Microbiology and Tumorbiology Center, MTC
Karolinska Institutet
Stockholm, Sweden

Assessing the toxic impact of chemicals using bacteria

Jenny Gabrielson

Stockholm 2004

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-143-1

Allting har sin tid.
Dansa har sin tid…
…arbeta har sin tid…
… älska har sin tid.

Predikaren 3:1-8

Abstract

There is a considerable backlog in the testing of new chemical compounds for their ecotoxic properties, mostly due to the lack of appropriate testing methods. In addition, there is a vivid debate today about the test endpoint for many of the existing methods. This thesis describes the Microbial Assay for Risk Assessment, MARA, which is a new method for ecological risk assessment and toxicity testing. MARA is based on the simultaneous reading of growth inhibition of eleven microbial strains, exposed to a concentration gradient of the tested chemical compound.

The eleven strains have been selected so that different strains exhibit different sensitivities to chemicals. A growth inhibition pattern – the toxic fingerprint – can be thus detected on the microplate in which the test is performed. The toxic fingerprint, rather than the eleven individual growth inhibitory concentrations, is the test result. It can be compared to toxic fingerprints from other tested chemicals in a database and thereby generate more information regarding the type of toxic effect and species specificity of the tested compound than a single-value result would on its own.

The toxic fingerprint for two groups of chemicals, disinfectants and chlorophenols, were compared. It was concluded that MARA can differentiate between different types of toxic effects and also between chemicals with similar molecular structure. MARA is comparable to other bacteria based tests both regarding sensitivity and reproducibility.

The toxic fingerprint is heavily dependent on the selection of the strains on which the assay is based. They must give a diversified answer to large groups of chemicals, and as bacteria have a larger genetic diversity than higher organisms, it would plausibly be advantageous with a high genetic diversity among the strains. It was shown that the strains should preferably belong to different genera, but not necessarily to different phyla, to yield the most differentiated response to different chemicals.

In order to make MARA as user-friendly as possible, a new method for detection of microbial growth/inhibition of growth in microplates using a flat-bed scanner was developed. The results obtained with the scanner were highly correlated to results obtained with a spectrophotometer, the classical device used for instant quantification of microbial growth, when using TTC (triphenyl tetrazolium chloride, tetrazolium red) or MTT (chelating tetrazole) as growth indicators.

Sammanfattning

Det föreligger en avsevärd eftersläpning i arbetet med att testa nya kemikaliers ekotoxiska egenskaper, i huvudsak beroende på bristen på lämpliga testmetoder. Dessutom pågår en livlig debatt om vilken parameter man bör studera i de tester som används. Denna avhandling beskriver ett Mikrobiellt Test för RiskBedömning (MARA), en ny metod för ekologisk riskbedömning. MARA är baserat på mätning av tillväxtinhibering av elva mikrobiella stammar som utsätts för en koncentrationsgradient av ett kemiskt ämne.

De elva stammarna har valts så att de skall uppvisa olika känslighet för olika kemikalier. Ett inhibitionsmönster – det toxiska fingeravtrycket – kan avläsas på mikroplattan som testet utförs i. Det är det toxiska fingeravtrycket snarare än de elva enskilda inhiberingsvärdena som är slutresultatet. Det kan jämföras med toxiska fingeravtryck från andra kemikalier i en databas och således kan ett mer informativt svar erhållas än om resultatet bestått av ett enda värde.

De toxiska fingeravtrycken från två kemikaliegrupper, desinfektionsmedel och klorfenoler, har jämförts. Det konstaterades att MARA kan skilja mellan olika typer av toxiska effekter och även mellan kemikalier med likartade molekylära strukturer. MARA är jämförbart med andra liknande tester m a p såväl känslighet som reproducerbarhet.

Urvalet av stammar i MARA påverkar i högsta grad de toxiska fingeravtrycken. Stammarna måste ha varierande känslighet för olika kemikalier, och emedan bakterier har en större genetisk variation än högre organismer torde en hög genetisk diversitet vara önskvärd. Det konstaterades att stammarna i MARA med fördel bör plockas från olika genus, men inte nödvändigtvis från olika fyla, för att största möjliga känslighetsvariation skall erhållas.

För att göra MARA så användarvänligt som möjligt har en ny metod för mätning av mikrobiell växt/inhibering av växt i mikroplattor m h a en skanner utvecklats. Plattorna skannades och ett speciellt utvecklat datorprogram användes sedan för att mäta växten genom att analysera bilderna. Resultaten som erhölls med skannern visade hög korrelation med resultat som erhållits med en spektrofotometer, vilken är den klassiska metoden för momentan avläsning av mikrobiell tillväxt, om TTC (trifenyltetrazoliumklorid, tetrazoliumrött) eller MTT (kelaterande tetrazol) användes som tillväxtindikatorer.

List of publications

This thesis is based on the following papers, which in the text will be referred to by their roman numerals

Paper I

Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates
Jenny Gabrielson, Mark Hart, Anna Jarelöv, Inger Kühn, Douglas McKenzie, Roland Möllby
Journal of Microbiological Methods, 2002 50:1 63-73

Paper II

A microplate based Microbial Assay for Risk Assessment (MARA) and (eco)toxic fingerprinting of chemicals
Jenny Gabrielson, Inger Kühn, Patricia Colque-Navarro, Mark Hart, Aina Iversen, Douglas McKenzie, Roland Möllby
Analytica Chimica Acta, 485 (2003) 121-130

Paper III

Bacterial diversity related to the toxic impact of chemicals
Jenny Gabrielson, Inger Kühn, Ruth de Karzow, Terry Dando, Roland Möllby
Submitted

Paper IV

Microbial Arrays and Pattern Recognition for analysis of toxicity of chemicals
Inger Kühn, **Jenny Gabrielson**, Ruth de Karzow, Patricia Colque-Navarro, Roland Möllby
Submitted

Abbreviations used in the thesis

ARDRA	Amplified Ribosomal DNA Restriction Analysis
BTB	Bromothymol Blue
DGGE	Denaturating Gradient Gel Electrophoresis
ECVAM	European Centre for Validation of Alternative Methods
FAME	Fatty Acid Methyl Ester
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IC_{50}	the Concentration at which 50% Inhibition is obtained
LC_{50}	the Concentration at which 50% Lethality is obtained
LOEL	Lowest Observed Effect Level
MARA	Microbial Assay for Risk Assessment
MEIC	Multicentre Evaluation of In vitro Cytotoxicity
MIC	Minimal Inhibitory Concentration
MTC	Microbial Toxic Concentration
MTT	3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide
NCIMB	National Collections of Industrial, Food and Marine Bacteria
NOEC	No Observed Effect Concentration
QSAR	Quantitative Structure-Activity Relationships
PLFA	Phospholipid Fatty Acid
RISA	Ribosomal Intergenic Spacer Analysis
SIR	Substrate Induced Respiration
TGGE	Temperature Gradient Gel Electrophoresis
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TTC	Tetrazolium red, 2,3,5-triphenyl tetrazolium chloride

Contents

Foreword

I would like to thank each and everyone who helped me during these years to finish my PhD-work– colleagues, family and friends. To name and express my gratefulness to all of you would require another thesis.

This study has been performed with financial support from the European Union's Fifth Framework Program, Project No. MAS3-CT98-0181, The Swedish National Board for Laboratory Animals, Vinnova (project number 14239-3) and The Swedish Animal Welfare Agency (project number 34-329).

Jenny

Introduction

There is an emerging need for fast, cheap and simple ecotoxicity tests. The number of chemicals used in our society is constantly increasing, and for most chemicals nothing or very little is known about their effects when being discharged into nature. Unanimous reports about damages to the environment due to toxic chemicals are regularly found in the media.

There are a number of (eco)toxicity tests in use today, most of them animal based. A system based on microbes would be an ideal model for use within an assay designed to assess potentially toxic compounds. In contrast to multi-cellular eukaryotic organisms, bacteria have rapid rates of growth and reproduction. Furthermore, there are a large number of individuals within a given population limiting the effects of cell-to-cell variability's, the use of naturally occurring strains has no ethical ramifications, and they interact rapidly with their environment. Bacteria-based tests are currently available, but they tend to utilise a single strain. The correlation of the toxicity of a chemical, as shown by its inhibitory effect upon a single bacterial strain, with overall environmental toxicity is tenuous, and has a potential for creating inaccurate and unnecessary responses. The use of a multiple strain assay, using metabolically and genetically diverse bacteria, could potentially provide a much more accurate and appropriate tool for the screening of chemicals with unknown ecotoxic properties.

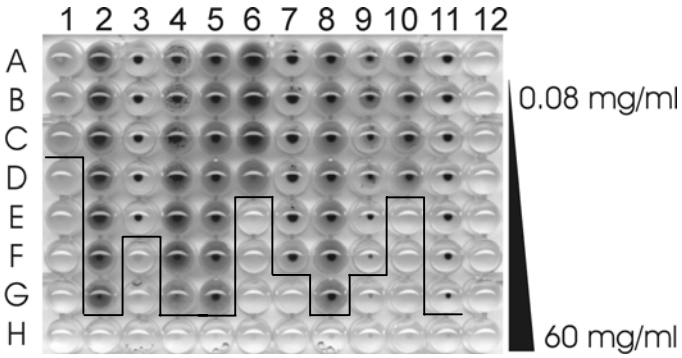


Figure 1 MARA-plate with a seven step concentration gradient of H_2O_2. One microbial strain in each of columns 1-11, column 12 is a control without microbes. H_2O_2 is added with the highest concentration in row H and the lowest in row B. Row A is the control. The dots seen on the plate indicate microbial growth. The toxic fingerprint is marked in the figure.

In the present thesis a Microbial Assay for Risk Assessment, MARA, has been developed (**Paper II**). The idea is to expose at least eleven microbial strains, lyophilised in a microplate, to a concentration gradient of the chemical to be tested. Since the different strains exhibit different sensitivities to chemicals, a growth pattern – the toxic fingerprint – can be seen on the plate (**Figure 1**). Our hypothesis is that the toxic fingerprint is unique for each chemical and that it is indicative for the mode of toxicity of the chemical. The importance of having a wide phylogenetic diversity among the strains in MARA for getting the optimal fingerprint was assessed in **Paper III**. In **Paper IV** the concept of toxic fingerprint was further tested and was proven to be successful. To be able to read the result of the assay in a simple way, a suitable system for capturing data using a flat-bed scanner was developed (**Paper I**). The plates were scanned and a software program developed in-house was used to analyse microbial growth. An appropriate growth indicator, such as tetrazolium red (TTC), was added to facilitate the reading.

Similar tests have been developed elsewhere, e g a toxicity test based on algae in microplates (3) or a test for detection of the antimicrobial activity of plant extracts (4). In both tests, microorganisms are exposed to a concentration gradient of a sample to be tested in the same way as in MARA. The major novelties with MARA are

1) The toxic fingerprints. It is not the inhibition values of a specific strain that give the result but rather the combination of the inhibition values from multiple strains that forms the toxic fingerprint. This toxic fingerprint can be compared to toxic fingerprints from other chemicals. In cases when the result is preferred as a single value, such as when comparing the result from MARA to that from other tests, either the mean inhibition value or the inhibition value of the most sensitive strain can be used.
2) The possibility to build a database of the toxic fingerprints. In this way more information can be retrieved from each test. If the toxic fingerprint of a tested chemical is similar to that of a tested chemical with known (eco)toxic effects it may also have a similar type of toxic effects. At this time, this correlation is just a hypothesis though.
3) The simple reading of the result. A flat-bed scanner can be found in almost every office these days. Together with the MARA-software it takes less than 24h from the start of the test to the completed result. Only one of those hours is working time, the rest is incubation time.
4) A new way to calculate growth inhibition was developed, the Microbial Toxic Concentration (MTC), which is based on the comparison of the areas under and above the growth inhibition curve.

Aims of the study

The aim of this study was to develop a cheap and simple (eco)toxicity test based on growth inhibition of microorganisms in a microplate. The hypothesis was that since different bacteria have different sensitivities towards different toxic chemicals, unique "toxic fingerprints" from the chemicals would be obtained from the test. In order to get the unique fingerprints, a wide diversity among the bacteria in the test is desirable and thus a study on the importance of phylogenetic diversity was needed. In addition, a method for capturing data by computerised reading of bacterial growth would enhance the possibilities for the test to reach outside the sphere of microbiological laboratories.

Ecotoxicity testing

Every day of our lives we meet a large amount of anthropogenic substances. Most of them pass unnoticed, some of them affect us temporarily (like ethanol) and others again have long-term harmful influence on our bodies even though they do not constitute any acute risk (like e g mercury). All these chemicals also pass through the environment: some of them without making any harm, some of them making temporary lesions and some of them making irreversible damages. It is obvious that efforts must be made to minimise the use of such chemicals that may damage the environment or our health. But to make those efforts, knowledge must first be gained about the effects of different chemicals.

> The ecotoxicological methodology include as well cell- and organo-physiological experiments in the laboratory as field studies and mathematical models.
>
> *National Encyclopedia*

The only known way to find out about the hazardous effects is to make assays on living materials, traditionally whole animals. During the past years there has been a vivid debate about the right of the animals – what gives the right to mankind to make painful tests of our innovations on innocent animals? As a result of this debate, a number of *in vitro*-tests on cell-lines, primitive animals and microorganisms have been developed. Much work has been done, and it has been proven that these alternative tests may give results just as valid as tests on whole animals (5),(6). See also next chapter, Alternative methods to animal toxicity testing.

New chemicals that are released on the European market today must be tested on fish, *Daphnia* (a crustacean) and algae according to the OECD Test Guidelines. These tests are about to be the worldwide standard for ecotoxicity testing. In special cases, or when the standard tests give doubtful results, other tests such as tests on activated sludge and multi-species tests are performed. If satisfying results are still missing, further assays such as tests on mesocosms (see next paragraph) may be necessary. These tests are very costly though and the chemical must be valuable for the manufacturer to proceed to this stage. (Alf Lundgren, National Chemicals Inspectorate (Kemikalieinspektionen), personal communication)

Tests for assessing the ecotoxic effects of a chemical have evolved later than tests for human health. The ideal test from an ecologist's point of view would be a lab-scale ecosystem (a so called micro- or mesocosm) where the fate of contaminants can be monitored. A number of such studies have been presented, but the amount of information retrieved from a mesocosm is enormous and is very difficult to interpret (7). Further, the knowledge of complex ecological systems is still limited and this makes the interpretation of data even more difficult. Criticism against the use of mesocosms has been expressed regarding the limited value of a test; even if full knowledge can be gained about the effects in this specific ecosystem not much can be said about the effects under other circumstances. Kraufvelin (8) showed that the repeatability of results from mesocosms is at an unacceptably low level and it would be unrealistic to make any predictive validations based on these data. On the other hand, Hall and Giddings (9) has shown that concentrations of a chemical exceeding the critical level found in single-species tests has no or little effect in an aquatic ecosystem. To make a full assessment of a chemical it would thus probably be necessary with a combination of the different testing methods. Much research is

needed though before a satisfying level of knowledge is reached regarding how to interpret the results, and before such knowledge is gained deficient but efficient methods must be used.

Most ecotoxicity tests used today are based on one single species. This is of course much simpler and cheaper than creating a complex mesocosm. Several studies (10),(11), have shown that a combined set of single species tests can give NOEC (No Observed Effect Concentration)-values in the same order of magnitude as a model ecosystem. It has been found though that single-species tests often give somewhat higher NOEC-values than multi-species tests and thus rather underestimate the risk of a chemical. There are a number of plausible explanations to this; variability of data collected in the test systems, lack of knowledge about the most appropriate test methods and –times, extrapolation from individual to population-level endpoints etc (11),(9). It has often been suggested that a battery of different single-species tests would make a good ecotoxicity test (12),(13),(14). Care must be taken though when selecting the species to be included in a battery. Henschel et al (15) found that of salicylic acid, paracetamol, clofibrinic acid and methotrexate tested with three acute standard tests alone (algae, *Daphnia* and fish), ecotoxic potentials were underestimated for all tested substances but salicylic acid.

Microorganisms, such as bacteria, are very suitable for ecotoxicity testing. Their small size makes it possible to make assays on millions of individuals simultaneously and thereby avoid uncertainties due to individual variations. As bacteria have short generation times, sometimes as short as 20 minutes, test times can be reduced considerably compared to tests on larger organisms. Bacteria are easy and cheap to handle, and staff without any advanced training are generally able to perform the tests. They incorporate toxicants in their metabolism faster than higher organisms do. Last, but not least, there are no ethical problems connected to the use of microorganisms in toxicity testing. (16),(17)

Microorganism based ecotoxicity testing has been explored both commercially and in academic research. The most commonly used commercial test is Microtox™, a test based on the inhibition of luminescent *Vibrio fischerii* (earlier known as *Photobacterium phosphoreum*) (18). Other common tests are Polytox™ and ToxAlert™, based on a cocktail of 12 aerobic strains (19) and bioluminescent *Vibrio fischerii* (Merck cat no 1.08931.0001) respectively. Alternative tests have been developed, such as a growth inhibition test based on *Rhizobium meliloti* (20), a test for nitrification inhibition on *Nitrosomonas* and *Nitrobacter* (21), a biosensor based on gene-modified *E.coli* attached to an optical fiber (22) etc. A common disadvantage for all of the above mentioned tests is that they only return one value each. This value gives very limited information about the toxicity of the sample. By analysing the toxic effect on a number of strains in parallel, as is done in the MARA-test, a more informative response is retrieved.

Chemicals affect bacteria in a number of ways, see fig 2. It is thus important to be aware of what to measure when basing a toxicity test on bacteria; Torslov showed that the choice of parameter to be measured is just as important as the selection of strains for the result (23). We have selected to measure viability in the MARA-test, both for the sake of simplicity and for the sake of covering a broad range of toxic mechanisms as most toxic mechanisms give reduced growth rate in the end. This choice might lead to a reduced sensitivity to specific types of damages, such as a low level of genotoxicity that can be detected via e g the Ames' test, but the advantages outweighs the disadvantages.

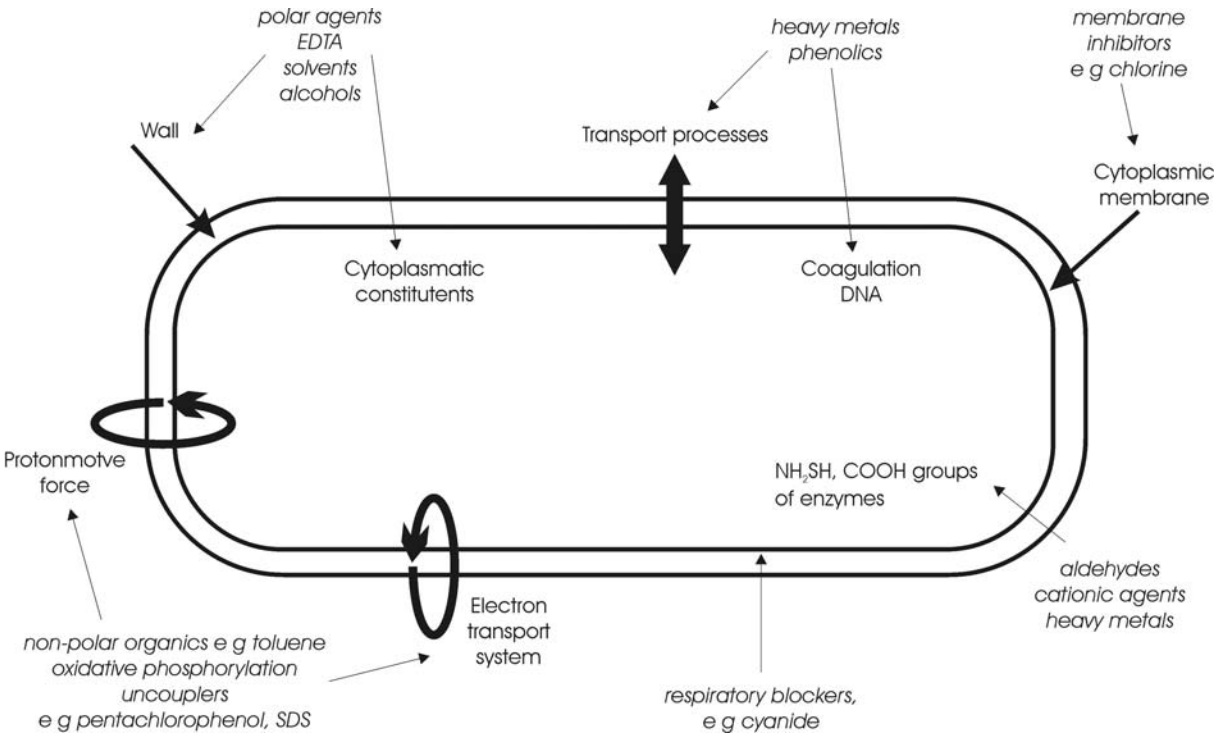


Figure 2 How different chemicals affect bacteria. Each of these toxic effects may lead to growth inhibition. After Merck's ToxAlert brochure.

Alternative methods to animal toxicity testing

There is a debate in society about the large number of animals used in toxicity tests of new chemicals. What right do we have to sacrifice all these animals in painful tests? There are also economical and scientific objections - animal tests are expensive, slow and difficult to interpret. The question has reached the political agenda, and in 1991 The European Union started a European Centre for Validation of Alternative Methods (ECVAM). The corresponding organisation in USA is the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Several research projects have been launched to reduce the number of test animals, both by the development of new *in vitro*-tests and by the refinement of existing animal tests.

An example of the latter is the replacement of the LD_{50}-test in the OECD-guidelines for oral toxicity. The LD_{50}-test aim to find the dose that is lethal for 50% of the tested population. When it first was introduced in 1927, it required up to 100 animals for each substance tested. In 1981 it was incorporated into the new OECD Test Guidelines for oral toxicity (OECD 401), and by then it was refined to 30 animals needed. Alternative Test Guidelines, where fewer animals was needed and/or the endpoint was no longer death of the animal, were adopted during the years to come (OECD 420, 423, 425), but were used to a lower degree than expected. In December 2002, the LD_{50}-test for oral toxicity was completely removed from the OECD guidelines for oral toxicity (24), (25).

One of the projects for the development of alternative test methods is the MEIC (Multicentre Evaluation of *In vitro* Cytotoxicity)-study (26). In MEIC, 50 selected chemicals with known human toxicity were tested in a number of *in vitro* tests. The goal was to find a correlation between the results from the *in vivo* and *in vitro* tests in order to replace the *in vivo* tests. By the end of the project, the 50 chemicals had been tested in 61 different *in vitro*-tests. Statistical analysis of the results gave by hand that a test battery consisting of three human cell lines gave a better prediction of acute lethal blood concentrations in humans than the LD_{50} results from assays on mice and rats (27). It is regarded as one of the most promising alternatives to *in vivo*-testing (24). MARA and other *in vitro*-tests under development has used the chemicals in the MEIC-list as reference chemicals, although they were not included in the evaluation mentioned above.

An alternative is the use of **stem cells**, a research field under heavy debate but with a large potential to interesting results. They can be used primarily for testing of developmental toxicants. A validation of the embryonic stem cell test in comparison to *in vivo*-results has been done, but further development as well as a political acceptance is needed before the tests can come to general use. The combination of the use of stem cells and microarray techniques offers a unique possibility to understand toxicological mechanisms. (28)

Animal tests for **skin irritation** are among the mostly debated tests as they are often used for the testing of cosmetics. For the general audience, those products are not important enough to mankind to make it ethically defensible with painful animal assays. In addition, the tests are not always predictive for damages in human. Alternative tests have been developed, and the most popular are the organotype skin equivalents due to their resemblance to human skin. There is still

a lack of standardised test protocols though (29). With *in vitro* assays, other and more objective endpoints than with *in vivo* assays can be measured, such as transcutaneous electrical resistance. (25)

In MARA, bacteria have been used as test organisms. Other alternative test organisms used are aquatic organisms, birds, invertebrates and yeasts (30). The less the resemblance to mammalians, the better the test is from an ethical point of view. The limit between an ethical acceptable test and a none acceptable test is purely objective. No ethical objectives have been raised towards the use of bacteria or other microorganisms in toxicity testing, they are classified as *in vitro*-assays.

It will take at least 10 years before all *in vivo*-tests can be replaced with *in vitro*-tests for acute toxicity testing (24). There are many projects in progress, but the validation and general acceptance takes time. To replace the chronic toxicity tests will take even longer as it requests large progress in the theoretical knowledge about the fate and destiny of foreign chemicals in the body. There is a general agreement that no single test is good enough but a test battery is required.

Effects of toxic chemicals on bacteria

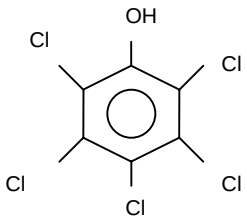
Chemicals can exert a number of different toxic effects on a bacterial cell (see **Figure 2**). It is difficult or even impossible, with our current knowledge, to deduce the toxic mechanism of a specific chemical by just looking at its molecular structure, although chemicals with similar structures and/or physico-chemical properties are expected to have similar modes of action (31). Several studies have been done on the Quantitative Structure-Activity Relationships (QSARs), but still knowledge is scarce. There are some general rules though, such as lipophilic chemicals being more prone to disturb the bacterial membrane than hydrophilic chemicals and electrophilic chemicals often form irreversible covalent bonds to their target site at nucleophilic entities in biological molecules, such as proteins and DNA (Escher 02). A chemical may have multiple modes of toxic action and at low concentration it may even be used as a nutrient.

The toxicity can be more or less species specific, many biological structures and functions are highly conserved between species (31) and thus it is of less importance than one could think whether the tested species are of prokaryotic or eukaryotic origin. This view is supported by the results from genome sequencing projects, showing that the human genome is far more similar to more ancient organisms than previously thought (30), (32). Certain toxic effects are very species specific though, such as inhibition of photosynthesis – a system found only in algae.

The target for the toxic action may depend on the concentration of the toxic chemical. When the target is found in the cytosol, the intracellular rather than the extracellular concentration should be considered. The two are not necessarily correlated, the intracellular concentration is rather correlated to e g the external pH. An example is the LC_{50}-values for chlorophenols on fish, which depend on the pH of rather than on the concentration in the media (33).

Thus, knowledge about the specific target site and mode of action is indispensable when making risk assessments and QSARs (34), (35). As this knowledge often is scarce, a screening method, such as MARA, can be used for getting an idea about the type of activity at the target site.

Chlorophenols



In **Paper IV**, the toxicity of chlorophenols was assessed in MARA. They are an interesting group of chemicals as they are well studied, they are commonly used in industry and their toxicity is depending on the number and distribution of chlorine atoms around the phenol ring; the higher the number of chlorines on the molecule, the higher the toxicity (33). The chlorophenols are believed to enter the cell by passive diffusion. This is well in line with the chemical structure and toxicity data; the higher the chlorine substitution, the less polar the molecule, the more lipophilic it becomes and the easier it can diffuse across the lipid membrane. *Ortho*-substituted chlorophenols are less toxic than expected considering the number of chlorines (33), (36) due to the limited effect on polarity chlorines in *ortho*-position has. Chlorophenols can only diffuse through the membrane in its undissociated form though and thus the diffusion rate is highly dependent on the pH of the media. Again, this is in line with the empiric toxicity results as the higher the number of substitute chlorines, the lower the pK_a-value of the molecule and thus the lower the pH of the surrounding media can be without causing dissociation of the molecule.

Disinfectants

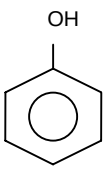
In **Paper IV**, the toxicity of some disinfectants was assessed in MARA. Disinfectants, or antiseptics, are chemicals used for deleting microbes from surfaces where they are not wanted, e g in hospitals, in toothpaste and in the food industry. They are similar to antibiotics in the sense that they should be toxic to micro-organisms but non-toxic to higher organisms, especially humans. The major difference is that whilst antibiotics should be as species-specific as possible, disinfectants should be effective against a wide array of microbes. Much research has been done on the exact mechanism and efficacy of antibiotics, but information is scarcer when it comes to disinfectants.(37)

The use of disinfectants is increasing in the society. We meet them in our everyday life in toothpaste, washing-up detergents, shoes etc. A risk with the increased use of disinfectants is the possible development of bacteria resistant to the disinfectants and, even worse, cross-resistance to antibiotics. E g, the development of the new group of antibiotics, the diazaborines, has been stopped as the target of this group, the enoyl reductase (an enzyme involved in bacterial fatty acid synthesis), is also a target for triclosan and hexaclorophene (38). It is debatable whether it is worth the risk to use disinfectants for none-medically purposes, such as reducing the smell in shoes, if it decreases the possibilities to use the same disinfectants in medically motivated areas such as wound cleansing.

Oxidative agents

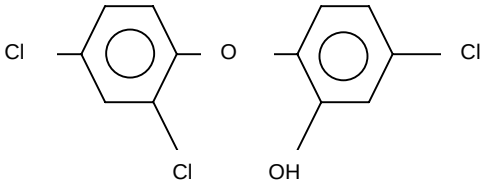
Three of the disinfectants used in **Paper IV**, hydrogen peroxide (H_2O_2), Perasafe and Virkon, are oxidative agents. The oxidisers damage cells by the formation of free hydroxyl radicals, •OH, which induce e g DNA-damage, lipid peroxidation and protein oxidation. The degradation products are harmless, e g hydrogen peroxide is reduced to water, and thus the peroxides are fairly environmental friendly. A problem is that they also damage eukaryotic cells, but they have more efficient protective mechanisms than bacteria and it has thus been possible to use hydrogen peroxide for wound sterilisation. An advantage with the oxidising disinfectants is their simple mechanism of action and their multiple targets, meaning that resistance towards these disinfectants is rarely, if ever, developed in bacteria.

Phenol



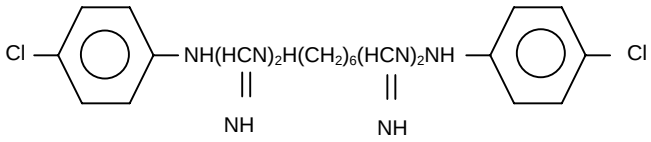
Phenol is a disinfectant that has been used since 1867. These days it has been replaced though by different phenol derivates, such as phenyl- or halogenphenols, due to its systematic toxicity and caustic effect on skin and tissues in humans (39). It is still used as the standard disinfectant towards which new disinfectants are compared though. The most commonly used derivate is hexachlorophene. Phenol causes membrane disruption in bacteria (40). It can also coagulate cytoplasmic constituents.

Triclosan



Triclosan is a bis-phenol, often used as skin disinfectant. It is also known for its anti-inflammatory activity. The exact antimicrobial mechanism is not known. It affects membrane structures and nutrition uptake, and inhibits fatty acid synthesis (41). The cumulative effect on multiple targets also contributes to the toxicity. The toxic efficacy can be increased substantially by combining triclosan with other chemicals.

Chlorhexidine



Chlorhexidine is a biguanide and is one of the, or maybe even the, most used disinfectants. It is very common in skin disinfectants as it has no or little adverse effect on the skin. In low concentrations it causes leakage in the membrane of bacteria but at higher concentrations it coagulates cytoplasmic constituents and thus leakage decreases. As it attaches to the bacterial surface due to its positive charge at physiological pH (42), the toxic effect is depending on the pH of the solution, and the presence of organic matters reduces the effect.

Other commonly used disinfectants, not tested in Paper IV

Hexachlorophene is similar to triclosan; they are both bis-phenols and they are both membrane disruptors and inhibit fatty-acid synthesis. *Silver-compounds*, such as silver nitrate and silver sulfadiazine, has long been used for antimicrobial purposes. E g in Sweden, children used to get a drop of silver nitrate in the eyes upon birth to prevent the transfer of gonorrhoea from the mother. Silver nitrate is also used for wart treatment. The silver ions interact with thiol (-SH) – groups, but also other toxic mechanisms are involved. *Ethanol* and other alcohols, such as isopropanol, are commonly used for skin cleaning

Reading of microbial growth

Quantification of microbial growth is an important feature when working with microorganisms, not least when dealing with inhibition of microbial growth. The quantification can be done in a number of ways, such as direct microscopic count, viable count (spreading on agar plates), cell mass measurement, measurement of turbidity or measurement of emitted light from certain bacteria (43).

To do **microscopic count (total count)**, a drop of the sample is placed on a special glass slide with microscopic chambers of known volume. The method gives a very exact answer, although there is a risk of counting dead cells together with vivid ones as they cannot be distinguished by eye.

Viable count is a simple technique that gives very accurate results. A known volume of the sample is plated onto an agar plate and the number of "colony forming units", CFU (i e the number of vivid cells in the sample), is counted. The main disadvantage of viable count is that it takes at least over-night incubation before the result is obtained and it is thus not an appropriate technique when a fast result is required.

To measure the **cell mass**, a sample is centrifuged and the cells form a pellet that can be weighed. This is useful when the mass of the cells is more important for the study than the number.

Turbidity measurement offers a simple way to estimate the amount of cells in a sample. As the cells grow the turbidity of the growth media increases and this can be measured by measuring the amount of light that is transmitted when a light beam passes through the sample. Turbidity measurements are made with a spectrophotometer. This method is often used when e g the growth curve of a culture is monitored.

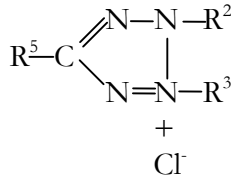
The quantification of luminescent bacteria (such as *Vibrio fischerii*) is easily done by measuring the amount of **light** emitted from the bacteria. This feature is utilised by several ecotoxicity tests, such as Microtox™ and ToxAlert™ (see "Ecotoxicity testing"-section). Gene-modified organisms, such as *E.coli* that have received the *lux*-gene to make it luminescent, have also been used. A disadvantage of luminescence measurements is that the necessary reading devices are either a luminometer, which is fairly expensive, or photofilm, which demand access to a dark room. As one of the aims of the MARA-project was to make the test as cheap and simple as possible, measurement of luminescence was considered as an inappropriate reading method.

Most of the methods described above are too labour-intensive to be a realistic alternative for reading of MARA-plates as 96 quantifications must be done simultaneously. Therefore a new reading method for quantification of microbial growth in microplates using a scanner and a growth indicator (in our case tetrazolium red (TTC)) was developed (**Paper I**). Reading of growth in the MARA-plates can be done with a plate microplate spectrophotometer, but a scanner is a far cheaper alternative even though it necessitates the use of a growth indicator.

Tetrazolium theory

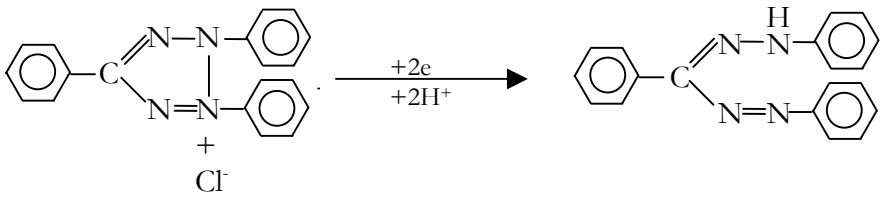
Different tetrazolium salts have been used to indicate enzymatic processes since the 1940's (44). The salts have been useful both for assays on larger organisms, such as seed, and for assays on bacteria. The salt precipitates to coloured formazan crystals upon enzymatic reduction. The reducing activity in an actively growing culture is proportional to the number of bacteria and thus the amount of bacteria may be quantified by measuring the amount of precipitated salt with an appropriate device, such as a spectrophotometer or a digital scanner (**Paper I**). A qualitative judgement can normally be done by eye. The reduction potential of the typical tetrazolium salts is about –0.08V and it may thus act as electron acceptors for many pyridine nucleotide linked enzyme systems. The tetrazolium salt competes with oxygen in receiving hydrogen ions from NADPH or NADH. The reduction of tetrazolium salts take place in the membrane of the bacteria, since this is where the enzymes mediating electron transport and oxidative phosphorylation are located (45). This was confirmed by microscopic studies of bacteria in the present study; the salt seemed to have precipitated on the bacteria and no free formazan crystals were seen in the solution.

A number of different tetrazolium salts have been used, more or less extensively. They all have the common basic structure, namely



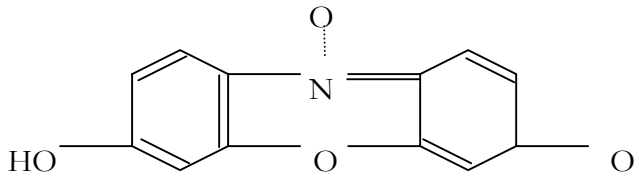
Depending on the properties of the R-groups, the formazans may be water-soluble or not. Other properties affected by the R-groups are light sensitivity, crystal size, crystal colour and speed of reduction. This influences the indicator of choice in a given situation.

In MARA, the tetrazolium red (2,3,5-triphenyl tetrazolium chloride, TTC) was used. It is one of the most commonly used tetrazolium salts and was also one of the first to be synthesized (46). It was chosen as it fulfilled a number of the criteria that were put on the assay: low price, a suitable reaction speed, easiness to read, detects growth of a wide variety of microbial strains etc. The chemical equation of the redox reaction is



In histology, TTC is used to detect cystein containing proteins as it is reduced by sulfhydryl compounds under specific conditions.

Resazurin is a compound closely related to the tetrazolium salts. The molecular structure is



The molecule is blue in its original form but changes colour in two steps as it is oxidised; first to the pink-coloured compound resorufin and next to the colourless hydroresorufin (47). Resazurin is used to detect the dehydrogenase activity in a sample containing microorganisms (23) and is widely used in the diary industry (48). The resorufin intermediate is fluorogenic and the reaction can thus be followed by fluorimetry. It can also be registered by eye as the colours are easily detected, but visual inspection always give room for a certain amount of arbitrariness.

Image analysis

The use of a flat-bed scanner is central in both papers included in this thesis. Scanners have become more and more common during the past years as the prices have gone down. The technology is similar to that of copying machines, but instead of transferring an image from paper to paper it transfers it from paper to the computer. It is still unusual to use a small office scanner for scientific purposes, but in **Paper I** it was shown that the scanner can be used for measurement of inhibition of bacterial growth. The technique was used to read the results in **Paper II, III** and **IV**.

The normal use of a scanner is to scan papers or other two-dimensional objects, such as overhead transparencies. Microplates are three-dimensional transparent objects and this may cause problems during scanning. As the plates are about 1 cm high the lid of the scanner cannot be closed properly. In some scanners, such as Agfa Snapscan 600 and HP Scanjet XPA, the lid is detachable and may thus be placed horizontally on top of the plate. Other models, such as UMAX Astra 6450, have a fixed lid and a certain angle between the lid and the plate is inevitable. When a reflective scanner is used, the plates must be covered with e g a white paper in order to enhance the contrast and it is thus preferable with an even pressure from the lid on the plate. If a scanner for transparencies is used (i e a scanner where the light pass through the item to be scanned) nothing should be put between the plate and the lid. It is still important though that the light source and the detector are parallel to each other. Empirical studies have shown though that as long as the angle between the lid and the plate is small enough, this is of minor importance.

Figure 3 Function of a reflective scanner (left) and a scanner for transparencies (right). The light goes from the light source L to the detector D. In the reflective scanner, the light beam is reflected in the lid whilst in the scanner for transparencies the light beam goes through the sample to the detector placed in the lid. Also, note the importance of the angle between the bed and the lid.

The microplates are transparent and as the light source is not limited to the area under the plate, additional light beams enter through the sides of the plates and affect the result for the outer wells. In a similar manner, light scatter out from the sides of the plates and affects the result. This is a minor problem when working with an indicator such as TTC that precipitates in the bottom of the wells. The pellet has the same size and colour regardless of some extra light that passes through the well. If a soluble indicator such as BTB (pH indicator used e g in the Phene Plate System™ for indication of bacterial fermenting capacity) is used, the result is heavily depending on the exact amount of light passing through each well. Different ways to minimise the scatter of light, such as a black paper silhouette around the plates, have been evaluated but none has been found to be satisfactory for a reflecting scanner. If a scanner for transparencies is used, the light

seem to get a more uniform distribution around the plate and no disturbing effect of the scattered light can be seen on the outer wells of plates with a soluble indicator.

A round-bottomed microplate is preferred when using an indicator that precipitates, such as TTC or MTT. The indicator assembles to a pellet in the bottom of the well and the pellet size is directly related to the amount of microbial growth. When scanning a round bottomed plate with a scanner for reflectives, smile-like reflections appears in the bottom of the wells. This seems to be inevitable, but is compensated for by the in-house software used to analyse the images as it measures the size of a pellet by calculating the width and mean intensity of the pellet over a diagonal cross section just above the smile-like reflection (see **Figure 4**). The reflections do not appear when a scanner for transparencies is used.

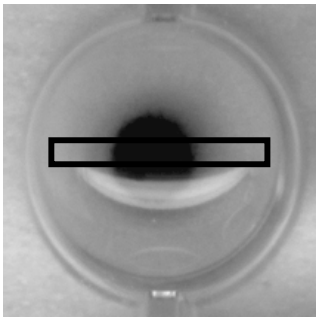


Figure 4 The pellet size is measured over a cross-section of the pellet

The software used to analyse the scans is designed for BMP-files. BMP is a memory-demanding storage format for images compared to e g JPEG, but with the JPEG-format much information is lost by default. TIFF is another commonly used format for images, but a disadvantage is that there are many versions of it and it may be problematic to transfer images between programs.

Calculation of inhibition values

There are many different methods for calculation of inhibitory concentrations. Commonly used values are LOEL (Lowest Observed Effect Level), NOEC (No Observed Effect Concentration), IC_{50} (the Concentration at which 50% Inhibition is obtained) and MIC (Minimum Inhibitory Concentration). All methods have their advantages and drawbacks.

LOEL is a rather straightforward way to "calculate" the inhibition value. It is simply the lowest concentration at which an effect of the chemical can be seen. It does not demand any mathematics and is easy to understand. The major problem is that per definition the only possible outcomes are the tested concentrations. Thus, if different concentrations are used in different studies the LOEL-value will vary. In addition, the result is sensitive to small changes in the raw data. If the obtained LOEL-value is close to the concentration at which no effect is seen, a minor change in the raw data from a repeated assay may give a LOEL-value that differ substantially. The "effect" itself is not obvious to detect and the choice of effect to observe and the sensitivity in the method of observation may cause large variations in the result. NOEC, which is the highest concentration at which no effect of the chemical on the model is observed, is obtained in an analogous way and it has the same advantages and disadvantages as LOEL. Both LOEL and NOEL

The IC_{50}-value is the concentration at which 50% inhibition of biological activity (growth, enzyme activity, number of living individuals etc) is found. In most cases this value must be interpolated from the obtained data, as much luck is needed if the exact IC_{50} should be among the tested concentrations in an assay. To make an interpolation between the points in a curve, the exact form of the curve must be known. Often an S-shaped curve is assumed, empirical data give by hand that this is the most common shape. Further, all the points in a curve are normally used to make the interpolation. When the IC_{50}-value was calculated in **Paper II**, the interpolation was made between the two concentrations closest under and above 50% inhibition and the inhibition curve is assumed to form a straight line between these two points. An interpolation over all points would be complicated as the curve does not follow the s-shape in all cases. In fact, empirical data from MARA give by hand that it is impossible to predict the form of the inhibition curve. It is a function of both the microbial strain and the tested chemical. Some of the irregularities in the curves may originate from the variation in target site depending on the chemical concentration exhibited by some chemicals (see the discussion under Effects of toxic chemicals on bacteria).

The MIC-value is the minimum concentration of a chemical needed to give full inhibition of growth. It is a term commonly used in the testing of antibiotics. Just as LOEL and NOEC it has the disadvantage that the only possible result is the concentrations actually tested.

A new method for the calculation if inhibition values was developed for MARA and presented in **Paper II**. The MTC (Microbial Toxic Concentration)-values were calculated by comparing the area under and above the growth curve, mathematically it was done according to the formula below

$$MTC = c_{\min} * d^{\frac{P_{tot}}{P_0} - 0.5}$$

c_{min} = lowest concentration in the gradient
d = dilution factor
P_0 = pellet size in the control well
P_{tot} = sum of the pellets in all wells exposed to the concentration gradient of the chemical to be tested

By making a calculation based on areas, the shape of the inhibition curve becomes irrelevant and thus a common source of error is eliminated. The calculation of the area under the curve is based on all available points on the curve and thus the tested range of concentration must cover the interval from no to full inhibition of growth. This is a disadvantage compared to e g the calculation of LOEL/NOEC-values.

Lyophilisation

Lyophilisation is a well known method to preserve bacteria for long-term storage. It is commonly used by culture collections since a strain can be kept in room temperature for years before revitalisation. In this study the microbes were to be lyophilised in microplates. Nothing similar was found in the literature and a new method had to be developed.

Theory

Lyophilisation, or freeze-drying, is a drying-process in which no additional heat above room temperature is necessary. A frozen sample is exposed to an elevated temperature (i e higher than the temperature it was frozen in) in a vacuum chamber. The water sublimes (i e goes directly from ice to gas without taking the liquid form in between) as the process is performed below the triple-point of water (0.01°C, 611.73Pa), and the sample dries. This is feasible when drying e g heat-sensitive products such as food or living organisms. A disadvantage in a normal drying process is that the water floats from the inner to the outer parts of the sample bringing salts and other soluble particles to the surface. The locally increased concentration of these substances is seldom wanted and is avoided in a lyophilisation process.

The first step in the lyophilisation process is to freeze the sample ①. The freezing-rate and temperature affects the result of the lyophilisation. For lyophilisation of microorganisms the freezing must be fast and in most cases the temperature is as low as –70°C. The samples can be placed directly into a freezer, when an even higher freezing rate is desired they may be frozen in a bath with dry ice. In other cases it is more appropriate with a lower freezing rate; the ice-crystals become larger and more regular the slower the freezing is.

The next step is the actual lyophilisation process ②. The frozen sample is moved into a vacuum chamber connected to a condenser. As the ice need some additional heat energy to sublime the vacuum camber must have a higher temperature than the frozen sample. In simpler lyophilisators room temperature is used, for more sensitive samples it might be necessary to start at a lower temperature. The condenser is requisite since the speed of the process is depending on a low vapour pressure in the chamber. When all the ice has sublimated the process is finished and the sample can be kept under normal temperature and pressure ③.

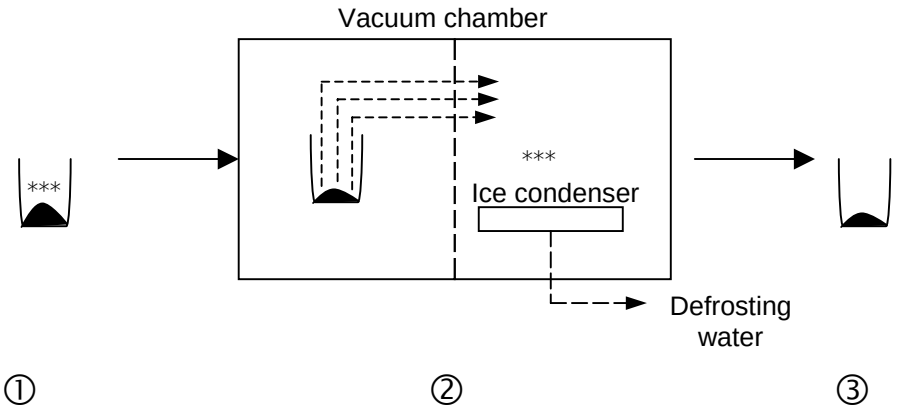


Figure 5 Schematic picture of the lyophilisation process

Results and Discussion

When lyophilising microorganisms for a toxicity test, commonly used lyophilisation techniques are not applicable as most known lyophilisation media contain some sort of serum as a protective agent. The serum would probably interfere with the interaction between the tested chemicals and the microbes and thus an alternative media was developed. A special feature when lyophilising in microplates is that the microorganisms in the wells must not swirl around and contaminate any other wells during the exposure to vacuum. In addition, the media must stick to the plastic in the plates after lyophilisation so that the plates can be transported without risk of contamination during the transport.

A lyophilisation medium without serum was developed that fulfilled all of the above demands. The plates with lyophilised microorganisms were packed into vacuum-sealed aluminium bags. In order to enhance the durability of the lyophilised microorganisms, nitrogen gas and dried silica-gel was added. The survival rate after one week's storage increased markedly, 100-fold for more than half of the strains when kept in +20°C, due to the addition of silica-gel. There are good reasons to believe that the same increase can be seen on plates kept in +8°C in a longer perspective. The positive effect of N_2 was not as strong but still evident.

Bacterial taxonomy and phylogeny

All living organisms can be classified and divided into different taxonomic groups. *Homo sapiens* is a well known species that also an amateur can recognise. Most of us can tell the difference between a dog and a goat, although the outer signs are similar – four legs and a tail. Identification of plants is trickier, but already 250 years ago (1753) Carl von Linnaeus wrote *Systema Naturae* by which most plants can be classified.

Bacterial taxonomy poses other problems than the taxonomy of higher organisms. Bacteria are too small to be seen with a naked eye, and even at a close look under the microscope many taxonomically different species are undistinguishable. Traditionally, bacterial classification is based on phenotypical traits, such as morphology and different biochemical reactions, but today DNA-analysis is regarded as the most prominent method. The phylogenetic tree of bacteria is continuously re-drawn as more information is gathered.

Some bacteriologists have argued that bacterial taxonomy is a meaningless task. Bacterial genomic differences are more or less a continuum that cannot be divided into entities. Although some species definitions may seem arbitrary, they may still fulfil practical purposes. Most studies in taxonomy and phylogeny today is based on 16S rRNA sequencing, although voices are raised for a polyphasic taxonomy, i e a taxonomy based on a combination of rRNA sequences and phenotypic characteristics (49). 16S based taxonomy has the advantages of giving an idea about the phylogenetic relation between bacteria as the molecule is regarded as a phylogenetic chronometer. It is not unambiguous though and other markers have been suggested. In addition, for practical purposes, a classification based on phenotypical traits may be of larger interest to the bacteriologist at the lab. The problem will diminish though as it gets easier to sequence the whole bacterial genome (see F**igure 5**), but today it would take at least a month for a well-equipped lab to do it. It is thus not an option for most purposes.

Definitions

"Taxonomy" is, in this thesis, defined as the science of classifying and naming organisms, particularly bacteria. "Phylogeny" is the science of systematising bacterial strains according to their evolutionary relationships. The word "phylogeny" was coined by the German philosopher-biologist Haeckel in 1866 (50). The two fields are closely related; to systemise undenominated strains is just about as meaningless as giving random names to all strains.

> "Bacteria show little interest in the matter of their classification. For the systematist, that is sometimes a very sobering thought."
>
> *Bergey's Manual*

The so-called "type strains" are the link between taxonomy and phylogeny. A type strain is a strain that has been selected to be representative for the species and to which all other bacteria in the species are related. It does not have to be a typical strain though, but it is the strain that will keep the species name if the species is divided. Due to the system with type strains, bacterial taxonomy keeps a rigid structure and it is always possible to follow the history of a species and relate newly found strains to strains described in older papers. Thanks to our collaboration with NCIMB, the majority of the strains used in **Papers III** and **IV** are type strains.

Some history of bacterial taxonomy

In the old days, one strain could be "discovered" several times. E g *Klebsiella pneumoniae*, which was published under six different genera between 1885 and 1928 (51). Prokaryotes were not recognised as a special type of organism but were traditionally regarded as fungi, and the taxonomic confusion increased as bacteriologists up to 1930 tried to follow the Botanical Code of Nomenclature, which did not allow some of the methods necessary for bacterial classification. In 1958, the first edition of International Code of Nomenclature of Bacteria and Viruses was published. This was a step forward, but still in the decades to come, a large proportion of the published bacterial names were useless due to deficient descriptions and lack of type strains. A new start was made 1 January1980. Instead of approving all names published since Linnaeus published the plant names in *Species plantarum* 1 May 1753, a new list of acceptably published names, the Approved List of Bacterial Names, was published. All names not found on the list lost their standing in nomenclature. From now on, all new names were to be published in the International Journal of Systematic Bacteriology (now International Journal of Systematic and Evolutionary Microbiology) or, if published elsewhere, be sent to the Validation Lists, administrated by the same journal. All bacterial names are therefore found in the same journal, which has made life easier for bacteriologists worldwide (www.the-icsp.org).

The species

The species is the basic unit in the system of bacterial taxonomy. Two genetic criteria for the separation of species are used; two strains of the same species should have at least 70% resemblance in DNA-DNA hybridisation (52) **and** 97% rRNA similarity (53). Another accepted criterion is at least 80-85% similarity between isolates based upon unweighted-pair-group-analysis data matrices containing independent covariant characters (51). These limits are arbitrary rather than based on strict scientific facts, and there are thus several examples of less successful classifications where the rules have been applied to the letter. Strains with similarities over the above-mentioned limits may differ substantially in their phenotypic traits and there are good reasons to divide them into different strains (54). Some species have been designed to different species due to an important property, such as pathogenicity, although they have over 70% DNA-similarity, such as *Yersinia pestis* (high pathogenicity) and *Yersinia pseudoturbercolosis* (low pathogenicity) (55). On the other hand, two *E.coli*-strains may differ 20-30% whilst some *Salmonella* and *E.coli* may share 70% of their DNA (56).

The phyla

In Paper III, the difference in sensitivity of bacteria from different phyla to a number of toxic chemicals is assessed. The phylum is the highest rank in bacterial classification within the domain of bacteria, but there is a remarkable lack of definitions on how the boarders between the phyla are drawn. According to Woese –87 (57) "bacteria separate into more or less naturally defined "phyla"". The phyla have been reorganised under the years though. The definition used in Paper III is from Woese -87 (although Woese's Purple bacteria are called by their new name, Proteobacteria) and is based on a total number of ten phyla. In Bergey's Manual of Systematic Bacteriology from 2001, 23 phyla are recognised, whilst Cavalier-Smith 2004 (58)

Bacterial ranks
Phylum
Class
Order
Family
Genus
Species
Subspecies

defines eight phyla. The differences are minor though; it mostly seems to be a question about the cut-off limit between the phyla (major exception: Cavalier-Smith defines the archae as a bacterial phylum whilst the others make a clear distinction between archae and bacteria). The phylum is not covered by the Bacteriological Code and should thus be regarded as a more informal classification than the other ranks.

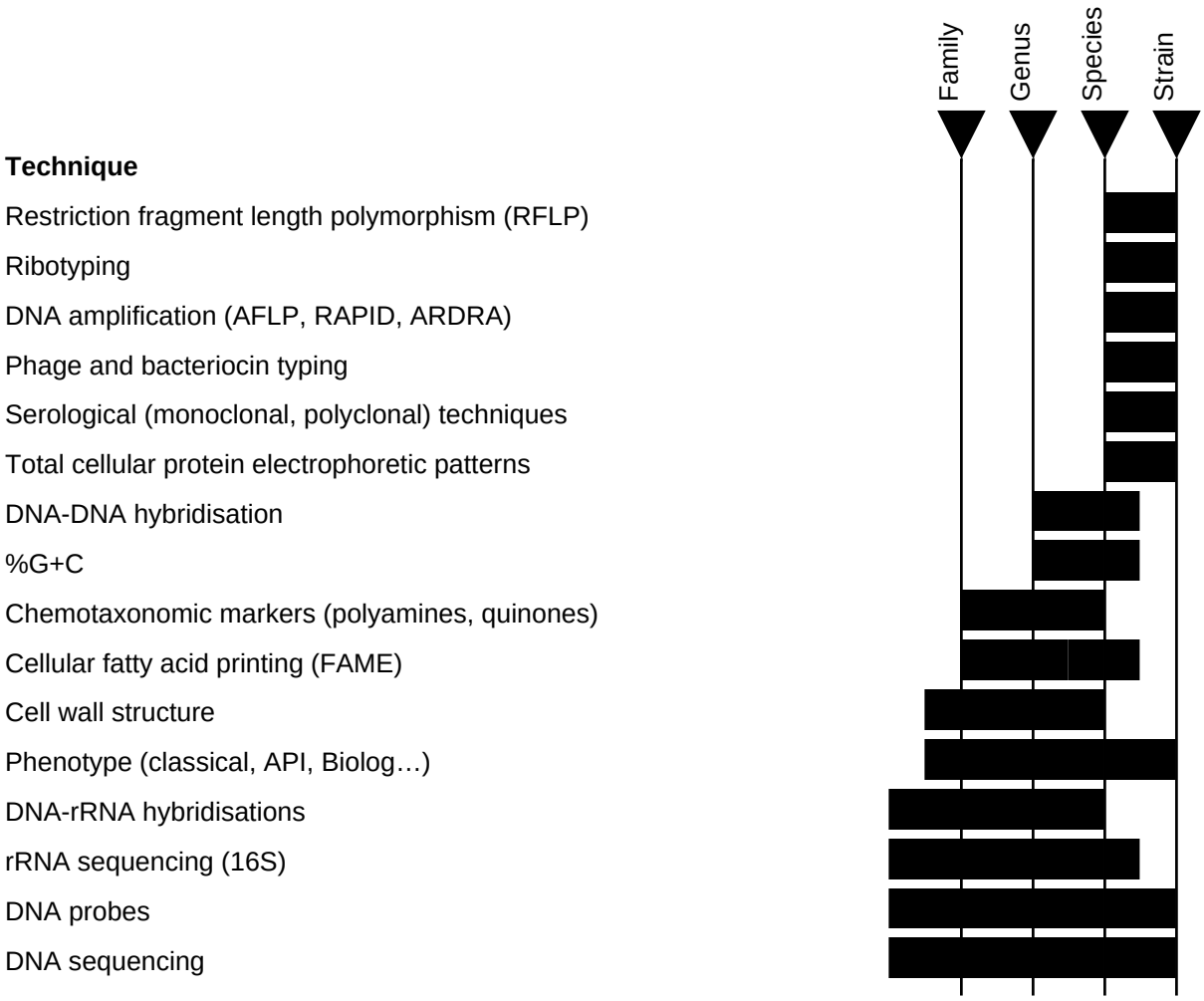


Figure 5 The figure describes the resolutions of some different methods used for bacterial classification and identification. The methods are used both for identification of pure strains and for analysis of whole communities. From Microbial Diversity and Ecosystem Function (1)

Biodiversity

Biodiversity is a general term for describing the abundance of different species and their distribution. Describing the biodiversity of higher organisms often includes the counting of different species, but this is difficult, or even impossible, to do in a bacterial community. The most commonly measured parameters are genetic, phylogenetic and functional diversity, but knowledge about the relation between genetic/phylogenetic and functional diversity is scarce. Most studies on the importance of microbial biodiversity is done on natural communities, which gives realistic data but suffers from the same problems as ecotoxicity studies made on micro- and mesocosms (see discussion under Ecotoxicity testing).

In **Paper III**, a comparison was done between the phylogenetic biodiversity and the functional (in this case, sensitivity towards different chemicals) diversity in an artificial system consisting of pure cultures of a number of strains grown under controlled conditions. The results are of importance when natural and more complex ecosystems, such as soil or activated sludge, are studied.

Importance of biodiversity

A general conclusion from the numerous studies done on biodiversity is that a high biodiversity correlates to a high stability and productivity in ecosystems (59). In a microbial community it does not seem to be the biodiversity *per se* that gives the increased stability though, but rather functional redundancy (60), (61). There is a general opinion that biodiversity is intrinsically good in an ecosystem. A problem when studying biodiversity though is that no two ecosystems are the same. In addition, the boundaries between different ecosystems and different groups of individuals in an ecosystem are often diffuse, not the least when looking at microbiological biodiversity. Thus, drawing general conclusions regarding the consequences of changes in biodiversity from single studies should be done with care. (59)

Definition of bacterial biodiversity

The simplest definition of biodiversity is probably the number of species present in a system (61), but to define and quantify all species in a microbial ecosystem is beyond what we can do with the methods available today. In one of the pioneer papers within the field of microbial biodiversity, Ronald M Atlas (62) states that "Diversity describes the heterogeneity of information within the community; it is a measurement of entropy and reflects the amount of energy required to maintain the organisation of the community. There are two components of diversity: the total amount of information, most often in terms of species number, and the way in which the information is appointed within the community". A number of papers describes and discuss biodiversity without defining it, e g Loreau 01 (63), Solé 04 (64), Loreau 04 (65). Martens et al (Martens 03) discuss the difficulties to define biodiversity – as the concept of biodiversity is so diffuse and includes so many complex processes we will always be surrounded by a certain amount of structural uncertainty when studying biodiversity.

> "Yet, diversity is rather like an optical illusion. The more it is looked at, the less clearly defined it appears to be."
> *Anne E Magurran*

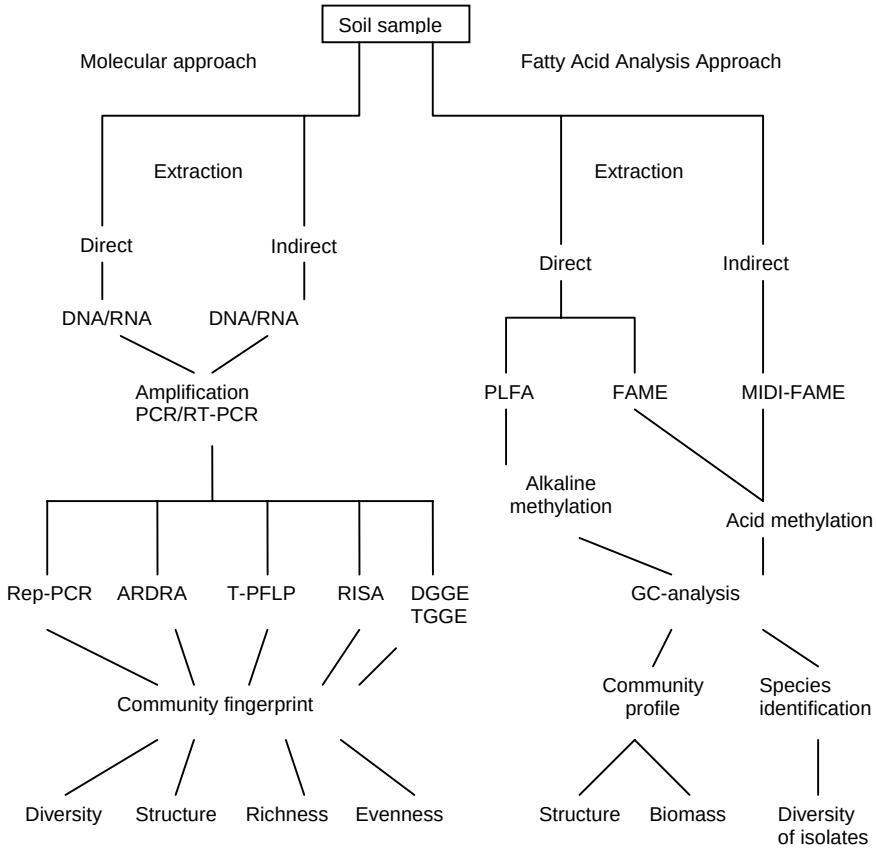


Figure 6 A map over the most commonly used techniques for microbial community fingerprinting in a soil sample. From Kozdroj (2)

Methods for measurement of bacterial biodiversity

In **Figure 6**, a map is drawn over the most commonly used techniques when measuring the bacterial diversity in a soil sample. The different endpoints are found in the last line. To describe each method is beyond the scope of this thesis, but of special interest is that one line only in the map leads to species identification, which is the most common method to assess biodiversity in communities consisting of higher organisms. Information regarding species composition of the community can be acquired also from the molecular approach by the application of different species- or group specific probes or sequencing of DNA/RNA fragment from the gel, but a complete mapping of the species composition is impossible as most species are not known and there are thus no probes that can be used to detect them or any databases that recognise the resulting DNA-sequence.

There is, however, nothing that supports the idea that species identification is the best unit to be used when measuring the diversity in a microbial community. Other alternatives are genes (56), (66) and fatty acid patterns (2).

Another commonly used parameter is the functional biodiversity. It is even regarded as more important than the genetic/phylogenetic biodiversity (60) as functional redundancy gives stability to ecosystems, according to the insurance hypothesis developed by Yachi and Loreau (67). It also has the advantage of being less laborious to measure (61). Examples of parameters that can be measured are the pattern of metabolisation of different sugars and amino acids in Biolog™ or PhPlate™, measurement of enzyme activities (68), mineralisation kinetics of compounds added to soils (61) and SIR – Substrate Induced Respiration (69).

A comparison of the toxic effect on a mixed and single species community

MARA is based on the testing of chemicals on single species. In "real life", bacteria always exist in mixed communities though where they interact, compete and protect each other. A study was done where the toxic effect of chemicals on single species was compared to the toxic effect on a mixed community.

Theory

It is known that bacteria affect each other and communicate when coexisting in a culture. They compete for space and nutrition, but they also collaborate e g by using one species' excretion as nutrition for another species (70). In a dense culture, communication via quorum sensing can occur both intra- and inter species (71). Bacteria fight each other with anti-microbial peptides, bacteriocins (72), (73), and an intense communication between bacteria of different species is needed in the formation of complex biofilms (74).

Due to these effects, there is a difference in behaviour between mono- and mixed cultures. Chen et al found that an *E.coli*-strain enhances the decolourisation ability of a *Pseudomonas luteola*-stran in a mixed culture in an azo-dye solution, although the *E.coli* is a non-decolouriser in monoculture (75). Others have found that single bacterial cultures have a lower degrading power for food industry waste water than the species taken together in a mixed community (76) but a mixed community can also collaborate in different ways to degrade an organic pollutant in several steps, each step performed by different species (77).

Results and Discussion

In this study, we have assessed the difference in sensitivity of six different bacteria (table 1) to four toxic chemicals, namely glyphosate (the active ingredient in the herbicide Round Up), 3,5-dichlorophenol, nicotine and acrylamide, in mixed and monocultures. The bacteria were added to sterile filtered brackish water in a concentration close to the natural concentration of culturable bacteria in the water, i e $2\text{-}9*10^4$ cells/ml. The result was obtained by plate count, and as the different strains had different colours they were easily distinguishable.

Name	Colour	NCIMB-number
Arthrobacter citreus	yellow	8915
Serratia rubridea	red	4
Planococcus citreus	orange	1493
Kocuria rosea	pink	9147
Burkholderia cerpacia	purple	9089
Bacillus licheniformis	white	9375

Table 1 Table over the strains included in the mixed community study

The four tested chemicals resulted in different patterns; nicotine and DCP gave enhanced growth in mixed culture whilst acrylamide promoted growth in monoculture for one of the strains. When growing in glyphosate, no difference was seen between the mixed and the monoculture.

The mechanisms behind these different outcomes cannot be revealed without further assays, but examples of protective mechanisms of a mixed culture are Pelz et al, showing that bacteria metabolising certain chemicals may be sensitive to the toxic metabolites it produces, but in a mixture with bacteria using these metabolites as carbon source, they can survive and together they constitute a stable community (78). Further, a mixed community can protect single species in a biofilm when exposed to a toxic chemical (79). On the other hand, e g competition of scarce nutrients leads to better growth in monoculture. The strains in our study apparently interact in spite of the fact that Malakar et al showed that bacterial concentrations of 10^8 cfu/ml are needed for bacterial interaction (80) i e about 10^4 times higher than the concentrations used in this assay should be needed for interaction.

Thus, interpretations of results from mono-strain toxicity tests should be interpreted with care.

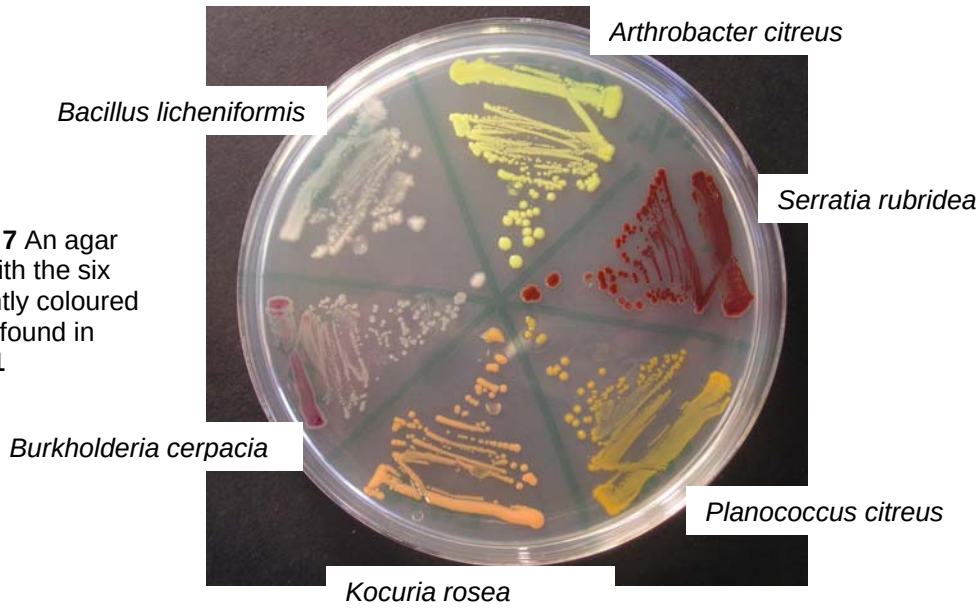


Figure 7 An agar plate with the six differently coloured strains found in **Table 1**

Results and future perspectives

The aim of this project was to develop an (eco)toxicity test based on the growth inhibition of microorganisms, mainly bacteria.

To be able to read the growth inhibition in a cheap and simple way, a method for reading microbial growth in a 96-well microplate using a simple growth indicator, tetrazolium red, and a flat-bed scanner, was developed. It was shown to give comparable results to readings with a microplate spectrophotometer, which is the most common, but expensive, device for reading bacterial growth in microplates.

A Microbial Assay for Risk Assessment, MARA, was developed where the growth inhibition of eleven microorganisms due to a toxic chemical was measured in parallel (**Paper II**). The test was performed in a 96 well microplate (12 columns x 8 rows) with one strain in each column and the possibility to test seven different concentrations of a chemical on each strain. The strains show different sensitivities to different chemicals and the resulting array of eleven inhibition values gives a "toxic fingerprint" of the chemical tested. It was shown that the toxic fingerprints from 13 different chemicals could be separated when being compared mathematically. MARA had the same reproducibility and sensitivity as other bacteria based assays. A further proof of concept was made in **Paper IV** where two groups of chemicals, disinfectants and chlorophenols, were tested in MARA. The disinfectants is a group with similar toxic effect but different chemical structure, whilst the chlorophenols is a group of chemically similar compounds with different toxic effects.

In order to get as diverse toxic fingerprints as possible, the selection of bacterial strains must be optimised. In **Paper III**, the importance of having a wide phylogenetic diversity among the strains in MARA for getting diverse toxic fingerprints was assessed. It was found that to include bacteria from different phyla did not increase the diversity in the sensitivity to different chemicals whilst having strains from different parts of the same phyla increased the diversity. The result is also important for the study of the importance of having a wide phylogenetic diversity in an ecosystem exposed to toxic chemicals.

Future work

It has been found that MARA is a promising method for (eco)toxicity testing. Work that remains to be done is

- optimisation of the array of microbial strains
- extension of the array of microbial strains. A better result can be obtained if more strains are included and measured in parallel
- optimisation of the assay so that not just pure chemicals but also e g polluted sewage water can be tested
- proof of concept; it is not yet clear if a specific toxic fingerprint really indicates a specific mode of toxic action

Abstracts of included papers

Paper I: "Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates"

The growth indicators TTC, INT, XTT, MTT and resazurin were tested for their ability to indicate bacterial growth/growth inhibition. Two reading devices were evaluated and compared, a microplate spectrophotometer and a digital flatbed scanner. The bacteria used in the study were cultivated in 96-wells microplates and readings were made after 24h. The scanned pictures were analysed with a software developed in-house to generate numerical values. It was found that resazurin was difficult to use since it shifts between three colours. MTT and TTC had a high correlation between the spectrophotometer data and the data from the scanned images. The reproducibility was similar for both reading devices. In no case was there a need to resuspend the pellets before reading. XTT and INT showed lower correlations.

It is concluded that bacterial growth/growth inhibition can easily and reproducibly be measured from microplate cultivations with a flatbed scanner or with a microplate spectrophotometer.

Paper II: "A microplate based Microbial Assay for Risk Assessment (MARA) and (eco)toxic fingerprinting of chemicals"

We have developed a multi-species microbial assay, MARA, for assessing the (eco)toxic risks of chemical compounds and for the determination of their toxic fingerprints. The toxic activity is measured in parallel on eleven microbial organisms lyophilised in a microplate. A concentration gradient of the chemical to be tested is added and growth was indicated through the reduction of tetrazolium red (TTC). The microplates are read by a common flatbed scanner or a microplate spectrophotometer. The eleven different inhibition values constitutes a toxic fingerprint, characteristic for each type of chemical compound. The sensitivity and reproducibility of the MARA-test was similar to that of other tests.

Paper III: "Bacterial diversity related to the toxic impact of chemicals"

Hypothesis: A group of bacteria having a high phylogenetic diversity displays a higher variation in sensitivity to toxic chemicals than a group having lower phylogenetic diversity.

Fifty-one bacterial strains belonging to different phyla were exposed to 14 different chemicals. It was found that the variation in sensitivity towards chemicals was at the same level in a group of bacteria from the same species as in a group with different species from the same genus. Increasing the phylogenetic diversity to a group of bacteria belonging to different species from the same phylum also increased the variation in sensitivity whilst increasing the phylogenetic diversity to bacteria from different phyla did not increase the variation in sensitivity any further. Thus, the phylogenetic diversity of a population is thus not *per se* a sufficient measurement of the plausible stability of a microbial population when being exposed to a toxic chemical.

Paper IV: "Microbial Arrays and Pattern Recognition for analysis of toxicity of chemicals"

A toxicity test based upon a standardised array of eleven bacterial strains has previously been developed (named MARA). The bacteria are dispensed in a microplate and exposed to a concentration gradient of the compound under test. The results are expressed as a toxic concentration of the compound for each bacterium, and these concentrations together form a "toxic fingerprint" of the compound, which can be subject to cluster analysis. In the present study, MARA was evaluated by testing a set of organic chemicals with a similar chemical structure (chlorophenols), a set of chemicals having a different structure, but used for similar purposes (disinfectants), and a set of inorganic chemicals (metal salts). The toxicity of the chlorophenols tested in MARA agreed well with findings for other organisms, and cluster analysis of the toxic fingerprints yielded further information on similarities in their mode of action. For some of the disinfectants there was a large range of the concentrations required to inhibit growth of different bacteria, whereas individual metal salts often showed similar toxicities for all bacteria tested. It was concluded that MARA could be a useful assay for dereplication of new chemicals that are produced e.g. in the pharmaceutical industry, as well as for evaluation of the toxicity and other biological effects of old and new chemicals.

Conclusions

A Microbial Assay for Risk Assessment of chemical compounds, MARA, was developed. It was based on the simultaneous testing of growth inhibition on eleven different microbial strains. The array of individual inhibition values from the tested strains constituted the "toxic fingerprint" of the chemical compound tested. The toxic fingerprint is a more informative result than the single value obtained from similar tests as it e g can be compared to other toxic fingerprints in a database and conclusions can be drawn regarding the type of toxicity and species specificity.

The importance of the phylogenetic diversity among the test strains for getting unique fingerprints from different chemical compounds was assessed. It was found that the optimal array preferably would contain strains from different genera, but not necessarily from different phyla. It was further shown that the toxic fingerprint can separate between a large number of chemicals and that it can be useful for dereplication of chemicals with similar molecular structure but different modes of toxic action.

The individual inhibition values were calculated as MTC (Microbial Toxic Concentration)-values, a new calculation method developed within the project. The toxic fingerprints could also be calculated using traditional calculation methods. A cheap and simple method for data capturing from MARA-plates using a flat-bed scanner and a growth indicator (tetrazolium red, TTC) was also developed. It was shown to give results comparable to those obtained with a spectrophotometer.

MARA has a sensitivity and a reproducibility that is comparable to similar assays. Further refinement and proof of the toxic fingerprint concept is needed, but it is believed that it can be a useful tool in screening of chemical compounds for their acute (eco)toxicity.

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