1. Introduction

1.1 Ecotoxicology

In the last two decades ecotoxicology evolved mainly from three different disciplines: toxicology, applied ecology and environmental chemistry. Ecotoxicology as an interdisciplinary environmental science deals with the interactions between environmental chemicals and biota, thereby focusing on adverse effects at different levels of biological organisation. Toxic effects of anthropogenic compounds in biota and ecosystems are investigated in close connection to their environmental chemistry and fate in the environment. The bioavailability of chemicals, which is dependent on biogeochemical processes, is an important factor often neglected in ecotoxicological evaluation and hazard assessment. The bioavailable fraction is the critical parameter for uptake and ultimately for the concentration at the target sites in organisms, which is the critical parameter for toxicity (Fig. 1). Ecotoxicological research on selected pollutants requires an interdisciplinary effort, considering physicochemical, molecular, toxicological, physiological and ecological processes. Whereas practical aspects of ecotoxicology are mainly focused on regulatory issues (registration of chemicals), and thus to testing of chemicals in standardized tests, the focus of ecotoxicological research is aimed at an understanding of toxicological phenomena in a variety of biota, populations and the ecosystem as whole. Thereby, diverse aspects such as mechanisms of toxic action and ecological processes in contaminated systems are regarded (Fent, 2003).

Ecotoxicological studies may also focus on ecological and toxicological effects observed in the field in retrospective studies, whereby a causative correlation between effects and chemical residue analysis is, however, often difficult to establish. Ecological investigations such as biomonitoring studies alone do not have sufficient resolving power to identify causative agents. Likewise, chemical analysis of pollutants in ecosystems alone cannot provide evidence for toxicological consequences in biota. Only an integrated approach considering environmental chemical, toxicological and ecological concepts may be suitable for understanding ecotoxicological effects in contaminated ecosystems (Fent, 2003). One strategy to at least assess the contamination and its potential effects is the use of biomarkers in ecological surveys to

verify the bioavailability and presence of relevant concentrations in biota (Bucheli and Fent, 1996). A selection of the type of biomarker allows a gross discrimination between certain groups of contaminants, e.g. polyaromatic and dioxin-like pollutants versus heavy metals, as well as toxicological mechanisms or biological functions affected, e.g. genotoxicity versus neurotoxicity or reproductive effects. A more prospective approach is based on investigations of potential toxicological effects in laboratory assays that may be used

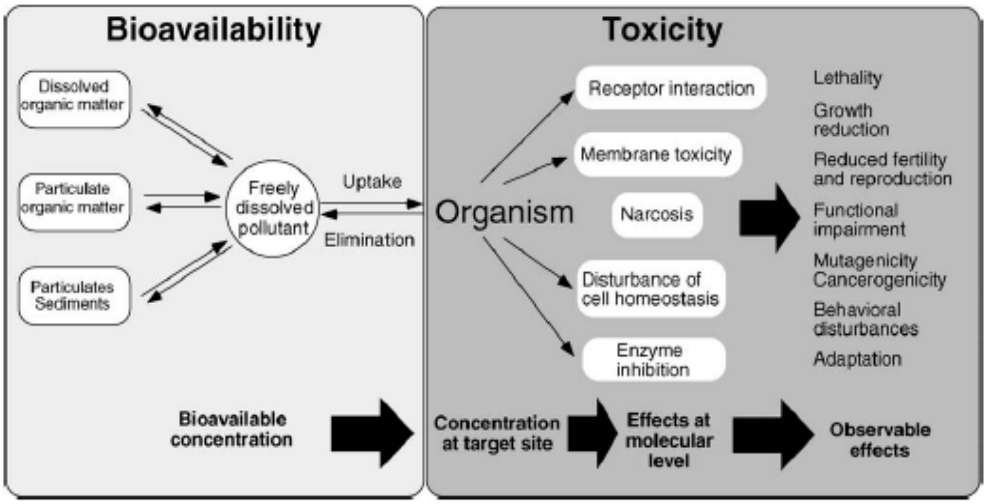


Fig. 1. Ecotoxicological effects are dependent on the bioavailable fraction of pollutants. Concentrations at the target sites induce molecular effects that propagate to a variety of toxic manifestations in organisms.

for extrapolation to the field. Bioassays play an important role in this process, however, more comprehensive studies on contaminated systems and ecotoxicological processes are needed in addition. Often, bioassays do not consider the processes in ecosystems, and neglect environmental factors that influence toxicity. However, they are valuable tools in the characterisation of the toxic action of chemicals, and in the understanding the associated toxicity. Despite the usefulness of these tools, it should be noted that the multitude of chemicals in ecosystems, species diversity, biological and ecological functions and structures makes extrapolations necessary for estimating possible effects in ecosystems. An important task therefore is the improvement of the predictive power and quality of experimental systems and risk assessment models.

In ecotoxicological research cellular effect studies including knowledge of mechanisms of toxic action are as important as studies in laboratory species,

because the primary interaction between chemicals and biota occurs at the surface of or in cells (Fig. 1). Whether chemical-induced alterations in cell structure and physiology will develop into an adverse toxic effect depends on many parameters, including adaptive responses. A cellular effect is often, but not necessarily, deterministic for adverse effects at higher levels of biological organization. Factors such as compensatory mechanisms and the presence of indirect effects may influence the relevance of the cellular toxicological response for overall ecotoxicological effects of a given chemical. The relation between cellular toxicological responses to toxicity at higher biological levels is a key question in ecotoxicology. The hypothesis that cellular changes may ultimately influence biological parameters important for populations such as growth, development, health, and reproduction is obvious. Hence, cellular toxicology provides an essential concept in understanding ecotoxicological processes, as it plays a key role in elucidating toxic modes of action, and diagnoses toxicological effects at higher biological levels, but it is not the only sufficient one. Its value will be strongly increased, when it can be integrated more closely with ecological effects. Here, the applicability of in vitro cell systems for the assessment of contaminated sites with pollutants having different toxicological modes of action such as general (narcotic) toxicity and dioxin-like toxicity is presented.

1.2. Ecological hazard and risk assessment

Whereas the ecotoxicological activity of single chemicals can reasonably be assessed by current standardized ecotoxicological tests, the evaluation of the ecotoxicological potential of contaminated sites is a much more difficult task. Environmental hazard posed by a contaminated site is a source of potential danger to humans and the environment. Hazard assessment refers to evaluation of inherent properties of contaminants at these sites to cause harm. Risk is defined as the probability that a hazard will be realized.

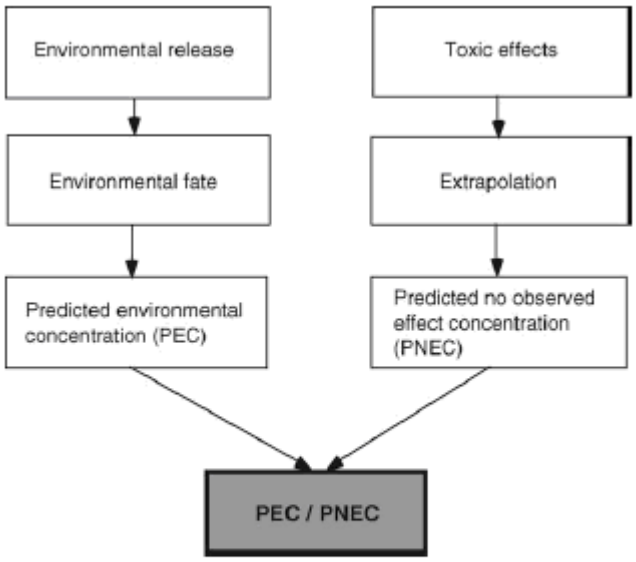


Fig. 2. Environmental risk assessment. PEC, predicted environmental concentration; PNEC, predicted no observed effect concentration. Risk analysis is necessary when PEC/PNEC >1. Adapted from ECETOC (1993).

In a first phase of an ecological risk assessment, in which sources and contaminants of potential concern are identified, toxicity testing is applied. In this regard, relatively simple ecotoxicity tests serve to identify hazard, and thus fit a first stage of an eco(toxico)logical risk assessment, the hazard identification. The aim is to search for potential causal relationships among contaminants, receptors and ecotoxicological endpoints. Further stages of risk assessment—exposure and effects assessment, then risk characterization require additional information and can include other tools such as measures of the health of residents at contaminated sites, exposed populations of animals and plants, longer-term laboratory or field bioassays, toxicity identification evaluations (TIE) approaches, etc. Thereby, analysis of the relationship between contaminants and both laboratory and field effects data is crucial. Finally, risk characterization builds upon the results of the analysis phase to develop an estimate of risk. Fig. 2 illustrates a concept, often applied in chemicals risk assessment, which can also be used for contaminated sites. Often, leachates from sediments, soil and groundwater at contaminated sites are being tested in whole effluent toxicity tests. Whole effluent toxicity and similar toxicity tests integrate interactions among complexmixtures of contaminants. They measure the total toxic effect, regardless of physical and chemical composition. This is a powerful integrative measure of the toxicity of chemicals not being achievable by analytical chemical measurements.

Usually, direct toxic effects on survival, growth or reproduction are being assessed using bacteria, algae or periphyton, water flea and fish. Although these tests have limitations and disadvantages such as not necessarily being environmentally realistic, they give important hints to the ecotoxicological potential of contaminated environmental media and can be adjusted based on site- and situation-specific conditions to be more predictive.

Often, however, there is a discrepancy between results derived from both standardized laboratory tests and whole effluent tests compared to known biological impacts. For example, a set of 250 discharges from contaminated river systems across the United States were tested in standardized Daphnia and fathead minnows tests, whole effluent toxicity tests, and instream biological condition as measured by benthic macroinvertebrate assessments (species composition and abundance). The results indicated that whole effluent toxicity testing was more predictive of biological effects in the rivers, if several tests addressing different types of endpoints were used (Diamond and Daley, 2000). Fish acute and chronic endpoints were most related to instream condition, but no one endpoint was capable of accurately reflecting conditions of all discharges. This indicates that whole effluent toxicity testing is a tool, which is useful for ecotoxicological evaluation of contaminated sites, but as all tools, has its limitations and imperfection. They include test variability, species differences and extrapolations from laboratory to the field. For instance, it was found that whole effluent tests underestimated field effects (Clements and Kiffney, 1994), in other cases these tests were good predictors of fish response, but were poor predictors of invertebrate response (Birge et al., 1989). With regard to reliability in predicting biological community responses laboratory single-species toxicity tests are suggested, in a majority of cases, reliable qualitative predictors. The significance will be increased when a variety of different assays using different biota of diverse evolutionary levels and ecological function are used.

Laboratory species are generally not the same as the resident species in the field aimed at protecting. Despite this fact, risk assessment is based on laboratory tests for mainly practical reasons (Burton, 1992; Linthurst set al., 1995). Extrapolating effects of toxicants from a limited number of test species to ecosystems as a whole is a difficult, but essential part of environmental risk assessment (Koller et al., 2000). If toxicity test results are available for very few species, the lowest toxicity value is divided by an application factor or safety factor that varies from 10 to 1000, depending on the number of species tested and whether the endpoint is based on acute mortality or effects (LC50 or EC50), or chronic no-observed-effect

concentrations (NOEC). A comparison of 248 studies on 34 substances involving both model ecosystems studies and chronic single-species studies indicate that an assessment factor of 8 would be appropriate in the extrapolation from the lowest chronic singe-species NOEC-value in a model ecosystem (ECETOC, 1993). Applying safety factors in the range of 10–1000 for extrapolation from the laboratory to field is therefore appropriate. Thisrisk assessment concept using the ratio between predicted exposure concentration (PEC) and predicted no observed effect concentration (PNEC), as outlined in Fig. 2, is widely accepted (EC, 1994, 1996; ECETOC, 1993). However, it has also its limitations and results of such analyses should be judged and interpreted with necessary caution with respect to ecological relevance (Koller et al., 2000). Analyses using ecotoxicological tests with laboratory species may provide an uncertain level of protection for several reasons. First, abiotic and biotic factors influence the bioavailability and toxicity of contaminants. Often, contaminant concentrations vary in time, which is not regarded in laboratory tests. Second, mixtures of a set of contaminants as generally found in contaminated sites may show different toxicity compared to single compounds. Third, bioconcentration and bioaccumulation in food webs is not regarded. Fourth, they do not reflect effects on populations or community responses and may differ from the situation in the field. In contaminated sites, adaptations of biota to contaminants can also occur. In general, adaptation has ecological costs such as energy consumption, which may reduce the organism's fitness. Adaptive reactions include actively pumping out incoming toxicants, detoxification mechanisms, damage repair and avoidance of contamination (Hansen et al., 1999).

1.3 General definitions of terms

Of a single dose of a substance.

(i) **Acute toxicity** comprises the adverse effects occurring within a given time (usually 14 days), after administration of a single dose of a substance.

(ii)**Evident toxicity** is a general term describing clear signs of toxicity following administration of test substance. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

(iii) **Dose** is the amount of test substance administered. Dose is expressed as weight (grams or milligrams) or as weight of test(i) **Acute toxicity** comprises the adverse effects occurring within a given time (usually 14 days), after administration substance per unit weight of test animal (e.g. milligrams per kilogram body weight), or as constant dietary concentrations (parts per million or milligrams per kilogram of food).

(iv) **Discriminating dose** is the highest out of the four fixed dose levels which can be administered without causing
compound-related mortality (including human kills).

(v) **Dosage** is a general term comprising of dose, its frequency and the duration of the dosing.

(vi) **LD50** (median lethal dose) is a statistically derived single dose of a substance that can be expected to cause death in 50 % of dosed animals. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram).

(vii) **LC50** (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during exposure or within a fixed time after exposure in 50 % of animals exposed for a specified time. The LC50 value is expressed as weight of test substance per standard volume of air (milligrams per litre).

(viii) **NOAEL** is the abbreviation for no observed adverse effect level and is the highest dose or exposure level where no adverse treatment-related findings are observed.

(ix) **Repeated dose/Sub-chronic toxicity** comprises the adverse effects occurring in experimental animals as a result of repeated daily dosing with, or exposure to, a chemical for a short part of their expected life-span.

(x) **Maximum Tolerated Dose** (MTD) is the highest dose level eliciting signs of toxicity in animals without having major effects on survival relative to the test in which it is used.

(xi) **Skin irritation** is the production of inflammatory changes in the skin following the application of a test substance.

(xi) **Eye irritation** is the production of changes in the eye following the application of a test substance to the anterior surface of the eye.

(xiii) **Skin sensitisation** (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance.

(xiv) **Dermal corrosion** is the production of irreversible tissue damage in the skin following the application of a test substance for the duration period of 3 minutes up to 4 hours.

(xv) **Toxicokinetics** is the study of the absorption, distribution, metabolism and excretion of test substances.

(xvi) **Absorption** is the process(es) by which an administered substance enters the body.

(xvii) **Excretion** is the process(es) by which the administered substance and/or its metabolites are removed from the body.

(xviii) **Distribution** is the process(es) by which the absorbed substance and/or its metabolites partition within the body.

(xix) **Metabolism** is the process(es) by which the administered substances are structurally changed in the body by either enzymatic or non enzymatic reactions.

1.4 Toxicity testing and identification

Biological systems exhibit great variability in their response to external influences such as exposure to a chemical. Even with a complete and adequate data set based on human tests, as provided for pharmaceutical products, it is impossible to predict the precise influence a chemical will have on each and every member of an exposed human population. Therefore, the aim of most toxicological testing is not to arrive at the best estimate of the magnitude of any risk but rather to determine whether or not there is sufficient reassurance of little or negligible risk under the relevant exposure situation.

Toxicity tests include assessments of acute toxicity, epithelial (skin) irritation and corrosion, immunological sensitisation, and toxicity expressed only on repeated dosing. Assessments are also made of the ability of chemicals to cause cancers (*carcinogenicity*), to induce permanent transmissible genetic changes (*mutagenicity* or *genotoxicity*), or to cause foetal abnormalities (*teratogenicity*). Other important manifestations of chemical toxicity investigated include damage to the nervous system (*neurotoxicity*), adverse effects on reproduction (*reprotoxicity*), and skin reactions on exposure to light (*phototoxicity*). (See appendix D for a list of toxicity tests required by EU regulations.)

Toxicity involving any system may manifest within hours or days (acute) or take weeks, months or the organism's lifetime (chronic). Toxicity tests are classified according to:

- the length of dosing – from acute studies lasting up to a few days to chronic studies spanning the lifetime of a test species (18-30 months in rodents);
- the route of administration – ingested, intravenous, oral, dermal, ocular or inhaled; and
- the end-point being studied – which may be death, appearance of a tumour, effects on reproduction or development, allergic sensitisation, or a neurotoxic or behavioural effect.

Where the end-point of a toxicity test is death, the results are expressed as either the *median lethal concentration* or LC50 (the concentration that brings about the death of 50% of the individuals in a test population) or the *median lethal dose* or LD50 (the single dose that brings about 50% mortality) within the duration of the test. On their own, these measures provide only a very rough idea of the relative toxicity of substances and the actual lethal dose will depend heavily on both the target species and the local environmental conditions. LC50 and LD50 data are rarely, if ever, the sole basis for regulation.

The results of tests for sub-lethal effects of a substance are expressed as the *median effective concentration* (EC50) or the *median effective dose* (ED50), the concentration or dose which, in a given time under given conditions, causes 50% of the test population to exhibit a particular response or in some cases, causes a 50% change in a specified response relative to unexposed controls.

A: Methods for the determination of toxicity

A.1bis ACUTE ORAL TOXICITY - FIXED DOSE PROCEDURE
A.1tris ACUTE ORAL TOXICITY - ACUTE TOXIC CLASS METHOD
A.2 ACUTE TOXICITY (INHALATION)
A.3 ACUTE TOXICITY (DERMAL)
A.4 ACUTE TOXICITY: DERMAL IRRITATION/CORROSION
A.5 ACUTE TOXICITY: EYE IRRITATION/CORROSION
A.6 SKIN SENSITIZATION
A.7 REPEATED DOSE (28 DAYS) TOXICITY (ORAL)
A.8 REPEATED DOSE (28 DAYS) TOXICITY (INHALATION)
A.9 REPEATED DOSE (28 DAYS) TOXICITY (DERMAL)
A.10 MUTAGENICITY - IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST)
A.11 MUTAGENICITY - IN VIVO MAMMALIAN BONE-MARROW CHROMOSOME ABERRATION TEST
A.12 MUTAGENICITY MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST
A.13/14 MUTAGENICITY - REVERSE MUTATION TEST USING BACTERIA
A.15 GENE MUTATION - *SACCHAROMYCES CEREVISAE*
A.16 MITOTIC RECOMBINATION - *SACCHAROMYCES CEREVISAE*
A.17 MUTAGENICITY - IN VITRO MAMMALIAN CELL GENE MUTATION TEST
A.18 DNA DAMAGE AND REPAIR - UNSCHEDULED DNA SYNTHESIS - MAMMALIAN CELLS *IN VITRO*
A.19 SISTER CHROMATID EXCHANGE ASSAY *IN VITRO*
A.20 SEX-LINKED RECESSIVE LETHAL TEST IN *DROSOPHILA MELANOGASTER*
A.21 *IN VITRO* MAMMALIAN CELL TRANSFORMATION TEST
A.22 RODENT DOMINANT LETHAL TEST
A.23 MAMMALIAN SPERMATOGONIAL CHROMOSOME ABERRATION TEST
A.24 MOUSE SPOT TEST
A.25 MOUSE HERITABLE TRANSLOCATION
A.26 SUB-CHRONIC ORAL TOXICITY TEST. REPEATED DOSE 90 - DAY TOXICITY STUDY IN RODENTS
A.27 SUB-CHRONIC ORAL TOXICITY TEST: REPEATED DOSE 90 - DAY TOXICITY STUDY IN NON-RODENTS
A.28 SUB-CHRONIC DERMAL TOXICITY TEST: 90-DAY REPEATED DERMAL DOSE STUDY USING RODENT SPECIES
A.29 SUB-CHRONIC INHALATION TOXICITY TEST: 90-DAY REPEATED INHALATION DOSE STUDY USING RODENT SPECIES
A.30 CHRONIC TOXICITY TEST
A.31 TERATOGENICITY TEST – RODENT AND NON-RODENT
A.32 CARCINOGENICITY TEST
A.33 COMBINED CHRONIC TOXICITY/CARCINOGENICITY TEST
A.34 ONE-GENERATION REPRODUCTION TOXICITY TEST

A.35 TWO GENERATION REPRODUCTION TOXICITY TEST
A.36 TOXICOKINETICS
A.37 DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES FOLLOWING ACUTE EXPOSURE
A.38 DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES 28 DAY REPEATED DOSE STUDY
A.39 UNSCHEDULED DNA SYNTHESIS (UDS) TEST WITH MAMMALIAN LIVER CELLS *IN VIVO*
A.40 SKIN CORROSION (IN VITRO)
A.41 PHOTOTOXICITY - *IN VITRO* 3T3 NRU PHOTOTOXICITY TEST
A.42 SKIN SENSITISATION: LOCAL LYMPH NODE ASSASY
A.43 NEUROTOXICITY STUDY IN RODENTSGENERAL INTRODUCTION

B: Methods for the determination of ecotoxicity

B.1 ACUTE TOXICITY FOR FISH
B.2 ACUTE TOXICITY FOR DAPHNIA
B.3 ALGAL INHIBITION TEST
B.4 BIODEGRADATION: DETERMINATION OF THE "READY" BIODEGRADABILITY
B.4-A DISSOLVED ORGANIC CARBON (DOC) DIE-AWAY TEST
B.4-B MODIFIED OECD SCREENING TEST
B.4-C CARBON DIOXIDE EVOLUTION TEST
B.4-D MANOMETRIC RESPIROMETRY TEST
B.4-E CLOSED BOTTLE TEST
B.4-F MITI TEST
B.5 DEGRADATION : BIOCHEMICAL OXYGEN DEMAND
B.6 DEGRADATION: CHEMICAL OXYGEN DEMAND
B.7 DEGRADATION: ABIOTIC DEGRADATION: HYDROLYSIS AS A FUNCTION OF PH
B.8 TOXICITY FOR EARTHWORMS : ARTIFICIAL SOIL TEST
B.9 BIODEGRADATION: ZAHN - WELLENS TEST
B.10 BIODEGRADATION: ACTIVATED SLUDGE SIMULATION TEST
B.11 BIODEGRADATION: ACTIVATED SLUDGE RESPIRATION INHIBITION TEST
B.12 BIODEGRADATION: MODIFIED SCAS TEST
B.13 BIOCONCENTRATION: FLOW-THROUGH FISH TEST
B.14 FISH JUVENILE GROWTH TEST
B.15 FISH, SHORT-TERM TOXICITY TEST ON EMBRYO AND SAC-FRY STAGES
B.16 HONEYBEES - ACUTE ORAL TOXICITY TEST
B.17 HONEYBEES - ACUTE CONTACT TOXICITY TEST
B.18 ADSORPTION/DESORPTION USING A BATCH EQUILIBRIUM METHOD
B.19 ESTIMATION OF THE ADSORPTION COEFFICIENT (KOC) ON SOIL AND ON SEWAGE SLUDGE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

(HPLC)
B.20 DAPHNIA MAGNA REPRODUCTION TEST
B.21 SOIL MICROORGANISMS: NITROGEN TRANSFORMATION TEST
B.22 SOIL MICROORGANISMS: CARBON TRANSFORMATION TEST
B.23 AEROBIC AND ANAEROBIC TRANSFORMATION IN SOIL
B.24 AEROBIC AND ANAEROBIC TRANSFORMATION IN AQUATIC SEDIMENT SYSTEMS

1.4.1 Acute toxicity - repeated dose

The acute toxic effects and organ or system toxicity of a substance may be evaluated using a variety of toxicity tests (Methods A.1 -A.5) from which, following a single dose, a preliminary indication of toxicity may be obtained. Dependant on the toxicity of the substance, a limit test approach or a full LD50 may be considered, although no limit test is specified in inhalation studies, because it has not been possible to define a single inhalation exposure limit value. Consideration should be given to methods, which use as few animals as possible and minimise animal suffering, for example the fixed dose method (Method A.1 bis) and acute toxic class (Method A.1 tris). In level 1 testing, a study in a second species may complement the conclusions drawn from the first study. In this case, a standard test method may be used or the method may be adapted for a smaller number of animals. The repeated dose toxicity test (Methods A.7, A.8 and A.9) includes assessment of toxic effects arising from repeated exposure. The need for careful clinical observations of the animals is stressed, so as to obtain as much information as possible. These tests should help to identify the target organs of toxicity and the toxic and toxic doses. Further in depth investigation of these aspects may be required in long term studies (Methods A.26 - A.30 and A.33).

1.4.2 Chronic toxicity

In ecotoxicological assessments, abbreviated chronic assays, such as the early life stage test in the aquatic environment and a variety of screening tests for mutagenicity and teratogenicity, can be used to assess chronic toxicity. But it is more usual to use a relationship known as the *acute to chronic ratio* to extrapolate between short-term and long-term results. The acute to chronic ratio is determined by dividing the acute LC50 by a measure of chronic toxicity such as the *maximum acceptable toxic concentration* , the geometric mean of the no effects and the low effects concentrations. The acute to chronic ratio is then used to extrapolate between different species and different chemicals to give an extremely crude estimate of chronic toxicity.

1.4.3 Mutagenicity

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes, 'mutations', may involve a single gene or gene segments, a block of genes, or whole chromosomes. Effects on whole chromosomes may be structural and / or numerical. The mutagenic activity of a substance, for the base set information, is assessed by *in vitro* assays for gene (point) mutations in bacteria (Method A.13/14) and for structural chromosome aberrations in mammalian cells, (Method A.10).

Acceptable are also *in vivo* procedures, e.g. the micronucleus test (Method A.12) or the metaphase analysis of bone marrow cells, (Method A.11). However, in the absence of any contraindication the *in vitro* methods are strongly preferred. Additional studies to investigate mutagenicity further or to pre-screen for carcinogenicity are required for higher production volumes and/or to conduct or follow-up a risk assessment, and these can be used for a number of purposes: to confirm results obtained in the base set; to investigate end-points not studied in the base set; to initiate or extend *in vivo* studies. For these purposes, methods A.15 to A.25 include both *in vivo* and *in vitro* eukariotic systems and an extended range of biological end-points. These tests provide information on point mutations and other end-points in organisms more complex than the bacteria used for the base set. As a general principle, when a programme of further mutagenicity studies is considered, it should be designed so as to provide relevant additional information on the mutagenic and/or carcinogenic potential of that substance. The actual studies which may be appropriate in a specific instance will depend on numerous factors, including the chemical and physical characteristics of the substance, the results of the initial bacterial and cytogenetic assays, the metabolic profile of the substance, the results of other toxicity studies, and the known uses of the substance. A rigid schedule for selection of tests is therefore inappropriate in view of the variety of factors which may require consideration. Some general principles for the testing strategy are laid down by Dir. 93/67/EEC, but clear testing strategies may be found in the technical guidance document for Risk Assessment, which nevertheless is flexible and can be adapted as appropriate to specific circumstances.

Methods for further investigation are however grouped below, on the basis of their principal genetic end-point:

Studies to investigate gene (point) mutations

a) Forward or reverse mutation studies using eukaryotic micro-organisms (*Saccharomyces cerevisiae*) (Method A.15)

b) *In vitro* studies to investigate forward mutation in mammalian cells, (Method A.17)

c) The sex-linked recessive lethal assay in *Drosophila melanogaster*, (Method A.20)

d) *In vivo* somatic cell mutation assay, the mouse spot test, (Method A.24)

Studies to investigate chromosome aberrations

a) *In vivo* cytogenetic studies in mammals; *In vivo* metaphase analysis of bone marrow cells should be considered if it

has not been included in the initial assessment (Method A.11). In addition, *in vivo* germ cell cytogenetics may be

investigated, (Method A.23)

b) *In vitro* cytogenetic studies in mammalian cells, if this has not been included in the initial assessment, (Method A.10)

c) Dominant lethal studies in rodents, (Method A.22)

d) Mouse heritable translocation test, (Method A.25)

1.4.4 Genotoxic effects - effects on DNA

Genotoxicity, identified as harmful effects on genetic material not necessarily associated with mutagenicity, may be indicated by induced damage to DNA without direct evidence of mutation. The following methods using eukaryotic micro-organisms or mammalian cells may be appropriate for such investigation:

a) Mitotic recombination in *Saccharomyces cerevisiae,* (Methods A.16)

b) DNA damage and repair - unscheduled DNA synthesis - mammalian cells - *in vitro*, (Method A.18)

c) Sister chromatid exchange in mammalian cells - *in vitro*, (Method A.19)

1.4.5 Carcinogenicity

Chemicals may be described as genotoxic or non-genotoxic carcinogens, dependant on the presumed mechanism of action. Pre-screening information for genotoxic carcinogenic potential of a substance may be obtained from the mutagenicity/genotoxicity studies. Additional information may be obtained from the repeated dose, subchronic or chronic toxicity tests. The repeated dose toxicity test, Method A.7 and longer repeated dose studies include assessment on histopathological changes observed in repeated dose toxicity

tests, e.g. hyperplasia in certain tissues which could be of concern. These studies and toxicokinetic information may help to identify chemicals with carcinogenic potential, which may require further in-depth investigation of this aspect, in a carcinogenicity test (Method A.32) or often in a combined chronic toxicity/carcinogenicity study (Method A.33)

1.4.6 Reproductive Toxicity

Reproductive toxicity may be detected in different ways e.g. impairment of male and female reproductive functions or capacity, identified as 'effects on fertility', or induction of non-inheritable harmful effects on the progeny, identified as 'developmental toxicity' where teratogenicity and effects during lactation are also included. For teratogenicity studies, as part of the developmental toxicity testing, the test method (Method A.31), is primarily directed to administration by the oral route. Alternatively, other routes may be used depending on the physical properties of the test substance or likely route of human exposure. In such cases, the test method should be suitably adapted taking into consideration the appropriate elements of the 28-day test methods. Where a three-generation reproduction (fertility) test is required, the described method for the two-generation reproduction test (Method A.35), can be extended to cover a third generation.

1.4.7 Neurotoxicity

Neurotoxicity may be detected in different ways e.g. functional changes and/or structural and biochemical changes in the central or peripheral nervous system. A preliminary indication of neurotoxicity may be obtained from acute toxicity tests. The repeated dose toxicity test, Method A.7, includes assessment of neurotoxicological effects, and the need for careful clinical observations of the animals is stressed, so as to obtain as much information as possible. The method should help to identify chemicals with neurotoxic potential, which may require further in-depth investigation of this aspect. Additionally, it is important to consider the potential of substances to cause specific neurotoxic effects that may not be detected in other toxicity studies. For example, certain organophosphorous substances have been observed to cause delayed neurotoxicity and can be evaluated in methods A.37 and A.38, following single or repeated-dose exposure.

2. PCBs & CBAs

2.1 Properties of PCBs

Polychlorinated biphenyls (PCBs) are made up of a biphenyl nucleus with 1–10 chlorine atoms having a chemical formula of $C_{12}H_{10-n}Cl_n$. The basic structure of PCBs is given by Wiegel and Wu and is shown in Fig. 3.

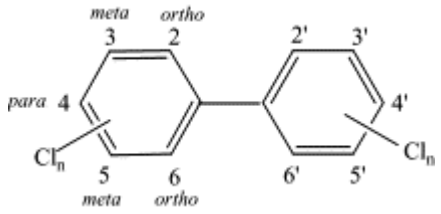


Fig. 3. Structural formula of PCB

Their manufacture produces a mixture of compounds with molecular weight ranging from 188 to 439.7 depending on the number of chlorine atoms attached to the biphenyl ring. Toxic congeners carry between 5 and 10 chlorine atoms, mostly in the *para*- and *meta*-positions , however, the congener substituted at the 3,4-*ortho* positions are considered the most toxic.

The properties of each PCB congener are dependent on the degree of chlorination. The industrially produced PCBs have properties ranging from highly mobile liquids that are colourless and oily to more viscous and increasingly darker liquids, to yellow and then black resins. Lower chlorinated PCBs (the mono-, di-, tri-, and tetra-chlorinated PCBs) tend to be colourless oily liquids. Pentachlorobiphenyls are heavy, honey-like oils. The most highly chlorinated PCBs are greases and waxy substances. Flash points can be as low as 140 °C to 200 °C; however, most have no flash points at all as measured by standard test. The vapour is invisible and has a characteristic strong odour.

PCBs are poorly soluble in water but extremely soluble in oils and fats. Their solubility in water decreases with increase in the degree of chlorination. It ranges from 6 ppm for monochlorobiphenyl to 0.007 ppm for octachlorobiphenyl. Decachlorobiphenyl, although it has higher chlorine content, has solubility twice that of octachlorobiphenyl. The solubility also varies among congeners of the same number of chlorine atoms.

The properties of PCBs that made them valuable for industrial applications include thermal stability, chemical inertness, non-flammability, high electrical resistivity or high dielectric constant and low acute toxicity.

2.2 Uses of PCBs

For several decades PCBs were used extensively in a wide range of industrial applications such as: oil in transformers, dielectrics in capacitors, hydraulic fluids in hydraulic tools and equipment and heat exchange liquids. PCBs also found widespread use as lubricants for turbines and pumps, in the formulation of cutting oils for metal treatment, and, to a lesser extent, in applications such as plasticizers, surface coatings, adhesives, pesticides, carbonless copy paper, inks, dyes, and waxes.

2.3 Sources of PCBs

There are no known natural sources of PCBs. They persist in the environment and are found in air, water, soil, and food. PCBs entered the air, water, and soil during their manufacture, use, and disposal; from accidental spills and leaks during their transport; and from leaks or fires in products containing PCBs. PCBs can travel long distances in the air and can be deposited in areas far away from where they were released. Municipal waste combustion, hazardous waste incineration, and medical waste incineration account for a significant portion of PCB emissions to air. Additional sources of PCB emissions include treatment, storage, and disposal facilities and landfills; hazardous waste sites; steel and iron reclamation facilities (e.g., auto scrap burning); accidental releases (PCB spills and leaks, and transformer fires); and environmental sinks of past PCB contamination.

In water, PCB concentrations are generally higher near human activity and near shorelines. The major source of PCBs in surface waters is from environmental cycling (i.e., from sediment, air and land). Sediments at the bottom of a water body can act as a reservoir from which PCBs can be released in small amounts to water. PCBs in fish can be hundreds of thousands of times higher than in water because they accumulate in the fish.

Polychlorinated biphenyls (PCBs) attach strongly to soil and may remain there for several years. Environmental cycling is suspected of being the current major source of PCBs in soil outside of disposal and spill sites. Another source of PCB exposure is the workplace. Workplace exposure can occur during repair and maintenance of PCB transformers, accidents, fires,

spills, or disposal of PCB containing material by breathing contaminated air and touching materials containing PCBs. Old appliances and electrical equipment are also believed to be the primary source of household contamination, since they may contain PCBs. PCB levels in indoor air are often much higher than in outdoor air.

2.4 Health and environmental effects of PCBs

Polychlorinated biphenyls (PCBs) have low-to-moderate toxicity. Treated samples of animals show an LD_{50} ranging from 0.5 g/kg to 11.3 g/kg of body weight. Most of the effects are the result of repetitive or chronic exposure.

Polychlorinated biphenyls (PCBs) are absorbed by humans and animals through the skin, the lungs, and the gastrointestinal tract. Once inside the body, they are transported through the blood stream to liver, to various muscles and adipose tissue where they accumulate. Research shows that PCBs cause a variety of adverse health effects depending on the route of exposure, age, sex, and area of the body where PCBs are concentrated. Studies on animals show conclusive evidence that PCBs are carcinogenic. Animals that ate food containing large amount of PCBs for short periods of time had mild liver damage and some died. PCBs have also been implicated as a cause of mass mortalities in seabirds. Environmental concerns over PCBs first surfaced in the late 1960s, some 30 years after PCBs were introduced. A Swedish scientist found eggshell thinning among seabirds due to bioaccumulation of PCBs, leading to reduced reproductive capacity. PCBs have anti-estrogen properties that can inhibit calcium deposition during eggshell development, leading to insufficiently strong shells and premature loss. Anti-estrogen effects of PCBs may lead to adverse effects on male reproductive capabilities of birds and animal species.

In addition to animal studies, a number of epidemiological studies of workers exposed to PCBs have been performed. Results of human studies show that PCBs are probable carcinogens. PCB workers were found to have increases in rare liver cancers and malignant melanoma. The presence of cancer in the same target organ (liver) was found in both humans and animals exposed to PCBs. Research also shows that exposure to PCBs in high concentration can have various acute effects that include a skin disease known as chloracne (skin lesions), liver damage including clinical hepatitis; other non-cancer short-term effects like body weight loss, impaired immune function; and clinically diagnosable damage to the central nervous system, causing headaches, dizziness, depression, nervousness, and fatigue. Other

adverse health effects of PCBs are liver, stomach and thyroid gland injuries, behavioural alterations, and impaired reproduction.

Polychlorinated biphenyls (PCBs) can affect the productivity of phytoplankton and the composition of phytoplankton communities. Phytoplankton is the primary food source of all sea organisms and a major source of oxygen in the atmosphere. The transfer of PCBs up the food chain from phytoplankton to invertebrates, fish, and mammals can result in human exposure through consumption of PCB-containing food source.

2.5 Metabolic products of PCBs in bacteria and plants

Microbial and plant species may posses enzymes capable of metabolizing certain environmentally persistent xenobiotics that contaminate soil and water. It has been shown that some bacteria metabolize different organic molecules including highly persistent polychlorinated biphenyls. Some plant species have been described to have also ability to metabolise PCBs and polyaromatic hydrocarbons. Generally, plant metabolism has been studied to a lesser extent than that in bacteria or mammals and little formation is available. Not much is known about the intermediates in plans, their toxicity and the effect, which such compounds may have on animals and other organisms. In plants, organic compounds are in the first phase transformed into more reactive ones, then conjugated with sugars, amino acids, etc. to less phytotoxic ones and deposited in vacuoles or lignified parts of the cell wall. Unfortunately this fact does not mean that metabolites or products have lower toxicity towards other living systems.

Different systems for ecotoxicity measurement were evaluated during the last fifteen years. Many of them are based on measurement of viability of different organisms and their ability to survive in presence of different toxicants. Bacteria and plants are mainly involved in transformation of toxic compounds in nature and they are responsible for the further fate of those xenobiotics and their intermediates in the environment.

The main products of bacterial degradation of PCBs are chlorobenzoic acids, which can be further degraded by other bacteria present in contaminated environment.

Analysis of the products of bacterial and plant metabolism has shown that plant products (hydroxychlorobiphenyls) of the first phase of PCBs transformation are similar to those detected in mammalian cells. While some bacteria further degrade PCBs to chlorobenzoic acids, plants are generally

unable to open the ring and degrade the chemical structure of biphenyl. To better understand the fate of PCBs (and all other toxicants) in the environment, the cooperation of plants and microorganisms living in the rhizosphere in contaminated soil should be evaluated. The ability of plant enzymes to further metabolize the products of bacterial PCB degradation (the chlorobenzoic acids) has been followed as well as the ability of bacterial enzymes to transform the plant formed hydroxychlorobiphenyls.

Commercial PCBs were produced as mixtures of different congeners varying in their degree of chlorination. The simplest PCBs are monochlorobiphenyls which are usually not present in commercial mixtures, but they can be formed in the natural environment by microbial dechlorination or degradation of more highly chlorinated congeners.

2.5.1 Biological transformation of PCBs

Organisms may modify organic pollutants such as PCBs in such a way that the negative effects are minimised. Microorganisms participate in the biodegradation by producing enzymes, which modify the organic pollutant into simpler compounds. Biodegradation is of two forms, mineralization and co-metabolism and. In mineralization, competent organisms use the organic pollutant as a source of carbon and energy resulting in the reduction of the pollutant to its constituent elements. Co-metabolism, on the other hand, requires a second substance as a source of carbon and energy for the microorganisms but the target pollutant is transformed at the same time. If the products of co-metabolism are amenable to further degradation they can be mineralized, otherwise incomplete degradation occurs. This may result in the formation and accumulation of metabolites that are more toxic than the parent molecule requiring a consortium of microorganisms, which can utilize the new substance as source of nutrients.

The effectiveness of biodegradation depends on many environmental factors. Rates vary depending on the conditions present in the environment. These factors include the structure of the compound, the presence of exotic substituents and their position in the molecule, solubility of the compound and concentration of the pollutant. For aromatic halogenated compounds, a high degree of halogenation requires high energy by the microorganisms to break the stable carbon–halogen bonds. Chlorine as the substituent alters the resonant properties of the aromatic substance as well as the electron density of specific sites. This may result in the deactivation of the primary oxidation of the compound by microorganisms. Additionally, the positions occupied by

substituted chlorines have stereochemical effects on the affinity between enzymes and their substrate molecule.

The water solubility of the compound has a vital role in its degradation. Compounds with high aqueous solubility are easily accessed by microorganisms than those with low solubility. For PCBs, highly chlorinated congeners are very insoluble in water. This could account for the resistance of highly chlorinated PCB congeners to biodegradation.

Pollutant concentration is also a major factor affecting biodegradation. In general, a low pollutant concentration may be insufficient for the induction of degradative enzymes or to sustain growth of competent organisms. On the other hand, a very high concentration may render the compound toxic to the organisms. At a low concentration range, degradation increases linearly with increase in concentration until such time that the rate essentially becomes constant regardless of further increase in pollutant concentration.

Other environmental factors affecting degradation are temperature, pH, presence of toxic or inhibitory substance and competing substrates, availability of suitable electron acceptors, and interactions among microorganisms. All these factors interplay and make the rates of biodegradation unpredictable.

The use of microorganisms, both anaerobic and aerobic, is the only known process to degrade PCBs in soil systems or aquatic environments. Anaerobic bacteria possess characteristics that are well adapted to pollutants with high carbon concentration because of the diffusional limitation of oxygen in high concentration systems. The environment of anaerobes is conducive to reductive transformations where chlorine is displaced by hydrogen. The dechlorinated compound is suitable for the oxidative attack of the aerobic bacteria. Aerobic bacteria grow faster than anaerobes and can sustain high degradation rates resulting in mineralization of the compound. Theoretically, the biological degradation of PCBs should result to give CO_2, chlorine, and water. This process involves the removal of chlorine from the biphenyl ring followed by cleavage and oxidation of the resulting compound.

2.5.2 Anaerobic transformation of PCBs

Anaerobic transformation of chlorinated organic compounds involves reductive dehalogenation where the halogenated organic compound serves as the electron acceptor, the halogen substituent is replaced with hydrogen.

$$R\text{—}Cl + 2e^- + H^+ \rightarrow R\text{—}H + Cl^-$$

Electron acceptors are generally the factors limiting metabolism in anaerobic environments. Thus, any microorganism that could use PCBs as terminal electron acceptors would be at a selective advantage.

Anaerobic dechlorination can attack a large array of chlorinated aliphatic and aromatic hydrocarbons. Several anaerobic dechlorinating bacteria have been isolated. These include *Desulfomonile tiedjei*, *Desulfitobacterium*, *Dehalobacter restrictus*, *Dehalospirillum multivorans*, *Desulforomonas chloroethenica*, *Dehalococcoides ethenogenes* and the facultative anaerobes *Enterobacter* strain MS-1 and *Enterobacter agglomerans*. Some of these microorganisms reductively dechlorinate the chlorinated compound in a co-metabolism reaction; others utilize the chlorinated compounds as electron acceptors in their energy metabolism. The characteristics common to dehalogenators are: (a) aryl reductive dehalogenation is catalyzed by inducible enzymes, (b) these enzymes exhibit distinct substrate specificity, (c) aryl dehalogenators function in syntrophic communities and may be dependent on such communities and, (d) aryl dehalogenators derive metabolic energy from reductive dehalogenation. Microorganisms with distinct dehalogenating enzymes each exhibit a unique pattern of congener activity.

Under anaerobic condition, reductive dechlorination of PCBs occurs in soils and sediments. Different microorganisms with distinct dehalogenating enzymes are responsible for different dechlorination activities and dehalogenation routes. The rate, extent, and route of dechlorination is dependent on the composition of the active microbial community, which in turn are influenced by environmental factors such as availability of carbon sources, hydrogen or other electron donors, the presence or absence of electron acceptors other than PCBs, temperature, and pH.

The enrichment of microorganisms from sediments with 2,3,4,5,6-pentachlorobiphenyls resulted in a sequential *meta*- and *para*-dechlorination of Aroclor 1260. The hexa- through nonachlorobiphenyls in the sediments was reduced from 66.3 mol% to only 16.7 mol% through *meta*-chlorine removal from serial transfers of actively dechlorinating slurries. The enrichment also fostered *para*-dechlorination that caused further conversion of the meta-dechlorination products to tri- and tetra-chlorobiphenyls.

Studies on the primary and enriching effect of several PCB congeners on the dechlorination of Aroclor 1260 are believed to be mediated by two distinct populations of PCB dechlorinators with different specificities. In addition, the primary congeners were good substrates for the respective

dehalogenases and support growth of the dechlorinators by acting as electron donors.

Studies on the effect of temperature on dechlorination show that temperature has a significant effect on the growth of the microorganisms and the catalytic activity of the enzymes. The investigation of the dechlorination of added 2,3,4,6-chlorobiphenyl and residual Aroclor 1260 in Woods Pond revealed that Aroclor 1260 was marginally dechlorinated at 8–34 °C and at 50–60 °C with an optimal temperature of 18–30 °C. Between 8–34 °C and 50–60 °C, it was observed that flanked *meta*-dechlorination occurred, whereas unflanked *para*-dechlorination was observed only between 18 and 34 °C. Dechlorination of doubly unflanked *para*-chlorines occurred only in the temperature range of 18–30 °C. For 2,3,4,6-chlorobiphenyl optimal temperature for overall chlorine removal was 20–27 °C. Unflanked *ortho*-dechlorination was observed at 8–30 °C. Wiegel and Wu proposed a temperature-dependent route of microbial reductive dechlorination of spiked 2,3,4,6-chlorobiphenyl in Woods Pond as shown in Fig. 4.

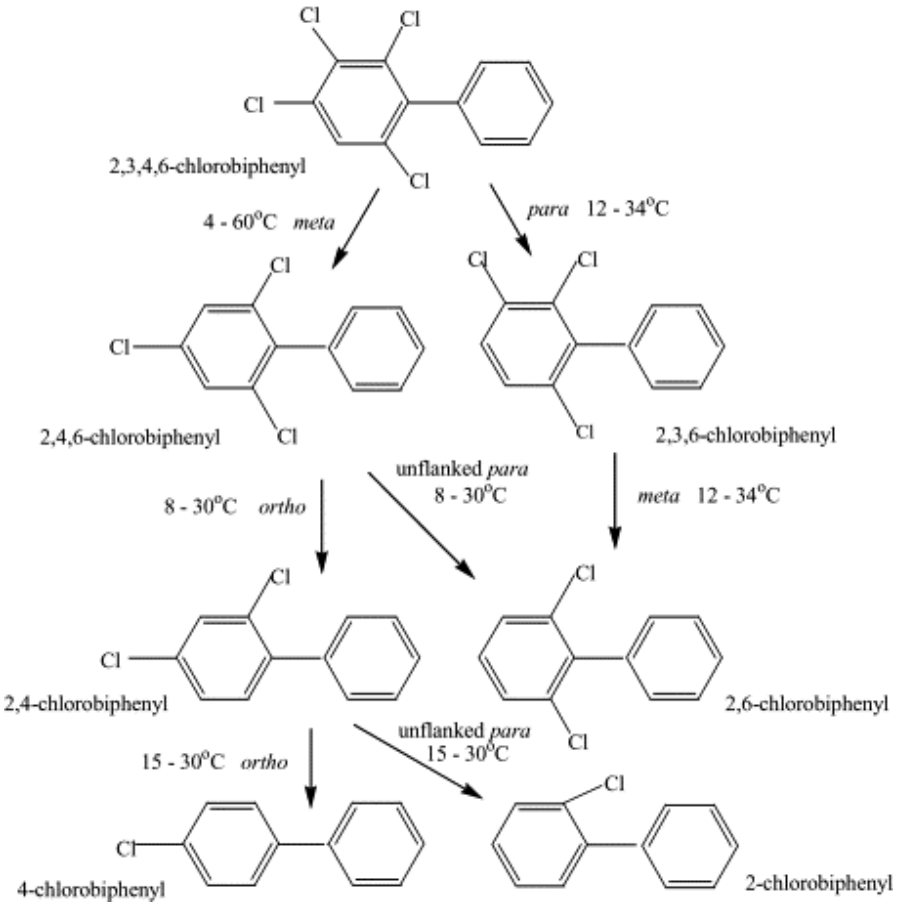


Fig. 4. Temperature-dependent routes of microbial reductive dechlorination

The effect of pH on the dechlorination of PCBs in sediments is complex because of the interaction of the different dehalogenating and non-dehalogenating microbial population. Further, the bioavailability of PCBs is affected by the equilibrium between PCBs that are dissolved and those that are adsorbed to organic matter. The dechlorination of residual PCBs in Woods Pond and of 2,3,4,6-chlorobiphenyl added as primer was studied at pH values between 5.0 and 8.0 at temperatures where dechlorination was observed. Some dechlorination was observed at all temperatures and pH except for 34 °C at pH 5.0. Overall optimal pH for chlorine removal was at 7.0–7.5. The flanked *meta*-dechlorination occurred at pH 5.0–8.0, unflanked *para*-dechlorination at pH 6.0–8.0, and *ortho*-dechlorination at 6.0–6.5. At pH 7.0 and 15 °C, *ortho*-dechlorination dominated, whereas at 18 and 25 °C, unflanked *para*-dechlorination outpaced the other dehalogenation reactions. The optimal pH for overall chlorine removal was at 6.0–7.5.

All laboratory investigations of microbial PCB dechlorination have been carried out using sediment slurries, both as a source of microorganism and growth matrix. These investigations show that microorganisms with different characteristic specificities for PCB dechlorination existed in PCB-contaminated sites. The microbial population present in the sediments have distinct dehalogenating enzyme, each exhibiting a unique pattern of congener selectivity resulting in various patterns of PCB dechlorination. However, a similarity between degradation patterns exists. The *para*- and *meta*-substituted congeners are more commonly degraded than *ortho*-substituted congeners as shown in Fig 5.

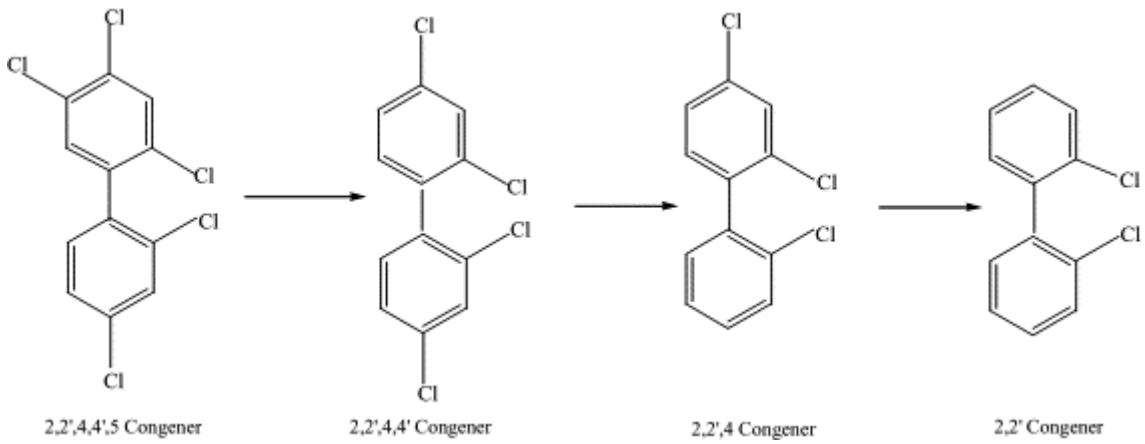


Fig. 5. Potential pathway for anaerobic dechlorination of a highly chlorinated congener

The decrease in risk is manifested in two ways. First, lightly chlorinated congeners produced by dechlorination can be readily degraded by indigenous bacteria. Second, dechlorination significantly reduces bioconcentration potential of the PCB mixture through conversion to congeners that do not significantly bioaccumulate in the food chain.

2.5.3 Aerobic biodegradation of PCBs

The lightly chlorinated PCB congeners resulting from the dechlorination of highly chlorinated congeners are substrates for aerobic bacteria. Aerobic oxidative destruction involves two clusters of genes. The first one is responsible for the transformation of PCB congeners to chlorobenzoic acid, and the second cluster is responsible for the degradation of the chlorobenzoic acid. A common growth substrate for PCB-degrading bacteria is biphenyl or monochlorobiphenyl. When biphenyl is utilized by bacteria yellow *meta*- ring cleavage product is produced. This has been observed in most bacteria studied especially by the *Pseudomonas* sp., which was also observed in *Micrococcus* sp.. The metabolic pathway used by this family of bacteria is illustrated in Fig 6.

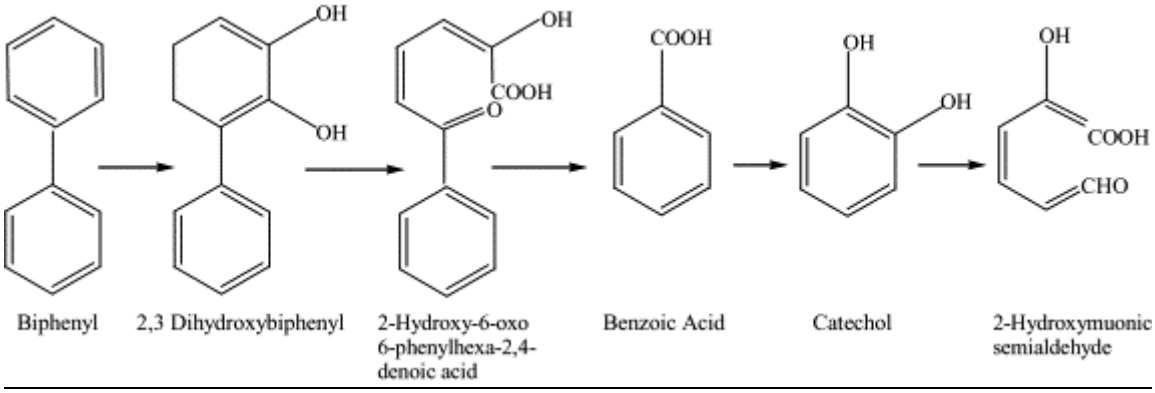


Fig. 6. Possible pathway for the aerobic oxidative degradation of biphenyl

By way of 1,2-dioxygenative ring cleavage, benzoate results as a common by-product of biphenyl degradation. Although different bacterial species seem to produce benzoate through PCB metabolism, further breakdown of benzoate seems to differ among the different microbes. Nevertheless, the by-products produced are less toxic compounds to people and the environment. Since PCBs are more persistent with increasing chlorination of the congener,

aerobic biodegradation involving biphenyl ring cleavage, is restricted to the lightly chlorinated congeners.

While biphenyl and monochlorobiphenyl can serve as growth substrates, the degradation of PCB congeners with more than one chlorine atom proceeds by a co-metabolic process in which biphenyl is used as carbon and energy source while oxidizing PCBs. Biphenyl also serve as an inductor of degrading enzymes. Ahmed and Focht first reported that two species of *Achromobacter* are capable of growing on biphenyl and 4-chlorobiphenyl. The degradation of PCBs by *Nocardia* sp. and *Pseudomonas* sp. increased upon addition of biphenyl. Clark et al. described the enhanced co-metabolism of Aroclor 1242 in the presence of acetate using mixed cultures of *Alcalegenes odorans*, *A. denitrificans*, and an unidentified bacterium. Focht and Brunner observed the increased mineralization of Aroclor 1242 by *Acinetobacter* strain P6 by the addition of biphenyl. Furukawa et al. reported that *Acinetobacter* strain P6 and *Arthrobacter* strain B1B grows well on biphenyl and 4-chlorobiphenyl. These microorganisms also co-metabolized Aroclor 1254 in the presence of biphenyl. *Alcalegenes* H850 utilized biphenyl and three monochlorobiphenyls as growth substrates and oxidized all detectable di-, tri-, and tetra-chlorobiphenyls in Aroclors 1242 and 1248, and partially degrading Aroclor 1254. The observed oxidation of PCBs in the presence of a second substrate was attributed to the increased biomass that even a slight oxidation by each microorganism would lead to a more complete degradation of PCBs for the system as a whole.

In a recent study, a new bacterium, *Janibacter*, MS3-02, was isolated from soil. It is interesting to note that the degradation of Aroclor 1242 was significantly higher in the liquid medium without biphenyl (70–100% after 7 days). When biphenyl was added in the medium, degradation was only 84%. For the studies on soil medium, it was observed that the soil native population was not able to degrade the PCBs present in Aroclor 1242. On the other hand, inoculation of the soil with MS3-02 produced a decrease in some of the chromatographic peaks. Comparison of the result obtained in the liquid medium with that obtained in soil shows that the degradation was less efficient in soil because of the lower bioavailability of PCBs and the interactions with the indigenous soil microorganisms.

Several studies on the microbial degradation of commercial PCB mixtures show that certain patterns of chlorine substitution seriously hinder PCB degradation. For lightly chlorinated PCB congeners, a sequential enzymatic steps involved in the degradation had been developed. The metabolic pathway is shown in Fig 7.

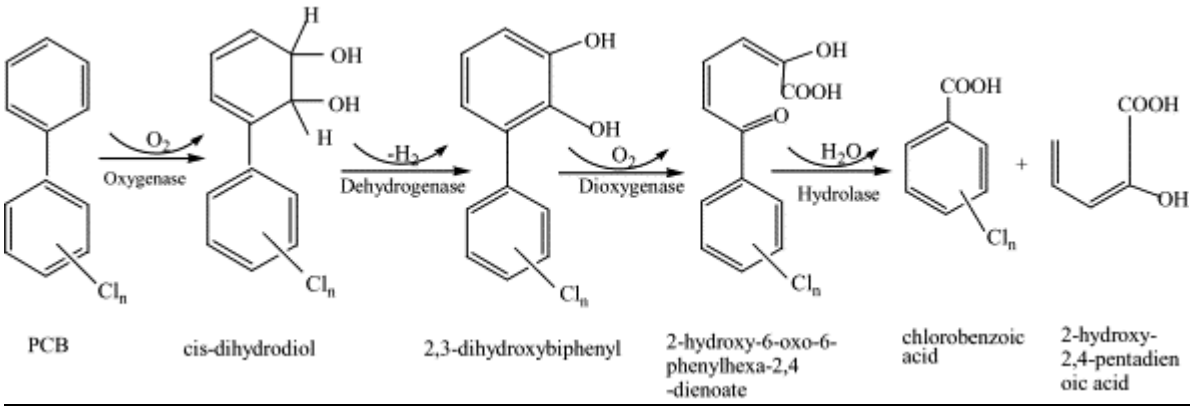


Fig. 7. Major steps in the conversion of PCBs into chlorobenzoates

Molecular oxygen is introduced at the 2,3 position of the non-chlorinated or lesser chlorinated ring of PCB to form cis-dihydrodiol compounds (2,3-dihydroxy-4-phenylhexa-4,6-diene) by the action of oxygenase. The dihydrodiols are then dehydrogenated to yield 2,3-dihydroxy-biphenyl by a dihydrodiol dehydrogenase. The 2,3-dihydroxy biphenyl is cleaved at the 1,2 position by a 2,3-dihydroxy biphenyl dioxygenase to produce the *meta*-cleavage compound, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. The *meta*-cleavage compound is hydrolyzed to the corresponding chlorobenzoic acid by a hydrolase. Congeners with chlorine at the 2,5,4′-, 2,3,2′,5′-; 2,4,5,2′,3′-positions are accessible to both 2,3- and 3,4-dioxygenase attack but would be increasingly difficult to degrade due to the degree of chlorination. The degradation of 4,4′-dichlorobiphenyl proceeds through a series of hydroxylation, the two rings are cleaved and cleaved products eventually enter the natural pathway. However, congeners blocked at the 2,3- and 5,6-positions can be degraded by *A. eutrophus* H850. On the other hand, *P. putida* LB400 has the ability to degrade 2,4,5,4′,5′-hexachlorobiphenyl, a congener with no adjacent unchlorinated carbon. Generally, highly chlorinated PCB congeners showed resistance to biodegradation.

The complete degradation of PCBs requires various microbial strains with specific congener preferences. In addition, the position and number of chlorines on the molecule can influence the rate of the first oxygenase attack. Unterman et al. proposed a mechanism for the oxidation of PCBs by *A. eutrophus*, *P. putida*, and a *Corynebacterium* sp. *Alcalegenes eutrophus* and *P. putida* strains degrade tetrachlorobiphenyl via 2,3- attack while

Corynebacterium degrades the compound via 3,4-attack. This is illustrated in Fig 8.

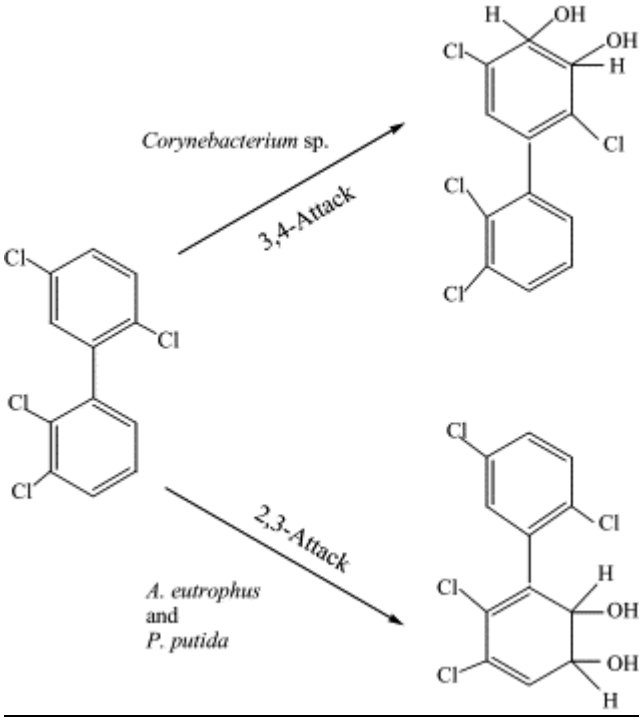


Fig. 8. Microorganism-specific nature of PCB degradation

The bulkiness of the chlorine atoms prevents access to the enzyme's active site. Furthermore, the chlorine atoms may prevent oxygenation if they occupy the carbon positions that are most susceptible to the oxygenase attack. The *ortho*-positions are also the most resistant to microbial attack.

Some aerobic bacteria have the capacity to degrade highly chlorinated PCB congeners using a different initial oxygenase reaction involving a 3,4-hydroxylation instead of 2,3-hydroxylation. This is the case for *P. putida* LB400 and *P. testosterone* B356. In *P. testosterone*, the oxygenase attack was always on the *ortho*- and *meta*-carbon while for *P. putida*, the oxygenase attack on PCB congeners with chlorine atoms on both rings was usually accompanied by a *para*- or *ortho*-dehalogenation of the molecule.

Furukawa summarized the relationship between chlorine substitution and the microbial breakdown of PCBs as follows:

1. The degradation rate of PCBs decreases as chlorine substitution increases.

2. PCBs containing two chlorines in the *ortho*-position of a single ring (i.e., 2,6-) and each ring (i.e., 2,2′) show a striking resistance to degradation.

3. PCBs containing all chlorines on a single ring are generally degraded faster than those containing the same number on both rings.

4. PCBs having two chlorines at the 2,3 position of one ring such as 2,3,2′,3′-, 2,3,2′,5′-, 2,4,5,2′,3′-chlorobiphenyls are susceptible to microbial attack compared with other tetra- and penta-chlorobiphenyls, although this series of PCBs is metabolized through an alternative pathway.

5. Initial dioxygenation followed by ring cleavage of the biphenyl molecule occurs with a non-chlorinated or less chlorinated ring.

The usual products from the co-metabolic degradation of PCBs are chlorinated benzoic acids and but other metabolites have been observed. *Acinetobacter* sp. rapidly degraded Kaneclor 200 (dichlorobiphenyl) after 4 h of incubation showing predominant accumulation of monochlorobenzoic acid. Kaneclors 300 (trichlorobiphenyl) and 400 (tetrachlorobiphenyl) produced various intermediate metabolites such as mono- and di-chlorobenzoic acid, di-hydroxybiphenyl compounds with two and three chlorines and the ring *meta*-cleavage compounds with two and three chlorines. In addition to these products large amounts of unknown compounds with two chlorines in the molecule were also produced. On the other hand, Kaneclor 500 was resistant to degradation and hardly metabolized. The degradation of 4-chlorobiphenyl by an *Achromobacter* sp. and a *Bacillus brevis* strain generated the same metabolites with 4-chlorobenzoic acid as the major metabolite. *Alcalegenes eutrophus* H850 can degrade dichlorobiphenyl yielding the corresponding chlorobenzoic acid and a novel metabolite 2′,3′-dichloroacetophenone while 2′,4′,5′-2′,4′,5′-hexachlorobiphenyl with no adjacent unsubstituted carbons was oxidized to 2′,4′,5′-trichloroacetophenone. In the metabolism of PCBs with four chlorine substituents by *Alcaligenes* sp. JB1, monochlorobenzoates and dichlorobenzoates were detected as metabolites. Resting cell assays with chlorobenzoates showed that JB1 could metabolize the monochlorobenzoates and dichlorobenzoates containing only *meta*- and *para*-chlorine substituents but not dichlorobenzoates possessing an *ortho*-chlorine substituent. The chlorobenzoates formed tend to accumulate in the reaction mixture together with other intermediate metabolites such as dihydroxy and trihydroxy compounds and other unidentified products. Cultures capable of co-metabolizing PCBs are usually unable to grow on these substrates. This suggests that no single organism is responsible for the degradation of multiple chlorinated biphenyls. Previous work have shown that a more efficient degradation of monochlorinated biphenyls by bacterial consortia occurs in the presence of a bacterial strain capable of degrading chlorobenzoates produced by the PCB-degrading members, either directly or by co-metabolism. This

may suggests that chlorobenzoates are in general involved in the regulation of the PCB aerobic degradation process. The incorporation of chlorobenzoate degraders such as *Pseudomonas aeroginosa* and *Pseudomonas putida* can greatly effect complete mineralization of PCBs. A mixed microbial consortium is also needed for microbial synergism and co-metabolism Synergism is also important in enhancing the overall rate of degradation of PCBs in mixed cultures. This increased rate is a result of combined metabolic attack at different sites on the organic compound, increasing overall degradation rate.

2.5.4 Sequential anaerobic–aerobic transformation of PCBs

The biodegradation of PCBs by aerobic bacteria had been well studied. However, it was observed that only lightly chlorinated PCB congeners, those with four or less chlorine atoms, was degraded. Highly chlorinated PCB congeners, those with five or more chlorine atoms, remain biorefractory to aerobic bacteria, although there had been few reports on the aerobic degradation of penta- and hexa-chlorobiphenyls.

Studies have also been conducted on the transformation of PCBs using anaerobic bacteria eluted from PCB-contaminated sediments. Under anaerobic condition, highly chlorinated PCB congeners have been found to be reductively dechlorinated through a preferential *meta*- and *para*-chlorine removal producing less chlorinated congeners that are amenable to aerobic biodegradation. This biotransformation pattern appears to be common among halogenated aromatic compounds.

The results of the studies conducted using solely aerobic or anaerobic microorganism suggests that mineralization of chlorinated organic compounds can be attained through sequential exposure to specialized anaerobic and aerobic microbial cultures. This was demonstrated by the degradation of hexachlorobenzene, tetrachloroethylene and chloroform. The chlorinated compounds were added to the influent of a constant flow anaerobic reactor containing mixed methanogenic cultures. The compounds were dechlorinated to the level of tri- and di-chlorinated products in the anaerobic reactor. These products were subsequently transformed into non-volatile intermediates and carbon dioxide in the aerobic reactor.

A sequential anaerobic–aerobic treatment of PCBs has been successfully tested in microcosms of HR sediment. Batch cultures were incubated anaerobically for 20 weeks in sealed serum bottles with methanol. The tri-, tetra-, penta-, and hexa-chlorobiphenyls were reduced and an increase in

mono- and di-chlorobiphenyls was observed. After 20 weeks of incubation the anaerobic cultures were purged with oxygen and inoculated with an aerobic bacterium isolated from HR. After 96-h incubation most of the mono- and about 25% of the di-chlorobiphenyl were degraded.

Rogers and Julia reported a sequential anaerobic–aerobic treatment of PCBs in soil microcosms. Results of the batch soil–slurry microcosm showed dechlorination of several hexachlorobiphenyl to penta- and tetra-chlorobiphenyl by indigenous microorganisms. The aerobic microcosm experiment demonstrated the presence of microorganisms capable of degrading the tri- and tetra-chlorobiphenyl.

A sequential anaerobic dechlorination, and subsequent aerobic degradation of PCB in soil spiked with Aroclor 1242 was reported by Anid et al. and Shannon et al. reported a sequential anaerobic–aerobic laboratory scale treatment of soil contaminated with weathered Aroclor 1260. Using an initial concentration of 59 ppm, the major components of Aroclor 1260 were either completely or partially transformed to less chlorinated PCB congeners within 4 months of anaerobic treatment. The major products of reductive dechlorination were 2,4,2′,4′-tetrachlorobeiphenyl and 2,4,2′,6′-tetrachlorobiphenyl. There was no decrease in the total PCB. These products were degraded in the subsequent aerobic treatment using *Bukholderia* sp. strain LB400. The concentration of PCBs was reduced to 20 ppm after 28 days of aerobic treatment.

2.6 CBAs

Benzoic acid and its derivatives may impact on the environment in a number of ways. They are widely used reaction intermediates and as such may be discharged into the environment. In addition they are often formed as the result of degradation of aromatic compounds. Notably, chlorinated benzoic acids are the most common metabolites following the degradation of polychlorinated biphenyls.

The first step in the degradation of most compounds by a microorganism is their transfer across the cell membrane(s) into the cytoplasm. Uptake of aromatic compounds has been studied from two different perspectives. For toxic compounds, control of their permeation into microorganisms unable to degrade them entails the evolution of physiological and structural modifications leading to restricted permeation. The capacity of cells to

develop these may be an important survival mechanism. For metabolisable aromatic compounds, uptake can occur by diffusion or via the evolution of highly specific permeases. Aromatic acids, being lipophilic weak acids, are generally assumed to traverse cell membranes by passive diffusion. Indeed, such compounds have been used for measuring pH gradients across membranes. Little is known, however, about the uptake mechanisms of chlorinated aromatic compounds. Microbial metabolism of polychlorinated biphenyls (PCBs) by pure cultures of PCB-degrading microorganisms is generally incomplete and results in the formation of isomeric mixtures of chlorinated benzoic acids (CBAs) as dead-end metabolites. More complete degradation of PCBs by cocultures consisting of both PCB- and CBA-degrading microorganisms, in which the rate of CBA removal from the growth medium dictated the rate of PCB degradation by the cells, has been reported. Organisms capable of degrading 2-CBA, 3-CBA, and 4-CBA have been isolated. Less is known about microorganisms with dichlorobenzoic acid (DCBA)-degradative capabilities.

Furthermore, these compounds are known to adversely affect the PCB degradation pathway. Chlorinated benzoic acids have a wide range of solubility and recalcitrance, ranging from the labile and soluble 3-chlorinated benzoic acid (3CBA) to the recalcitrant and poorly soluble 2,5-dichlorobenzoic acid (25diCBA). The metabolism of these components includes a variety of pathways with one common aerobic degradation route illustrated in Fig 9.

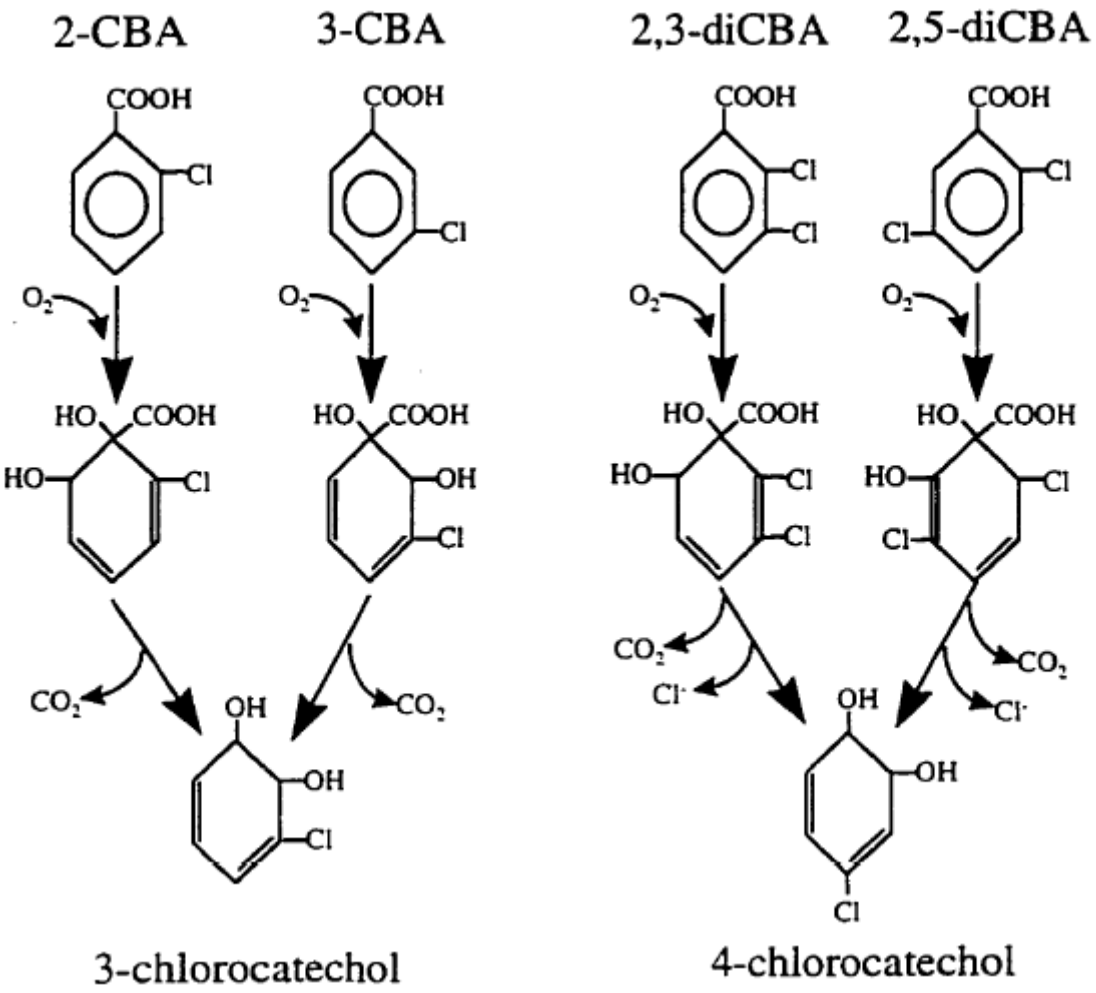


Fig.9. A common aerobic degradation route of di- and mono-chlorinated benzoic acids. ZCBA is 2-chlorobenzoic acid; 3-CBA is 3-chlorobenzoic acid; 2.3-diCBA is 2.3-dichlorinated benzoic acid; 2.5-diCBA is 2.5-dichlorinated benzoic acid.

A number of studies have demonstrated benzoic acids to be toxic to a range of biota in the environment including bacteria, invertebrates and fish. Toxicity assessment of such compounds is thus vital to appreciate environmental effects. Furthermore toxicity assessment of acids is important to establish the effect of properties such as ionisation on uptake and toxicity.

3. Luminescence Bioassay

3.1 Luminescent bacteria-microtox test

A review of aquatic toxicity *in vitro* assays reveals an abundance of bacterial test systems. Bacterial toxicity tests tend to fall into one of five categories: (1) population growth, (2) energy, (3) substrate consumption, (4) respiration, and (5) bioluminescence assays. The popularity of bacterial assays is based on the facts that bacteria are an integral part of ecosystems and the assays are quick and simple and on the assumption that most chemicals exert their effects by interfering with common cellular processes (i.e., energetics, macromolecular synthesis). Among the bacterial assays, the luminescent bacteria inhibition assay is one of the most popular.

The main characteristic of Luminescent bacteria is the conversion of chemical energy into light, which allows them to glow in the dark. Most luminescent bacteria are found in the ocean, and are associated with marine animals like shrimps, clams, or fish. Some fish, such as the flashlight fish, possess a special gland in their body, called the light organ, in which luminescent bacteria can grow. In the flashlight fish, the luminescent bacteria are closely packed in the light organ, and so the fish appears to glow in the dark. Is not yet known why these bacteria produce light. One idea is that producing light gives them an advantage for survival. By glowing, they attract fish, which they then use as a host organism the bacteria are able to live in the fish's body. These fish, which are attracted to the bacteria's light, pick up the bacteria and prevent them from settling on the bottom of the ocean, where there is less oxygen available. The fish might also benefit from allowing the bacteria to live in their body the fish are able to glow due to the presence of luminescent bacteria, and this light might help them to spot food more easily in the dark ocean.

The luminescence reaction constitutes a branch of the electron transport chain at the level of flavin. In this reaction, reduced flavin mononucleotide and a long-chain aldehyde are oxidized by molecular oxygen to give an electronically excited flavin and the corresponding fatty acid. Measurement of bacterial luminescence assesses the flow of electrons in the respiratory chain and the metabolic state of the cell. For this reason it can be used for monitoring toxic effects.

Scientists published a paper in 1979 entitled "*The Use of Luminescent Bacteria for Determining Toxicity in Aquatic Environments*" establishing the technology and a commercial basis for a rapid, low cost and standardized aquatic toxicity test system. The technology breakthrough described in this paper utilized a special freeze-drying technique to provide shelf stable toxicity test organisms that could be instantly reconstituted and used whenever toxicity testing was needed. This technology has provided the basis for producing and distributing standardized test organisms in a vial with a shelf life of up to 18 months.

During the last years there has been considerable interest in the development of luminescence based microbial bioassays, as these offer an inexpensive and rapid assessment of environmental samples. While they do not reveal what specific contaminants are present, they indicate the presence of bacterial toxicants and, therefore, can be utilized as an environmental screening system to detect a wide range of contaminants in the ecosystem. This approach is useful where potential pollutants are unknown or diverse requiring a whole suite of analytical approaches for screening.

Bioassays using luminescent bacteria are routinely used to assess the acute toxicity of environmental samples. Luminescent bacteria possess several attributes, which support their practical use for toxicity testing. Their small cell size provides a high surface to volume ratio, which maximizes exposure potential. This structural characteristic plus lack of membrane aided compartmentalization, the presence of most respiratory pathways (including enzymes required for bioluminescence) on or near the cell membrane, a relatively short division cycle, an inducible luciferase pathway and a metabolic rate 10 to 100x that of mammalian cells, provides a dynamic metabolic system which can be easily quantitated by measuring the rate of light output.

A number of marine bacterium possess the capacity to luminesce, however, bioluminescence in terrestrial environments is rare. Plasmid insertion of the lux genes encoding for bioluminescence into the bacterial genome of *Pseudomonas fluorescens,* produced a terrestrial bacterium capable of luminescing. Luminescence is dependent on metabolic activity and, therefore, any chemical which causes metabolic stress may result in a decline in bacterial luminescence. *The P. fluorescens* bioassay has been shown to be sensitive to heavy metals and organic contaminants such as benzenes, phenols, catechols and chlorobenzenes.

The use of living test organisms is the only reliable way to measure the potential biological impact (toxicity) of a water or soil sample. This type of

testing has been in use for many years but has suffered from common deficiencies such as cost per test, time to obtain test results (usually days), and the inherent variability of test data. AZUR Environmental has combined the advantages of whole organism toxicity testing and instrumental precision to produce biological test systems, which possess the features of instrumental based analytical test systems. One of these test systems is Microtox Test system.

The Microtox test uses the luminescent marine bacterium *Vibrio Fischeri* (formerly *Photobacterium phosphoreum*) specifically the strain *Vibrio fischeri* NRRL B-11177, which carries lux genes to express respiratory controlled natural luminescence. Lux genes encode for luciferase and aldehyde production, which are major components of *the P. phosphoreum* metabolic system. *Vibrio Fischeri* is a Gram-negative, marine prokaryote. When properly grown, luminescent bacteria produce light as a by-product of their cellular respiration. Bioluminescence is a complex regulated phenomenon in *Vibrio fischeri.* There are several regulatory circuits which control the amount of light emission. Three main factors are known: First of all the bioluminescence is dependent on the energy status of the cell as the luciferase reactions are ATP and $FMINH_2$ dependent. Secondly the bioluminescence - as it is an enzyme catalyzed process – is dependent on biosynthetic processes and thirdly the bioluminescence is regulated through the external concentration of a small organic molecule, the autoinducer. Cell respiration is fundamental to cellular metabolism and all associated life processes. Bacterial bioluminescence is tied directly to cell respiration, and any inhibition of cellular activity (toxicity) results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. The more toxic the sample, the greater the percent light loss from the test suspension of luminescent bacteria. During tests cells are harvested in the late logarithmic stage of growth, which means the enzyme, apparatus for bioluminescence is fully developed and the cells show maximum bioluminescence. Therefore, no additional protein synthesis is necessary during the exposure time of 30 minutes in the short term ISO-Test, which is reflected by the extremely low effects of chemicals specifically interfering with this process (chloramphenicol, nalidixic acid and tetracycline). Bacterial bioluminescence has proved to be a convenient measure of cellular metabolism and consequently, a reliable sensor for measuring the presence of toxic chemicals in aquatic samples. Strain 11177 was originally chosen for the acute and chronic tests because it displayed a high sensitivity to a broad range of chemicals.

The Microtox Acute Test has been the subject of many reproducibility evaluations including multiple laboratory round robin test evaluations. These published studies have confirmed coefficient of variation (CV) values averaging about 20% which is similar to chemical analysis methods and much better than other whole organisms bioassays.

It has been successfully used for a variety of environmental, process monitoring and educational applications. The most common environmental applications include:

- Wastewater treatment plant influent testing for protection of activated sludge
- Wastewater treatment plant effluent testing for protection of receiving waters
- Toxicity Reduction Evaluations (TRE's) and Toxicity Identification Evaluations (TIE's)
- Surface water monitoring for identification of point source and non-point source pollution
- Monitoring raw drinking water for contamination due to spills, point source or non-point source pollution, or sabotage
- Sediment testing
- Soil contamination testing and monitoring of remediation processes
- Biocide monitoring of industrial process waters

Fig 10. Microtox device

Microtox is a standard government agency ecotoxicological bioassay in Canada, The Netherlands, France, Germany, Spain, and Sweden. It is also a bioassay widely used to assess the acute toxicity, cytotoxicity, and irritation of environmental contaminants and utilizes the decline in bacterial luminescence on exposure to toxic compounds as a means of quantification. In the United States, it is involved in American Society for Testing and Materials (ASTM) Method D-5660 (ASTM, 1996), and in the *Standard Methods for the Examination of water and wastewater* as Part 8050. Has been also approved in the final ISO Draft (11348-3) entitled "Water Quality-Determination of the Inhibitory Effect of Water Samples on the Light Emission of *Vibrio fischeri* (Luminescent Bacteria) Test". It is sensitive to heavy metals, organics, and their mixtures. Toxicity reduces enzyme activity and thus reduces light output. The test can be quite sensitive and can detect low concentrations of some toxicants.

The original test is supplemented by chronic, genotoxic, and acute solid-phase bioassays, using the same instrumentation. Several sample dilutions are incubated with luminescent bacteria for 5, 15, and/or 30 min at 15°C. Light output of serial dilutions of the test solutions form the basis for a dose-response curve. If a dose-response relationship exists between the concentration of test solution and light output, then the concentration that reduces the light output by 50% over the control is reported as the EC_{50}, (Effective Concentration for 50% light reduction).

Quantitative structure–activity relationships of the EC_{50}'s of several organic chemicals with structural parameters have been carried out. The inhibition of bioluminescence can be correlated successfully with the partition coefficient between *n*-octanol and water (*P*OW) as a model of the influence of hydrophobicity of molecules on the permeation of the bacterial membrane. Introduction of molar refractivity (MR)—a function of the index of refraction, density, and molecular weight—and molar volume (MW/d) improved the quality of the relationships. The influences of MR or MW/d could be related with an interaction of the tested chemicals to the enzyme luciferase or the reduced flavin mononucleotide
(FMNH2)-donating system. The EC50s of 55 chlorinated aromatic compounds were correlated with log *P*OW and the hydrophilic effect parameter (*V*H).

3.2 Short Description of the Microtox test

First we resuspend the frozen bacteria by adding 0.5 ml of 2% NaCl to the vial and then we make a solution of the luminescent bacteria in NaCl which gives luminescence 20.000-60.000. After we make different dilutions of the toxicant we want to test with NaCl, the first solution usually contained 1% of the toxicants and in case it was not clear if the samples were toxic we increased this percentage to 5%. From the first solution we make two successive dilutions, first one consists 50% of the initial solution and 50% of NaCl and the second contains some of the previous dilution and NaCl again in ratio 1:1. We add the bacteria to ten small vials (5 pairs), we count the initial luminescence and then we add 0.5 ml NaCl to each one of the first couple and 0.5 ml of the other solutions to the other vials so as to have 2 samples for each dilution. These samples are stored in cold water (15°C) because bacteria must be preserved in low temperature. Fifteen minutes after the first measurement we measure again the luminescence of the samples and the reduction, which may be observed, indicates the toxicity of the substance we want to test.

After we process the experimental data to calculate the EC_{50} through the formulas, which can be seen below:

$KF = IC_{15} / IC_0$

$INH\% = 100 - (IT_{15} / KF * IT_0) * 100$

Where:

KF: Correction factor
IC_{15}: Luminescence intensity of control after 15 min
IC_0: Initial luminescence intensity of control sample
IT_{15}: Luminescence intensity of test sample after 15 min
IT_0: Initial luminescence intensity of test sample

Finally the EC_{50} value is determined by using standard linear regression analysis of the formula:

$$Inh = \frac{C^n}{C^n + EC50^n}$$

If the range of value pairs can not be linearized, the EC_{50} value can be determined graphically using a double logarithmic co-ordinate system. The INH% is plotted on the y-axis and the concentration on the x-axis.

4. Ames test

4.1 Introduction

The identification of substances capable of inducing mutations has become an important procedure in safety assessment. Chemicals that can induce mutations can potentially damage the germ line leading to fertility problems and to mutations in future generations. Mutagenic chemicals are also capable of inducing cancer, and this concern has driven most of the mutagenicity testing programs. Mutations can occur as gene (point) mutations, where only a single base is modified, or one or a relatively few bases are inserted or deleted, as large deletions or rearrangements of DNA, as chromosome breaks or rearrangements, or as gain or loss of whole chromosomes.

Gene mutations are readily measured in bacteria and other cell systems when they cause a change in the growth requirements of the cell, whereas chromosome damage in mammalian cells is typically measured by observing the cell's chromosomes under magnification for breaks or rearrangements. The *Salmonella typhimurium*/microsome assay (*Salmonella* test; Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test uses a number of *Salmonella* strains with preexisting mutations that leave the bacteria unable to synthesize the required amino acid, histidine, and therefore unable to grow and form colonies in its absence. New mutations at the site of these preexisting mutations, or nearby in the genes, can restore the gene's function and allow the cells to synthesize histidine. These newly mutated cells can grow in the absence of histidine and form colonies. For this reason, the test is often referred to as a "reversion assay."

The *Salmonella* strains used in the test have different mutations in various genes in the histidine operon; each of these mutations is designed to be responsive to mutagens that act via different mechanisms. Additional mutations were engineered into these strains to make them more sensitive to a wide variety of substances.

The *Salmonella* mutagenicity test was specifically designed to detect chemically induced mutagenesis. Over the years its value as such has been recognized by the scientific community, and by government agencies and corporations. The test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs because there is a high

predictive value for rodent carcinogenicity when a mutagenic response is obtained. International guidelines have also been developed (e.g., Organisation for Economic Co-operation and Development (OECD); International Commission on Harmonization (ICH)) for use by corporations and testing laboratories to ensure uniformity of testing procedures prior to submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides.

4.2 Historical aspects

The Ames *Salmonella*/microsome mutagenicity assay evolved over the years from the initial screening of a number of histidine mutants which led to the selection of mutants that were highly sensitive to reversion by a variety of chemical mutagens. Because bacteria are unable to metabolize chemicals via cytochromes P450, as in mammals and other vertebrates, a key component for making the bacterial mutagenicity test useful was the inclusion of an exogenous mammalian metabolic activation system. At the same time, the development of the plate incorporation assay protocol to replace spot test or liquid suspension procedures was a major contributing factor to the success of the Ames test because it made the test easier to perform and reduced its cost.

4.3 Screening of histidine mutants and development of the plate incorporation assay

Studies performed to identify and map the genes responsible for histidine biosynthesis produced a large number of spontaneous, radiation-, and chemical-induced histidine mutants of *Salmonella typhimurium* LT-2. Some of the mutants contained single base changes (base-pair substitution mutants), and others contained additions or deletions of one or more bases (frameshift mutants). It was later realized that some of these mutant strains could be used to identify and characterize mutagenic chemicals by their ability to revert to wild-type (histidine-independence) in the presence of mutagens. In 1966, Ames and Whitfield proposed a set of histidine mutant strains for screening chemicals for mutagens using a spot test procedure that was previously used by Szybalski and Iyer and Szybalski for mutagen screening with an *E. coli* strain. The spot test consists of applying a small amount of the test chemical directly to the center of a selective agar medium plate seeded with the test

organism. As the chemical diffuses into the agar a concentration gradient is formed. A mutagenic chemical will give rise to a ring of revertant colonies surrounding the area where the chemical was applied. If the chemical is toxic, a zone of growth inhibition will also be observed. Fig.11 depicts a spot test with methylmethane sulfonate and strain *Salmonella* strain TA100.

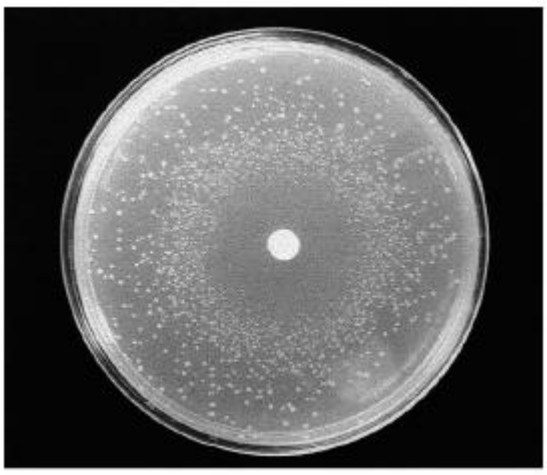


Fig. 11. Spot test with strain TA100 and methyl methanesulfonate (10 μl).

In 1973, Ames et al. developed the plate incorporation assay procedure which is more sensitive and quantitative than the spot test. The procedure consists of adding the buffer or S-9 mix, the histidine dependent bacteria (about 10^8) and test chemical to 2 ml of top agar containing biotin and a trace amount of histidine (0.05 mM each). The mixture is then gently mixed and poured on glucose minimal (GM) agar plates. When the top agar has solidified the plates are incubated in an inverted position in a 37°C incubator for 48 h at which time the histidine revertant colonies are counted. The assay procedure is depicted in Fig. 12.

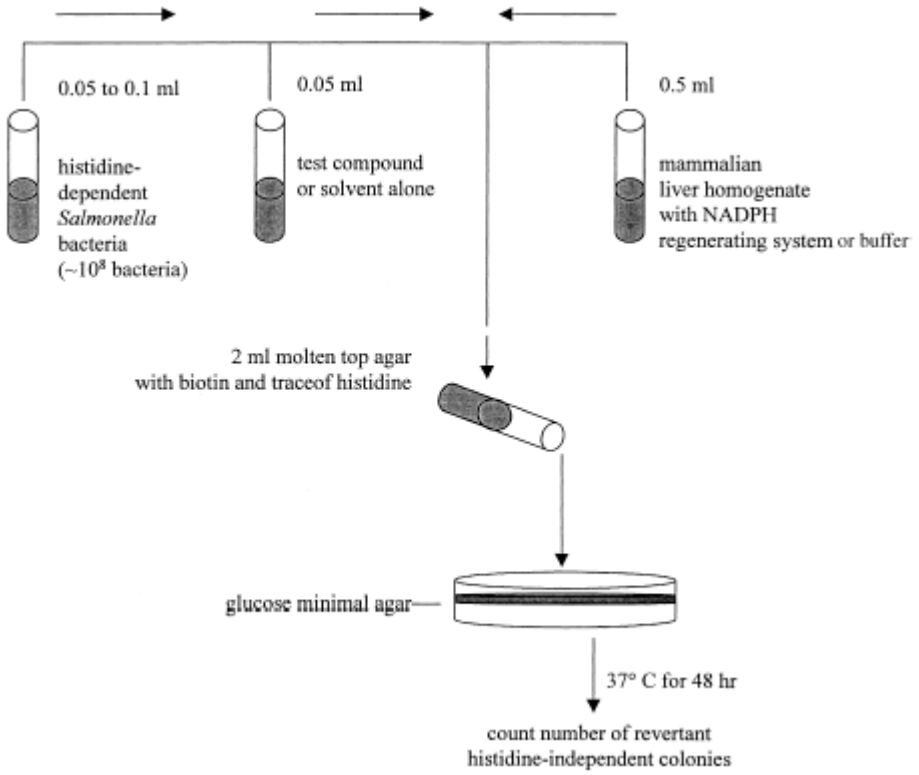


Fig.12. Diagram depicting the steps involved in the plate incorporation assay.

When the histidine dependent bacteria are grown on a glucose minimal (GM) agar plate containing a trace amount of histidine, only those cells that revert to histidine independence (His^+) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few cell divisions; in many cases, this growth is essential for mutagenesis to occur. The His^+ revertants are easily scored as colonies against the slight background growth. The number of spontaneously induced revertant colonies is relatively constant for each strain. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose related manner as depicted in Fig. 13.

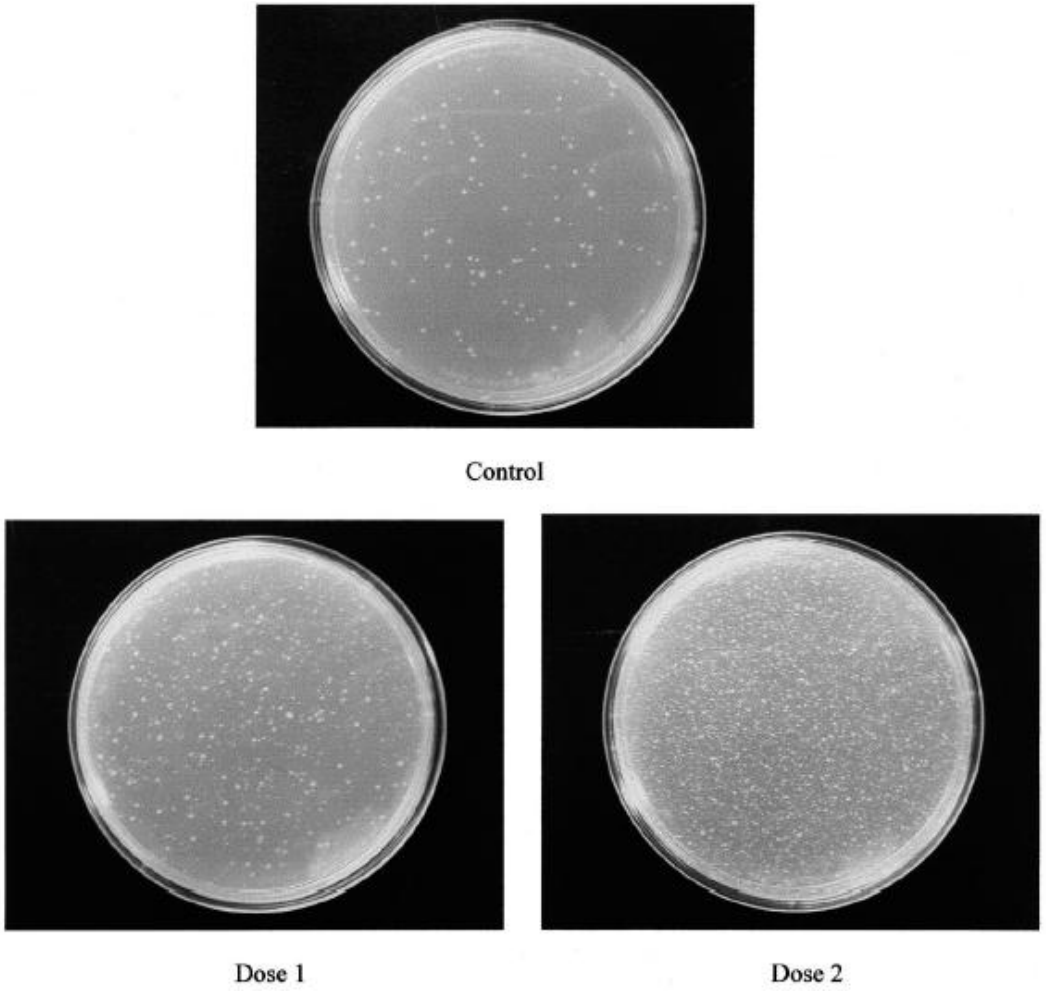


Fig. 13. Mutagenic dose response with strain TA100 and sodium azide. Control: spontaneous revertants; dose 1:2.5 μg/plate; dose 2:5 μg/plate.

The plate incorporation test does not permit the enumeration of the total number of surviving cells because of bacterial growth on the plate and competing toxicity due to the chemical treatment. Also, because of the extra cell divisions that take place after adding the bacteria to the plate, it is not possible to estimate the number of cells at risk for mutation. Therefore, the mutation values obtained can only be expressed as number of mutants/plate, or number of mutants/amount of chemical added.

A plate test that can provide quantitative toxicity information is the "treat-and-plate" suspension procedure but takes longer to perform than the plate

incorporation assay. In this procedure, the bacteria are washed free of growth medium, resuspended in non-nutrient growth medium and treated with the test substance for various time intervals. Separate samples of the bacteria are then plated on selective medium for mutant determination and on complete medium for survival determination. The results establish the mutation frequency by calculating the number of mutants per surviving fraction of bacteria. However, the plate incorporation assay offers the advantages that limited steps are required to expose the bacteria to the test chemical with no need for washing or resuspending the bacteria prior to or after treatment. In addition, the bacteria are allowed to undergo a few cell divisions in the presence of the test substance, which increases their sensitivity to mutation induction.

4.4 Metabolic activation systems

1) Oxidative metabolism

Some carcinogenic chemicals, such aromatic amines or polycyclic aromatic hydrocarbons, are biologically inactive unless they are metabolized to active forms. In humans and lower animals, the cytochrome-based P450 metabolic oxidation system, which is present mainly in the liver and to a lesser extent in the lung and kidneys, is capable of metabolizing a large number of these chemicals to DNA-reactive, electrophilic forms. Some of the intermediate metabolites are potent mutagens in the Ames *Salmonella* assay. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system needs to be added to the petri plate together with the test chemical and the bacteria. For this purpose, a rodent metabolic activation system was introduced into the test system. The metabolic activation system usually consists of a 9000×*g* supernatant fraction of a rat liver homogenate (S-9 microsomal fraction), which is delivered to the test system in the presence of NADP and cofactors for NADPH-supported oxidation (S-9 mix). To increase the level of metabolizing enzymes, the animals are retreated with the mixed-function oxidase inducer Aroclor 1254. Other inducers, such as phenobarbital and -naphthoflavone, can also be used.

The mixed function oxidase enzymes (S-9 fraction) can also be obtained from animal species other than rat such as mouse, hamster, guinea-pig and monkey and organs other than liver such as kidney and from human liver. In comparative studies in which coded compounds were tested, induced and uninduced Syrian hamster or mouse liver S-9 offered no overall advantage over Aroclor-1254 induced rat liver S-9 for the induction of mutagenesis.

However, there are chemicals that may be more efficiently detected as mutagens with rat, mouse, or hamster liver S-9.

2) Reductive metabolism

The metabolic activation system can also consist of a reductive enzyme system for classes of chemicals containing azo and diazo bonds. Reduction of chemical substances can occur in mammals, including humans, by anaerobic intestinal microflora, and very likely by mammalian reductases in the intestinal wall or in the liver. Two types of reductive in vitro metabolic activation systems have generally been used, those based on a liver homogenate supplemented with FMN, and those that are based on rat intestinal microflora preparations.

4.5 Construction of base-specific Salmonella tester strains

A set of 6 base-specific *Salmonella* tester strains was developed by Gee et al., with the unique property that each strain can be reverted by a unique transition or transversion event thereby enabling the identification of specific base-pair substitutions. In addition to the *his* mutation all strains carry the following genetic markers:

• the *rfa* mutation that affects the permeability of the cell wall

• the *uvrB-bio* deletion that affects the accurate DNA repair pathway and which makes the cells biotin dependent

• the mutagenesis-enhancing plasmid pKM101.

Each strain carries a unique missense mutation with the base change indicated below in the histidine biosynthetic operon:

Strain	Operon	Mutation
TA7001	*hisG1775*	AT to GC
TA7002	*hisC9138*	TA to AT
TA7003	*hisG9074*	TA to GC
TA7004	*hisG9133*	GC to AT
TA7005	*hisG9130*	CG to AT
TA7006	*hisC9070*	CG to GC

Table 1. Results of a validation study published by Gee et.al

4.6 Modifications of the standard plate incorporation assay

Over the years, modifications to the standard plate incorporation assay (Fig.12) have been developed by different researchers that enhanced the sensitivity of the test and allowed the testing of a wider range of chemicals,

including gases and volatile chemicals. The most commonly used modifications are described below.

1) The preincubation assay

In the preincubation assay, the tester strains are exposed to the chemical for a short period (20 to 30 min) in a small volume (0.5 ml) of either buffer or S-9 mix, prior to plating on glucose agar minimal medium (GM agar) supplemented with a trace amount of histidine. With few exceptions it is believed that this assay is more sensitive than the plate incorporation assay, because short-lived mutagenic metabolites may have a better chance reacting with the tester strains in the small volume of preincubation mixture, and the effective concentration of S-9 mix in the preincubation volume is higher than on the plate.

2) The Kado salmonella microsuspension assay for testing small sample volumes

This procedure was designed to detect mutagenic metabolites in urine samples obtained from animals treated with test chemicals because of the relatively small sample volumes obtained in these studies. In this procedure, the overnight cultures are centrifuged to obtain a 10-fold higher than normal cell density (i.e., about 1 to 2×10^{10} bacteria per ml, which results in about 1 to 2×10^{9} bacteria per tube when delivered in a 0.1 ml inoculum) for use in the preincubation assay. The exposure of a higher number of bacteria to the urine samples is believed to enhance the detection of mutagenic metabolites present in urine samples. This procedure is also well suited for testing small quantities (about 20 mg) of samples where the sample quantities are limited, and for testing complex mixtures other than urine samples.

3) Testing of chemicals in a reduced oxygen atmosphere

Anaerobic environments, such as anaerobic chambers, have been used to study mutagenicity of chemicals and fecal samples under reduced oxygen levels. Highly reduced oxygen levels may lead to a reduction in both spontaneous and induced revertant colonies and may interfere with the interpretation of the results. A complete absence of revertant colonies is observed when a strict anaerobic environment and prereduced and anaerobically sterilized (PRAS) media are used. It is therefore important to perform preliminary experiments to determine whether the spontaneous reversion rate is affected by the intended reduced oxygen level.

4.7 Validation studies

Over the years, many validation studies have been performed to determine the reproducibility of test results on an intra- and inter-laboratory level. In addition, many studies have been performed to determine the sensitivity and correlation of the Ames test with animal carcinogenicity studies. It has indeed been established that there is a high predictivity of a positive mutagenic response in the Ames test for rodent carcinogenicity, ranging from 90% to 77%, the primary differences being the chemical composition of the compiled databases. The test therefore is in many instances used as a first screen to determine the mutagenic potential of new chemicals and drugs. In addition, data from the test is submitted to regulatory agencies in support of registration or acceptance of many chemicals, including drugs and biocides. International guidelines have been developed for use by corporations and testing laboratories to ensure uniformity of testing procedures.

4.8 Spontaneous control values

Each tester strain has a characteristic spontaneous mutant frequency. There is usually some day-to-day and laboratory-to-laboratory variation in the number of spontaneous revertant colonies. Choice of solvent may also affect the spontaneous mutant frequency. Each laboratory has a characteristic range of revertant colonies for each strain which is referred to as "historical control values". The spontaneous mutant frequency obtained when the strain check is performed should be compared to the laboratory's historical control values. Table 2 presents a range of spontaneous histidine revertant (negative solvent) control values per plate with and without metabolic activation considered valid in the authors' laboratories. The values obtained in the presence of a metabolic activation system includes both rat and hamster liver S-9. The spontaneous values presented for S-9 were from 10% S-9 in the S-9 mix. Some of the strains (e.g., TA97, TA102, TA104) are highly sensitive to S-9 concentrations and their spontaneous reversion values will increase with the S-9 concentration. Other acceptable ranges of background revertant counts have been published.

Strain	Number of revertants[a]	
	Without S-9	With S-9
TA97	75–200	100–200
TA98	20–50	20–50
TA100	75–200	75–200
TA102	100–300	200–400
TA104	200–300	300–400
TA1535	5–20	5–20
TA1537	5–20	5–20
TA1538	5–20	5–20

[a]Range considered valid in the authors' laboratories.

Table 2. Spontaneous revertant control values

4.9 Toxicity determination

Toxicity determination in the Ames *Salmonella* test requires the evaluation of characteristics of the final population on the GM agar plate after the 48-h incubation instead of a quantitative survival determination. These characteristics are:

• thinning of the background lawn which may be accompanied by a decrease in the number of revertant colonies

• absence of background lawn (i.e., complete absence of growth)

• presence of pinpoint non-revertant colonies (generally in conjunction with an absence of background lawn)

The overnight *Salmonella* nutrient broth cultures largely consist of histidine-dependent bacteria with just a few pre-existing histidine-independent (His^+) bacteria that arose during the overnight incubation. The addition of a small amount of histidine to the top agar allows all the plated bacteria (approximately 1×10^8 cells) to undergo between six and eight cell division before the histidine is depleted. In many cases, this limited growth is essential for mutagenesis to occur by allowing fixation of the mutational lesions. The pre-existing His^+ bacteria as well as the His^+ revertants that arose on the plate (plate revertants) will continue to grow in the absence of histidine and will give rise to visible colonies. These His^+ colonies are easily scored against a slightly hazy looking background lawn which is made up of the microcolonies of the histidine-dependent bacteria.

Microscopic (40×) examination of the background lawn in the absence of toxicity will reveal the presence of densely packed microcolonies which form a uniform, though somewhat granular thin film. In such cases, all the plated histidine-dependent bacteria were able to undergo six to eight cell divisions. However, when a chemical is toxic there may be "thinning" or complete absence of the background lawn compared to the negative or solvent control.

Partial toxicity of the chemical will give rise to thinning since not all the plated bacteria were killed or had their growth inhibited. In this case, the surviving bacteria still form microcolonies but they are not densely packed and may appear as single sparsely spaced microcolonies which results in the "thinning" effect; these colonies will not be visible to the naked eye. A decrease in the number of revertant colonies to levels below the spontaneous reversion level may on occasion be seen with thinning. A complete absence of background lawn indicates a high level of toxicity with the inability of the bacteria to grow and form a lawn. Such a toxic dose should not be used.

Occasionally numerous small non-revertant colonies are present on the plate. The colonies are referred to as "pinpoint colonies" and consist of histidine-dependent bacteria that survived high chemical toxicity. These colonies are readily visible by the naked eye and may be mistaken for revertant colonies. Microscopic inspection of the plates will, however, reveal that there is a total absence of background lawn. The pinpoint colonies arise due to the fact that the high level of toxicity resulted in more histidine being available to the surviving His^- bacteria on a per cell basis. Therefore, these bacteria can undergo additional cell divisions until the depletion of the histidine. The histidine dependency can be readily checked by streaking a few pinpoint colonies on GM agar plates supplemented with biotin but without histidine in the absence of the test chemical. A preliminary toxicity experiment is usually performed to determine the top dose that can be tested

It is important that the same number of bacteria be used in the preliminary toxicity assay as well as in the definitive mutagenicity assay. Dilution of the tester strain to plate <1000 cells to enable a quantitative toxicity determination could lead to an erroneous result. On a per cell basis, more chemical would be available to each bacterium than would be available if $1–2\times10^8$ bacteria are exposed to the substance, as happens in the definitive mutagenicity test procedure.

4.10 Minimal defined agar medium

1) Top agar

The top agar, a solution of 5 g NaCl and 6 g noble agar diluted in 1000 ml of destilled water, is one of the most critical medium components in the Ames test because it contains the trace amount of histidine (0.05 mM) for limited growth. It also contains biotin at a concentration of 0.05 mM which is in excess of what is needed for the growth of the *Salmonella* strains. Because

the His^- bacteria stop growing when the histidine is depleted, the final population of His^- bacteria is dependent on the histidine concentration. In turn, the final population on the plate will affect the number of spontaneous revertant colonies. It is therefore important that utmost care is taken to accurately supplement the top agar with 0.05 mM histidine. Too little histidine may result in the background lawn looking sparse, which might be taken as evidence that toxicity is present even on the solvent negative control plates. Too much histidine will cause heavy growth that may obscure the revertant colonies. For the same reason, it is also important to ensure that consistent techniques are used to deliver 2 ml of the top agar to the GM agar plates.

2) GM agar plates

The Ames test uses minimal defined agar medium consisting of Vogel-Bonner E medium (V/B salts), supplemented with glucose (between 0.5% and 2%, w/v) and agar (1.5%, w/v). This medium is generally referred to as glucose minimal agar medium (GM agar). Plates usually contain between 20 and 30 ml of the medium and may be stored for several months in the refrigerator in sealed plastic bags to prevent dehydration. Ideally, each laboratory should work with plates that have a constant volume of agar medium. The plates should be used fairly soon after they have been poured before excessive dehydration of the agar medium occurs. Dehydration of the agar medium will increase the concentrations of the V/B salts, glucose and agar which may affect the spontaneous and induced mutagenesis process. As described above for the top agar, small variations in the medium composition may increase variability in test results.

4.11 Assay procedures

1) Standard plate incorporation assay

The standard plate incorporation assay consists of exposing the tester strain(s) to the test chemical directly on a minimal glucose agar plate (GM plate) usually in the presence and absence of a metabolic activation system (Fig 12). The different components are first added to sterile test tubes containing 2 ml of molten top agar supplemented with limited histidine and biotin. It is important to maintain the top agar at a temperature between 43°C and 48°C and to minimize prolonged exposure to avoid killing of the tester strains. The contents of the tubes are mixed and poured on glucose minimal agar plates. After the top agar has hardened the plates are inverted and

incubated at 37°C for 48 h, at which time histidine-revertant colonies are counted on all plates. The number of colonies on the test plates are compared to those on the (negative) solvent control plates.

Experimental procedure

1. Steps taken prior to performing the experiment

• Inoculate *Salmonella* cultures 15–18 h prior to performing the experiment.

• Label an appropriate number of GM agar plates and sterile test tubes for each test chemical

• Prepare metabolic activation system and keep on ice until use

• Prepare chemical dilutions

• Melt top agar supplemented with 0.05 mM histidine and biotin and maintain at 43°C to 48°C.

2. To the 13×100 mm sterile glass tubes maintained at 43°C, add in the following order with mixing (e.g., vortexing) after each addition.

• 2 ml of molten top agar

• 0.50 ml of metabolic activation (S-9) mix or buffer

• 0.05 ml of the test chemical dilution

• 0.05–0.10 ml overnight culture of the *Salmonella* strain (about $1–2\times10^8$ bacteria per tube).

3. The contents of the test tubes are then mixed and poured onto the surface of GM agar plates.

4. When the top agar has hardened (2–3 min), the plates are inverted and placed in a 37°C incubator for 48 h.

5. The colonies are then counted and the results are expressed as the number of revertant colonies per plate.

2) Preincubation assay

The preincubation assay is a modification of the standard plate incorporation assay and involves exposing the tester strains for a short period (usually 20 min) in a small volume (usually 0.50 ml) containing the test agent with buffer or S-9 mix prior to plating on GM agar medium. It is believed that short-lived mutagenic metabolites have a better chance of reacting with the tester strains in the small volume of the preincubation mixture compared to when the incubation mixture is plated immediately on minimal defined

agar plates. In addition, the smaller preincubation volume results in a higher effective concentration of the S-9 and cofactors.

Experimental procedure

The steps taken prior to performing the experiment are the same as those for the standard incorporation assay described above. The preincubation assay is performed as follows:

1. To the 13×100 mm sterile glass tubes maintained at room temperature add in the following order with mild mixing after each addition

• 0.50 ml of metabolic activation (S-9) mix or buffer

• 0.05 ml of the test chemical dilution

• 0.05 to 0.10 ml overnight culture of the *Salmonella* strain (about 1–2×10^8 bacteria per tube).

2. Incubate the mixture at 37°C for 20 min.

3. To each tube add 2 ml of molten top agar maintained at 43°C to 48°C. The contents of test tubes are then mixed and poured onto the surface of GM agar plates.

4. When the top agar has hardened (2–3 min), the plates are inverted and placed in a 37°C incubator for 48 h.

5. The colonies are then counted and the results are expressed as the number of revertant colonies per plate.

4.12 Experimental design

The Ames *Salmonella* test is a versatile assay as evidenced by the different procedural variations that are available, in addition to the numbers of strains and types of metabolic activation systems that can be used. Before initiating an Ames test, a number of critical parameters will need to be evaluated.

1) Assay procedure

For liquid chemicals the assay of choice is the preincubation assay because many of these chemicals may be volatile. For most other chemicals the standard plate incorporation assay or the pre-incubation assay may be used. However, some researchers believe the pre-incubation assay to be somewhat more selective in detecting mutagens compared to the plate incorporation assay. The reason may be that short-lived mutagenic metabolites have a better chance of reacting with the tester strains in the small volume of the preincubation mixture. For general screening purposes the laboratory should adopt one testing procedure that will help in developing a

historical database for the negative control, and the solvent and positive control chemicals.

The modified (Kado) *Salmonella* microsuspension assay is recommended for testing samples that are available in only small (i.e., mg) amounts or complex mixtures such as urine. The reductive metabolism assay is recommended for chemicals that require reduction for their activation such as azo dyes. The desiccator assays are recommended for volatile liquids and for gaseous substances.

2) Solvents

The solvent of choice is sterile distilled water. Chemicals that do not dissolve in water should be dissolved in dimethyl sulfoxide (DMSO). Other solvents that may be considered are: acetone, ethyl alcohol (95%), tetrahydrofuran, dimethylformamide and methyl ethyl ketone (MEK). These other solvents may be toxic to the bacteria at higher concentrations. Therefore, a toxicity assay with these solvents should be determined in a preliminary assay to determine the maximum concentration that can be used without interfering with bacterial growth and survival. Some of the solvents may also interfere with the metabolic activation system. In such case, the concentration of the S-9 fraction may have to be adjusted. For a comprehensive study on the compatibility of solvents with the Ames *Salmonella* test see Maron et al..

3) Positive and negative (solvent) controls

Each experiment should include solvent controls and diagnostic positive control chemicals specific for each strain and for the metabolic activation system Table 3 lists the representative positive controls.

Table 4
Representative positive control chemicals

Strain	Control chemical (μg/plate)[a]	
	Without activation	With activation
TA97	9-Aminoacridine (50)	2-Aminoanthracene (1–5)
TA98	4-Nitro-*o*-phenylenediamine (2.5)	2-Aminoanthracene (1–5)
TA100	Sodium azide (5)	2-Aminoanthracene (1–5)
TA102	Mitomycin C (0.5)	2-Aminoanthracene (5–10)
TA104	Methyl methane sulfonate (250)	2-Aminoanthracene (5–10)
TA1535	Sodium azide (5)	2-Aminoanthracene (2–10)
TA1537	9-aminoacridine (50)	2-Aminoanthracene (2–10)
TA1538	4-Nitro-*o*-phenylenediamine (2.5)	2-Aminoanthracene (2–10)

[a]Concentration based on 100×15-mm petri plate containing 20 to 25 ml of GM agar.

Table 3. Representative positive control chemicals

4.13 Dose selection

1) Preliminary toxicity determination

It is recommended that a preliminary toxic dose range experiment be performed to determine an appropriate dose range for the mutagenicity assay. If more than one tester strain will be used in the mutagenicity assay, it is sufficient to perform the toxicity assay with strain TA100 with and without metabolic activation. Otherwise, the toxicity assay should be performed with the strain that will be used in the definitive assay. The toxicity determination can also be performed using an alternate toxicity assay developed by Waleh et al..

The chemical should be tested for toxicity over a wide range of concentrations with a total of eight concentrations, spaced in half-log intervals, with the highest dose limited by solubility, or by an arbitrary value (usually 5000 or 10,000 μg/plate). For volatile liquids that are tested in desiccators, 0.5 and 5 ml should be used as the low and high dose, respectively. For gases tested in desiccators, the low and high dose should be 0.5 and 4.5 l, respectively.

The preliminary toxicity assay should be performed in the absence and in the presence of the metabolic activation system that is to be used in the definitive mutagenicity assay. Positive and solvent control chemicals need to be included in the assay; one plate per dose level should be sufficient. It is important that the same number of bacteria be used in the preliminary toxicity assay as well as in the definitive mutagenicity assay. Exposing a diluted culture to the chemical could result in an erroneous higher toxicity level.

2) Doses for definitive assay

A minimum of five dose levels covering a range of at least three logs should be selected for the definitive test. Two or three plates should be used for each dose level and for the controls. For toxic chemicals, only the highest dose used should exhibit toxicity. For non-toxic chemicals, a high dose of 5000 or 10,000 μg/plate is acceptable. Non-soluble chemicals may be tested as a suspension up to a dose level that does not interfere with handling of the suspension with pipets or pipet tips. For non-toxic volatile liquids that are tested in desiccators, 5 ml should be used as the high dose. For non-toxic gases tested in desiccators, the high dose should be 4.5 l.

Must benoted that each test should be performed using a single batch of reagents, media, and agar. The use of different batches of bottom agar plates, top agar, buffer, solvents, cofactors, or S-9 batches within an experiment can

lead to excess variation in the results, or clear demarcations of results between the different batches.

4.14 Strain selection and testing strategies

There has been no evidence to show that the specific tester strains, or the number of tester strains mutated by a chemical, is related to the chemical's potency in other test systems, or the predictivity of the test result for cancer induction or other effects. Therefore, it may not be necessary to test all chemicals with the full complement of strains. Although a single strain is sufficient to demonstrate a mutagenic response, it is generally accepted that a negative result can be defined using 4–5 tester strains. Strains TA98, TA100, and TA97 or TA1537 are always considered necessary. The other strains usually used are TA1535 and/or TA102.

When planning to test a large number of chemicals, it may be most time and cost efficient to perform the testing in stages. For example, it has been shown that testing with strains TA98 and TA100 without metabolic activation and with S-9 from rats or hamsters was sufficient to identify approximately 90% of the mutagens in a population containing about 35% mutagenic chemicals. Using such a strategy, the chemicals not mutagenic or equivocal in these two strains can be tested in additional strains. The higher the anticipated number of mutagens among the chemicals to be tested, the more cost effective the staged testing strategy will be.

If there is prior information about the chemical or class of chemicals to be tested that indicates the need for specific treatment conditions, or that the chemical is likely to induce a certain type of DNA damage, the testing strategy should be directed appropriately. For example, if the chemicals are of a structure that is known to require metabolic activation, the initial screen could be performed only with S-9 mix, or if the chemicals are structurally related to a class known to produce only base-pair substitutions, the initial testing could use only the appropriate target strains.

1) Tier approach

Unless required by specific guidelines, it is recommended that for general screening purposes a tier approach be used using strains TA98 and TA100 with and without metabolic activation

If a positive response is obtained the assay is repeated only using the condition(s) and strain(s) that elicited the positive response. If needed, the dose range may be adjusted to better clarify a dose response. If equivocal or weak positive responses are obtained, the corresponding plasmid-free strains,

TA1538 and TA1535, respectively, should be used. Justification for the use of TA1535 comes from a number of reports about unique positive results in TA1535. Demonstration of a unique response in strain TA1538 is more challenging because few databases have data available compared to databases that have strain TA1535 included. However, specific examples of strain TA1538 giving a unique response exist.

If negative results are obtained in the initial mutagenicity test with strain TA98 and TA100, other strains such as TA1535 and TA97 are used with and without metabolic activation. A confirmation experiment is performed if a positive response is obtained using only the condition(s) and strain(s) in which the mutagenic response was observed. If equivocal results or a weak response are obtained in strain TA97, strain TA1537 can be used in an attempt to clarify the results.

Strains TA102 and TA104 may be used if it is suspected that the chemical may induce oxidative damage (i.e., free radical production), or be a DNA cross-linking agent. Strain TA102 is especially sensitive for detecting DNA cross-linking damage because it has an intact (wild-type) DNA excision repair mechanism which is required for the repair of such damage.

If prior to testing information is available about the potential mutagenicity of the chemical or chemical class, the initial testing is performed with the strain(s) most likely to yield a positive response.

2) Battery approach

A battery approach employs a fixed set of tester strains for testing a chemical in the presence and absence of a metabolic activation system. For general mutagenicity testing it is recommended that strains TA97, TA98, TA100 and TA102 be used. However, some laboratories use strain TA1535 instead of strain TA102 when a tier approach is used in the selection of tester strains. As in the tier approach, strains TA1538 and TA1535 are used when equivocal results are obtained with their respective pKM101-carrying strains, TA98 and TA100. Strain TA1537 can detect mutagens not detected by strain TA97. Strain TA104, though in some laboratories tested in parallel with TA102, is recommended when negative results are obtained in strain TA102 especially if it is suspected that a chemical may cause oxidative DNA damage.

4.15 Positive control chemicals

Diagnostic mutagens (positive control chemicals) must be included in each experiment to confirm the reversion properties and specificity of each

tester strain, and the efficacy of the metabolic activation system. Table 3 lists the positive control chemicals with their respective concentrations/plate that have been routinely used in the authors' laboratories. Other chemicals can be selected as positive controls. Where the test chemicals are members of a specific chemical class, a positive control chemical with a similar structure, or requiring similar metabolism, should be used. The optimum positive control concentration should be determined for each new batch of metabolic activation (S-9). Some researchers have suggested that 2-aminoanthracene should not be recommended as the only positive control to evaluate the metabolizing activity of the S-9 fraction, because it has been shown that 2-aminoanthracene may be activated by enzymes other than the microsomal cytochrome P450 family.

5. Seed germination-Root elongation tests

5.1 Introduction

Plant seed germination and root elongation tests have been used as simple and sensitive techniques for detection of the toxicity of various environmental pollutants such as heavy metals, phenolic compounds, refuse compost, industrial effluents from heavy machinery, agricultural product utilization and specialist chemical industries. Plant seeds make excellent bioassay test organisms. As long as they are kept dry, they remain dormant and can be stored for long periods of time without losing viability. Once seeds are hydrated, they enter the germination (sprouting) phase during which they undergo rapid physiological changes and become highly sensitive to environmental stress.

Many plant species, including cabbage, lettuce, carrot, radish, cucumber, tomato and oats, have been recommended for seed germination and root elongation tests. Radish seeds work well as they are easy to handle, not sensitive to light, and germinate in 2-3 days. Lettuce seeds germinate in 3-5 days; however, they are light sensitive and must be incubated in the dark. Lettuce sprouts tend to be relatively straight making it easier to measure length.

Seed germination test is considered to be used as a rapid, simple, reliable and reproducible technique to indicate the damaging effects of land application of animal manure, compost or sewage sludge on plant growth. Root elongation was found to be a more sensitive parameter than seed germination, as a reduction in root growth was observed throughout the study while inhibition of seed germination was found only towards the end of the experiment. Ratsch (1983) concluded that inhibition of root elongation was a valid and sensitive indicator of toxicity. Germination index (GI) which combines germination and root growth, has proved to be a very sensitive parameter. GI is able to account both for low toxicity, which affects root growth and increased toxicity which affects germination.

Before describing the test method it’s useful to give some definitions:

ECX means the experimentally derived chemical concentration that is calculated to affect X percent of the test criterion.

Embryo means the young sporophytic plant before the start of germination.

Germination means the resumption of active growth by an embryo. The primary root should attain a length of 5 mm for the seed to be counted as having germinated.

Hypocotyl means that portion of the axis of an embryo or seedling situated between the cotyledons (seed leaves) and the radicle.

Radicle means that portion of the plant embryo which develops into the primary root.

Test solution means the test chemical and the dilution water in which the test chemical is dissolved or suspended.

5.2 Summary of the test

Seed should be separated into appropriate size classes, and that size class containing the most seed should be used exclusively for the test. Fresh test solutions should be added to Petri dishes that have been completely filled with either precleaned quartz sand, 200 mm glass beads, or other inert material. The seed should then be positioned on the substrate allowing adequate room for anticipated growth. It is recommended that the radicle end of the seed be aligned in the direction of this growth. Petri dish lids should be used to hold the seed in place, and the dishes sealed with tape. Deionised or glass-distilled water should be added to the substrate prior to positioning the seed for those chemicals that are insoluble in water and that should be sorbed to the substrate.

The dishes should be placed in a seed germinator or other growth facility at a slight angle to facilitate linear root growth. Seed should be incubated in the dark until at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. The dishes must be incubated at room temperature for 1 - 5 days. The number of days to germination will depend on the type of seeds used. If the paper begins to dry out, should be added a few drops of water distilled or test water to the appropriate filter paper. The number of seed that germinate should be counted, and root lengths

measured. Concentration response curves, EC10s, and EC50s for seed germination and root elongation should be determined and reported for each of the species tested.

Test chemicals that are soluble in water should be dissolved in deionised or glass-distilled water and added to the substrate in the petri dishes at the start of the test. Test chemicals that are insoluble in water but which can be placed in aqueous suspension with a carrier should be suspended in deionised or glass-distilled water with the carrier and then added to the Petri dishes. The carrier should be soluble in water, relatively nontoxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol, and others have been used extensively in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants. Tests of the carrier effect should be included in the test experimental design and conducted simultaneously as controls. Water-insoluble chemicals for which no nontoxic water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution and substrate should be placed in a rotary vacuum apparatus, and evaporated, leaving a uniform coating of test chemical on the substrate. A weighed portion of the substrate should be extracted with the same organic solvent and the chemical assayed before the containers are filled. Solvent controls should be included in the experimental design and tested simultaneously. Deionised or glass-distilled water should be added to the treated substrate prior to positioning the seed on the substrate.

A range-finding test should be conducted to establish if definitive testing is necessary and to determine test solution concentrations for the definitive test. The seed should be exposed to a chemical concentration series (e.g., 0.01, 0.1, 1.0, 10, 100, and 1,000 mg/L. The lowest concentration in the series, exclusive of controls, should be at the detection limit of the chemical. The upper concentration, for water soluble compounds, should be the saturation concentration. The test consists of one run for each of the recommended plant species or selected alternates. A minimum of 15 seeds per species should be exposed to each chemical concentration and control. The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. The exposure period may be shortened if data suitable to establish the test solution concentration series for the definitive test can be obtained in less time and if the definitive test is to be conducted. No replicates are required and nominal concentrations of the chemical are acceptable unless definitive testing is not

required. Definitive testing is not necessary if the highest chemical concentration tested results in less than a 50 percent inhibition of germination or reduction in root growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent inhibition of germination or reduction in growth. Graphical analysis of the range-finding data facilitates selection of chemical concentrations for the definitive test.

The purpose of the definitive test is to determine the concentration-response curves, the EC10s and EC50s for seed germination and root elongation for each species tested, with the minimum amount of testing beyond the range-finding test. The seed of each species tested should be exposed to at least 6 concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/L). The concentration ranges should be selected to determine the concentration response curves between the EC10 and EC50 for both germination and root elongation. Test solutions or substrate extracts should be analyzed to determine chemical concentration prior to use. Selection of seed from the size class lot to be exposed to each test concentration should be unbiased. At least three replicates, each with at least 10 seed per species should be tested for each concentration and control. Every test should include controls consisting of the same dilution
water, conditions, procedures and seed from the same lot used in the exposure group, except that none of the chemical is added. If a carrier (solvent) is needed to suspend or disperse the chemical, a separate carrier control should also be used. The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. When both conditions are satisfied, the mean number of seed germinating and mean root length per treatment (and control) can be determined. If the test chemical concentration series does not bracket the EC10 through EC50, for both germination and root elongation, the test should be repeated (at a higher or lower concentration series). Concentration response curves, EC10s and EC50s for germination and root elongation should be determined for each species tested and reported along with their 95 percent confidence limits. Any abnormal seedling development or appearance such as lesions,
enhanced root growth (measured), discoloration, swelling, loss of turgor, etc., should also be reported. A randomized complete block design is recommended for the definitive test with blocks delineated within the seed germinator or growth chamber. If, for any reason, blocking is not feasible, total randomization within chambers is acceptable. Temperature in the

germination facility should be recorded hourly. The pH of the test solutions should be recorded at the initiation of the definitive test.

Stock solutions should be diluted with glass-distilled or deionised water to prepare test solutions. Standard analytical methods should be used (if available) to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use. The number of seeds that germinate should be counted and root lengths measured for each definitive test species. All root elongation measurements for a given species should be made sequentially before proceeding to the next species. Root length should be measured from the transition point between the hypocotyl and root to the tip of the root. Means and standard deviations should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves.

Test plants recommended for use include:

(A) *Lycopersicon esculentum* (tomato)
(B) *Cucumis sativus* (cucumber)
(C) *Lactuca sativa* (lettuce)
(D) *Glycine max* (soybean)
(E) *Brassica oleracea* (cabbage)
(F) *Avena sativa* (oat)

(G) *Lolium perenne* (perennial ryegrass)
(H) *Allium cepa* (common onion)
(I) *Daucus carota* (carrot)
(J) *Zea mays* (corn)

Other species of economic or ecological importance to the region of impact may also be appropriate for testing. A minimum of 10 species should be tested. Information on seed lot, the seed year or growing season collected, and germination percentage should be provided by the supplier of the seed. Only untreated seed (not treated with fungicides, repellents, etc.) taken from the same lot and year or season of collection should be used in a given test. In

addition, all seed of a species used in a test should be from the size class, which contains the most seed. Damaged seed should be discarded. Standard seed dockage sieves should be used to size seed.

For the test is needed a seed germinator, or other controlled environment chamber capable of maintaining a uniform testing temperature of 25 ±1 °C is required. In addition, the facilities should include work areas for sizing, counting, and exposing seed for root measurement. If possible, these areas should be isolated from other activities. A fume hood may be needed when testing substances potentially hazardous to human health. Apparatus for distilling and deionising water are needed unless reagent grade water is used. Refrigeration facilities to hold the seed in cold storage (5 °C) in moisture-proof containers at seed moisture contents of less than 10 percent are also needed.

Disposal facilities should be adequate to accommodate spent glassware, sand, beads, and test solutions at the end of each run and any bench covering, lab clothing, or other contaminated materials. Petri dishes and sand or glass beads, or other inert substrate to fill them are needed. Large (200 mm) glass Petri dishes are recommended. Perlite, vermiculite, or native soils should not be used as substrates. All glassware and the substrate should be cleaned before each test following standard good laboratory practice. The substrate should be washed in 7.5 N nitric acid and rinsed with a mild base followed by washes of glass-distilled or deionised water. The pH of the washed substrate should be near neutral. If the glass beads are to be reused, they should be heated to 100 °C for 8 to 12 hours prior to acid washing. A dichromate solution should not be used for cleaning beads or Petri dishes. Sand and plastic Petri dishes should not be reused. If fungal or other microbial contamination interferes with seed germination so that germination is less than 65 percent in the controls, glassware should be sterilized and/or the seed surface sterilized prior to use, e.g., the seed may be soaked for 10 minutes in a 10 percent sodium hypochlorite solution, then rinsed and soaked for 1 hour in glass-distilledwater.

Environmental conditions should be controlled to maintain incubation temperature at 25 ±1 °C in complete darkness. Incubation conditions may have to be adjusted to meet germination and root length criteria in the controls if species other than the 10 recommended for use are tested.

The sponsor should submit all data developed during the test that are suggestive or predictive of phytotoxicity.

In addition to the general reporting requirements, the following should be reported:

(1) Information on the source and history of the seed, germination percentage reported by the supplier, and the seed size class used for testing.

(2) The number of seed of each species per treatment, the number of replicates, carriers, incubation conditions, and seed sterilization procedures.

(3) The concentration of the chemical added to each treatment dish and its pH (pH is optional).

(4) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1,000 mg/L) had no effect on the test species, report the results by species and concentration and a statement that the chemical is of minimum phytotoxic concern.

(5) If the range-finding test showed greater than 50 percent inhibition of germination or root elongation at a test concentration at the analytical detection limit, the results by species and concentration and a statement that the chemical is phytotoxic below the analytical detection limit.

(6) For each species included in the definitive test, means and standard deviations for germination and root length in each treatment. In addition, concentration response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and EC10s and EC50s identified.

(7) Methods and data records of all chemical and numerical analyses including method validation and reagent blanks.

(8) The data records of the incubation temperature, germination counts, and root length measurements.

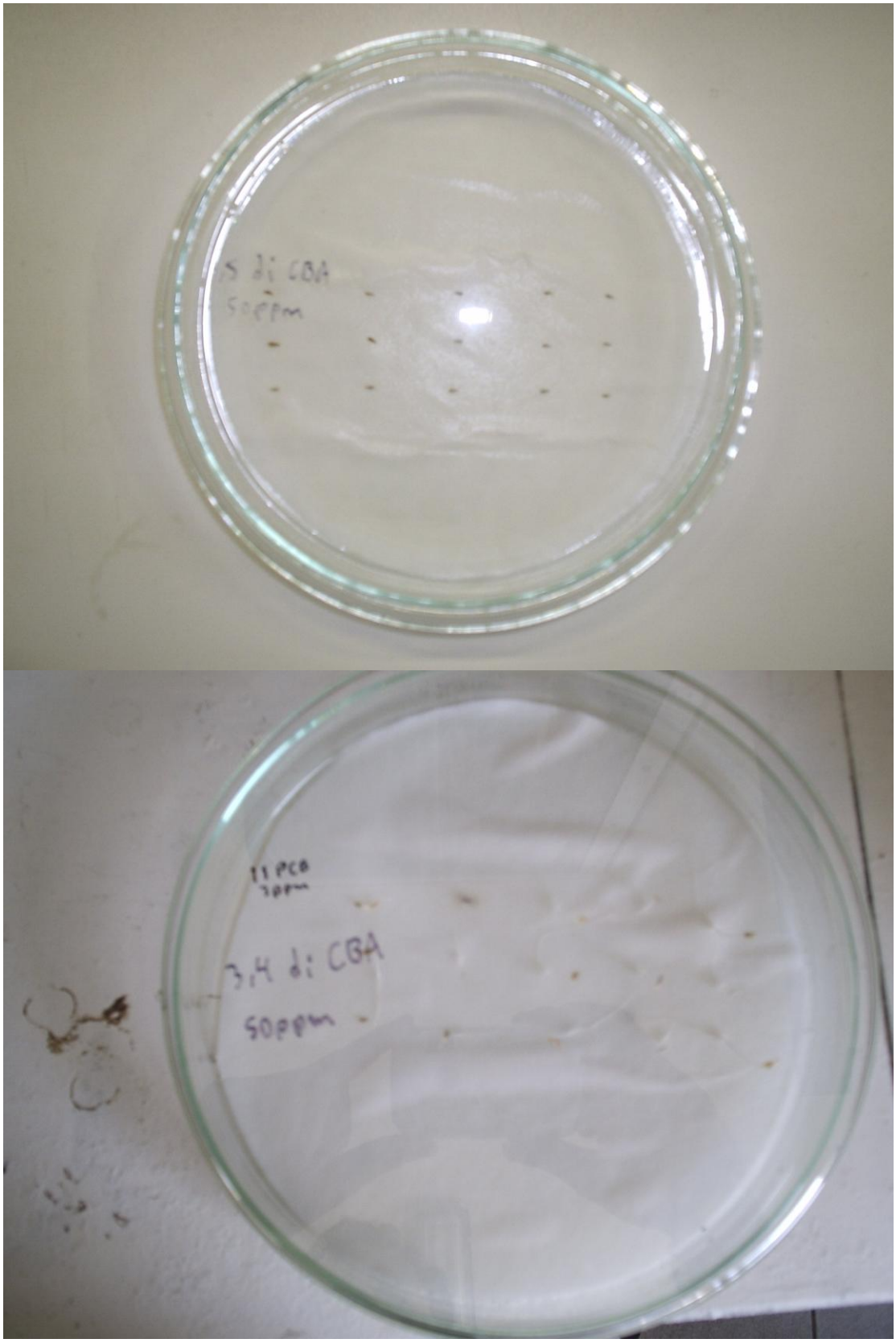


Fig14. Seeds of lettuce before and after cultivation (4 days)

5.3 Special features of my experiments

For my experiments I used seeds of lettuce (Lactusa Sativa) and I cultivated 15 seeds evenly spaced in each Petri dish. The seeds were placed over two layers of blotter paper with referent medium which consisted of 18,5 g/l $CaCl.2H_2O$, 2,3 g/l KCl, 49,3 g/l $MgSO_4$. $7H_2O$, 25,9 g/l $NaHCO_3$ (2,5 ml for 1000ml H_2O). The Petri dishes were folded with aluminium foils and incubated for 4 days at the temperature of 22°C without light. I had two control Petri dishes and two for each concentration of the substance tested, all PCBs and CBAs were tested at the concentrations of 3 and 50 ppm.

5.4 Experimental data analysis

First I calculate the average length of the roots for each Petri dish and then the average for both Petri dishes of the same concentration of testing substance; I do the same for the control solution. The coefficient of inhibition is given by the formula:

$$I= (L_C - L_S) / L_C*100$$

Where

L_C is the length of root for control solution
L_S is the length of root of the sample

In that formula instead of the length of each one of the roots I use the values of the average length of the roots of the control solution and the samples. The sample is considered to be toxic in case that the I-value is greater than 30%.

If fewer than 80% of the seeds in the control dish germinate, this indicates a problem with the assay (*ie*. poor quality seeds, poor incubation conditions) and the test should be rerun.

6. Bioscreen toxicity test-Growth curves

6.1 Growth rate-kinetics

The growth rate of a microorganism is the time that it takes for the cell to reproduce. This characteristic of the cell can be quite variable and depends on several physiological parameters. As a result of microbial growth there is an increase in the number of cells and the biomass.

Reproduction by binary fission results in doubling of the number of viable bacterial cells. Therefore during active bacterial growth the bacterial population is continuously doubling. The time required to achieve a doubling of the population size, known, as the doubling time or generation time is the unit of measure of the bacterial growth rate.

Because the bacterial population doubles every generation, if the initial population size is N_0, after n generations of growth:

$N_n=2^n * N_0$

The growth rate of a bacterial culture can also be expressed as a function of the reciprocal of the doubling time, K:

$$k = \frac{n}{t} = \frac{\log N_t - \log N_0}{0.301 * t}$$

This mathematical formula for the bacterial growth rate is based on the premise that the rate of increase is proportional to the number, or mass of cells present at any given time and that the doubling time is constant during a period of growth.

By determining cell numbers during the period of active cell division, the generation time can be estimated. In comparing generation times, one finds that bacteria reproduce more rapidly than higher microorganisms. A bacterium such as *E. coli* can have a generation time as short as 20 minutes under optimal conditions, although in nature many bacteria have generation times of several hours. One cell of a bacterium with a 20 minute generation time could multiply to 1,000 cells in 3.3 hours and to 1,000,000 cells in 6.6 hours. The generation times for many archaea is 20 to 30 minutes but some

reproduce more slowly and have doubling times of 45-90 minutes. *Methanococcus* for example has a doubling time of 20 minutes in 85°C.

6.2 Phases of bacterial growth

If an old culture of bacteria is inoculated or added to a fresh medium and the cell concentration is periodically measured, a curve describing the change in cell number against time can be drawn. This curve, called the growth curve, will be hyperbolic due to exponential nature of bacterial growth.

6.2.1 Lag phase

The typical growth curve of a bacterial culture begins with the lag phase. During the lag phase there is little increase in cell numbers. Rather, during this phase the bacteria are transporting nutrients inside the cell from the new medium, preparing for reproduction and synthesizing DNA and various inducible enzymes needed for cell division. They increase in size during this process but the number of cells does not increase.

6.2.2 Exponential phase

In the log growth phase, also called the exponential growth phase, bacterial cell division begins and proceeds as a geometric progression. One cell divides to form two, each of these cells divides to form four, and so forth in a geometric progression.

During the log phase of growth , so-named because the logarithm of the bacterial biomass increases linearly with time, bacterial reproduction occurs at a maximal rate for the specific set of growth conditions. This growth phase is better called the exponential growth phase because the number of cells is increasing as an exponential function of time. Growth during much of the exponential growth phase is said to be balanced, that is, the concentrations of all macromolecules of the cell are increasing at the same rate. The average composition of the cells therefore remains constant. During the log phase of the growth curve, the growth of a bacterium is proportional to the biomass of bacteria that is present.

The growth rate during the log phase is described by the equation:

$$\frac{dB}{dt} = \alpha * B$$

where B is the bacterial biomass, t is time, and α is the instantaneous growth rate constant. During this period the generation time of the bacterium is determined. If a bacterial culture in the exponential growth phase is inoculated into an identical fresh medium, the lag phase is usually bypassed ad exponential growth continues. This occurs because bacteria are already actively carrying out the metabolism necessary for continued growth. If, however, the chemical composition of the new medium differs significantly from that of the original growth medium, the bacteria go through a lag phase wherein they synthesize the enzymes needed for growth in the new medium before entering the logarithmic growth phase.

6.2.3 Stationary phase

In a cultivation, logarithmic growth cannot be endless. In fact there is a slight decrease and finally nullification of the growth rate. In this point we consider that the cultivation is in the stationary phase. The passing from the logarithmic to the stationary phase involves a period of non-balanced growth of the cultivation, during which the percentage of cells that participate in the division process is continuously decreasing. This reduction can be explained by the lack of some substances, which are totally consumed during the previous phases or by the presence of by-products of the growth which eliminate the development of the cultivation. During the stationary phase the number of cells is constant while these cell functions which are related with the maintenance still go on (active transition through membrane, ATP composition). The availability of nutrients (especially the carbon sources) decrease under the limit for the survival of the cells which start dying and give proteins,fat and hydrocarbons as products of their decomposition. These nutrients are consumed for the growth of the rest of the microorganisms (cannibalism). These two procedures finally equilibrate and so there is neither development nor decrease of the number of cells.

6.2.4 Death (decline) phase

The death of the cells means their decomposition. The decline phase can also be described by a logarithmic function although the death rate is lower than the growth rate. There are big differences of the death rate among microorganisms which depend mostly on environmental factors and on the specie of the microorganisms.

6.3 Bioscreen

Bioscreen uses the well-known biological phenomena that all microorganisms increase a turbidity of a liquid growth medium during growth and multiplication. Almost any experiment where the growth of bacteria, yeast, fungi, cell or phage are wanted to follow kinetically can be performed with it. Bioscreen C is an automated turbidimeter able to measure optical density and plot microbiological growth curves. As microorganisms grow, they increase the suspension turbidity of their growth medium. The test length is from a single measurement to three months. Bioscreen C is extremely versatile; any microorganism (aerobic or anaerobic) can be grown with any liquid growth medium and with any chemical by using user-created test protocol.

By measuring the turbidity of this medium over time, an optical density (OD) curve can be generated via export into Excel. Growth curves produced by the Bioscreen are presented as OD plotted against time. A detection time (determined from a point where there is a rapid change in OD) can be related to cell number. The detection time for cells grown in the test broth can be compared to the detection time for cells grown in the control broth. The detection times can be converted to cell numbers using calibration graphs. The pass limit of the QC test was taken to be <1 $\log_{10}$ difference between cell numbers in test and control media. This is a more stringent criteria than that of the tube dilution method.

The curve reflects the growth (increased concentration) of the organism. Specimens are manually dispensed using a 5 or 10 channel pipette into the wells of a special microplate. A 10 by 10-well plate (400µl per well) is designed to provide uniform temperature across each of the 100 wells in the plate. A fitted plate lid with special characteristics eliminates troublesome condensation, which can interfere with OD readings.

The device includes an incubator to provide constant temperature and a linear shaker to assure good mixing. The instrument is equipped with a sensitive reader that measures turbidity, a technique more sensitive than a spectrophotometer. With 8 filters installed to measure specific wavelengths. Two 100 well sterile honeycomb plates with covers are handled by the instrument at one time, making it possible to run 200 samples simultaneously. Turbidometric measurements are made kinetically during the course of the run. This information is processed to generate microbiological growth curves,

plotting turbidity vs. time and exported to a PC for report generation via Excel.

Bioscreen test allows evaluation of the effect of various concentrations of toxicants on bacterial viability and growth in comparison with controls cultivated without toxicants. Values of OD_{400nm} and the time dependence of growth are monitored in parallel samples incubated with or without toxicants for 2 days. Minimal medium containing glycerol (5g/l) and toxicants were used for the incubation.

The device has software, which drives the workstation, records results and collects OD data. All experimental data (OD, test parameters, sample names, growth curves, and results) can be exported to Excel or Word for preparation of reports. It can perform up to 200 microbiological experiments (each well is one test) simultaneously. Bioscreen assay is fast, one is able to complete as many tests in just a few weeks as would normally take a year by using traditional manual techniques. Bioscreen work covers all main areas of microbiology, food microbiology, pharmaceutical/medical microbiology, environmental microbiology and university research microbiology. The range of tests is virtually endless - miniaturized bioassay (of vitamins, antibiotics, amino acids, etc.), effect of chemicals on pure and mixed cultures, development of antibiotics, QC of food (total count - CFU/g) and others. Bioscreen gives to the user the ability to monitor the growth of pure or mixed cultures and check the effect of single or multiple parameters (e.g. pH, temperature, chemicals) of different microorganisms.

Some examples of the thousands of tests that can be performed by Bioscreen C: toxicity measurement, stimulant and inhibitor screening, growth media development (wide range or selective), testing and rapid development of pharmaceutical, optimization of fermentation process and microbiological production, optimization of wastewater treatment process (sludge or biofilm) and single-cell-protein (SCP) production, development of preservative and antifungal, development of milk substitute, mutagenicity research, research microbiology, bacteriophage study, measurement of antimicrobial effect, water activity study, development of biosensor, in vitro testing, viability tests, ammonification, nitrification and denitrification studies, waste detoxification, phenol and heavy metal removal studies, bacteria measurement, theoretical study of metabolic processes (mathematical modeling), and many others.

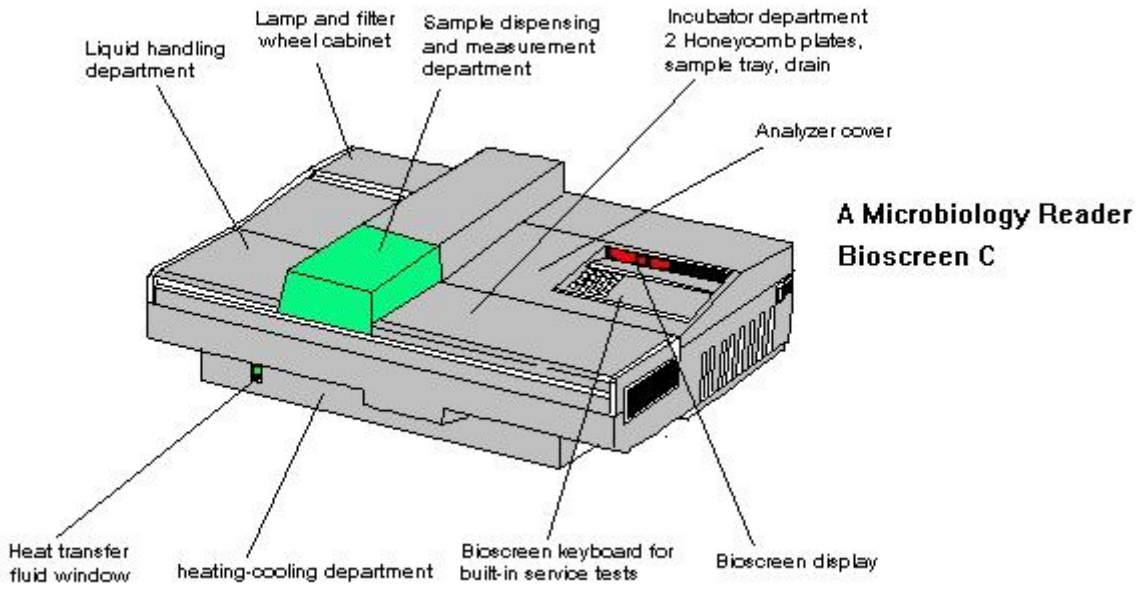


Fig.15 Bioscreen device

Bioscreen consists of:
1)Sterile multiwell plates Bioscreen uses disposable, sterile 100-well Honeycomb plates with covers. Two Honeycomb plates can be placed for the same run in the incubator/reading chamber. The volume of each well is 400 µl.
2)Turbidity and colour reader Bioscreen C measures culture turbidity, which indicates bacterial count better than the measurement of culture colour. Turbidity is measured by a wide band filter which is rather insensitive to color change in the sample. For color measurement Bioscreen C has 7 other filters with special wavelengths.
3)Incubator and optional dispenser. A heating/cooling system provides a wide range of incubation temperatures (1.0-60.0 °C), and the temperature can be changed during the experiment. The special technology guarantees that there is no condensed liquid on the lower side of the multiwell plate cover. Bioscreen has an optional automated sterile dispensing feature. Sterilization of tips and tubing can be programmed for each step.

Below are mentioned the Key Features of the device

- Kinetic measurement of turbidity of up to 200 samples simultaneously.
- Open system for almost any bacteria, fungi, yeast, cell or phage.

- Is an automation tool for all research microbiologists and for many clinical/industrial users.
- Shaking, incubating, OD measurement and reporting.
- Incubation temperature range is 1.0-60.0 °C in steps of 0.1 °C.
- Result Modes: 1) Color Growth Curves, 2) Assay Test Calculated Results, 3) OD Matrix
- Software for data process
- A lot faster compared to manual experimentation

7. Results & Discussion

7.1 Microtox test

PCBs were tested with the microtox test, the luminescent bacteria I used was *Vibrio Fisheri*. In the next table there are values of EC50, which is the concentration of the toxicant in mg/l for which we have 50% reduction of the luminescence. Therefore high values of EC50 stand for low toxicity. For each PCB is presented the average EC50 from several number of tests that have been completed.

PCBs	**3**	**2,4'**	**2,5**	**2,6**	**3,3'**	**3,4'**
average EC50	2,76	2,06	1,84	11,81	1,97	27,23

Table 4. EC50 results for PCBs

3,4' exhibited low toxicity, the lowest values of EC50 were given from 2,4', also low are the values for 2,5-3,3' and 2,4'. Results from older tests that were made in the microbiology lab of VSCHT are presented in table 5. It is interesting to mention that in this test a variance of the results was more usual compared to the other tests I used. That can be seen in the older results for 3,4 PCB.

PCBs	**2,4'**	**2,5**	**3,3'**	**3,4**	**3,4'**
meas.1	2,27	0,59	1,75	166,7	30,57
meas.2		1,46	2,23	19,52	
meas.3		1,18		121,73	
meas.4				24,59	
meas.5				13,16	

Table 5. Older tests of EC50 for PCBs

In the table below there are presented the results of microtox test, toxicity is expressed again by the average EC50.

CBAs	2	3	4	2,3	2,4	2,5	2,6	2,3,5	2,4,6	3,4	3,5
Average EC50	7,63	4,92	8,2	5,56	9,74	4,12	21,28	9,86	5,18	6,25	7,16

Table 6. EC50 results for CBAs

2,6-2,3,5 and 2,4 CBAs seem to be the less toxic whereas 2,5-3 and 2,4,6 caused a sudden decrease of luminescence to the samples.
In table 7 are presented few values of EC50 from older tests that other student completed in the laboratory of microbiology.

CBAs	2	3	4	3,4
meas. EC50	8,4	4,6	7,16	7,8

Table 7. Table 5. Older tests of EC50 for CBAs
Generally PCBs have lower values of EC50 than CBAs, which indicates that are more toxic for *Vibrio Fisheri*.

7.2 Germination of seeds

In the seed germination ecotoxicity test we used seeds of lettuce (*Lactusa Sativa*). In the table below are presented the results for PCBs. The toxicity is expressed by the inhibition coefficient; if we have more than 30% of inhibition we consider that the samples are toxic.

		3 ppm	50 ppm
PCB 8	2,4’	18,8	71,1
PCB 9	2,5	15,0	65,1
PCB 10	2,6	11,8	55,7
PCB 11	3,3’	16,4	64,6

Table 8. Inhibition coefficient for PCBs

Among the samples of 3ppm no one exhibited high levels of toxicity, all of them showed inhibition lower than 20%. On the other hand all the samples of 50ppm PCBs caused inhibition more than 30%, 8 gave the highest value of

inhibition as in the microtox test and 10 the lowest, 9 and 11 gave similar values also in this test. In CBAs we find higher values of inhibition because of the highest solubility in the water that seeds use to grow up.

Older results from other tests of microbiology lab are presented in the table 9.

	3 ppm	**50 ppm**
2,2'	7,9	84,6
2,3	38,9	54,2
2,3'	8,9	26,9
2,4'	27,9	63,5
2,5	34,7	67,2
2,6	48,0	61,2

Table 9.Older test results of inhibition coefficient for PCBs

In these results 2,4'-2,5 and 2,6 caused more inhibition in the concentration of 3ppm compared to the previous test but didn't showed a high increase of inhibition in the concentration of 50 ppm. It's possible that samples of 3 ppm showed in the second test for some reason an excess of inhibition. The values for 50 ppm are similar for both tests.

CBAs were also tested with the seed germination assay, the results are presented in table 10.

CBAs	**2**	**3**	**4**	**2,4,6**	**2,5**	**3,4**	**2,3**	**3,5**	**2,4**
3ppm	32,9	58,6	14,3	41,4	48,6	48,1	43,2	32,1	27,2
50ppm	72,1	84,3	28,1	93,4	98,8	96,1	88,4	90,1	81,6

Table 10. Inhibition coefficient for CBAs

All samples except for 4 CBA showed high degree of inhibition to the roots of *lactusa sativa.*
2,5 is again the most toxic as in microtox assay, followed by 3,4-2,4,6 and 3,5 CBAs exhibited again the highest levels of toxicity whereas 4 is again less toxic.

7.3 Ames test

In ames test I used the strains TA98 and TA100of the bacteria S. *typhimurium*. Ames test is used to assess the mutagenic effect of substances. A sample can be considered mutagenic in case that the ratio Rc/Ro is greater than 2.

Below are presented the results of CBAs on the strain TA98.

TA 98 2 CBA			**average**	**Rc/Ro**	**TA 98 3 CBA**		**average**	**Rc/Ro**
control	25	18	21,5	1,00	6	1	3,5	1
200ppm	23	28	25,5	1,19	20	20	20	5,71
100ppm	23	24	23,5	1,09	14	24	19	5,43
50ppm	15	19	17	0,79	12	10	11	3,14
20ppm	13	12	12,5	0,58	9	11	10	2,86

TA98 4CBA		**average**	**Rc/Ro**		**TA 98 2,5CBA**		**average**	**Rc/Ro**
6	1	3,5	1,00	control	6	1	3,5	1,00
16	15	15,5	4,43	200ppm	4	3	3,5	1,00
13	10	11,5	3,29	100ppm	9	4	6,5	1,86
11	9	10	2,86	50ppm	12	7	9,5	2,71
12	8	10	2,86	20ppm	11	17	14	4,00

	TA 98 2,4,6 CBA		**average**	**Rc/Ro**
control	6	1	3,5	1,00
200ppm	20	16	18	5,14
100ppm	15	15	15	4,29
50ppm	12	10	11	3,14
20ppm	11	5	8	2,29

Tale 11. Mutagenicity factor of CBAs on strain TA98

2 CBA didn't show any sign of mutagenic effects in any of the concentrations, 3-4-2,4,6 were mutagenic for all the concentration tested and 2,5 CBA was mutagenic for the concentrations below 100 ppm.

In table 12 are presented the results of the test for the strain TA100. The samples of CBAs didn't show any mutagenic effects on that strain of the bacteria S. *typhimurium*.

TA 100 4 CBA			**average**	**Rc/Ro**	**TA 100 2,5 CBA**			**average**	**Rc/Ro**
control	182	163	172,5	1,00	control	182	163	172,5	1,00
200ppm	148	148	148	0,86	200ppm	151	172	161,5	0,94
100ppm	109	104	106,5	0,62	100ppm	140	181	160,5	0,93
50ppm	118	150	134	0,78	50ppm	149	159	154	0,89
20ppm	153	137	145	0,84	20ppm	147	141	144	0,84

TA 100 2,5 CBA			**average**	**Rc/Ro**	**TA 100 2 CBA**			**average**	**Rc/Ro**
control	182	163	172,5	1,00	control	126	90	108	1,00
200ppm	172	168	170	0,99	200ppm	92	106	99	0,92
100ppm	155	173	164	0,95	100ppm	99	133	116	1,07
50ppm	158	160	159	0,92	50ppm	138	132	135	1,25
20ppm	150	110	130	0,75	20ppm	152	110	131	1,21

TA 100 3 CBA			**average**	**Rc/Ro**
control	182	163	172,5	1,00
200ppm	162	177	169,5	1,11
100ppm	149	138	143,5	0,94
50ppm	132	148	140	0,92
20ppm	129	120	124,5	0,81

Tale 12. Mutagenicity factor of CBAs on strain TA100

The tests on strain TA97 were also negative regarding the mutagenicity of 2-3-4 and 2,5 CBA.

TA 97 2 CBA			**average**	**Rc/Ro**	**TA 97 3 CBA**			**average**	**Rc/Ro**
Control	152	140	146	1,00	Control	152	140	146	1,00
200ppm	155	140	147,5	1,01	200ppm	182	166	174	1,19
100ppm	172	143	157,5	1,08	100ppm	155	163	159	1,09
50ppm	159	122	140,5	0,96	50ppm	144	152	148	1,01
20ppm	106	126	116	0,80	20ppm	120	171	145,5	1,00

TA 97 4 CBA			average	Rc/Ro	TA 97 2,5 CBA			average	Rc/Ro
control	152	140	146	1,00	control	152	140	146	1,00
200ppm	149	148	148,5	1,02	200ppm	120	150	135	0,92
100ppm	151	158	154,5	1,06	100ppm	142	140	141	0,97
50ppm	155	157	156	1,07	50ppm	132	133	132,5	0,91
20ppm	120	106	113	0,77	20ppm	135	149	142	0,97

Tale 13. Mutagenicity factor of CBAs on strain TA97

Also the results on strain YG1042 don't indicate mutagenic effects of 2-3-4 and 2,5 CBA except for the concentration of 5 ppm for 2CBA which exhibited marginal mutagenicity and the concentration of 10 ppm for 4 CBA for which the value of Rc/Ro is very close to the limit (2,00). The fact that these two CBAs didn't give higher values of the Rc/Ro ratio in higher concentrations indicates that the two results which are above the limit can be attributed to an error of the experimental process. Strain YG1042 is constructed for detection of the same mutation type as strains TA 98 and TA 100 respectively. YG strains were selected from TA strains and have enlarged enzyme complements enabling them to metabolize tested substances in a different way than TA strains. YG strains can be more sensitive.

YG 1042 2 CBA			average	Rc/Ro	YG 1042 3 CBA			Average	Rc/Ro
control	26	21	23,5	1,00	control	26	21	23,5	1,00
20ppm	31	22	26,5	1,13	20ppm	33	37	35	1,49
10ppm	33	35	34	1,45	10ppm	37	36	36,5	1,55
5ppm	51	43	47	2,00	5ppm	38	27	32,5	1,38
2,5ppm	34	21	27,5	1,17	2,5ppm	29	27	28	1,19

YG 1042 4CBA			average	Rc/Ro	YG 1042 2,5CBA			average	Rc/Ro
control	26	21	23,5	1,00	control	26	21	23,5	1,00
20ppm	33	20	26,5	1,13	20ppm	30	59	44,5	1,89
10ppm	40	55	47,5	2,02	10ppm	35	39	37	1,57
5ppm	35	33	34	1,45	5ppm	39	42	40,5	1,72
2,5ppm	41	28	34,5	1,47	2,5ppm	29	40	34,5	1,47

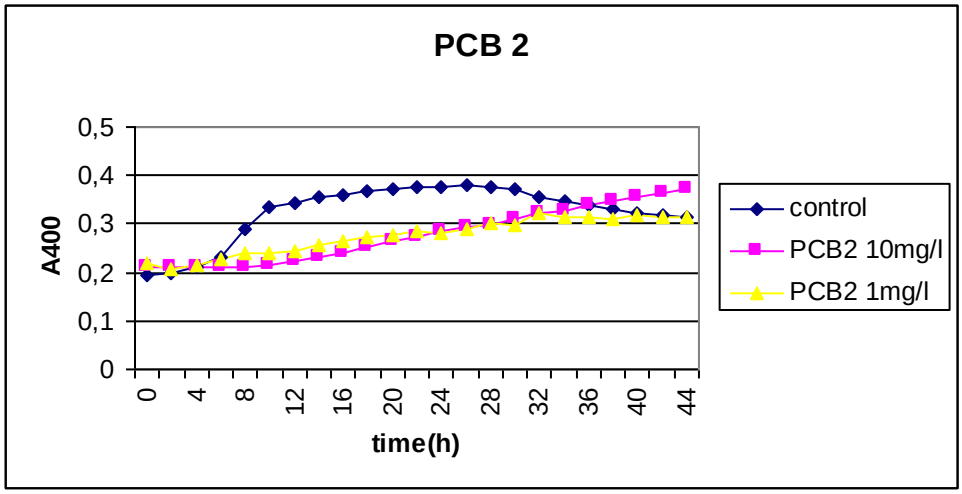
Tale 14. Mutagenicity factor of CBAs on strain YG1042

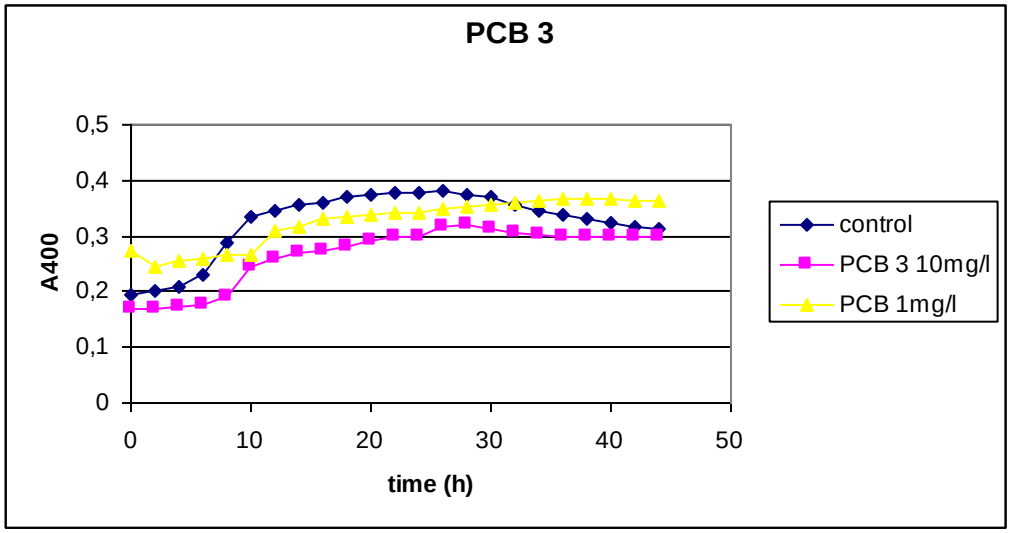
Generally CBAs didn't exhibited mutagenic effects on the different strains which were tested except for the strain TA98 for which almost all the samples were mutagenic.

7.4 Bioscreen test

The ecotoxic effects of CBAs and PCBs were tested at first on the bacteria *Bacillus subtilis*. PCBs were tested at the concentrations of 1 and 10 mg/l whereas CBAs were tested at first at the concentrations of 10,20,40 and then on the higher concentrations of 100 and 200 mg/l because the first results didn't indicate toxicity. Finally the tests didn't show significant toxic effect for the concentrations below 100mg/l, and the curves were similar for the different concentrations of the samples but in the last test the concentrations of 100 and 200 mg/l affected obviously the growth of *Bacillus subtilis*. That indicates that *Bacillus subtilis* is quite resistant to the toxicity of the substances tested. The next tests on *Escerichia Coli* and *Pseudomonas sp.* R9 gave clear results about the inhibition on the growth of the bacteria from CBAs, the concentrations tested were 10,20 and 40 mg/l.

In the next graphs are presented the results for *Bacilus subtilis* on PCBs and CBAs

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0,4
0,3
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0
0
10
20
30
40
50
time (h)
control
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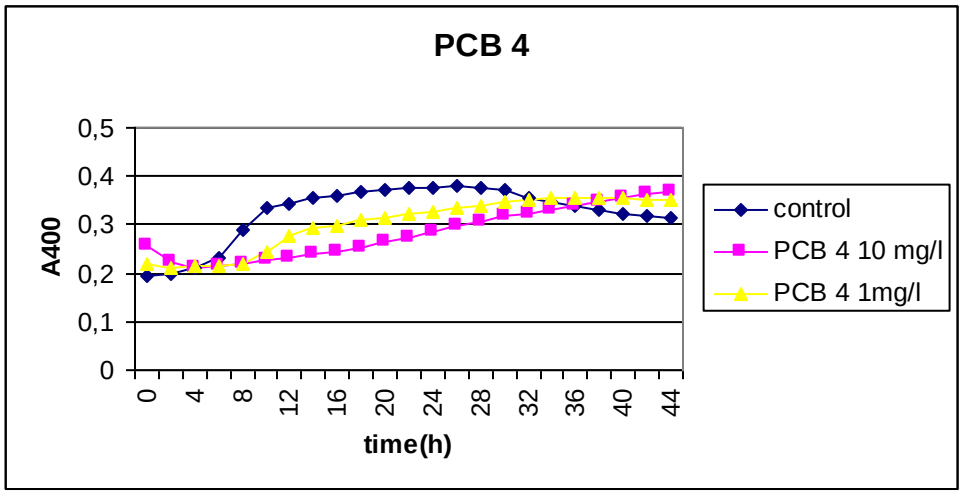
PCB 1mg/l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0
0
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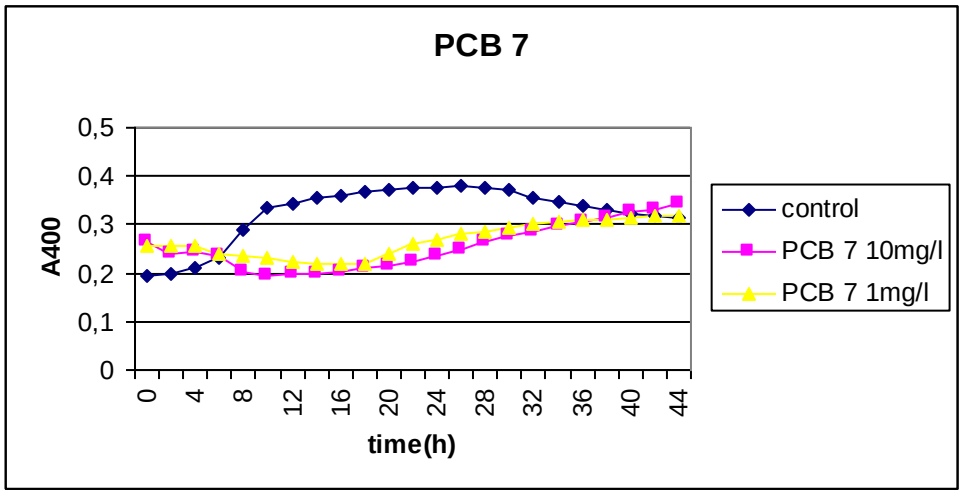
control
PCB 4 10 mg/l
PCB 4 1mg/l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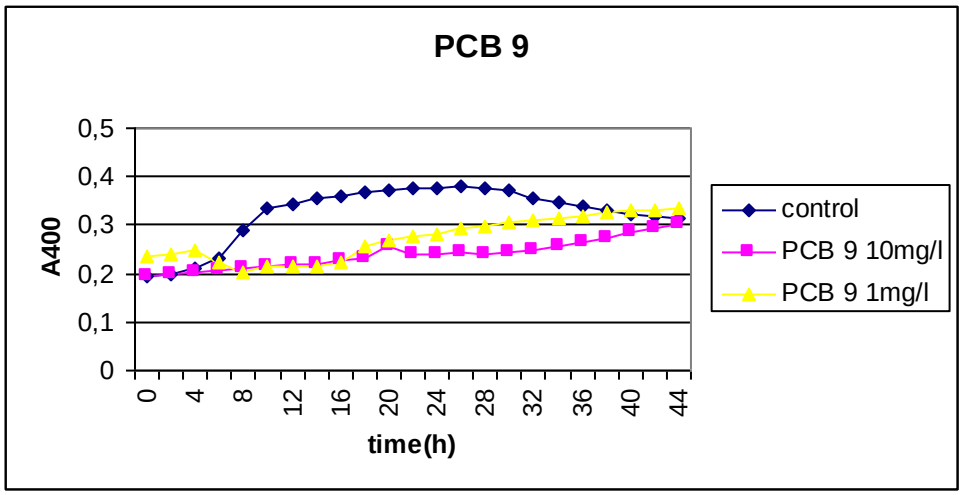
0,4
0,3
0,2
0,1
0
0
4
8
12
16
20
24
28
32
36
40
44
time(h)
control
PCB 7 10mg/l
PCB 7 1mg/l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A400
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0,4
0,3
0,2
0,1
0
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time(h)
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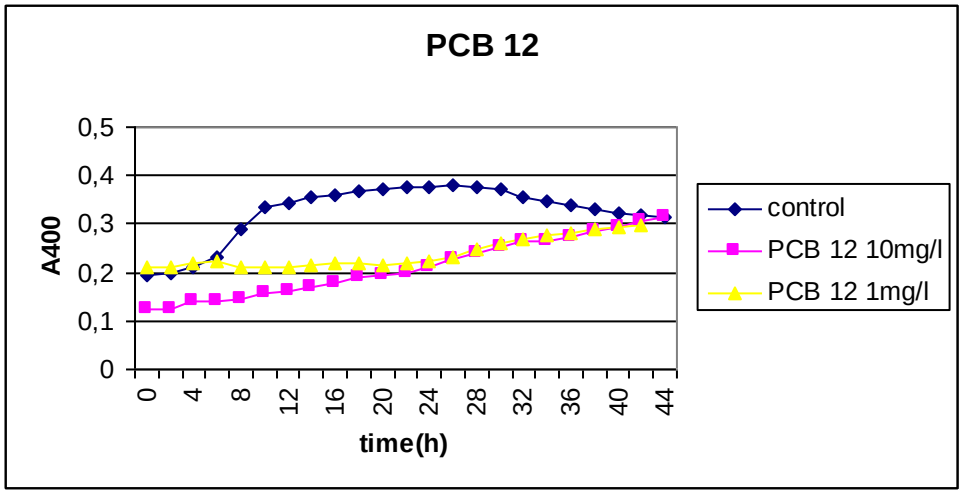
PCB 9 1mg/l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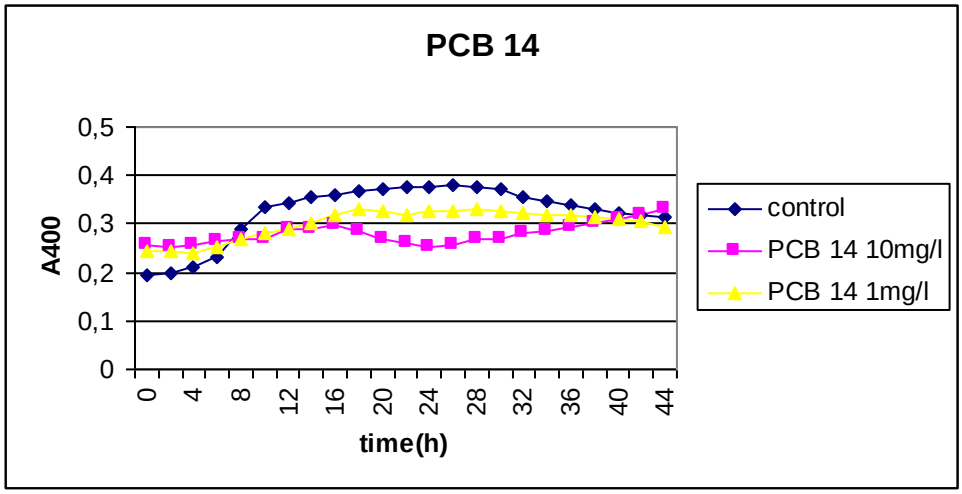
A400
0,5
0,4
0,3
0,2
0,1
0
0 4 8 12 16 20 24 28 32 36 40 44
time(h)
control
PCB 12 10mg/l
PCB 12 1mg/l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0,4
0,3
0,2
0,1
0
0 4 8 12 16 20 24 28 32 36 40 44
time(h)
control
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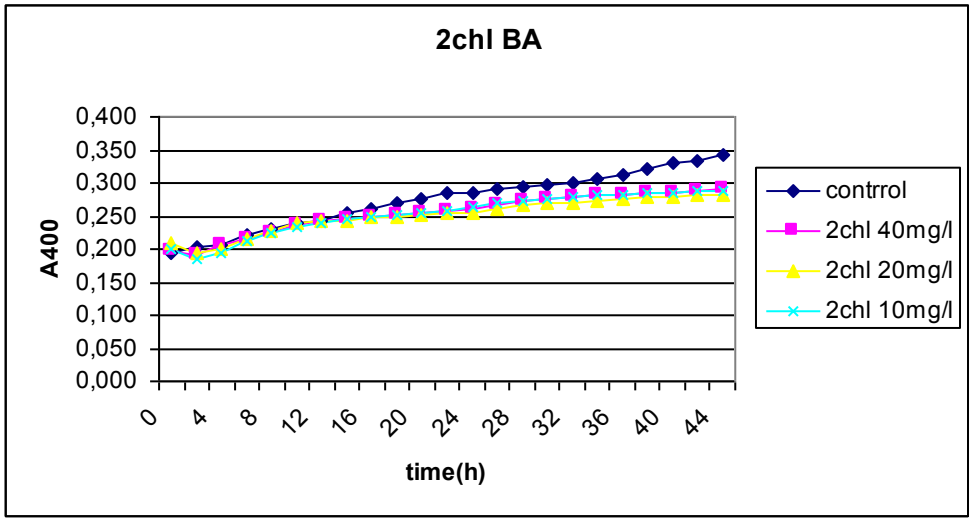
PCB 14 1mg/l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4
8
12
16
20
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32
36
40
44
time(h)
controol
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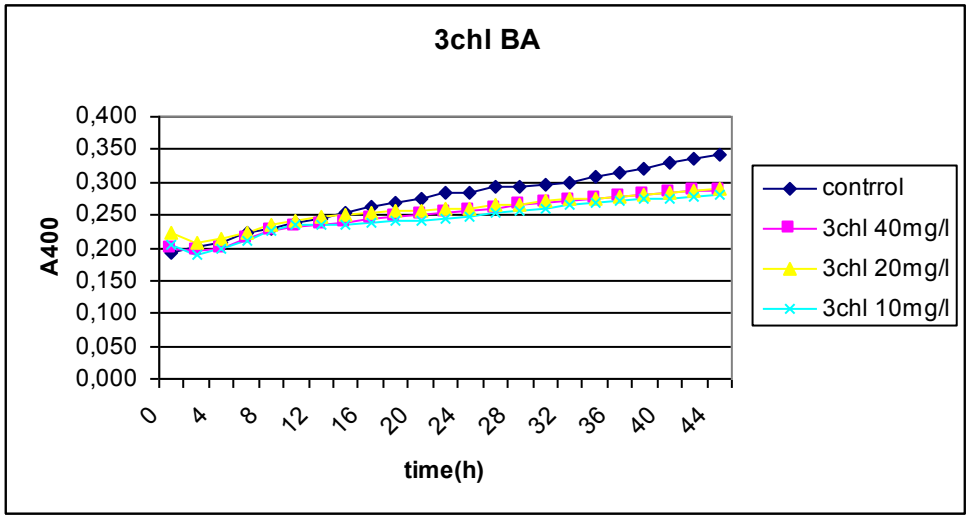
2chl 20mg/l
2chl 10mg/l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A400
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0,150
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0
4
8
12
16
20
24
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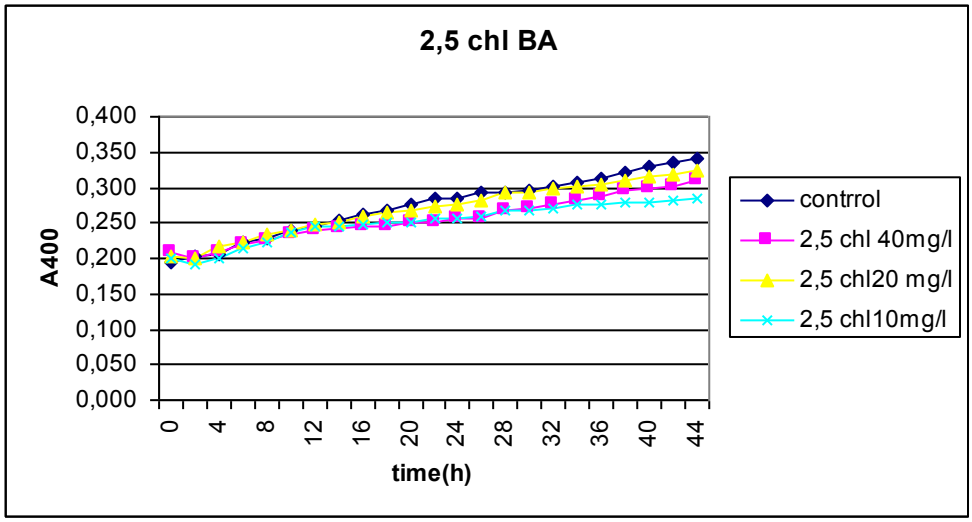
32
36
40
44
time(h)
controol
3chl 40mg/l
3chl 20mg/l
3chl 10mg/l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0,150
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0,000
0
4
8
12
16
20
24
28
32
36
40
44
time(h)
controol
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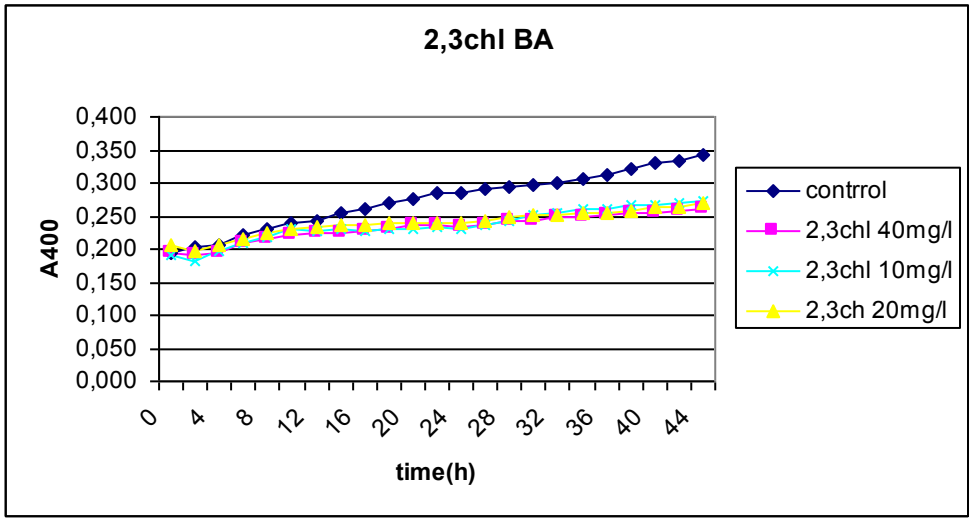
2,5 chl20 mg/l
2,5 chl10mg/l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0,200
0,150
0,100
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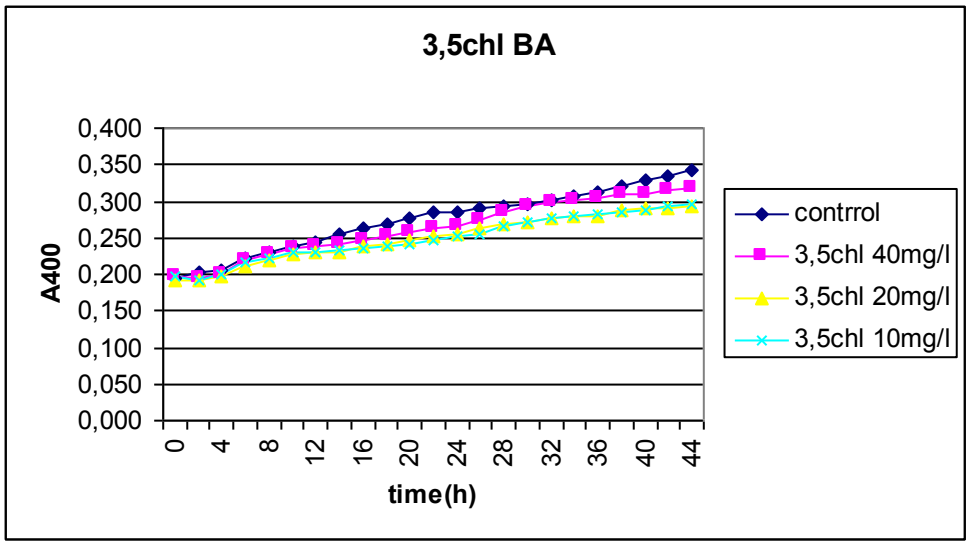
0,000
0
4
8
12
16
20
24
28
32
36
40
44
time(h)
contrrol
2,3chl 40mg/l
2,3chl 10mg/l
2,3ch 20mg/l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0,150
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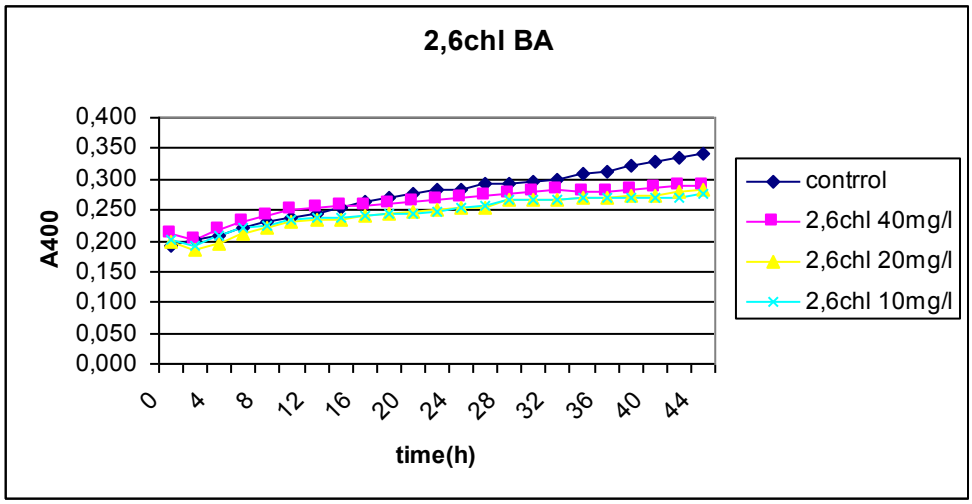
0,000
0
4
8
12
16
20
24
28
32
36
40
44
time(h)
contrrol
3,5chl 40mg/l
3,5chl 20mg/l
3,5chl 10mg/l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0,150
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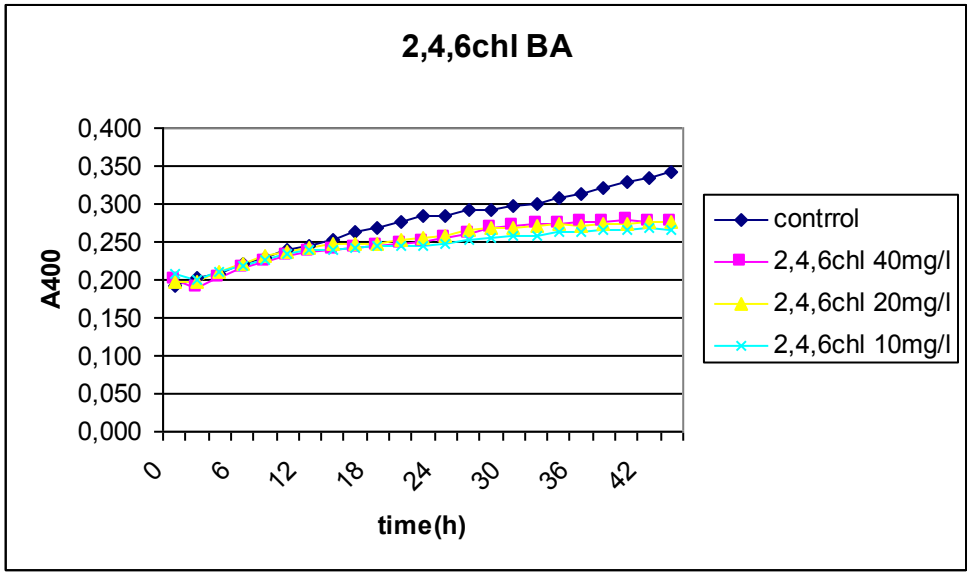
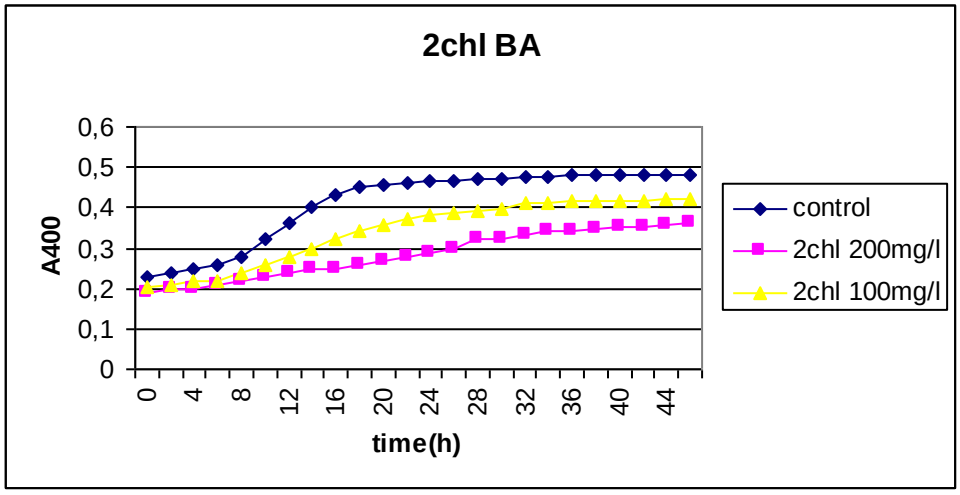
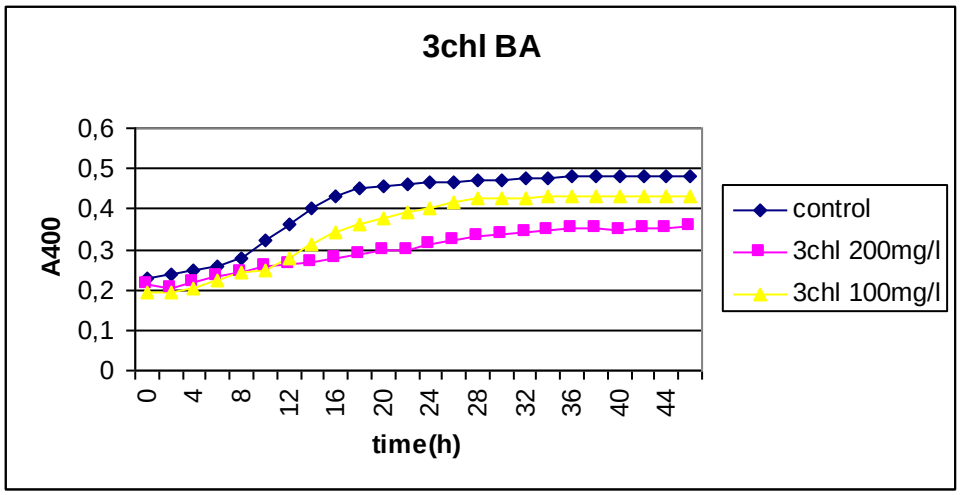
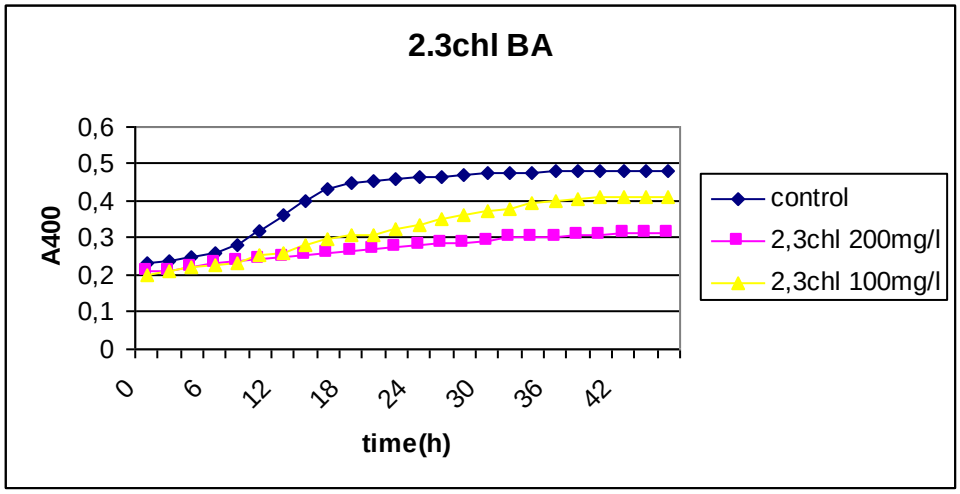
0,000
0
4
8
12
16
20
24
28
32
36
40
44
time(h)
contrrol
2,6chl 40mg/l
2,6chl 20mg/l
2,6chl 10mg/l



In the last set of experiments at higher concentrations the inhibition in growth is clear.




3chl BA
A400
0,6
0,5
0,4
0,3
0,2
0,1
0
0 4 8 12 16 20 24 28 32 36 40 44
time(h)
control
3chl 200mg/l
3chl 100mg/l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0,1
0
0 6 12 18 24 30 36 42
time(h)
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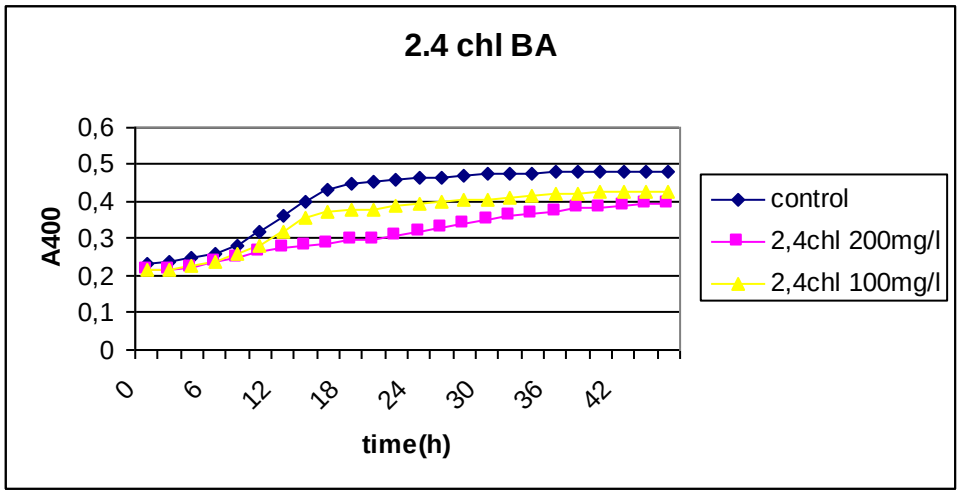
2,3chl 200mg/l
2,3chl 100mg/l


2.4 chl BA
A400
0,6
0,5
0,4
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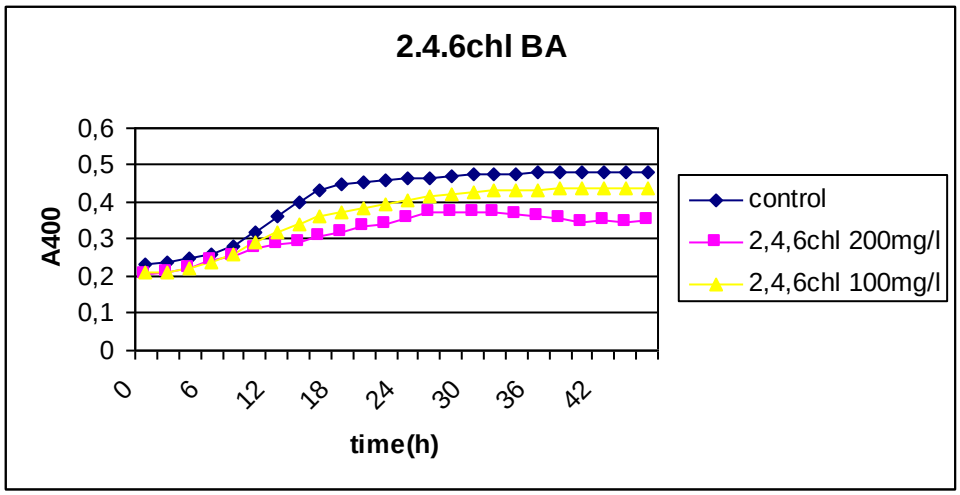
0,2
0,1
0
0 6 12 18 24 30 36 42
time(h)
control
2,4chl 200mg/l
2,4chl 100mg/l


2.4.6chl BA
A400
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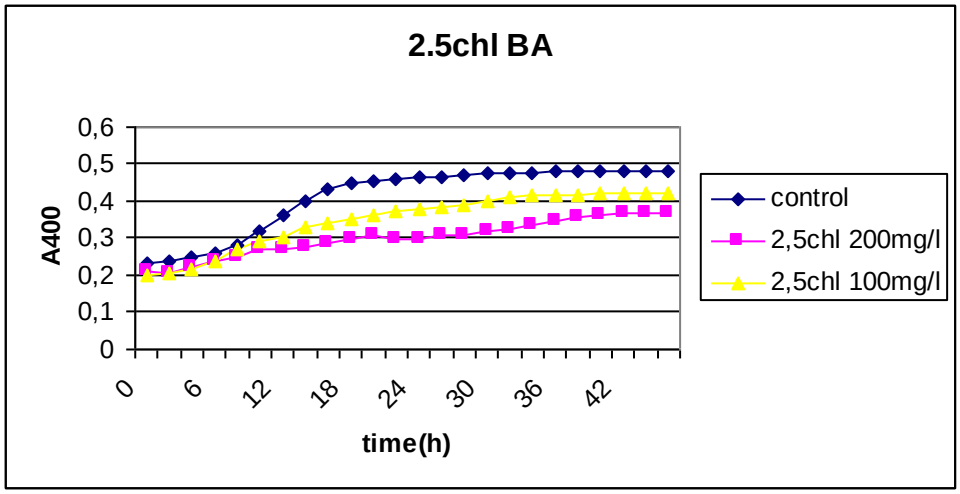
0,4
0,3
0,2
0,1
0
0
6
12
18
24
30
36
42
time(h)
control
2,4,6chl 200mg/l
2,4,6chl 100mg/l


2.5chl BA
A400
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0,5
0,4
0,3
0,2
0,1
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0
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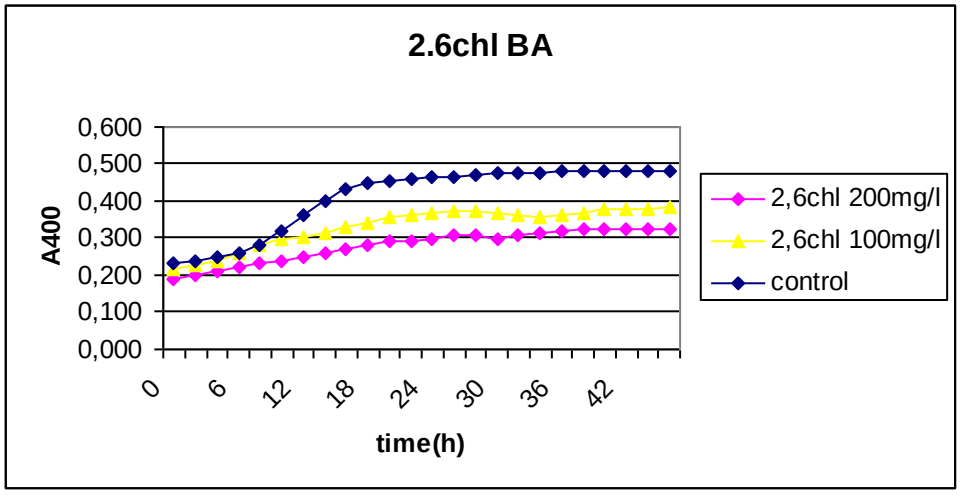
12
18
24
30
36
42
time(h)
control
2,5chl 200mg/l
2,5chl 100mg/l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0,600
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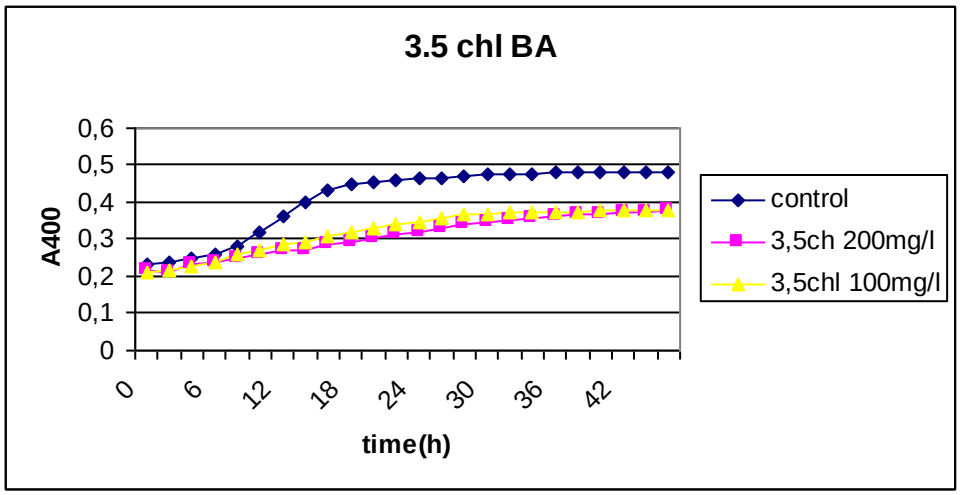
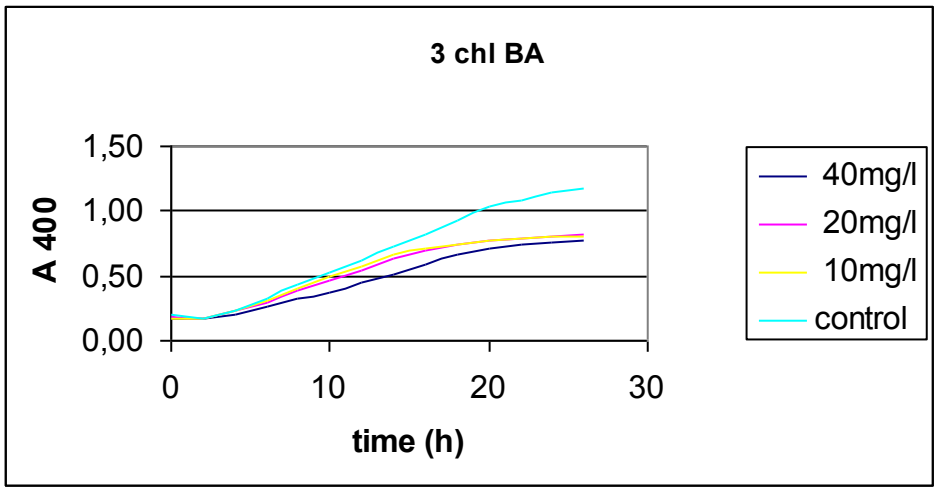
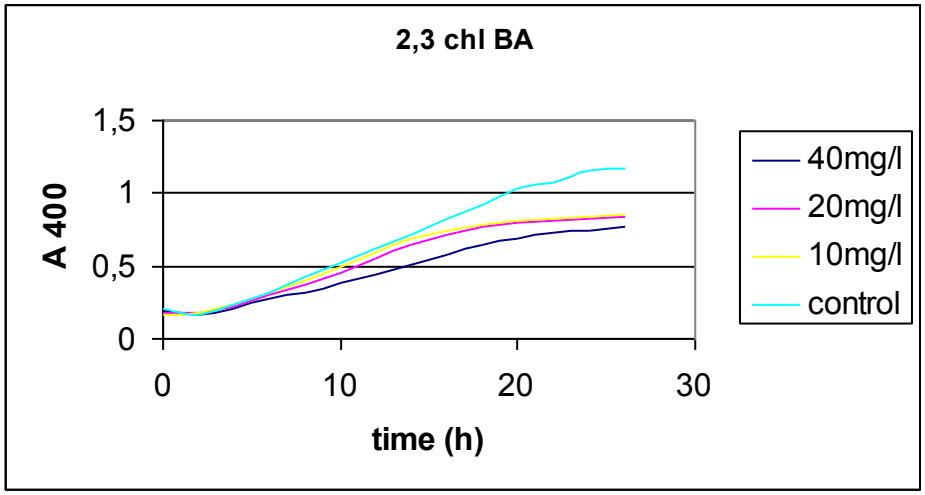
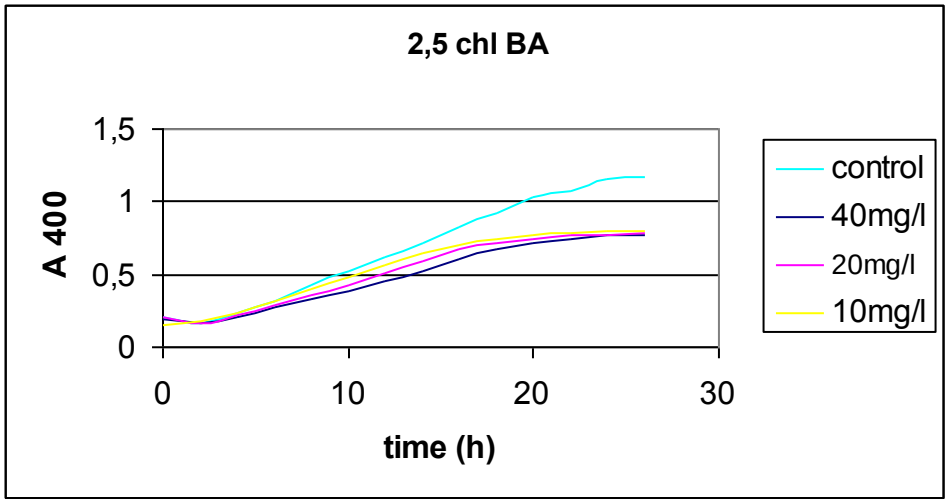
0,200
0,100
0,000
0
6
12
18
24
30
36
42
time(h)
2,6chl 200mg/l
2,6chl 100mg/l
control



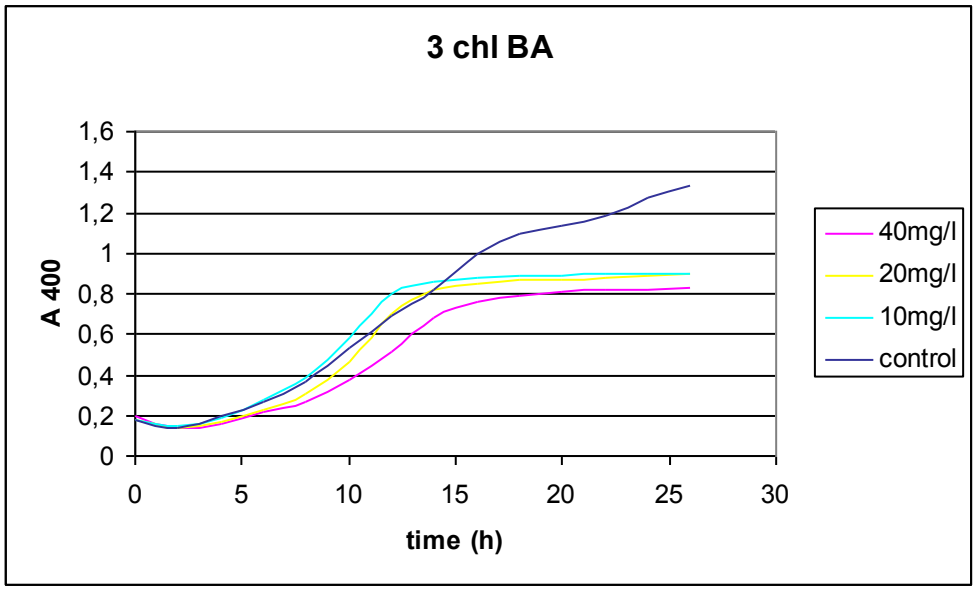
In the next graphs are presented the results for 3-2,3 and 2,5 CBAs on *Escerichia Coli*, the effect of CBAs on the growth curves is clear and dependent on the concentration of the CBAs in the samples. The inhibition seems to start after 8 hours of growth.

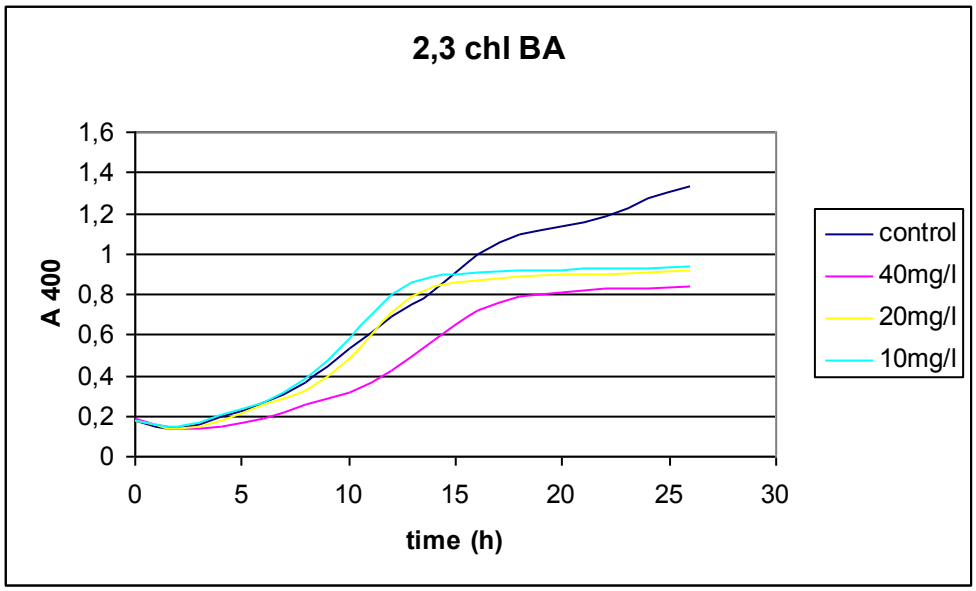


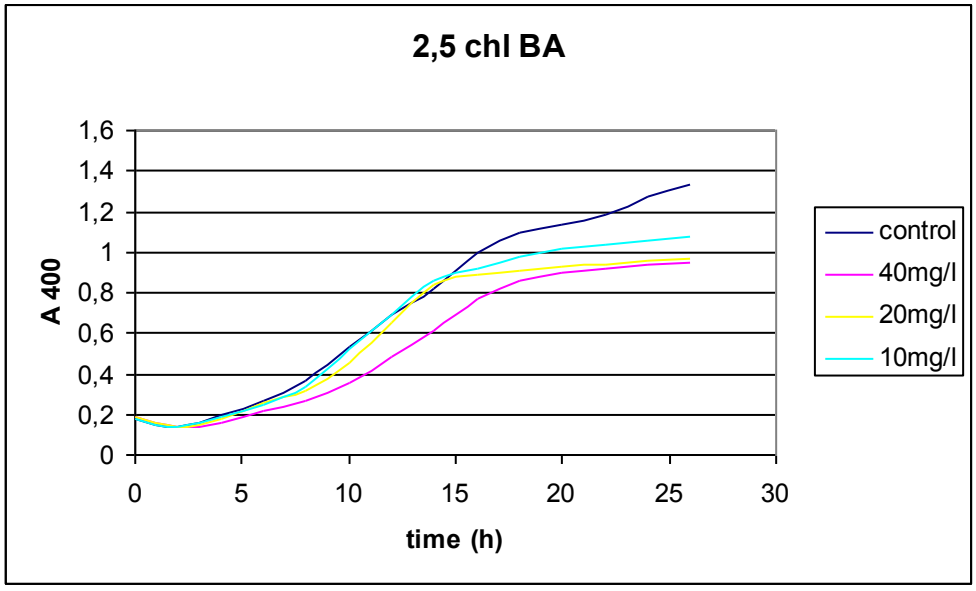




The next graphs show the results of the same CBAs (3-2,3-2,5) on *Pseudomonas sp.* R9 where the inhibition is also obvious after the first fifteen hours of growth for all the CBAs tested.




2,3 chl BA
A 400
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
25
30
time (h)
control
40mg/l
20mg/l
10mg/l


2,5 chl BA
A 400
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
25
30
time (h)
control
40mg/l
20mg/l
10mg/l

7.5 Other toxicity studies-Metabolic products of PCBs in bacteria and plants-comparison of their toxicity and genotoxicity

(Petra Lovecka, Petra Prouzova, Martina Mackova and Katerina Demnerova)

In this paper are tested the compounds:PCB1, PCB2, PCB3, 3CLA, 2,3CLA and 2,5 CLA (chlorobenzoic acids) with 4 different tests. The toxicity assays used are described below:

1) Toxicity assay using luminescent bacteria

A working suspension of luminescent bacteria was prepared by reconstituting a vial of lyophilized cells of *Vibrio fisheri*, using 0,5 ml of 2% NaCl aqueous solution at 2-5°C. The bacterial suspension was added to 0,5 ml dilution series of toxicant (chlorobenzoic acids, congeners of PCB, hydroxychlorobiphenyls) in 2% NaCl. Luminescence was measured after 15 minutes incubation.

2) Toxicity assay using the Bioscreen test

This test allowed the evaluation of the effect of various concentrations of toxicants on bacterial viability and growth in comparison with controls cultivated without toxicants. Values of OD_{400nm} and the time dependence of growth are monitored in parallel samples incubated with or without toxicants for 2 days. The effect of three different concentrations of toxicants (PCB, chlorobenzoic acids and hydroxychlorobiphenyls) at three concentrations (10mg/l, 20mg/l and 40mg/l) was followed using three different bacterial strains (*Pseudomonas* sp. R9 - isolated from the soil contaminated with polyaromatic hydrocarbons, *Pseudomonas* sp. P2 - isolated from the soil contaminated with PCB and *E. coli* from the Collection of the Department of Biochemistry and Microbiology). Minimal medium containing glycerol (5g/l) and toxicants as described above was used for the incubation.

3) Toxicity assay using germination of seeds

This method is based on measuring of the root lenght of *Lactuca sativa* in presence of toxicants. The temperature of incubation is 22°C without light for 4 days and referent medium consists of 18,5 g/l $CaCl.2H_2O$, 2,3 g/l KCl, 49,3 g/l $MgSO_4.7H_2O$, 25,9 g/l $NaHCO_3$ (2,5 ml for 1000ml H_2O). Results are expressed as coefficient of inhibition I (%). The sample is interpreted to be toxic, when I – value is greater than 30 %.

4) Measurement of genotoxicity using Ames test

The Ames test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs. The test is also used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides.

First of all the toxicity of the substances to *Salmonella* strains was tested. The Ames test has a range of specific modifications and enables detection of a wide variety of mutagens. The detection system using *Salmonella typhimurium* His^- differs in mutations within histidine operon compared with the original strain. Mutations in the histidine operon are induced by mutagens leading to reversion to protothrophy, e.g. the ability to synthesize histidine. To enhance the sensitivity of indicator strains differing in mutation type with the tested substance, markers were added. Additionally, markers are also used to monitor genetic stability. *uvrB* mutation originally eliminates the excise reparation system, but also targets neighbouring gene for biotin synthesis. Thus biotin must necessarily be added. *rfa* and *gal* mutations suppress synthesis of o-antigen polysaccharide chains of lipopolysaccharide leading to a higher outer membrane permeability for tested toxicants. Strains can also bear several plasmids encoding antibiotic resistance when the opportunity to use more selective media can be exploited.

Due to the differences in the enzymatic capacities between prokaryotic and eukaryotic organisms, some substances do not have any mutagenic effect on bacterial detection systems and show mutagenicity after metabolic activation in mammalian organisms. Thus a test based on metabolic activation *in vitro*, which uses liver either rat or hamster homogenate S9 was developed. The exogenous enzymatic activation fraction has to be added to bacteria together with the tested substances.

The test without metabolic activation showed a direct mutagenic effect of tested chemical substances and was evaluated as a rate of generation of histidine protothrophy colonies (CFU). The number of induced colonies was compared with a blank (consisting of bacterial strain and solvent), because there is always a constant number of colonies reverting spontaneously to protothrophy.

The test with metabolic activation *in vitro* showed the effect of metabolism on the mutagenic activity of the tested substances. Plates were only considered as being positive when the number of reverting colonies increased to more than twice of the controls in both sets of the test.

Finally, the results were compared using the parameter Rt/Rc, where. Rt represents the total number of revertants of a particular concentration of a tested substance, where Rc is a total number of spontaneous revertants on control plates.

The main products of bacterial degradation of PCB are chlorobenzoic acids which, can be further degraded by other bacteria present in contaminated environment.Analysis of the products of bacterial and plant metabolism has shown that plant products (hydroxychlorobiphenyls) of the first phase of PCB transformation are similar to those detected in mammalian cells. While bacteria degrade PCB to chlorobenzoic acids, plants are generally unable to open the ring and degrade the chemical structure of biphenyl.

PCBs were produced as mixtures of different congeners varying in their degree of chlorination. The simplest PCBs are monochlorobiphenyls which are usually not present in commercial mixtures, but they can be formed in the natural environment by microbial dechlorination or degradation of more highly chlorinated congeners. For the tests were used monochlorobiphenyls and their products identified in bacterial and/or plant cells as models for the toxicity measurements of compounds which can exist naturally in contaminated soils and increase the toxicity of polluted areas. As was previously described bacterial and plant intermediates of PCBs are structurally different. Toxicity of bacterial (chlorobenzoic acids) and plant intermediates (hydroxychlorobiphenyls) of PCB transformation compared to initial monochlorobiphenyls measured using luminescent bacteria is shown in

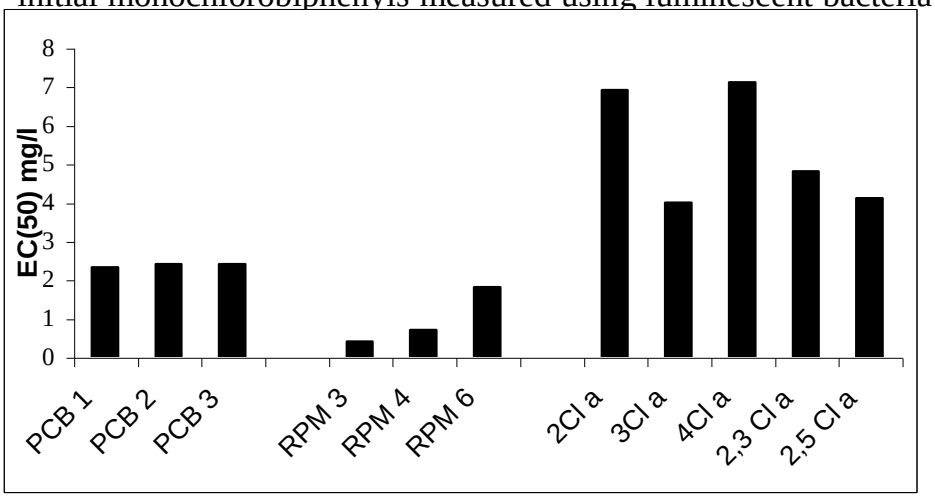


Fig.16 Toxicity of monochlorobiphenyls and products of bacterial and plant metabolism measured by luminescent bacteria *Vibrio fischeri*

Toxicity of the same compounds was tested using an independent method-namely Bioscreen measurement. Using this method, the growth of three different bacterial strains incubated in medium with and without toxicants was followed over 2 days.

The Bioscreen test showed a different response of the selected model organisms. *Pseudomonas sp*. R9, which is not able to degrade PCBs, exhibited the strongest response to individual congeners of polychlorinated biphenyls, while hydroxychlorobiphenyls and chlorobenzoic acids were less toxic. The opposite effect was followed with *Pseudomonas sp.* P2 which is able to degrade PCBs and thus it was not susceptible to PCBs but to products of PCB degradation - chlorobenzoic acids. *E coli* exhibited a similar response to all three groups of tested compounds.

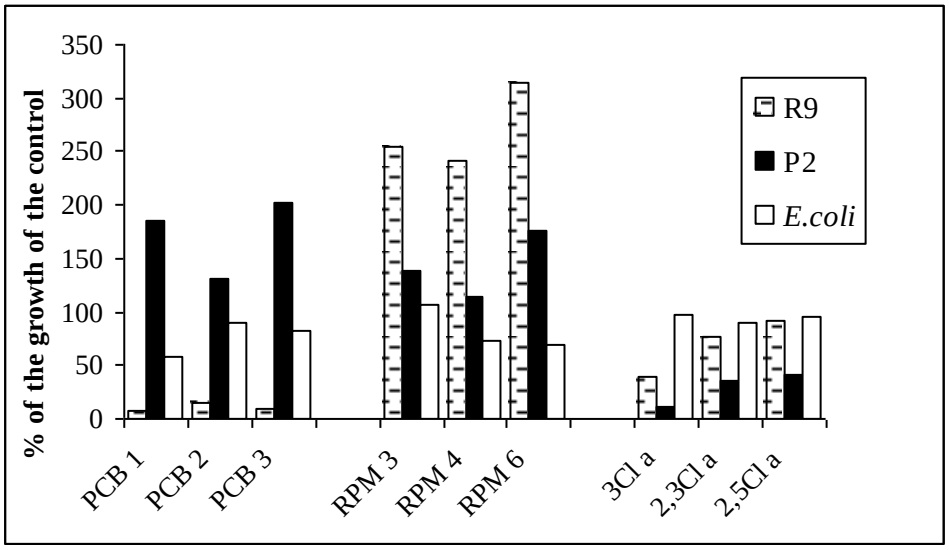


Fig.17 Evaluation of specific growth rates of three bacterial strains incubated with PCBs, chlorobenzoic acids and hydroxychlorobiphenyls at a concentration of 10 mg/l

Results of measuring with seeds of *Lactusa sativa* are shown in table 15. Monochlorbiphenils are not toxic for plant system, but chlorobenzoic acids and hydroxychlorobiphenyls are more toxic then PCB. It is probably due to their better solubility in water.

I(%)	PCB 1	PCB 2	PCB 3	RPM 1	RPM 3	RPM 4	3 Cl a	2,3 Cla	2,5 Cl a
3 mg/l	19,1	16,5	7,8	4,7	2,0	15,7	55,5	44,5	53,7
50mg/l	25,8	27,3	7,5	86,9	81,8	93,6	79,1	93,7	99,6

Table 15. Toxicity of products of bacterial and plant metabolism measured by seeds of *Lacuta sativa*

Measurement of genotoxicity by Ames test

Due to the toxicity of the tested substances for our tested microorganisms it was necessary to establish an appropriate concentration, which did not kill the bacterium itself. The lowest concentration showing any mutagenic effect was chosen and a concentration gradient was prepared. Genotoxic effect was proved in case of chlorobenzoic acids. Original PCBs and hydroxychlorobiphenyls showed less genotoxicity than chlorobenzoic acids, only with TA 100 cells weak genotoxic effect was measured.

7.6 Comparison of measurements

The values of EC50 and the Inhibition coefficient are similar between the results reported in the paper and the values I found out. Almost in all cases the values of EC50 are slightly higher in my results as well as the values of inhibition. In the germination test the results agree regarding the order of toxicity of the different samples, for example in both researches 2,5 CBA gives the highest inhibition coefficient among the tested CBAs. The inhibition in the growth of *E. Coli* and *R9* in the Bioscreen test has similar value for both bacteria in both researches. In Ames test comes up a contradiction as in my results there is no evidence for mutagenicity on the strain TA100 whereas in the paper is mentioned weak genotoxic effect for the same strain.

7.7 Conclusion

In this study I presented the different behavior of organisms to the presence of pollutants, namely of some PCBs and their bacterial products. These data show the phenomena that could appear with low soluble contaminants transformed by any living system, unfortunately cannot be generalized to properties of all contaminants polluting the environment.

In each case basic data documenting behavior of pollutants and their products should be evaluated using proper system for ecotoxicity analysis and compared with analytical results determining chemical origin of pollutants.

From the results it can be concluded that chlorobenzoic acids as products of PCB biodegradation are more toxic for plants (higher inhibition

coefficients) but PCBs are more toxic on bacteria. Chlorobenzoic acids are more soluble in water than the original PCB and thus are more available and toxic for living organisms. Comparing both systems for the ecotoxicity measurement luminescent bacteria are susceptible to lower concentrations of tested compounds. Also chlorobenzoic acids didn't exhibit any mutagenic effects except for one strain (TA 98). It is also important to mention the great difference in the response to ames test of different strains of the same bacteria to the same toxicants, which indicates that slightly different microorganisms can give completely different response to the same substance.