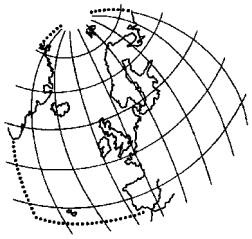
Hazardous Substances Series

Survey on Genotoxicity Test Methods for the Evaluation of Waste Water within Whole Effluent Assessment



OSPAR Commission
2002

The Convention for the Protection of the Marine Environment of the North-East Atlantic (the "OSPAR Convention") was opened for signature at the Ministerial Meeting of the former Oslo and Paris Commissions in Paris on 22 September 1992. The Convention entered into force on 25 March 1998. It has been ratified by Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, Netherlands, Norway, Portugal, Sweden, Switzerland and the United Kingdom and approved by the European Community and Spain.

La Convention pour la protection du milieu marin de l'Atlantique du Nord-Est, dite Convention OSPAR, a été ouverte à la signature à la réunion ministérielle des anciennes Commissions d'Oslo et de Paris, à Paris le 22 septembre 1992. La Convention est entrée en vigueur le 25 mars 1998. La Convention a été ratifiée par l'Allemagne, la Belgique, le Danemark, la Finlande, la France, l'Irlande, l'Islande, le Luxembourg, la Norvège, les Pays-Bas, le Portugal, le Royaume-Uni de Grande Bretagne et d'Irlande du Nord, la Suède et la Suisse et approuvée par la Communauté européenne et l'Espagne.

ISBN 1-904426-02-6

contents

GLOSSARY

Cell culture:	Cells from different organisms, e.g. from Chinese hamster or human, are cultivated in artificial media under constant environmental conditions. Permanent cultures: cultures maintained for years, cells often transformed or from cancer biopsis, used for *in vitro* biotests. Primary cells: isolated from an organism, e.g. primary hepatocytes (liver cells). Used in biotests *in vitro* and *in vivo*.
Chromosomal aberration:	Structural aberration: change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division; numerical aberration: change in the number of chromosomes from the normal number characteristic of the cells utilised.
DNA adducts:	Electrophilic substances or metabolites react covalently with nucleophilic sites on the purine or pyrimidine bases of the DNA and are attached to the DNA: chemical modification of the bases may lead to mispairing; as bulky adducts they may prevent replication.
DNA damage:	Refers to any change of structure and composition of DNA, includes the different forms of mutations, DNA strand breaks, loss of bases, as well as DNA adducts.
DNA repair:	Refers to enzyme systems, recognizing and eliminating damaged strands of DNA, modified bases, adducts, or simply DNA breaks. Different systems display various error probabilities. SOS system: bacterial, error prone repair system. Repair capacity differs considerably between organisms of different evolutionary levels.
DNA strand breaks:	Break in the sugar-phosphate backbone of the DNA; as the DNA molecule is a duplex, single and double strand breaks can be distinguished.
DNA:	Deoxyribonucleic acid, genetic material of most living organisms. Double-helical polynucleotide chain, one nucleotide consists of sugar (deoxyribose), phosphate, and one out of four bases (adenine, guanine, thymine, cytosine). The genetic code employs just four "letters" arranged in codons, each consisting of three bases therefore 4^4 (64) different codons are possible.
Eucaryonts:	From unicellular organisms, plants, fungi, to mammals and man. Complex cellular organisation, membrane enclosed organelles, cellular compartimentation.
Gene pool:	Pool of genetic information of individual species or populations in ecosystems.
Genotoxicity:	Potentially harmful effects on genetic material not necessarily associated with mutagenicity, may be indicated by induced damage to DNA without direct evidence of mutation.
In situ:	Animals, plants originating from the ecosystem under consideration are examined in the laboratory, e.g. fish from a river in a monitoring study of water quality.
In vitro:	Cells are exposed to chemicals or environmental samples in the laboratory and reactions are evaluated according to the test design.
In vivo:	Animals, plants are exposed to chemicals or environmental samples in the laboratory and reactions evaluated according to the test design.
Micronucleus:	Small nucleus, separate from and additional to the main nucleus, produced during telophase of mitosis (meiosis) by lagging chromosome fragments or whole chromosomes.
Microplate test:	Microplates are available in formats from 6 wells to 384 wells. Biotests usually use 24 and 96 well plates (volume 2 ml and 300 µl respectively); each well represents one experimental point.
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.

Neoplasm:	An abnormal tissue that grows by cellular proliferation more rapidly than normal and continues to grow after the stimuli that initiated the new growth cease. Neoplasms show partial or complete lack of structural organization and functional coordination with the normal tissue, and usually form a distinct mass of tissue which may be either benign (tumor) or malignant (cancer).
Phage:	Virus which uses bacteria as its host; often used in molecular biology as a vector to introduce genetic material into bacteria.
Plasmid:	Circular DNA found besides the main DNA molecule in certain bacteria. Genes responsible for antibiotica resistance are often found on plasmids.
Procaryonts:	Bacteria and cyanophyceae, characterised by having no membrane enclosed nucleus, DNA circular organised as nucleoid, no chromosomes, no cell organelles like mitochondria, endoplasmatic reticulum and cilia.
Replication:	Doubling of the DNA, as a prerequisite for cell division. Mechanism semi-conservative, one strand of the double-stranded DNA molecule as a matrix to synthesize a new strand. Due to different organisation of the genetic material mechanistic differences between procaryonts and eucaryonts exist.
Reporter gene:	The gene product of the reporter gene can be measured through a biochemical reaction, and as it is functionally fused to a target gene, it also represents the activity of the target gene. For example the activity of the umu genes in the umu test is expressed by the amount of ß-galactosidase, the product of the corresponding reporter gene.
Reverse mutation-test:	This test detects mutations in an amino acid requiring strain in either *Salmonella typhimurium* or *Escherichia coli* (histidine or tryptophan, respectively) to produce a strain able to grow in the absence of the amino acid.
S9:	Supernatant 9000 g. Microsomal fraction of liver homogenate usually from mammals containing biotransformation enzymes. Animals, usually rats, treated with enzyme-inducing agents such as Aroclor 1254. Added to *in vitro* biotests to mimic mammalian liver metabolism.
Sister chromatid exchange:	Reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. Exchange process presumably involves DNA breakage and repair.
Teratogenicty:	Malformations or other deviations from the normal embryonic development, induced by chemicals or physical agents.
Transgenic animals:	Refers to genetically modified organisms.
Unscheduled DNA repair synthesis:	DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damage induced by chemical substances or physical agents.

EXECUTIVE SUMMARY

This survey on genotoxicity test methods for the evaluation of waste water within whole effluent assessment (WEA) supplements the OSPAR Background Document concerning the Elaboration of Programmes and Measures relating to Whole Effluent Assessment (2001).

Genetic hazard assessment deals with changes in genetic material of organisms, either human or other natural origin. Although considered an important element of the basic mechanisms of evolution, mutations often have a more detrimental effect on individuals and their offspring, and may adversely affect populations. There is consensus about a close association of DNA damage, mutations and the induction of various types of cancer. In eco-genotoxicity, possible effects of mutagenic/genotoxic substances on populations and ecosystems are investigated. This report gives an overview on genotoxocity test methods and their application to monitoring and assessment of waste water.

Mutagenicity testing has been performed with all types of organisms. For monitoring purposes higher organisms (eukaryotes) were exposed to the environmental compartment "*in situ*" or in laboratory tests "*in vivo*". Mutagenicity represents permanent changes to single genes or chromosomes, while genotoxicity focuses on primary damage of DNA. Some of the methods applied to environmental samples are based on corresponding OECD and EC guidelines used for chemical assessment, but others have not yet been standardised.

The bacterial Ames, *umuC* and SOS chromo assays have been applied predominantly to waste water samples. Tests with eukaryotic cells or organisms might be more relevant for human and ecological risk assessment, but generally they are much more time-consuming. Several tests have been developed using the integrity of DNA as an unspecific endpoint of genotoxicity e.g. Comet Assay, Alkaline DNA-eluation assay, DNA alkaline unwinding assay, UDS-assay; the Comet Assay probably the most cost-efficient test among these. Most eukaryotic mutagenicity tests detect macro damage of chromosomes in the visible light microscope following appropriate staining (Chromosomal aberration, Micronucleus assay, SCE assay). Plants, amphibians, fish and permanent mammalian cell lines such as V79, CHO or CHL, but also marine and fresh water mussels have been used as test organisms.

For genotoxicity testing, surface water samples were often highly concentrated in order to enhance sensitivity. However, this can lead to unrealistically high and ecologically irrelevant exposure concentrations, and comparison of different study results remains therefore difficult.

Genotoxicity test results are reported for a broad range of industrial and municipal effluents and results from some exemplary sectors are described in this report. As a rule, no genotoxic and mutagenic effects can be measured in domestic waste water in the inlet and outlet of municipal treatment plants. Mutagenic effects have been found in waste water from the textile industry and hospitals as well as in waste water from the pulp and paper and chemical industry.

"Genotoxicity backtracking" has been applied successfully, i.e. to assess the relative contribution of disinfectant by-products to the total mutagenicity of drinking water. Further more, the origin of genotoxity in river water has been assigned to single substances (chromium, nitroarenes, aromatic amines, PBTA-1). Also azo dyes have been determined as the principal source of mutagenicity in waste water of textile finishing. Fluoroquinolone antibiotics were found to cause genotoxicity in waste water from hospitals. Numerous studies are available on the ability of eliminating genotoxins by treating municipal waste water.

Although the potential hazard of genotoxins to the environment needs further clarification, the need to consider genotoxicity and mutagenicity testing in WEA is widely acknowledged. It is accepted that an individual test covers only one definite endpoint. Several researchers have advocated to use a test battery of one bacterial and one eukaryotic test system following the approach used in chemical risk assessment. From a scientific point of view, further studies considering genotoxicity backtracking and/or higher test organisms should be performed in particular in those cases that show positive results in a first survey with bacterial tests.

RÉCAPITULATIF

Cette étude des méthodes de test de génotoxicité destinées à l'évaluation des eaux usées dans le contexte de l'évaluation des effluents entiers complète le Document de fond OSPAR relatif à l'élaboration des programmes et mesures visant l'évaluation des effluents entier, 2001.

L'évaluation des risques génétiques traite des modifications qui se produisent dans le matériel génétique des organismes, qu'ils soient humains ou d'autres origines naturelles. Bien qu'elles soient considérées comme un important élément des mécanismes de base de l'évolution, les mutations ont souvent un effet dommageable sur les individus et leurs descendants, et peuvent porter atteinte aux populations. Il y a consensus sur le fait qu'il existe un rapport étroit entre les dommages causés à l'ADN, les mutations et l'apparition de diverses formes de cancer. Dans le domaine de l'éco-génotoxicité, l'on étudie les effets que les substances mutagènes/génotoxiques sont susceptibles d'avoir sur les populations et les écosystèmes. Le présent rapport donne une vue d'ensemble des méthodes de test de génotoxicité et de leur application à la surveillance et à l'évaluation des eaux usées.

Des tests de mutagénicité ont été effectués sur tous les types d'organismes. Aux fins de la surveillance, des organismes supérieurs (eukaryotes) ont été exposés soit *« in situ »* à un compartiment environnemental, soit à des tests *« in vivo »* en laboratoire. La mutagénicité est constituée par les modifications irréversibles d'un gêne ou d'un chromosome, tandis que la génotoxicité concerne les dommages primaires de l'ADN. Certaines des méthodes appliquées à des échantillons prélevés dans l'environnement sont basées sur les lignes directrices correspondantes de l'OCDE et de la CE applicables à l'évaluation chimique, les autres méthodes n'ayant toutefois pas encore été normalisées.

Les analyses d'ames bactérienne, *umuC* et de SOS chromatiques ont été surtout pratiquées sur des échantillons d'eaux usées. Quoique les tests sur des cellules ou des organismes eukaryotiques soient peut-être mieux adaptés à l'évaluation des risques pour l'homme et des risques écologiques, ils prennent en revanche en général beaucoup plus de temps. Plusieurs tests ont été mis au point, sur la base de l'intégrité de l'ADN pris comme point final non spécifique de la génotoxicité, par exemple la méthode d'analyse Comet, la méthode de l'analyse de l'ADN par élution alcaline, la méthode du déroulement de l'ADN en milieu alcalin, la méthode UDS ou de synthèse non programmée de l'ADN, la méthode Comet étant probablement celle qui offre le meilleur rapport coût-efficacité parmi celles-ci. Les tests de mutagénicité des organismes eukaryotiques permettent pour la plupart de déceler les gros dégâts des chromosomes au microscope optique, ceci après une coloration adéquate (aberrations chromosomiques, analyse du micro-noyau, analyse SCE). Des végétaux, des amphibiens, des poissons et des cultures permanentes de cellules de mammifères telles que V79, CHO ou CHL, quoique également des moules d'eau de mer et d'eau douce, ont été utilisés comme organismes test.

Pour les tests de génotoxicité, les échantillons d'eau de surface ont souvent été ultra concentrés pour accroître la sensibilité. Toutefois, cette méthode peut donner lieu à des teneurs si fortes qu'elles ne correspondent pas à la réalité et qu'elles ne sont pas pertinentes sur le plan écologique, d'où le fait qu'il soit très difficile de comparer les résultats des diverses études dans ces conditions.

Les résultats des tests de génotoxicité d'un vaste éventail d'effluents industriels et municipaux ont été obtenus, et les résultats qui concernent certains secteurs exemplaires sont donnés dans le présent rapport. En règle générale, aucun effet de génotoxicité et de mutagénicité ne peut être mesuré dans les eaux usées domestiques, que ce soit à l'entrée ou à la sortie des stations d'épuration municipales. Des effets mutagènes ont été constatés dans les eaux usées de l'industrie textile et des hôpitaux ainsi que dans les eaux usées de l'industrie du papier et de la pâte à papier et de l'industrie chimique.

La méthode du « traçage de la génotoxicité » a été appliquée avec succès à l'évaluation de la contribution relative de la mutagénicité totale des méthodes de désinfection de l'eau potable. La génotoxicité de l'eau fluviale a été imputée individuellement à certaines substances (chrome, nitro-arènes, amines aromatiques, PBTA-1). Il a été constaté que les colorants azoïques étaient la principale source de la mutagénicité des eaux

usées des opérations de finissage des textiles. L'on a par ailleurs constaté que les antibiotiques à la fluoroquinolone rendaient les eaux usées des hôpitaux génotoxiques. De nombreuses études sur les possibilités d'éliminer les génotoxines lors du traitement des eaux usées municipales sont disponbiles.

Bien que le danger que les génotoxines sont susceptibles de présenter pour l'environnement reste à clarifier, il est largement reconnu qu'il est nécessaire d'envisager des tests de génotoxicité et de mutagénicité dans l'évaluation des effluents entiers. Il est considéré que chacun des tests ne couvre qu'un seul et unique point final défini. Plusieurs chercheurs se sont avérés favorables à un système de tests composé d'un test sur des bactéries et d'un test sur un organisme eukaryotique dans les mêmes conditions que dans l'évaluation des risques chimiques. D'un point de vue scientifique, il conviendrait de procéder à d'autres études de traçage de la génotoxicité et/ou sur des organismes tests supérieurs, en particulier dans les cas où une première série de tests sur des bactéries a donné des résultats positifs.

1. INTRODUCTION

An Intersessional Expert Group (IEG) on Whole Effluent Assessment (WEA) has been established by the OSPAR Working Group on Point and Diffuse Sources to assess the use of effect-based methods for the evaluation of waste water, the application of WEA in different industrial sectors and to implement WEA in OSPAR's strategy to hazardous substances.

Within this working group, the consultants of Hydrotox GmbH supported this work by collecting and evaluating (un)published studies by industry, governmental agencies and experts from other institutions and in setting up a common strategy for the use of WEA in evaluating and controlling industrial waste waters.

The project for this report was "to make a survey of applied methods and those under development on tests for genotoxicity and mutagenicity". In its present form it summarises the methods for detecting genotoxicity and mutagenicity currently used both for waste water evaluation (mainly "*in vitro*" assays) and for monitoring of fresh and marine water environments ("*in vitro*" and "*in vivo*" methods) as taken from numerous publications. Exemplary case studies about genotoxicity identification and backtracking are reported. In addition experience with genotoxicity testing in different waste water sectors is summarised.

2. FUNDAMENTALS

2.1 Objectives of Genetic Toxicology

In the field of genetic toxicology, all kinds of changes to the genetic material of an organism are evaluated in order to identify genetic risks after exposure to chemical substances or certain environmental conditions (e.g. solar radiation). Mutagenic agents might be man-made or of natural origin. Especially plants have developed chemical interactions with their environment and many of these substances have mutagenic properties. Beside exogenous sources also endogenous ones as reactive intermediates of cell metabolism contribute to "spontaneous" induced DNA damage. Therefore complex systems of DNA repair evolved - from enzymes detecting and eliminating damage at the affected DNA strand or chromosome to different proof-reading mechanisms during the processes of replication and transcription/translation. Still, all these DNA repair processes are to a certain degree error-prone, so there remains a chance that induced DNA damage might lead to permanent changes (mutations) of the genetic make-up of an organism.

Although considered as an important part of the basic mechanisms of evolution, mutations more often have a detrimental effect for individuals and their offspring. Furthermore, increased mutation rates, e.g. due to environmental pollution, might even negatively affect populations (see 2.3) There is consensus about a close proximity of DNA damage, mutations and the induction of various kinds of cancer. *It is the dominant paradigm in genetic toxicology that the ability of a chemical to cause mutation presages its ability to cause cancer* (Zeiger 2001). Even though carcinogenesis is a complex, multi-step process, that is still not fully unravelled, growing evidence shows that it involves multiple mutations eventually leading to uncontrolled cell proliferation.

In genetic toxicology two testing strategies can be distinguished by the endpoint used (figure 1):

- mutagenicity testing, endpoint mutation (single gene, chromosome, or genome mutations);
- genotoxicity testing with different endpoints representing primary DNA damage like e.g. DNA strand breaks, DNA adducts, induction of the SOS repair system, and chemically altered DNA bases.

Mutagenicity testing has been done with all kinds of organisms from bacteria, invertebrates, mammals, fishes to plants. For monitoring purposes higher organisms (eucaryotes) were exposed to the environmental

Genetic damage	Bacterial tests - S9 / + S9	Eucaryotic tests *in vitro* / *in vivo*
genotoxic effects	umu-test SOS-chromo-test	Comet-assay DNA alkaline unwinding assay Alkaline DNA-elution assay DNA-repair synthesis (UDS-assay) Sister chromatid exchange (SCE-assay)
mutagenic effects **germ cells** **somatic cells**	Ames-test *E.coli* -test Mutatox-test	(Currently somatic cells are preferred for water samples) Chromosome aberration assay Micronucleus assay

Figure 1: Evaluation of genotoxicity of water samples

compartment "*in situ*" or in the laboratory "*in vivo*". In vitro test systems usually use bacteria or unicelllular eucaryotes (e.g. yeast), primary cultures of tissues, blood cells as well as permanent cell lines originating from eucaryotic organisms.

Although mutation represents the indisputable endpoint, the proof is often difficult and time-consuming in higher eucaryotic test systems. As primary DNA damage is one of the important prerequisites for exogenously induced mutations, results from genotoxicity tests can be used as an indicator for an interaction of test substance and DNA with the potential to induce mutations.

The main objectives of genetic toxicology testing are:

- identifying mutagenic/genotoxic substances in order to minimize the risk of exposure to these compounds with suspected carcinogenic properties;
- genotoxic/mutagenic substances may also induce hereditary defects through mutations in germ cells and they often exhibit teratogenic properties;
- in an ecological context, mutagenic/genotoxic compounds might induce substantial reproductive loss in exposed populations and could further influence individual fitness by a toxicity-related phenomenon described as *genotoxic disease syndrome* (Kurelec 1993).

2.2 Testing strategy in chemical risk assessment

For the notification on new substances according to EC Directives the extent of genotoxicity/ mutagenicity testing is mainly determined by the amount of the chemical introduced onto the market every year. From 1 tpa (tons *per annum*) at least two *in vitro* tests are required: one bacterial gene mutation test (usually the *Salmonella* reverse mutation [Ames] test) and one mammalian cell line test, capable of detecting chromosome damage (usually *in vitro* chromosomal aberration test or *in vitro* mammalian cell gene mutation test). According to the *in vitro* test results and/or the production level, further *in vivo* tests must be performed. Within the OECD guidelines 16 tests for the detection of mutagenic/genotoxic properties of test compounds are listed, all of which have been adopted by EC legislation (Annex V of Directive 67/548/EEC, table 1 and figure 1). Classification and labelling of the tested chemicals are laid down in Annex VI of Directive 67/548/EEC (EEC 1992). A general summary of genotoxicity tests for chemical substances regulation in the European Community has been presented by Broschinski *et al*. (1998).

With respect to mutagenicity, three categories have been distinguished:

- category 1 substances proved to be mutagenic to man, which has to be followed up by epidemiological studies. Up to now no substance has been classified as category 1. For methodological reasons substances of this kind can scarcely be identified;
- category 2 chemicals are substances inducing either mutations in germ cells *in vivo* in animal tests or somatic cell mutation *in vivo*, with evidence that these substances might reach the germ cells;
- category 3 substances are classified by positive *in vivo* somatic cell mutation tests. As a rule, the identification of mutagenic/genotoxic properties of a substance only in tests *in vitro* is not sufficient for a classification in category 3, nevertheless it indicates an urgent need for further *in vivo* testing.

Therefore according to the EU directives mutagenicity testing is focussed mainly on *in vivo* germ cell tests. But it is of fundamental importance to note that genotoxicity testing of new substances should not only be regarded in conjunction with the possible induction of germ cell mutations, but also serves as an initial screening for possible carcinogenic effects (EEC 1967, European Commission 1996, Broschinski *et al*. 1998).

The terms "mutagenic" and "genotoxic" depend on the test systems applied (figure 1). In chemical hazard assessment positive results in "in vitro" assays have to be confirmed by "in vivo" assays. Therefore these terms should not be considered as intrinsic properties of a waste water sample or chemical compound but should always be connected to the test system utilised (e.g. "mutagenic in the Ames-test").

Table 1: Genetic toxicology test methods for the evaluation of chemicals

	OECD	Annex V Directive 67/548/EEC	Principle / tester strains
Bacterial Reverse Mutation Test (Ames test)	**471 / 472**	**B.13/14**	**Reverse mutation of amini-acid requiring strains of *Salmonella thyphimurium* TA1535, TA1537, TA97, TA97a, TA98, TA100, TA102 and *E.coli* WP2 uvrA, *E.coli* WP2 uvrA(pKM101)**
***In vitro* Mammalian Chromosomal Aberration Test**	**473**	**B.10**	**Induction of structural chromosome aberrations in different cell lines, cell strains or primary cell cultures**
Mammalian *in vivo* Erythrocyte Micronucleus Test	**474**	**B.12**	**Formation of micronuclei in bone marrow and/or peripheral blood erythrocytes**
Mammalian *in vivo* Bone Marrow Chromosomal Aberration Test	**475**	**B.11**	**Induction of structural chromosome aberrations in bone marrow cells**
***In vitro* Mammalian Cell Gene Mutation Test**	**476**	**B.17**	**Mutation of thymidine kinase and hypoxanthine-guanine phosphoribosyl transferase in suitable cell lines**
Sex-Linked Recessive Lethal Test in *Drosophila melanogaster*	**477**	**B.20**	**Mutations in the germ line which cause absence of males in the next generation**
Rodent Dominant Lethal Test	**478**	**B.22**	**Mutations in the germ line of rats or mice which are lethal to fertilised eggs or developing embryos**
In vitro Sister Chromatid Exchange Assay in Mammalian Cells	**479**	**B.19**	**Detection of SCE in primary cultures or cell lines**
***Saccharomyces cerevisiae*, Gene Mutation Assay**	**480**	**B.15**	**Haploid strain XV 185-14C, dipoloid strain D7**
Saccharomyces cerevisiae, Miotic Recombination Assay	**481**	**B.16**	**Detection of mitotic crossing-over of DNA segments**
DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells *in vitro*	**482**	**B.18**	**Uptake of radioactively labelled thymidine in primary mammalian cells or cell lines**
***In vivo* Spermatogonial Chromosome Aberration Test**	**483**	**B.23**	**Detection of structural aberrations in spermatogonial mitoses of male Chinese hamsters or mice**
Mouse Spot Test	**484**	**B.24**	***in vivo* exposition of developing embryos in mice strains changes colour in the coat**
Mouse Heritable Translocation Assay	**485**	**B.25**	**Detection of structural and numerical chromosome changes in germ cells**
Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo*	**486**	**B.39**	**Detection of DNA repair in liver cells by uptake of tritium-labelled thymidine**

2.3 Eco-genotoxicity of waste water

Several reviews demonstrate the presence and potency of genotoxins from a broad range of industrial and municipal effluents (De Raat *et al*. 1990, Stahl 1991, Helma *et al*. 1994, White *et al*. 1996a, Helma and Knasmüller 1997, Claxton *et al*. 1998). In eco-genotoxicity, possible effects of mutagenic/genotoxic substances, present in the environment, are investigated on the population and ecosystem level (De Raat *et al*. 1990, Würgler and Kramers 1992). Contrary to human toxicology studies which focus on the fate of the individual, eco-genotoxicity tests evaluate the consequences for population size and structure. Investigations showing high prevalence of hepatic tumours in different fish species from contaminated areas initiated studies in the aquatic environment (Murchelano and Wolke 1991, McMahon 1994, Moore and Myers 1994). Several examples of neoplasms in fish due to waste water effluents have been described (Metcalfe and Sonstegard 1985, Metcalfe *et al*. 1985, Kimura *et al*. 1989).

Furthermore, exposure to DNA damaging agents may result in the formation of carcinogen-DNA adducts. These adducts as possible indicators for carcinogens have been detected in mussels (Harvey *et al*. 1997) and fish from contaminated sites (Dunn 1991, Weisburger and Williams 1991, Pfohl-Leszkowicz *et al*. 1993, El Adlouni *et al*. 1995, Ericson and Larsson 2000).

Increased mutation rates, e.g. due to environmental pollution, might negatively affect populations. This is still controversially debated in the scientific community (Anderson and Wild 1995, Würgler and Kramer 1992) but evidence is growing that environmental mutagens can reduce the reproductive success of populations. Lynch *et al*. (1995) developed a mathematical theoretical approach for evaluating the risks of small populations to extinction via the accumulation of mutations. Even though an increasing number of studies involving eco-genotoxicity are available (Hose and Brown 1998, Hutchinson *et al*. 1998, Theodorakis *et al*. 1998, Rodgers and Baker 2000), the identification of clear cause-effect relations is increasingly complicated, the higher the level of biological organisation.

For example Hose *et al*. (1998) performed a large-scale genotoxicity assessment in a coastal marine environment following the Exxon Valdez oil spill in 1989 using the anaphase aberration test with newly hatched herring fish eggs. Aberration rates were significantly elevated in the fish larvae from heavily contaminated sites and correlated with PAH concentration. In the following years the population of herring was reduced to one-third of the expected value. Nevertheless it could not be conclusively demonstrated that the spilled oil caused significant long-term damage to the herring population. While Anderson and Wild (1998) stated that genotoxicity could be correlated with reproductive effects using a polychaete worm, Hutchinson *et al*. (1998) found, that municipal sewage effluent disinfected with sodium hypochlorite, although causing strong developmental effects, did not increase chromosome aberration in larval stages of a marine polychaete worm.

According to Anderson and Wild (1994) endpoints in eco-genotoxicity studies include frequencies of gamete loss due to cell death, embryo mortality caused by lethal mutations, abnormal development, cancer, and mutation frequencies affecting the gene pool of exposed populations. Up to now only endpoints like gamete loss or teratogenic effects as well as cancer incidences can be measured. Effects for exposed populations might be estimated, in cases where these populations are ecologically characterised. But knowledge about consequences of genotoxic exposure on the gene pool of exposed species is still scarce. As mentioned earlier Kurelec and coworkers described a *genotoxic disease syndrome* that, in combination with loss in reproduction, can pose a threat to population survival. For populations with a large reproductive surplus loss of individuals due to mutational changes might not be critical (Würgler and Kramers 1992). Newer approaches to describe genetic effects of contaminants on the population level focus on the genetic diversity, examining the current status and the history of populations by molecular genetic techniques. But these effects were not necessarily caused by mutagenicity. They depend also on chronic effects and population size (Bickham *et al*. 2000, Belfiore *et al*. 2001).

The use of mutagenicity/genotoxicity tests in effluent testing is clearly focussed on hazard assessment:

- genotoxic effects detected in surface water indicate a massive input of genotoxic substances from point (and diffuse) sources. Therefore waste water samples should be measured for genotoxicity in order to identify the sources;

- as surface water serves multiple purposes in modern societies e.g. as drinking water source, recreation area or drainage of effluents, routine monitoring of waste water for mutagenic/genotoxic properties minimizes the risk of exposure of humans to compounds with suspected carcinogenic properties;
- it is a further step in reducing the anthropogenic impact on natural biota if the risk of imposing eco-genotoxic effects on the ecosystem is minimized;
- genotoxic effluents might indicate that humans are exposed to hazardous substances at the workplace or as consumers (e. g. mutagenic textile dyes).

Nevertheless, the scientific interpretation of genotoxicity data for complex waste water samples, and especially extracts of such samples must be done with care.

3. METHODS FOR GENOTOXICITY TESTING OF WASTE WATER

Some of the methods applied to the assessment of genotoxicity with environmental samples are derived from the corresponding OECD and EC guidelines (table 1) used for chemical assessment, but others (e.g. the Comet assay) have not been standardised up to now. Generally the tests have to be adapted for the application with waste water. In Germany the Ames and the umu assays have been standardized for assessing waste water samples (DIN 38415-4: 1996, DIN 38415-3: 1999). For the umu assay an international standard (ISO 13829: 2000) also exists. To our knowledge further standards do not exist. Test kits that are commercially distributed, like the SOS chromo assay, the Mutatox assay, the Vitotox assay or the Gentronix test, although applied widely, cannot generally be included in international standardisation as organisms/test kits must not be dependent on one commercial source only.

3.1 Bacterial Test Methods

All bacterial test methods have some common characteristics. Most tester strains contain mutations which increase sensitivity to genotoxins. The *rfa* mutation for example causes a partial loss of cell wall and therefore increases permeability to larger molecules such as benzo[a]pyrene. The *uvrB* mutation of most Ames-tester strains deletes a gene coding for the DNA excision repair system and therefore hinders the repair of DNA damage. Often a test battery of several tester strains is applied in order to characterise specific genotoxic spectra or get hints on the origin of genotoxins. As bacteria do not possess the metabolic capacity of eucaryotes the tests are usually performed in the absence and the presence of S9 liver homogenate (supernatant of rat liver extract centrifuged at 9000 g).

3.1.1 Bacterial Genotoxicity Tests

3.1.1.1 UMUC-ASSAY

The umuC-assay was originally developed by Oda *et al*. in 1985. A microplate version of the test is available (Reifferscheid *et al*. 1991). The assay is based on the use of a genetically modified *Salmonella typhimurium* strain TA 1535 that contains the plasmid pSK1002. Here the umuC gene, as a part of the SOS system, is fused in a reporter gene, lacZ, that encodes for ß-galactosidase. If genotoxins induce the SOS function, the reporter gene is also activated and the formation of ß-galactosidase is quantified photometrically at 420 nm by its ability to form a yellow-coloured metabolite (Oda *et al*. 1985). The test is carried out with and without S9. Bacterial growth is measured as turbidity at 600 nm and biomass factors are considered in the test results. A reduction of cell growth by more than 50% is considered as a toxic effect and ß-galactosidase should not be evaluated for those wells. National (DIN 38415-4: 1996) as well as international standards (ISO 13829: 2000) exist.

Practical experience with the umu-test is available on extracts of bleached kraft mill effluents in Canada (Rao *et al*. 1995). In Switzerland and Germany hospital, municipal and various industrial waste waters have been investigated (Fenn and Popp 1996, Giuliani *et al*. 1996, Miltenburger 1997, Zipperle 1997, Hartmann *et al*. 1998, Siersdorfer *et al*. 1998, Hartmann *et al*. 1999, Gartiser 2000, Gartiser *et al*. 2001). The test method has been introduced for routine regulatory testing of chemical and pharmaceutical effluents (Waste

water Ordinance of Germany). Extracts from suspended particulate matter of river water have also been tested (Vahl *et al*. 1997).

Recently several other tester strains which overexpress specific activation enzymes (acetyltransferase, nitroreductase) have been developed in order to increase the sensitivity against specific genotoxins like nitroarenes and/or aromatic amines (Oda *et al*. 1992, Oda *et al*. 1993, 1995). But these tester strains have been applied to environmental samples only in a few studies (Ohe 1996, 1997). The application of a fluorometric *umu*-test system has been developed in order to increase the sensitivity of the test for the detection of genotoxic compounds in surface water (Reifferscheid and Zipperle 2000).

3.1.1.2 SOS CHROMOASSAY

The SOS chromotest originally was developed by Quillardet *et al*. (1982, 1985). The test detects induction of the SOS genes, which are involved in DNA repair in *Escherichia coli* K12 bacteria. The principle is similar to that of the umuC-test (SOS genes are fused in the *lacZ* reporter gene). There is some evidence that the umuC test detects lower genotoxic responses than the SOS chromotest for two reasons: firstly, the outer wall of the *Salmonella* tester strain used is made more permeable to genotoxins, and secondly, the umuC reporter gene is placed on a multicopy plasmid while in the SOS chromotest it is placed on a single bacterial chromosome (De Maagd 2000). But there are only few comparative studies about the sensitivity of tests.

Waste water studies using the SOS chromotest were performed in Canada (Legault *et al*. 1996, White *et al*. 1996a, White *et al*. 1996b, White and Rasmussen 1998, White *et al*. 1998b, White *et al*. 1998a), Austria (Helma *et al*. 1996), Finland (Suominen *et al*. 1998), and Germany (Janz *et al*. 1990). Sorption of genotoxins to effluent suspended particulate or detection of genotoxic substances in bivalve molluscs has also been studied (White *et al*. 1996b, White *et al*. 1997).

3.1.1.3 MICROSCREEN PHAGE-INDUCTION ASSAY

The Microscreen phage-induction assay with *E. coli* strains was developed by Rossmann *et al*. (1984). The activation of the SOS system results in the release of lytic phages from *E. coli* [WP2s], which are detected following their infection of a second (indicator) *E. coli* strain [TH-008]. The genotoxic potency is evaluated by counting the plaques in the bacterial layer. The DNA-repair assay with *E. coli* K12 strains enables the detection of (repairable) DNA-damage by comparison of the differential survival of strains differing in their DNA-repair capacity.

With waste water only few comparative data of the Microscreen phage-induction assay with other genotoxicity tests exist (Rank and Nielsen 1994, Vargas *et al*. 1995, Helma *et al*. 1996, Vargas *et al*. 2001). In some cases the microscreen phage-induction was more sensitive than the Ames-test, which was explained by metal contamination (Vargas *et al*. 2001).

3.1.1.4 OTHER BACTERIAL GENOTOXICITY TESTS

Newer genetically modified tester strains have been developed using the luminescence gene of *Vibrio fischeri* as reporter for the activation of the SOS answer. A SOS bioluminescence test with the Ames tester strain *Salmonella typhimurium* TA104, which is commercially available, shows considerable higher sensitivity toward several chemicals than the Ames test and the SOS chromotest (van der Lelie *et al*. 1997). Similary Davidov *et al* (2000) fused *S. typhimurium* TA94 with the lux gene of *Vibrio fischeri*. Currently no application in waste water evaluation has been reported for these tests.

3.1.2 Bacterial Mutagenicity Tests

3.1.2.1 AMES ASSAY

The Ames assay, originally developed by Ames *et al*. in 1973, uses mutant *Salmonella typhimurium* strains that have lost their ability to grow in the absence of histidine. Reverse mutations caused by exposure to mutagenic compounds can reactivate their ability to synthesise histidine and thus can grow in the absence of histidine. The number of colonies at different concentrations of the test compound is compared with that of the negative controls and indicates the degree of mutagenicity (Ames *et al*. 1973, Ames *et al*. 1975, Maron and Ames 1983). For the evaluation of chemicals according to OECD and EC standards five tester strains are needed. The most commonly used *Salmonella* strains in waste water screening are TA 98 and TA 100, designed for detecting frame shift mutations and point mutations respectively. The Ames test has been the most widely used method in waste water mutagenicity testing (Stahl 1991, Houk 1992) but in the last decades other genotoxicity tests have been established, which are faster and easier to use. In Germany an adapted version of the Ames test is used for waste water evaluation (DIN 38415-3: 1999). A commercial microplate version of the Ames-test based on colour changes has been developed (Hubbard *et al*. 1984).

3.1.2.2 E. COLI WP2

The *Escherichia coli* WP2 test is similar to the Ames test and both have been adopted in one EC test guideline (67/548/EEC, B.13/14). The principle of the test is that an *E. coli* strain deficient to synthesise tryptophane reverts to its "wild" type and recovers its ability to grow on tryptophane free agar plates under the influence of mutagens. Compared with others this test has not achieved any considerable importance, neither in chemical nor in environmental evaluation of mutagenicity. Therefore only few data with waste water are documented (Fracasso *et al*. 1992, Codina *et al*. 1994).

3.1.2.3 MUTATOX ASSAY

The Mutatox assay uses a non luminescent variant of the luminescent saltwater bacteria *Vibrio fischeri* (*Photobacterium phosphoreum*), which is also used for the determination of acute bacterial toxicity. Genotoxic damage induces the re-establishment of luminescence, which indicates the degree of genotoxicity. In contrast to the SOS chromotest and the umuC test the activation of the SOS pathway the formation of a protease is measured , that degrades a repressor protein of the lux pathway thus leading to luminescence (De Maagd 2000). The test is used especially in the United States (Johnson 1992).

3.2 Test with eucaryotic cells and organisms

In vitro and *in vivo* testing of genotoxicity at a higher level of biological organisation with eukaryotic cells or organisms might be more relevant for human and ecological risk assessment. But generally test performance is much more time-consuming compared with the bacterial tests.

3.2.1 Eucaryotic Genotoxicity Tests

Several genotoxicity tests have been developed which use the integrity of DNA as an unspecific endpoint of genotoxicity. Hereby different techniques are used to measure DNA fragmentation as a result of DNA strand breaks. Alkaline DNA denaturating conditions are added to the test protocols in order to detect, besides double strand breaks, single strand breaks, alkali-labile sites and repair-enzyme-mediated incisions. In contrast, the UDS assay, as described under Section 3.2.1.4, measures repair activity after exposure to genotoxins.

3.2.1.1 COMET ASSAY

In recent years the Comet assay has gained broad attention, because the test is relatively easy to handle and can be applied with cells from different organisms and tissues (see table 2). The alkaline version of the comet assay has been developed by Singh *et al.* (1988). In general cells are mixed with low-melting agarose, placed on microscope slides and lysed by an alkaline buffer with ionic detergents. The liberated DNA is resolved in an electrophoresis chamber, stained and evaluated by fluorescence microscopy. Cells with increased DNA damage display increased migration from the nuclear region towards the anode (Singh *et al*.

1988). The resulting comet-like structure is quantified by measuring the length of the tail and/or the tail moment (the intensity of the migrated DNA multiplied by the respective tail length (integral) with respect to the nuclear DNA). A review about the applicability of the comet assay in environmental monitoring has been provided by Mitchelmore and Chipman (1998b). The test has been applied to a broader range of aquatic organisms such as algae (Erbes *et al*. 1997), mussels (Mitchelmore *et al*. 1998, Pavlica *et al*. 2001), amphibians (Ralph and Petras 1998) and fish (Pandrangi *et al*. 1995, Devaux *et al*. 1997, Belpaeme *et al*. 1998, Mitchelmore and Chipman 1998a, Villarini 1998, Risso-de Faverney *et al*. 2001, Schnurstein *et al*. a, Schnurstein *et al*. b). Advantages of the test are the possibility to choose a broad range of test organisms and tissues, the use of even non-proliferating cells, and that results can be obtained within one day. On the other hand there are still no standard test protocols and a certain degree of handling skills is a necessary prerequisite to routinely performing the test. Although no international accepted standard exists many researchers refer to a test protocol of Tice (1998). The use of defined permanent cell lines such as the human-derived Hep G2 hepatoma cells has been proposed because these are well known cells used in mutagenicity testing (Kosz-Vnenchak and Rokosz 1997, Slamenova 1997, Uhl *et al*. 1999).

3.2.1.2 ALKALINE DNA-ELUATION ASSAY

The alkaline elution assay detects single- and double strand breaks in the DNA. The test measures the rate of elution of DNA through a membrane filter after tissue digestion and DNA denaturation in a buffer with detergents and protease. The content of DNA in the filtrate is measured by fluorimetry. The elution rate increases with the reduction of the molecular weight of the DNA fragments. The test has been applied with wild living clams (*Corbicula fluminea*) for the detection of genotoxic potentials in native surface waters (Waldman *et al*. 2000) and with the Chinese hamster cell line V79 for assessing waste water from a paraquat manufacturing plant (Kuo and Lin 1993).

3.2.1.3 DNA ALKALINE UNWINDING ASSAY

The level of DNA strand breaks with respect to total DNA can be determined by following a time-dependent alkaline unwinding assay. Unwinding of DNA takes place at single-stranded DNA breaks, hence the amount of double-stranded DNA remaining after a given period of alkaline expoure will be inversely proportional to the number of strand breaks. The amounts of these two types of DNA are measured by fluorescence analysis after interaction with appropriate dyes. *In situ* investigations for the detection of genotoxic potential in selected surface water with the DNA alkaline unwinding assay have been reported using fish cells, early life stages of fish, crustaceae and mussels (Wittekindt *et al*. 2000). Everaarts and Sarkar (1996) studied DNA damage in seastars (*Asterias rubens*) in order to assess the state of pollution of the North Sea.

3.2.1.4 DNA-REPAIR SYNTHESIS (UDS-ASSAY)

The unscheduled DNA synthesis assay measures the incorporation of radioactively labelled nucleosides (usually tritium-labelled thymidine) in cells that are not undergoing scheduled (S-phase) DNA synthesis. The test therefore detects DNA repair synthesis after excision and removal of a DNA stretch damaged by mutagenic agents. The uptake of thymidine usually is determined by autoradiography. Both an OECD and an EC standard guideline exist. The DNA repair synthesis UDS test has been applied using primary hepatocytes from rats and fish to assess genotoxicity in surface water (Müllerschön 1989, Grummt 2000b). Equipment costs and time effort for test performance are high.

3.2.2 Eucaryotic Mutagenicity Tests

Eucaryotic chromosomes are large enough to be visible in the light microscope following appropriate staining. This is the basis of various mammalian cytogenetic assays described below. Test guidelines for eucaryotic gene mutation tests are also available (e.g. mouse spot assay, mouse lymphoma assay) but only the yeast test has been performed with waste water samples.

3.2.2.1 SACCHAROMYCES CEREVISIAE GENE MUTATION ASSAY

The test performance of the gene mutation assay with unicellular yeast (*Saccharomyces cerevisiae*) is more comparable with the bacterial assays than with other eucaryotic tests. The test principle is the detection of forward or reverse mutations in a variety of haploid and diploid strains leading to specific properties such as

"red mutants", loss of antibiotic resistance or establishment of a metabolic defect (Zimmermann 1984). An OECD test protocol is available. The yeast assay only occasionally has been applied to waste water samples (Kamra *et al*. 1983, Miadokova *et al*. 1999). In assessing the toxicity of aquatic pollutants the yeast test has been found to be less sensitive than cell culture systems (Mochida *et al*. 1988).

3.2.2.2 CHROMOSOME ABERRATION ASSAY

Chromosomal mutation is a macrodamage of chromosomes. Chromosome aberration include structural aberrations such as fragments or intercalations and numerical aberrations (unequal segregation of homologous chromosomes during cell divisions, which leads to a loss or surplus of chromosomes (aneuploidy and polyploidy). Cytogenetic effects can be studied either in whole animals (*"in vivo"*) or in cells grown in culture (*"in vitro"*). Generally the cell culture is exposed to the test substance and then afterwards treated with a metaphase-arresting substance (Colcimide). Following suitable staining the metaphase cells are analysed microscopically for the presence of chromosomal aberrations. Although considerable effort in test performance limits the applicability in routine measurements, numerous publications from different research projects are available (see table 3).

Plants have been especially used for the evaluation of chromosome aberration (Grant *et al*. 1992, Rank and Nielsen 1994, Rank and Nielsen 1998, Cabrera and Rodriguez 1999, Grover and Kaur 1999), fish have been applied only occasionally (Hayashi *et al*. 1998). Most of the experience gained is available with permanent cell lines such as Chinese hamster lung cells (V79) (Göggelmann *et al*. 1989, Jäger and Meyer 1995, Gartiser and Brinker 1996, Gartiser *et al*. 1996, Jäger *et al*. 1996a, Miltenburger 1997), Chinese hamster ovary cells (CHO) (Strniste *et al*. 1982, Waters *et al*. 1989, Venegas and Garcia 1994) and Chinese hamster lung cells (CHL) (Nobukawa and Sanukida 2000). The detection of aberrations in fish cells is difficult due to the high number and small size of chromosomes in most species.

3.2.2.3 MICRONUCLEUS ASSAY

Micronuclei are chromosome fragments or whole chromosomes that were not incorporated in the daughter cell nuclei and appear in the cytoplasm. For the measurement of micronuclei cell division must be allowed to continue up to the interphase. A mammalian erythrocyte micronucleus assay with bone marrow has been standardized by OECD and EC. Micronucleus formation along with the sister chromatid exchanges and chromosome aberration assays is considered as a clastogenic endpoint. In principle flow cytometric measurement of micronuclei is possible (Kohlpoth *et al*. 1999, Sánchez *et al*. 2000) but equipment costs are high.

Environmental biomonitoring with micronuclueus assays usually has been performed "*in vivo*" by exposure of relevant aquatic organisms for several days followed by microscopic analysis of erythrocytes, gill cells (animals) or roots (plants). But permanent fish (RTG-2) and human derived cell lines have also been used "*in vitro*" (Chung *et al*. 1997, Kohlpoth *et al*. 1999, Sánchez *et al*. 2000). Table 4 gives an overview of different research projects carried out.

Especially amphibians (Jaylet *et al*. 1986, Krauter *et al*. 1987, Fernandez *et al*. 1989, Fernandez *et al*. 1993, Gauthier *et al*. 1993, Fernandez and l'Haridon 1994, Godet *et al*. 1996, Djomo *et al*. 2000), fish (Odeigah *et al*. 1995, Hayashi *et al*. 1998, Marlasca *et al*. 1998, Tuvikene *et al*. 1999) and plants (Panda *et al*. 1988, Sandhu and Lower 1989, Sandhu *et al*. 1989, Grant *et al*. 1992, Panda *et al*. 1992, Ma *et al*. 1995, Smaka-Kincl *et al*. 1996, Cabrera and Rodriguez 1999, Duan *et al*. 1999a, Grover and Kaur 1999, Ma 1999, Miao *et al*. 1999, Steinkellner *et al*. 1999, Wang 1999, Yang 1999) have been used as organisms. Some results for marine and fresh water mussels (Wrisberg and van der Gaag 1992, Burgeot *et al*. 1995) and nematodes (Arkhipchuk *et al*. 2000) are also available.

3.2.2.4 SCE ASSAY

The Sister chromatid exchange (SCE) assay detects reciprocal exchanges of DNA segments between two sister chromatids of a duplicating chromosome. SCEs represent the interchange of DNA at apparently homologous loci. This process involves DNA breakage and repair but as this process does not necessarily lead to permanent mutations some researchers classify the SCE assay as a genotoxicity test. Although little is known about its molecular basis, the SCE frequency is elevated under the influence of mutagenic agents and therefore serves as a model for mutagenicity. OECD and EC standards are available. The detection of

sister chromatids is achieved by incorporation of e.g. bromodesoxyuridine into chromosomal DNA for two cell cycles followed by fluorescence microscopy. For genotoxicity assessment in environmental samples SCE assays have been performed with mussels (Jha *et al*. 2000a, Jha *et al*. 2000b), fish cells (Kligerman *et al*. 1984, Zakour *et al*. 1984, Sahoo *et al*. 1998); and mammalian cells (Chinese hamster lung, CHL, Chinese hamster ovary cells, CHO) (Ohe *et al*. 1993, Pérez-Alzoa and Santos 1997).

3.3 Recent developments

In the field of genotoxicity evaluation of environmental samples similar developments as in classical toxicology have been undertaken. Amanuma established a transgenic zebrafish for the detection of mutagens; it carries plasmids that contain the rpsL gene of *Escherichia coli* as a mutational target gene (Amanuma *et al*. 2000). Winn *et al*. (2000) prepared a transgenic fish that carries multiple copies of a bacteriophage lambda vector that harbours the cII gene as a mutational target, a technique originally developed for lambda transgenic rodents. The p53 tumour supressor gene, which is known to be implicated in cancer development, has been investigated as a possible biomarker for genotoxins in fish cells (McMahon 1994, Bhaskaran *et al*. 1999, 2000). The amplification of DNA by the Polymerase Chain Reaction technique enabled the detection of mutations at specific sites and the development of electrochemical DNA-based biosensors (Kennerley and Parry 1994, Parsons and Heflich 1998, Mascini *et al*. 2001). Polyak *et al.* (2000) developed a whole-cell biosensor with genetically engineered bacteria. The reaction to target toxicants is detected by the induction of a selected promoter and subsequent bioluminescent light through a recombinant lux reporter. A genotoxicity and cytotoxicity test kit based on genetically modified yeast (*Saccharomyces cerevisiae*) which uses the green fluorescent protein as reporter system has been developed for drug screening (Anonymous 2001).

4. EXPERIENCE ON GENOTOXICITY TESTING WITH WATER SAMPLES

4.1 Surface Water

For genotoxicity testing of surface water samples were often highly concentrated on solid phase or extracts in order to enhance sensitivity. But that might lead to unrealistically high and ecologically irrelevant exposure concentrations, and comparison of different studies remains difficult. In Germany a comprehensive study with 17 genotoxicity/mutagenicity tests or test variants and native surface water samples from the Rhine and Elbe rivers has been performed. Following a validation study with well-known mutagens, four sampling stations along the Elbe and three on the Rhine were alternatingly probed monthly for one year. 16 % of the tests performed detected genotoxicity in Rhine water and close to 15 % in Elbe water samples. Most frequently positive water samples were identified with the comet assay and protozoa, algae and permanent cell lines. The bacterial assays (Ames test and umu test) also detected genotoxicity but only with newly established test variants. In general, the distribution of genotoxic water samples reflected the situation of the rivers, with concentrations of positive signals on more polluted sample sites. However a correlation between biotest results and chemical analysis, identifying 41 potentially genotoxic substances, could not be established (Grummt *et al*. 2000).

4.2 Waste water Sectors

Genotoxicity test results are reported for a broad range of industrial and municipal effluents in several reviews (De Raat *et al*. 1990, Stahl 1991, Helma *et al*. 1994, White *et al*. 1996a, Helma and Knasmüller 1997, Claxton *et al*. 1998). Some exemplary sectors are described below. As concentration techniques might overestimate possible risks of genotoxins this chapter focuses on native waste water samples.

Municipal sewage
The genotoxicity in the in- and outlet of municipal treatment plants has been measured by Fenn and Popp (1996) and Zipperle *et al*. (1997). Further studies with the umu test have been summarised by Diehl and Hagendorf (1998). The conclusion is that no genotoxic and mutagenic effects can be measured in native waste water in the in- and outlet of municipal treatment plants using the Ames and/or umu test.

Textile Industry
For native textile waste water mutagenic effects have been found using the Ames- and the V79 chromosome aberration tests (Jäger and Meyer 1995, Jäger *et al*. 1996a) (see also section 4.3). In several "*in vivo*" tests with fish elevated micronucleus rates have been found (Odeigah *et al*. 1995, Marlasca *et al*. 1998). The Comet assay with fish also gave positive results (Sumathi *et al*. 2001). However no significant genotoxic effect was measured in waste water of several plants (Cordova Rosa *et al*., 2001).

Hospitals
Several native samples of hospital waste water were mutagenic in the Ames- and V79 chromosome aberration tests, but sources have not been identified (Gartiser and Brinker 1996). The genotoxicity of native waste water as measured in the umu test has been attributed to fluroquinoline antibiotics (Hartmann *et al*. 1998, Hartmann *et al*. 1999), (Gartiser, 2000) (see also section 4.3).

Pulp and paper industry
With the Ames test (TA 98, TA100), strong effects in TA100 have been determined but those were almost completely removed by multistage sewage water purification (Glazer *et al*. 1990). *Tradescantia* stamen hair and micronucleus assays and *Vicia faba* CA bioassay (Grant *et al*. 1992) have also been used as test system. Effluents of pulp and paper mills (one effluent obtained from a conventional radiata pine kraft-bleaching process and one derived from a biobleaching process with hemicellulase) were tested without purification steps in the Ames *Salmonella* assay (TA100) and MN and SCE tests in CHO cells. In these cases, no positive Ames and MN results were found, however, increased SCE frequencies in CHO cells were determined (Pérez-Alzoa and Santos 1997).
Genotoxicity effects have been detected in waste water from a wheat and rye straw paper pulp factory using *in vivo* test systems in mussels (*Mytilus edulis*) and sister chromatid exchange in fish (*Nothobranchius rachowi*) (Wrisberg and van der Gaag, 1992).

Chemical industry
Fenn and Popp (1996) used the Ames and umu assays to evaluate waste water from several sectors. About 10% of the samples were positive. In 1992 - 1996 a joint programme of the German Environmental Agency (UBA) and the Association of the German Chemical Industry (VCI) on the selection of appropriate methods for assessing the mutagenic potential of native industrial waste waters showed, that eucaryotic test systems often gave non-reproducible and unsystematic results. Therefore the steering group of the joint UBA-VCI project focussed on the umu test for practical reasons (Miltenburger 1997). The study of Sundvall *et al*. (1984) in which nitroaromatic compounds were identified as the source for mutagenicity in waste water from a nitrobenzoic acid and nitrotoluene production plant is described in section 4.3.

4.3 Genotoxicity identification/backtracking

"Toxicity backtracking" is defined as a methodology to identify the source or group of substances causing an undesired biological effect. This could be done by Toxicity Identification Evaluation (TIE) or by testing tributary streams of the mixed sample (OSPAR Commission, 2000). In this capture some examples are given which underline the usefulness of the application of genotoxicity tests within Whole Effluent Assessment.

Disinfection by-products
For drinking water treatment the application of genotoxicity testing has a long tradition. It is generally known that chlorine disinfection of drinking water that is derived from surface water leads to the formation of mutagenic compounds. Mutagenicity tests have been used to assess the relative contribution of disinfectant by-products to total mutagenicity as measured in drinking water (see table 5). Thus a significant portion (in some cases over 60%) of the mutagenicity has been attributed to the presence of various chlorohydroxyfuranones, and one of the compounds, namely 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), was shown to account for up to 60% of the overall activity (Kronberg 1999).

Kinae *et al.* (2000) identified the compound MX also in river water highly polluted by industrial and domestic chemicals. The contribution ratio of MX to the total mutagenic activity of river water was estimated as 5% to 30%. To elucidate the origin of MX, several chemicals contained in domestic sewage such as catechin and diosmin were treated with sodium hypochlorite, and MX was isolated and identified. The results suggested that domestic sewage was a new source of MX.

Genotoxicity in river water upstream and downstream of discharges
One method to identify genotoxic sources is to compare genotoxicity in river water upstream and downstream of the discharges examined. In theory the "distance from pollution" should be proportional to the decrease of genotoxicity. In table 6 some examples are summarised.

As shown in table 6, chromium (Al-Sabti *et al*. 1994), nitroarenes and aromatic amines (Cerna *et al*. 1996, Ohe 1996) have been correlated with genotoxicity in river water. In one profound study Shiozawa *et al*. (1998) succeeded to identify textile effluents with AZO DYE-1 as the principal source of mutagenicity in the Nishitakase River in Japan. The compound identified has been determined to be 2-[2- (acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-1).
They found that AZO DYE-1 changed to the dechlorinated derivative of PBTA-1 with sodium hydrosulfite during industrial processes, which reacted with sodium hypochlorite during wastewater treatment to produce PBTA-1. PBTA-1 showed potent mutagenic activities in *S. typhimurium* TA98 and YG1024 (Shiozawa *et al*. 1998).

Another case study related high incidences of chromosome mutations in a natural population of the fern *Osmunda regalis* with pollution from paper recycling wastes (Klekowski and Levin 1979). Vargas *et al*. (1993) found that mutagenicity effects detected in the Ames test in the Cai River, Brazil, were due to waste water from petrochemical industries (Vargas *et al*. 1993).

White *et al*. (1998) performed probably one of the most comprehensive studies at the St. Lawrence River in Canada using the SOS Chromo assay. Firstly they studied the genotoxicity of several industrial and municipal waste water effluents and found that a substantial fraction of genotoxicity was adsorbed to suspended solids (White *et al*. 1996a). Afterwards they investigated the presence and potency of particle bound genotoxins as well as genotoxins accumulated by macroinvertebrates and fish downstream of the source. Discharges from foundries, aluminium and petroleum refineries were the most genotoxic samples. In taking into account the genotoxic equivalents measured in the tissue, bioconcentration factors of 100-1000 were found (White *et al*. 1998b, White *et al*. 1998a).

Identification of genotoxic substances in waste water
In several case studies the sources of genotoxicity in the waste water has been attributed to specific chemicals (see table 7).

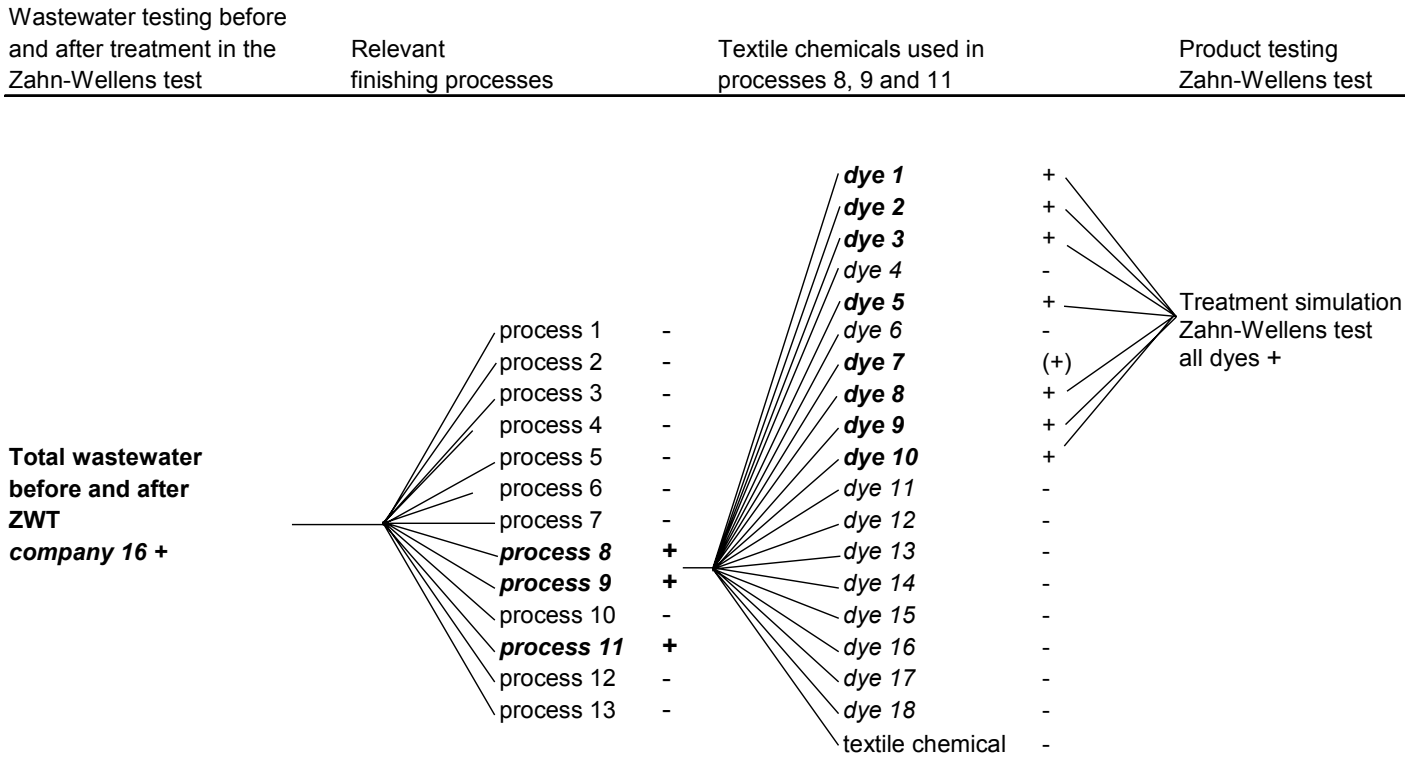
In Germany Jäger *et al*. (1995, 1996b) identified azo dyes as the principal source of mutagenicity in total waste water of a textile finishing plant by systematic backtracking. From 18 plants included in this survey, one native waste water sample was found to be mutagenic in the Ames test with strain TA 98 (figure 2). The mutagenicity of the waste water from one plant with an extraordinarily high maximal induction rate (IR_{max} 12,3) was only partly removed after treatment with activated sludge (Zahn-Wellens test with 7 days test duration). After excluding that pH regulation of the waste water by an acid recycling product before discharging caused mutagenicity, 13 partial streams from textile finishing processes were tested, with three of them showing mutagenic effects. Afterwards all textile dyes and auxiliary chemicals which were used exclusively in that process were examined in the Ames test. Seven out of 18 dyes, which were used in polyester colouring, were identified as principal sources of mutagenicity in total waste water. The results were proven by additional investigation of the elimination of those dyes in the Zahn-Wellens test. As observed with the total waste water the Ames mutagenicity of all dyes was only partly removed during treatment with activated sludge.

Another example of genotoxicity backtracking is the source of genotoxicity in hospital waste water as observed in the umu test. Hartmann *et al*. (1998) calculated theoretical sewage concentration of drugs in a Swiss hospital by using consumption data and compared them with genotoxicity data for several drugs. Thus fluoroquinolone antibiotics (ciprofloxacine, norfloxacin) were identified as the principal source of genotoxicity in native hospital waste water. Additionally HPLC-determined ciprofloxacine concentrations found in waste water samples confirmed these results (Hartmann *et al*., 1998). Afterwards this study was extended to German hospital waste water and similar results were found. Nevertheless the sources of mutagenicity in German wastewaster as measured in the Ames test (TA98, TA100) and in the chromosome aberration test with Chinese hamster cell line V79 remained unexplained (Gartiser and Brinker, 1996, Hartmann *et al*., 1999). Disinfectants are unlikely as the main source of genotoxocity and Ames mutagenicity in hospital waste water (Gartiser *et al*. 2001).

Manabe *et al*. (1984) investigated waste water from oil-water separating tanks of ten petrol stations after fractionation into neutral, acidic, and basic fractions. Mutagenicity was measured with *S. typhimurium* strains. The neutral fractions showed high mutagenicity in the Ames test with TA98 and TA100. Each neutral fraction was subjected to HPLC fractionation and analysed by GC-MS HPLC. The amount of 1-nitropyrene in 36 samples of waste water accounted for 0,3-58,5% of the total mutagenicity of the neutral fractions .

In some studies the specific mutagenic spectra of Ames or umu tester strains was used to identify the substantial chemical group of mutagens/genotoxins. Thus Sundvall *et al*. (1984) found out that half of the mutagenicity in waste water from the production of nitrobenzoic acids and nitrotoluenes was accounted for by one single compound, 3,5-dinitrobenzoic acid, by using nitroreductase-proficient and -deficient tester strains complemented with single substance analysis.

Figure 2 Mutagenicity backtracking in waste water of a textile finishing plant (Data from Jäger et al., 1995, 1996)



"+" = wastewater, finishing processes and dyes with positive results in the Ames test, TA 98

4.4 Application of test methods in the framework of BAT

Drinking water treatment
Several studies reveal the influence of different disinfection agents for mutagenity. Nobukawa *et al*. (2000) evaluated the genotoxic characteristics of chlorinated and brominated substances produced by ozonation and chlorination of river waters. Mutagenic activities of the drinking waters produced by chlorination were observed to be higher than those by ozonation. In another study the effect of different disinfectants and granular activated carbon filtration on the removal of disinfection by-product precursors was determined. The results showed that disinfection with chloramine produced lower trihalomethane, AOX, MX and mutagenicity levels compared to chlorine disinfection (Vahala *et al*. 1999).

Also different steps during drinking water treatment were assessed with mutagenicity tests. Liu *et al*. (1999) found out that raw water from Lake Chao, China, induced mutagenicity in XAD-extracts in the Ames test, the SCE test with mammalian CHL cells and the micronucleus induction test in fish. After coagulation and sedimentation the settled water gave negative results, but finally after sand filtration and chlorination the water was again positive. On the other hand Mei *et al*. (2001) investigated the drinking water treatment processes of the Beijing Water Works using the Ames test with water extracts and found that addition of coagulant increased mutagenic effects. Sand and granular activated carbon filtration effectively removed most of the formed mutagens and rechlorination did not obviously increase the mutagenic effects.

Zhou *et al.* (1997) stated that only 1/8 of the observed mutagenicity in tap water originated from chlorination of humic acids isolated from raw water, and claimed that the contamination from industrial waste and human excretions was an important source of mutagenicity in chlorinated drinking water.

Elimination of genotoxic substances during waste water treatment
Numerous studies deal with the capacity of municipal waste water treatment to eliminate genotoxins, thus indicating that treatment with activated sludge corresponds to BAT for such indirectly discharged waste water if genotoxins were effectively removed. Often laboratory batch tests (Zahn-Wellens test) or flow through tests (Coupled Units test) were used as a model to assess the behaviour of partial waste water streams during activated sludge treatment (see table 8).

Consequently it was found that while genotoxicity of hospital waste water was completely removed during waste water treatment, mutagenicity of textile waste water and medical laboratory waste water might still be present in some cases (Gartiser *et al*., 1997, Gartiser, 2000). Mutagenicity of waste water from a pulp and paper plant was almost practically removed by multistage sewage water purification (Glazer *et al*. 1990). Legault *et al*. (1996) used a different approach as they investigated the genotoxic activity of industrial effluents after a 5-day aeration treatment with the SOS chromo assay. They found only minor reduction of genotoxicity compared to native (unaerated) samples, suggesting that soluble genotoxicants were relatively persistent to oxidation.

The adsorption behaviour of genotoxic substances has also been assessed in order to evaluate possible emission routes or treatment possibilities. White *et al*. (1996b) proposed a genotoxicity sorption partition coefficient to describe the affinity of genotoxins for particulate matter.

4.5 Risk assessments of chemicals

Environmental risk assessment of chemicals including genotoxicity/mutagenicity tests might be an additional tool to optimise the selection and use of chemicals in different areas such as industrial or water treatment processes. Takigami *et al*. (1998) evaluated organic polymer flocculants used for municipal sludge dewatering with the *Bacillus subtilis* rec-assay and found direct DNA damage in eight out of ten cationic flocculants. Gartiser *et al*. (2001) identified isothiazoline biocides used in cooling water treatment as causing genotoxic effects in the umu assay while other biocides did not explain genotoxicity in the concentrations applied. Kümmerer *et al*. studied the biodegradation of several antibiotics using the closed bottle test and the SOS chromotest. None of the test compounds was biodegraded and genotoxicity was not eliminated.

4.6 Application within discharge permits

In Germany in 1999 the umuC-assay has been included in the Ordinance on Requirements for the Discharge of Waste water into Waters, Waste water Ordinance based on the German Water Management Act. The test performance follows the DIN 38415-3 Standard from 1999. The assay is a requirement for direct discharges of waste water from the chemical and pharmaceutical industry with a Lowest Ineffective Dilution (LID) ratio of 1,5. That means that a negative genotoxicity test result determined by the umu test is generally accepted. The chemical industry supports this conclusion (Skalicky 1996). A waste water variant of the Ames test with the testing strains TA98 and TA100 has also been standardised (DIN 38415-4: 1999) but up to now the test has not yet been included in the Waste water Ordinance. The regulatory requirements also favoured the application of test series with the umu assay, a strategy that is supported by the German Association of the Chemical Industry (VCI). BASF AG reported results of 172 two-hour samples from the department in Ludwigshafen with the umu assay. Only one sample proved to be genotoxic (Andreae, 2000). Other applications within discharge permits of Contracting Parties have not been reported yet.

The application of genotoxicity bioassays for discharge permits requires a statistically robust design with reproducible and unambiguous test results in order to treat all discharges equally by law. Tests should always be carried out by approved laboratories with quality control accreditation.

5. CONCLUSIONS

5.1 Sample preparation

For the purposes of standardisation a test strategy has to be defined carefully. There should be agreement on sampling procedures, the handling of samples (e.g. use of extraction/concentration procedures) as well as on the test system/test battery. Often effluent as well as surface water samples are highly concentrated on solid phase or in extracts in order to enhance sensitivity. But this may lead to unrealistically high and ecologically irrelevant exposure concentrations and there is no agreement as to what concentration factor would be acceptable. Also, each concentration procedure recovers different fractions of the sample, and volatile substances may be lost. So testing crude samples should be favoured to get a realistic estimate of the genotoxicity of an effluent (De Maagd, 1998, OSPAR Commission, 2000, De Maagd, 2000, Grummt *et al*., 2000). If extraction/concentration procedures are considered necessary, those procedures should be harmonised in order to improve comparability of test results.

For all bacterial or cell culture systems at least sterile filtration is required, whereby a large part of particulate matter is removed from the sample, which means that effects of particle-associated xenobiotica might remain undetected. In contrast "*in vivo*" assays allow an integrated approach as living organisms might also be affected by adsorbed genotoxins incorporated by ingestion.

5.2 Test strategy

The need to consider genotoxicity and mutagenicity testing in whole effluent assessment is widely acknowledged although the potential hazard of genotoxins to the environment remains unclear. It is accepted, that an individual test covers only one definite endpoint. A test battery is called for (OSPAR Commission 2000). The test strategy has to be defined carefully. There should be agreement on sampling procedures, the handling of samples (e.g. use of extraction/concentration procedures) as well as on the test system/test battery.

Bacterial tests
Bacterial mutagenicity tests are in widespread use, highly standardised and comparably cheap. The Ames mutagenicity test as well as the umuC-test and SOS-chromo genotoxicity tests have been applied to a wide range of waste water samples. The Ames test has undoubtedly proven its applicability for waste water testing. Its main advantages are the unambiguous endpoint and the existence of a large data base due to its importance in chemical substance assessment. Due to the shorter incubation time test results might be obtained within several hours for the umuC and SOS chromo assays while for the Ames test at least two days are needed. There are some indices that the umu test is more sensitive than the SOS chromo test

although few comparative data exist (De Maagd, 1998). Negative genotoxicity should be an acceptable result in native waste water samples. In case of positive genotoxicity results, hazard assessment should be performed considering data with eucaryotic tests and the causes for the toxicity should be explained.

Tests with eucaryotic cells
The need for other test systems on a higher organism level such as plants, mussels, fish or mammals for waste water evaluation is recognised. Although numerous convincing studies have been published, no internationally accepted guideline for waste water assessment currently exists. Genotoxicity and mutagenicity tests with mammalian cells could better predict human risks, but are highly time and cost intensive. "*In situ*" and *"in vivo*" assays with test animals retrieved from wildlife or bred/kept in laboratories are even more time consuming and more expensive than "*in vitro*" assays with defined cell lines.

Several researchers call for a test battery of one bacterial and one eucaryotic test system following the approach in the EU chemical risk assessment guidance. Göggelmann *et al*. (1989) proposed a test battery of the Ames test, the V79 chromosome aberration assay and the micronucleus assay with Xenopus larvae. on Grummt *et al*. (2000a) favoured an "*in vitro*" basis examination with the Ames- or umu test and the Comet assay.

The time, effort and therefore costs to assess genotoxicity/mutagenicity of waste water with eukaryotic cells are comparatively high and that might hinder a broad acceptance for routine measurement within waste water surveillance. Nevertheless at least in those cases in which positive results were found in a first survey with bacterial tests, from a scientific point of view further studies considering genotoxicity backtracking and/or higher test organisms should be required. Additionally monitoring programs with test systems at higher organism levels should be performed by industry and governmental authorities in order to obtain a better linkage from bacterial tests to hazard and risk assessment of waste water corresponding to the accepted test strategy for chemical risk assessment.

From the literature it can be noted that two strategies for waste water testing with eucaryotic cells are used: the micronucleus assay for detecting mutagenic agents and the Comet assay as a genotoxicity test, the latter probably requiring the lowest time effort of all eucaryotic tests. Organisms or cell cultures from plants, mussels, fish, mammals or even human cell cultures have been used in both strategies. Plants are increasingly used for genotoxicity/mutagenicity testing, the large evolutionary distance to humans being a drawback but with the advantage that in some of the test systems no manipulation of the native environmental sample is needed.

5.3 Possibilities for the application of genotoxicity test methods in Whole Effluent Assessment

- Genotoxicity and mutagenicity are important endpoints for human and environmental hazard evaluation.
- Bioassays for detecting genotoxic and mutagenic effects provide additional information about the quality of waste water and should be implemented in whole effluent assessment.
- Negative genotoxicity should be an acceptable result in native waste water samples unless the origin has been explained and further tests show harmlessness of effluents.
- In case of positive genotoxic results in surface water, from a scientific point of view, extensive monitoring programmes should be performed in order to identify industrial or municipal sources of genotoxic substances.
- Genotoxicity tests should be implemented in discharge permits for those industrial or municipal sectors which are supposed to use, process or discharge genotoxic substances.
- A test battery of bacterial (umu assay or SOS chromo assay and Ames test) and eucaryotic cells (micronucleus or Comet assay with permanent cell lines or suitable organisms) should be considered.

Table 2 Application of the Comet assay with aquatic organisms and/or waste water

Organism	Samples	Results	Author
Algae			
Chlamydomonas reinhardii	Surface water Rhine, Elbe		Glos *et al*. 2000
Chlamydomonas reinhardtii	Well known reference mutagens	Dose-dependent DNA damage.	Erbes *et al*. 1997
Mussels			
Dreissena polymorpha gill cells	Surface water Rhine, Elbe		Glos *et al*. 2000
Mytilus edulis gill cells	*in vitro* exposure with H2O2 and N-nitroso-dimethylamine	The assay has potential for use in an *in vitro* context for the screening of agents destined for release or disposal into the marine environment.	Wilson *et al*. 1998
Mytilus edulis gland cells	Reference contaminants	DNA strand breakage at subtoxic concentrations of a range of agents, some of which require metabolic activation.	Mitchelmore *et al*. 1998
Mytilus edulis hematocytes	Naval Station, San Diego Bay	After exposing mussels for 30 days the Comet assay responded rapidly to genotoxic contaminants. Chemical analysis of bioaccumulated metals and PAH suggest that photoactivation of PAH might be associated with the effects.	Steinert *et al*. 1998
Dreissena polymorpha haemocytes	Pentachlorophenol (PCP) and Sava River downstream from Zagreb	Detection of DNA damage in zebra mussel using the comet assay. Significant increase in DNA damage after exposure to PCP (80 µg/l) and after in situ exposure in the Sava River downstream of the municipal waste water outlet.	Pavlica *et al*. 2001
Amphibians			
Rana clamitans (green frog) and *Bufo americanus* (American toad)	11 sites in southwestern Ontario, Canada	Amphibians were caged for either 7 or 14 days. Significantly increased levels of DNA damage, relative to the controls, were observed in tadpoles caged at three sites draining a large petrochemical installation, and for a part of the St. Clair River.	Ralph and Petras 1998
Fish			
Cyprinus carpio	Textile dye effluent		Sumathi *et al*. 2001
*Danio rerio (*zebrafish) Primary hepatocytes and gill cells	Native surface waters Rhine, Elbe		Schnurstein *et al*. 2001
Oncorhynchus mykiss (rainbow trout) hepatocytes		Cadmium induces apoptosis and genotoxicity in trout liver cells.	Risso-de Faverney *et al*. 2001
Permanent cell lines			
Chinese hamster cells V79 RTG-2, RTL-1 fish cells	Surface water Rhine, Elbe		Glos *et al*. 2000
human hepatoma cells (Hep G2)	Thermal-electric power station and the Institute of Metal Cutting	A greater number of cells with comets were observed in those treated *in vitro* with the polluted water samples (70%-88%) than in those in the control (22%, 33%).	Kosz-Vnenchak and Rokosz 1997
Plants			
Vicia faba	surface water of Yangzhong city		Zhong *et al*. 2001

Table 3 Application of the chromosome aberration test to aquatic organisms and/or waste water

Organism	Samples	Results	Author
Fish			
Rhodeus ocellatus (rose bitterling) embryos		Development of method.	Hayashi *et al*. 1998
Primary cultures of mammals			
Human lymphocytes	Industrial effluents, river waters	No significant cytogenetic effect.	Cerna *et al*. 1996
Human lymphocytes	Cai River water at a petrochemical complex in Brazil	Detection of mutagenicity.	Torres de Lemos and Erdtmann 2000
Permanent cell lines			
Chinese hamster lung cells (V79)	Domestic and industrial waste water		Göggelmann *et al*. 1989
Chinese hamster lung cells (V79)	Hospital waste water	Mutagenicity in raw samples.	Gartiser and Brinker 1996, Gartiser *et al*. 1996, Hartmann *et al*. 1999
Chinese hamster lung cells (V79)	Textile finishing waste water	Mutagenicity in raw samples due to azo dyes.	Jäger and Meyer 1995, Jäger *et al*. 1996a
Chinese hamster lung cells (V79)	Waste water from chemical industry		Miltenburger 1997
Chinese hamster ovary cells (CHO)	Bio-Bio River, Chile	Genotoxic effects induced by contaminated river water.	Venegas and Garcia 1994
Chinese hamster ovary cells (CHO)	Eight waste water samples from five different sites	Comparative study; Ames test was the more sensitive.	Waters *et al*. 1989
Chinese hamster ovary cells (CHO)	Sunlight-activated oil shale retort process water	Genotoxic effects detected.	Strniste *et al*. 1982
Chinese hamster lung cells (CHL)	Chlorinated and brominated substances produced by ozonation and chlorination	Comparative study; mutagenic activities of the drinking waters produced by chlorination were observed to be higher than those by ozonation.	Nobukawa and Sanukida 2000
Plants			
Allium cepa	Soil irrigated with waste water, leachates from a landfill and extracts from compost		Cabrera and Rodriguez 1999
Vicia faba	Pulp and paper effluents		Grant *et al*. 1992
Allium cepa	Sewage and industrial effluent		Grover and Kaur 1999
Allium cepa	Two municipal waste water treatment plants and twelve industries		Nielsen and Rank 1994
Allium cepa			Rank and Nielsen 1994
Allium cepa	Sludges from three Danish municipal waste water treatment plants	Only two sludge samples from the smallest plant with the lowest industrial load induced significant chromosome aberrations.	Rank and Nielsen 1998

Table 4 Application of the Micronucleus assay with aquatic organisms and/or waste water

Organism	Samples	Results	Author
Mussels			
Crassostrea gigas haemocytes	Seawater		Burgeot *et al*. 1995
Mytilus edulis	Wheat and rye straw paper pulp factory	Study revealed that genotoxins are produced in the chlorine dioxide bleaching process as well as in the pulping process, also indicating genotoxic activity of non-chlorinated compounds.	Wrisberg and van der Gaag 1992
Fish			
Carassius sp., *Zacco platypus Leiognathus nuchalis, Ditrema temmincki,* Gill cells	Tomio River and Mochimune Harbor, Japan	Fish collected upstream tended to have lower frequencies than midstream samples. The marine fishes showed seasonal differences.	Hayashi *et al*. 1998
Oncorhynchus mykiss erythrocytes	Wool shrinkproofing effluent	After exposure for 30 days significant increase of micronuclei.	Marlasca *et al*. 1998
Clarias lazera	Brewery and textile-mill effluent	After exposure for 14 days dose-dependent micronuclei induction was significantly higher for textile than for brewery effluent.	Odeigah *et al*. 1995
Oncorhynchus mykiss *Perca fluviatilis* *Rutilus rutilus*	Narva River near oil shale industry, Estonia	Micronucleus test did not show any evidence of genotoxicity.	Tuvikene *et al*. 1999
Amphibians			
Pleurodeles waltl, erythrocytes of larvae	Petrochemical waste water		Djomo *et al*. 2000
Pleurodeles waltl larvae erythrocytes	19 compounds	Newt micronucleus could be used to monitor aquatic pollution.	Fernandez *et al*. 1989
Pleuodeles waltl, Ambystoma mexicanum, Xenopus laevis, erythrocytes	Testing freshwater pollutants and radiations	The very low radiation dose of 6 rad gave positive results. 47 chemicals tested with 8 and/or 12 days exposure.	Fernandez *et al*. 1993
Pleurodeles waltl newt	Benz(a)pyrene and oil refinery effluent	Genotoxicity of BaP was detected under different lighting. The effluent itself was not genotoxic.	Fernandez and l'Haridon 1994
Jaylet Test:	Waste water from tanneries and petrochemical industries	Genotoxicity was detected after dilution.	Gauthier *et al*. 1993
newt larvae red blood cells	Electroplating effluents	Induction of micronuclei by metals.	Godet *et al*. 1996
		Cytogenetic study of organic and inorganic toxic substances on *Allium cepa, Lactuca sativa*, and *Hydra attenuata* cells	Arkhipchuk *et al*. 2000
Rana catesbeiana erythrocytes	Radiation	A 3-fold increase was obtained with a dose of 3,0 Gy radiation.	Krauter *et al*. 1987
Ambystoma mexicanum larvae erythrocyte	Benzo[a]pyrene ethylmethane sulphonate (EMS)	Positive results after 8 days of treatment 0,025 ppm (BaP) and 24 ppm EMS.	Jaylet *et al*. 1986

Permanent cell lines			
RTG-2 fish cell line	Model genotoxic substances	Flow cytometric detection of micronuclei	Sánchez *et al*. 2000
RTG-2 fish cell line	38 industrial waste waters from 11 different branches, Bavaria	14 samples showed significant increase of micronuclei measured by flow cytometry.	Kohlpoth *et al*. 1999
HeLa/S3 mammalian cells	Tamagawa River, Japan	Genotoxicity was observed in methanol while not in dichloromethane extracts, suggesting polar micropollutants.	Chung *et al*. 1997
Plants			
Allium cepa *Vicia faba*		Meristem micronucleus assay has been adopted in the US EPA Gene-Tox programme since 1980.	Ma *et al*. 1995, Ma 1999
Tradescantia paludosa	Leachates from a landfill and of extracts from compost	Trad-micronucleus assay more sensitive than the Trad-stamen hair mutations assay.	Cabrera and Rodriguez 1999
Vicia faba	Xiaoqing River, China	8 samples polluted with industrial waste and municipal sewage were positive.	Miao *et al*. 1999
Tradescantia paludosa	Panlong River, China	The peak frequencies were observed in samples near industrial and municipal waste water influents.	Duan *et al*. 1999b
Tradescantia paludosa	Pulp and paper effluents	After 24 h exposure positive responses.	Grant *et al*. 1992
Allium cepa	Sewage and industrial effluent	Positive responses of industrial effluents both in the micronucleus and aberration assays.	Grover and Kaur 1999
Eichhornia crassipes	Mercury	Low levels of mercury (0,001 ppm) induced micronuclei	Panda *et al*. 1988
Eichhornia crassipes	Rushikulya estuary, India	Genotoxicity of low levels of mercury in the vicinity of a chloralkali plant; highly correlated with bioconcentrated and aquatic mercury.	Panda *et al*. 1992
Tradescantia paludosa	Chemicals commonly found at the industrial waste sites	Bioassay could be effectively utilized for assessing the potential clastogenicity of the chemicals.	Sandhu *et al*. 1989
Allium cepa	Undiluted industrial and municipal waste water, Drava River water	The most polluted liquids caused inhibition of root growth, decrease of mitotic index, increase of micronuclei and increase of presence of aberrant cells.	Smaka-Kincl *et al*. 1996
Tradescantia paludosa	Urban, river and ground water samples from Austria	Samples collected near an industrial waste dump were positive.	Steinkellner *et al*. 1999
Vicia faba	Soil samples collected at chromium processing plant in Tianjin, China	Clastogenicity of chromium contaminated soil samples. Micronuclei frequencies inversely proportional to the distance from the source.	Wang 1999
Tradescantia paludosa	Lake Hongzhe, China	Water quantity bears some relationship with the genotoxicity. High nitrates and nitrogen content were the main contributing factors observed.	Yang 1999

Table 5 Elimination or induction of genotoxicity within water treatment by chlorination

Organism	Endpoint	water providence	Extraction	Results	Author
Ames test	M bacteria	Surface water		Chlorine disinfection of drinking water that is derived from surface water leads to the formation of mutagenic compounds. A significant portion has been attributed to various chlorohydroxyfuranones. The compound MX accounted for up to 60% of the overall activity.	Kronberg 1999
TA100	M bacteria	Polluted river water (Katsura River, Japan)	XAD-2000	The mutagenic compound MX has been identified in river water highly polluted by industrial and domestic chemicals. The contribution ratio of MX to the total mutagenic activity of river water was 5–30%. Several chemicals contained in domestic sewage were treated with sodium hypochlorite. Contribution ratios to the total mutagenicity of each reaction mixture were 4% - 96%. The results suggest that domestic sewage is a new source of MX.	Kinae *et al*. 2000
Tradescantia	MN	Groundwater near hazardous waste landfill		Groundwater collected near a waste landfill was treated in a purification plant (activated charcoal filtration, UV irradiation). UV irradiation of activated charcoal-filtered water resulted in an enhancement of MCN frequencies, which decreased with a half-life of approximately 1 day.	Helma *et al*. 1994
TA98 TA100		Beijing Ninth Water Works	XAD-2	Water from different treatment processes was concentrated and assessed by Ames test. Prechlorination caused mutagenicity, addition of coagulant increased mutagenic effects greatly, sand and coal filtration and granular activated carbon filtration could effectively remove most of the formed mutagens, the rechlorination did not obviously increase the mutagenic effects.	Mei *et al*. 2001
Ames test	M bacteria	Raw water; Tap water	China	Only 1/8 of observed direct frameshift mutagenicity in tap water originated from chlorination of humic acid isolated from raw water; contamination from industrial waste and human settlement (night soil) are important potential sources of mutagenicity in chlorinated drinking water.	Zhou *et al*. 1997
Ames test CHL Silver carp erythrocytes	M bacteria SCE MN	Chao Lake, China	XAD-2	Chlorinating during water treatment produced mutagenic activity in several tests. Raw water induced positive responses in the Ames test which was eliminated after coagulation and sedimentation; the treated water (after sand filtration and chlorination) was mutagenic.	Liu *et al*. 1999
TA98 TA100 CHL	M bacteria CA	River water containing bromide		Evaluation of the genotoxic characteristics of chlorinated and brominated substances produced by ozonation and chlorination of the river waters containing bromide. Mutagenic activities of the drinking waters produced by chlorination were observed to be higher than those by ozonation.	Nobukawa and Sanukida 2000
Ames test	M bacteria	Pilot plant at Pitäkoski, Finland		Pilot plant study in order to determine the effect of water treatment on the removal of disinfection by-product precursors. Disinfection with chloramine produced lower mutagenicity and levels of the halogenated furanone MX compared to chlorine disinfection.	Vahala *et al*. 1999

TA and YG: Ames-tester strains; NM: umu tester strains; CHL: Chinese hamster lung cell line; V79: Chinese hamster cells; M: mutagenicity; MN: micronucleus; SCE: sister chromatid exchange; CA: chromosome aberration; G: genotoxicity

Table 6 Genotoxicity in river water above and below discharges

Organism	Endpoint	Locality	Extraction	Results	Author
Fish *Carassius auratus gibelio*	MN Erythrocytes	Ljubljanica River	None	MN of samples 100 m above and 400 m below the discharge of leather waste products correlated with chromium concentration under both field and laboratory conditions.	Al-Sabti *et al*. 1994
TA98, YG1021, YG1024; YG1041	M bacteria	Labe River		Total and fractionated organic extracts from industrial effluents river water, indication of possible presence of nitroarenes and aromatic amines in industrial effluents, river waters, and their fractions.	Cerna *et al*. 1996
Natural population of the fern *Osmunda regalis* / Ames-test	Chromosome. damage / M bacteria	Millers River, USA	Waste extracts	A high incidence of chromosome mutations in a river heavily polluted with paper recycling wastes was found, samples of this solid waste were extracted with various solvents and tested in the Ames test; a majority was mutagenic with S-9 activation; results document the presence of mutagens in the solid waste generated by a paper recycling industry and the genetic impact of these mutagens on the local biota.	Klekowski and Levin 1979
CHL	SCE	Tributaries of the Yodo River, Japan	Blue rayon	Higher SCE frequencies in river waters downstream a waste water treatment plant than upstream suggesting that the effluents were possible pollution sources of genotoxic chemicals in the rivers.	Ohe *et al*. 1993
TA98 and YG1024	M bacteria	Nishitakase River, Japan	Blue cotton	Azo Dye-1 was identified as source of mutagenic novel aromatic amine PBTA-1 isolated from water samples, taken at sites below the municipal sewage plants. AZO DYE-1 from dyeing factories' effluents reacts with sodium hydrosulfite and sodium hypochlorite during the treatment of waste water, to form PBTA-1.	Shiozawa *et al*. 1998
TA100, TA98	M	Cai River, Brazil	none	Identification of petrochemical industries as source of river water mutagenicity; 82% of the sample at sites closest to the industrial complex were positive.	Vargas *et al*. 1993
NM2009 NM2000	G bacteria	Yodo River, Japan	XAD-2, blue rayon	With NM2009 extracts collected downstream from waste water treatment plants showed higher inducing activity than those upstream, indicating presence of nitroarenes and/or aromatic amines such as 1-nitropyrene in river water.	Ohe 1996
NM2009	G bacteria	Yodo River, Japan	blue rayon	The total amounts of four heterocyclic amines, MeIQx, Trp-P-1, Trp-P-2 and PhIP, in river water accounted for 24% of the genotoxicity of extracts.	Ohe 1997
SOS Chromo test	G bacteria	St Lawrence River, Canada	Dichlor-methane	Particle bound genotoxins and genotoxins accumulated by macroinvertebrates and fish downstream. Discharges from foundries, aluminium and petroleum refineries yielded the most genotoxic samples.	White *et al*. 1998b, White *et al*. 1998a
SOS Chromo test with extracts from *Mya arenaria* and *Mytilus edulis*	G bacteria	Saguenay Fjord, Canada	on dichloromethane extracts	Genotoxicity analyses were performed with bivalve molluscs collected downstream from several aluminum refineries and forestry products industries using the SOS chromotest. In several cases, SOS response induction factors exceeded 3,0. The results failed to reveal a clear downstream trend of decreasing genotoxicity with increasing distance, thus the accumulated direct-acting genotoxins may be of municipal origin.	White *et al*. 1997

TA and YG: Ames-tester strains; NM: umu tester strains; CHL Chinese hamster lung cell line; M: mutagenicity; MN: micronucleus; SCE: sister chromatid exchange; G: genotoxicity

Table 7 Identification of genotoxic sources in waste water

Organism	Endpoint	Sector	Extraction	Results	Author
Ames test	M bacteria	Municipal incinerator		About 90% of all mutagens produced in a municipal incinerator are discharged into the atmosphere as emission gases, and 10% are disposed of in the waste water treatment plants.	Kamiya and Ose 1987
TA 98 TA100 V79	M bacteria CA	Textile industry, Germany	None	Azo dyes were identified as main source of mutagenicity in total effluent of a textile plant by backtracking. The mutagenicity of the Azo dye and the total effluent was not removed completely in the Zahn-Wellens-test.	Jäger *et al*. 1996b Jäger and Meyer 1995
TA1535/pSK1002	G bacteria	Hospitals, Switzerland	None	Identification of fluoroquinolone antibiotics as the main source of *umuC* genotoxicity in native hospital waste water.	Hartmann *et al*. 1998
TA1535/pSK1002 TA98, TA100 V79	G bacteria M bacteria CA	Hospitals, Germany	None	UmuC test but not mutagenicity (Ames, V79 CA) correlates with ciprofloxacin concentrations.	Hartmann *et al*. 1999 Gartiser *et al* 1996
TA 98 TA 100	M bacteria	Oil-water separating tanks		Waste water was fractionated into neutral, acidic, and basic fractions. The neutral fractions showed high mutagenicity. By GC-MS HPLC analysis 1-nitropyrene accounted for 0,3-58,5% of the total mutagenicity.	Manabe *et al*. 1984
Allium	MN	Chloralkali plant, Ganjam estuary, India	None	Concentration and genotoxicity of mercury in the industrial effluent and contaminated water was highly correlated not only with bioconcentrated mercury (root mercury) but also with the levels of aquatic mercury.	Panda *et al*. 1992
Ames test with 5 tester strains		Production trinitro-toluene		The mutagenicity of 36 polynitroaromatics indicated that polynitroaromatic compounds in TNT waste waters possess a potential for biological activity.	Spanggord *et al*. 1982
TA1535 TA1538 TA98 TA100 TA100NR	M bacteria	Production of nitrobenzoic acids and nitrotoluenes		The mutagenicity of nitrobenzoic acids and other components detected in the waste water was determined with nitroreductase-proficient and -deficient tester strains, 30-40% of the mutagenicity could be related to the 16 identified nitroaromatic compounds. One single compound, 3,5-dinitrobenzoic acid, was responsible for more than 80% of mutagenicity.	Sundvall *et al*. 1984
Ames test	M bacteria	Loujia Channel Xindun Channel, Tongji.	H-103 resin	Waste water samples from three industrial waste water channels and 12 related factories were tested and analysed by GC/MS/DS techniques. Results showed that mutagenic compounds mainly come from dyestuff factory, and that pharmaceutical factory is a main pollution source of mutagenic compounds for the Xindun Channel.	Tang *et al*. 1991
Ames test	M bacteria	Urban highway runoff		Characterization of heavy metals and polycyclic aromatic hydrocarbons in urban highway runoff samples. Mutagenicity was appreciably associated with PAHs in the particulate fraction of runoff water. The dissolved fraction also showed positive mutagenic response by unknown aromatic compounds.	Shinya *et al*. 2000
Mytilus edulis *Nothobranchius rachowi*	MN SCE	Paper pulp factory		In vivo detection of genotoxicity in waste water in mussels and fish revealed that genotoxins are produced in the chlorine dioxide bleaching process as well as in the pulping process.	Wrisberg and van der Gaag 1992

TA and YG: Ames-tester strains; NM: umu tester strains; CHL: Chinese hamster lung cell line; V79: Chinese hamster cells; M: mutagenicity; MN: micronucleus; SCE: sister chromatid exchange; CA: chromosome aberration; G: genotoxicity

Table 8: Elimination or induction of genotoxicity within waste water treatment

Organism	Endpoint	Waste water sector	Extraction	Results	Author
TA 98		Municipal treatment plant		Mutagenicity of two waste water samples was removed or inactivated during treatment.	Filipic and Toman 1996
TA 98 TA100	M bacteria	Baikal Paper and Pulp Combine		Mutagenicity in effluents from pulp and paper industry after cellulose chlorination was almost completely removed by multistage sewage water purification.	Glazer *et al*. 1990
Tradescantia	MN	Groundwater near hazardous waste landfill		Groundwater collected near a waste landfill was treated in a purification plant (activated charcoal filtration, UV irradiation). UV irradiation of activated charcoal-filtered water resulted in an enhancement of MCN frequencies which decreased with a half-life of approximately 1 day.	Helma *et al*. 1994
				Mutagens in waste waters renovated by advanced waste water treatment.	Saxena and Schwartz 1979
SOS Chromotest	G bacteria	Metal surface treatment, inorganic and organic chemical production		The affinity of extracted genotoxins for suspended particulate matter in effluent was expressed as a genotoxicity sorption partition coefficient. The percent of organic genotoxins adsorbed to effluent suspended particulate matter ranged from 2,3% to 99,8%. High values (>70%) were obtained for metal surface treatment and inorganic and organic chemical production, low values (>30%) for sewage treatment facilities and pulp and paper mills.	White *et al*. 1996b
SOS Chromotest	G bacteria	Industrial effluents		Of 48 effluent samples 37 elicited a significant induction of SOS response. The genotoxic activity of whole effluents subjected to a 5-day aeration treatment was as high as that of native (unaerated) samples, suggesting that soluble genotoxicants are relatively recalcitrant to oxidation.	Legault *et al*. 1996
TA98	M bacteria	Textile industry	None	Elimination of ecotoxic and mutagenic effects in the Zahn-Wellens-test. While the toxicities as measured in the luminescent bacteria and Daphnia test were essentially eliminated, two out of 12 mutagenic samples retained their mutagenicity. The elimination of mutagenic effects was much more effective after treatment with the Zahn-Wellens-test than with the OECD confirmatory test probably due to different adsorption capacities.	Gartiser *et al*. 1997
TA1535/pSK1002	G bacteria	Hospitals	None	The ecotoxic (luminescent bacteria and Daphnia test) and genotoxic effects observed in total hospital waste water were completely eliminated after a treatment in the Zahn-Wellens-test.	Gartiser 2000
TA100	M bacteria	Hospital laboratories		The mutagenicity of three laboratory waste water samples only partly was removed after a treatment in the Zahn-Wellens-test indicating persistency of mutagens as sodium azide.	Gartiser and Brinker 1996
Ames test CHL Silver carp, erythrocytes	M bacteria SCE MN	Chao Lake, China	XAD-2	Chlorinating during water treatment produced mutagenic activity in several tests. Raw water induced positive responses in the Ames test which was eliminated after coagulation and sedimentation; the treated water (after sand filtration and chlorination) was mutagenic.	Liu *et al*. 1999

TA and YG: Ames-tester strains; NM: umu tester strains; CHL: Chinese hamster lung cell line; V79: Chinese hamster cells; M: mutagenicity; MN: micronucleus; SCE: sister chromatid exchange; CA: chromosome aberration; G: genotoxicity

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