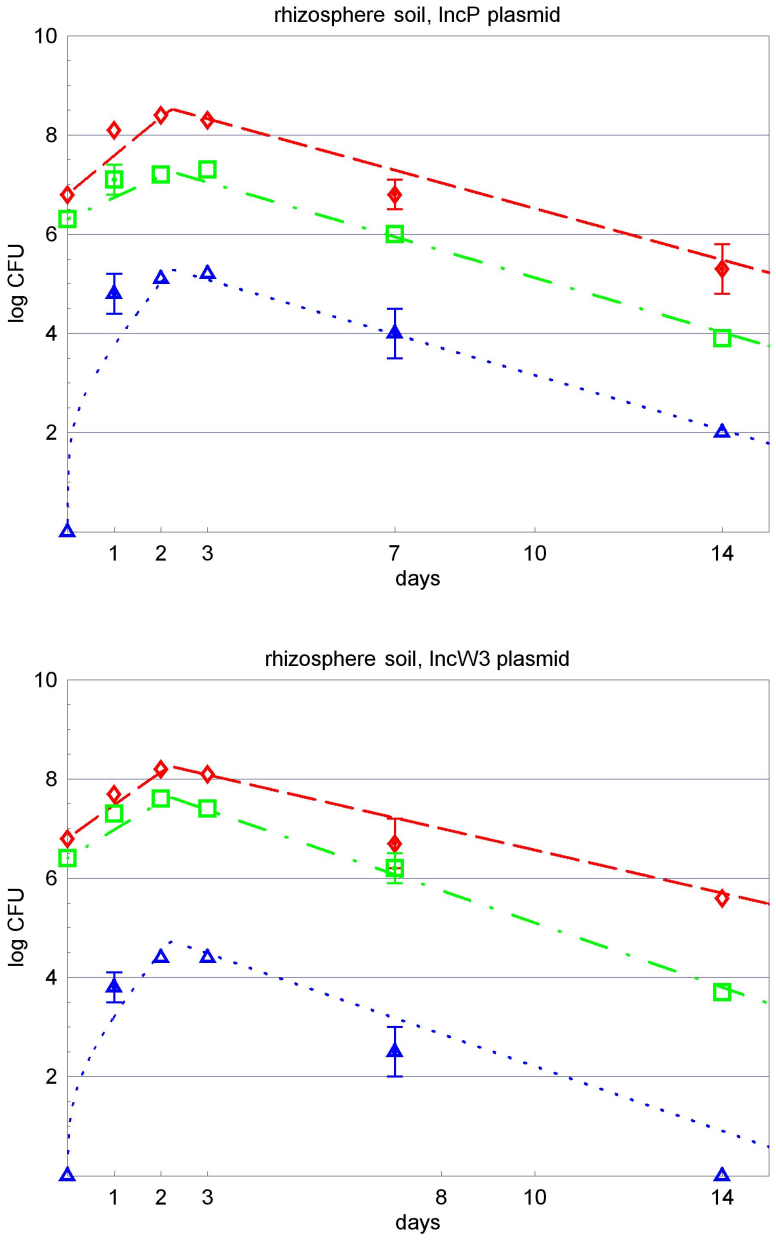


Figure 5.3: Data points and simulations for nutrient amended rhizosphere soil, IncP1 (top) and IncW3 (bottom) plasmids. Measured values for donor, recipient and transconjugant cells are marked by diamonds (⋄), boxes (□), and triangles (△), respectively. Panels depict the IncP1 and IncN plasmid scenarios. Simulation results for donor, recipient, and transconjugant densities are displayed with dashed, dot-dashed, and dotted lines, respectively. Error bars are shown, if the experimental values deviate from their mean value more than 0.2 [log CFU/g]. For parameter values, see table 5.4.

and $10^{7.2}$ CFU/g, respectively. From the second day on, cell densities display a monotonous decline. This holds as well for the transconjugant cells. In comparison to the experiments in sterile and non-sterile soil, the maximum values of transconjugants are about equal in rhizosphere soil. Simulation results for this scenario fit the data very well, even if the data points show greater deviations compared to sterile and non-sterile soil. In the IncP1 scenario in rhizophere soil five data points show a deviation greater than 0.2 log CFU/g from their respective mean values.

Table 5.4: Parameter values estimated for scenarios with nutrient amended rhizosphere soil.

	μ^D_{max} [d^{-1}]	δ^D [d^{-1}]	μ^R_{max} [d^{-1}]	δ^R [d^{-1}]	γ [g CFU d^{-1}]	N_0 [μg g^{-1}]
IncP1	1.84	0.59	1.04	0.63	$5.9{\cdot}10^{-11}$	232
IncN	0.92	0.90	0.35	0.75	$4.9{\cdot}10^{-11}$	34
IncW3	1.61	0.50	1.38	0.75	$6.7{\cdot}10^{-12}$	144

Deviations in data points obtained from the IncW3 scenario are similar to those from the IncP1 scenario (cf. figure 5.3(b)). In both scenarios, the agreement between simulations and data points is very well. The time course of donor and recipient densities has been met nearly exactly. The development of transconjugant densities is underestimated by the simulations in both scenarios on the first day, in contrast to the sterile and non-sterile scenarios. This suggests, that the rhizosphere influences plasmid transfer and/or transconjugant growth positively. This supposition is confirmed by plasmid transfer rates calculated for all rhizosphere scenarios, which are higher in comparison to those rates calculated from the sterile and non-sterile scenarios (cf. table 5.4).

Differences between the development of transconjugant densities in the IncP1 and the IncW3 scenarios can be observed. Donor and recipient cells have been inoculated at similar densities and show an approximately equal development, as it is reflected in the similar growth and death rates (cf. table 5.4). In contrast, the transconjugant cells in the case of the IncP1-bearing strain exceed a density of 10^5 CFU/g on the second and the third day, the IncW3-bearing strain does not exceed 10^5 CFU/g at all. This development continues, as on the fourth day in the IncP1 scenario the transconjugant density is above 10^4 CFU/g and on the fourteenth day above 10^2 CFU/g, while in the IncW3 scenario the transconjugant density is below 10^3 CFU/g and on the fourteenth day transconjugants have not been detected at all. Together with the higher plasmid transfer rate this observations suggest, that the IncP1 plasmid is more successful in transferring than the IncW3 plasmid. Since IncP1 plasmids are known to be rather promiscuous, the experiments approve that this is the fact in soil, too.

The experimental results from the IncN scenario are not considered in this extent

for reasons of strong variations (cf. figure 5.4). Nine of eighteen data points display a deviation greater than 0.2 log CFU/g from their respective mean values. The data values obtained from the rhizosphere soil show an inhomogeneity greater than in the sterile and non-sterile scenarios. This could be due to a greater spatial variability of growth processes in rhizosphere soil, possibly caused by root influences. However, the way of inoculation of rhizosphere microcosms has been carried out different from the other scenarios. Bacterial suspension has been dripped onto on the soil surface covered by grass with a pipette. In comparison to the homogenized sterile and non-sterile soil, already the initial experimental conditions have been different for rhizosphere soil (cf. Pukall (1996), p. 80). Thus, the variations in data points obtained from rhizosphere scenarios can be traced back to the experimental methods and do not directly indicate a greater spatial variability in rhizosphere soil.

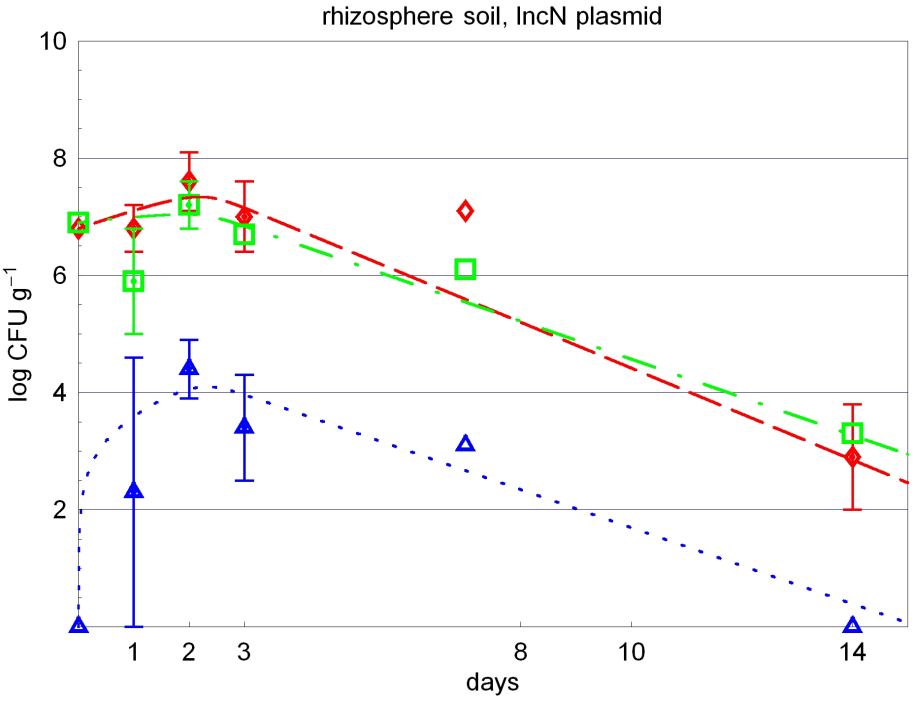


Figure 5.4: Data points and simulations for nutrient amended rhizosphere soil, IncN plasmid. Measured values for donor, recipient and transconjugant cells are marked by diamonds (⋄), boxes (□), and triangles (△), respectively. Simulation results for donor, recipient, and transconjugant densities are displayed with dashed, dot-dashed, and dotted lines, respectively. Error bars are shown, if the experimental values deviate from their mean value more than 0.2 [log CFU/g]. For parameter values, see table 5.4.

Influence of rhizosphere on growth

The attempt to investigate the influence of the rhizosphere on the observed dynamics seems to be difficult, keeping in mind that measured values are resolved at the level of average cell densities. A comparison of nutrient turnover values suggests, that there is a negative influence of the rhizosphere on growth, since the values estimated for N_0 within the sterile and non-sterile scenarios are about ten times higher than those estimated in the rhizosphere scenarios. However, the shift in the amount of nutrient turnover can be attributed to the differences in the initial donor and recipient densities.

A more meaningful information about growth dynamics can be gained through the determination of the direct differences between initial and maximal values of cell densities as measures for growth activity relative to the initial density via

$$RGI = \sum_{X \in \{D,R,T\}} log_{10} \frac{X_{max}}{X_0} . \tag{5.1}$$

Values for the relative growth index indicate the orders of magnitude, the *E. coli* densities rise during the experiments. Rating the values calculated by equation 5.1, the observation of a negative influence obtained from the consideration of the nutrient turnover rates can be validated false. There seems to be a rather positive influence of the rhizosphere on cellular growth in comparison to the homogenized soils (cf. table 5.5). In the case of the IncP1 plasmid, the RGI values range within the dimension of 10^7 CFU/g. The positive effect of rhizosphere is not too striking, possibly due to the general good growth of the IncP1-bearing strains in all scenarios.

In the case of the IncN plasmid scenarios, the positive effect becomes more clearly. The RGI value increases from $10^{4.8}$ CFU/g in sterile soil to $10^{5.5}$ CFU/g in the rhizosphere scenario. In the case of the IncW3-bearing strain, the RGI values show a rise from $10^{3.5}$ CFU/g to 10^7 CFU/g, so that the positive impact of the rhizosphere cannot be dismissed.

Table 5.5: Relative population growth values [log CFU/g] in homogenized and rhizospheral soil in comparison as calculated with equation 5.1.

	IncP1	**IncN**	**IncW3**
sterile soil	7	4.8	3.5
non-sterile soil	7.5	4	3.2
rhizosphere soil	7.8	5.5	7

The rhizospheral influence on the obtained numbers of transconjugants and therefore on the plasmid transfer is partially commented in section 5.4. It would be

interesting to compare transconjugant densities obtained from scenarios with homogenized and structured soil inoculated with cells at about identical densities, because under these conditions it might be possible that transconjugant densities in rhizospheral soil exceed those in homogenized soil by far.

Scenarios without nutrient amendment

Representative for scenarios, in which donor and recipient populations displayed only decreasing cell densities, the IncP1-scenario in rhizospheral soil without nutrient amendment is shortly valuated. Cell decomposition rates have been estimated using equation 4.15d. Resulting values are presented in the caption of figure 5.5. No nutrient flow through the system has been considered, so the plasmid transfer rate has been estimated from population densities at the third day of sampling.

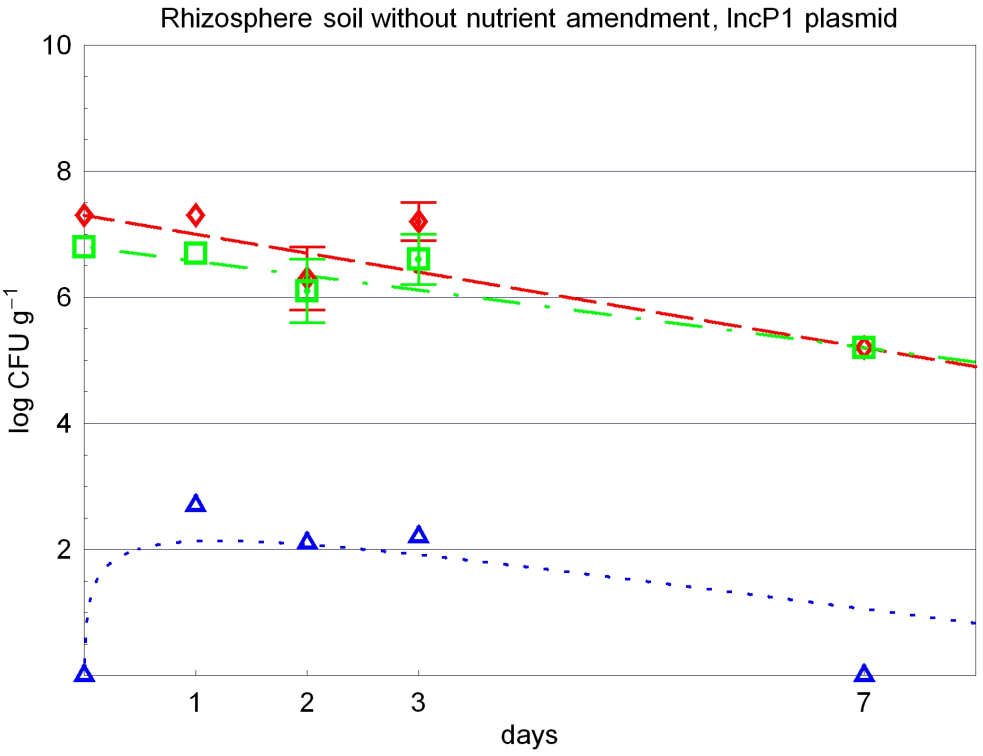


Figure 5.5: Data points and simulations for rhizosphere soil, IncP1 plasmid, without nutrient amendment. Labelling of data and simulation results as in formerly figures. The death rates for donor and recipient population have been estimated to 0.69 and 0.52, rsp. The plasmid transfer rate determined by the ratio between transconjugant cells and the product of donor and recipient cells at the third day of sampling was estimated to $2.5 \cdot 10^{-12}$.

The continuously declining cell densities of donor and recipient cells are met

well by the simulations performed under the assumption of a simple exponential decay. The comparison between transconjugant densities from experimental data and simulation results suggest, that the estimation of plasmid transfer rates by equation 4.25 is an appropriate tool, since the transconjugant's order of magnitude is met by the simulation results. This is remarkable, because this method seems to be rather simple compared to equation 4.24. Equation 4.25 obtains the advantage, that it can be applied even in the case of declining cell densities. It is not only a measure for plasmid fertility, as has been suggested by Sudarshana and Knudsen (1995), but can additionally be used for parameter estimation of a simple plasmid transfer model.

5.3 Nourseothricin-spiked soil

The impact of antibiotic selective pressure on plasmid transfer rates was in the focus of the third basic experimental set-up. Nourseothricin was added to non-sterile, nutrient amended, homogenized soil, resulting in concentrations of 100, 750 and 2500 μg of the antibiotic per gram of soil (μg/g). For the purpose of comparison, an additional scenario without antibiotic load has been investigated.

Nourseothricin is a mixture of different streptothricines. No rate constants such as e.g. K_{OC} are known, so an evaluation of the chemodynamical behavior of this antibiotic in soil is not possible, apart from the qualitative results obtained from the agar diffusion tests. These tests showed, that the effectiveness of the antibiotic declined in soil possibly due to adsorption (cf. section 3.2), so that effective concentrations of nourseothricin in soil have been lower than the denoted concentrations.

All set-ups have been sampled at any of the first three days and additionally at the eighth day. In all scenarios, the development of cell densities follows a typical batch dynamic achieving maximal values at the first day after inoculation. After the maximum was achieved, the cell densities showed a monotonous decline. The time course of all experiments looks very similar. Parameters for the single scenarios could be estimated without complications. Resulting parameter values are presented in table 5.6 on page 62. Experimental values reflect that the samples have been taken from homogenized soil, since the corresponding deviations of data points from their respective mean values are rather low. So, data points obtained from these scenarios offer ideal conditions for the evaluation of the influence of antibiotic pressure on plasmid transfer in soil.

Pertaining to the donor and recipient cells in the IncP1 scenario, simulation results hit data points well (cf. figure 5.6). The difference between the cell densities of donor and recipient cells is small, at the eighth day the recipients even outnumber the donor cells. This situation could not be observed in any scenario before. With increasing antibiotic concentration, the donor cell's densities rise and show at 2500 μg g^{-1} values above the recipients concentration at any day after the first. The selective pressure of the antibiotic can clearly be observed. The donor strains carry resistance genes and are not affected by the antibiotic, since the resistance is maintained as modification of the antibiotic by acetylation. The resistance to nourseothricin is achieved by the donor cells with small effort only.

In contrast, the recipient's cell densities decline and reach a value below 10^4 CFU/g on the eighth day with the highest nourseothricin concentration . Transconjugant cell densities are simulated well on the first day after inoculation in all four IncP1 scenarios. Simulation results are close to the measured cell densities also on the second and third day of the experiment. In all scenarios the simulation results overestimate the data points in the later stage of the experiments by a factor between 10 and 100. These experimentally observed transconjugant densities of about zero

after eight days are remarkable, because in rhizosphere soil the comparable densities have been above 10^4 CFU/g. The overestimation by the simulations should not be overvalued regarding the uncertainty of countings of low cell numbers.

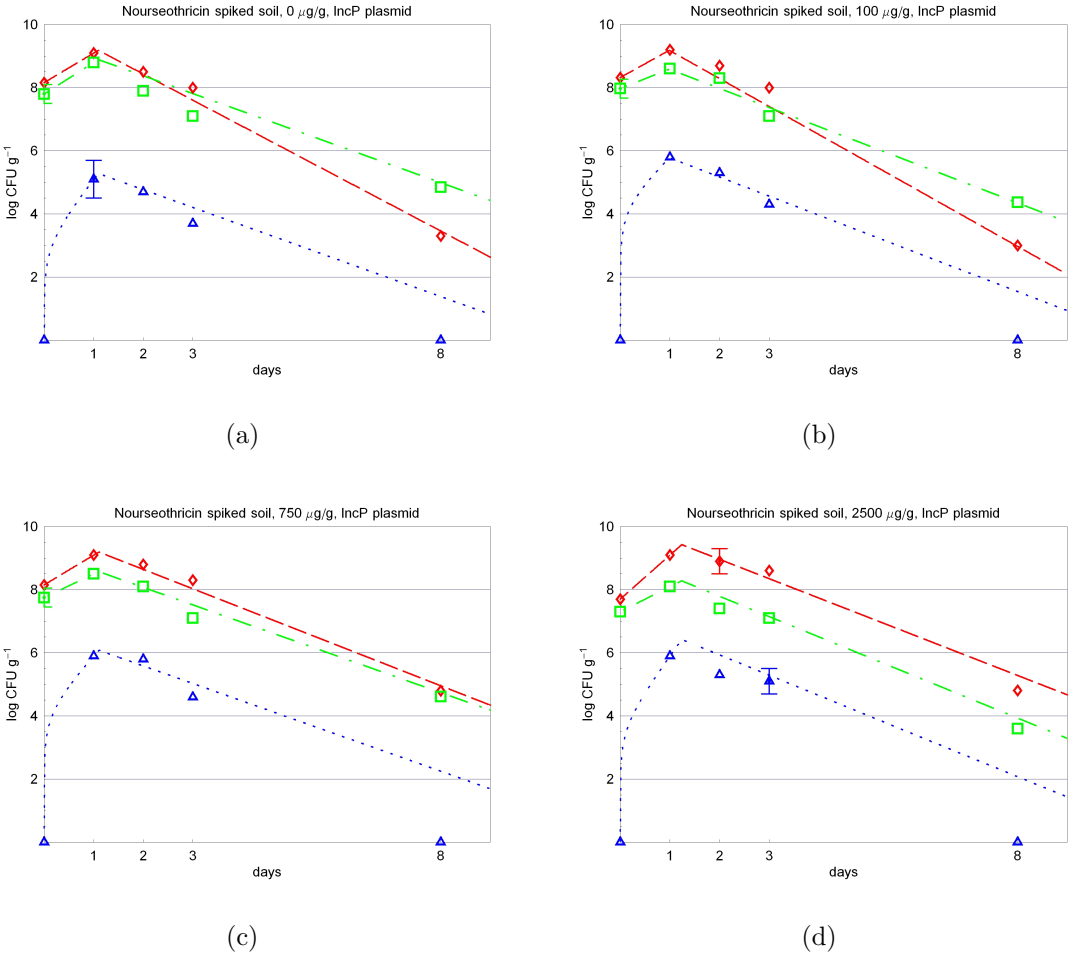


Figure 5.6: Data points and simulations for nutrient amended non-sterile soil, IncP1 plasmid. Panels a) to d) depict rising concentrations of nourseothricin of 0, 100, 750, and 2500 μg g^{-1} soil, respectively. Measured values for donor, recipient and transconjugant cells are marked by diamonds ($\diamond$), boxes ($\square$), and triangles ($\triangle$), respectively. Simulation results for donor, recipient, and transconjugant densities are displayed with dashed, dot-dashed, and dotted lines, respectively. Error bars are shown, if the experimental values deviate from their mean value more than 0.2 [log CFU/g]. For parameter values, see table 5.6.

The situation is nearly identical in the IncN scenario. Even the transconjugant density at the first day of sampling approximately equals the one observed in the IncP1 scenario (cf. figure 5.7). So, the hypothesis given with the IncP1 scenario is corroborated: with increasing antibiotic concentration, the fitness of resistant donor cells relative to the sensitive recipient cells increases due to the benefit from their plasmidic genes.

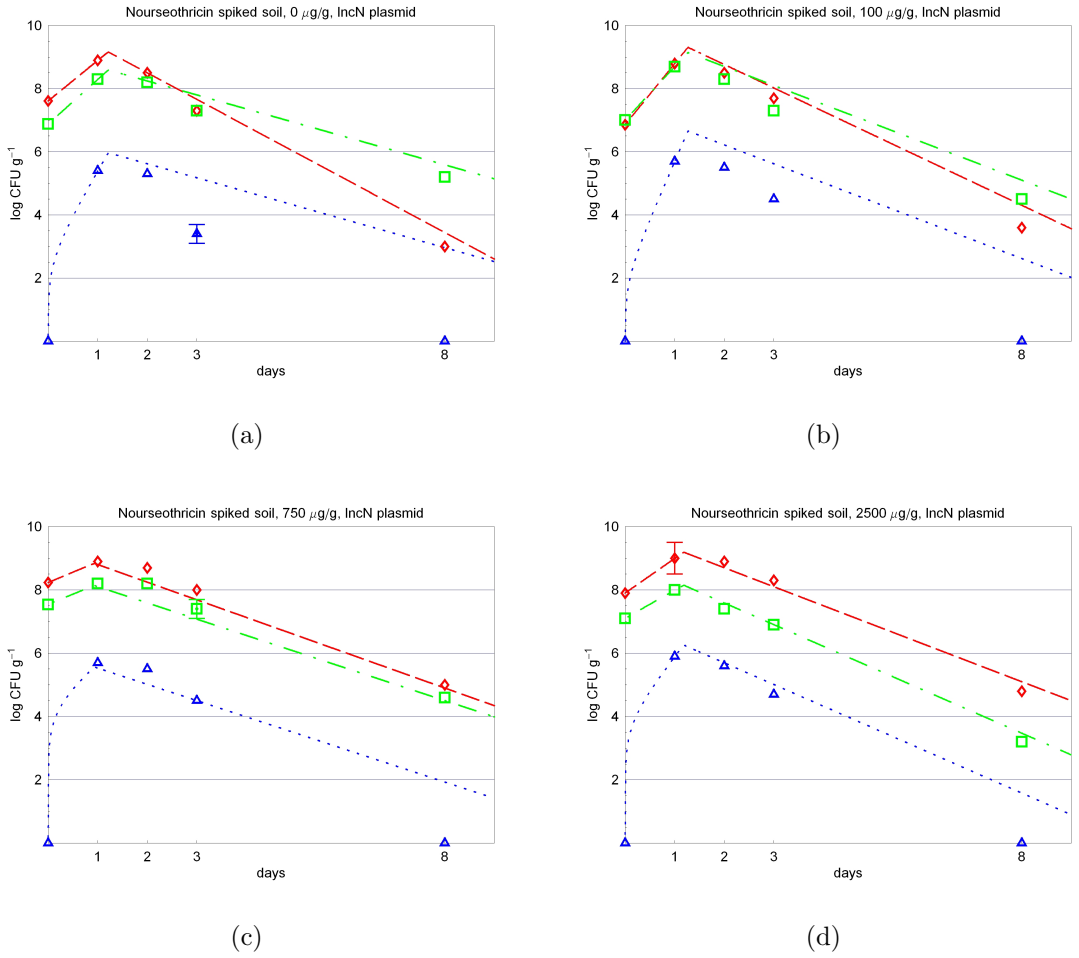


Figure 5.7: Data points and simulations for nutrient amended non-sterile soil, IncN1 plasmid. Panels a) to d) depict rising concentrations of nourseothricin of 0, 100, 750, and 2500 μg g^{-1} soil, respectively. Measured values for donor, recipient and transconjugant cells are marked by diamonds ($\diamond$), boxes ($\square$), and triangles ($\triangle$), respectively. Simulation results for donor, recipient, and transconjugant densities are displayed with dashed, dot-dashed, and dotted lines, respectively. Error bars are shown, if the experimental values deviate from their mean value more than 0.2 [log CFU/g]. For parameter values, see table 5.6.

In contrast, the IncW3 plasmid seems to be handicapped in transferring to or maintaining itself within the hosts (cf. figure 5.8), since the absolute densities of transconjugants are about one hundred times lower than those observed in the IncP1 and IncN scenarios. This is the fact in spite of the maximal cell densities of donor and recipient populations in the IncW3 scenarios achieving values similar to the ones in the two other scenarios. Thus, the lower transconjugant counts cannot be caused by a lack of the facility for transfer.

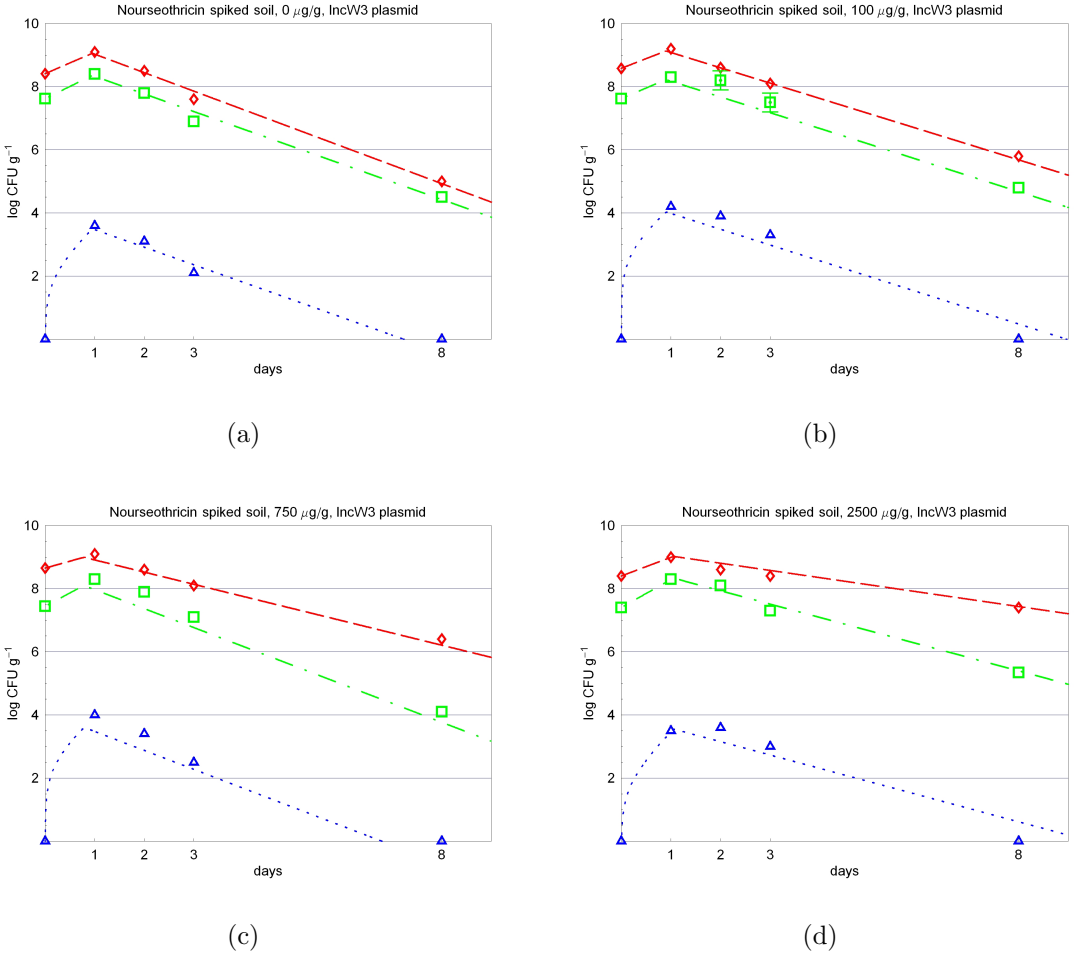


Figure 5.8: Data points and simulations for nutrient amended non-sterile soil, IncW3 plasmid. Panels a) to d) depict rising concentrations of nourseothricin of 0, 100, 750, and 2500 μg g^{-1} soil, respectively. Labeling of data and simulation results as in figures 5.6 and 5.7. For parameter values, see table 5.6.

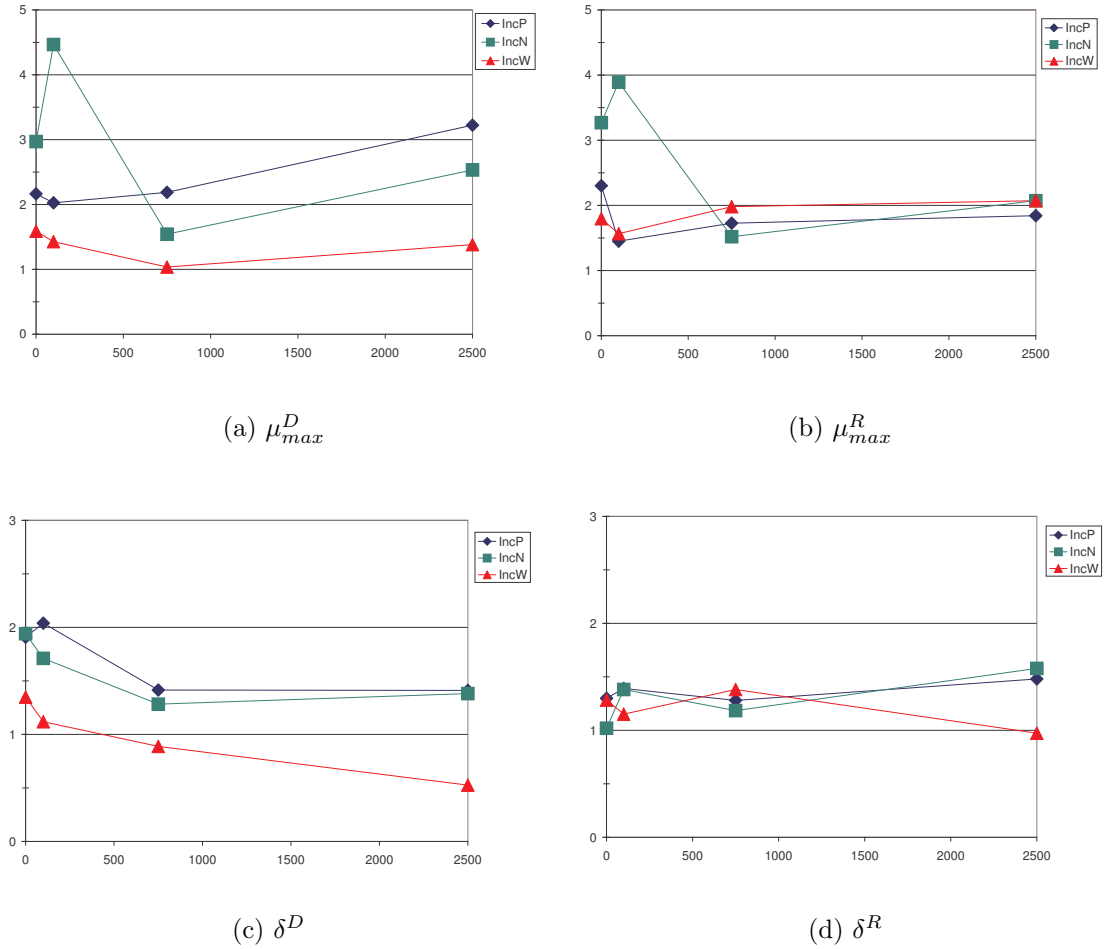
Table 5.6: Estimated parameter values for all scenarios with nourseothricin-spiked soil.

		μ^D [d^{-1}]	δ^D [d^{-1}]	μ^R [d^{-1}]	δ^R [d^{-1}]	γ [g CFU^{-1} d^{-1}]	N_0 [μg g^{-1}]
IncP1 [a]	0	2.16	2.30	1.91	1.30	$3.8 \cdot 10^{-13}$	1860
	100	2.03	1.45	2.04	1.39	$2.4 \cdot 10^{-12}$	1616
	750	2.19	1.73	1.41	1.28	$5.0 \cdot 10^{-12}$	1448
	2500	3.22	1.84	1.41	1.48	$1.7 \cdot 10^{-11}$	2047
IncN [a]	0	2.97	3.27	1.94	1.30	$4.9 \cdot 10^{-12}$	1433
	100	4.47	2.89	1.71	1.39	$7.1 \cdot 10^{-12}$	2349
	750	1.54	1.52	1.28	1.18	$7.8 \cdot 10^{-12}$	576
	2500	2.53	2.07	1.38	1.48	$2.2 \cdot 10^{-11}$	1257
IncW3 [a]	0	1.59	1.80	1.35	1.28	$2.5 \cdot 10^{-14}$	984
	100	1.43	1.57	1.12	1.15	$9.4 \cdot 10^{-14}$	983
	750	1.04	1.98	0.89	1.38	$6.1 \cdot 10^{-14}$	591
	2500	1.38	2.07	0.53	0.97	$2.9 \cdot 10^{-14}$	698

[a]The numbers in the second column denote the nourseothricin concentrations in soil [μg g^{-1}].

An attempt to gain insight into more detailed processes within the *E. coli* cells under increasing selective pressure can be made by investigating the growth and death rates of donor and recipient cells. Figure 5.9 depicts the dependence of both donor and recipient death rates on the antibiotic concentration. First the values of donor and recipient growth rates in the IncN-scenarios spiked with zero and 100 μg g^{-1} are outstanding. Some disturbances in these scenarios are likely to have happened, since the growth rates obtained from all other scenarios give a more consistent picture. The nutrient amendments of these two IncN scenarios could have been greater than those of the other scenarios, because only the growth rates seem to be concerned.

Apart from these shown four values, slight trends in the dependency of the growth and death rates of donor and recipient cells on the antibiotic concentration can be recognized. The growth rates of the donor population increase with rising antibiotic concentration by about 1 d^{-1} in the IncP1 scenario, and by a value of about 0.5 d^{-1} in the IncW3 scenario, respectively (cf. fig. 5.9(a)). Except for the first two IncN-values of recipient growth rates, other values for the maximal growth remain approximately slight below 2 d^{-1} (cf. fig. 5.9(b)). The death rates of donor cells decrease by about 0.5 d^{-1} in the IncP1 and IncN scenario, and by 0.8 d^{-1} in the IncW3, respectively (cf. fig. 5.9(c)). Finally, the death rates of recipient cells increase in the case of the IncP1 and IncN plasmid slightly by values of 0.3 d^{-1}, whereas the death rates in the IncW3 scenarios show a nonuniform course, totaling decrease by a value of 0.3 d^{-1} (cf. fig. 5.9(d)).



(a) μ^D_{max} (b) μ^R_{max}

(c) δ^D (d) δ^R

Figure 5.9: Maximum growth and death rates of donor and recipient cells with different plasmids under antibiotic pressure. Unit of x-axis is in all figures μg nourseothricin g^{-1}, unit of y-axis is d^{-1}. Panels illustrate the dependence of a) the donor's growth rate, b) the recipient's growth rate, c) the donor's death rate, and d) the recipient's death rate on the antibiotic concentration.

A general evaluation of these observations calls for some simplifications: (*i*) the first two rate values for the IncN plasmid in figures 5.9(a) and 5.9(b) cannot be taken into account. (*ii*) changes occurring between proximate rate values should not be overvalued, it should rather be considered the overall trend in the rate values. Under these preconditions, the development of growth and death rates in dependence on antibiotic concentration can be summarized:

1. The growth rates of the resistant donor strains rise, possibly due to a decreasing competitive pressure.

2. The death rates of the resistant donor strains decrease, for the same reason as in 1.

3. The growth rates of the sensitive recipient strain stay about constant. This could be traced back to the relatively impassiveness of the recipient strain to the antibiotic (cf. figure 3.1) under simultaneously decreasing counts of microbial competitors.

4. The death rates of the sensitive recipient cells stay about constant. This results from an overlay of two different influences: decreasing microbial competition takes effect in form of falling death rates, and the rising antibiotic concentration impacts the death rate positively.

Besides a detailed analysis of growth dynamics within the antibiotic spiked soil, it becomes clear that the applied concentrations of nourseothricin caused no complete inhibition of non-resistant *E. coli* K-12 (CV601). The antibiotic agent led to displacements in the relative abundance of resistant and sensitive strains, but its effective concentrations were not sufficient to enable the resistant donor strain to crowd out the sensitive recipient strain clearly.

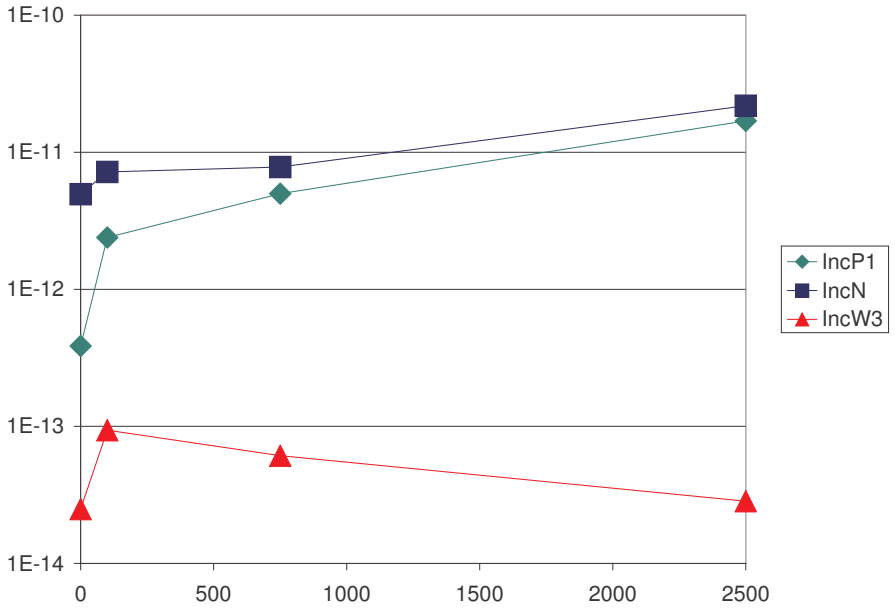


Figure 5.10: Plasmid transfer rates for nutrient amended, non-sterile soil, with antibiotic pressure. Unit of x-axis is in all figures μg nourseothricin g^{-1}, unit of y-axis is g CFU d^{-1}. Curves depict the development of the plasmid transfer rate γ in dependency on the antibiotic concentration for the IncP1, IncN, and IncW3 scenario.

Thus far, the scenario analyses focussed on the evaluation of simulation results and the examination of observed growth dynamics. Since the plasmid transfer rates are one of the most interesting model parameters, the scenarios with increasing selective pressure afford the opportunity to investigate the influence of antibiotic concentrations in soil on transfer rates. Figure 5.10 resumes the plasmid transfer rates observed in antibiotic spiked soil under increasing selective pressure. A clear difference between the IncP1 and IncN scenarios on the one hand and the IncW3 scenario on the other hand becomes obvious. While the IncW3 plasmid transfer rate increases from an antibiotic concentration of 0 to 100 μg g^{-1}, to decrease in the following, the transfer rates or the IncP1 and IncN plasmids show a monotonous rise. Here, the type of dependency on the antibiotic pressure reminds of a saturation dynamic. The IncN plasmid transfer rates are slightly higher than the IncP1 ones.

Subsuming the evaluation of nourseothricin influence on plasmid transfer, the main results are (*i*) growth of resistant donor cells is affected positively by rising nourseothricin concentrations, (*ii*) growth of sensitive recipient cells is more or less unaffected by the antibiotic, and (*iii*) this and the observed moderate enhancement of plasmid transfer rates in the case of the IncP1 and the IncN scenarios leads to the conclusion, that the impact of nourseothricin concentration on plasmid transfer is not so high as expected and is mediated mainly by the reduction of microbial competitors.

5.4 Review of plasmid transfer and growth

In the former sections, the results of single experimental set-ups have been analyzed without comparing the different results among each other. Now, the comparison between results from different set-ups is supposed to give a review about relevant characteristics of all experiments. Under consideration of plasmid transfer dynamics, the plasmid transfer rates (eq. 4.24), the nutrient turnover values (eq. 4.22), and the relative growth values (eq. 5.1) are important indicator values.

Figure 5.11 shows transfer rates already obtained from the basic set-ups with sterile, non-sterile, and rhizosphere soil. Within those three basic set-ups, transfer rates increase from the IncW3 plasmid over the IncN to the IncP1 plasmid. Microbial competition within the non-sterile soil impacts the transfer rates of all three plasmids positively. This can be seen from the comparison of the values from sterile and non-sterile soil among each other.

The highest plasmid transfer rates can be observed in rhizospheral soil. These values are for all plasmids about two to three orders of magnitude above those from non-sterile and sterile soil, respectively. Thus, the rhizosphere positively affects plasmid transfer. This can be traced back to stimulation of microbial growth by root exsudates and in particular to the function of rhizoplane as contact area for *E.*

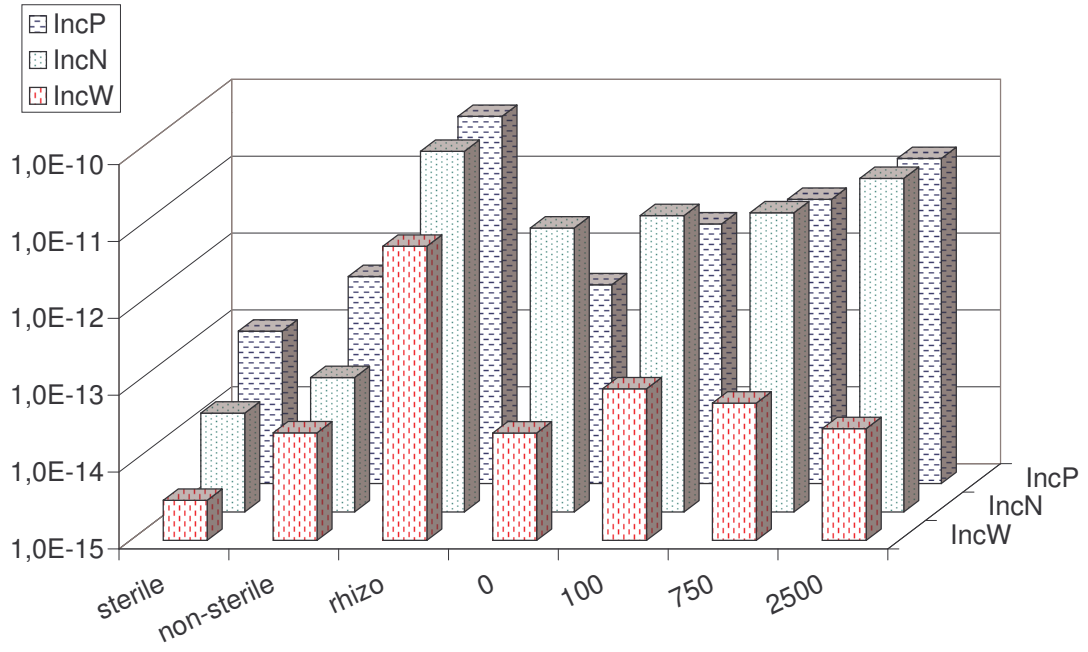


Figure 5.11: Plasmid transfer rates for all scenarios. Values on the logarithmic y-axis numeralize the transfer rates calculated with eq. 4.24, while the three plasmid types are plotted on the z-axis. The x-axis refers to the seven scenarios with nutrient amended sterile, non-sterile, and rhizospheral soil, and accordingly to nutrient amended, non-sterile soil spiked with nourseothricin resulting in concentrations of 0, 100, 750, and 2500 μg nourseothricin g^{-1}.

coli (and other) cells.

Within figure 5.11, the two scenarios denoted as "non-sterile" and "0" are identically with respect to the experimental set up. This allows for a check of the transfer rates calculated for the IncN plasmid in the scenarios denoted as "0" and "100" (see figure 5.9). For the IncP1 and the IncW3 plasmid the transfer rates in the "non-sterile" and the "0" scenario are very similar, while the respective values for the IncN plasmid differ by a factor of about one hundred. Thus, results for the IncN scenario with antibiotic pressure at amounts of zero and one hundred are suspected to be subject to unidentified changes in experimental conditions.

Though, the most important conclusion is that plasmid transfer rates within the rhizospheral soil are higher than in all other scenarios including the experiments under selective antibiotic pressure.

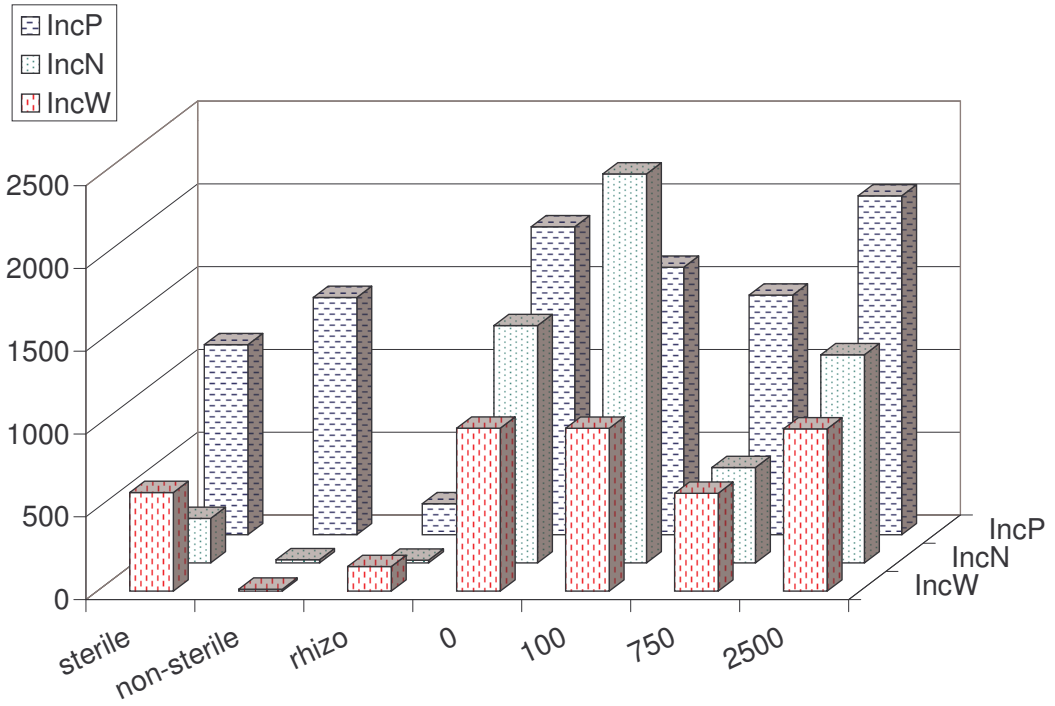


Figure 5.12: Nutrient turnover values for all scenarios. Values on the logarithmic y-axis numeralize the nutrient turnover values calculated with eq. 4.22, while the three plasmid types are plotted on the z-axis. The x-axis refers to the seven scenarios with nutrient amended sterile, non-sterile, and rhizospheral soil, and accordingly to nutrient amended, non-sterile soil spiked with nourseothricin resulting in concentrations of 0, 100, 750, and 2500 μg g^{-1} nourseothricin.

Regarding nutrient turnover and relative growth rates, the main question is, if the results obtained from plasmid transfer dynamics can be confirmed. At first, the difference between the nutrient turnover and the relative growth values is outstanding. An inconsistent picture is obtained from the nutrient turnover values (see fig. 5.12). Primarily, the rhizosphere-scenarios appear underestimated, but also within the values calculated from antibiotic spiked soil no trend can be detected. Thus, the nutrient turnover values are inappropriate for drawing conclusions with respect to growth and transfer dynamics. It is obvious, that the differences in the initial densities of donor and recipient cells are responsible for this.

In contrast, the relative growth rates (see fig. 5.13) have been determined by relating maximal cell densities to the initial densities. These values display a clear reference to the plasmid transfer rates. In particular three conclusions arise from the obtained relative growth values under consideration of the results of plasmid transfer rate analysis: first, the two mentioned IncN scenarios strike here as well. Second,

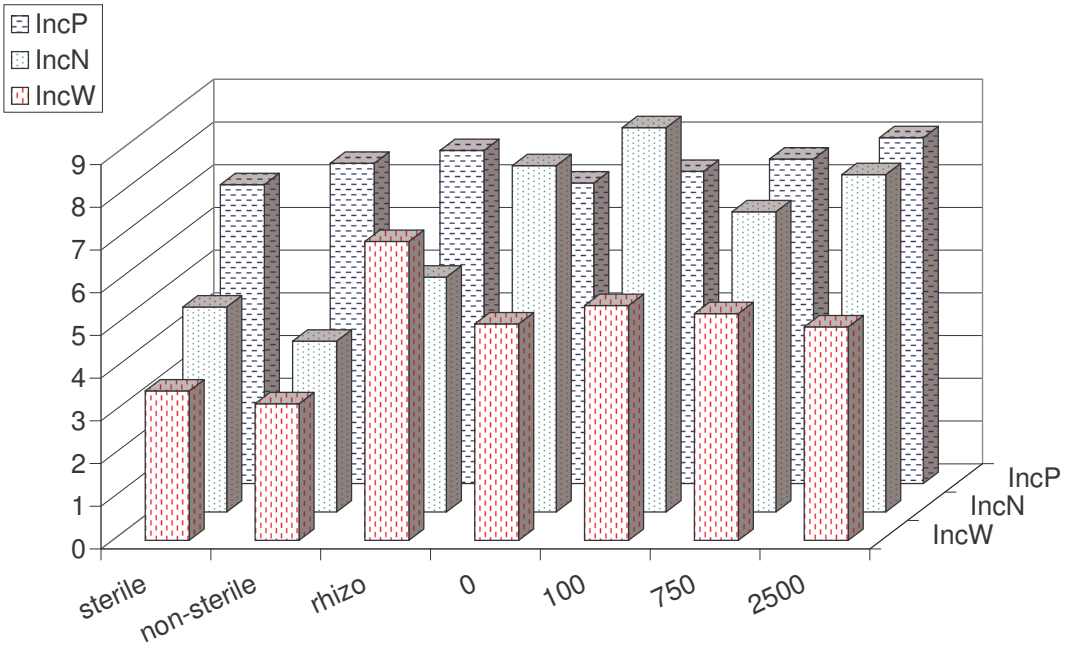


Figure 5.13: Relative growth values for all scenarios. Values on the logarithmic y-axis numeralize the relative growth values calculated with eq. 5.1, while the three plasmid types are plotted on the z-axis. The x-axis refers to the seven scenarios with nutrient amended sterile, non-sterile, and rhizospheral soil, and accordingly to nutrient amended, non-sterile soil spiked with nourseothricin resulting in concentrations of 0, 100, 750, and 2500 μg g^{-1} nourseothricin.

comparing the values of the IncN and IncW3 plasmids detected in sterile and non-sterile soil, the relative growth values in the non-sterile soil are lower than those in the sterile soil. This suggests, that the increase observed in the transfer rates from sterile to non-sterile soil are not caused by a stronger growth of cells, but rather by a direct stimulation of plasmid transfer in presence of microbial competitors. Third, the relative growth values in the rhizosphere scenarios do not reflect the outstanding plasmid transfer rates in these scenarios, since the relative growth values do not appear much higher than the other values. Thus, root exsudates or any nutrient-related influences are not likely to be responsible for the higher transfer rates in rhizospheral soil. Rather the function of roots as contact area for the bacterial cells can be supposed as a factor for plasmid transfer stimulation.

Chapter 6

Summary and outlook

A mathematical model was developed within the scope of this diplom thesis which describes the horizontal transfer of antibiotic drug resistance genes between *E. coli*-strains. This model covers growth and transfer dynamics within batch experiments. The model was modified to enable its application to experiments, which show no batch dynamic, but a simple exponential decay of cells. Methods for parameter estimation from experimental data were derived from the model equations for growth parameters and nutrient turnover. For the estimation of the plasmid transfer rate, the end-point method of Simonsen *et al.* (1990) has been applied.

The developed model is not completely new, since the coupling of nutrient limited growth dynamics and a mass-action transfer dynamic has been performed previously by several authors. The progression of the model developed in this thesis consists in the inclusion of additional processes, governing the decline of cellular densities after nutrient depletion. So, not only the maximum densities of transconjugant cells and thus the fertility of the plasmid can be investigated, but it is also possible to compare the plasmids according to their behavior in decreasing microbial populations.

Model and estimation methods were used to simulate the time course of those experiments performed with IncP1, IncN, and IncW3 plasmids. The influence of microbial competition and rhizosphere could be evaluated by the comparison of experiments from sterile, non-sterile, and rhizospheral soil. The effect of nourseothricin on microbial growth has been examined more precisely by the inspection of cellular growth and death rates of donor and recipient cells. A reduction of microbial competitors in parallel to an increasing selective pressure led to increasing densities of the resistant donor cells for all plasmids. The transfer rates react slightly on an increasing antibiotic pressure in soil. This reaction can be traced back to better growth dynamics of plasmid bearing cells.

The time course of transconjugant populations has been simulated under the assumption, that transconjugant cells are subject to the same growth and death processes as the recipient cells. That means, bearing a plasmid has neither beneficial

nor adverse effects on cell growth in the model. Under this assumption the simulated dimension of transconjugants agrees well with data points. The inclusion of processes affecting growth of transconjugants or plasmid transfer like segregation, transitory derepression, or the consideration of a plasmidic burden on the host cell is not necessary. On the other hand, the data points had not enabled a more detailed analysis of the mentioned influence factors, if some of these would be included into the model.

Comparing plasmid transfer rates for all 21 scenarios, one of the most impressive results is that transfer rates within the rhizospheral soil exceed all others, even those for the scenarios with the highest antibiotic load. R. Pukall observed the highest plasmid transfer rates in rhizospheral soil as well (Pukall (1996), p. 80). However, he used the ratio between transconjugant and recipient cells to measure the transfer frequencies. This measure depends on the absolute numbers of recipients. Since the recipient densities differed strongly between the single scenarios, he was not shure if the observed high transfer frequencies can be traced back to the influence of rhizosphere. By application of the end-point method of Simonsen, a measure independent of absolute recipient numbers has been applied in this thesis, so that the positive influence of plant roots on transfer frequency can be proved. The importance of plant roots to plasmid transfer seems to be immense.

From the comparation between plasmid transfer rates and relative growth index, one can suspect that the nutrient influence exerted from the root exsudates are not the most significant reason for the high plasmid transfer rates in rhizospheral soil. A more detailed, spatial analysis of the microbial community in the surrounding of roots could elucidate, which factors are responsible. Since roots provide a large surface area by their rhizoplane, the high transfer rates in rhizospheral soil might be attributed to the function of plant roots as contact plane for bacterial cells rather than to root exsudates.

Another result of this thesis is the adequacy of the relation between transconjugant density and the product of donor and recipient densities for estimating the plasmid transfer rate. Usage of this relation as a measure for transfer frequency was already supposed by Sudarshana and Knudsen (1995), but they have not used this measure for differntial equations model parametrization. Results presented in figure 5.5 show, that the mentioned relation estimates the plasmid transfer rate well. This enables in particular model parametrization in situations without growth of cells. Keeping in mind, that the dynamics of microbial cells in soil are not likely to match a typical batch dynamic, the parametrization of models regarding long-term dynamics in soil is enabled with this the measure proposed by Sudarshana and Knudsen (1995).

Plasmid assessment

An assessment of the three investigated plasmids raises the question, if there exists a connection between high transfer rates and good host growth. The IncW3-plasmid showed the lowest transfer rates and, in virtually all scenarios, the lowest relative growth values, while both the IncP1 and the IncN plasmids displayed high transfer rates and high relative growth values (see fig. 5.11 and 5.13). In addition, the IncW3 plasmid failed to take advantage of the selective pressure obtained from antibiotic charge of the soil, in spite of bearing the identical antibiotic resistance gene (transposon Tn 1826). Plasmid replication, plasmid copy number control or the regulation of conjugation and pilus synthesis appears to be regulated resulting in a lower metabolic burden for the *E.coli* cells by the IncP1 and the IncN plasmids. If a connection between effective transfer and high parental growth exists, this would mean a mutual amplification of transfer events and parental growth in the case of "successful" plasmids.

The IncP1 plasmid has been observed to transfer even in the case of declining donor and recipient populations. From Turner (2004), it can be stated that the IncP1 plasmid displayed an infectious transfer, since it optimizes its horizontal mode of transmission regardless of the host's physiological state. Plasmids performing an infectious transmission are of outstanding interest in risk assessment of antibiotic resistance gene transfer. Here, it must be noted that the model 4.12 is only feasible for the description of transfer taking place in growing colonies or colonies showing a simple exponential decay. This holds as well for other models using logistic growth dynamics (Clewlow *et al.*, 1990) or nutrient limited exponential growth (Stewart and Levin, 1977; Simonsen *et al.*, 1990). These models can only be used for the description of short-term dynamics, spanning over a period of a couple of days. The simulation and evaluation of long-term plasmid transfer dynamics is not possible with the proposed and other models. There is the need for extended models regarding trade-off between horizontal and vertical modes of plasmid transmission and as well consideration of the activity state of cells in soil. An integration of plasmids as separate state variables seems to be reasonable, similar to the consideration of more detailed nutrient turnover processes as it has been described by Blagodatsky and Richter (1998).

Evaluation of model simplifications

Two central model assumptions should critically be evaluated: first, the model's homogeneity, and second, the constriction of plasmid copy number in the model to one plasmid per cell. To deal with the model's homogeneity, one can think about the consequences of choosing a spatial heterogeneous model. Possible approaches for explicit inclusion of space into a plasmid transfer model are the application of partial differential equations, cellular automata or models assuming a radial colony

growth. Regardless which method is applied, it would be necessary to determine values for the velocity of spatial spread not only of plasmids or resistance genes, but also of donor, recipient and transconjugant populations. If these values are guessed instead of being experimentally determined, the value of the model and of the simulations declines because simulation results become arbitrary in this case. On the other hand, when experimentally based values for the parametrization of spatial spread are used, laborious experimental work has to be done.

Lagido *et al.* (2003) developed a model considering radial colony growth. They carried out experiments to determine values for the maximum specific growth rate, the maximum radial growth rate and the maximum cell yield. Their model is based on the assumption, that all cells in a colony of recipient cells become transconjugant cells as soon as colonies of donor and recipient cells come into contact. This assumption is at least as strong as considering spatial homogeneity, so that simulations of plasmid transfer using this model are restricted as well.

The data obtained from the microcosm experiments do not provide information about the explicit spatial development of plasmid transfer. Under these conditions, it is helpful that a spatial modeling approach is necessary only if the numbers of donor and recipient cells show great differences. This has been elucidated by Simonsen (1990) and Lagido *et al.* (2003). Since the inoculation densities within Pukall's experiments were always about the same for donor and recipient cells, the necessity for spatial modeling is not given here. The results of this thesis show, that in the special case of equal initial densities of donor and recipient cells horizontal transfer of plasmids in soil can be described appropriately by the application of a homogeneous model.

The second central model assumption deals with the plasmid copy number. The copy number can determine the resistance level of a cell. This holds for the resistance to e.g. β-lactam antibiotics, tetracycline, and spectinomycin (Schumann, 1989, p. 15). In these cases, a linear correlation between the number of plasmids per cell and the level of resistance can be noted. In the case of nourseothricin resistance, such a correlation is not yet known. Additionally, apart from the correlation between copy number and resistance level, the transconjugant cells in the underlying experiments have been selected by their phenotypical resistance by plating on a nourseothricin-containing medium. The plasmid number per cell has not been determined within the experiments. So, a differentiation between different plasmid counts per cells is difficult.

The consideration that a transconjugant cell contains maximal one plasmid can be modified to the assumption, that a transconjugant cell always contains the average plasmid number. A discrimination of copy numbers ought to be regarded considering e.g. segregational processes, but within the objectives of this thesis, it is more reasonable to leave the plasmid number per cell at a constant value. The good agreement between simulated transconjugant densities and data points confirms,

that it is not necessary to include a variable plasmid copy number into the proposed model under consideration of the objectives of this thesis.

With the model developed in this diplom thesis, it was possible to describe the plasmid transfer of *E. coli* in the analyzed experiments with reasonable agreement between simulations and measurements. However, extrapolation to natural habitats and prognosis of transconjugant number development with a mathematical model are not possible to date. Such a model had to take into account a lot of different factors as nutrient concentration in the surrounding, the relevant aspects of the considered plasmid, and the interactions between the plasmid and the host cells, where information is not known in sufficient detail. If modelers and microbiologists work hand in hand in the development of models and the design and performance of experiments, their work can fertilize each other in order to improve the knowledge about critical combinations of resistance genes and environmental influence factors.

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Appendix A

Mathematica code

In this section, `mathematica` code used for the numerical solution of the model and the visualization of the solutions and data points is presented. The code is structured as a block expecting three lists and two integer values as parameters. The lists should be the logarithmic values of donor, recipient and transconjugant cells. The parameter `tmax` represents the index of that day, the maximum cell densities can be observed. The parameter `tend` should be the number of data points within the lists, text is used for plot labeling.

```
PEANS[logD_, logR_, logT_, tmax_, tend_, text_] := Block[{},
  t0 = 1;
  tEnd = logD[[tend]][[1]][[1]] + 1;
  gd = 1;
  δD = -N[(Log[10^logD[[tend]][[1]][[2]]] - Log[10^logD[[tmax]][[1]][[2]]])/
      (logD[[tend]][[1]][[1]] - logD[[tmax]][[1]][[1]])];
  δR = -N[(Log[10^logR[[tend]][[1]][[2]]] - Log[10^logR[[tmax]][[1]][[2]]])/
      (logR[[tend]][[1]][[1]] - logR[[tmax]][[1]][[1]])];

  μD = N[(Log[10^logD[[tmax]][[1]][[2]]] - Log[10^logD[[t0]][[1]][[2]]])/
      (logD[[tmax]][[1]][[1]] - logD[[t0]][[1]][[1]])] + δD;
  μR = N[(Log[10^logR[[tmax]][[1]][[2]]] - Log[10^logR[[t0]][[1]][[2]]])/
      (logR[[tmax]][[1]][[1]] - logR[[t0]][[1]][[1]])] + δR;
  μT = μR;
  δT = δR;
  e = 0.0000005;
  HN = 4;
  N0 = ((μD - δD) (10^logD[[tmax]][[1]][[2]] - 10^logD[[t0]][[1]][[2]])
      + (μR - δR) (10^logR[[tmax]][[1]][[2]] - 10^logR[[t0]][[1]][[2]])
      + (μT - δT) * 10^logT[[tmax]][[1]][[2]]) * e;
  Cep = 10^logT[[t0 + gd]][[1]][[2]] + 10^logR[[t0 + gd]][[1]][[2]] + 10^logD[[t0 + gd]][[1]][[2]];
  Cep0 = 10^logD[[t0]][[1]][[2]] + 10^logR[[t0]][[1]][[2]];
  γ = (μD - δD) * Log[1 + (10^logT[[t0 + gd]][[1]][[2]])/(10^logR[[t0 + gd]][[1]][[2]]) *
      Cep/(10^logD[[t0 + gd]][[1]][[2]])] * 1/(Cep - Cep0);
```

...continued next page.

The block performs a parameter estimation routine (previous page) and calculates the numerical solution of the four differential equations. Simulation curves and data points are visualized at the end of the block (this page).

```
Print["Parameter values:"];
Print[TableForm[Transpose[{{"μD", μD - δD}, {"δD", δD}, {"μR", μR - δR}, {"δR", δR}, {"N0", N0},
                          {"γ", γ}}]]];
sol = NDSolve[{
   DON'[t] == (μD * NUT[t]/(NUT[t] + HN) - δD) * DON[t],
   REC'[t] == (μR * NUT[t]/(NUT[t] + HN) - δR) * REC[t] - γ * (DON[t] + TRA[t]) * REC[t] * NUT[t]/(NUT[t] + HN),
   TRA'[t] == (μT * NUT[t]/(NUT[t] + HN) - δT) * TRA[t] + γ * (DON[t] + TRA[t]) * REC[t] * NUT[t]/(NUT[t] + HN),
   NUT'[t] == -e * (μD * DON[t] + μR * REC[t] + μT * TRA[t]) NUT[t]/(NUT[t] + HN),
   DON[0] == 10^logD[[1]][[1]][[2]], REC[0] == 10^logR[[1]][[1]][[2]], TRA[0] == 0, NUT[0] == N0},
  {DON, REC, TRA, NUT}, {t, 0, tEnd}, AccuracyGoal → 10, PrecisionGoal → 10, WorkingPrecision → 15,
  MaxSteps → 10000];
Dsim = Table[{t, Lg[First[Evaluate[DON[t]] /. sol]]}, {t, 0, tEnd, 0.01}];
Rsim = Table[{t, Lg[First[Evaluate[REC[t]] /. sol]]}, {t, 0, tEnd, 0.01}];
Tsim = Table[{t, Lg[First[Evaluate[TRA[t]] /. sol]]}, {t, 0, tEnd, 0.01}];
$TextStyle = {FontFamily → "Arial", FontSize → 52};
l1 = MultipleListPlot[logD, logR, logT, PlotJoined → False,
  SymbolShape → {PlotSymbol[Diamond, 18, Filled → False], PlotSymbol[Box, 18, Filled → False],
    PlotSymbol[Triangle, 18, Filled → False]},
  SymbolStyle → {{RGBColor[1, 0, 0], Thickness[0.005]}, {RGBColor[0, 1, 0], Thickness[0.005]},
    {RGBColor[0, 0, 1], Thickness[0.005]}},
  ErrorBarFunction → {mybarfunc1, mybarfunc2, mybarfunc3}, DisplayFunction → Identity];
l2 = MultipleListPlot[Dsim, Rsim, Tsim, PlotJoined → True, SymbolShape → {None},
  Ticks -> {{0, 1, 2, 3}, Automatic},
  PlotStyle → {{RGBColor[1, 0, 0], Dashing[{Dash}], Thickness[0.0035]},
               {RGBColor[0, 1, 0], Dashing[{Dash, Dot}], Thickness[0.0035]},
               {RGBColor[0, 0, 1], Dashing[{Dot}], Thickness[0.0035]}}, DisplayFunction → Identity];
Show[l2, l1, DisplayFunction → $DisplayFunction,
 PlotRange → {{0, logD[[tend]][[1]][[1]] + 1}, {0, 10}},
 GridLines → {{16}, {2, 4, 6, 8, 10}}, AspectRatio → 210 / 297, Frame → True,
 FrameLabel → {"days", "log CFU", text, None},
 FrameTicks -> {{0, 1, 2, 3, 8, 10, 14}, Automatic, None, None}, ImageSize → 2000];
];
```

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