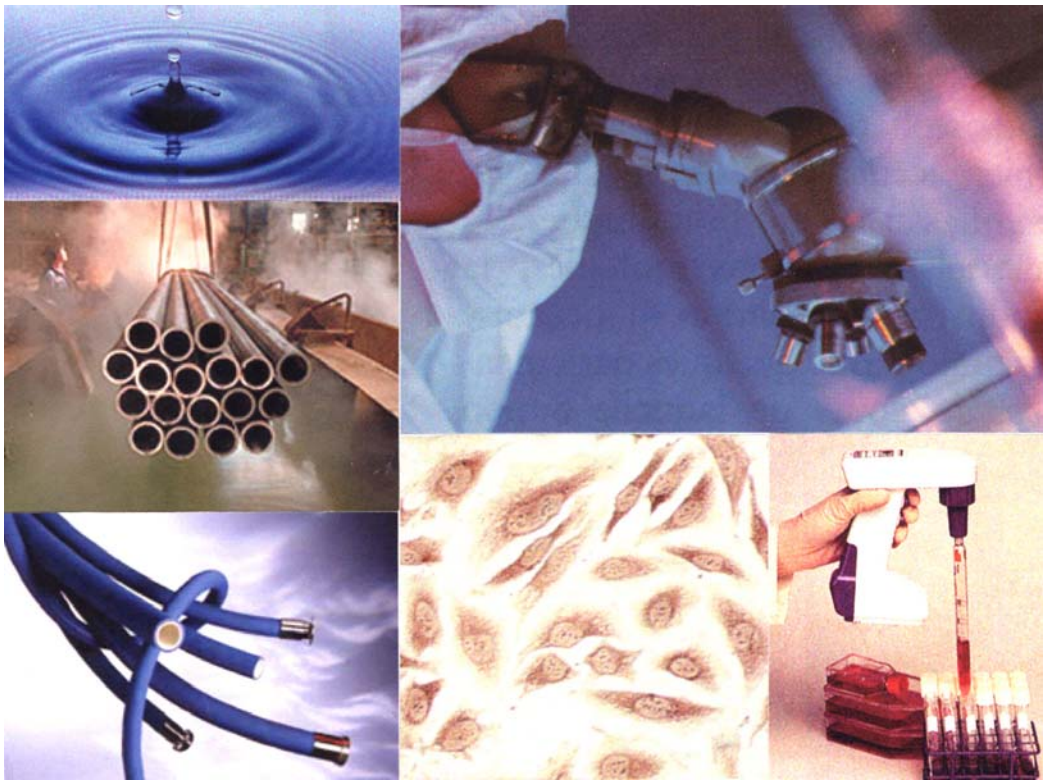
CPDW project

Assessment of cytotoxicological potential of products in contact with drinking water

F.Enkiri[1], D. Colle[1], T. Grummt[2], C. Matthews[3], R. Marcos[4], H. Westphal[5], E.J. Hoekstra[6], M. Philippe[1], P. Neveu[1]



[1] Centre de Recherche et de Contrôle des Eaux de Paris, France

[2] Umweltbundesamt, Germany

[3] Thames Water, United Kingdom

[4] Universitat Autònoma de Barcelona, Spain

[5] Danish Toxicology Centre, Denmark

[6] European Commission, DG JRC

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Assessment of cytotoxicological potential of products in contact with drinking water

Development of a harmonised test to be used in the European Acceptance Scheme concerning CPDW

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Authors
F.Enkiri, D. Colle (CRECEP), T. Grummt (UBA), C. Matthews (TW), R. Marcos (UAB), H. Westphal (DTC), E.J. Hoekstra (EC DG JRC), M. Philippe, P. Neveu (CRECEP)

PREFACE

The investigations described in this report were conducted as part of the European Project "Development of Harmonised tests to be used in the European Approval Scheme (EAS) concerning Construction Products in contact with Drinking Water (CPDW)", under Contract no. EVK1-CT2000-00052. This project is financially supported by the European Commission, the national authorities of Denmark, France, Germany, Portugal and the United Kingdom and the material suppliers in these countries and Europe, respectively. Work Package 2 concerned the cytotoxicity properties of materials of this project. The institutes participating in the investigations and discussions in this work package are listed below.

UBA, Germany	T. Grümmt, A. Jaeger
TW, United Kingdom [1]	J. Ashworth, C. Matthews, D. Holt
CRECEP, France	D. Colle (convenor), F. Enkiri, M. Philippe, P. Neveu
UAB, Portugal	R. Marcos
DTC, Denmark	H. Westphal
EC, DG JRC	E.J. Hoekstra

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[1] The Water Quality Centre, Materials Testing Service, Thames Water were involved in the laboratory development and evaluation of a cytotoxicity method for the project "Development of harmonised tests to be used in the European Approval Scheme (EAS) concerning construction products in contact with drinking water". The data analysis and reporting of the trials has been undertaken by other partners of the project and do not represent those of The Water Quality Centre. Any views or opinions expressed are solely the views of the other authors and do not represent those of Thames Water Utilities Limited or Thames Water Utilities Employees. Thames Water Utilities Limited gives no representation or warranty as to the accuracy, completeness, quality or otherwise of the data or information contained in the report and excludes all liability in this respect (to the extent permitted by law).

ABBREVIATIONS

acid Rnases	acid ribonucleases
AFNOR	Association Française de Normalisation
ATP	adenosine triphosphate
CEN	European Organisation for Standardisation
CPDW	construction products in contact with drinking water
cpm	counts per minutes
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAS	European Acceptance scheme
EPDM	Ethylene propylene diene monomer
EPDM RB	EPDM rubber O seal
EPDM RW	EPDM rubber washer
GCMS	gas chromatography – mass spectrometry
^{3}H uridine	tritiated uridine
IC 50	inhibitory concentration 50 %
mRNA	messenger ribonucleic acid
PE-X	crosslinked polyethylene
NRB	Nitrile Rubber O seal
PE-Xc	crosslinked high density polyethylene
PTFE	polytetrafluoroethylene
PVC-C	chlorinated-polyvinylchoride
PVC-R	polyvinylchoride rigid
RG	regulators group
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RSD	relative standard deviation
SD	standard deviation
SDS	sodium dodecyl sulfate
S/V	surface area to volume contact ratio (cm^{-1})
TAC	"titre alcalimétrique complet" = HCO_3^- [note: titration volume (in ml or °F) of 0.02M H_3O^+ necessary to add to 100 ml of sample in order to change the colour of phenolphtalein into red]
TCA	trichloroacetic acid
TOC	total organic carbon
VC_R	coefficient of variation of reproducibility (%)
WP	work package

ABSTRACT

A standardised test method is needed for assessing the potential toxicity of construction products in contact with drinking water (CPDW) in Europe. Two types of toxicity towards human cells exist: a metabolic toxicity and a genetic toxicity. Metabolic toxicity refers to the ability of a molecule to interact reversibly with cellular functions and to create disturbances, which can eventually lead to the death of the cell. Genotoxic agents have the capacity to interact directly or indirectly with the genetic material of the cell and to generate changes within it. Also, it is of public interest to prevent consumers from being exposed to toxic chemicals and to widely develop toxicity analysis. To achieve this goal, the characteristics, the advantages and the drawbacks of the two existing European Standards have been discussed. The British Standard (BS 6920) measures the degree of inhibition of growth of monkey kidney cells, whereas the French Standards (XP P 41-250-3 and XP P 41-260-3) measure the inhibition of ribonucleic acid (RNA) synthesis in human cells in relation to that obtained with a control. It was decided that the RNA synthesis inhibition assay was the best candidate for use as an European cytotoxicity test. A limited variety of CPDW, viz. EPDM, PE-X, PVC-r, PVC-C, NRB, stainless steel and organic cementitious, was selected to determine the cytotoxicity potential.

Several instruction days at CRECEP and a first inter-calibration comparison exercise were conducted at the beginning of the project. Differences in the test conditions between the three laboratories and difficulties in the implementation of the test in TW lead the WP2 partners to reconsider some issues. The effect of various parameters was studied to try to make the method robust and reliable for any laboratory. The following topics were investigated: the leachates preparation and storage, the cell viability, the cell growth, the "cell-leachate" incubation time, the uridine batches.

The experiments at UBA and at CRECEP clear showed that the leachates should be tested immediately after preparation and can't be stored. The cytotoxic effect may change during storage of the leachate.

An assessment of cell viability (trypan blue dye) prior to RNA synthesis determination was decided as well as a calculation of the percentage of cell growth.

An acceptance limit of 80 % of viable cells after incubation of the cells for the blank was set to allow the experiments to be pursued with the kinetics. A threshold of "greater than 50 % of growth" in the blank was set too.

To improve and insure the quality of the cell growth in the different laboratories, various parameters were checked (degree of cell confluence, incubation time, cell viability, quality of the reference water, etc.).

In order not to modify the results, uridine batches of high quality grade were selected. Taking into account the information deduced from these surveys, a harmonised cytotoxicity procedure was obtained and was used to test the selected CPDW.

Eleven materials were tested with the final draft protocol. Three methods of calculation were applied to the data, complied with the French acceptance criteria of "greater than 70 %". A good intra- and inter- laboratory reproducibility could be pointed out for 8 materials (RSD max = 5.08 % and VC_R max = 11.7 %, first mode of calculation, RSD max = 5.35 % and VC_R max = 19 %, second mode of calculation, RSD max = 10.55 % and VC_R max = 22.6 %, third mode of calculation).

Two types of materials seemed to vary depending on the migration water and, consequently, the assessment of toxicity. This variability might not be due to the cytotoxicity protocol itself but be dependent of two major factors, i.e. the nature of the material and the absence of a washing step prior to leachate preparation. This phenomenon should be studied in more detail.

Therefore, the reproducibility of the test, defined as the coefficient of variation of reproducibility VC_R, ranged from 1.52 % to 141.42 % with a median value of 10.52 % (first mode of calculation), from 0.89 % to 141.42 % with a median value of 11.25 % (second mode of calculation) and from 1.74 % to 138.24 % with a median value of 7.32 % (third mode of calculation).

Different materials were tested successfully by the cytotoxicity test, supporting the fact, that this test system is a useful screening test for exposures, which may be cytotoxic. Moreover, the test seems to satisfy many criteria for routine use in regulatory practices. It is very sensitive, quantitative, reliable, reproducible and requires no concentration procedures of the sample to be tested (UBA and CRECEP findings). However, no robust positive material was found among the selected CPDW and positive materials are necessary to well-establish the test.

In conclusion, the validation of the test system should be achieved to include it as a CEN standard. A number of aspects need further investigations, including:

- a first agreement about the influencing factors of cell cultures; the storage, handling and quality of uridine; the nature of the culture vessel and its cleaning
- the test automation (use of 96-well microplate equipped with GF/C glass filters and direct radioactivity counting in a top count microplate reader)
- an inter-validation of the assay with known chemical and/or biological toxicants
- a second agreement for material investigation : the storage of the materials, the material preconditioning, the preparation and handling of the leachates, the mode of treatment of the results
- an inter-validation of the test by analysing a wide variety of CPDW, which should cover a large range of toxicity levels in order to assess the discriminatory power of the method. The results of the tests should meet specific requirements e.g. repeatability < 25% and reproducibility < 30%.

Further studies to establish pass-fail criteria for CPDW, based on RNA synthesis inhibition in human cells. A 70% acceptance limit was previously set because, below this level, cellular sublethal changes occur. But no instructions are clearly established concerning materials which give percentages of RNA synthesis ranged from 70 to 100 %.

Once the cytotoxicity assay will be presented as a CEN standard, recommendations could be made for the RG-CPDW concerning acceptance criteria for the European Acceptance Scheme (EAS).

The next step would be the implementation of a set of genotoxic assays. As it was shown, at the end of this work, the detection of mutagenic effects (Ames test) provides additional information about the quality of materials.

The cytotoxicity assay associated with a suitable battery of genotoxicity tests should be, in the future, able to assess the global cytotoxicity of compounds leached from CPDW and give a sound answer concerning the health risk incurred by the consumers.

CONTENTS

INTRODUCTION

Necessity of an European approval system for material in contact with drinking water

An increasing number of (synthetic) products are used in the construction of drinking water storage, transportation and water distribution systems. Construction Products in contact with Drinking Water (CPDW) may affect water quality by releasing compounds that could have toxic properties, undesirable taste/odour, or promote microbial growth.

CPDW selection is regulated in various EC countries. CPDW must pass tests included in the national approval schemes in order to protect the health of the consumer. These national approval schemes differ significantly in their concept, their methodologies and approval criteria.

In order to reduce CPDW testing in Europe and to open the European Market for CPDW, a working group was set up to prepare a European Approval System (EAS). This Regulator Group for these products (RG-CPDW) identified the necessity to harmonise four tests, which could become part of the EAS. The aim of this research project was to determine if four current test methodologies were suitable for inclusion in the proposed EAS. And therefore, it was logical to divide the project into four work packages (WP):

- WP 1: enhancement of microbial growth
- WP 2: assessment of cytotoxicity / genotoxicity
- WP 3: GC-MS of non-target compounds
- WP 4: potential to form disinfectant by-products

The scope of the present report is to describe and summarise the work (both bibliographic and experimental) performed by the laboratories involved in WP2.

Cytotoxicity tests for CPDW

Maintaining the quality of drinking water during its storage and its distribution is a main concern in water supply. A broad range of chemical substances may be present in drinking water, essentially as trace amounts.

These chemicals may originate from two different sources: the tap water and/or the CPDW. A few of them can present a risk for public health. Organic chemicals will be regulated in the EAS by a Positive List (PL), which will contain all substances known from the formulation. Limits for drinking water at the consumers' tap will be set in the PL. However, it is unclear up to what detail industry should know the formulation of their products including the toxicity of the substances. More specifically, the role of impurities of raw materials and reaction products should be clarified. This gap can be covered by toxicity tests.

Two types of toxicity towards human cells can be described: metabolic toxicity and genetic toxicity. Metabolic toxicity refers to the ability of a molecule to interact reversibly with cellular functions and to create disturbances, which can eventually lead to the death of the cell. Genotoxic agents have the capacity to interact directly or indirectly with the genetic material of the cell and to generate changes within it. The metabolic toxicity can be assessed by a cytotoxicity test. The aim of this kind of assays is to give an overall judgement on the toxicity of the product, not only synergetic or antagonistic effects of compounds but also the presence of unexpected chemicals such as impurities or reaction products. It is of public

interest that consumers are not exposed to toxic chemicals and that toxicity analysis is widely developing.

Among the available chemical methods, the GCMS is applied to CPDW testing to detect unsuspected substances. This technique aims to identify and to semi-quantify the organic compounds that are present in the migration water, by comparison with existing standards. The GCMS method is obviously necessary in order to control that the levels of detected substances are not higher than the authorised limits. But unsuspected substances are, by nature, unknown and only a small percentage can be detected, identified and quantified. Actually, the technique requires a concentration step of the leachate and only a part of organic substances are extracted depending on the solvent. The efficiency of the extraction is, in addition, related to the nature of the compounds themselves. More important is that the toxicity of different compounds is not comparable at the same concentration. Even if the compounds are identified, their effects on the human health can not be completely predicted because of synergetic or antagonistic effects between individual compounds. Keeping in mind those limitations, GCMS appears not to be enough adequate to make sure a CPDW is not hazardous for consumers.

The low concentration and the large diversity of toxic agents make them difficult to identify and, therefore, several different methods are, indeed, needed to correctly assess the toxic potential of these leached substances. Only global biological tests, including both cytotoxicity and genotoxicity tests, are able to ensure a sufficient consumer's protection.

Currently, cytotoxicity tests are only used in two European countries. In the United Kingdom, CPDW leachates are tested for cytotoxicity using the British Standard (BS 6920) procedure. This method is subjective as it measures the degree of inhibition of growth of monkey kidney cells on the basis of morphological modifications. This test must be performed in triplicate and if growth of more than one of the test leachates causes inhibition of the culture the CPDW is not approved.

In France, the normalised tests (Association Française de Normalisation or AFNOR, XP P 41-250-3 and AFNOR XP P 41-260-3) measure the inhibition of the RNA synthesis in human cells. In these assays, the effects of a compound on the cellular RNA synthesis are followed by kinetic uptake of radiolabelled uridine. The synthesis of RNA is one of the vital cellular functions and is directly proportional to the square of cellular growth rate. Furthermore, it is sensitive to any kind of disturbances. Thus, it indicates early toxic effect at cellular level even before any other detectable cellular damage has occurred: decrease in the rate of RNA synthesis reflects sub-lethal toxic effects. Moreover, due to its high sensitivity, this test does not require preliminary concentration of the toxic chemicals leached from materials in contact with drinking water. The noxious effect of poorly controlled extractions is well known as toxicity varies according to the concentration procedure (Fauris *et al*, 1985 a and 1985 b; Annual report Biosafe Paper 2001-2002; Lefebvre, 1994).

The validation of this method by the French Department of Health was based on the

- close correlation found with the attachment inhibition tests (Fauris *et al*, 1986)
- qualitative and quantitative analysis of a large variety of toxics (potassium dichromate, colchicin, dimethyl sulfoxide (DMSO), cupper sulfate, cadmium chloride…) (PhD of Omaya Hiddeh (1987))
- deep knowledge of the test from studies of numerous water cytotoxicity evaluations (surface water, drinking water, underground water, treated water, mineral water), studies of a large number of materials in contact with drinking water or foodstuffs.

During the initial planning stages of this research project, the experts considered the two existing cytotoxicity methods and agreed that the existing RNA synthesis inhibition test (the French test) was the best candidate for use as a European cytotoxicity standard.

Genotoxicity tests for CPDW

Cytotoxicity tests only measure the potential metabolic effect of products released by the material but do not allow conclusions to be drawn about the potential genotoxic effects of those.

To make sure of the innocuity of a CPDW, genotoxicity tests should be applied along with the cytotoxicity test. For the moment, no genotoxicity test is used in national approval scheme for CPDW in Europe, despite the fact that a wide range of assay systems exists (from simple systems as bacteria to the most complex eukaryote cell as whole mammalian individuals). To select the suitable genotoxicity test or "battery of tests", further investigations are needed and should take into account the following aspects:

- preparation of leachates should represent the actual genotoxicity of the tested sample
- the various mechanisms, in which genotoxic agents interact with DNA, inducing different types of lesions on DNA (changes at the bases' level, at the structure of individual genes' level, on the chromosomes).

Research objectives

The objectives of the project are:

- to make a review of the available cytotoxicity and genotoxicity tests
- to have endpoints relevant to actual consumer safety
- to set up and to cross-validate the RNA synthesis inhibition method among different partners
- to propose an harmonised procedure to test the cytotoxicology of leachates from CPDW which can serve as a basis for a CEN standard in the EAS program
- to gather information to provide international approved pass/fail criteria regarding cytotoxicity

The final procedure must fulfil the following properties:

- fast response
- high sensitivity towards a wide range of toxic
- discriminatory power
- reproducibility
- good adaptability to routine analysis
- low cost

MATERIALS AND METHODS

Project structure

The research was divided into five stages in which the participants were involved in relation to their specific knowledge and competence.

Stage 1: review of applied cytotoxicity and genotoxicity tests

Cytotoxicity
DTC, UAB, CRECEP reviewed and compared the existing cytotoxicity tests. The scope of this part was to summarise the existing tests, practices, guidelines and regulations in order to determine the improvements which could be applied on the RNA synthesis inhibition test. These participants were to review information from their own country and establish a network of information about other countries not directly involved in this research.

Genotoxicity
DTC, UAB, CRECEP carried out the review of existing genotoxicity tests in the same manner as for the cytotoxicity test review. The main aim was to determine if one of the presently available tests could be applied to CPDW.

Stage 2: harmonisation of the cytotoxicity test

part 1: Intercalibration of the cytotoxicity test
Detailed instructions were given by CRECEP to the participating institute (TW and UBA) about the cell culture procedures and about the cytotoxicity method. A training stay was, also, performed in CRECEP.

Then, an initial intercalibration comparison exercise was carried out between the three laboratories testing leachates from three organic materials (two EPDM seal type and a PE-X pipe).

part 2: Study of the impact of various parameters upon the cytotoxicity test
The selection of appropriate conditions is relevant for a correct determination of cytotoxicity. Therefore, the effects of factors introducing variability in the analysis were investigated by the three laboratories (cell viability, cell growth, incubation time, preparation of the migration water, S/V ratio…).

Stage 3: Application of the harmonised method on various types of CPDW

Further inter-laboratory tests were conducted by CRECEP, UBA and TW with a number of selected materials to assess the applicability and the reproducibility of the method wherever it is proceeded.

Materials tested in these investigations were :
- PVC-R pipe, PVC-C pipe, EPDM rubber hose, PE-Xc pipe, nitrile rubber O seal, stainless steel, EPDM rubber O seal, organic cementitious, EPDM rubber washers
- Each test included if possible, a positive control, i.e. potassium dichromate (2 mg/L) and a blank or reference control called reference water (autoclaved purified and pyrodistilled water for TW and CRECEP, autoclaved purified water for UBA).

A negative control was only realised by CRECEP and consisted of a borosilicated glass jar filled with reference water without any material.

Stage 4: Statistical interpretation of the results

Statistical analysis of the results was performed mainly by CRECEP on the basis on the findings that had been obtained.

Stage 5: Drafting the cytotoxicity protocol

A procedure for an harmonised cytotoxicity test that could be considered as a potential CEN standard was developed. Recommendations might be made for the RG-CPDW concerning acceptance criteria for the EAS.

Materials

Nature and S/V ratio

The materials selected by the project group are listed in Table 1. A picture of the different selected materials is given in Figure 1. The materials were sent pre-cut in order to have the same S/V ratios in the 3 laboratories.

Table 1 Materials tested

category	number	material	S/V ratio (cm^2/l)
Organic materials	1	EPDM seal type 1	4.24
	2	EPDM seal type 2	4.24
	3	PE-X pipe (black)	240
	4	PVC-R pipe (grey)	398
	5	PVC-C pipe (light-yellow)	240
	6	EPDM rubber hose	315
	7	PE-Xc pipe (white)	470
	8	Nitrile rubber O seal	200
	10	Nitrile rubber O seal	200
	11	EPDM O seal	319
	13	EPDM rubber washer	1064
Metallic materials	9	Stainless steel	1140
Cementitious materials	12	Organic cementitious	740

Preconditioning step

As no guideline was given to the participants on this issue, it was first decided to realise a pre-conditioning treatment of the materials to test. Before any cytotoxicity analysis, materials 1, 2, 3, 4, 5 and 6 were thus submitted to the following cleaning:

- introduce the needed number of pieces into a borosilicated glass jar filled with 500 ml of reference water
- close the jar and incubate for 24 hours at 23°C, in darkness, without agitation
- remove and discard this water

No preconditioning step was done for the other tested materials later on, except for the organic cementitious product (12), which required a special treatment according to its nature.

The treatment for material 12 was:

- place the material upon a monolayer glass beads in a convenient glass container.
- put the material under a "preconditioning water" (1000 ml).
- close the container with a glass cover and incubate at 23°C during 24 hours, in darkness, without agitation (incubation n° 1)
- remove and discard this water and re-immerse into the preconditioning water (1000 ml).
- incubate at 23°C during 24 hours (incubation n° 2)

The sequence was repeated three times (until incubation n° 5). The preconditioning water is composed of demineralised water supplied with (222 ± 2) mg/l of $CaCl_2$ and (336 ± 2) mg/l of $NaHCO_3$ (the pH was adjusted to 7,4 ± 0,1).

Figure 1 Picture of the various tested materials.

Preparation of the leachates

It is important that the preparation of the migration water fulfils the following criteria:

- the surface exposed to obtain the leachates should represent as much as possible the surface exposed in practice
- the S/V ratio must be clearly defined to enable the comparison of the results
- the exposure in the cytotoxicological testing procedure should resemble those in chemical/microbial testing

All leachates were obtained in the same manner except the one, which came from the organic cementitious product.

Organic and metallic materials

Volumes of 1000 ml of reference water were introduced into borosilicated glass jars. The needed number of test pieces was, then, added. After closing with inert lids, the test containers were incubated for 24 hours at 23°C in darkness, without agitation. The jars and lids differed from laboratory to laboratory. For instance, CRECEP used borosilicated glass jar and lid and TW “the kilner glass jar, without seals”.

cementitious material
The material was placed upon a monolayer glass beads in a convenient glass container and put under mineral water (1000 ml). The container was closed with a glass cover and incubated at 23°C during 24 hours, in darkness, without agitation. The mineral water should fulfil the following recommendations:

- conductivity = 50µS/cm
- pH = 8 ± 0,2
- oxidability to $KMnO_4$ < 0,5 mg/l O_2
- TOC < 0,5 mg/l C
- TAC = 5 ± 0,1 °F or $HCO3^-$ = 61 ± 1.2 mg/L
- silica = 25 -30 mg/l SiO_2

These leachates were immediately investigated in cytotoxicity.

Waters composition/quality

The composition /quality of the different waters used in the test are described below

Purified water

Purified water is produced from tap water by successively conducting the following treatments: softening, reverse osmosis, passing through granular activated carbon, then through mixed bed ion exchange micro-resin cartridges, ultra-filtration (cut-off threshold at 10kd) and UV photo-oxidation. This purified water is used for the cleaning of the glassware and for the production of the reference water.

Reference water

Pyrodistilled water or any water in glass bottle, which respects characteristics as follow:

- resistivity (at 20°C) 6250-6750 ohm.cm
- pH 6.9-7.4
- SiO_2 30-38 mg/l
- Ca 10-11 mg/l
- Mg 6.9-7.5 mg/l
- Na 10-11 mg/l
- K 5.5-6.2 mg/l
- Cl 10-11 mg/l
- NO_3 5.5-6.2 mg/l
- SO_4 6-9 mg/l
- HCO_3 67-70 mg/l

The water shall be free from pyrogens and from any other organic substances, which could interfere with normal cell growth. It corresponds to the blank in the experiments and it was used to prepare the culture media and leachates (with the exception of the experiments where cementitious materials were tested).

UBA used purified water as reference water. The blanks, which were performed whenever an experiment was carried out, always gave similar and good results showing that this water did not affect the results of the test.

Migration water

Reference water that has been in contact with a specimen of the material under specified conditions.

Cell line

HeLa S3 cells (ATCC CCL 2.2) are derived from human cervical carcinoma cells. It is the first aneuploid, epithelial-like cell line to be derived from human tissue and maintained by serial cell culture. The three laboratories used the same cell line provided by CRECEP, thus all the partners performed the cytotoxicity using cells from a common origin and history. When a stock culture of the cell line was needed, it was stored in liquid nitrogen, after being preserved in the culture medium supplemented with DMSO (10 % V/V final) or glycerol (10 % V/V final).

Reagents

Media and reagents for cell culture

The culture media, the foetal serum and the solutions used for the cell culture were sterile. Media should be stored in accordance with the manufacturer's instructions.
All the reagents must be of high quality grade.

Medium for maintenance of the monoloyer cells (1X medium)
The following ingredients were mixed in a sterile bottle containing 800ml of sterile reference water:

- 100 ml of 10X Eagle minimal essential (Ref. Gibco-BRL 21430-020)
- 30 ml of 7,5% sodium bicarbonate solution
- 10 ml of 200mM glutamine solution
- 10 ml of 100X solution of non-essential amino acids (Ref. Gibco-BRL 11140-035)
- up to 1000 ml with reference water
- 50 ml of bovine foetal serum
- pH adjusted to 7.2± 0.1 with a solution of NaOH 1N (or HCl)

The HeLa S3 cells were maintained and cultivated without antibiotics as their presence could interfere with the assessment of CPDW leachate cytotoxicity. So, the absence of an eventual contamination by mycoplasma was periodically controlled.

Concentrated culture medium (5,25X) for the cytotoxicological evaluation:

- 50ml of 10X Eagle minimal essential medium
- 15ml of 7.5% sodium bicarbonate solution
- 5ml of 200mM glutamine solution
- 5ml of 100X non essential amino acids
- 25ml of bovine foetal solution

This last medium was not kept more than 3 days. When it was possible, fresh medium was prepared for each experiment.

Solution for rinsing the monoloyer cells : PBS 1X

Dissociation reagent : 1 : 5000 versene solution

Chemical products

All the reagents must be of high quality grade.

- 96% ethanol
- Whatman paper 3MM for chromatography (46x57 cm).
- Trypan blue 0.4% (P/V)
- scintillation liquid for the counting of the tritium on dry filter.
- a 3% (P/V) solution of sodium dodecyl sulfate (SDS) prepared in reference water
- a 5% (P/V) trichloroacetic acid (TCA) prepared in reference water
- potassium dichromate

Radio-labelled tracer

The measure of the RNA synthesis requires the use of a sterile radioisotope [5,6-^{3}H] uridine (1.29 – 1.85 TBeq/mmol; 37 MBeq/ml). The tritium is a beta-particle emitter and as such is submitted to regulation. Laboratories must get a specific authorisation and licences for its handling and its disposal. Nevertheless, the tritium is included in the group 4 (low radioactivity): it does not present any risk of irradiation or of external contamination, but could lead to a risk in case of internal contamination (after accidental consumption).

Equipment

Equipment for the migration test

- Incubator or room, capable of maintaining the requested test temperature

Equipment for the preparation of the leachates

- Borosilicated glass jars with borosilicated glass lids
- Glass containers
- kilner glass jars without seals

The jars should have wide openings to allow the introduction of the samples.

Equipment for cell culture

- sterile culture flasks 150 cm^2 (products Corning, Nunc, Falcon)
- sterile bottles (1000 ml and 100 ml) made in borosilicated glass with lid in polypropylene, used for the preparation of the culture media
- sterile borosilicated glass tubes (incubation tube) with round-bottom and polypropylene screw cap (16x100 mm), used for the incubation of the cells with the migration water
- sterile PTFE magnetic bars (10x6 mm) (for the incubation tubes)
- sterile centrifuge polypropylene conical-bottomed tubes (50 ml, single use and suitable for the cell culture)
- sterile pipettes (1, 2, 5, 10 and 25ml), (single use)
- a laminar airflow work area, integrated, as far as possible, in a controlled dust room
- an incubator capable of maintaining a temperature of (37±1)°C
- a contrasting phase inverted microscope
- usual laboratory equipment for cell culture

Equipment for the cytotoxicological inspection

- a centrifuge capable of centrifugation at 1000 X g
- a stirring water bath calibrated to (37±0.5)°C
- a multiposition (15-60 positions) magnetic stirrer without a motor
- sterile polypropylene round-bottomed tubes with cap (6ml, for single use), used for realisation of the kinetics
- sterilised syringe filters 0.2 µm (Sartorius, Minisart NML etc…)
- sterile syringes (5 and 20 ml) and needles for mixing the cells
- variable volume micropipettes (10 or 20 µl, 100 µl, 1000 µl)
- sterile tips for micropipettes
- descending chromatography tank
- vortexes
- liquid scintillation counting system for measuring the tritium (β radioactivity)
- scintillation vials
- quartz drying-lamp (epiradiator) (this equipment is not essential)
- pH meter (pH Boy type)
- thermometer
- Bunsen burner
- cell counter (automated particle counter, haemocytometer, etc…)

- radioactive waste containers
- a stop watch
- an autoclave
- refrigerator and freezers (-20°C and –80°C)
- apparatus for purified water production
- apparatus for pyrodistilled water production
- an oven (550°C and 180°C)
- 1.5 ml microtubes

Cleaning of the glassware

General information

- The cleaning of the glassware must be done very properly as it could affect the results.
- When the tubes or the bottles become damaged or scratched, they should be replaced at once. They are dedicated to this test and must not to be used for any other purpose. Moreover, they must never be in contact with any kind of non-ultra-pure water.

Cleaning liquids for glassware

- laboratory detergent : 5% (V/V) RBS 25 or 1% (V/V) Aquet, prepared in the reference water or in purified water
- nitric acid bath : 5% (V/V) solution, prepared by dilution of 65% analytical grade nitric acid in the reference water or in purified water
- rinsing water for the glassware :
- purified water or reference water
- a calcium chloride solution (3,30g of analytical grade $CaCl_2.2H_2O$ in 20 l of reference water or purified water)

Glassware cleaning procedure

first step: rinsing and soaking
Rinse the glassware with reference water then soak it for 12 hours into detergent.
Note : the incubation tubes lids should not come into contact with detergent. The lids are just rinsed with reference water and air dried.

second step (excluding magnetic bars)
Two methods can be chosen for cleaning glassware at this stage: by hand or with a washing machine supplied with purified water.

a) Use of a washing machine
The washing up is taken out of the detergent bath, drained and washed in the machine using the following cycle:
- warm rinsing at 85°C
- cold rinsing
- rinsing with a 20% acetic acid solution
- cold rinsing
- warm rinsing at 80°C.

The washing up is dried at room temperature.

b) hand washing
The washing up is taken out of the detergent bath, drained and rinsed carefully with calcium chloride solution until the detergent is completely removed. It is dipped into a 5% nitric acid bath for at least 2 hours. Then it is rinsed with reference water and dried at room temperature.

third step
- The incubation tubes are baked for 4 hours in a 550°C oven. After cooling, the magnetic bars are added and the caps are fastened and autoclave for 20 min at 120°C under 3 bars pressure.

- The lids are loosely screwed on the bottles for the preparation of the culture media. The whole is sterilised by autoclaving at 120°C under 3 bars pressure, during 20 minutes.
- The borosilicate glass containers and others jars are heated for 2 hours in a 180°C oven.

Special case: magnetic bars

The magnetic bar should only be washed by hand using the following method:

- soak in detergent for 12 hours
- drain and rinse with calcium chloride solution
- rinse with reference water
- air dry.

The cytotoxicity test method

Principle

Human cells (HeLa S3) are incubated, in suspension, in a culture medium reconstituted with the leachate. After (19±2) hours, tritiated uridine is added to the culture and its speed of incorporation into the cellular RNA is measured. After a lag phase of approximately 4 minutes (which represents the time required by the uridine to penetrate into the cells), the RNA synthesis rate is linear. This linear phase lasts for at least 30 minutes and can be used to estimate cytotoxicity. The RNA synthesis rate is determined by calculating the slope of the straight regression line corresponding to the experimental values of the kinetic of incorporation of uridine. This rate is then plotted against that obtained for the blank, arbitrarily fixed to 100%. The results are expressed as a percentage of RNA synthesis in relation to the blank. Thus, the total absence of toxicity of a sample is equal to 100%. This test was originally developed by Fauris *et al* (1985) to monitor raw water quality.

Blank or reference control

The blank or reference control consists of autoclaved purified pyrodistilled or only purified water

Positive control

A positive control was analysed in all experiments in order to check the test system. It allows to show, on the one hand, that the test truly measures the effect of toxic compounds on the cells and, on the other hand, that the test gives similar responses to a known toxic. It was agreed to use potassium dichromate as positive control at the concentration of 2 mg/L (CI50), as it is done routinely by CRECEP (Hiddeh, 1987; Fauris and Vilaginès, 1998).

Description of the cytotoxicity test

Each leachate was submitted to a cytotoxicity investigation (or test) that was carried out simultaneously in triplicate (three suspension cultures were prepared). Moreover, it was strongly recommended to test each leachate twice, aiming to perform two cytotoxicity investigations on the same leachate. Therefore, the RNA synthesis rate was measured twice, each measure being the average of three straight lines. CRECEP and UBA followed this instruction, if possible. The initial protocol described below concerned one cytotoxicity investigation (or test). The procedure has undergone modifications during this work.

Part one: Incubation of the cells with a potential toxic
HeLa S3 cells were grown in a 1X medium in culture flasks at 37°C until confluent monolayers were obtained. The cells were detached from monolayer cultures and counted precisely. $12x10^6$ cells were placed into a 50ml tube and were centrifuged. The resulting supernatant was decanted and the cells re-suspended in 20ml of media containing the sample (dilution of the 5,25X medium with the negative control, the blank, the positive control or the leachate, resulting in a 1X medium), giving final concentration of about 600.000 cells/ml. 5ml of this cellular suspension were transferred into 3 glass tubes with a bar magnet, prior incubation for about 19 hours at 37°C, under agitation. The agitation must be homogeneous from one tube to another and must be adapted so that the cells are maintained in suspension without any alteration of their integrity.

Part two: Measurement of RNA synthesis
After homogenisation of the cellular suspension, a 500µl aliquot of these cells was treated with the 5,6 ^{3}H uridine to allow the RNA synthesis rate to be measured (at 37°C) over a 30 minutes times period. This was done by sub-sampling the 500µl aliquot after the uridine is added. In practice, 40µl sub-samples were immobilised on chromatography paper (pre-treated with SDS that lyses cells) 5, 10, 15, 20, 25 and 30 minutes after the addition of the uridine. The descending chromatography was then realised in TCA in order to separate the various components of the cells: those that are acido-soluble were swept along by the TCA. Nucleic acids (RNA and DNA) and proteins only remained on the area of the deposits. The amount of uridine in the areas where each aliquot had been deposited was measured by scintillation counting.

Duplicate 40µl sub-samples were also immobilised on un-treated chromatography paper. These samples did not undergo chromatography and were counted directly by scintillation counting to determine the total amount of radioactivity that had been introduced in each tube. This allows to compare the amount of radioactivity incorporated by the cells and the initial amount of radioactivity introduced into their contact.

Part three: Treatment of the results
The scintillation counter gave a reading of uridine activity in counts per minute (cpm) units. The cpm values of uridine incorporated into the cells were compared to the total radioactivity introduced in the tube and the results were expressed as a percentage of tritium incorporation.

In addition to the original French method of calculation, two new methods were applied to the data (only for the cytotoxicity assessment of the various materials) in order to compare the different ways of calculation. The participants wanted to ascertain that the original French method of calculation do not have an influence on the results.

a) First mode of calculation: French calculation
Percentage incorporation of uridine in each sample was plotted on a graph against time and a line of best fit was added. The equation of the straight line (y=ax+b) and the correlation coefficient (R^2) were determined. Three replicates were performed for each cytotoxicity investigation, therefore three straight lines of best fit were produced.

The data of the different kinetics are given in an excel document to make the calculations (linear regression equation, average of rna synthesis percentage…) and to draw the corresponding graphs. Information for easy reading and understanding of these excel files are presented in Annex 1.

The kinetics are validated when:
- "a" is greater than 1,75 in the blank tubes. In absence of a toxic, cells must be in perfect health. In this case, the incorporation of the uridine in the cellular RNA is not affected. The study of blanks during 10 years in CRECEP showed that a good incorporation of the radio-element was obtained only if the slope of the straight line of the blank reaches at least 1.75. This criteria was integrated in the French Standard XP P 41-250-3 in 1996.
- "b" is negative in all the experimental tubes. After labelled uridine was added, a few minutes is required for it to enter the cells. b represents this lag period and, accordingly, it must be negative.
- the coefficient correlation (R^2) is ≥ 0.98 (a perfect straight line has a R^2 equal to 1).
- at least two out of the three slopes (a) values are in the same accuracy range

But two additional comments must be taken into account for the treatment of the results:

1) Usually a kinetic is plotted taking the 6 experimental values (points 5, 10, 15, 20, 25 and 30 minutes), but sometimes, it happens that one value is obviously outside the straight line of best fit. This point is, therefore, omitted and the linear regression equation is determined according to the 5 other time points. Under certain circumstances, the last data (30 minutes) is omitted because the limit of linearity of the kinetic is reached.
2) One of the three straight lines can be excluded from the calculation, because of a different accuracy of range and/or of the pointing out of disturbing events in an incubation tube such as white clumps (bad physiological condition of the cells, inadequate agitation etc…) or red colour (changes of the medium pH due to scratched vessels or insufficient cleaning of the glassware).

The slope of the validated straight lines for each sample (RNA synthesis rate) was determined. The average slope value of the leachate, positive control and the blank were, then, calculated. The percentage of RNA synthesis of the blank is arbitrarily fixed at 100 % (absence of toxicants). The percentages of RNA synthesis of the leachate or the positive control were expressed in relation to the blank: (average slope value of the leachate or the positive control / average slope value of the blank) x 100. One further criteria for satisfactory cytotoxicity test performance should be met : Cell concentration should increase by 50% during incubation in the blank tubes (proof of a sufficient growth of the cells during the incubation)

b) Second mode of calculation

As previously, the cpm values of uridine incorporated into the cells were compared to the total radioactivity introduced in the tube and the results were expressed as a percentage of tritium incorporation. For each cytotoxicity investigation, 3 (or 6) replicates were obtained. So, for each time of a kinetic, the average of these 3 (or 6) values and the associated standard deviation were calculated using Graph Pad Prism 4 software. The replicate values can be averaged because they represent replicate measurements from the same organism. This software is currently used for toxicity assessment (IC 50 etc…). Further analysis was conducted by plotting the mean of percentage incorporation of uridine of the sample as a function of time. Thus, only one straight line of best fit was produced. The best fit for this linear regression was chosen by the software under the option "robust to outliers". This robust method basically replaces the assumption of a Gaussian distribution with a Lorentzian one. Therefore the possible effects of outliers are minimised. It shall become part of a new version of Prism along with detailed explanation.

Validation of the regression line was done when "a" was greater than 1.75 in the blank (perfect cell fitness) and "b" was negative in all the samples (correct uridine incorporation into the cells). Data points were only excluded based on experimental grounds (a tube looking funny, abnormal microscopic blank cell aspect, bacterial contamination of the cells, vessel's problem…).

c) Third mode of calculation

As previously described, the cpm values of uridine incorporated into the cells were compared to the total radioactivity introduced in the tube and the results were expressed as a percentage of tritium incorporation. For each cytotoxicity investigation, 3 (or 6) replicates were obtained. So, for each time of a kinetic, the average of these 3 (or 6) values and the associated standard deviation were calculated using Excell software. Further analysis was conducted by plotting the mean of percentage incorporation of uridine of the sample as a function of time. Thus, only one straight line of best fit was produced. The best fit for this linear regression was chosen by the software using a Gaussian distribution (linear regression option), where the

effects of outliers are not minimised. The slope and the corresponding standard deviation were determined taking into account all the data, and not by using the average values.

The validation of the regression line was made as mentioned above [$a > 1.75$; $b < 0$; absence of experimental problems]. Data points were only excluded based on experimental grounds.

The pass/fail criteria for first mode of calculation

The RNA synthesis rate results were used to determine whether each material was suitable or unsuitable for use in water distribution systems.

- materials with ≥ 70% RNA synthesis rate compared to the blank pass the test (French Standard).
- materials with ≤ 70% RNA synthesis rate compared to the blank fail the test (French Standard).

The 70 % limit is set because, below this level, cellular modifications are observed such as increased granulations, lysosomal hypertrophy, release of proteolytic and nucleolytic enzymes (acid RNAses) or decrease of rRNA /m RNA. It signifies that this parameter measures a degree of dysfunction of the cell, which occurs before cell death (Fauris and Vilaginès, 1998; Fauris *et al*, 1988). Comparison between this test and the cellular attachment inhibition method confirms this limit too (Fauris *et al*, 1986).

STAGE 1: REVIEWS OF APPLIED CYTOTOXICITY AND GENOTOXICITY TESTS

Objectives

The main purposes of this stage were:

- to make a review of the cytotoxicity tests that are currently applied in various countries (description, and guidelines, regulations, practices if possible). That bibliographic work might lead us to point out the improvements that could be achieved on the selected cytotoxicity test, the RNA uptake inhibition test.
- to make a review of the presently available genotoxicity tests to determine if one or a set of them could be adapted for assessing CPDW.

During the last decades, numerous toxicity tests have been developed based on

- animal experiments
- studies on a whole organism
  - aquatic organisms as daphnia, algae, mollusc, protozoan, phytoplankton, fish etc…
  - yeast
  - bacteria
- studies on animal or human tissue or cell.

In the environmental field, hazard identification and risk evaluation were related to results derived from animal experiments. This reliance on animal data , however, brings up a number of problems and concerns. Such traditional tests are time consuming, expensive and associated with ethical questions. Moreover, the results must be extrapolated from animal models to humans in order to assess their relevance and this can't be accomplished without introducing a significant degree of uncertainty (Balls *et al*, 1990 ; Koëter, 1995). Thus, new alternative approaches have been proposed (Koëter, 1995 ; Ekwall, 1983). They are summarised in this bibliographic review. We have not attempted to give an exhaustive account of the published literature, but have instead focused on tests, which either have been frequently used in aquatic ecosystems (waste water, surface water…) or could be candidates for the assessment of CPDW toxicity.

Cytotoxicity review

Cellular toxicity mechanism interferes with membrane integrity and metabolic pathways. It inhibits or stimulates enzymatic reactions leading to cellular damage and death (Fauris *et al*, 1997).

Toxicity evaluation by aquatic organisms

The assays described in this chapter are of great interest to detect the degree of pollution of an aquatic ecosystem. Nevertheless, the main drawback is often a lack of reproducibility because of the variability in maintenance conditions and the differences in tested organisms (health status, age, species, genetic uniformity…) (Buikema *et al*, 1982; Fauris *et al*, 1997). Special attention is given to these tests due to their use at large scale in environmental toxicology, even though they are not suitable for the detection of toxics in trace amounts as required for CPDW (low sensitivity).

Phytoplankton
Due to its crucial role in aquatic systems, several surveys have been concerned with the use of phytoplankton as model organisms for assessment of the toxicity of chemicals. The tests determine the mortality increase of the exposed populations. However, the sensibility of phytoplankton varies widely, depending upon both the species and environmental conditions. Actually, phytoplankton show a high degree of diversity and the composition and structures of the cell wall (which mainly govern the entry of xenobiotics into the cell) differ among the different species. Moreover, the results can't be easily extrapolated from these aquatic organisms to humans. The predictive value of these toxicity tests in a sanitary point of view is therefore limited (Isomaa *et al*, 1994).

Algae
Bioassays with single-celled algae have been exploited extensively over the past 20 years for the determination of toxicity of complex effluents, polluted freshwater aquatic systems and specific chemicals. The standard test measures the inhibition of algal growth rate over 72 hr exposure to a toxic (Kooijman *et al*, 1996; El Jay, 1996).

These growth inhibition tests are time-consuming, rather costly and present a low reproducibility. They can't detect sub-acute endpoints and therefore show a lack of sensitivity. Alternative toxicity test based on enzymatic activity in algae has been developed but their use is also limited to effluents and freshwater aquatic systems (Peterson and Stauber, 1996).

Protozoan
Toxicity tests utilising Protozoans differ strongly in their methodologies : The techniques either measure the growth inhibition, the decrease of respiration, the inhibition of chemoattraction or the loss of coordinated movements (Isomaa *et al*, 1994).
These methods are quite adequate to monitor the quality of polluted waters but are not sufficiently sensitive to detect toxic agents at concentrations within the limits specified for drinking water and accordingly for CPDW (Slabbert and Morgan, 1982).

Molluscs
Many different species of molluscs have frequently been used in water quality screening tests and many different methods have been applied in toxicity testing (enzymatic inhibition, activity behaviour, lethality…).The same drawbacks, as those described above, can be acknowledged (Isomaa *et al*, 1994).

Fishes
Fishes as rainbow trout can act as indicators of sub-lethal toxicity. Four measures of their behaviour can be easily tested (spontaneous swimming activity, swimming capacity, feeding and vulnerability towards *Micropterus salmoides*). These behavioural responses can yield a more comprehensive assessment than would be provided by mortality alone. Thus, this method can predict the toxicity of single chemicals (present in adequate amounts) or complex effluents very well (Little *et al*, 1990; Lee *et al*, 1995).

Daphnia
Daphnia magna has been extensively used as indicator of toxicity for several decades and tests to assess both acute and chronic toxicity have been standardised (European Standard NF EN ISO 6341, 1996 - OECD guideline 202). The assays are usually performed with less than 24 hour-old *Daphnia* neonates.

In the acute toxicity tests, immobility after 24h or 48h of contact with potential toxics is used as an endpoint equivalent to death, as death may be difficult to ascertain. An effective

inhibitory concentration, called CE50, is calculated. It corresponds to the initial concentration of products which causes a 50 % of immobility of the tested *Daphnia* neonates (Isomaa *et al*, 1984 ; Lilius *et al*, 1995).

Under good culture, Daphnia females reproduce parthenogenetically, and a parthenogenetic clone derived from a single female is genetically uniforme. Cultures with genetically homogenous animals are, therefore, obtained. Nevertheless, the sensitivity of *Daphnia* towards toxic agents can vary according to their age and their origin (Isomaa *et al*, 1994).
In the chronic tests, survival time and number of young produced per female are used as endpoint. The female neonates are exposed to a range of concentrations of the potential toxics during a period of 21 days. Survival and number of offspring are observed on a daily basis when food is supplied. Variability in the results can be noticed because of lack of reproducibility in *Daphnia* reproduction. Reproduction can, indeed, be affected indirectly via effects on feeding, growth or maintenance (Kooijman and Bedaux, 1996).

Toxicity evaluation by yeast

Another approach for assessing toxicity is to monitor sensitive, non-specific, subcellular target sites such as mitochondria. Changes in mitochondrial function (respiratory activity) can indicate a toxic effect. It was found that the respiratory function of the yeasts is a physiological function very sensitive to the action of the toxic substances. As this function is dependent on the concentration of oxygen in solution, it can be assessed by means of an amperometric oxygen sensor (Haubenstricker et al, 1990; Campanella et al, 1995). Methods based on the changes of the respiratory activity of Saccharomyces cerevisiae, immobilised on an agar gel containing the culture medium have been developed.
Several toxic substances (heavy metals, organic pollutants) can be detected by this test. However, biosensor sensitivity to agents clearly varies related to the chemical nature of the pollutant. This method can be adapted to industrial waste water control (Campella et al, 1995).

Toxicity evaluation by bacteria

Bacteria, as a prokaryotic organism, differs strongly from eucaryotic cells (lack of nucleus, presence of a cell wall and/or a capsule, absence of numerous intra-cellular structures…). Furthermore, they possess a high adaptive ability. For these reasons, bacteria may be less sensitive to toxicants than animal or human cells, and their reliance as models in toxicity assessments may be limited.

Though, much attention has been paid to the effects of pollutants on bacteria, because they are cheap to culture, easy to handle, have a rapid growth rate and give reproducible results under the same experimental conditions. Measurements can be taken quickly, and automation of the procedures is often possible (Isomaa *et al*, 1994; Fauris *et al*, 1997). Several tests have been proposed and divided into five categories, according to the kind of parameter used to assess the toxic effect. The sensitivity of the different assays differ greatly accordingly with the parameter used (Torslov, 1992).

Bacterial growth assays
Liu *et al* (1989) developed a simple and rapid method, known as "the growth zone inhibition test", for determining the toxicity of a chosen chemical or a mixture of substances, using. *Bacillus cereus* as the test organism. The potential toxicity can be easily demonstrated at a low concentration and a linear relationship between the concentration of toxicant and the diameter of the clear inhibition zone on the agar plate can be established. Variations in the

results may be due to differences in experimental conditions and to differences in the assessment of the diameter of the halos (Ghosh *et al*, 1996).

Other methods detect the inhibition of growth by measuring the increase in cell density with a spectrophotometer (optical density values) after a 16h incubation time (Torslov, 1992).

Cell energy assays
ATP is an energy carrier in all living bacterial cells, linking catabolism and biosynthesis. The turnover time for the intracellular ATP pool in bacteria is very short (< 1s) enabling a rapid response of the micro-organism to changes. Its rate decreases rapidly when the cell dies or is subjected to an harmful compound which creates injury of membrane functions and /or respiratory system. Bacterial ATP content is determined by the ATP-dependent firefly-luciferase reaction which is known to be the most rapid, sensitive and reproducible assay for quantitation of this nucleotide (Steinberg *et al*, 1995).

Motility assays
Spirillum volutans (ATCC 19 554) exhibits a characteristic reversing motility pattern due to the presence of a fascilce of polar flagella at each end of the cell. In the presence of pollutants, the bacteria loses its coordination and motility. Three endpoints (loss of reversing motility, loss of forward motility and total loss of both flagella movement) are used to determine effective concentration of a toxic agent required to remove typical motility in 90 % or above of bacterial cells (Gosh *et al*, 1996).

Enzyme activity assays
Inhibition or activation of enzymes which are linked to metabolic pathways can assess damages occurred in the cell. Esterase activity is involved in intra- and extracellular degradation of organic substances and act as indicator of general heterotrophic activity. This method is not sensitive to all chemicals reflecting their specific modes of action. Thus, it can be applied for detecting specific pollutants but not for giving a global health risk assessment (Torlov, 1993).

Bioluminescence assays
Bacterial luminescence has been extensively used as a tool in toxicity assessment of aquatic ecosystems. A bioassay is commercially available under the trade name Microtox which measures the inhibition of bioluminescence of *Vibrio fisheri* (formerly identified as *Photobacterium phosphoreum*). The experimental procedure has been adapted for the official standards of several countries (NF EN 11348-1,2 or 3…).

This bioassay is a direct test measuring bioluminescence reduction or stimulation in living cell suspension of *Vibrio fisheri*. The process of bioluminescence involves luciferase and several other related enzymes (e.g. electron transport). These enzyme systems are modified by one, or both, of two possible mechanisms : a direct action on bioluminescence activity or an indirect action by general cytoplasmic poisons which interfere with essential and related metabolic processes. The changes in the light output of the luminescent bacteria are detected photometrically and are proportional to the degree of toxicity. The method is simple and rapid (the incubation time is usually between 5 and 15 minutes (Thomulka *et al*, 1993; Ribo, 1997). It has been validated by comparison with other bioassays and by inter-laboratory surveys, testing a large variety of products : it appears to be sensitive, reproducible and suitable for the assessment of the global toxicity of polluted waters (Kahru and Borchardt, 1994; Ribo, 1997).

However, the very short incubation time may be a drawback. This time could be too short for some compounds to equilibrate between the external medium and the cytoplasm and, also, for

certain cellular damage to manifest itself (Isomaa *et al*, 1994). CRECEP compared the microtoxicity of pesticides assessed by the Microtox method and the uridine uptake inhibition assay. Measurements performed with the Microtox kit appeared to be less sensitive and less reproducible. The lack of reproducibility could be explained by the difficulties encountered to detect the toxicity of low water soluble compounds ((Somarundaram *et al*, 1990; Lefebvre, 1994).

This procedure has been adapted to create a biocaptor that uses the immobilised luminescent *Vibrio fischeri* bacteria as a continuous biological reagent in order to follow *in situ* the quality of complex effluents (Osbild et al, 1998). Another possible application has been the construction of recombinant *Escherichia coli* cells, containing stress specific promoters or constitutive promoters fused to luciferase genes originating from *Vibrio fischeri*. These genetically engineered cells are immobilised in 96 well plates. Bioluminescence outputs decreased or increased dose-dependently upon adding test chemicals. This new biosensor have not yet been compared enough with other toxicity tests, but it could be further harmonised and could become an adequate tool for the evaluation of toxicity (Kim *et al*, 2003).

Toxicity evaluation by animal or human cell

A number of cell types are capable of survival and division in vitro. Monolayer cell cultures exhibit a morphology that is related to tissue architecture and allow cell migration, adhesion and contact regulation to be studied. Suspension cultures, on the other hand, are mostly used when large cell population are required, e.g when low enzymatic activities is to be checked, metabolic pathways studied.

Cell cultures are usually classified as primary cultures, cell lines or cell strains. Primary cell cultures are obtained by culturing, for more than 24h, dispersed cells from tissues or organs taken directly from organisms. Cell lines are subcultures derived from a primary culture ; they may be diploid, established or clonal. For cell line to be diploid means that no less than 75 % of all its cells must be of the same standard karyotype as the origin species. Established cell lines derive from primary cultures or diploid cell lines by transformation processes which are either spontaneous or induced by viruses, chemical or physical agents.

Clonal cell lines derive from the mitosis of a single cell. They can be obtained from primary culture, diploid or established lines by several techniques. A cell strain is obtained from a primary culture, diploid or established cell line by selecting a small number of cells that have a common biological characteristic. This characteristic must persist during subsequent culturing but the ability of specialized cells to perform specific functions in culture is sometimes difficult to maintain (Paganuzzi Stammati et al, 1981).

Because they are far easier to handle and give more reproducible results, cell lines are rather preferred to primary cultures. Consequently, only toxicity tests performed with cell lines are described in this chapter.

Some factors are known to be important to the quality of cell culture response to toxic agents:

- the way the cells are exposed to compounds as well as the physical and chemical environment in which the experiment is carried out:
  - The sample to be tested may be present in a solid form : a preparation of migration water or an extraction are therefore required. These procedure should be clearly defined, because toxicity varies according to the concentration or extraction techniques, for they may distort the actual sample chemical composition

  - Testing volatile chemicals leads, on the one hand, to a significant loss of the chemical during the test, the results becoming thus altered, and, on the other hand, to a possible cross contamination if other tests are realised in the same incubator.
  - The choice of the best exposure time period should be also taken into consideration. As there is no standard by which these criteria can be decided and as it is related to the growth rate (an adequate number of cells in perfect health is necessary to obtain reliable and reproducible results), a preliminary study of this last parameter should be undertaken before any analysis.
  - Experiments should be performed in conditions where environmental factors (temperature, sterility, humidity, gas composition…) are closely controlled.
- the cell culture medium may modify the behaviour of the toxicant. The most important component in the medium is the serum, which, through binding the test substance, may cause a masking of toxicity (Syversen, 1994; Frasier and Bradlaw, 1989). Metabolism modifications can be acknowledged with diverse organic buffers such as Hepes (Claude Danglot, personal communication).
- The use of plastic dishes and containers can interfere with the sample (adsorption onto the plastic surface), with the culture medium (adsorption of proteins onto the plastic surface) or can release chemicals (Syversen, 1994).
- The cell culture conditions (cells in suspension, monolayers) can also affect the results. When cells are in suspension, their surface in contact with the toxic compounds is more important than the one available in monolayer cells (Claude Danglot, personal communication).
- The selection of the adequate biological system should reflect the purpose of the research as numerous cell lines are available : HeLa cells, HepG2 cells, human lymphocytes, CHO cells etc…. The cell line must be subjected to a constant scientific evaluation (cell characterisation biochemically and morphogenically, absence of contamination, origin, number of passages etc…). Confounding factors can affect cell growth and therefore should be closely controlled : pH of the culture medium after addition of the sample to be tested, osmolarity of the culture medium, cleanliness of the glassware… Thus, it is essential that these tests are carried out and supervised by scientific staff experienced with culture techniques and cell line morphology (Syversen, 1994; Frasier and Bradlaw, 1989).

It is often advantageous to select human cell lines that are closer to harmful human response. One should, also, keep in mind that the results must be extrapolated from cells to an entire complex organism in order to assess their reliance.

The aim of these cytotoxicity tests is to show the degree of cellular damage caused by exposure to a sample which contains potential toxicants. A control cell population is tested in parallel and the objective is to determine the difference between the control and treated population. Many endpoints are possible and they do not necessary provide comparable information. The range includes loss of membrane integrity, release of cytoplasmic enzymes, loss or decrease in metabolic processes, cessation / reduction of DNA or RNA synthesis, inability to continue cell replication etc. (Harbell *et al*, 1997).

Counting methods

a) Direct cell-counting assays

Methods of direct cell counting have been extensively used since their set up in 1959 (Harris, 1959). Cell number determination can be achieved using a contrasting phase inverted microscope and a haemocytometer or using an automated particle counter.

Dose-response curves to pollutants can be plotted, the values of IC50 are deduced showing potential toxic effect of the substances. Most reproducible results are obtained when counting is performed with a multisizer particle counter (Jantova *et al*, 1996).

b) The cellular attachment inhibition method

Cells in perfect physiological condition possess the ability of anchorage on any surface. It has been demonstrated that cells incubated with a toxic present a delay in their adhesion property by changes in the cytoskeletal organisation (Sit, 1996). The importance of the delay is closely related to the concentration of the toxics.

Typically, cells are grown in suspension in a culture medium reconstituted with the sample to analyse. After growth, they are placed in a flask filled with culture medium at a controlled temperature and the cells still in suspension are counted at regular periods of time. The percentage of non anchored-cells is compared to the one obtained with the blank and gives an estimation of cellular damages.

This method has been applied for the measurement of the amounts of toxic compounds leached from materials used in water distribution networks. Measurements has been performed on a great number of different materials and allowed to classify the materials from a sanitary point of view. Though, this technique is labour intensive, is not easily adaptable for routine analysis and presents a wide inter-laboratory variability which affect the results (Fauris *et al*, 1986).

c) Colony forming ability (CFA) assays

This technique determines the proportion of single cells in a cellular population able to give rise to growing colonies. Actually it measures cell recovery or cellular sublethal damages / cell death after exposure to a toxicant. The cells are generally treated with the sample to be tested and then subcultured at low density in absence of the toxicants. Only the really surviving cells are able to divide and form colonies (Wite *et al*, 1996 ; Biosafe Paper report 2002). A number of considerations limit the usefulness of the CFA test:

- single cell suspensions are not always obtained after trypsinization and clumps may form with the consequence of survival being either under or overestimated
- the plating efficiency of each cell types tested should be determined because it varies widely among cell lines ranging from 1 to 90 %
- the results of an experiment may require waiting from 1 to 3 weeks depending on the cells (different growth rates)
- this test can be expensive, time consuming and labour intensive, particularly when many samples are being processed.

However, when used properly, the CFA assay is highly sensitive (Cook and Mitchell, 1989).

Biochemical methods

These tests mainly include the determination of the major intracellular components (DNA and proteins) which reflects the cells number and the measurement of DNA or proteins synthesis.

a) DNA and total protein content assays

When a toxic substance interferes with growth or causes the death of all or a part of the exposed cell population, a decrease occurs in the cell number and therefore a decrease in global DNA and protein amounts. The total protein or DNA contents are compared to those of control cultures (Clemedson *et al*, 1996).

Cells are treated with the test agents for 24, 48 or 72h. Then the total protein content is determined, after lysis of the cells, according to Lowry or Bradford procedures, using bovine serum albumin as a standard. The method can be adapted into an automated microplate assay in order to be able to test numerous samples at one and the same time. Dose-response curves

tend to be very different for various types of cells and for different classes of toxicants. (Clemedson *et al*, 1996; Jantova *et al*, 1996 Harbell *et al*, 1997). Moreover, this method is not sensitive enough to detect trace amounts of toxicants (Fauris and Vilaginès, 1992).

An automated micro-method has been set up for quantitation of DNA using the Hoechst 33342 dye that possesses the ability to bind DNA.

b) DNA and protein synthesis assays

These tests are based upon the measurement of the inhibition of DNA or protein synthesis in cells in presence of toxicants. DNA synthesis and protein synthesis are known to be directly proportional to growth rate (Witte et al, 1996). The synthesis can be determined either by an end-point method or by a kinetic assay. The first method is not described in this review because of its obvious lack of reproducibility.

For DNA synthesis investigations, cells are incubated in a culture medium prepared with the toxics or blank solution. The reaction is then started by addition of [^{3}H]thymidine to the cells. The DNA synthesis is measured by kinetic uptake of the radiolabelled thymidine. The results are expressed as a percentage of DNA synthesis in relation to the blank. As DNA synthesis just takes place in the S-phase of the cell cycle, the sensitivity of the assay strongly decreases when the cells divide randomly. A synchronous cultivation system should be introduced in the procedure.

Protein synthesis is determined as described for DNA by replacing [^{3}H]thymidine by [^{3}H]leucine. This method requires, for cell growth, the preparation of a specific culture medium without any leucine. It has been shown that this method possesses a low reproducibility (Fauris *et al*, 1985).

Protein precipitation can also measure a chemical-induced cytotoxicity. The endpoint of this new method corresponds to the minimum effect concentration (MEC) that induces protein precipitation in 5 hours exposure with respect to the negative control. Its low sensitivity is possibly due to its shorter exposure period and because precipitation is the ultimate event in the sequence of a protein disturbance. However, this assay is simple, inexpensive and rapid but further investigations are required to obtain a complete characterization of the test (Novillo *et al*, 2001).

Viability assays

Viability assays are based on the integrity of the cell membrane. Different kinds of dyes can be used to assess the membrane damages of cells exposed to toxics. According to its characteristics, the dye may enter and be retained into viable or dead cells. The cell population viability is directly related to the dye concentration.

The counting of the two populations (dead and alive) can be assessed by microscopy examination, by flow cytometric technique, or more often, by microplate spectrophotometric or spectrofluorimetric reader (Cooke and Mitchell, 1989).

a) Exclusion or lethal dyes

Membrane damages can allow large bulky, charged molecules which are normally excluded by cells to access to the cytoplasm. If the molecules have special light-absorbing or fluorescent properties, then damaged cells can be readily identified by conventional microscopy or by spectrofluorimetric techniques (Fauris *et al*, 1997).

The classical example is the trypan blue exclusion assay. Under bright field microscopy, dead or damaged cells are stained light purple violet whereas undamaged cells appear translucide. Other variations include fluorescent dyes, for instance, propidium iodide that in the case of membrane damage can bind to nucleic acids and becomes highly fluorescent, the cell counting is therefore carried out using flow cytometric reader (Marsteinstredet *et al*, 1997).
Several serious drawbacks of die exclusion essays have been figured out:

- the subjectivity of microscopic methods : the operator counts dead, damaged and viable cells according to his own appreciation under microscopic observation
- the lack of reproducibility of the tests : the procedure needs to be closely standardized to prevent the interaction of factors, such as dye concentration, pH, dye contact period, presence of serum, on the results (Tolnai, 1975).
- the low sensitivity of the technique : a pre-concentration of the sample for CPDW cytotoxicity assessment is required and can modify its toxic properties (Fauris *et al*, 1997)
- the possible underestimation of cellular damage : the detection of toxicants does not occur until membrane integrity is lost within the population. This implies that cytotoxic events that produce damage at other sites of the cells may go unnoticed (Cook and Mitchel, 1989).

b) Inclusion or vital dyes

These dyes only enter living cells. Thus, after treatment with a toxicant, viable cells appear stained whereas damaged or dead cells are still translucide.

The neutral red assay (NR assay) has been extensively used and is based on incorporation of the dye into lysosomes of viable cells by passive transport across the plasma membrane due to the differential pH between the inside of the lysosome and the surrounding cytoplasm.
The amount of neutral red taken up by the cell population is directly proportional to the number of viable cells in the culture. A test using 96 wells microplates has been developed and allow an automated spectrophotometric reading (Harbell *et al*, 1997; Zuang, 2001).

The rhodamine 123 gives information concerning the total mitochondrial activity and therefore can be used to evaluate the cytotoxicity of samples. The method is sensitive but labour intensive and not easily adaptable to routine analysis (Biosafe Paper, 2002).

Enzymatic activities assays

Techniques that measure isolated enzymatic activities often present a good sensitivity towards specific toxic agents but, a lack of sensitivity towards other toxicants. Consequently they are too selective and not suitable for global cytotoxicity assessment (Fauris and Vilaginès, 1992). Two pesticides (lindane and an organochloride pesticide), for instance, are known to induce free radical toxicity which can be specifically detected by following the activity of two enzymes involved in xenobiotics detoxication process : superoxide dismutase and glutathion transferase (Descampiaux *et al*, 1999).

a) The MTT (3 (-4,5-dimethylthiazol-2-yl) 2-5 diphenyl tetrazolium bromide) activity assay

This assay is the most widely used for cytotoxicity assessments regarding cosmetic industry or materials safety (polymers, cements, ceramic…) (Chiba *et al*, 1989; Taniguchi *et al*, 1994; Schweikl and Schmalz, 1996; de Souza Costa *et al*, 2003). It has been developed by Mosmann in 1983 and improved by Denisot and Lang in 1986 (Sauvant *et al*, 1997).

It measures the cell ability to produce ATP by evaluation of mitochondrial succinate dehydrogenase activity with a tetrazolium dye.. In this NADH-dependent reaction catalysed by succinate dehydrogenase, MTT dye serves as hydrogen acceptor and is reduced to formazan. In its oxydized form, MTT is yellow and somewhat water-soluble, on reduction the

colour turns blue-black and the dye precipitates. This reduced form can be measured by spectrophotometry. Under defined conditions, the amount of reduced MTT per unit of time is proportional to the cell number.

Typically, two sets of cells, treated and untreated, are allowed to grow for several days. Then, the dye is added and the quantity of formazan produced is determined at 565 nm. The treated cells exhibit a lower production of formazan (Cook and Mitchell, 1989 ; Harbell *et al*, 1997). This test is rapid, reliable, inexpensive, with an automated processing that allows the treatment of a large number of samples in a short time (Schweikl and Schmalz, 1996). Though, it should be noted that diverse toxicants can interfere with the mitochondrial activity by increasing the reduction of MTT (as surfactants at doses which increase membrane permeability without lysis the cells). Moreover, an extraction step is required to solubilize the formazan in an organic solvent (DMSO, isopropanol). This step introduces a factor of variability in the results (Ishiyama *et al*, 1996).

Lastly, although cells may be mortally wounded by a drug, they may still retain membrane integrity for a relatively long time. Under these circumstances, this method misses critical cytotoxic effects, so feared in cytotoxicity screening (Slater, 2001).

b) Lactate dehydrogenase (LDH) activity assay

This test is based on the release of a cytoplasmic enzyme , the LDH, after the loss of cell membrane integrity in presence of a toxic agent. The release of LDH into the culture medium is detected quantitatively by a chromogenic substrate. This method has been improved by using 96-well microplates for growth and measurement of the enzyme activity; However, as the membrane leakage is required, this assay often only detects the last stages just before cell death (Harbell *et al*, 1997).

c) EROD (7-ethoxyresorufin-O-deethylase) activity assay

The main pathways of detoxication are processed through liver cells. This process transforms hydrophobic compounds to more hydrophilic forms which are readily excreted from the body (elimination in the urine and/or the bile). Two steps (functionalisation and conjugaison) are required for this metabolism, but occasionally may lead to increased toxicity.

Across this process, diverse enzyme systems are activated, for example, cytochrome P450 monooxygenase (CYPs) family. Consequently, the increase in CYPs activity can be regarded as an indicator of subacute toxic effects. (Dubois *et al*, 1996; Biosafe Paper 2002). The activity of CYP 4501 A1 isoenzyme can be easily measured using EROD as a diagnostic catalytic marker. Immortalized human or murine liver cell lines (HepG2, Hepa-1) which have retained some essential components of the hepatic xenobiotic metabolising enzyme system are selected for these surveys (Radice *et al*, 1997).

Typically, liver cells are cultured in multi well plates and exposed to samples for 24 to 72h. Subsequently, ethoxyresorufin is added and the EROD reaction is started by adding NADPH. Fluorescence of resofurin is measured using excitation/emission wavelength of 530/590 nm, and total proteins by using a wavelength of 405/460 nm respectively. Resorufin and bovine serum albumin standard curves are realised in parallel.

If inducers of CYP 4501 A1 are present in the tested sample, the enzymatic activity indicated by EROD is higher in exposed culture compared to the control culture. This technique appears to be rapid and reliable (Dubois *et al*, 1996; Biosafe Paper 2002).

d) Bioluminescent assays

The previously described assay (bioluminescence assay - micotox) use a prokaryotic system where as these assays are performed in an eukaryotic system. ATP plays a central role in energy exchanges in eukariotic systems. It serves as the principal donor of free energy and is present in all metabolically active cells. Cell injury and death result in the rapid decrease of ATP. An high-throughput screening has been developed using a 384-well microplate and the luciferase-luciferine bioluminescence reaction to quantify ATP cell content. It appears to be a simple, sensitive and reliable method (Slater, 2001). But, the ATP extraction procedures may modify the results, as yields are not controlled, and lead to a lack of reproducibility of the test.

Boar spermatozooan motility test

Sperm cells are known to be very sensitive to toxic agents. This test measures damage to the mitochondria and signalling systems and plasma membrane integrity. Boar semen is exposed to the chemical substances for 1, 2, 3 and 4 days. Sperm motility is measured and compered to that of control sperm cells. An EC50 can be calculated and is defined as the concentration where motility is decreased by 50 % as compared to the control. The results must be extrapolated from animals to humans to assess their reliance and this can't be easily accomplished (Biosafe Paper, 2002).

Morphological modifications assays

A number of large-scale morphological changes that occur in the cytoplasm or at the cell surface can be followed and related to cell viability (cell size, irregular shaping of the cells, signs of rounding-off of the cells, granular inclusions, changes in the cytoskeleton …) (Cook and Mitchell, 1989).

The British Standard (BS 6920) for the assessment of CPDW toxicity is based on this parameter. Material is immersed in the test water for 24h. The extract is collected and together with a control (test water) it is utilised in the preparation of culture media used for the growth of African green monkey kidney cells (VERO ATCC CCL81). After incubation for 24h, the cells are observed microscopically.

If no initial signs of toxicity are observed, the material may be approved for use in contact with potable water. If granular inclusions, irregular shaping of the cells or other signs of toxicity are detected, the cells are re-examined at daily intervals for three to four days. In parallel the confluence of the cell line is investigated.

If confluent growth of the cell line does not occur in the presence of the extract of the test material and if signs of morphological changes are still observed, this is interpreted as indicating cytotoxicity. One problem that limits morphological tests is the transient nature of morphological changes. Moreover, the method is subjective as it takes into account the degree of growth inhibition (microscopic evaluation) which can vary according to the operator.

Cell morphology can also be assessed by scanning electron microscopy (de Souza Costa *et al*, 2003).

The RNA synthesis inhibition assays

The test has been extensively described in the previous chapters of this report. Briefly, it is based on the RNA synthesis inhibition of human cells in the presence of toxic compounds with regard to blank control. The RNA synthesis is one of the major cellular functions and can be correlated with the cellular growth rate (RNA synthesis is proportional to the square of cellular growth rate). It indicates early toxic effects at cellular level (sublethal toxic effect) even before any other detectable damages have occurred (Fauris *et al*, 1986; Descampiaux *et*

al, 1994). The RNA synthesis inhibition assay was set up in 1981 by Fauris *et al* and has been closely related to the cellular attachment inhibition assay (Fauris et al, 1986) or to the Draize test used in pharmaceutical and cosmetic industry (Descampiaux et al, 1994).

As cellular RNA synthesis is sensitive to a wide range of pollutants, this method is, therefore, very useful for global health risk evaluation. Actually, uridine is involved in several cellular pathways (Figure 2), and the inhibition of its incorporation in RNA can point out numerous dysfunctions in the cell (membrane modifications, changes in cell regulations, metabolism disturbance etc…).

A great number of measures were carried out for 25 years to assess safety or toxicity of waters (drinking and polluted water, sea waters, mineral waters and renal dialysis waters), of materials in contact with potable water (normalised tests AFNOR, XP P 41-250-3 and AFNOR XP P 41-260-3), of products used in pharmaceutical and cosmetic industry and of paper and board for food contact (Fauris , 1982; Fauris *et al*, 1986; Fauris *et al*, 1988; Descampiaux et al, 1994; Biosafe Paper 2002). Consequently, this test shows a wide application field.

Moreover, it requires a lower number of cells than the other generally applied methods. It presents a better sensitivity, a higher reproducibility, a faster response and a better adaptability to routine analysis than most other assays (morphological modifications, cell growth, cloning efficiency, protein, DNA and RNA contents, cellular ATP concentration, the Microtox method) (Fauris et al, 1985; Descampiaux et al, 1994; Lefebvre, 1994; Valentin-Severin *et al*, 2002). In fact, its sensibility is so high that all the samples can be tested without any concentration. The drawbacks of concentration are serious : yields are not controlled and the compounds could be modified during the process. Consequently, the extract does not reflect the actual chemical composition of the sample (Fauris and Vilaginès, 1992).

The reproducibility of the method allows the results of seven independent measures of the same sample to fall within a 2 % standard deviation. Its response is fast enough to require only 2h to 24h incubation for the samples according to their toxicity (Fauris *et al*, 1985). It is easily adaptable to routine analysis and appears both sensitive and rapid enough for high-thoughput daily screening.

Modifications of the original test has been introduced using hepatic cells which retains a certain capacity towards metabolism of xenobiotics (Valentin-Severin *et al*, 2002).

Finally, another European project, Biosafe Paper aims at the development, validation and intercalibration of a short-term biological test battery for safety assessment of food contact paper. It is a pre-normative research effort, which will be used to launch regulatory harmonisation at EU level on the safety of food contact paper and board. The emphasis will be on cost effective tests with toxicological relevant end points.

The chosen methods for assessing cytotoxicity were the RNA synthesis inhibition test, the boar spermatozooan motility test, the *Photobacterium* test, the EROD induction test, the total protein content test, the colony forming ability test, the viability test (neutral red assay). The first part of the research program was to evaluate the relative sensitivities of the different proposed test systems.

For this purpose, positive controls were prepared and sent to the various laboratories involved in the project. The first conclusions seem to confirm that RNA synthesis inhibition is a rather

sensitive early indication of toxic effects at cellular level and that this method is the best candidate for an European cytotoxicity standard.

Apoptosis detection assays

Of great importance to the study of cell death is the differentiation between the two principle modes, namely, apoptosis and necrosis. Necrosis is the passive result of cellular injury leading to complete loss of cell integrity and the release of cellular components into the surrounding environment (membrane breakdown, DNA dissolution (karyolysis), increase of cell size etc…). Apoptosis, on the other hand, is an active process that is accompanied by a series of distinct cellular and molecular events that form an integral part of normal physiological reactions (reduction of cell size, condensation of nuclear chromatin, DNA fragmentation, activation of endogeneous endonucleases and proteases etc…) (Slater, 2001).

Numerous methods exist to quantify cells in apoptosis. Some of them refers either to electronic microscopy to reveal ultrastructural changes in the cells (Searle et al, 1995) or to fluorescent microscopy to visualise DNA structure after staining with specific dyes (eosin, DAPI, Hoechst 33342 etc…) (Marsteinstredet et al, 1997).

Other techniques for assessment of apoptosis are based upon the internucleosomal DNA fragmentation. The most widely used test consists of the DNA analysis by agarose gel electrophoresis. After gel staining, a apoptosis characteristic ladder pattern is obtained. Nevertheless, this method has a low sensitivity.

The DNA clivage can also be detected by labelling the end of the DNA strands *in situ*. Two sensitive methods are available : the TUNEL and the ISEL techniques, but they are too labour intensive.

Flow cytometric analysis combined with microscopic examination remains the method of choice for the study of apoptosis. It reveals membrane modifications as a loss of phospholipid asymmetry (Marstrienstredet *et al*, 1997).

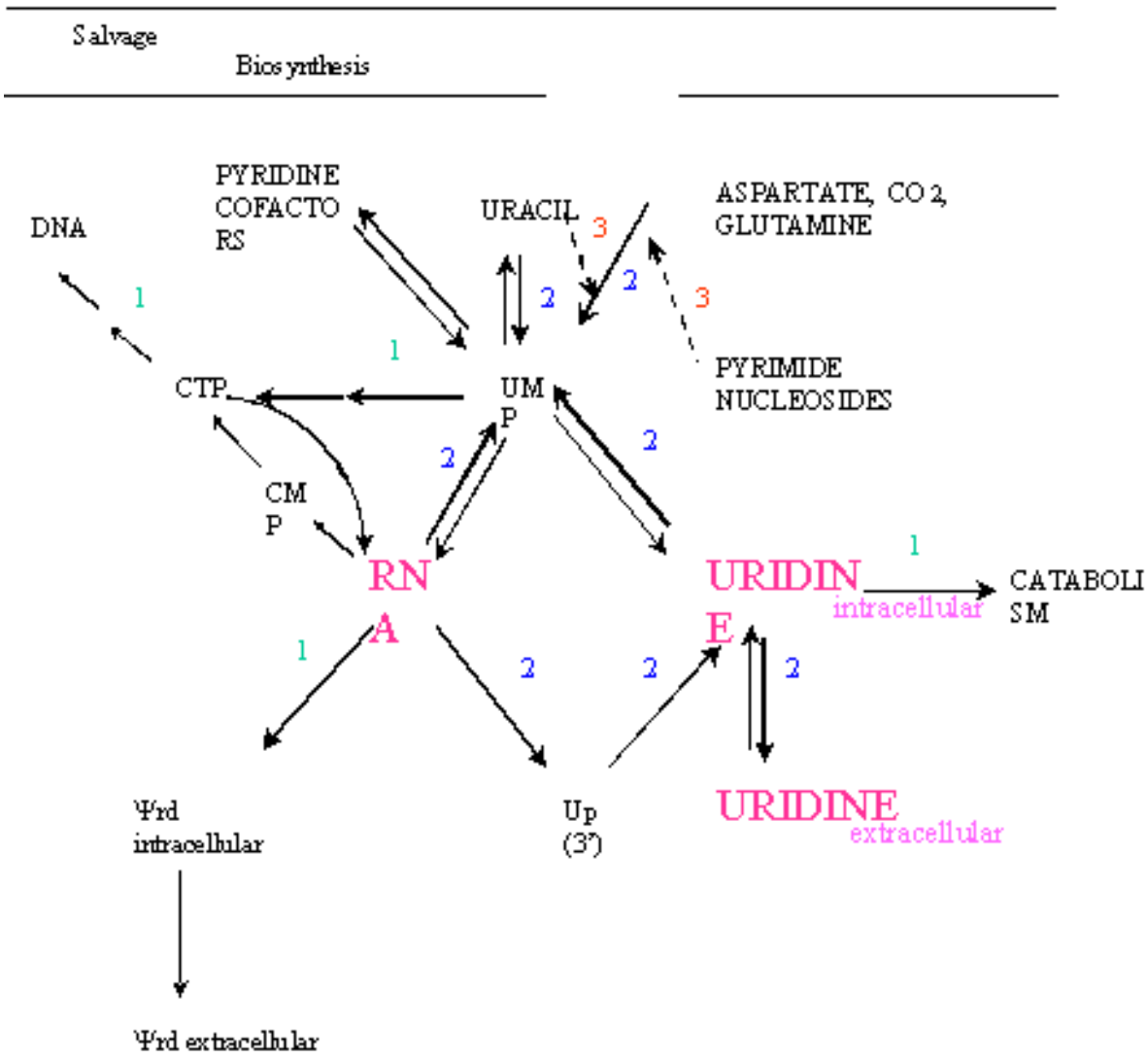

Figure 2 Summary of uridine metabolism. The several sources of salvage and the biosynthesis pathways are illustrated. The dotted lines indicate regulatory blocks and the putative inducer. The numbers refer to the utilisation of uridine. 1. Irreversible loss of uridine through conversion. The parenthesis indicates the process may not be complete. 2. Sources of metabolism dependent isotope dilution. 3. Regulation of biosynthesis.

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Genotoxicity review

Agents that possess the ability to interact, directly or indirectly, with the genetic material of the cell are considered to be genotoxic. Genotoxicity and mutagenicity are two related terms, sometimes difficult to discriminate, which would indicate the multiplicity of ways in which changes in the genetic material can be effected.

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes may involve a single gene or a gene segment, a block of genes or whole chromosomes. Effects on chromosomes may be structural and/or numerical.

Genotoxicity is a broad term that refers to potentially harmful effects on genetic material, which are not necessarily associated with mutagenicity.

Thus, test for genotoxicity include tests which provide an indication of induced damage to DNA (but not direct evidence of mutation) as well as test for mutagenicity.

Knowing the transcendence of the genotoxic (mutagenic, teratogenic or carcinogenic) effects, the detection of those agents is of paramount importance. In contrast with the toxic (generally speaking) agents that have many possible targets in the whole organism, the genotoxic agents have (most of the time) only one target: DNA.

Nevertheless, proteins involved in chromosome segregation or in repair, must also be taken into account. Thus, if DNA is the most common target for genotoxic agents, in principle any type of cell/organism can be used in the evaluation of the genotoxic effects.

In fact, there exist a wide range of assay systems detecting genotoxicity, ranging from simple systems as bacteria to the most complex eukaryote cell of whole mammalian individuals. Nevertheless, and in spite of the simplistic view above designed, genotoxic agents can interact with DNA in different ways, inducing different types of effects and, as consequence, different assays can measure particular ways of how a defined compound can show its genotoxic potential.

The genotoxicity assays can be classified according to different aspects:

- according to the test organism used: prokaryotes *vs* eukaryotes
- according to the complexity of the test system: *in vitro* vs *in vivo*
- according to the level: genic *vs.* chromosomic

Taking into account the above aspects, the following table gives a complete view of the types of assays that can be used in the detection of genotoxicity:

In vitro	Primary DNA damage in prokaryotes Primary DNA damage in low eukaryotes Primary DNA damage in mammalian cells Gene mutation in prokaryotes (Ames test) Gene mutation in low eukaryotes Gene mutation in mammalian cells Aneuploidy in low eukaryotes Aneuploidy in mammalian cells Sister chromatid exchanges in mammalian cells Chromosome aberrations in mammalian cells (AC test) Cell transformation in mammalian cells Micronucleus test (MN test) Comet test
In vivo	DNA repair in mammalian somatic cells Somatic gene mutation in *Drosophila* Spot test in mice Micronuclei in mammals Germinal damage in *Drosophila* Locus specific test in mammals Dominant lethal in mammals Heritable translocations in mammals Sperm morphology in mammals

Although each one of the assays by itself can measure genotoxicity, not all them measure the same type of genetic damage. Thus, to solve this apparent dilemma, different assays are used together to have a complete picture on the genotoxicity risk associated with the exposure in front of a particular compound. This approach is denominated as a battery approach.

In spite that all the assays are able to measure genotoxicity, the complexity of the indicated tests is very different. It is not the same to use whole animals than cells culture; it is not the same to detect germinal damage than somatic damage; and so on.

Several genotoxicity assays are widely used as routine in many laboratories worldwide (see Table 2). Thus, it is easy to select those which fulfil the appropriate requirements: reliance, rapidity and inexpensiveness. Among the most used assays, those that can accomplish such requirements are as follows:

- The Ames test. This is perhaps the most used test. It measures the induction of point mutation in a prokaryote organism (*Salmonella thyphimurium*). Among its advantages must be indicated the rapidity, sensitivity, and the wide database on the response on front

many compounds. Among its disadvantages, it must be indicated the simplicity of its genome far away from the complexity of the human genome.

- The in vitro chromosomal aberration (AC) test. To avoid the problems associated with the simplicity of the bacterial genome, the detection of genetic damage on chromosomes (a structure lacking in prokaryotes) can be used. In this case, human cells can be used, which give special reliance to the obtained data. One objection to this assay is its tediously.

In fact, for many authors both assays can constitute a simple battery in which both assays complements very well.

- The in vitro micronucleus (MN) test. To solve the tediously of the chromosome aberration test, that also requires well training personnel, the micronuclei test have been proposed. This assay that also can be carried out by using human cells, has the advantage of its simplicity and the facts that can measure aneuploidy induction, of particular relevance in genotoxicity testing. It has the disadvantage that, contrarily to the two other assays, it is not yet a validated test. Nevertheless, the large amount of existing data, as well as the different inter-laboratory studies (mainly in comparison with the chromosome aberration test), indicates the advantages of its use. It must be indicated that in the new proposal of the UE Technical Guidance Document for testing chemical compound, this assay is proposed as alternative to the AC test.
- the Comet test. This assay, that can use also human cells, can compete with the Ames test in rapidity and simplicity. These advantages have make to this assay a very popular one, and its use in genetic toxicology testing is increasing over world. The possibility to use an automated system to score the effects, which increases the objectivity and rapidity, is another of its advantages. Nevertheless, it is not yet a validated study.

Perhaps the above four tests are those that, by their probed advantages, can constitute the set of assays to be used in the discussion on which can be the tests used in the detection of the genotoxicity potential of those compounds used in CPDW.

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TEST SYSTEM	TEST ORGANISM	BIOLOGICAL ENDPOINT	VALIDATION/ GUIDELINES	AVAILABLE DATABASE	REGULATION	REF.
Salmonella/microsome Mutagenicity assay (Ames Test)	Battery approach Stains : TA97, TA98, TA100, TA102, TA1525, TA1527, TA1538	Point mutation	OECD Guideline for testing of Chemicals; Test guideline 471. Bacterial reverse mutation test, OECD Paris, 1997	http://toxnet.nlm.nih.gov/servlets/simplesearch?1.25.0.3876 http://ntp-server.nih.gov/cgi/iHIndexes/Res.Start/iH.Res.Stat.Frames.html	Legal requirement for substances and products used in different fields (e.g. Pharmaceuticals, plant protection products)	1,2
In vitro chromosome Aberration assay	Established cell lines: Human peripheral blood Lymphocytes	Structural chromosomal mutations	OECD Guideline for testing of chemicals; Test guideline 473	http://toxnet.nlm.nih.gov/	Legal requirement for substances and products used in different fields (e.g. Pharmaceuticals, plant protection products)	3
Gene mutation assay in cultured mammalian cells	Established mammalian; Cell lines: V79, CHO, L5178Y	Point mutation at the hprt and tk loci	OECD Guideline for testing chemicals; Test guideline 476	http://toxnet.nlm.nih.gov/	Legal requirement for substances and products used in different fields (eg. Pharmaceuticals, plant protection products)	4,5
In vitro micronucleus test	Established cell lines Human peripheral blood Lymphocytes	Structural chromosomal mutations and aneuploidy	Without guidelines	http://ehs.sph.Berkeley.edu/Holland/HUMN/	Included in the new EU TGD; Proposal for the genotoxicity testing of chemicals	6,7
The Comet assay	Established cell lines Human peripheral blood lymphocytes	DNA breaks and alkali-labile sites	Without guidelines	http://www.cometassay.com		8

Table 2 short-term tests for genotoxicity

STAGE 2: HARMONISATION OF THE CYTOTOXICITY TEST

This stage was divided into two parts.

Part1: Inter-calibration of the cytotoxicity test

Introduction

The main tasks of this part were to introduce the cytotoxicity test to the participating institutes (TW and UBA), and to check the quality of the set up of the test in the different laboratories in an inter-calibration comparison exercise.

Instruction days

CRECEP set up the test in 1981, optimised it afterwards and has used it for more then 20 years. Training stays were organised at CRECEP in the beginning of the project. Detailed instructions were given to the two other participants about the culture of HeLa S3 cells in suspension, the preparation of leachates, the precautions to be taken for using ^{3}H-uridine, the measurement of RNA synthesis, the information regarding the treatment of the results and the critical points and difficulties of the method.

During these days, various samples (negative controls, a CPDW and a paper for food contact) were analysed simultaneously by UBA and CRECEP or TW and CRECEP. No particular difficulties were encountered and the results obtained by TW, UBA and CRECEP were in the same range of values (results not shown).

Inter-calibration comparison exercise

Once the training stays were over, a cross validation of the protocol was realised by testing 3 organic materials manufactured as seals or pipes.

Materials
The materials used in stage 2 - part 1 are listed in Table 3. They had been prepared and distributed by CRECEP.

Table 3 Materials used in inter-calibration comparison exercise

Material number	Material	Product	S/V ratio cm^2/L
1	EPDM type 1	seal	4.24
2	EPDM type 2	seal	4.24
3	PE-X	pipe	240

The RNA synthesis inhibition test
The test (leachate preparation and cytotoxical assessment) was conducted as described in Materials and Methods.

Results and discussion
The raw data regarding materials 1, 2 and 3 are reported in the Annex 2. The slopes values of the regression lines and the percentages of RNA synthesis are summarised in

Table 4 and Table 5, respectively, with the corresponding standard deviations.

Various replicates or points were omitted in the first way of calculation:

- concerning UBA, one replicate for material 1 was excluded because of a different accuracy of range and one data of a replicate was omitted (last point of the straight line) for material 3.
- regarding TW, two points were omitted for the blank, two replicates were excluded for material 1 (first replicate : positive intercept, $R^2 < 0.98$, second replicate : different accuracy of range), one replicate for material 2 was not taken into account ($R^2 < 0.98$), one replicate was omitted (positive intercept, $R^2 < 0.98$).
- concerning CRECEP, one data point (30 minutes) was excluded for material 1 and one for material 2 too.

The required slope value of "higher than 1.75" was obtained by the three institutes. The constant b was for all calculation methods negative, as required. The blanks gave similar results for CRECEP, TW and UBA. The relative standard deviation RSD of the slopes was for all replicates and calculation methods smaller than 10 %. This low RSD value clearly show the good reproducibility of the measures.

Analysis of positive controls revealed a toxic effect of potassium dichromate only in CRECEP experiments. TW shows a relatively high SD of 22 %, whereas for the blank and the migration waters of the three materials the SD values were in the same range and much lower. One disturbing factor could be that no information about the preparation of the dichromate potassium solutions and their conditions of storage was given to the participants. CRECEP observed that the standard solution should be kept at room temperature, as a 4°C storage of the solution leads to a decrease in cell toxicity. The protocol should include a checking of the accuracy of the dichromate potassium dilutions and the validity of the standard solution, by the determination of an IC50 curve.

As the experiments were not performed at the same time in the three institutes, comparison of the results obtained for the materials was not easy. Some materials can be unstable over a long period storage and external effects (air quality, dust …) can occur. However the selected materials did not seem to behave significantly differently in the tests, the percentage of RNA synthesis was always greater than 70% (from 81 to 122), pointing out the absence or the very low toxicity of the 3 leachates. The inter-laboratory reproducibility was good with a coefficient of variation of reproducibility (VC_R) of 11.8 % for material 1, 10.5 % for material 2 and 10.8 % for material 3 (first calculation method), of 6.9 % for material 1, 12.2 % for material 2 and 19% for material 3 (second calculation method) and of 7.2 % for material 1, 9.8 % for material 2 and 11.5 % for material 3 (third calculation method).

The results in Table 5 show that the omission of data according to the French calculation method may cause a deviation of up to 15 % with a median value of 7% and up to 20% with a median value of 2% in relation to the second method of calculation and the third one respectively.

Nevertheless, whatever the mode of calculation was, no big differences in the percentages of RNA synthesis could be observed for the three tested materials (values always higher than 70%). Further materials should be analysed, with a back up by statistical treatment, to make sure the three ways of calculation give identical assessment of cytotoxicity.

Table 4 The linear regression parameters for materials 1, 2 and 3 calculated by the three methods

			Slope a (cpm) ± SD *(R^2 or Rf)* [a]		
Material	**Institute**	**Date**	**First method French**[b]	**Second method Graph Pad**[c]	**Third method Excel**
Blank	TW	14/12/01	2.46 ± 0.01	2.54 ± 0.05 *(0.99)*	2.48 ± 0.11 *(0.98)*
	UBA	05/07/01	2.52 ± 0.25	2.67 ± 0.10 *(0.98)*	2.52 ± 0.13 *(0.96)*
		12/07/01	2.49 ± 0.17	2.54 ± 0.05 *(0.99)*	2.49 ± 0.10 *(0.99)*
	CRECEP	31/05/01	2.06 ± 0.04[c]	2.10 ± 0.02 *(0.99)*	2.06 ± 0.04 *(0.99)*
EPDM seal (1)	TW	14/12/01	2.99[d]	2.81 ± 0.24 *(0.87)*	2.41 ± 0.38 *(0.97)*
	UBA	12/07/01	2.44 ± 0.06[e]	2.44 ± 0.10 *(0.96)*	2.18 ± 0.21 *(0.99)*
	CRECEP	31/05/01	2.11 ± 0.11[f]	2.12 ± 0.03 *(0.99)*	2.07 ± 0.04 *(0.99)*
EPDM seal (2)	TW	14/12/01	2.22 ± 0.09[e]	2.26 ± 0.10*(0.97)*	2.41 ± 0.19 *(0.99)*
	UBA	12/07/01	2.09 ± 0.18	2.06 ± 0.08 *(0.98)*	2.10 ± 0.11 *(0.99)*
	CRECEP	31/05/01	2.15 ± 0.08[f]	2.15 ± 0.03 *(0.99)*	2.11 ± 0.04 *(0.99)*
PE-X pipe (3)	TW	14/12/01	2.09 ± 0.18	2.20 ± 0.16 *(0.94)*	2.33 ± 0.28 *(0.97)*
	UBA	12/07/01	2.17 ± 0.10	2.14 ± 0.05 *(0.99)*	2.01 ± 0.10 *(0.99)*
	CRECEP	31/05/01	2.11 ± 0.08[f]	2.45 ± 0.03 *(0.99)*	2.11 ± 0.05 *(0.99)*
$K_2Cr_2O_7$ (2 mg/L)	TW	14/12/01	2.13 ± 0.47[e]	2.60 ± 0.22 *(0.90)*	2.46 ± 0.34 *(0.97)*
	UBA	05/07/01	2.24 ± 0.16[e]	2.24 ± 0.16 *(0.92)*	1.62 ± 0.43 *(0.46)*
		12/07/01	2.07 ± 0.19	2.12 ± 0.06 *(0.99)*	2.07 ± 0.09 *(0.99)*
	CRECEP	31/05/01	1.35 ± 0.10	1.28 ± 0.02 *(0.99)*	1.35 ± 0.05 *(0.99)*

[a] Each result is the average of 3 replicates
[b] R^2 per definition ≥ 0.98
[c] Rf (robustness factor) was calculated automatically by Graph Prism software (second method). It replaces the usual R^2 as a witness of goodness of fit.
[d] 1 replicate, of which one data point was omitted (30 minutes)
[e] 2 replicates
[f] 6 replicates
Note: CRECEP observed cell growth of 68 ± 7.3 % during incubation of the blank; TW and UBA did not measured cell growth at this stage.

Table 5 Percentages of RNA synthesis for materials 1, 2 and 3 calculated by the three methods. The percentages of RNA synthesis of the leachates were expressed in relation to the blank: (average slope value of the leachate/average slope value of the blank)*100. The percentage of RNA synthesis of the blank is fixed at 100 % (total absence of toxicants).

			% of RNA synthesis		
Material	**Institute**	**Date**	**First method French**	**Second method Graph Pad**	**Third method Excel**
EPDM seal (1)	TW	14/12/01	122	110 ± 9	97 ± 15
	UBA	12/07/01	98 ± 2	96 ± 4	87 ± 8
	CRECEP	31/05/01	103 ± 6	101 ± 2	100 ± 2
EPDM seal (2)	TW	14/12/01	90 ± 4	89 ± 4	97 ± 8
	UBA	12/07/01	84 ± 7	81 ± 3	84 ± 4
	CRECEP	31/05/01	104 ± 4	103 ± 2	102 ± 2
PE-X pipe (3)	TW	14/12/01	85 ± 9	87 ± 6	94 ± 11
	UBA	12/07/01	87 ± 4	84 ± 2	81 ± 4
	CRECEP	31/05/01	102 ± 4	117 ± 2	102 ± 2

Different experimental conditions in the three laboratories were pointed out, after discussion:

- UBA and TW checked cell viability with Trypan blue. UBA fixed the acceptance limit at 80 % viability.
- CRECEP verified the percentage of cell growth in the blank tubes between the beginning and the end of the incubation time: an adequate number of cells in perfect health is required to obtain reliable and reproducible results, 50 % growth rate should be reached.
- CRECEP performed a negative control of the cleanness of glassware and of the surrounding environment: a borosilicated glass jar identical to those used for leachates preparation was filled with reference water and kept at 23°C for 24 hours, the resulting water was tested for cytotoxicity. No deviation was observed compared to the reference control.

TW carried out the assays six month later than the two other participants did. Actually, it underlined difficulties in controlling cell cultures in suspension. Consequently, actions and advice were given by CRECEP (and UBA) in order to figure out exactly what the problems were and afterwards to remedy cell growth issue (Annex 3).

1) Further training periods were planned and undertaken at CRECEP, at UBA and at TW to explain the procedure again and to try to understand the encountered difficulties. It is important to note that inter-comparison, at the same place and with exactly the same procedure, did not show a difference in performance of the technician.
2) TW was supplied with some equipment to check if it could improve the results.
3) Detailed glassware cleaning procedures were provided by CRECEP (see materials and method) as this parameter can affect greatly the cell growth rate.
4) A new stirring apparatus was purchased by TW to suspend cell cultures during the incubation period. At the beginning of the project, a Bibby monoposition magnetic stirrer served for agitation. As the cells in all these tubes must exactly be subjected to the same conditions of agitation, it is more convenient and reproducible to use a multiposition magnetic stirrer on which agitation is homogenous.
5) Centrifugation conditions were closely defined to avoid the high loss of cells observed in this step by TW: an increasing centrifuge speed was applied (300X g for 5 minutes) with a Sorvall apparatus. Thus, the correct number of cells (approximately 600.000 cells/ml) could be introduced into the glass tubes.
6) The suspension culture conditions were also checked by CRECEP as several parameters are known to be essential for cell growth (the state of confluence of the cells, media pH, initial number of cells : approximately 600 000 / ml, incubation time…).
7) Different cell counting methods can be applied to determine cell concentrations: automatic particle counters or haemocytometers. Automatic particle counters give the most accurate cell counts, but they are very expensive. These apparatus should be calibrated once a year. This point was achieved at CRECEP by cell counts comparison between the automatic counter and the Nageotte cell counter. UBA used an automatic particle counter too. Alternative cell counting method can consists in using haemocytometers but this method can't be easily adaptable to routine analysis. The Nageotte haemocytometer appears to be more accurate (lower standard deviation) than the Neubauer one, but the grids do not contain subdivisions and consequently the work is far more tedious. TW used the Neubauer counting method all along the project.

Conclusion

TW problems and discussions with WP2 partners led us to fix diverse requirements and to study the effect of various parameters in order to make the assay robust and reliable for any laboratory:

- the preparation and the storage of the leachates

- the assessment of cell viability and cell growth
- the best incubation prior to RNA synthesis determination
- the choice of uridine batches (importance of a convenient supplier) etc…

Moreover, the laboratories were requested by CRECEP to record further information regarding each experiment: cell counting before and after incubation, colour of the culture media after incubation.

Part 2: Study of the impact of different test variables

Introduction

The aim of this chapter was to define diverse factors in order to harmonise the cytotoxicity assay and to obtain reproducible and reliable results when testing CPDW where ever it is proceeded. These experiments were mainly conducted by CRECEP due to its advanced knowledge of the technique, but UBA and TW actively participated on some of them too. A part of these investigations was carried out in parallel to the stage 3.

The following topics were studied :
- the leachates preparation
- the leachates storage
- the cell viability
- the percentage of cell growth in the blank tubes
- the best "cell-leachate" incubation time
- the use of glass or plastic tubes for "cell-leachate" incubation
- the uridine batches

In this chapter, the raw data were treated only with the first mode of calculation.

Preparation of the leachates

Objectives
The procedure to follow to prepare leachates may influence their resulting cytotoxicity. Various factors may modify the characteristics of the migration waters such as the vessel, the air/water ratio in the flask, the S/V ratio of the material, the reference water, the experimental conditions etc…Different experiments were conducted to try to draft a detailed procedure for the preparation of the leachates.

Verification of the absence of impact of the jar on the cell response towards toxicity
CRECEP performed a set of negative controls that can validate the cleanness of glassware and of the surrounding environment. A borosilicated glass jar is filled with reference water without any material and kept at 23°C for 24 hours without agitation, the resulting water is tested for cytotoxicity. No deviation was observed compared to the reference control (Table 6), pointing out the quality of the cleaning of the glassware.

Effect of S/V ratio
CRECEP selected two different S/V ratios for material 8 (Table 7), which is known to release toxic agents as shown in WP 1, in order to investigate the possible variation in the cytotoxicity response. The first S/V ratio corresponded to 200 cm^2/L (WP 1 ratio) and the second to 10 cm^2/L. Table 6 presents the obtained results.

Table 6 Comparison between the negative controls and the blanks.

Date	Blank (reference control) (average of 6 tubes)		Negative control (average of 3 tubes)	
	Slope (cpm)	% RNA synthesis	Slope (cpm)	% RNA synthesis
20/12/01	2.60 ± 0.14	100 ± 6	2.63 ± 0.05	101 ± 2
31/05/01	2.8 ± 0.06	100 ± 6	2.88 ± 0.06	102 ± 2
13/08/02	2.94 ± 0.19	100 ± 2	2.9 ± 0.2	97 ± 7
21/08/02	3.12 ± 0.05	100 ± 6	3.15 ± 0.11	101.5 ± 3.5
29/11/02	3 ± 0.17	100 ± 6	3.04 ± 0.22	101 ± 7
20/12/02	3.05 ± 0.17	100 ± 6	3.19 ± 0.16	104 ± 5

Table 7 Effect of S/V ratio using leachates of material 8 on the result of the assay.

S/V ratio cm^2/L	% of RNA synthesis (average of 6 tubes)
200	2.8 ± 4
10	96 ± 6

The leachate 8 toxicity decreased strongly in relation to the S/V ratio, and no effect compared to the reference control was found with a S/V ratio of 10 cm^2/L. This observation is not surprising as the quantity of released toxicants from the material is correlated to the S/V ratio. Thus, defined appropriate S/V ratios is of great importance for the cytotoxicity assessment, keeping in mind that a non-cytotoxic material remains safe even at high S/V ratios.

Comparison between two different procedures for leachate preparation

Other criteria for the preparation of leachate were defined by UBA and compared to the usual ones described in materials and methods. The experimental procedure was changed in terms of temperature, i.e. incubation at 20°C instead of 23°C, and handling, i.e. incubation under agitation instead of static conditions. Materials no. 1-8 (listed in Table 1) were subjected to those two modes of preparation in parallel.

Leachates were prepared immediately before performing the test series. The totality of the detailed results is presented in Annex 4. The RNA synthesis rate was determined, as usual, by the slope of the regression straight line obtained from the test values and expressed in relation to that obtained with the blank which is considered to be 100 %. The results regarding materials 7 and 8 are given in Table 8.

Table 8 RNA synthesis percentage of materials studied in the cytotoxicity assay

		RNA synthesis	
		Slope	%
Material 7	20°C, with agitation	2.53 ± 0.2	102 ± 8
	23°C, without agitation	2.28 ± 0.17	92 ± 7
Material 8	20°C, with agitation	1.03 ± 0.4	50 ± 19
	23°C, without agitation	1.84 ± 0.07	89 ± 3

No difference in cytotoxicity was pointed out for material 7 whatever the mode of preparation was. But, cytotoxic effects were detected with the leachate 8 prepared under agitation at 20°C whereas no cytotoxicity was found with the other leachate preparation. These findings were reproducible in triplicate on one migration water. Although diffusion inside organic materials is the controlling factor that determines the concentration in the migration water, agitation perhaps allows a better diffusion of the toxic agents released by material 8. Further

investigations are necessary to choose the best way to prepare the leachate. The results in Table 8 should be considered together with the data in Table 23 and Table 24.

Effect of the leachate storage on the cytotoxicity test

Objectives
The aim of this survey was to know if leachates could be prepared days in advance and be kept at 4°C until analysis. Actually, the chemical composition of a leachate can change over time. For instance, during storage, volatile compounds can be lost or diverse products can be transformed into less or more toxic agents.

CRECEP investigations
The effect of leachate storage was studied using the material 8 (nitrile rubber O seal) which has been shown to be toxic for HeLa cells. One migration water was prepared as described in materials and methods. The potential toxicity of the leachate was detected by the RNA synthesis inhibition assay immediately, or after 3 and 8 days long of conservation at 4°C. The results are reported in Table 9.

Table 9 Comparison of the cytotoxicity of the leachate 8 dependently on its storage time.

Date	Material 8 (average of 6 tubes)	
	Slope a (cpm)	% RNA synthesis
1/10/02 (day 0)	0.08 ± 0.1	2.8 ± 4
4/10/02 (day 3)	0.1 ± 0.1	3.4 ± 4
9/10/02 (day 8)	0.46 ± 0.1	17 ± 4

The cytotoxicity in the leachate of material 8 decreased during the time of storage (from 2.8 % to 17 % of RNA synthesis), but the material toxicity remained positive, considering the threshold defined in the French standard (70 %). It should have been of interest
1) to go on with a longer time of storage and to determine if the toxic properties of the leachate continues to become lower
2) to practice the same experiments with other potential toxic materials.

UBA surveys
UBA tested for cytotoxicity the leachates of materials 10 and 11 after 0, 2 and 15 days of conservation at 4°C. One migration water was prepared per material, as CRECEP did, and the RNA synthesis was determined as described in the first way of calculation. The results of these three consecutive experiments are given in Table 10.

The cytotoxicity in the leachate 10 decreased strongly during the storage period, and no effect compared to the reference control was found after 15 days of conservation (see Annex 4). The material 11 appeared to be not toxic and this property did not change over time.

Table 10 Comparison of the cytotoxicity of the leachates 10, 11 accordingly to their storage.

Date	Material 10		Material 11	
	Slope a (cpm)	% RNA synthesis	Slope a (cpm)	% RNA synthesis
29/1/03 (day 0)	0.03 ± 0.03	0.7 ± 1	2.5 ± 0.59	92 ± 22
31/1/03 (day 2)	0.19 ± 0.13	8 ± 6	2.18 ± 0.14	98 ± 6
13/2/03 (day 15)	2.68 ± 0.43	110 ± 17	2.78 ± 0.13	113 ± 5

Conclusion
These experiments clearly show that the leachates should be tested at once and could not be kept over time without taking the risk to introduce modifications in the toxicity assessment.

Study of the cell viability, the cell growth and the incubation time

Objectives
In order to obtain reproducible and reliable results in each laboratory, HeLa cells in suspension must be in a constant healthy physiological state. Consequently different aspects might be taken into account to assess this constant cell physical fitness. To identify if the cell viability and the ability to divide, were relevant for this purpose, a set of experiments was conducted testing simultaneously the microscopic cell aspect, the percentage of viability, the percentage of growth and the percentage of RNA synthesis in blanks and positive controls.

Methods
Cell viability can easily be assessed by an exclusion dye method. As UBA and TW routinely used Trypan blue as a lethal colorant, it was decided to transpose this technique to the CRECEP institute. Briefly, after cell coloration with Trypan blue (0.4 % P/V), dead or damaged cells are stained light purple violet whereas undamaged cells appear uncoloured under a conventional microscopic observation. The ratio of counted coloured cells and total cells is determined before and after incubation and the percentages of viable cells can be deduced. A criterion of cell viability was set to ± 80% in the blank tubes by UBA: this threshold should be reached to allow the experiment to be pursued with the kinetics.

Percentage of growth can be established by counting the number of cells before and after incubation using an automatic particle counter (UBA and CRECEP) or a Neubauer haemocytometer (TW). A 50 to 100% of growth is generally reached, conditionally with the incubation time and with the initial number of cells. The effect of different incubation times were investigated in parallel (from 17h to 23h).

Results
Prior to any investigation, the macroscopic and microscopic cell aspects of each blank has to be examined before and after incubation. White clumps must not be observed and the medium must be yellowish. Cellular aggregates and/or fragments must be absent in the sample.

Results from TW and CRECEP are reported in Table 11 to Table 13 and Figure 3, which was drawn with Prism 3.03 software. The raw data corresponding to the CRECEP experiments are detailed in Annex 5.

No correlation between the percentage of viable cells and the percentage of growth can be established. For instance, in Table 11 the percentage of growth of the blank is strongly altered (7 %) while the percentage of viable cells still remains at 97 % (above the UBA acceptance limit of 80 %). Moreover, the percentage of cell viability can remain high (close to 90 %) in the presence of the known toxic (potassium dichromate) whereas the cell growth is strongly inhibited (close to 10 %) (Table 13).
Exclusion dye methods only detect membrane modifications of the cell. but other harmful events (sublethal effects) can occur in the cell leading to an injured physiological state which won't be revealed by those methods. Consequently, assessment of cell viability can't provide sufficient information concerning the cell physical fitness.

High SD values were found for the assessment of growth rate in Table 12 that confirm the difficulties TW encountered in getting a correct growth of the HeLa cells (lack of

repeatability of the growth, observation of white clumps, pink colour of the medium, too low cell increases, cell contamination in a few tubes).

Table 11 Measurements of cell viability and growth enhancement of the blank (TW results)

Incubation time	% viable cells before incubation	% viable cells after incubation	Culture appearance After incubation	% cell growth
17 h	98.7	95.4	yellow/ orange with	38
18 h	99.4	97.6	white clumps in every	37
19 h	99.4	99.2	tube	36
20 h	98.7	97.1		7

Table 12 TW results regarding blank and positive control after a 19h exposure time.

Date	Blank			Positive control (2 mg/L)		
	cell growth %	viable cells %	% RNA synthesis	cell growth %	viable cells %	% RNA synthesis
31/10/02	71 ± 57	89 ± 4	100	-3 ± 11	57 ± 3	3
14/11/02	67 ± 20	95 ± 2	100	17 ± 22	37 ± 9	2

Table 13 CRECEP results regarding blank and different concentrations of potassium dichromate ($K_2Cr_2O_7$) after various exposure times.

	i.t. h	Blank	Concentration				
			1.5 mg/L	1.8 mg/L	2.1 mg/L	2.5 mg/L	IC 50
Cell growth (%)	19	84 ± 12	13	12	13	17	
	21	93 ± 7	15	14	17	10	
	23	84 ± 6	20	15	6	5	
viable cells %	19	96	n.m.	n.m.	n.m.	91	
	21	95	n.m.	n.m.	n.m.	92	
	23	96	n.m.	n.m.	n.m.	87	
Slope a (cpm)	19	3.12 ± 0.09	2.31 ± 0.25	1.56 ± 0.12	1.09 ± 0.2	0.57 ± 0.01	
	21	3.00 ± 0.16	2.27 ± 0.06	1.25 ± 0.3	0.78 ± 0.01	0.40 ± 0.04	
	23	3.03 ± 0.11	2.16 ±0.02	1.38 ± 0.16	0.74 ± 0.1	0.28 ± 0.01	
% RNA synthesis	19	100 ± 3	74 ± 8	50 ± 4	35 ± 6	17 ± 1	1.84 ± 0.11
	21	100 ± 5	76 ± 2	42 ± 9	26 ± 0	14 ± 1	1.86 ± 0.11
	23	100 ± 4	72 ± 1	46 ± 5	24 ± 4	9 ± 0	1.75 ± 0.11

i.t., incubation time; n.m., not measured

The additional CRECEP investigations give raise to the following comments:

- The percentages of growth obtained for the blanks after 19. 21 and 23 hours were quite similar and higher than 80 %, which proves that the cells had divided properly over incubation period. To slacken incubation time (time range of 21h ±2) does not significantly change the RNA synthesis rate in the blanks. This means that the cells have already reached the stationary phase of the growth curve after 19h of incubation and that increasing incubation time is not necessary in these conditions.
- Dose-response curves of potassium dichromate were plotted for the three exposure times (Figure 3). The corresponding values of IC50 were calculated from the linear regression. These IC50 values for potassium dichromate are 1.84 mg/L, 1.86 mg/L and 1.75 mg/L for 19, 21 and 23 hours of incubation respectively. The associated SD proves that these IC50 values are not significantly different. Thus, increasing the exposure time from 19 to 23 hours in the experimental conditions does not modify the cell response towards the toxic agent.

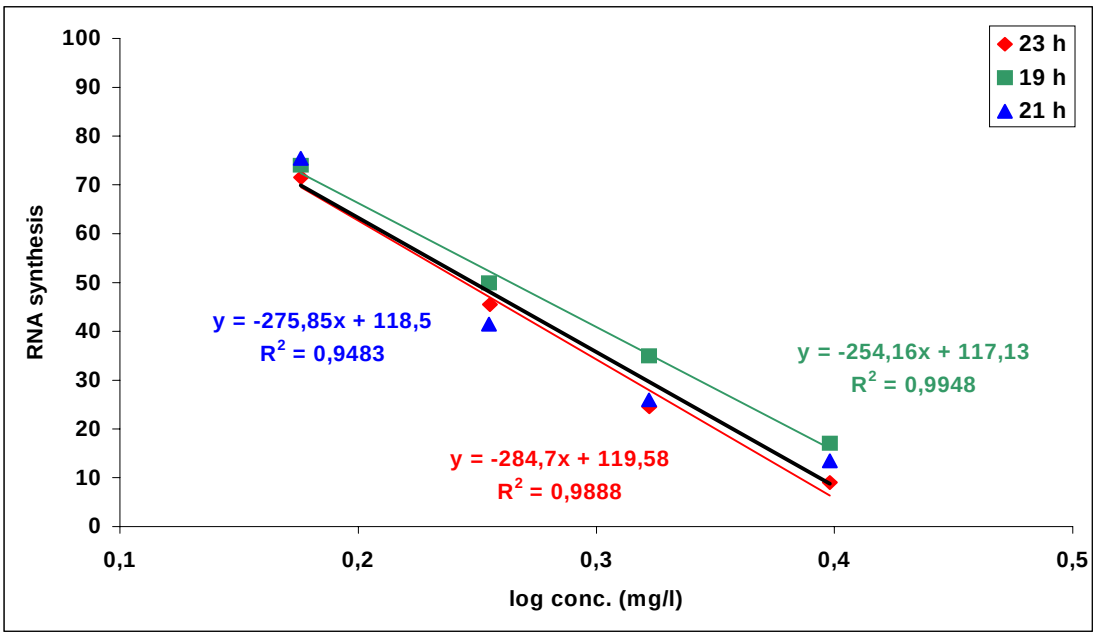

Figure 3 Dose-response curves with potassium dichromate after different exposure times.

Moreover, 26 independent measurements of RNA synthesis after cells exposure to potassium dichromate at 2 mg/L were realised by CRECEP. The results are summarised in Table 14. An average of RNA synthesis of 52.65 % was deduced from these raw data with a SD of 6.58 % and a relative standard deviation (RSD) of 12.5 %, proving that the method is very reproducible (Table 12).

The conclusions on these surveys are as follow:

- the measurement of viable cells gives an additional information on the physiological state of the cells. But only considering this criterion is far from being sufficient to verify the healthy status of the cells. Beside this limitation, it was decided to check cell viability before and after the incubation step in the blanks, with an acceptance limit of 80 % of viable cells.
- Dose-response curves of potassium dichromate clearly show the toxic effect of this product, with an IC50 value of approximately 1.8 mg/L, proving the ability of the RNA synthesis inhibition assay to give a quantitative and repeatable response towards toxic chemicals. Independent measurements of RNA synthesis after cells exposure to potassium dichromate at 2 mg/L point out the high reproducibility of the method (RSD = 12.5 %).
- The determination of the percentage of growth gives not only another data about the health state of the cells but also an indication on the cell culture conditions. Actually, an inadequate temperature or pH medium, for instance, leads to a poorly percentage of growth whatever was the healthy status of the cells at the beginning of the incubation. A required cell growth of 50 to 100 % is supposed to be reached in the blank tubes after incubation (CRECEP acceptance limit). This threshold was added to the final procedure.
- Slackening incubation time from 19h to 23h does not cause the death of the cells: the percentage of viable cells remains close to 95 %. Neither does it cause any change in the RNA synthesis rate in the cells. Thus, if the cells are not injured and the conditions of growth are correct, an incubation time close to 21 hours is quite appropriate.
- If the expected number of cells is not reached after a sufficient incubation period, different factors may have affected the cell growth: insufficient glassware cleaning, use of scratched or not tightly tubes, temperature changes in the incubator, unsuitable speed of the stirrer, inappropriate culture medium (pH, bacterial contamination), bad cell physical fitness etc. These parameters must be carefully checked. Problems in the cell counting can also occur with a not enough accurate method, leading to false cell number assessments.

- Each laboratory has to search for its doubling cell time and afterwards has to select the best incubation time to measure RNA synthesis rate. Actually, the incubation time varies according to the length of the lag phase before the growth starts in suspension. This lag phase is correlated to the degree of confluence of the cells used in the survey. This could be an explanation of the difficulties encountered by TW, and sometimes, by UBA. This incubation time should be included in a time range of 21 ± 2 h for the HeLa S3 cells, as previously shown.

Table 14 Cytotoxicity measurements after cells exposure to dichromate potassium 2 mg/L.

Date	% of RNA synthesis	Average of % of RNA synthesis	SD	RSD *RSD = (100xSD)/avg*
26/04/01	57			
15/05/01	57 - 57			
31/05/01	65			
1/06/01	69			
15/06/01	41 - 47			
20/06/01	56			
10/08/01	52	52.65	± 6.58	12.5
14/09/01	57			
26/09/01	63			
3/10/01	51			
5/10/01	55			
12/10/01	52			
17/05/02	46			
24/05/02	53			
14/06/02	41			
21/08/02	54			
20/09/02	48			
6/12/02	50			
19/12/02	50			
28/02/03	50			
21/03/03	46			
23/05/03	51			
3/06/03	53			
6/11/03	48			

Importance of the nature of the incubation vessel on the cytotoxicity test

The three institutes did not use the same vessel for the incubation step: UBA took polypropylene round bottom tubes (Falcon) whereas CRECEP and TW realised the incubation of the cells in borosilicated glass tubes. Therefore CRECEP decided to verify that plastic tubes did not interfere with the test results (release of compounds. adsorption of toxicants…). One sample, a toxic leachate from material 8 (nitrile rubber O seal), was prepared as usual. Cells were incubated in the culture medium reconstituted with the leachate, either in plastic tubes, or in glass tubes. The percentage of RNA synthesis was measured as already described. The resulting information is presented in Table 15.

Table 15 Comparison of two types of vessel for incubation on 8/10/02.

	Slope a	% RNA synthesis
Blank in glass	2.7 ± 0.33	100 ± 12
Material 8 in glass	0.46 ± 0.1	17 ± 4
Blank in plastic	2.74 ± 0.33	100 ± 12
Material 8 in plastic	0.93 ± 0.2	34 ± 7.5

The blanks show that the plastic containers do not release toxic compounds relative to glass. The toxicity of material 8 appears to be higher in glass tubes than in plastic ones : the percentage of RNA synthesis is twice superior in case of plastic tubes. This might confirm the possibility of adsorption effects of the toxic compounds to plastic. But further investigations must be planned to validate this hypothesis.

Study of the tritiated uridine decomposition over time

Radiolabelled uridine decreases in aqueous solution. The decomposition rate of radiolabelled uridine is determined by a combination of chemical, radiolytic and microbiological processes.

CRECEP investigated the radiolytic decomposition of tritiated uridine (*Amersham Bioscience*) by kinetics of uridine uptake in the HeLa cells and by carrying on a chromatography on 3MM paper in n-butanol-ethanol-water (104-66-30) as solvent. The results are summarised just below (Figure 1) and show a decrease in the peak of uridine related to the storage time.

Figure 4 shows the decrease of the peak of undamaged uridine overtime. As, the percentage of incorporated uridine in the cells is proportional to the quantity of undamaged uridine, the assessment of the decomposition rate should be checked by chromatography or more easily by the slope values of the blanks (keeping in mind that other factors can affect these slope values).

No experiment was conducted to evaluate the chemical and bacterial degradation of uridine, as uridine can be considered as a suitable metabolite by bacteria. Initially, TW sampled its uridine with sterile pipette tips but not under sterile conditions (problem of laboratory functionality). This repetitive contamination of the uridine solution over a long period of time could lead to a diminution of its incorporation percentage in the HeLa cell. Thus, after the initial experiments. TW used new batches of uridine for each experiment to reduce the chance of the results being affected by bacterial contamination.

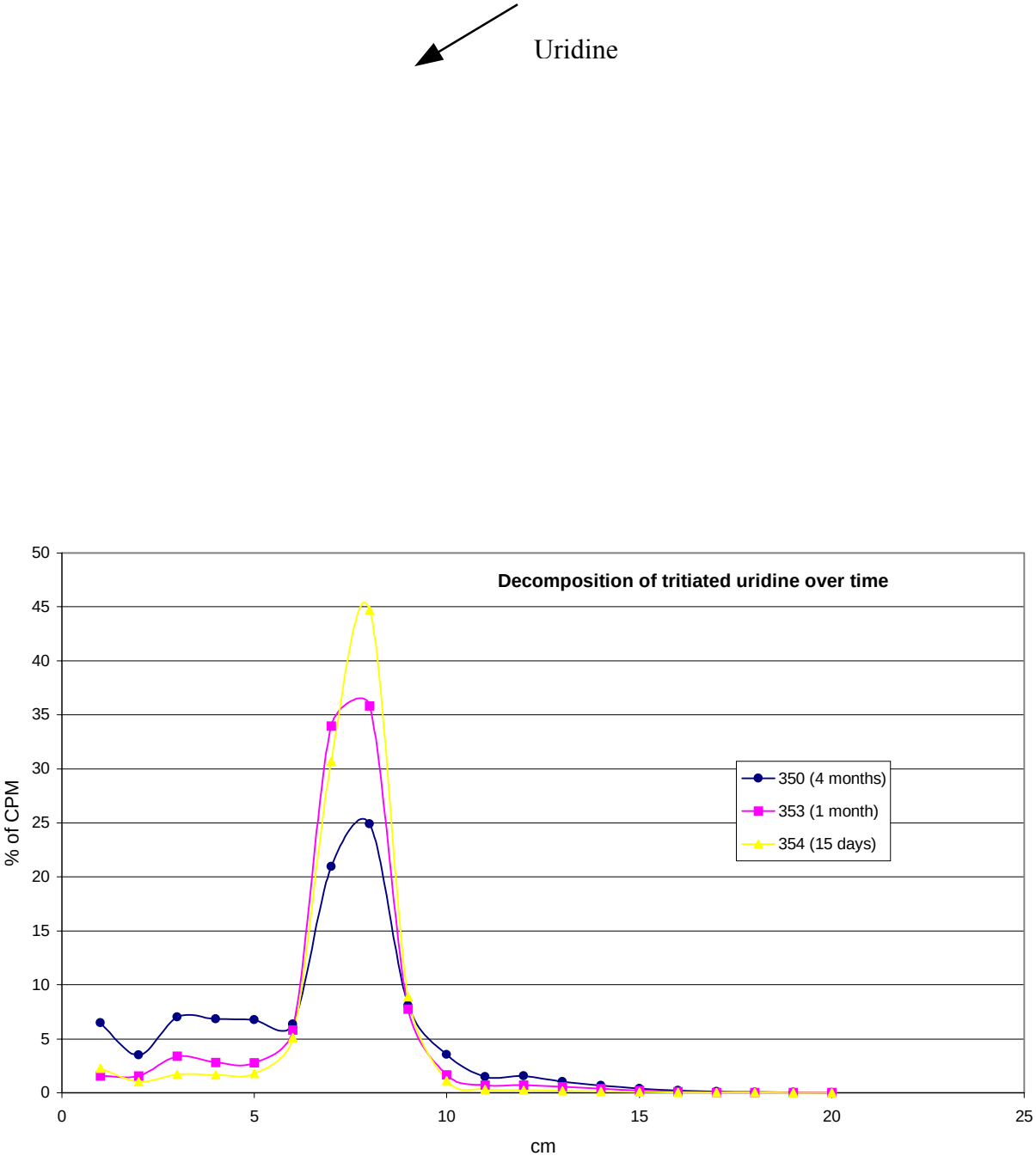

Figure 4 Decomposition of tritiated uridine over time

Importance of uridine batches of high quality grade

Objectives
As the quality of the ^{3}H-uridine can affect the cytotoxicity test response (low cpm, low incorporation rate, contamination), it seemed of interest to check the variability in the uridine batches applied in this work. The degree of quality of the ^{3}H-uridine can differ from batch to batch, from supplier to supplier or can change over time after the opening of the bottle.

Methods
The blanks were analysed in relation to the use of various uridine batches at CRECEP and UBA over a one year period.

Results
Table 16 and Table 17 summarise the available results at CRECEP and UBA, respectively. A decrease in the slope value of the linear regression would reveal the bad cell physiological state as well as the quality of ^{3}H-uridine.

Table 16 Impact of the uridine batches (*Amersham Bioscience*) on the cytotoxicity assay at CRECEP

Uridine batches (n°)	Number of blank measurements	Blank Average slope cpm	Standard Deviation cpm
364	30	3	± 0.3 (10 %)
362-1. -2. 364. 365. 366. 367. 368. 369	271	3.01	± 0.34 (11 %)

Table 17 Impact of the uridine batches purchased from *ICN Biomedicals Inc.* on the cytotoxicity assay at UBA

Number of tested urdine batches	Number of blank measurements	Blank Average slope cpm	Standard deviation cpm
> 2	41	2.5	± 0.4 (16 %)

Using 8 different uridine batches of high quality grade (*Amersham Bioscience*) did not significantly change the results (Table 16). This point indicates that the use of various ^{3}H-uridine batches from this supplier did not influence the cytotoxicity measurement.

Moreover, the absence of contamination of the uridine solution over time was checked: thirty measures were performed at CRECEP with a single uridine batch. The average slope value was greater than 1.75 with a SD of 10 %, which proves not only the absence of contamination of the uridine batch, but also the constant fitness of the HeLa cell cultures and the good reproducibility of the blank control measurements. The sampling of the radiolabelled compound is always done under sterile conditions at CRECEP, thus avoiding any contamination as mentioned before.

Similar investigations were undertaken at UBA and similar results were obtained (Table 17). Actually, the average slope values differ slightly between CRECEP and UBA: this reflects the proper but different growth culture conditions of each laboratory.

Conclusion
Each laboratory should maintain a Shewart chart of the blank average slope values, which serves as a tool to detect deviating blank slopes. Laboratories should check the purity (absence of microbial contamination) and the quality (choice of a good supplier) of the ^{3}H-

uridine batches. They should also study the time they could use a batch properly (decomposition over time).

Additional CRECEP experiment

The ability of the RNA synthesis inhibition test to give a quantitative reply to toxicants was also shown conducting the cytotoxicity test under two different concentrations of a toxic leachate. The migration water was prepared from the material 8 (a nitrile rubber O-seal with a S/V ratio of 200 cm^2/L) as described previously and measurements of RNA synthesis were performed as usual. The cytotoxicity response is reported in Table 18.

Table 18 CRECEP results obtained with a toxic leachate and its half on 4/10/02.

	Slope a	% RNA synthesis
Blank	2.92 ± 0.2	100 ± 7
Material 8; Leachate 8	0.08 ± 0.1	2.8 ± 4
Material 8; Leachate 8 diluted to 1/2	2.6 ± 0.1	98 ± 10

The results confirm that the method gives a dose response towards toxicants released from the material as it does with a known chemical toxic agent (see Table 13). This finding points out that a quantitative and reliable information regarding the cytotoxicity of a material can be assessed with this assay.

Conclusions of stage 2

- The first intercalibration exercise gave promising results. The three selected materials did not behave significantly differently in the three laboratories (RSD = 10.3 %). Moreover, the percentage of RNA synthesis was always greater than 84 %, pointing out the absence of toxicity of the 3 leachates.
- A procedure for leachate preparation should be described after further investigations, as it appears that various factors (temperature, agitation, S/V ratio etc.) can have an impact on the assay.
- Leachates should not be kept after preparation. Storage can greatly affect the cytotoxicity of the migration waters.
- An assessment of cell viability prior to RNA synthesis determination was decided in order to get an additional data on the physiological state of the cells. An acceptance limit of 80 % of viable cells for the blanks was set to allow the experiments to be pursued with the kinetics. However, the results show that this criterion is not sufficient to check the fitness of the cells.
- A determination of the percentage of growth should be applied prior to RNA synthesis measurement. The threshold of "greater than 50% of growth" in the blank tubes is perhaps too drastic and could be revised after additional researches. Actually it should be of interest to study if a lower cell enhancement allows the test to keep its characteristics.
- Each laboratory should fix its best incubation time, which is tightly related to the culture conditions. It should be included in a time range of 21 ± 2 hours for the HeLa S3 cells. Thus, each institute should try to have under control its cell growth conditions. For this purpose, the preliminary steps must be tightly checked (the degree of cell confluence of the culture, the quality of reference water, the use of a clean vessel only devoted to toxicity assays, etc.) as well as the incubation step (cell growth, incubator temperature, medium characteristics, etc.).

- Uridine batches of high quality grade should be selected not to modify the cytotoxicity measurement and uridine samplings should be performed under sterile conditions to avoid contamination.
- Each laboratory should maintain a Shewart chart of the blank average slope values, which could serve as a tool to detect deviating blank slopes.

Taking into account this information, a revised test method (Annex 6) was drafted. The main steps are described in Table 19.

Table 19 Tritiated uridine uptake inhibition assay: flow chart.

ASSAY STEPS	PROCEDURE
Leachate preparation (according to European Standards) subjected to modifications	introduce the material in a glass flask add 1L of reference water incubate (23°C, 24h, darkness, no agitation) test the leachate at once
Cell growth prior to incubation	inoculate a 1X medium with HeLa S3 cells incubate at 37°C until confluent monolayers obtention
Incubation step	remove culture medium prepare a new medium with the leachate inoculate 3 tubes with 5 ml of cells (~ 600 000 cells/ml) incubate (37°C, 21 h ± 2, under agitation) prepare the reference, positive controls and negative control in the same manner (the negative control should be added in the future)
Preliminary requirements	evaluate the morphological alterations macroscopically and microscopically determine the cell viability before and after incubation (> 80%) Calculate the percentage of growth (> 50%)
Uridine Uptake Inhibition Assay	treat 500 µl of cells with 3 µl tritiated uridine (1.29-1.85 TBq/mmol, 37MBq/ml) sub-sample this aliquote by immobilising 40 µl of the sample on chromatography paper pretreated with SDS to stop the reaction 5, 10, 15, 20, 25, 30 min after the addition of uridine use TCA for the descending chromatography to precipitate nucleic acids cut the spot areas of the sheet immerse them in ethanol (10 - 15 min) dry the paper strips introduce each spot area in a scintillation vial add scintillation liquid count directly radioactivity in cpm in a liquid scintillation counter determine of the total introduced radioactivity in each tube in parallel
Data analysis	RNA synthesis is determined by the mean slope of the regression straight line obtained from the experimental values It is then expressed in relation to that obtained with the control and converted to a percentage value

STAGE 3: APPLICATION OF THE HARMONISED CYTOTOXICITY METHOD TO VARIOUS TYPES OF CPDW

Objectives

The participants of WP2 decided that an harmonised protocol was needed for a number of reasons:

- leachate preparation and storage (based on the results of stage 2)
- similarity of the cell growth conditions (same cell line. same state of confluence. evaluation of cell growth. assessment of cell viability. detailed glassware cleaning procedures…)
- uridine batches (the batches should be of high quality grade and the sampling should be performed under sterile conditions).
- a negative control should be added to the experiments in a near future.

These aspects were included in the assays as conducted in stage 3 of the WP2 in which the RNA synthesis test with the harmonised protocol was tested in the three involved institutes with a number of selected materials. The main task of this last stage was to evaluate the harmonised cytotoxicity protocol: applicability to a wide variety of CPDW, inter-laboratory comparison of the results, assessment of the inter-laboratory variability, ability to discriminatory power etc.

Selected materials

Ten different materials (organic, cementitious and metallic types) were tested by the harmonised assay. Their nature and S/V ratio are given in Table 20.

Table 20 Materials investigated in stage 3.

category	number	material	S/V ratio (cm^2/l)
Organic materials	4	PVC-R pipe	398
	5	PVC-C pipe	240
	6	EPDM rubber hose	315
	7	PE-Xc pipe	470
	8	Nitrile rubber O seal	200
	10	Nitrile rubber O seal	200
	11	EPDM O seal	319
	13	EPDM rubber washer	1064
Metallic materials	9	Stainless steel	1140
Cementitious materials	12	Organic cementitious	740

Cytotoxicity investigation

The different leachates were prepared as described in
Annex 6, except for materials 4, 5 and 6 that underwent a precondition step as previously described in materials and method. The cytotoxicity measurements of the resulting leachates were performed using the harmonised protocol (
Annex 6).

Results

Both the average slope values of the straight lines and the percentage of RNA synthesis for each sample are noted in Table 21 to Table 26 using the three modes of calculation. The

EPDM rubber washer and the organic cementitious were received by CRECEP at the end of the project and, therefore, were only investigated by this institute; the obtained results are summarised in Table 27 and Table 28. The raw data for the whole materials are presented in Annex 7 to Annex 10.

Various replicates or points were discarded in the first way of calculation, however, for the other two methods of calculation these data points were not excluded:

- concerning UBA, one data of two replicates was omitted (last point of the straight line) for material 4 and material 5 (9/11/01), one data of one replicate was not taken into account (last point of the straight line) and one replicate was excluded ($R^2 < 0.98$) for material 6, one duplicate was removed ($R^2 < 0.98$, $a < 1.75$) for the blank (6/9/02), one data of two replicates was not taken into account (last point of the straight line) for the blank (20/9/02), one duplicate was removed ($R^2 < 0.98$) for the blank (11/9/02), one data of one replicate was omitted for material 7 (20/9/02), one data of one replicate was omitted (13/2/03) and one replicate was discarded (different accuracy of range) (31/1/03) for material 11.
- concerning TW, one replicate was excluded for material 5 (positive intercept. $R^2 < 0.98$).
- concerning CRECEP, one of the six replicates was not taken into account ($R^2 < 0.98$) for material 4, one data of two replicates was removed (last point of the straight line) and one replicate was omitted ($R^2 < 0.98$) for the blank (13/8/02), one data of two replicates was omitted (last point of the straight line) for the blank (29/11/02), one data of three replicates was omitted (last point of the straight line) and one replicate discarded ($R^2 < 0.98$) for the blank (29/11/02), one data of five replicates was discarded (last point of the straight line) and one replicate removed ($a < 1.75$) for the blank (21/8/02), one data of three replicates was not taken into account (last point of the straight line) for material 7 (13/8/02), one data of six replicates was omitted (last point of the straight line) for material 7 (21/8/02), one replicate was removed (red tube) for the blank (20/12/02), one data of one replicate was discarded for material 9, one data of one replicate was not taken into account (last point of the straight line) and one replicate was excluded ($R^2 < 0.98$) for material 10 (29/11/02), one data of five replicates was omitted (last point of the straight line) for material 10 (6/12/02), one data of one replicate was discarded (20 minutes point) and one duplicate was removed (different accuracy of range) for material 11 (6/12/02), one data of two replicates was not taken into account (last point of the straight line) for material 12.

Table 21 The linear regression parameters for materials 4, 5 and 6 calculated by the three methods.

Material	Institute	Date	Slope a (cpm) ± SD *(R^2 ou Rf)* [a]		
			First method French [b]	Second method Graph Pad [c]	Third method Excel
Blank	TW	13/11/02	2.73 ± 0.15	2.84 ± 0.06 *(0.99)*	2.73 ± 0.10 *(0.99)*
	UBA	09/11/01	3.57 ± 0.24	3.57 ± 0.11 *(0.98)*	3.41 ± 0.14 *(0.98)*
		06/12/01	2.68 ± 0.18 [e]	2.72 ± 0.07 *(0.99)*	2.56 ± 0.12 *(0.99)*
	CRECEP	20/12/01	2.57 ± 0.13 [f]	2.64 ± 0.04 *(0.99)*	2.57 ± 0.07 *(0.99)*
		31/05/02	2.68 ± 0.22 [f]	2.81 ± 0.04 *(0.99)*	2.68 ± 0.09 *(0.99)*
PVC-R pipe (4)	TW	13/11/02	2.88 ± 0.04	2.86 ± 0.07 *(0.99)*	2.88 ± 0.08 *(0.99)*
	UBA	09/11/01	3.37 ± 0.26	3.27 ± 0.11 *(0.98)*	3.12 ± 0.15 *(0.97)*
		06/12/01	2.70 ± 0.17	2.71 ± 0.06 *(0.99)*	2.64 ± 0.09 *(0.99)*
	CRECEP	20/12/01	2.64 ± 0.09 [f]	2.69 ± 0.04 *(0.99)*	2.60 ± 0.06 *(0.99)*
PVC-C pipe (5)	TW	13/11/02	2.58 ± 0.01 [e]	2.61 ± 0.07 *(0.99)*	2.55 ± 0.10 *(0.98)*
	UBA	09/11/01	3.49 ± 0.03	3.44 ± 0.09 *(0.99)*	3.22 ± 0.15 *(0.98)*
		06/12/01	2.73 ± 0.15	2.72 ± 0.05 *(0.99)*	2.74 ± 0.10 *(0.99)*
	CRECEP	20/12/01	2.45 ± 0.18 [g]	2.53 ± 0.04 *(0.99)*	2.45 ± 0.09 *(0.99)*
EPDM rubber hose (6)	TW	13/11/02	2.70 ± 0.14	2.76 ± 0.05 *(0.99)*	2.70 ± 0.07 *(0.99)*
	UBA	09/11/01	3.56 ± 0.25 [e]	3.40 ± 0.11 *(0.98)*	3.25 ± 0.15 *(0.97)*
		6/12/01	2.85 ± 0.10	2.78 ± 0.04 *(0.99)*	2.81 ± 0.07 *(0.98)*
	CRECEP	31/05/02	2.70 ± 0.05	2.71 ± 0.03 *(0.99)*	2.70 ± 0.05 *(0.99)*
$K_2Cr_2O_7$ (2 mg/L)	TW	14/12/01	0.07 [d]	0.03 ± 0.01 *(0.92)*	0.05 ± 0.05 *(0.65)*
	UBA		ND	ND	ND
	CRECEP		ND	ND	ND

ND, not determined

[a] Each result is the average of 3 replicates

[b] R^2 per definition ≥ 0.98

[c] Rf (robustness factor) was calculated automatically by Graph Prism software (second method). It replaces the usual R^2 as a witness of goodness of fit.

[d] 1 replicate, of which one data point was omitted (30 minutes)

[e] 2 replicates

[f] 5 replicates

[g] 6 replicates

Note: CRECEP and TW observed cell growth of 86 ± 9.7 % (20/12/01) and 76.7 ± 6.8 % (31/5/02), respectively, during incubation of the blank; UBA did not measured this parameter.

Table 22 Percentages of RNA synthesis for materials 4, 5 and 6 calculated by the three methods.

Material	Institute	Date	% of RNA synthesis		
			First method French	Second method Graph Pad	Third method Excel
PVC-R pipe (4)	TW	13/11/02	105 ± 2	100 ± 2	105 ± 3
	UBA	9/11/01	94 ± 8	92 ± 3	91 ± 4
		06/12/01	101 ± 6	99 ± 2	103 ± 3
	CRECEP	20/12/01	103 ± 4	102 ± 1	101 ± 2
PVC-C pipe (5)	TW	13/11/02	95 ± 1	92 ± 3	93 ± 4
	UBA	9/11/01	98 ± 1	96 ± 3	94 ± 4
		06/12/01	102 ± 6	100 ± 2	107 ± 4
	CRECEP	20/12/01	95 ± 7	96 ± 2	95 ± 3
EPDM rubber hose (6)	TW	13/11/02	99 ± 5	97 ± 2	99 ± 3
	UBA	9/11/01	100 ± 7	95 ± 3	95 ± 4
		06/12/01	104 ± 3	102 ± 2	110 ± 3
	CRECEP	31/05/02	101 ± 1	97 ± 1	101 ± 2

The percentages of RNA synthesis of the leachates were expressed in relation to the blank: (average slope value of the leachate/average slope value of the blank)/100. The percentage of RNA synthesis of the blank is fixed at 100 % (total absence of toxicants).

Table 23 The linear regression parameters for materials 7 and 8 calculated by the three methods.

Material	Institute	Date	Slope a (cpm) ± SD *(R^2 ou Rf)*		
			First method French[e]	Second method Graph Pad[f]	Third method Excel
Blank	TW	30/10/02	3 red tubes	3 red tubes	3 red tubes
	UBA	11/9/02	2.11 ± 0.11[c]	2.14 ± 0.03 *(0.99)*	2.10 ± 0.07 *(0.99)*
		06/9/02	3.26 ± 0.06[c]	2.27 ± 0.05 *(0.98)*	1.98 ± 0.22 *(0.99)*
		20/9/02	3.15 ± 0.24	3.10 ± 0.15 *(0.97)*	3.04 ± 0.21 *(0.99)*
	CRECEP	13/08/02	2.94 ± 0.19[c]	2.90 ± 0.05 *(0.99)*	2.74 ± 0.09 *(0.98)*
		21/08/02	3.12 ± 0.05[c]	2.90 ± 0.01 *(0.96)*	2.59 ± 0.21 *(0.98)*
PE-Xc pipe (7)	TW	30/10/02	*	*	*
	UBA	06/9/02	2.15 ± 0.05	2.19 ± 0.03 *(0.99)*	2.15 ± 0.05 *(0.99)*
		20/9/02	2.93 ± 0.24	2.78 ± 0.08 *(0.99)*	2.82 ± 0.11 *(0.99)*
	CRECEP	13/08/02	3.16 ± 0.15[d]	3.13 ± 0.05 *(0.99)*	2.89 ± 0.11 *(0.98)*
		21/08/02	3.20 ± 0.08[d]	3.06 ± 0.08 *(0.98)*	2.72 ± 0.11 *(0.95)*
NRB (8)	TW	30/10/02	*	*	*
	UBA	11/9/02	1.84 ± 0.07	1.86 ± 0.02 *(0.99)*	1.84 ± 0.05 *(0.99)*
	CRECEP	13/08/02	0.53 ± 0.43[d]	0.23 ± 0.09 *(0.70)*	2.45 ± 0.13 *(0.99)*
		21/08/02	0.17 ± 0.41[d]	0.00 ± 0.00 *(0.98)*	0.01 ± 0.00 *(0.88)*
$K_2Cr_2O_7$ (2 mg/L)	TW	30/10/02	*	*	*
	UBA		ND	ND	ND
	CRECEP	13/08/02	1.42 ± 0.53	1.11 ± 0.08 *(0.96)*	1.42 ± 0.20 *(0.99)*
		21/08/02	1.67 ± 0.08	1.60 ± 0.03 *(0.99)*	1.63 ± 0.05 *(0.99)*
		01/10/02**	0.08 ± 0.1	ND	ND

Each result is the average of 3 replicates . Otherwise : [a] 1 replicate. [b] 2 replicates . [c] 5 replicates. . [d] 6 replicates

[e] R^2 per definition ≥ 0.98

[f] Rf (robustness factor) was calculated automatically by Graph Prism software (second method). It replaces the usual R^2 as a witness of goodness of fit.

ND : Not determined

*For TW. no value was established because of the blanks (3 red tubes)

** These results were obtained from the investigation on leachate storage (see Table 9)

Note: CRECEP and TW observed cell growth of 68 ± 16 % (13/08/02) and 97 ± 6 % (20/08/02) respectively during incubation of the blank; UBA did not measured this parameter.

Table 24 Percentages of RNA synthesis for materials 7 and 8 calculated by the three methods.

Material	Institute	Date	% of RNA synthesis		
			First method French	Second method Graph Pad	Third method Excel
PE-Xc pipe (7)	TW	30/10/02	*	*	*
	UBA	6/09/02	95 ± 3	96 ± 1	108 ± 2
		20/09/02	93 ± 8	89 ± 2	93 ± 4
	CRECEP	13/08/02	107 ± 5	108 ± 2	105 ± 4
		21/08/02	102 ± 2	105 ± 3	105 ± 4
NRB (8)	TW	30/10/02	*	*	*
	UBA	11/09/02	88 ± 3	87 ± 1	88 ± 2
	CRECEP	13/08/02	18 ± 15	0	2 ± 0.1
		21/08/02	0	0	0
		01/10/02	2.8 ± 4	ND	ND

The percentages of RNA synthesis of the leachates were expressed in relation to the blank: (average slope value of the leachate/average slope value of the blank)/100. The percentage of RNA synthesis of the blank is fixed at 100 % (total absence of toxicants).

* For TW, no value was established because of the blanks (3 red tubes)

Table 25 The linear regression parameters for materials 9, 10 and 11 calculated by the three methods.

Material	Institute	Date	Slope a (cpm) ± SD *(R^2 ou Rf)*		
			First method French[e]	Second method Graph Pad[f]	Third method Excel
Blank	UBA	?	2.79 ± 0.17	2.87 ± 0.08 *(0.99)*	2.78 ± 0.12 *(0.99)*
		29/1/03	2.71 ± 0.05	2.72 ± 0.03 *(0.99)*	2.71 ± 0.05 *(0.99)*
		31/1/03	2.24 ± 0.12	2.19 ± 0.36 *(0.99)*	2.24 ± 0.06 *(0.99)*
		13/2/03	2.45 ± 0.17	2.50 ± 0.10 *(0.97)*	2.45 ± 0.14 *(0.99)*
	CRECEP	29/11/02	3.00 ± 0.17[d]	2.99 ± 0.05 *(0.99)*	2.93 ± 0.07 *(0.99)*
		6/12/02	3.29 ± 0.08[c]	3.23 ± 0.07 *(0.99)*	3.10 ± 0.09 *(0.98)*
		20/12/02	3.05 ± 0.17[c]	3.20 ± 0.04 *(0.99)*	3.05 ± 0.08 *(0.99)*
Stainless steel (9)	UBA	?	2.42 ± 0.13	2.32 ± 0.07 *(0.98)*	2.42 ± 0.12 *(0.99)*
	CRECEP	20/12/02	3.02 ± 0.18[d]	3.04 ± 0.05 *(0.99)*	2.94 ± 0.07 *(0.99)*
NRB (10)	UBA*	29/1/03	0.03 ± 0.03	0 *(0.86)*	0.03 ± 0.01 *(0.99)*
		31/1/03	0.19 ± 0.13	0.12 ± 0.02 *(0.93)*	0.19 ± 0.05 *(0.99)*
		13/2/03	2.68 ± 0.43	2.39 ± 0.13 *(0.96)*	2.68 ± 0.21 *(0.99)*
	CRECEP	29/11/02	2.53 ± 0.29[c]	2.51 ± 0.07 *(0.97)*	2.35 ± 0.13 *(0.99)*
		6/12/02	2.99 ± 0.10[d]	2.98 ± 0.05 *(0.99)*	2.70 ± 0.10 *(0.98)*
EPDM O seal (11)	UBA*	29/1/03	2.50 ± 0.59	2.13 ± 0.11 *(0.97)*	2.49 ± 0.26 *(0.99)*
		31/1/03	2.18 ± 0.13	2.17 ± 0.1 *(0.96)*	1.82 ± 0.25 *(0.99)*
		13/2/03	2.78 ± 0.13	2.80 ± 0.08 *(0.99)*	3.02 ± 0.17 *(0.99)*
	CRECEP	29/11/02	0[d]	0 *(0.99)*	0 *(0.98)*
		6/12/02	2.50 ± 0.60[b]	2.6 ± 0.1 *(0.97)*	2.34 ± 0.20 *(0.99)*
		20/12/02	2.07 ± 0.26	2.09 ± 0.09 *(0.97)*	2.07 ± 0.13 *(0.99)*
$K_2Cr_2O_7$ (2 mg/L)	UBA		ND	ND	ND
	CRECEP	6/12/02	0.96 ± 0.07	0.93 ± 0.03 *(0.98)*	0.95 ± 0.03 *(0.99)*
		20/12/02	1.61 ± 0.15	1.59 ± 0.04 *(0.99)*	1.60 ± 0.06 *(0.99)*

Each result is the average of 3 replicates . Otherwise : [a] 1 replicate. [b] 2 replicates . [c] 5 replicates. . [d] 6 replicates

[e] R^2 per definition ≥ 0.98

[f] Rf (robustness factor) was calculated automatically by Graph Prism software (second method). It replaces the usual R^2 as a witness of goodness of fit.

ND : Not determined

***** The leachate was prepared on 29/1/03 and kept at 4°C for 15 days. Cytotoxicity assessments were performed immediately and after 2 and 15 days long of storage.

Note : CRECEP observed cell growth of 73 ± 10 % (20/12/02). 62 ± 15 % (29/11/02) and 60 ± 7 % (6/12/02) during incubation of the blank; UBA did not measured this parameter.

Table 26 Percentages of RNA synthesis for materials 9. 10 and 11 calculated by the three methods.

Material	Institute	Date	% of RNA synthesis		
			First method French	Second method Graph Pad	Third method Excel
Stainless steel	UBA	?	87 ± 5	81 ± 3	87 ± 4
(9)	CRECEP	20/12/02	99 ± 6	95 ± 2	96 ± 2
NRB	UBA	29/01/03	0	0	0
(10)		31/01/03	8 ± 6	0	8 ± 2
		13/02/03	110 ± 17	96 ± 5	109 ± 8
	CRECEP	29/11/02	84 ± 10	84 ± 2	80 ± 4
		6/12/02	91 ± 3	92 ± 2	87 ± 3
EPDM O seal	UBA	29/01/03	92 ± 22	78 ± 4	92 ± 10
(11)		31/01/03	97 ± 6	99 ± 5	81 ± 11
		13/02/03	113 ± 5	112 ± 3	123 ± 7
	CRECEP	29/11/02	0	0	0
		6/12/02	76 ± 18	80 ± 3	75 ± 6
		20/12/02	68 ± 9	65 ± 3	68 ± 4

The percentages of RNA synthesis of the leachates were expressed in relation to the blank: (average slope value of the leachate/average slope value of the blank)/100. The percentage of RNA synthesis of the blank is fixed at 100 % (total absence of toxicants).

Table 27 The linear regression parameters for materials 12 and 13 calculated by the three methods.

Material	Date	Slope a (cpm) ± SD *(R^2 ou Rf)*		
		First method French[e]	Second method Graph Pad	Third method Excel
Blank	20/12/02	3.05 ± 0.17[c]	3.20 ± 0.04 *(0.99)*	3.05 ± 0.08 *(0.99)*
	28/03/03	3.23 ± 0.06	3.25 ± 0.07 *(0.99)*	3.23 ± 0.1 *(0.99)*
Organic cementitious (12)	28/03/03	2.90 ± 0.30[d]	2.99 ± 0.04 *(0.99)*	2.85 ± 0.07 *(0.99)*
		2.98 ± 0.09[d]	2.99 ± 0.03 *(0.99)*	2.86 ± 0.08 *(0.99)*
EPDM rubber washer (13)	20/12/02	2.54 ± 0.03[d]	2.57 ± 0.03 *(0.99)*	2.54 ± 0.03 *(0.99)*
$K_2Cr_2O_7$ (2 mg/L)	20/12/02	1.61 ± 0.15	1.59 ± 0.04 *(0.99)*	1.61 ± 0.06 *(0.99)*

Each result is the average of 3 replicates . Otherwise : [a] 1 replicate. [b] 2 replicates . [c] 5 replicates. . [d] 6 replicates

[e] R^2 per definition ≥ 0.98

Rf (robustness factor) was calculated automatically by Graph Prism software (second method). It replaces the usual R^2 as a witness of goodness of fit.

Note: CRECEP observed cell growth of 73 ± 10 % (20/12/02) and 49 ± 4.7 % (28/03/03) during incubation of the blank. CRECEP only investigated these two materials.

Table 28 Percentages of RNA synthesis for materials 12 and 13 calculated by the three methods.

Material	Date	% of RNA synthesis		
		First method French	Second method Graph Pad	Third method Excel
Organic cementitious (12)	28/03/03	90 ± 9	91 ± 1	88 ± 2
		92 ± 3	91 ± 1	88 ± 2
EPDM rubber washer (13)	20/12/02	84 ± 1	80 ± 1	83 ± 1

The percentages of RNA synthesis of the leachates were expressed in relation to the blank: (average slope value of the leachate/average slope value of the blank)/100. The percentage of RNA synthesis of the blank is fixed at 100 % (total absence of toxicants).

An overview of the results for different materials analysed in WP2 is given in Table 29, including also the findings obtained with the first tested materials (stage 2, materials 1, 2, 3). The percentages of RNA synthesis for the 13 selected materials are reported associated with their respective SD in Table 30.

Table 29 Assessment of cytotoxicity of all the tested materials using the three ways of calculation. (French pass / fail criteria: 70% of RNA synthesis)

Sample	S/V ratio (cm^2/L)	UBA	TW	CRECEP
Material 1 EPDM seal type 1	4.24	non-cytotoxic	non-cytotoxic	non-cytotoxic
Material 2 *EPDM seal type 2*	4.24	non-cytotoxic	non-cytotoxic	non-cytotoxic
Material 3 PE-X pipe	240	non-cytotoxic	non-cytotoxic	non-cytotoxic
Material 4 PVC-R pipe	398	non-cytotoxic	non-cytotoxic	non-cytotoxic
Material 5 PVC-C pipe	240	non-cytotoxic	non-cytotoxic	non-cytotoxic
Material 6 EPDM rubber hose	315	non-cytotoxic	non-cytotoxic	non-cytotoxic
Material 7 PE-Xc pipe	470	non-cytotoxic	*	non-cytotoxic
Material 8 Nitrile rubber O seal	200	non-cytotoxic	*	cytotoxic
Material 10 Nitrile rubber O seal	200	cytotoxic non-cytotoxic	nd	non-cytotoxic
Material 11 EPDM O seal	319	non-cytotoxic	nd	cytotoxic non-cytotoxic
Material 13 EPDM rubber washer	1064	nd	nd	non-cytotoxic
Material 9 Stainless steel	1140	non-cytotoxic	nd	non-cytotoxic
Material 12 Organic cementitious	740	nd	nd	non-cytotoxic

Table 30 Comparison between the three methods of calculation

Material	Date	RNA synthesis French calculation %	RNA synthesis GraphPad Calculation %	RNA synthesis Excel Calculation
(1) EPDM seal type 1	14/12/01	122	110 ± 9	97 ± 15
	12/07/01	98 ± 2	96 ± 4	87 ± 8
	31/05/01	103 ± 6	101 ± 2	100 ± 2
(2) EPDM seal type 2	14/12/01	90 ± 4	89 ± 4	97 ± 8
	12/07/01	84 ± 7	81 ± 3	84 ± 4
	31/05/01	104 ± 4	103 ± 2	102 ± 2
(3) PE-X pipe	14/12/01	85 ± 9	87 ± 6	94 ± 11
	12/07/01	87 ± 4	84 ± 2	81 ± 4
	31/05/01	102 ± 4	117 ± 2	102 ± 2
(4) PVC-R pipe	13/11/02	105 ± 2	100 ± 3	105 ± 3
	9/11/01	94 ± 8	92 ± 3	91 ± 4
	6/12/01	101 ± 6	99 ± 2	103 ± 3
	20/12/01	103 ± 4	102 ±1	101 ± 2
(5) PVC-C pipe	13/11/02	95 ± 1	92 ± 3	93 ± 4
	9/11/01	98 ± 1	96 ± 3	94 ± 4
	6/12/01	102 ± 6	100 ± 2	107 ± 4
	20/12/01	95 ± 7	96 ± 2	95 ± 3
(6) EPDM rubber hose	13/11/02	99 ± 5	97 ± 2	99 ± 3
	9/11/01	100 ± 7	95 ± 3	95 ± 4
	6/12/01	104 ± 3	102 ± 2	110 ± 3
	31/05/02	101 ± 1	97 ± 1	101 ± 2
(7) PE-Xc pipe	30/10/02	*	*	*
	6/09/02	95 ± 3	96 ± 1	108 ± 2
	20/09/02	93 ± 8	89 ± 2	93 ± 4
	13/08/02	107 ± 5	108 ± 2	105 ± 4
	21/08/02	102 ± 2	105 ± 3	105 ± 4
(8) Nitrile rubber O seal	30/10/02	*	*	*
	11/09/02	88 ± 3	87 ± 1	88 ± 2
	13/08/02	18 ± 15	0	2 ± 0.1
	21/08/02	0	0	0
(9) Stainless steel	?	87 ± 5	81 ± 3	87 ± 4
	20/12/02	99 ± 6	95 ± 2	96 ± 2
(10) Nitrile rubber O seal	?	0	0	0
	?	8 ± 6	0	8 ± 2
	?	110 ± 17	96 ± 5	109 ± 8
	29/11/02	84 ± 10	84 ± 2	80 ± 4
	6/12/02	91 ± 3	92 ± 2	87 ± 3
(11) EPDM O seal	?	92 ± 22	78 ± 4	92 ± 10
	?	97 ± 6	99 ± 5	81 ± 11
	?	113 ± 5	112 ± 3	123 ± 7
	29/11/02	0	0	0
	6/12/02	76 ± 18	80 ± 3	75 ± 6
	20/12/02	68 ± 9	65 ± 3	68 ± 4
(12) Organic cementitious	28/03/03	90 ± 9	91 ± 1	88 ± 2
	28/03/03	92 ± 3	91 ± 1	88 ± 2
(13) EPDM rubber washer	20/12/02	84 ± 1	80 ± 1	83 ± 1

TW results: in black. UBA results: in blue. Crecep results: in green

* no results: no determination of the % of RNA synthesis as the blank was invalidated (3 red tubes)

As the experiments for the same material, were not carried out the same day (or in a short time delay) in the three institutes, inter-laboratory comparison of the results was not easy.

Some materials do not have a stabilised formulation and some others can release unstable or volatile compounds or be contaminated by inadequate storage conditions. In fact, over a long period storage, different events can occur and can either modify the properties of the tested material or lead to its contamination by undesirable substances.

Materials 1, 2, 3, 4, 5, 6, 7 and 9 did not behave differently in the laboratories. The percentage of RNA synthesis was in the range of 81 to 122, indicating the non-cytotoxicity of the leachates. Those materials complied with the French acceptance criterion (> 70%). For instance, percentages of RNA synthesis in the HeLa cells were 100 and 104 % (for UBA), 99 % (for TW) and 101 % (for CRECEP) after exposure to material 6 leachate (first mode of calculation).
When these various materials were tested twice or more in independent experiments in the same laboratory, very low RSD values were found (first mode of calculation; Table 31 and Table 32). For instance, material 7 gave an RSD of 1.5 % and 3.38 % in UBA and in CRECEP, respectively.

Table 31 Relative standard deviation for materials 4, 5, 6 and 7 in UBA (first mode of calculation)

Material	UBA		
	Average (%)	SD (%)	RSD (%)
4 : PVC-R pipe	98	4.95	5.08
5 : PVC-C pipe	100	2.83	2.83
6 : EPDM rubber hose	102	2.83	2.77
7 : PE-Xc pipe	94	1.41	1.5

Table 32 Relative standard deviation for material 7 in CRECEP (first mode of calculation)

Material	CRECEP		
	Average (%)	SD (%)	RSD (%)
7 : PE-Xc pipe	104	3.54	3.38

The main objective of stage 3 was to test the inter-laboratory reproducibility of the harmonised protocol using a number of selected materials. From these percentages of RNA synthesis, the reproducibility of the test, defined as the coefficient of variation of reproducibility VC_R, was calculated. These VC_R values ranged from 1.52 % to 141.42 % with a median value of 10.52 % (first method), from 0.82 % to 141.42 % with a median value of 11.25 % (second method), from 1.74 % to 138.24 % with a median value of 7.32 % (third method) (see Table 33 to Table 35).

The lowest VC_R values were obtained for EPDM rubber hose (1.52 % first method, 0.87 % second method, 1.74 % third method). PVC-C pipe (2.99 % first method, 3.20 % second method, 4.04 % third method) and PVC-R pipe (4.22 % first method, 3.36 % second method, 3.96 % third method).

Regarding NRB, statistical analysis revealed that the VC_R values for this material were the highest. Two NRB materials, provided by the same supplier, were tested: material 8 and material 10. The first NRB (material 8) was sent by TW on July 2002, the second one (material 10) arrived at the laboratory on November 2002. Material 8 was considered cytotoxic by CRECEP in three independent experiments and non-cytotoxic by UBA (only one experiment) (VC_R = 141.42 % first and second methods, 138.24 % third method).

Table 33 Percentages of RNA synthesis of selected materials (first mode of calculation). NR, no results; ND, not determined

Material	% RNA synthesis TW	% RNA synthesis UBA	% RNA synthesis Crecep	Average	SD	VC_R (%)
				107.67		
EPDM seal type 1	122	98	103		12.66	11.76
EPDM seal type 2	90	84	103	92.33	9.71	10.52
PE-X pipe	85	85	102	90.67	9.81	10.83
				102.17		
PVC-R pipe	106	97.5	103		4.31	4.22
PVC-C pipe	95	100	95	96.67	2.89	2.99
				100.67		
EPDM rubber hose	99	102	101		1.53	1.52
PE-Xc pipe	NR	94	105	99.50	7.78	7.82
						141.42
NRB (1)	NR	88	0	44.00	62.23	
Stainless steel	ND	87	99	93.00	8.49	9.12
NRB (2)	ND	39	88	63.65	34.44	54.10
EPDM O seal	ND	101	48	74.50	37.48	50.30

Table 34 Percentages of RNA synthesis of selected materials (second mode of calculation) NR, no results; ND, not determined

Material	% RNA synthesis TW	% RNA synthesis UBA	% RNA synthesis Crecep	Average	SD	VC_R (%)
EPDM seal type 1	110	96	101	103.30	7.09	6.93
EPDM seal type 2	89	81	103	91.00	11.13	12.24
PE-X pipe	87	84	117	96.00	18.25	19.00
PVC-R pipe	100	96	102	99.17	3.33	3.36
PVC-C pipe	92	98	96	95.33	3.05	3.20
EPDM rubber hose	97	98	97	97.50	0.87	0.89
PE-Xc pipe	NR	92	106	99.50	9.90	9.95
NRB (1)	NR	87	0	43.50	61.52	141.42
Stainless steel	ND	81	95	98.00	9.90	11.25
NRB (2)	ND	32	88	60.00	39.60	66.00
EPDM O seal	ND	96	48	72.30	33.94	46.94

Table 35 Percentages of RNA synthesis of selected materials for third mode of calculation. NR, no results; ND, not determined

Material	% RNA synthesis TW	% RNA synthesis UBA	% RNA synthesis Crecep	Average	SD	VC_R (%)
EPDM seal type 1	97	87	100	94.67	6.81	7.19
EPDM seal type 2	97	84	102	94.33	9.29	9.85
PE-X pipe	94	81	102	92.33	10.60	11.48
PVC-R pipe	105	97	101	101.0	4.00	3.96
PVC-C pipe	93	100	95	96.17	3.88	4.04
EPDM rubber hose	99	102	101	100.83	1.75	1.74
PE-Xc pipe	NR	100	105	102.75	3.18	3.10
NRB (1)	NR	88	1	44.50	61.52	138.24

Stainless steel	ND	87	96	91.75	6.72	7.32
NRB (2)	ND	39	84	61.25	31.47	51.37
EPDM O seal	ND	102	44	73.00	41.72	57.15

So, a second investigation was carried out three or four months later with material 10 in order to confirm or invalidate the toxicity of this type of NRB. The same inter-variability was pointed out (VC_R = 54.1 % first method, 66 % second method and 51.37 % third method). CRECEP found that material 10 was not cytotoxic in two independent experiments whereas UBA detected a cytotoxicity (29/1/03). UBA went on studying the cytotoxicity of this leachate and measured it after 2 and 15 days long of storage at 4°C. The cytotoxicity in the leachate 10 decreased strongly during the storage period and no effect compared to the reference control was perceptible after 15 days (see Table 10). This tends to prove the presence of volatile and/or unstable toxic compounds in the leachate. Therefore, the variability in the cytotoxicity measurement could be caused by:

- differences in the material storage (storage time and storage conditions) leading to potential modifications in the material; no particular instructions were given concerning this topic during the project. At CRECEP, the materials were kept at room temperature, in the dark and protected from dust and contamination. The way the material must be kept before its analysis should be studied and closely defined. Furthermore, the cytotoxicity assessment should be planned between institutes in order to conduct the experiments at a given time and, consequently, to simplify the inter-laboratory comparisons.
- heterogeneity in the material production: one piece of material could differ from another.
- unstable formulation of the material. This material was tested in WP1 (microbial growth) and similar variability was observed: NRB also gave large differences in growth promoting properties (VC_R =107 %).
- absence of a washing step prior to preparation of migration water. This step is important since the initial leaching from fresh materials is rather variable and the leaching stabilizes by performing the washing procedure. In case materials do not leach cytotoxic compounds, there is no problem. On the contrary, when materials contain cytotoxic products, the absence of the washing procedure may be the cause of the variability in the results.

No toxicity was detected concerning material 11 (EPDM O seal) in experiments carried out in UBA laboratory (RSD = 10.9 %). Three independent experiments were conducted at CRECEP. The institute could not conclude to a toxic effect systematically (RSD = 87 %, first method). The same kind of hypothesis as above might be formulated, but further investigation should be conducted to conclude.

Percentages of RNA synthesis of material 12 (organic cementitious) and of material 13 (EPDM rubber washer) indicates the absence of toxicity of the corresponding leachates (90 and 84 % respectively, first method). Two different migration waters were produced from material 12, the associated results were not significantly different (RSD = 1.95 %. first method), pointing out the homogeneity of the material and the repeatability of the method.

The blanks and the positive controls were studied too. The RSD for the blanks are 16.16 % for UBA, 13.46 % for CRECEP and 7.36 % for TW. The RSD value for TW is lower but less experiments were performed by the laboratory (2 instead of 10 measures). An inter-laboratory coefficient of variation of reproducibility was calculated: VC_R = 5.69 % (first method). This low VC_R suggests a good UBA - CRECEP reproducibility of the blanks. The positive controls were subjected to the same analysis. The corresponding RSD were calculated and were reported in Table 36.

More data should be available to allow an accurate assessment of intra- and inter- variability. A VC_R of 38.79 % on the mean of the positive controls could be deduced from the results. The main factors affecting the reproducibility of the positive control are the preparation of the solutions, the conditions of storage of the toxicant and the cell fitness. Those observations suggest an improvement in the similarity of the test conditions in the three laboratories.

Table 36 Relative standard deviation of the positive controls in the three institutes (first method of calculation).

UBA		CRECEP		TW	
Data % RNA synthesis	RSD %	Data % RNA synthesis	RSD %	Data % RNA synthesis	RSD %
83 89	4.93	65 49 52 29 53	26.27	87 3	131.99

Conclusions

The main goals of stage 3 were the validation of the harmonised cytotoxicity test and the assessment of its reproducibility. The three laboratories should have tested thirteen materials but materials 12 and 13 arrived lately in the course of the project and were only investigated by CRECEP. Therefore they can't be included in the comparison assay.

The inter-calibration shows that eight materials (EPDM seals type 1 and 2, PE-X pipe, PVC-R pipe, PVC-C pipe, EPDM rubber hose, PE-Xc pipe, stainless steel) behave similarly in the three institutes. Those materials comply with the French acceptance limit of "greater than 70 %". A good intra and inter- laboratory reproducibility of the method can be pointed out with these selected materials (RSD max = 5.08 % and VC_R max = 11.7 %. first method).

Concerning materials 8, 10 (NRB) and 11 (EPDM O seal), the results vary and are dependent on the migration water (VC_R of 141.42, 54.1 and 50.3 % respectively according to first method calculation). Similar problems occurred when NRB was investigated for microbial growth. even after a washing step. Thus, the observed variability can't be due to the cytotoxicity protocol itself but might be dependent of two factors: the type of material (unstabilised formulation, heterogeneity in the production etc.) and the absence of a washing step. To confirm or reject these explanations, the same materials should be sent again and analysed at once and after a defined storage time, by the different laboratories, with or without the washing step.

Regarding the complete results. UBA and CRECEP conclude that the harmonised protocol appears to be sensitive and reliable to assess the toxicity of a CPDW.

A VC_R of 38.79 % (first method) on the mean of the positive controls indicates that further standardisation of the test is needed. The preparation of the solutions. the conditions of storage of the toxicant and the cell fitness are the main factors affecting the reproducibility of the positive control. Nevertheless. more data must be available to allow an accurate assessment of intra- and inter- variability of this control.

Three methods of calculation were applied to the data : the "French calculation". the "Graph Pad calculation" and the "Excel calculation". No particular effect on the results were detected. To treat objectively the data in any laboratory. further standardization is needed. A larger

number of materials should be analysed using the three modes of calculation and the corresponding results should be subjected to a statistical treatment.

To enhance the test reproducibility. the similarity in the test protocol should be improved. An agreement on the following topics are necessary:
- cell cultures
- storage. handling and quality of the uridine
- nature of the glassware and its cleaning
- storage of materials
- preconditioning of the material
- preparation and handling of the leachate (the pr EN 12873-1 standard might be a good basis)
- test automatization (use of 96-well microplate equipped with GF/C glass filters and direct radioactivity counting in a top count microplate reader)

Once those parameters will be settled. the validation of the test should be achieved by testing. in the first place. a wide range of chemical and/or biological toxicants and. afterwards. various CPDW. The selected CPDW should cover a large range of toxicity levels in order to assess the discriminatory power of the method. Furthermore. an adequate number of laboratories should be involved in the experiments to obtain an accurate evaluation of the inter-laboratory reproducibility of the assay.

Genotoxicity tests

Outside the scope of this project, UBA also performed a preliminary study on the use of the AMES test to check for genotoxicity effects. The same materials and protocol for preparation of the migration water were used. The summarising results are shown in Table 37 and Annex 4.

Table 37 genotoxicity of materials studied in Ames assay

	Material	Ames test results
1	EPDM seal type 1	non-genotoxic
2	EPDM seal type 2	non-genotoxic
3	PE-X pipe (black pipe)	genotoxic
4	PVC-R pipe (grey pipe)	non-genotoxic
5	PVC-C pipe (light-yellow pipe)	non-genotoxic
6	EPDM rubber hose (black hose)	non-genotoxic
7	PE-Xc pipe (white pipe)	genotoxic
8	nitrile rubber seal (O)	genotoxic
9	stainless steel	non-genotoxic
10	nitrile rubber seal (O)	genotoxic
11	EDPM rubber seal (O)	non-genotoxic

Materials 3, 7, 8 and 10 present genotoxic properties using Ames assay. Comparison with the cytotoxicity assessment lead to the following comments:
- a material can be both cytotoxic and genotoxic: materials 8 and 10 (NRB)
- a material can be only cytotoxic: material 11 (EPDM O seal)
- a material can be only genotoxic: materials 3 or 7 (PE-X pipe, PE-Xc pipe)
- a material can be both non-cytotoxic and non-genotoxic: materials 1, 2, 4, 5 …

Thus, bioassays for detecting genotoxic effects provide additionally information about the quality of materials and should be considered in the global test strategy.

GENERAL CONCLUSION

The cytotoxicity assay, selected to check the safety of CPDW, measures the inhibition of RNA synthesis in human cells in relation to that obtained with a control. Thirteen CPDW were investigated.

Eleven materials complied with the French acceptance criteria of "greater than 70 %". A good intra- and inter- laboratory reproducibility could be pointed out for eight materials (RSD max = 5.08 % and VC_R max = 11.7 % first mode of calculation; RSD max = 5.35 % and VC_R max = 19 % second mode of calculation; RSD max = 10.55 % and VC_R max = 22.6 % third mode of calculation).

Two types of materials seemed to vary depending on the migration water and, consequently, the assessment of toxicity too. This variability seems not to be due to the cytotoxicity protocol itself but mainly dependent on the nature of the material and on the absence of a washing step prior to leachate preparation. Therefore, the reproducibility of the test, defined as the coefficient of variation of reproducibility VC_R, ranged from 1.52 % to 141.42 % with a median value of 10.52 % (first mode of calculation), from 0.89 % to 141.42 % with a median value of 11.25 % (second mode of calculation) and from 1.74 % to 138.24 % with a median value of 7.32 % (third mode of calculation).

Three methods of calculation were applied to the data: the "French calculation", the "Graph Pad calculation" and the "Excel calculation". No particular effect on the results could have been detected. The behaviour of the different tested materials remained similar (same order of magnitude of the results. same materials accepted or refused) whatever the calculation mode was. An appropriate statistical technique should be selected with which it can be decided if certain data of the kinetics are outliers.

In conclusion, different materials were tested successfully by the cytotoxicity test, supporting the fact, that this test system is an useful screening test for exposures which may be cytotoxic. Moreover, the test seems to satisfy many criteria for routine use in regulatory practices. It is very sensitive, quantitative, reliable, reproducible and requires no concentration procedures of the sample to be tested (UBA and CRECEP findings). However, no robust positive material was found among the selected CPDW and positive materials are necessary to well-establish the test.

Contrarily to traditional short term tests for cellular toxicity, the present cytotoxicity test measures sublethal effects: therefore it provides sensitive means for early detection of potentially harmful compounds at sublethal cellular levels rather than after cell death. Additionally, as RNA synthesis can be inhibited by numerous mechanisms it provides a general target for multiple toxic effects. The RNA synthesis inhibition assay can be regarded as a robust safety test, keeping in mind the difficulty to extrapolate the significance of such a cellular toxicity test to a specific organ or even to a whole human being.

Though, the RNA synthesis inhibition assay requires a highly trained staff regarding cell culture, a special care to the glassware cleaning, a specific equipment and licence for the handling and disposal of radioactivity. The use of tritium does not involve a special health risk for humans as far as basic precautions are followed. But, using radioactivity is a part of the test sensitivity. Moreover no other alternative marker is available nowadays.
The validation of the test system should be achieved to include it as a CEN standard. Further investigations must be performed:

- setting a first agreement about the following topics:

  - the influencing factors of cell cultures (percentage of confluence of the monolayer cells, passage from monolayer cells to cells under suspension conditions, constant cell fitness, cell growth etc.)
  - the storage, handling and quality of uridine
  - the nature of the vessel and its cleaning
  - the test automation (use of 96-well microplate equipped with GF/C glass filters and direct radioactivity counting in a top count microplate reader)
- inter-validation of the assay with known chemical and/or biological toxicants
- setting a second agreement afterwards for material investigation:
  - the storage of the materials (conditions and time)
  - the material preconditioning before the leachate preparation
  - the preparation and handling of the leachate (negative control, temperature, agitation, S/V ratio, air/V ratio etc...)
  - the time of contact between the potential toxicant (leachate) and the cells (a longer period of contact simulates a potential chronic toxicity effect whereas a short time of contact takes only into account the accurate toxicity)
  - the best treatment of the results (mode of calculation), with a statistically design in order to treat all materials equally under regulation.
- the inter-laboratory validation of the test, by analysing a wide variety of CPDW, which cover a large range of toxicity levels.

Once the cytotoxicity assay will be presented as a CEN standard, recommendations could be made for the RG-CPDW concerning acceptance criteria for the EAS (instructions concerning materials which give percentages of RNA synthesis ranged from 70 to 100 %).

The next step would be the implementation of a set of genotoxic assays. As it was shown, at the end of this work, the detection of mutagenic effects (Ames test) provides additional information about the quality of materials.

The cytotoxicity assay associated with a suitable battery of genotoxicity tests should, in the future, be able to assess the global potential toxicity of compounds in leachates of CPDW and give a correct answer concerning the risk borne by the consumers.

ANNEXES

Annex 1 Explanation tables

Annex 2 Results materials 1, 2 and 3 with 3 calculation methods

Annex 3 Final report TW, 24 February 2003

Annex 4 Final report UBA, 21 February 2003

Annex 5 Effect of incubation time and concentration of potassium dichromate on percentage of RNA synthesis

These annexes are presented in a separate document

CYTOTOXICITY EVALUATION

RNA SYNTHESIS INHIBITION TEST

Harmonised protocol

A- CELL LINE :

1- Cell line providers

The cell line HeLa S3 is purchased from the American Type Culture Collection (HeLa S3 CCL 2.2).

2- Maintenance of the cell strain

The cells are cultivated without antibiotics as their presence could interfere with the assessment of CPDW leachate cytotoxicity. So the absence of an eventual contamination by mycoplasma must be periodically checked.

3- Monolayer cells

HeLa S3 cells are grown in a 1X medium in culture flasks at 37°C until confluent monolayers are obtained. The medium is removed and the monolayer sheet is rinced twice with PBS. The monolayer sheet is covered with a versene solution and the flask is placed at (37±1)°C until the cells can be separated by gentle shaking. Then a few ml of 1X medium are added in order to stop the reaction. The cells are homogenised by repeated pipetting and counted precisely.

4- Preservation of the cell strain

When a stock culture of the cell line is needed, it is stored in liquid nitrogen, after being preserved in the culture medium with dimethylsulfoxide (10% V/V final) or glycerol (10% V/V final).

B- PREPARATION OF THE MIGRATION WATER :

1- Pre-conditioning step

No pre-conditioning step is conducted, except for organic cementitious products which requires a special treatment according to its nature.

The material is placed upon a monolayer glass beads in a convenient glass container, then put under the "preconditioning water" (1000 ml). The container is closed with a glass cover and is incubated at 23°C during 24 hours, in darkness, without agitation (incubation n° 1). This water is removed and discarded and the material is re-immersed into the preconditioning water (1000 ml). The container is incubated again at 23°C during 24 hours (incubation n° 2)

The sequence is repeated three times (until incubation n° 5).

The preconditioning water is composed of demineralised water supplied with (222 ± 2) mg/l of $CaCl_2$ and (336 ± 2) mg/l of $NaHCO_3$ (the pH was adjusted to 7,4 ± 0,1).

2- Preparation of leachates

* Organic and metallic materials :

1000 ml of reference water (pyrodistilled water for TW and CRECEP, purified water for UBA) are introduced into borosilicated glass jars. The needed number of test pieces are, then, added. After closing with inert lids, the test containers are incubated for 24 hours at 23°C in darkness, without agitation.

* Cementitious material
The material is placed upon a monolayer glass beads in a convenient glass container and put under mineral water (1000 ml). The container is closed with a glass cover and incubated at 23°C during 24 hours, in darkness, without agitation.
The mineral water should fulfil the following recommendations:

- conductivity = 50µS/cm
- pH = 8 ± 0,2
- oxidability to $KmnO_4$ < 0,5 mg/l O_2
- TOC < 0,5 mg/l C
- TAC = 5 ± 0,1 °F
- silice = 25 -30 mg/l SiO_2

Once the migration water is prepared, it should be immediately investigated in cytotoxicity.

C- CYTOTOXICOLOGICAL EVALUATION :

1- Definitions :

- *Blank/ Reference control* :
It consists of the cytotoxicity assessment of a culture medium prepared with reference water.

- *Negative control* :
The negative leachate is prepared by introducing 1000 ml of reference water in a borosilicated glass jar and by incubating the flask at 23°C for 24 hours, in darkness without shaking.
The negative control consists, therefore, of the cytotoxicity assessment of a culture medium prepared with the negative leachate.

- *Positive control* :
It consists of the cytotoxicity assessment of a culture medium prepared with a standard solution of potassium dichromate (final concentration : 2 mg/L).
Note : a fresh solution of potassium dichromate should be prepared every time a cytotoxicity test has to be performed. The standard solution must be kept at room temperature.

- *Sample evaluation* :
It consists of the cytotoxicity assessment of a culture medium prepared with a material leachate.

2- Preparation of the different culture media :

- *blank/Reference media*
The reference water is delivered into 50ml centrifuge tubes on the basis of 21,25 ml per tube. 5 ml of 5,25X medium is added to each tube. The medium is then sterilized through a single use syringe fitted with a filter into a second sterile tube.

- *positive control media*

The standard solution of potassium dichromate is sampled into 50ml centrifuge tubes on the basis of 21,25 ml per tube. 5 ml of 5,25X medium is added to each tube, the final concentration of potassium dichromate must be of 2mg/L. The medium is then sterilized through a single use syringe fitted with a filter into a second sterile tube.

- *negative and sample media*
The negative or material leachate are distributed into 50ml centrifuge tubes on the basis of 21,25 ml per tube. 5 ml of 5,25X medium is added to each tube. The medium is then sterilized through a single use syringe fitted with a filter into a second sterile tube.

3- Incubation of the HeLa S3 cells with the different media :

After dispersion of the cells from confluent monolayer cells, the cell viability * and the cell number are assessed. Then the cell suspension is dispensed into 50ml centrifuge tubes on the basis of 12.10^6 cells per tube. The cells are centrifuged at 300g, 5 min. The supernatant is discarded and the cells re-suspended in 20 ml of either the sample media, the positive control media, the negative control media or the blank media, giving final concentration of about 600.000 cells/ml. 3 x 5 ml of this cellular suspension are transferred into three glass tubes with a bar magnet. The tubes are closed tightly and incubated for 21 h ± 2 at 37°C, under agitation. The agitation must be homogenous from one tube to another and must be adapted so that the cells are maintained in suspension without any alteration of their integrity.

* The cell viability is determined by microscopic observation, after cell staining. The cell staining can be carried out as described below :

Trypan blue 0,4% in PBS 1X	250µl
PBS 1X	200µl
Cell suspension	50µl.

4- Preparation of the chromatography paper

This should be done prior to the kinetics to allow the paper to dry.

The chromatography paper sheets are cut into 4 pieces (23x28,5 cm).Rectangles (h=3 cm ; b=1,5cm) are drawn on each piece of paper with a soft pencil. They are then soaked with a SDS 3% P/V solution either with a pipette or by immersion into a tray containing the SDS solution.

*preparation of paper for measuring the total radioactivity (TR)
Another sheet of paper is prepared in the same way , but without SDS treatment. Rectangles (h= 3 cm and b = 2 cm)are drawn on this sheet.

5- Kinetics of incorporation of the uridine

The tubes appearance is checked in order to detect an obvious culture problem (red tubes, white clumps…) before performing the kinetics.

Sterile materials must be used even if the experiment is conducted outside the sterile work area. Because of the use of radioactive tracer, the work surface must be protected from radioactive contamination with either aluminium foil or BENCHKOTE type paper.

Each 3MM paper sheet, prepared as described previously, is folded to avoid the spot area to be in contact with the work area :

The incubation tubes are removed from the magnetic stirrer and each cell suspension is homogenised with a syringe and needle. 500µl of each cell suspension is transferred with a micropipette into a 6ml propylene tube. The tubes are closed and put into a water bath at (37 ± 0,5°C). The required volume of uridine (1,29 1,85 TBq/mmol, 37MBq/ml) is taken under sterile conditions and put into a tube. 3µl of uridine are introduced in the first 6 ml tube and the chronometer is started up. The introduction of the uridine into the subsequent tubes is performed successively at equal time ranges using the same micropipette tip. The time range is a function of the total number of tubes and of the experience of the operator. The kinetics are performed by spotting 40 µl from each tube onto 3MM pretreated paper sheet at 5, 10, 15, 20, 25 and 30 minutes.
In order to determine the total radioactivity (TR) introduced in the different tubes, two samples (40µl) are taken from each tube, at the end of the kinetics, and are spotted onto the 3MM non pre-treated sheet.

6- Observation of the cells after the incubation :

After the kinetics, an aliquot fraction of the cell suspension is removed from every blank. The aspect of the cells is examined under a microscope and the cells are counted with an automatic counter or an heamocytometer. An ideal growth of at least 50% is expected, but this issue is subject to changes. The percentage of cell viability is, also, assessed after staining with Trypan blue. An acceptance limit of 80 % of viable cells has been set.

7- Measurements of the RNA synthesis

When the spots are dry, the chromatography sheets is put into the descending chromatography tank. The descending chromatography is realised in TCA with a migration time of approximately 1h30 to 2h. After migration, the spot area should be free from any trace of medium. The sheets are removed from the tank. The spot area of each sheet is cut and immersed into a glass dish containing ethanol, for 10 to 15 min. Then the paper is air-dried or dried using a heater. Each rectangle is cut and introduced into a scintillation vial on the same manner, the spot side being outside. The scintillation vial is then filled up with the scintillation liquid and closed. The radioactivity is assessed by a scintillation counter.

8- Graphic exploitation of the results

- *generalities* :
The excel table (*annex n*° 01) is used to calculate the percentage of RNA synthesis. The count obtained for each tube is reported on the Excel table by referring to the serial number you attributed to it.
The two counts of total radioactivity (TR) obtained per tube is averaged for total radioactivity evaluation. Each count of the kinetic is then recalculated towards the average of the total radioactivity which is equal to 100 cpm. Thus corrected values are obtained which represents the quantity of uridine incorporated into the cells by comparison to the 100 cpm initially introduced in each tube.
For each kinetic, the six corrected values are used to draw the straight regression line of best fit (only five values are sometimes taken into account if one of them turns out to be aberrant). The equation of each straight regression line : $y=ax+b$, and its correlation coefficient R^2 are determined.

- *validity of the test* :
The following conditions must be respected to satisfactory cytotoxicity test performance :

♦ regarding the blank, positive control and samples :

The three conditions below are simultaneously required before taking into account any of the straight regression line obtained :

- the ordinate on the origin (b). must be negative
- the coefficient of correlation R^2 must be greater than 0.98.
- At least two of the three values of the slopes (a) must be in the same accuracy range. If one slope appears to be outside this accuracy range, it is rejected and the mean slope only corresponds to two resulting values.

♦ regarding the blank :

An additional criteria must be respected :

- the slope (a) must be greater than 1,75

If the previous criteria are met, the mean slope of the straight lines of best fit of the blank is calculated, and an arbitrary value of 100% is attributed to this average.
The mean slopes of the straight lines of best fit of the positive control and the samples are calculated and each mean value is compared to that obtained for the blank. The results are expressed as a percentage of RNA synthesis in relation to the blank.

9- Interpretation of the results :

If the percentage obtained for the sample by comparison to those obtained for the blank is less than 70%, the material is not accepted (French standard acceptance criteria). If event, another cytotoxicity test has to be conducted in order to confirm (or not) the first result obtained. This threshold of 70 % should be discussed in the future.

Annex 7 Results materials 4, 5 and 6 with 3 calculation methods

Annex 8 Results materials 7 and 8 with 3 calculation methods

Annex 9 Results materials 9, 10 and 11 with 3 calculation methods

Annex 10 Results materials 12 and 13 with 3 calculation methods

Annex 11

Annex 12

These annexes are presented in a separate document