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NORMAN Collaborative Trial on Bioassays for Genotoxicity Testing

KWR

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Summary

The aim of the interlaboratory study was to explore the performance of different bioassays for genotoxicity and related mechanisms and to generate communication, discussion and inspiration within the NORMAN network on the use of bioassays that detect (potential) genotoxicity of mixtures of chemicals. In the end, 18 of 24 registered participants blindly tested samples prepared and shipped by KWR. Samples contained a mixture of three genotoxic chemicals from different classes of compounds; polycyclic aromatic hydrocarbons, aromatic amines, and a pesticide precursor. The samples were dissolved in either sewage treatment plant (STP) effluent or dimethyl sulfoxide (DMSO). Samples in the STP effluent represented realistic environmental water samples, while the DMSO mixtures represented concentrates thereof.

Participants were encouraged to use their in-house assays and analysis methods to test samples. As a result, there was great variety in the number and variation on the assays tested. Overall, more than half of participants used the Ames Test (10 out of 18 participants), which resulted in the most variation and largest number of samples tested with the Ames (~600 replicates including different dilutions). For all assays, the sample representing a water DMSO concentrate including 3 genotoxic substances at ~10^5 times the (provisional health-based) guideline value [(p)GLV] triggered the most positive responses, followed by the sample representing a water DMSO concentrate with the same genotoxic substances at ~10^3x (p)GLV. No consistent difference in assay response was observed between the samples of STP effluent, regardless of the absence or presence of the 3 genotoxic substances at 10x (p)GLV.

Some participants observed interference in the Ames, SOS-Chromo, UMU-Chromo and Comet assays from the DMSO solvent in the NORMAN-KWR-DMSO-Blank sample, which calls into question the results for the samples dissolved in DMSO for those assays. In the future, the suitability of solvent for use in particular assays should be considered.

It was outside of the scope of this report to investigate in detail the effects of other variables on the test data, including the choice of positive and negative controls, dilution, pretreatment, choice of organism, strain or detection method within an assays. These variables can be analyzed in follow-up research to contribute to the growing body of research of the use of bioassays for detecting genotoxicity and related mechanisms.

Contents

1 The NORMAN collaborative trial on bioassays for genotoxicity

1.1 Set-up of the collaborative trial

Inspired by ongoing discussions and questions about bioassays for genotoxicity in the Dutch water sector, KWR initiated a collaborative trial on bioassays for genotoxicity within the NORMAN network (WG-2 on bioassays). The aim of this study was to explore the performance of different bioassays for genotoxicity and related mechanisms and to generate communication, discussion and inspiration within a large consortium of participants, including both NORMAN network members and other organizations, on the use of bioassays for the detection of (potential) genotoxicity.

Participants blindly test water-relevant micropollutants mixtures using their own methods for sample preparation and genotoxic bioassays. The outcomes provide a qualitative evaluation of which bioassays are responsive to a representative set of water pollutants and may thus be suitable for water quality monitoring. The performance of testing procedures were not compared quantitatively.

The invitation for participation in the study (Appendix A) were distributed in the NORMAN network in the first half of 2018. After confirmation of participation (Appendix B), KWR prepared samples to be tested blindly. Samples were distributed during the summer, A template was provided by KWR to the participants to report the results. Given that participants were expected to provide study results without providing them funding for this effort (i.e. in kind contribution), the deadline for results were moved to the end of 2018. The preliminary results were presented by KWR at the 2018 General Assembly. The results of the study are presented here, although the data has not been analysed exhaustively, and additional analyses can be pursued in follow-up research. Suggestions are made throughout this report.

1.2 Participating Institutions

In total, twenty four institutions from nine countries registered to participate in the collaborative trial (Table 1). Participants included members of the NORMAN network, as well as outside organizations. Participants included academia, research institutions, bioassay companies, water utilities and environmental agencies.

Table 1 Participating institutions in the NORMAN collaborative trial on bioassays for genotoxicity testing.

Institution	Country
Bavarian Environmental Agency (Bayerisches Landesamt fuer Umwelt)*	Germany
BioDetection Systems (BDS)	Netherlands
Bundesanstalt für Gewässerkunde*	Germany
Environmental Bio-Detection Products Inc.	Canada
German Environment Agency (Umweltbundesamt – UBA)*	Germany
H1 Research Team, Bulgarian Academy of Sciences	Bulgaria
Helmholtz Centre for Environmental Research – UFZ (Leipzig)*	Germany
Het Waterlaboratorium	Netherlands
Hydrotox GmbH	Germany
Italian National Institute of Health (Istituto Superiore di Sanità)	Italy
IWW Rheinisch-Westfälisches Institut für Wasser*	Germany
KWR Watercycle Research Institute*	Netherlands
Landesamt für Natur, Umwelt und Verbraucherschutz NRW	Germany
LUBW Landesanstalt für Umwelt Baden-Württemberg	Germany
LW Zweckverband Landeswasserversorgung	Germany
National Institute of Biology	Slovenia
Niedersächsischer Landesbetrieb für Wasserwirtschaft, Küsten- und Natruschutz	Germany
RWTH Aachen*	Germany
SARL TOXEM	France
SYKE*	Finland
Technische Universität München*	Germany
Toxys B.V.	Netherlands
University of Belgrade	Serbia
Vrije Universiteit Amsterdam*	Netherlands

*Member of the NORMAN network

1.3 Summary of Bioassays

Participating institutions were invited to use their in-house bioassays to test the samples for genotoxicity and related mechanisms. No restrictions on the type or number of bioassays tested were imposed. Institutions tested the samples blindly and sent the results to KWR. Some institutions were not able to complete the tests, and in the end 18 of the 24 institutions submitted results. In total, seven different bioassays and their variations were tested (Table 2). A brief overview of the different bioassays is provided below.

Table 2 Bioassays tested and the number of the corresponding participants who privided results

Assay	No. Participants
Ames Mutagenicity	10 participants
p53 CALUX	1 participant
SOS-Chromo	2 participants
UMU-Chromo	7 participants
Comet	2 participants
Micronucleus	4 participants
ToxTracker	1 participant

Ames Mutagenicity

The Ames test is one of the most commonly applied used bioassays for water quality (Heringa et al. 2013). The Ames test uses strains of *Salmonella typhimurium* with mutations which inhibit the bacteria's production of histidine (auxotrophic mutants). The bacteria are therefore unable to grow without the addition of histidine to the growth medium. When the auxotrophic bacteria are exposed to test samples which contain mutagenic compounds, the bacteria can revert back to being able to grow on medium without histidine (prototrophic). Often (rat) liver enzyme is added to test the metabolic activation of test components. Revertant bacteria are often detected by a change in color of sample wells. The color change is a result of bacterial metabolism reducing the pH of the medium in the well (Tejs, 2008).

p53 CALUX

The p53 CALUX assay detects activation of the tumor suppressing gene, TP53. Increased p53 levels are indicative of genotoxicity, as the p53 protein responds to DNA damage, and is a transcription factor for genes related to DNA-damage repair, cell-cycle arrest and apoptosis (Van der Linden et al., 2014). Chemical activated luciferase gene expression (CALUX) is a bioassay used to detect specific chemicals in a sample. This is done through a modified cell line with a luciferase reporter gene and response elements which induce transcription of the light generating enzyme (BDS, 2014). The p53 CALUX test uses human osteosarcoma cells (U2OS cells).

SOS-Chromo

The SOS-Chromo test detects DNA damage by quantifying the expression of the sfiA gene, which is a part of the SOS repair system. In *Escherichia Coli* PQ37 the lacZ gene is controlled by the sfiA promoter (EBDP, 2018). When DNA damage occurs due to genotoxic samples, the SOS repair system is activated, the lacZ gene is induced and the synthesis of β-galactosidase is quantified by a color change (optical density).

UMU-Chromo

The UMU-Chromo test detects DNA damage by quantifying the expression of the umuC gene, which is a part of the SOS repair system. In *S. typhimurium* TA 1535 [pSK 1002] the umuC gene is fused to the lacZ reporter gene (Nakamura et al., 1985). Similar to the SOS-Chromo test, when DNA damage occurs, the SOS repair system is activated, the lacZ gene is induced and the synthesis of β-galactosidase is quantified by a color change (optical density).

Comet

The Comet Test, also called the single cell gel electrophoresis (SGCE) test, detects DNA damage in cells (Collins, 2004). In the test, cells are suspended in thin agarose gel and exposed to a sample containing a potentially genotoxic chemical. After exposure, cells

are lysed removing all cellular protein so that only DNA remains. The DNA is allowed to unwind under alkaline conditions, then electrophoresis is applied. Under electrophoresis, smaller DNA fragment travel faster than larger, more intact DNA fragments, forming an image of a comet, with intact DNA at the head of the comet and a tail of DNA fragments (Collins, 2004). The extent of DNA damage is directly proportional to the size of the comet tail.

Micronucleus

The micronucleus test is used to identify the (chemical-induced) formation of micronuclei (small membrane bound DNA fragments) in the cytoplasm of cells. These micronuclei contain lagging chromosome fragments or whole chromosomes. The test often uses the Chinese hamster ovary (CHO) cell line and can be performed with and without metabolic activation (±S9). Cells are visually scored for the presence of micronuclei. Increased frequency of micronuclei is indicative of induced chromosomal damage (OECD, 1997).

ToxTracker

The ToxTracker test is a green fluorescent protein based genotoxicity assay consisting of different mouse embryonic stem (mES) reporter cell lines which are responsive to compounds which are genotoxic or induce oxidative stress (Hendriks, et al., 2012). The ToxTracker assay is also able to provide insight into the primary toxic properties of compounds through integrated evaluation of the results from the different reporter cells in the test (Hendriks, et al., 2012). In this trial, genotoxicity, oxidative damage, cellular stress and protein damage were assessed using the ToxTracker.

1.4 Sample preparation and shipment

The samples distributed to participants consisted of a mixture of 3 genotoxic chemicals from different classes of compounds (Kirkland et al. 2016) selected based on their relevance for the aquatic environment (Baken et al., 2018; Sjerps et al. 2016; Table 3).

The mixtures was dissolved either 1) in sewage treatment plant (STP) effluent (undisclosed) at ~10x (provisional health-based) guidance values [(p)GLV] or 2) in dimethyl sulfoxide (DMSO) at ~10^3 times and ~10^5 times (p)GLV. The mixtures in STP effluent represent realistic environmental water samples, while the mixtures in DMSO represent concentrates thereof. In the DMSO samples, 2.5x concentrated water (1:1 mixture of STP effluent and surface water) has also been included. Control samples were also included.

Sample codes and constituents:

NORMAN-KWR-STP1:	STP effluent (NL) + DMSO solvent (0.03% v/v DMSO)
NORMAN-KWR-STP2:	STP effluent (NL) + Mixture of 3 genotoxic substances at ~10x pGLV (0.03% v/v DMSO)
NORMAN-KWR-DMSO1:	Mixture of 3 genotoxic substances at ~10^3 times (p)GLV + unknown mixture of STP effluent / surface water micropollutants in DMSO
NORMAN-KWR-DMSO2:	Mixture of 3 genotoxic substances at ~10^5 times (p)GLV + unknown mixture of STP effluent / surface water micropollutants in DMSO
NORMAN-KWR-DMSOBL:	DMSO solvent control

Table 3 Substances tested in the collaborative trial and their (provisional) health-based guideline values (Baken et al. 2018).

Substance Name	CAS	Substance Type	(Provisional health-based) Guidance Value
benzo[a]pyrene	50-32-8	Polycyclic aromatic hydrocarbon	0.01 µg/L
dimethylnitrosamine	62-75-9	aromatic amine	0.0007 µg/L
p-chloroaniline—free base	106-47-8	pesticide precursor	12 µg/L

1.5 Data Collection and Analysis

Participants were given an excel reporting template (Appendix B) and asked to indicate the assay used, the organism or cell type, the endpoint/mode of action, the detection method and an assay name to be used in the report. For each sample tested, participants reported the (numerical) assay results, whether a sample was diluted, if any pretreatment (e.g. filtration) took place and the positive and negative control chemicals used. If necessary, participants were asked to clarify results after the receipt of the data. For example some participants were asked to specify if results were positive/negative, whether metabolic activation was used etc.

As participants were encouraged to perform the analysis using in-house methods, the assay methodology, type of organism and/or strain varied considerably. As a result, the data were simplified to facilitate comparison within and between the different assays. To simplify the data, samples were classified as positive if at least one replicate/dilution of the sample had a positive response in the assay. Where possible, the different strains of organisms/bacteria used and other variations of the assay (e.g. metabolic activation) have been reported for the individual assay tables. A summary table has been prepared which reports the assay results per participant. In this case, if at least one replicate/dilution of the sample was positive for at least one variant of the assays tested, then the sample was listed as positive. Where possible, additional notes on sample results provided by participants have been added as footnotes to the relevant data tables.

It was outside of the scope of this report to exhaustively analyze the impact of other test variables. For example, the influence of sample pretreatment, detection method or choice of positive and negative control substances, and the expected response of different genotoxic chemicals included in the samples has not been analyzed. This can be pursued in follow-up research.

2 Results

2.1 Summary

A summary of the results from all bioassays is presented in Table 4. Results are reported using the assay name provided by participants in the reporting template. Samples were classified as positive if at least one replicate of the sample was reported as positive for at least one strain or variant in the assay. Table 5–Table 11 summarize in more detail the variation in responses within assays for different strains/organisms, whether metabolic activation was used and other relevant variation in the assays.

Overall, more than half of participants used the Ames Test (10 out of 18 participants), which resulted in the most variation and largest number of samples tested with the Ames (~600 replicates/dilutions). For all assays, the DMSO2 sample triggered the most positive responses, followed by DMSO1 sample. No consistent difference in assay response was observed between STP1 and STP2. No consistent pattern was observed for assay responses with or without metabolic activation for the different samples.

The DMSO Blank sample unexpectedly triggered responses in a number of assays (Ames, SOS-Chromo, UMU-Chromo, Comet). Responses were either positive for genotoxicity or cytotoxicity. These responses emphasize that solvent and procedure controls need to be included while testing environmental samples, to distinguish effects of micropollutants from that of the solvent itself.

Large variation was observed in responsiveness for a particular sample within groups of related assays. This could be due, in part, to the variation in organism/strain used, pre-treatment or choice of negative and positive controls. However, even when the same strain are compared for the same samples the results are not consistent, indicating that other variables can be important as well.

Table 4 Summary of results from the collaborative trial, organized by assay type and labelled per the assay name provided by institutions (in grey).

	NORMAN-KWR Sample Code				
Ames Mutagenicity	**DMSO Blank**	**DMSO1**	**DMSO2**	**STP1**	**STP2**
Ames Test MOD ISO method	-	+	+	+	-
Ames fluctuation	-	-	-	-	-
Ames Fluctuations Test ISO 11350 no S9-mix	-	-	-	-	-
Ames test	-	-	-	-	-
Salmonella/microsome fluctuation test (Ames fluctuation test) ISO 11350:2012*	-	-	-	-	-
Ames fluctuation test	-	-	-[1]	-	-
Ames plate incorporation assay	-	-	+[1]	-	-
AMES	-	-	-	-	-
AMES fluctuation assay**	-	-	-	-	-
Ames II fluctuation test (TA98+/-S9, TA100 +/-S9)‡	+[1]	+[1]	+[1]	+[1]	+[1]
P53 CALUX	**DMSO Blank**	**DMSO1**	**DMSO2**	**STP1**	**STP2**
P53 CALUX (without S9)	-	-	-	+[2]	+[2]
SOS-Chromo	**DMSO Blank**	**DMSO1**	**DMSO2**	**STP1**	**STP2**
SOS-Chromotest	+[3]	+[3]	+	+	+[3]
SOS Chromotest	-	-	-	-	-
UMU-Chromo	**DMSO Blank**	**DMSO1**	**DMSO2**	**STP1**	**STP2**
umu assay[5]	+,c	+,c	+,c	-	-
umu assay	+,c	+	+	-	-
Umu-test (ISO 13829:2000(E))	-	-	-	-	-
umu-test - DIN 38415-3:1996-12*	-	-	-	-	-
umu genotoxicity test	c	c	c	-	-
umu-c Test (DIN 38415-3)	NT	+	+	-	-
SOS-umu-Test, ISO 13829	-	-	-	NT	NT
COMET	**DMSO Blank**	**DMSO1**	**DMSO2**	**STP1**	**STP2**
COMET assay	-	-	-	-	+
COMET ASSAY*	+	+	+	+	+
Micronucleus	**DMSO Blank**	**DMSO1**	**DMSO2**	**STP1**	**STP2**
Micronucleus assay	-	-	-	-	-
in vitro Micronucleus Test	-	-	-	-	-
Micronucleus test	-	+	+	-	-
Micronucleus	-	-	-	-	-
ToxTracker	**DMSO Blank**	**DMSO1**	**DMSO2**	**STP1**	**STP2**
ToxTracker	-	-	+[4]	-	-

+ indicated a positive response in at least one sample replicate/variation, - indicates a negative response, c: cytotoxicity may have resulted in false positive response, NT: not tested, *samples thawed during transit, **contamination resulted in loss of half of sample volume (potential source of error), ‡Bioluminescent read-out, [1]see notes in Ames Table 5 for specific positive responses, [2]Waste water enriched, without S9, [3]Weak positive result, [4]positive for protein damage, [5]See notes in UMU Table 8 for notes response of DMSO Blank

2.2 Ames Assay Results

Ten participants used the Ames assay with eight different strains of *Salmonella typhimurium,* including two luminescent strains; TA100, TA100lux, TA98, TA98lux, TAMix, YG1024, YG1041, YG1042 (Table 5). In addition, metabolic activation (+/-S9) and the use of plasmid GST T1-1 led to a total of 17 different variations of the test. As few as one participant to as many as nine participants tested the same variant. Most participants visually scored or used the colorimetric detection method, though absorption and luminescence (TA100lux/TA98lux strains) were also used.

The majority of the results from the Ames test were negative, though there was at least one positive result for each of the 5 NORMAN-KWR samples. Only 3 participants had positive results and in each case at least one replicate of the DMSO2 sample was positive. One participant found interference from DMSO in the blank, causing a genotoxic response which could have triggered positive responses in both the DMSO1 and DMSO2 samples.

Responses of samples between the strains varied. All samples tested using strains TAMix, YG1042, YG1041, with or without metabolic activation, were negative. The strain TAMix was tested by two participants, though strains YG1041 and YG1042 were only tested by one participant. The strains YG1024 and TA98, without metabolic activation, were all negative, while at least one sample was positive with metabolic activation (DMSO1 and STP1 for YG1024 +S9 and DMSO2 and STP2 for TA98 +S9). The luminescent strains TA100lux and TA98lux, tested by one participant, were the most sensitive of all the strains. However, the DMSO Blank sample tested positive in both the TA100lux and TA98lux with metabolic activation.

Table 5 Ames Mutagenicity test results listed as per the assay name provided (in grey), horizontal lines divide results between institutions.

Assay Name	Organism or cell type	Strain	Variation	DMSO Blank	DMSO1	DMSO2	STP1	STP2
Ames Test MOD ISO method	Bacteria, Salmonella typhimurium	YG1024	-S9	-	-	-	-	-
			+S9	-	-	+	+	-
		TA100	-S9	-	+	+	+	-
			+S9	-	-	+	+	-
			plasmid GST T1-1	-	+	-	-	-
Ames fluctuation	Salmonella Typhimurium	TA98	± S9†	-	-	-	-	-
		TAMix	± S9	-	-	-	-	-
Ames Fluctuations Test ISO 11350 no S9-mix	Salmonella Typhimurium	TA100	± S9	-	-	-	-	-
		TA98	± S9	-	-	-	-	-
		TAMix	± S9	-	-	-	-	-
		YG1042	± S9	-	-	-	-	-
		YG1041	± S9	-	-	-	-	-
Ames test	Salmonella typhimurium	TA98	± S9	-	-	-	-	-
		TA100	± S9	-	-	-	-	-

Assay Name	Organism or cell type	Strain	Variation	DMSO Blank	DMSO1	DMSO2	STP1	STP2
Salmonella/ microsome fluctuation test (Ames fluctuation test) ISO 11350:2012*	S. typhimurium	TA100	± S9	-	-	-	-	-
		TA98	± S9	-	-	-	-	-
Ames fluctuation test	Salmonella typhimurium	TA98, TA100	± S9	-[1]	-	-[2]	-	-[3]
Ames plate incorporation assay	S. typhimurium	TA98	-S9	-	-	-	-	-
			+S9	-	-	+	-	-
		TA100	-S9	-	-	-	-	-
			+S9	-	-	+	-	-
AMES	Salmonella	TA98	± S9	-	-	-	-	-
		TA100	± S9	-	-	-	-	-
AMES fluctuation assay**	*n.s.*	TA98	± S9	-	-	-	-	-
		YG1024	-S9	-	-	-	-	-
Ames II fluctuation test (TA98+/-S9, TA100 +/-S9)	Salmonella typhimurium	TA98	-S9	-	-	-	-	-
			+S9	-	-	-	-	+[4]
		TA100	-S9	-	-	-	+[5]	-
			+S9	-	-	-	-	-
		TA98lux	-S9	-	+[7]	-	+[7]	+[7]
			+S9	+[8]	+[9]	+[9]	-	+[7]
		TA100lux	-S9	-	-	+[7]	+[7]	+[7]
			+S9	+[7]	-	+[9]	-	-

*samples thawed during transit, **contamination resulted in loss of half of sample volume (potential source of error)†
Results reported as ± if no difference was observed with or without metabolic activation for all samples

[1] unreplicated positive response observed in 1/3 replicates in TA 98 +S9

[2] potential false negative in TA98 +S9, unreplicated potential positive response observed in 1/3 replicates in TA 100 ±s9 due to cytotoxicity

[3] unreplicated positive response observed in 1/3 replicates in TA 100 -S9

[4] Result is non-significant if historical background is considered

[5] Lowest dilution positive, potential error

[7] (Very) low background response

[8] Remarkably high, statistically significant result

[9] Response may be due to response of DMSO Blank

2.3 P53 CALUX Results

The p53 CALUX assay was tested by one participant using p53 CALUX osteosarcoma cells, with or without metabolic activation. The assay had two positive results, for STP1 and STP2 without metabolic activation (Table 6). In both cases, only the undiluted sample was positive, the 2x and 4x diluted samples tested were negative. All samples with metabolic activation were negative (Table 6).

Table 6 P53 CALUX Assay results.

Assay Name	Organism or cell type	Variation	DMSO Blank	DMSO1	DMSO2	STP1	STP2
P53 CALUX	osteosarcoma cells	-S9	-	-	-	+	+
		+S9	-	-	-	-	-

2.4 SOS-Chromo Assay Results

The SOS-Chromo assay was tested by two participants using *E. coli* PQ37. Both participants tested the strain with or without metabolic activation by rat S9 metabolic enzymes (+/-S9), while one also tested the strain with or without the addition of human glutathione s-transferases (SOS GST T1-1), for a total of four variations on the assay (Table 7). Only one of the two participants found (weak) positive responses for any samples. Weak positive responses were found for STP1 (-S9), DMSO1 (+S9 and +SOS GST T1-1/-S9), STP2 (+/-S9), DMSO Blank (+SOS GST T1-1/-S9) and positive responses for DMSO2 (-S9) and STP1 (+S9). *E. coli* PQ37 strain with SOS GST T1-1 without metabolic activation was negative for all samples.

Table 7 SOS-Chromo bioassay results.

Assay Name	Organism or cell type	Variation	DMSO Blank	DMSO1	DMSO2	STP1	STP2
SOS-Chromotest	Bacteria *E. coli* PQ37 strain	-S9	-	-	-	+[1]	+[1]
		+S9	-	+[1]	-	+	+[1]
	Bacteria *E. coli* PQ37 strain with SOS GST T1-1	-S9	+[1]	+[1]	+	-	-
		+S9	-	-	-	-	-
SOS Chromotest	Bacteria *E. coli* PQ37 strain	± S9†	-	-	-	-	-

[1] Weak positive response

† Results reported as ± if no difference was observed with or without metabolic activation for all samples

2.5 UMU-Chromo Assay Results

The UMU-Chromo assay was performed by seven participants. Not all participants specified a particular strain of *S. typhimurium* used, though strains TA1535/pSK1002 were specified. Positive responses were found in the UMU-Chromo test *only* for the DMSO samples (DMSO Blank, DMSO1 and DMSO2, Table 8). Metabolic activation had no effect on the results. Three of the seven institutions found positive test results, three found cytotoxic responses and three found no positive results for any sample.

One institution observed cytotoxicity in the DMSO samples, prompting them to sample their own, independent (lab) samples of DMSO. Cytotoxicity was also observed in the lab DMSO and the institution specified their in-house samples are always tested in pure water. A second institution also observed cytotoxicity in the DMSO Blank sample.

A third institution found negative results for the DMSO samples, though did find that the DMSO Blank had higher values than the negative control. The institution did not test the STP1 or STP2 samples. Dose-dependency could not be observed when comparing the effects of the DMSO samples. The UMU-Chromo test therefore may not be compatible

with DMSO extracts and the positive genotoxic responses may be due to the DMSO solvent.

Table 8 UMU-Chromo bioassay results.

Assay Name	Organism or cell type	Variation	DMSO Blank	DMSO1	DMSO2	STP1	STP2
umu assay[1]	*Salmonella Typhimurium* TA1535/pSK1002	± S9†	+,c	+,c	+,c	-	-
umu assay	*salmonella thyphimurium*	*n.s.*	+, c	+	+	-	-
Umu-test (ISO 13829:2000(E))	*Salmonella* enterica subsp. enterica TA1535 (*Salmonella typhimurium*)	± S9	-	-	-	-	-
umu-test - DIN 38415-3:1996-12 *	*S. typhimurium* TA1535/pSK1002	± S9	-	-	-	-	-
umu genotoxicity test	*Salmonella typhimurium* TA1535pSK1002	± S9	c	c	c	-	-
umu-c Test (DIN 38415-3)	pSK1002, *Salmonella* enterica subsp	± S9	NT	+	+	-	-
SOS-umu-Test, ISO 13829[2]	*Salmonella Typhimurium*	± S9	-	-	-	NT	NT

*samples thawed during transit

† Results reported as ± if no difference was observed with or without metabolic activation for all samples

[1] Enriched samples in DMSO and independent samples of DMSO caused genotoxic effects

[2] No dose dependent response measured, DMSO Blank response > negative control

c: cytotoxicity observed, NT: not tested, *n.s.* not specified

2.6 Comet Assay Results

The comet assay was performed by two participants, using two different organisms (Table 9). One institute used Chinese hamster ovary (CHO) cells, with and without metabolic activation. In the CHO cell assay, only STP2 with metabolic activation had a positive response, while all others were negative. The second institution used HeLa S3 ATCC® CCL-2.2™ and tested three endpoints/modes of action. The first endpoint tested for double and single stranded DNA breaks and alkali labile sites using the alkaline Comet assay. This assay was positive for all undiluted and 10x diluted samples of DMSO2 and DMSO Blank only. The second endpoint tested for double stranded DNA breaks using the neutral assay. The assays was positive for all NORMAN-KWR samples for all dilutions. The final endpoint tested for cytostatic modes of action (blockage of cells in different phases of the cell cycle) using fluorescence activated cell sorting (FACS). The results were positive for the undiluted DMSO Blank, DMSO1, DMSO2 samples.

It is important to note that all samples for the second institution had thawed during transit, potentially affecting the results. From the responses of the second institution, DMSO may also be cytotoxic in the Hela cell line used in the Comet assay. STP1 and STP2 were negative for cytotoxic effects, though STP1 did contain DMSO, but positive for single

and double stranded DNA breaks. The use of different organisms in the Comet assay leads to very different responses for the same samples.

Table 9 Comet assay results from two institutions.

Assay Name	Organism or cell type	Variation	DMSO Blank	DMSO1	DMSO2	STP1	STP2
COMET assay	CHO cell line	-S9	-	-	-	-	-
		+S9	-	-	-	-	+
COMET ASSAY*	HeLa cells (HeLa S3 ATCC® CCL-2.2™)	alkaline	+	+	+	+	+
		neutral	+	+	+	+	+
		FACS	+	+	+	-	-

* all samples thawed during transit

2.7 Micronucleus Assay Results

Four participants used the Micronucleus assay with three different organisms, for a total of 6 variations on the assay; CHO-9 cells, with and without metabolic activation, HepG2, (human liver cancer cell line) measured by fluorescence/visual scoring and FACS and finally V79 (Chinese hamster lung cel), with and without metabolic activation. Only the HepG2 cells measured by fluorescence/visual scoring were positive for the DMSO1 and DMSO2 samples, all other tests/variations negative (Table 10).

Table 10 Micronucleus assay results.

Assay Name	Organism or cell type	Variation	DMSO Blank	DMSO1	DMSO2	STP1	STP2
Micronucleus assay	CHO cell line	± S9†	-	-	-	-	-
in vitro Micronucleus Test	CHO cell line	*n.s.*	-	-	-	-	-
Micronucleus test	HepG2	Fluors./ Vis.	-	+	+	-	-
		FACS	-	-	-	-	-
Micronucleus	V79	± S9	-	-	-	-	-

n.s. not specified

2.8 ToxTracker Assay Results

One participant used the ToxTracker assay and tested four different endpoints; genotoxicity, oxidative damage, cellular stress and protein damage. The assay used mouse embryonic stem cells and detection method was flow cytometry. Only DMSO2 was positive for protein damage, in all but the highest dilution (16x), all other samples and modes of action were negative (Table 11).

Table 11 ToxTracker assay results.

Assay Name	Organism or cell type	DMSO Blank	DMSO1	DMSO2	STP1	STP2
ToxTracker	mES cells	-	-	+*	-	-

*positive for protein damage, negative for genotoxicity, oxidative damage, cellular stress

3 Conclusions and discussion

Besides target chemical monitoring, non-targeted approaches based on chemical non-target screening and effect-based monitoring using bioassays are increasingly being used to assess chemical water quality (Brunner et al. 2018; Dingemans et al. 2018). The aim of the interlaboratory study was to explore the performance of different bioassays for genotoxicity and related mechanisms and to generate communication, discussion and inspiration within the NORMAN network on the use of bioassays for (potential) genotoxicity. In the end, 18 participants blindly tested five water samples prepared by KWR. Participants were encouraged to use their in-house assays and data analysis methods to test samples. As a result, there was great variety in the number and variation of the assays tested.

Seven assays were tested in total; Ames, p53 CALUX, SOS-Chromo, UMU-Chromo, Comet, Micronucleus and ToxTracker. The number of participants testing the assays varied from one up to ten participants. The Ames test was the most popular, with 10 of 18 participants using a variation of the assay. The Ames test, as a result, also had the most variation in test set-up. Eight different strains of bacteria were tested, with and without metabolic activation, for a total of ~600 dilution/replicate samples responses submitted.

Between the assays, the NORMAN-KWR-DMSO2 sample had the most positive responses, followed by the NORMAN-KWR-DMSO1 sample, as expected based on the concentrations of the genotoxic chemicals spiked in these samples. No substantial difference in responses between the NORMAN-KWR-STP1 and the NORMAN-KWR-STP2 samples was observed in the assays. This may be the result of speciation of the spiked chemicals to solids present in the STP effluent. Moreover, the actual exposure of bioassays to the spiked chemicals has likely differed between tests due to differences in water solubility (in particular BaP has low water solubility), degree of dilution in a bioassay and *in vitro* kinetics. For sensitivity and performance analyses of bioassays, it is therefore important to include different concentrations and preferably also chemical-analytical measurements of free concentrations. No clear pattern in responses with or without metabolic activation for all samples was observed.

It should be noted that some participants observed interference in the Ames, SOS-Chromo, UMU-Chromo and Comet assays from the DMSO solvent in the NORMAN-KWR-Blank sample. DMSO is known to trigger a positive response in the UMU-Chromo assays, as reported by Nakamura et al. (1990). The potential interference of DMSO (or other solvents, at different concentrations) can be studied in follow-up research. It was outside of the scope of this report to analyze the effects of all variables in the interlaboratory study. Future research could include investigation into, for example, the effects of different positive and negative control substances used by participants for the same assay or the effect of different pretreatments, for example filtration. In addition, the effects of dilution and the choice of organism and/or strain should be more robustly investigated.

Various studies on the performance of sets of bioassays for genotoxicity have been published earlier (e.g. DiPaolo et al. 2018; Prant et al. 2018). Unfortunately, it was outside the scope of this study to compare the outcomes of this study with literature and this is

pursued in follow-up research. Furthermore, the expected response of different assays to the genotoxic chemicals included in the samples has also not been analyzed but should be pursued in follow-up research.

Relatively simple mixtures were prepared. The individual chemicals in the mixture are included at similar (provisional) guideline values, in analogy to toxic units, although different methods and variables (uncertainty factors) can be applied for the derivation of guideline values. In follow-up research is recommended to also include 1) extracts of surface water; 2) extracts of treated water (in which transformation products can be expected) and 3) concentration series of individual genotoxic model chemicals (Kirkland et al. 2016) and water-relevant chemicals (Busch et al. 2016). A similar approach has also been used to evaluate bioassays for hormone modulation (Leusch et al. 2017, 2018). To quantitatively compare the performance of different bioassays, also other test parameters, such as dilution of concentrate should be considered and possibly standardized. The analysis of complete concentration series of water relevant chemicals can also aid in the development of trigger values for the interpretation of bioassay responses induced by complex mixtures in water samples, as this information is currently lacking, e.g. for the Ames fluctuation test (Escher et al. 2018). New, emerging chemicals with suspected genotoxicity may be selected based on mechanism-of-action as collected in the U.S. EPA ToxCast database (Sobus et al. 2019).

Also, more *in vitro* test systems to analyze (potential) genotoxicity have been and are being developed. For example, the high-throughput luminescent version of the Ames fluctuation test (included in this study) was developed recently (Zwart et al. 2018), and the ToxTracker assay (Hendriks et al. 2016) has been developed to further discriminate clastogens from aneugens. Numerous protocols and methodological approaches are available to test environmental samples for mutagenicity (Umbuzerio et al. 2017). To expand even further, the set-up of this NORMAN interlaboratory study can be used also to explore possibilities and experiences with bioassays for another water-relevant endpoint related to human health or the environment (Barron et al. 2015; Escher et al. 2014; Neale et al. 2017). Interlaboratory studies on varying sets of bioassays have also been described (Di Paolo et al. 2016).

Before it can be considered to include a particular bioassay in a test battery for water quality, it needs to be established whether a test is fit-for-purpose. To this aim, selection criteria have been developed in the FP7 DEMEAU project. Selection of a candidate bioassay should be followed by empirical research, for example to confirm that the bioassay can be applied for water sample concentrates, that the sensitivity is sufficient with acceptable intra- and inter-day variability.

Overall, the interlaboratory study has generated a large and variable data set to begin to analyze the performance of different bioassays for genotoxicity and related mechanisms. The variability of the data meant that detailed analysis was not possible within the scope of this report, however, general conclusions have been presented along with suggestions for future research.

4 Acknowledgements

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Appendix A

NORMAN Collaborative Trial

BIOASSAYS FOR GENOTOXICITY TESTING

Background

There are several *in vitro* bioassays available to test for genotoxic activity and related mechanisms. As one of the scientific activities of the NORMAN Joint Programme of Activities 2018 (WG-2), a collaborative trial to compare the performance of different bioassays for genotoxicity and related mechanisms is organised. NORMAN members and organisations outside the network will be invited to participate.

Objective

The aim of this study is to compare the performance of different bioassays for genotoxicity and related mechanisms for evaluation of chemical water quality. Water-relevant mixtures of micropollutants will be produced by KWR Watercycle Research Institute and sent to the participants. Different types of bioassays will be used by the participants to test these samples (blindly) in the assay(s) in use at their laboratories. The results will be evaluated by KWR and disseminated to the participants and the NORMAN network.

Time schedule

Deadline for registration	(KWR)	June 15th 2018
Preparation and distribution of samples	(KWR)	July 2018
Test results reported to KWR	(all participants)	November 2018
Dissemination of results	(KWR)	December 2018

Outline

Laboratories are invited to use their in-house methods to analyse the samples; these in-house methods will not be subject to restrictions. If your laboratory wishes to participate, the following procedure will be followed:

- You will receive four samples from KWR in July 2018: (i) a stock concentration of a model compound, (ii) a concentrated reconstituted mixture of micropollutants, (iii) a vial with solvent (used for preparation of the former two samples), and (iv) a representative polluted water sample. The samples can be frozen until analysis. We ask you to at least test the samples as delivered to you. You are welcome to additionally analyse dilutions or concentrated samples.

- Your laboratory will test the samples blindly using the genotoxicity assay(s) of your choice according to your own test protocols, including appropriate controls, and quality assurance procedures. Analysis and statistical evaluation of raw data should be performed by your laboratory. No financial compensation will be provided for these analyses; an in kind contribution of your laboratory is requested to this end. Final test results need to be reported to KWR in November 2018 ultimately, using a standardized template that will be distributed by KWR.
- KWR will evaluate the results and disseminate them to the NORMAN network by December 2018.

We intend to evaluate *qualitatively* which bioassays are responsive to a representative set of water pollutants and may thus be suitable for water quality monitoring, not to *quantitatively* compare the performance of each testing procedure. Quality and validity of the test results will not be checked by KWR. The test results will be anonymized in the study report. Selected organisations outside of the NORMAN network will be invited to participate as well and will receive the study report upon participation. If you would like us to invite organisations from your own network, please let us know. We reserve the right to select participants (based on NORMAN membership and inclusion of the largest diversity of bioassays) or ask for a contribution to the shipping costs in case a number of organisations disproportionate to the budget would apply for participation.

Registration

Please register at your earliest convenience, at the latest on **June 15th**, by sending an email to
Kirsten.Baken@kwrwater.nl.

We kindly ask you to include the following information in your application:

- We will apply the following assay(s):
- Our assay(s) requires the use of a specific solvent: **NO** / **YES**, i.e.:
- As a positive control we will use the following substance(s):
- The requested total volume per sample is (µl):
- We are able to process waste water samples: **YES** / **NO**
- Our contact person for receipt of the samples is (name, email, telephone):
- Our delivery address is

Appendix B

NORMAN Collaborative Trial

BIOASSAYS FOR GENOTOXICITY TESTING

Nieuwegein, July 19th 2018

Dear participant,

I would like to inform you on the distribution of the samples for the NORMAN Collaborative trial on genotoxicity testing, and provide some further information on the trial:

- We expect the samples to arrive at your laboratory on **Thursday July 26th**. Samples will be shipped to your contact person as previously indicated in the registration. If you are **not** able to receive the samples at this date, please let me know at your earliest convenience.
- The samples will be shipped **frozen**, please keep them frozen after receipt until analysis.
- You will receive 5 samples: NORMAN-KWR-STP1&2 representing waste water, NORMAN-KWR-DMSO1&2 representing concentrated water in DMSO, and NORMAN-KWR-DMSOBL as a blanc.
- Your laboratory is asked to test the samples using the genotoxicity assay(s) of your choice according to your own test protocols, including appropriate controls, and quality assurance procedures (as already indicated in the registration). Data analysis and statistical evaluation of raw data should be performed by your laboratory.
- Please analyse all samples at least once **without any extraction or concentration**. If your standard testing procedure requires sample pretreatment such as filtration of waste water, this can be applied. You are welcome to additionally analyse duplicates or dilutions.
- Please **report** your analysis results to KWR via Milou.Dingemans@kwrwater.nl before November 15th 2018 using the Excel template attached. Please use a separate worksheet for each assay that you apply. KWR will confirm the receipt of your test results.
- You can discard any remaining sample material after completion of your tests.
- The test results will be anonymized in the study report, which will be compiled by KWR after receipt of all test results.

Milou Dingemans (Milou.Dingemans@kwrwater.nl) will be your contact person for this trial after shipment of the samples, do not hesitate to contact her in case you have any questions. Thank you very much for your participation and enjoy the summer!

Kind regards,

Milou Dingemans & Kirsten Baken

Scientific researcher, toxicologist - Chemical Water Quality and Health | KWR Watercycle Research Institute | Groningenhaven 7, P.O. Box 1072, 3430 BB Nieuwegein, the Netherlands | T +31 30 606 9703 | E Kirsten.Baken@kwrwater.nl | W www.kwrwater.nl | Follow KWR on | Follow me on | Chamber of Commerce Utrecht e.o. 27279653 | KWR is WHO Collaborating Centre on Water Quality and Health

Reporting template

Please return this file to Milou.Dingemans@kwrwater.nl before November 15th

Participant	
Institution or company	
Contact person	
Assay information	
Assay name to be included in NORMAN report :	
Organism or cell type :	
Endpoint or mode of action :	
Detection method* :	

Graph

			Dilution of sample in the test		
Samples	**Test result****	**If this sample was diluted, please indicate dilution factor:**	Which sample volume (uL) was added to the test?	To which volume of medium (uL) was the sample added?	**Did sample pretreatment take place (filtration, ..)?**
NORMAN-KWR-STP1		0			
NORMAN-KWR-STP2		0			
NORMAN-KWR-DMSO1		0			
NORMAN-KWR-DMSO2		0			
NORMAN-KWR-DMSOBL		0			
insert rows for replicates or dilutions, if tested					
positive control: *[please specify substance and concentration]*		N.A.			
add rows for additional positive controls, if tested					
negative control: *[please specify substance and concentration]*		N.A.			
add rows for additional negative controls, if tested					

Detection method:	****Test result:***
- visual scoring	*- preferably numerical result*
- fluorescence (plate reader)	*- otherwise positive/negative indication*
- luminescence (plate reader)	*- graphical representation when possible (use field above or refer to separate worsk sheet)*