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PAPER

Biotests for hazard assessment of biofuel fermentation

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To meet the increasing demand for energy, development of alternative and renewable energy sources, such as bioenergy, has accelerated during the last decade. In this context, biofuels are one potential replacement for fossil fuels, although their impact on the environment has not been widely studied. Only a few studies are available on toxicity of biofuels and biofuel combustion. Furthermore, for a complete understanding of the environmental impact, the entire life cycle of a biofuel has to be analyzed. This study is an exemplary ecotoxicological investigation of a biomass-to-biofuel production process with respect to the generation of environmentally relevant contaminants either by means of biomass pretreatment or microbial activity. Our aim is the demonstration of the suitability of ecotoxicological biotests as part of a comprehensive hazard assessment of biofuels and related samples or processes. Five ecotoxicological endpoints were assayed to determine the impact of four different biomass pretreatments on process substrates and effluent toxicities. Four different test organisms (bacterium, yeast, fish cell line, and fish embryo) from different trophic levels as well as a combination of acute and mechanism-specific biotests were applied to strengthen the ecotoxicological relevance of this investigation. Biotest results revealed cytotoxic, acute embryotoxic and mutagenic effectiveness, and weak estrogenic activity, with biomass toxicity depending on the mechanism of substrate pretreatment. Open microbial communities (reactor microbiomes) involved in the production process decreased the toxicity considerably to levels of the product *n*-butyric acid due to degradation of inhibiting by-products, verifying their simultaneous biomass conversion and detoxification potential. Our results demonstrate that ecotoxicological biotests are useful tools for the biofuel industries to gain environmental friendliness as a selling point.

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1 Introduction

Dependence on non-renewable energy sources, such as crude oil, natural gas, and coal, has several major disadvantages compared to the use of energy from alternative and renewable energy sources. While the global demand – and, thus, the price – for crude oil increased overall during the last decade, crude oil

Broader context

The replacement of established fossil fuels by alternative and renewable biomass-derived fuels, in particular for the transport sector, poses a significant risk to the environment. Current discussion regarding the effects of biofuels on the environment focuses primarily on potentially negative GHG emissions and energy balances. However, release of these novel compounds into the environment due to leakages or handling accidents cannot be excluded. Unfortunately, little is known on the hazard potential of biofuels, because toxicological investigations have been largely neglected. Ecotoxicological biotests are a potentially powerful tool that can be applied in hazard assessment of biofuels. An assessment of the ecotoxicological hazard potential of biofuels and biofuel production streams should be conducted very early in the biofuel life cycle in parallel to the biofuel development. This could be used as a unique selling point by identifying the least toxic compounds at a very early stage and in an integrated biorefinery that could identify toxic intermediates from specific processes. The present study reports on a novel and innovative approach for an environmental quality assessment of biomass conversion processes within biofuel development.

production has not risen since 2005.[1] As a consequence of high oil prices and as a reaction to the impact on global climate, fossil fuels are being replaced by or supplemented with renewable energy sources, such as biomass, that are expected to lower the emission of green house gases (GHG).[2] Another important driving force is the strong governmental support for increased application of biofuels. By 2022, the European Union plans to have replaced 10% of the total fuel consumption with biomass-derived biofuels (EU (COM (2006)) 848).[3] Due to the need to develop alternative energy sources in combination with governmental support, biofuel technology and industry are expected to grow continuously within the next few years.[4] Much of this growth will likely be derived from a large number of economically available and environmentally low-impact feedstocks, including organic wastes and agricultural or industrial residues.[5–9] These will supplement biomolecules including alcohols (*e.g.* ethanol or butanol) and fatty acid methyl esters (FAME) derived from plant oil, such as rapeseed oil.[10,11]

From an environmental point of view, the increased deployment of biomass-derived biofuels also has some downsides. Most of the current controversy on environmental implications of biofuels has focused on land-use changes and a subsequent loss of habitats and biodiversity or competition with food production.[12] While these are important considerations, other potential negative effects of large-scale deployment of biofuels must not be neglected in research. For example, after many years of gasoline storage, the society has now to deal with the effects of continuous release of the toxic gasoline additive methyl *tert*-butyl ether (MTBE) to the environment. Investigations should, hence, identify and consider the ecological and toxicological effects of biofuels and biofuel-related streams before similar problems are encountered.[13] Bluhm *et al.* have reported that a considerable lack of data on the ecotoxicological hazard potential of biofuels exists to date. The review elucidates and concludes that ecotoxicological studies should be conducted within the development of biofuels.[14] Biotests are bioanalytical techniques used to assay the ecotoxicological hazard potential of chemicals or complex mixtures of substances on animals, plants, fungi or bacteria as test organisms. An advantage of these in the investigation of biofuel production streams is the ability to assay the effects of all compounds in a given sample, even if these compounds are unknown or unidentified. These so-called "biotest batteries" have been successfully applied in many fields before, such as the investigation of chemicals,[15,16] contaminated industrial sites,[17,18] sediments,[19–22] effect-directed analysis,[23] and also combustion products of biodiesel.[24–26] Since the informative value of a single biotest is often limited, a combination of acute and mechanism-specific biotests can be used to increase the ecotoxicological representativity of a study.[27,28]

In this proof-of concept study, we demonstrated the use of a battery of biotests to determine the ecotoxic hazard potential of intermediates from *the first two* processing steps in biological conversion of agricultural waste biomass to *n*-butanol, which is being considered an alternative to bioethanol and biodiesel because of its better energy and mixing characteristics.[10,29] The first step of this process is a chemical biomass pretreatment and is needed to break down the recalcitrant lignocellulosic material in agricultural biomass for subsequent biological processing steps. Thus, chemically pretreated biomass is an intermediate not only in *n*-butanol production, but also in most advanced biofuel and biochemical production processes, including cellulosic bioethanol production.[30–32] Certain chemical pretreatments have the known major disadvantage of generating compounds toxic to downstream (micro)biological steps (*i.e.*, conversion to *n*-butyrate, yeast fermentation for ethanol production, or fermentation of *n*-butyrate to *n*-butanol with pure bacteria cultures[29]) and also potentially to the environment. The second step we investigated is the biological conversion of chemically pretreated biomass to *n*-butyrate using versatile reactor microbiomes (*i.e.* open cultures of microbial consortia).[8,33] These microbiomes are central to the carboxylate platform,[33] and might become important contributors to biofuel and biochemical production processes due to their ability to convert a broad spectrum of substrates to bioproducts, and because they can be directed to produce a range of useful products and potential intermediates in biorefineries.[34] As the versatile microbiomes should degrade some toxic compounds, but potentially not others, they also offer an opportunity to test a battery of assays for their sensitivity to partial changes in toxicity potential.

To test the suitability of ecotoxicological biotests as part of a comprehensive hazard assessment of biofuels or biofuel-related samples, we conducted a battery of acute and mechanism-specific *in vitro* and *in vivo* biotests to successfully determine the cytotoxicity, embryotoxicity, aryl hydrocarbon receptor (AhR) agonist activity, mutagenicity, and estrogenic activity of the process streams. Acute cytotoxicity and acute embryotoxicity were investigated for a first screening of the samples. On the one hand, the Neutral red retention (NR_x) assay and the MTT assay for investigation of acute cytotoxicity were applied to get a first insight into the toxic potentials of the samples. The fish embryo toxicity (FET) test, on the other hand, is widely used for, *e.g.*, assessing water quality and is an established and standardized biotest in ecotoxicological test batteries. Correlation between embryo toxicity and *in vitro* cytotoxicity, at least for sublethal effects, has been reported before, with the embryo toxicity assays being more sensitive to many chemicals than the cell-based cytotoxicity tests.[35] Important for the evaluation of the fish embryo toxicity test is that this biotest was performed using fish embryos, and thus complex organisms, whereas the cytotoxicity assays used a permanent cell line. Due to their higher complexity, results from the FET test on embryotoxicity are better suited for transfer from the laboratory into an ecosystem than cell-based results. Measurements of AhR agonist activity and of the estrogenic activity were carried out due to reports indicating the possible formation of dibenzo-*p*-dioxins and dibenzo-furans (PCDD/Fs)[36] and estrogenic active compounds,[37,38] respectively. Last but not least, mutagenicity was investigated by means of the Ames fluctuation assay as an important endpoint in ecotoxicological hazard assessment.

2 Material and methods

2.1 Biomass-to-biochemical sample pretreatment and preparation

The biotests looked at a total of nine samples derived from various stages of the carboxylate platform, including chemical/physical pretreatment and bioprocessing with open microbial

Table 1 Pretreatment conditions of the five substrate samples (R1-S, R2-S, R3-S, R4-S, and CS)

Sample (bioreactor)	Treatment designation	Chemical treatment	Biomass concentration	Temperature	Treatment time
R1-S (R1)	Dilute-acid	0.5% v/v H_2SO_4	15% w/w	160 °C	20 min
R2-S (R2)	Alkaline	1 : 10 CaO to biomass w/w	15% w/w	160 °C	20 min
R3-S (R3)	Hot-water	Water only	15% w/w	160 °C	20 min
R4-S (R4)	Unpretreated	—	15% w/w	—	—
CS	Dilute-acid	0.7% v/v H_2SO_4	10% w/w	180 °C	10 min

cultures (*i.e.* reactor microbiomes) to convert agricultural waste biomass to biofuel precursors (Agler *et al.* 2012 (ref. 39)). Five samples represent substrates for the biofuel production process: one sample was pretreated corn stover (primarily the stalk and leaves of the corn plant [CS, Table 1]) and four additional samples were made from corn fiber (primarily the pericarp [*i.e.* outer skin] of corn kernels). Three of these corn fiber substrates (R1-S, R2-S, and R3-S, Table 1) were pretreated in three different regimes whereas one corn fiber substrate was unpretreated (R4-S, Table 1). All pretreatments with corn fiber substrates were performed in a fluidized sand-bath reactor under similar temperature regimes but with different added chemicals (dilute-acid; dilute-alkaline; and hot-water pretreatment). The other four samples were process effluent complementary (R1-E, R2-E, R3-E, and R4-E) to the corn fiber substrates that were taken after reactor microbiomes had converted them to *n*-butyrate and other carboxylates.

In short, the four effluent samples were prepared by inoculating semi-continuous bioreactors with microbiomes for the purpose of evaluating conversion of the complex corn-fiber substrate to *n*-butyrate. The effluent samples were collected from the four bioreactors simultaneously on day 70 of bioreactor operation. The CS sample was not converted to *n*-butyrate by reactor microbiomes, but is included as a reference sample due to its relatively higher lignin content, and therefore higher anticipated toxicity. All nine samples were collected and shipped frozen from Cornell University to RWTH Aachen University. Prior to biotesting, each sample was filtered using a glass fiber filter (MN85/70; Ø 25 mm; 0.6 μm; Macherey-Nagel GmbH & Co. KG, Düren) to remove any remaining granulate or microorganisms and to focus on soluble chemical intermediates. To limit complications caused by variable pH in the test medium due to the biomass pretreatments, the pH value of all samples was adjusted to 7.4 ± 0.2 using 0.5 M NaOH prior to biotest investigation. A low pH value interferes, because it causes toxic effects or false positives in the pH sensitive mutagenicity assay. Finally, the samples were aliquoted in 1.5 ml vials and stored at −24 °C (Fig. 1).

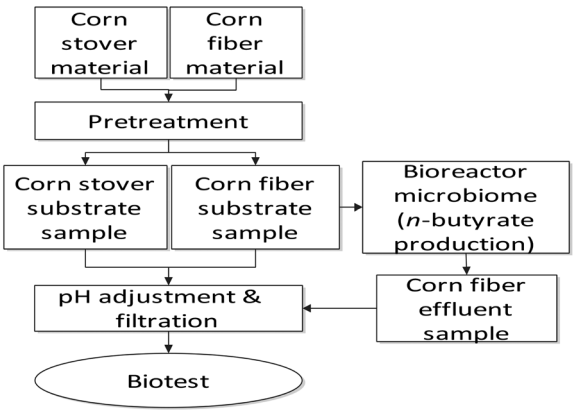

Fig. 1 Overview of the sample pretreatment.

2.2 Biotests

2.2.1 Cell culture with the permanent cell line RTL-W1. Cytotoxicity and Ah-receptor agonist activity were investigated using the permanent cell line RTL-W1 derived from rainbow trout liver (*Oncorhynchus mykiss*).[40] Cells were maintained in 75 cm^2 tissue culture flasks (Techno Plastic Products (TPP), Trasadingen, Switzerland) in L-15 Leibovitz's medium (Sigma-Aldrich), which was supplemented with 9% fetal bovine serum (Biowest) and 1% penicillin–streptomycin solution (Sigma-Aldrich).[40] Cells were incubated at 20 °C in darkness.

2.2.2 Acute cytotoxicity on fish cell lines – NR assay and MTT assay. The Neutral red retention (NR) assay was carried out according to Borenfreund[41,42] modified as published by Keiter *et al.*[20] and Klee *et al.*[43] while the MTT assay was performed according to Mosmann.[44] Each of the nine biomass samples (Table 2) was assayed by these biotests. For exposure, samples were serially diluted seven times on a 96-well microtiter plate (TPP), resulting in concentrations from 50% to 0.78% v/v in six replicates per concentration. As a positive control, 40 mg ml^{-1} 3,5-dichlorophenol (DCP) was added in six wells. Confluent RTL-W1 cells were trypsinized and the cell suspension was adjusted to 4×10^5 to 5×10^5 cells per ml and subsequently added to the 96-well plate. The 96-well plate was incubated for 48 h at 20 °C. Afterwards, exposure medium was discarded and cells were incubated for 3 h with a 0.005% Neutral red (2-methyl-3-amino-7-dimethylamino-phenanzine) solution or for 1 h with a 0.5 mg ml^{-1} MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution, respectively. The number of viable cells was determined either by the amount of incorporated Neutral red (NR assay) or by conversion of the yellow MTT to the purple formazan (MTT assay). The amount of extracted Neutral red and the amount of the formed formazan are directly correlated to the number of viable cells. Neutral red and formazan were determined by means of fluorescence measurement at 540 nm and a reference wavelength of 690 nm (NR assay) and at 492 nm (MTT assay) using a multiwell plate reader (TECAN infiniteM200; Tecan Austria GmbH, Grödig, Austria), respectively. Concentration–response curves were fitted with a nonlinear 'log(agonist) *vs.* response – variable slope' regression

Table 2 An overview of the investigated endpoints and the applied biotests for each sample. Also given is the number of replicates for each sample in each biotest

	CS	R1-S	R1-E	R2-S	R2-E	R3-S	R3-E	R4-S	R4-E
Cytotoxicity (NR)	$n = 4$	$n = 3$	$n = 3$	$n = 3$	$n = 3$	$n = 4$	$n = 3$	$n = 4$	$n = 2$
Cytotoxicity (MTT)	$n = 5$	$n = 3$	$n = 4$	$n = 3$	$n = 3$	$n = 3$	$n = 3$	$n = 3$	$n = 2$
Acute embryotoxicity (FET)	$n = 3$	$n = 3$	$n = 3$	$n = 4$	$n = 4$	$n = 4$	$n = 4$	$n = 4$	$n = 4$
AhR agonist activity (EROD)	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$
Mutagenicity (Ames)	$n = 5$	$n = 4$	$n = 3$	–	–	–	–	–	–
Endocrine activity (YES)	$n = 2$	$n = 2$	$n = 2$	–	–	–	–	+	–

(eqn (1)) using GraphPad Prism 5.01 (GraphPad Inc., San Diego, USA).

$$x = C - \left(\frac{\log 10 \dfrac{(B - A)}{(y - A)} - 1}{D} \right) \quad (1)$$

where x is the concentration, y is the percentage of cell vitality, A is the bottom plateau value, B is the top plateau value, C is the log EC_{50}, and D is the unitless slope factor of the curve. Concentrations resulting in cell viability of 50% and 80% were calculated and identified as $NR_{50/80}$-values for the NR assay and $EC_{50/80}$-values for the MTT assay, respectively. Cytotoxic potentials were calculated for each sample by setting each NR_{50}/EC_{50}-value relative to the lowest NR_{50}/EC_{50}-value. This procedure allows a comparison of the results obtained by both biotests. For detection of significant differences between the samples tested in the same bioassay and between the results of the same sample obtained with the two bioassays, the *t*-test following square root transformation was performed using SigmaStat 3.5 (Systat Software Inc., Chicago USA).

2.2.3 Acute embryotoxicity – fish embryo toxicity test. Acute embryotoxicity after 48 h was assayed by means of the fish embryo toxicity (FET) test according to Nagel (2002)[45] and modifications published by Braunbeck *et al.*[46] and Seiler *et al.*[47] Fertilized eggs of the zebrafish *Danio rerio* were collected and exposed to each of the nine biomass samples (Table 2) at concentrations of 1% v/v and 0.5% v/v. Additionally, the samples CS and R1-S were investigated at a concentration of 0.25% v/v. The test was performed on a 96-well microtiter plate with one egg in each well with 20 replicates per sample concentration. As a positive control, 20 eggs were exposed to 3.7 mg l^{-1} 3,4-dichloroaniline (DCA). After 48 h, eggs were inspected using a microscope (Nikon Eclipse TS100) and the percentage of mortality was evaluated. Mortality criteria according to DIN EN ISO15088 (2009) were coagulation of embryos, a lack of heart function or a non-detachment of the tail. A test was considered valid if the mortality of the negative control (artificial water[19]) did not exceed 10% and the positive control induced effects in more than 20% of the embryos. Samples that induced higher mortality than 10% were embryotoxic.

2.2.4 AhR agonist activity and dioxin-like activity – EROD assay. AhR agonist activity, so-called dioxin like activity, was investigated by means of the EROD assay according to Behrens *et al.*[48] and modifications published by Gustavsson *et al.*[49] Hydrophobic, aromatic compounds with a planar structure cause the AhR to act as the transcription factor for CYP1A induction.[50–52] Compounds that can fit the binding sites of the intracellular AhR are prominent pollutants, such as polychlorinated dibenzo-*p*-dioxins and dibenzo-furans (PCDD/F) and a number of polycyclic aromatic hydrocarbons (PAH).[51,52] Therefore, many ecotoxicological studies rely on the EROD assay as an important biomarker for dioxin-like substances (for example, in ecotoxicological investigations of contaminated industrial sites).[17,18] Here, it could be an indicator of aromatics that are a result of lignin degradation during pretreatment. Similar to the NR assay, confluent RTL-W1 cells were trypsinized and seeded in a 96-well microtiter plate. Cells were allowed to grow confluent for 72 h at 20 °C before beginning of exposure. For each of the nine biomass samples (Table 2), eight serial dilutions were prepared in L-15 Leibovitz medium beginning with the NR_{80}-value as determined in the NR assay. Each concentration was tested in six replicates per 96-well plate. Furthermore, a positive control, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was serially diluted (3.25–100 pM) in two replicates in L-15 Leibovitz medium. Cell exposure was terminated after 72 h. Exposure medium was discarded and cells were frozen at −80 °C for at least one hour before measurement of EROD activity. For measurement of EROD activity, the frozen 96-well plate was thawed and 100 µl of 7-ethoxyresorufin (1.2 µM, in phosphate buffer) was added to each well. EROD reaction was activated by adding 50 µl of NADPH (90 µM, in phosphate buffer) after 10 min and the reaction terminated after 10 min by adding 100 µl of 0.54 mM fluorescamine in acetonitrile. EROD activity was determined as pmol resorufin per mg protein per min reaction time. Therefore, resorufin was measured fluorometrically after 15 min at an excitation wavelength of 544 nm and emission at 590 nm using a microplate reader (TECAN infiniteM200; Tecan Austria GmbH, Grödig, Austria). Measurement of the flourescamine-bound protein was carried out at an excitation wavelength of 355 nm and an emission at 460 nm. Concentration–response curves were fitted using a nonlinear 'log(agonist) *vs.* response – variable slope' regression (*c.f.* eqn (1); GraphPad Prism 5.01) and TEQs (toxicity equivalent quotients) were calculated according to Keiter *et al.* (2008).[53]

2.2.5 Mutagenicity – Ames fluctuation assay. For measurement of the mutagenic potential, a modification of the standard Ames assay[54] was applied.[55] Mutagenicity was investigated for both dilute-acid pretreated samples CS and R1-S, as well as the corn fiber effluent sample R1-E using the *Salmonella typhimurium* strain TA100 (Table 2). This tester strain carries a variation in the histidine-coding region depriving the ability to synthesize histidine (histidine auxotroph). Thus, this bacterium is not able

to grow in histidine-free medium. However, the presence of mutagenic compounds in a sample leads to reverse mutation in the histidine-coding region. Detection of the reverse mutation was realized by using a pH indicator dye in the test media and counting the number of positive wells (wells with revertant growth). Sensitivity of the test system can be increased by adding a metabolic activation system, such as the so-called S9 mix. Addition of a metabolic activation system results either in an increased mutagenicity due to activation of progenotoxic substances or a decreased mutagenicity due to the detoxification of genotoxic compounds. Therefore, the S9 mix is best used for qualitative detection of progenotoxic compounds.[56] For test execution, bacteria were inoculated in growth medium 24 h before onset of the test. Prior to seeding into a 24-well test plate, optical density (OD) was measured photometrically at a wavelength of 595 nm and bacteria were adjusted to 450 FAU (Formazine Attenuation Units). For exposure, either samples or controls were added to a 24-well plate. As positive controls, 4×10^{-4} mg l^{-1} 2-aminoanthrancene (2-AA; in DMSO) for the approach with the S9 mix and 2.5×10^{-4} mg ml^{-1} nitrofurantoin (NF; in DMSO) for the approach without the S9 mix were used in the test. The negative control consisted of Millipore water. For experiments with metabolic activation, the S9 mix was added to each well. The three investigated samples (CS, R1-S, and R1-E) were diluted with Millipore water, before bacteria in exposure medium were transferred into each well. Test concentrations therefore resulted in 80%, 50%, 25%, and 12.5%. After 100 min, exposure was terminated by adding the reverse indicator medium to each well. Subsequently, 50 µl of each sample or control in indicator medium were added to 48 wells of a 384 well plate. Bacterial growth lasted 48 h, before results were evaluated. For evaluation all wells with revertant growth per concentration were counted, which is indicated by a yellow discolouration of the reverse indicator medium. A test was considered valid if the number of revertant wells in the negative control was less than 10%, while at least 25% of the positive control wells contained revertant bacterial growth. The number of positive wells allows an assessment of the mutagenic potential for each sample. Statistical analyses were conducted using the Toxrat software package (Toxrat Solutions GmbH, Alsdorf, Germany). Shapiro Wilk's test and Levene's test were performed for testing of normal distribution and variance homogeneity, respectively. Significant differences to the negative control were calculated by means of the Williams' test.

2.2.6 Endocrine activity – yeast estrogenic screen assay. The yeast estrogenic screen (YES) assay was performed according to Routledge and Sumpter,[57] using a novel protocol for lysing the cells.[58] The test was carried out with a genetically engineered yeast strain of *S. cerevisiae* according to the methods detailed by Grund *et al.*[59] The utilized tester strain contains the human estrogen receptor α (ER α) as well as expression plasmids with estrogen responsive elements operating the *lacZ* reporter gene, which encodes the enzyme β-galactosidase. Induction of the ERα leads to expression of β-galactosidase, which is, therefore, used as an indicator for estrogenic activity. Only the dilute-acid pretreated samples CS and R1-S, the effluent sample R1-E, and the unpretreated substrate sample R4-S were applied in this bioassay (Table 2). Prior to investigating the estrogenic activity, the range of test concentrations of the biomass processing samples were determined by means of photometric measurements of yeast cytotoxicity. The method involved preparing 17β-estradiol (E2) (480 pM to 480 nM in ethanol, abs.) as the positive control and applying it in a serial dilution from 1 pM to 1 nM. For test execution, 75 µl of each sample, 25 µl of minimal medium[60] (supplemented with ampicillin, streptomycin and $CuSO_4$) and 20 µl of yeast suspension were added in the respective wells on a 96-well plate, with eight replicates per concentration and eight concentrations per sample. The plate was sealed using breathe-easy® membrane (Diversified Biotech, Boston, USA) and incubated on a shaker for 24 h at 30 °C and 750 rpm. Exposure was terminated after 24 h. Subsequently, the cell number was determined photometrically by means of optical density at a wavelength of 595 nm. Quantification of β-galactosidase induction was measured *via* transformation of the artificial substrate chlorophenol-red-β-galactopyranoside (CPRG; yellow) to chlorophenol red (red) by β-galactosidase and subsequent photometrical measurement at 540 nm. The measurement was continued every 30 min until either of these three criteria was met: (1) the negative control showed higher values than 1.5; (2) positive control (E2) values were staying constant or were declining; and (3) the positive control showed higher values than 3.0 (data was shown as 'overflow'). Statistical analyses were not performed because only two replicates were available.

3 Results

3.1 Acute biotests: acute cytotoxicity and embryotoxicity

The NR assay and the MTT assay were applied for determination of the cytotoxic potential of the reactor samples (Fig. 2). Good concordance between the normalized data of both cytotoxicity tests as reported by Hollert *et al.* (2000)[61] was found, with the MTT assay being slightly more sensitive than the NR assay. The strongest cytotoxic potential was revealed by both *in vitro* biotests for the corn stover (CS) reference sample, followed by the dilute-acid pretreated substrate sample (R1-S). Whereas mean values of the results from the NR assay

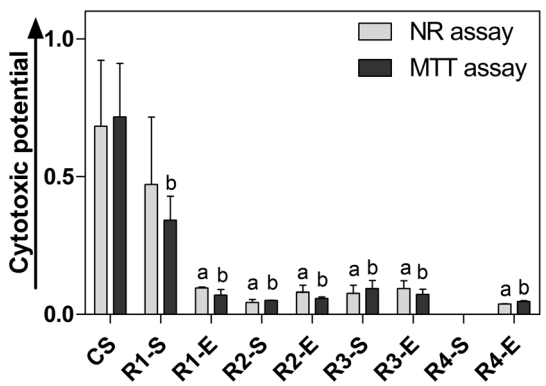

Fig. 2 Cytotoxic potential of each sample determined using NR assay and MTT assay, respectively. Data are given as means (bars) and standard errors of the means (error bars) of the cytotoxic potential. No NR_{50}/EC_{50}-values could be determined for sample R4-S. Small letters denote significant differences ($p < 0.05$) to sample CS, (a = NR assay; b = MTT assay). $n = 2$–5 (see Table 2).

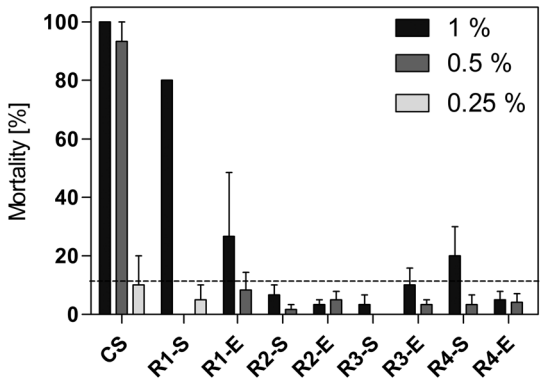

Fig. 3 Embryotoxicity of each sample in the FET test with *D. rerio*. Data are given as means (bars) and standard deviations (error bars). Presented are the mortalities in percent [%] for concentrations of 1% and 0.5% after 48 h of exposure. A sample is defined embryotoxic when exceeding 10% mortality (dotted line). $n_{R1\text{-}S} = 2$; $n_{rest} = 3$.

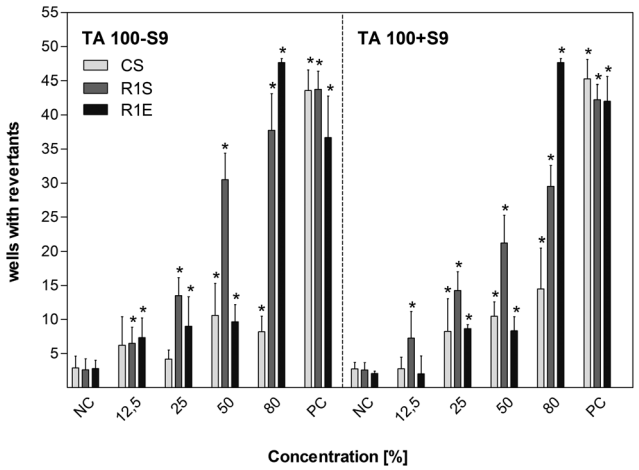

Fig. 4 Mutagenicity of the samples CS, R1-S, and R1-E as well as the positive control (PC) as determined by the Ames fluctuation assay using the *S. typhimurium* tester strain TA100. Data are given as means (bars) and standard deviation (error bars) of the number of positive wells with and without S9 supplementation. PC for tests with S9: 2-amino-anthracene, PC for tests without S9: nitrofurantoin (TA100). $n_{CS} = 5$; $n_{R1\text{-}S} = 4$; $n_{R1\text{-}E} = 3$.

gave evidence for a stronger toxicity of the sample CS than R1-S, no significant differences were found due to high data variability. The MTT assay, on the other hand, revealed significant differences between CS and R1-S ($p < 0.05$) with CS showing a higher cytotoxic potential. Both assays, however, revealed a significant decrease of the cytotoxic potential of the reactor effluent sample R1-E in comparison to the complementary substrate sample R1-S ($p_{NR} < 0.05$; $p_{MTT} < 0.001$). The substrate and effluent samples for both dilute-alkaline (R2-S and R2-E) and hot-water treatments (R3-S and R3-E) gave weak cytotoxic effects, which were significantly lower compared to CS and R1-S. The unpretreated substrate sample of reactor 4 (R4-S) gave no NR_{50}/EC_{50}-values, and hence no cytotoxic potential could be calculated. In contrast, the effluent sample R4-E showed a significantly lower cytotoxic potential compared to CS and R1-S. Although only raw data are presented and no EC_{50}-values could be calculated due to the low number of tested concentrations, the raw data obtained by the FET test (embryotoxicity; Fig. 3) showed similar patterns as the NR assay (Fig. 2). Highest mortality was determined for the dilute-acid pretreated samples CS and R1-S, with CS being slightly more toxic than R1-S. However, unlike with the cytotoxicity tests, embryotoxic effects were also found to be observed for samples R1-E and R4-S.

3.2 Mechanism-specific biotests: AhR agonist activity, endocrine activity, and mutagenicity

AhR agonist and dioxin-like activity was investigated by means of the EROD assay for each of the nine biomass samples. However, the EROD assay revealed no EROD induction and no differences in comparison with the negative control. Because of their relatively high cyto- and embryotoxicity, we investigated mutagenicity with the dilute-acid pretreated substrate samples CS and R1-S, as well as the reactor effluent sample R1-E, using the *S. typhimurium* strain TA100 with and without S9 supplementation. For these samples the corresponding number of positive wells (wells with revertant growth) is shown in Fig. 4. Mutagenic potentials could be demonstrated for all three samples, with the samples R1-S and R1-E showing equal or higher numbers of positive wells compared to sample CS both with and without the addition of a metabolic activation system (S9 mix, supplemented to increase the sensitivity of the assay). Both samples were mutagenic (significantly higher than the negative control) when tested without the S9 mix at concentrations higher than 12.5%. Whereas sample R1-S also revealed mutagenicity with S9 supplementation when tested in concentrations higher than 12.5%, the sample R1-E had to be applied in higher concentrations than 25% before causing mutagenic effects. Mutagenicity for the sample CS, on the other hand, could not be detected below a concentration of 50% without metabolic activation and 25% with metabolic activation.

The same three samples plus sample R4-S for comparison with untreated biomass were investigated for estrogenic activity in the YES assay, with results presented as Estradiol Equivalent Quotients (EEQs). EEQs were calculated according to Wagner and Oehlmann[60] and represent the induction of each sample compared to the activity of the potent ERα-inductor 17β-estradiol (E2). To keep sample concentrations below the threshold for cytotoxic effects on the yeast cells, we tested the substrates R1-S and R4-S at 62.5%, and the substrate CS at 20.8%. For the sample R1-E strong cytotoxic effects were observed; consequently, this sample could only be investigated at an initial concentration of 0.4%. For a better comparison, the sample concentrations were back-calculated to 100% v/v and the calculated EEQs are shown in Fig. 5. The dilute-acid pretreated substrate samples gave EEQs of 40.4 ng l^{-1} and 12.5 ng l^{-1} for CS and R1-S, respectively. The effluent sample R1-E showed the highest EEQ of 900 ng l^{-1}, while the untreated substrate sample R4-S showed an EEQ of 8.6 ng l^{-1}. However, the back-calculated EEQs are not based on the significant effect of the original EEQs compared to the negative control (4.6 ng l^{-1}).

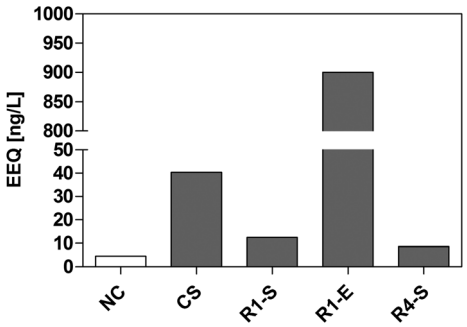

Fig. 5 Estrogenic activity of the samples CS, R1-S, R1-E and R4-S. Displayed is the highest induction of each sample (CS = 20.8%, R1-S and R4-S = 62.5%, R1-E = 0.4%) back-calculated to a test concentration of 100% v/v. Data are given as means (bars) and standard errors of the means (error bars) of the estradiol equivalent quotient (EEQ) in ng l^{-1}. The back-calculation is not based on significant differences. $n_{all} = 2$.

4 Discussion

4.1 Ecotoxicological biotests are an important tool to gain insight into generation of toxic compounds during biomass pretreatment

Due to the necessity of biomass pretreatment, the formation of a myriad of toxic compounds may not be able to be completely prevented nor can all possible products be easily identified by means of chemical analyses for process optimization. This study showed that biotests can be easily applied to identify where the generation of toxic compounds during biomass pretreatment occurs, thus leading to greater process optimization. Specifically, generally increased toxicity depending on the pretreatment could be monitored by the NR and MTT assays (acute cytotoxicity) and the FET test (acute embryotoxicity). We found that acidic pretreatments were related to generation of toxicity, with the dilute-acid pretreatment of CS exhibiting the highest cyto- and embryotoxicity. Compared to dilute-acid pretreated R1-S, CS was treated with a higher temperature and acid concentration and was from a different biomass feedstock with a different biomass composition (including higher levels of lignin). Sample R1-S showed the second highest cytotoxic and embryotoxic effects, while the hot-water pretreated R3-S (which also acts *via* weak acid hydrolysis) exhibited a small, albeit statistically significant (compared to R4-E), cytotoxic effect. Acidic biomass hydrolysis (even in very weak acidic pretreatments like hot-water) is known to generate toxic by-products from sugars and lignin which could cause the observed effects.[62–65] Among these are phenolic and furan compounds that were reported to be toxic for both fish cells as well as *D. rerio* embryos, and which are particularly environmentally relevant due to their estrogenic, genotoxic, hepatotoxic, and AhR agonist effects on various organisms, as well as effects on human health.[35,51,66,67] In comparison to acid-pretreated substrates the dilute-alkaline and untreated corn fiber substrates (R2-S and R4-S) exhibited no cytotoxic effects, although R4-S did result in significant embryotoxicity. The reason for this is unclear, but dissolved compounds from the unpretreated biomass could have negatively affected the embryos. Nevertheless, both assays are able to determine whether toxic components are generated and how strong the toxic potential is in relation to a different pretreatment, *e.g.* R1-S in comparison to R2-S.

While the ability of acute biotests to give insight into the actual composition of the investigated samples is limited, combined with mechanism-specific biotests, such as the EROD assay, the test battery can also provide more detailed information on the sample composition. For example, the lack of EROD induction can be an indicator for the absence of a known group of environmental hazardous contaminates. The EROD assay is a widely used indicator for induction of the CYP1A-biotransformation system. This enzyme system represents an important detoxification mechanism in the phase-I metabolism of hydrophobic organic toxicants in various organisms.[51] It converts hydrophobic substances into more hydrophilic – and, thus, more easily excretable – metabolites.[68] The lack of EROD activity could, therefore, be a result of investigating aqueous samples and indicate that the samples' acute toxicity was dominated by hydrophilic compounds, such as phenolics and furans. The presence of furan and hydroxymethylfuran, in particular in the dilute-acid pretreated samples, supports this hypothesis. Further, because the samples were produced by relatively weak pretreatment methods, lignin may not have been significantly degraded. More substantial degradation of lignin, which occurs in more stringent pretreatments, could release hydrophobic aromatic compounds that would cause EROD induction. A study by Tame *et al.*[36] revealed that more dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) from lignocellulosic materials are produced during combustion at low temperatures. Thus, although no EROD induction could be found for these pretreatments, dioxin-like activity should be considered for investigation of pretreated samples that are more likely to contain degraded lignin, such as bio oil from slow pyrolysis.

This proof of concept biotest battery demonstrates that by combining tweaks in biomass pretreatment choice and strategy (*i.e.*, stringency), the biofuel researcher could optimize the pretreatment method for a certain type of biomass feedstock. The specific optimization could be dictated by potential downstream environmental or human interactions or by the choice of a downstream biological conversion platform. For example, while our reactor microbiomes could decrease the toxicity of R1-S *in situ* (Agler *et al.* 2012 (ref. 39)), in pure culture conversion with, for example, yeast cells, the dilute-acid pretreatment could inhibit fuel production.[62,63]

4.2 Ecotoxicity assays sensitively demonstrate changes in ecotoxicological potentials during biomass processing steps

During chemical or (micro)biological biomass processing, the composition of the process streams, and, thus, their toxicity, is expected to change. Biotests were able to monitor this change by assessing the acute toxic potential (NR assay, MTT assay, and FET test) of a sample after specific biomass processing steps. The acute biotests revealed a reduced cytotoxic and embryotoxic potential of the effluent sample R1-E in comparison to the toxic potential of the substrate sample R1-S. The result indicates that the reactor microbiome was able to metabolise these compounds, and, thus, detoxify the sample. However, a low-level baseline toxicity was detected in all effluent samples that were complementary to the biomass pretreatments. Chemical analysis

showed that the formation and resulting increased concentration of carboxylate products from fermentation, such as acetate and *n*-butyrate, were most likely responsible for this weak toxic potential in each effluent sample. Furans are the most likely toxic compounds resulting from the dilute-acid pretreatment, and we found previously that they were completely removed in similar *n*-butyrate-producing acidogenic microbiomes (data not shown). Further cytotoxicity (NR) assays ($n = 3$) were conducted for an investigation of the cytotoxic potential of (sodium) *n*-butyrate and (sodium) acetate. Similar to the reactor samples, the pH value was adjusted to 7.4 ± 0.2. For acetate, an NR_{50}-value of 15.51 ± 2.02 mg ml^{-1} was determined, while the NR_{50}-value for *n*-butyrate was found to be 14.09 ± 10.05 mg ml^{-1} (details not shown). Although acetate and *n*-butyrate concentrations in the reactor effluent were lower than these levels (Table 3), the observed cytotoxic potential of the effluent samples is assumed to be at least partly elicited by the fermentation products, and synergistic or additive effects could have increased the toxic effectiveness. Nevertheless, besides toxicity of the carboxylate intermediates, effluent toxicity might be caused by other components, such as accumulated bacterial toxins.

In addition to acute toxicity, the mutagenic potentials of the samples were monitored by the Ames assay. Contrary to the results from the acute biotests (cytotoxicity- and embryotoxicity-tests), mutagenic potency was probably not related to acidic pretreatment stringency and biomass type because the substrate sample CS was not the strongest inducer. Instead, samples R1-S and R1-E showed the highest mutagenic potentials when tested without and with the S9 mix, respectively. Similarly to acute toxicity, the mutagenic potential was affected by microbiome fermentation as can be shown by comparison of R1-S and R1-E. At low sample concentrations R1-E exhibited less mutagenic effectiveness than R1-S when tested with S9 supplementation (Fig. 4) and we suspect that this was the result of a reduction of mutagenic compounds during the reactor microbiome fermentation. At the highest concentration (80%) we observed a seemingly contrary result, in which the number of positive wells for R1-E was higher than for the sample R1-S (Fig. 4). The reason for this observation is not clear, but could be due to the interactions between remaining hydrophobic compounds in R1-E and carboxylate fermentation intermediates, which required a certain threshold concentration to be reached before mutagenicity induction. The presence of hydrophobic compounds in R1-S could be indicated by a significantly lower mutagenic effectiveness of that sample ($p < 0.05$) with S9 supplementation. Specifically, the hydrophobic compounds are more likely activated or – in this case – deactivated by S9 enzymes.[69,70] The effluent sample R1-E, on the other hand, showed no significant differences between the approaches with and without the S9 mix. Therefore, the hydrophobic compounds might have already been degraded by the microbial community during the fermentation process.

Table 3 Average acetate and *n*-butyrate concentrations on days 50, 58 and 64 ($n = 3$ for all) measured in reactor effluent

Sample	Acetate (mg ml^{-1})	*n*-Butyrate (mg ml^{-1})
R1-E	7.03 ± 0.59	3.94 ± 0.94
R2-E	8.65 ± 0.60	3.73 ± 0.45
R3-E	4.69 ± 0.60	2.26 ± 0.48
R4-E	4.86 ± 0.41	0.73 ± 0.29

By means of these discussed biotests, changes in the actual composition of a sample during the biomass processing could be sensitively demonstrated. These results can be used in addition to the previous presented applications (*c.f.* Section 4.1) for biotests and be used for optimization of downstream processes and the dealing with intermediates that may interrupt the biomass processing. Further, because it has already been shown that the microbial communities responsible for microbiome fermentations can be shaped to achieve specific goals, it follows that these shaping techniques can be used in conjunction with biotest batteries to optimize toxicity reduction for downstream applications in parallel with product formation.

4.3 Potential issues and alternative endpoints

Ecotoxicological *in vitro* and *in vivo* biotests are adaptable test systems that can be modified for many purposes or challenges. However, complex samples, such as those investigated in this study, require a carefully considered choice of biotests, endpoints, and test organisms. Moreover, adaptations or modifications of standard test designs might be required to avoid misinterpretations of the obtained data. In the case of the Ames assay, the presence of histidine in samples investigated in the mutagenicity assay represents an issue that has to be considered prior to any investigation.[71] Histidine is known as a by-product of hydrolysis of proteins,[62] and therefore might have been present in the samples because they contained concentrations of proteins of up to 5 g l^{-1} (data not shown). Further, the stringency of the pretreatment might affect the amount of histidine in the sample. In this study, the absence of considerable histidine concentrations in the sample – and therefore the validity of the mutagenicity assay – could be confirmed by evaluating the mutagenic results for R1-S and CS. Since both corn stover (CS) and corn fiber (R1-S) samples are expected to contain protein and the CS pretreatment was more stringent than for R1-S (*i.e.*, higher temperature and acid concentration), a larger amount of histidine as a by-product to hydrolysis could be expected, thus leading to a higher count of revertants. A reliable indicator for the presence of histidine in the sample in quantities sufficient to cause false positives is the comparison of results obtained with *vs.* without metabolic activation. Histidine should cause similar false positive results independently from any metabolic activation. Instead, for samples R1-S and CS, significant differences between both test approaches were found for the concentrations 50% and 80%. It can, therefore, be concluded that the observed effects were due to the mutagenic effectiveness of sample compounds rather than a result of the presence of histidine. Nevertheless, since a significant mutagenic potential due to dilute-acid pretreatment was found, but the potential impact of histidine complicates interpretation of the data on mutagenicity, a biotest not depending on histidine-free samples, such as the Umu test, should be considered for future studies. To the best of our knowledge, no studies on this topic are available.

Another example for the importance of a careful selection of the test system is the YES assay for investigation of endocrine disruptors. Endocrine disruptors are molecules that affect the hormone system of organisms and affect a large number of

processes, such as the immune system.[72] The YES assay, however, investigates the induction of the human estrogen receptor α (ER α) by estrogenic active compounds, such as estradiol.[57] As described above (*c.f.* Section 4.1), the aqueous samples were expected to contain a large amount of hydrophilic and polar compounds. Therefore, the YES assay for investigation of estrogenic activity was applied subsequently to the EROD assay since a number of relevant estrogenic compounds are known to be polar.[73] In particular, the increase of the estrogenic activity induced by sample R1-E (900 ng l^{-1} when back-calculated to 100% v/v) in comparison to the complementary substrate sample R1-S was expected, because previous studies indicated that anaerobic treatment of an organic plant material by intestinal bacteria can increase the estrogenic potential of this material in bioreactors.[37,38] However, even if this value indicates an extremely high estrogenic potential of R1-E due to bacterial metabolites, it is not based on significant differences to the negative control. For example, the estrogenic potential of sample R1-E might be influenced by the comparably low test concentration of sample R1-E, which had to be reduced to 0.4% due to strong cytotoxic effects in comparison to 62.5% (R1-S and R4-S) and 20.8% (CS). Any putative estrogenic effects in R1-E were, therefore, presumably masked by strong cytotoxic effects of the carboxylate intermediates and the resulting necessary dilution of the test sample since it was shown that acetate and *n*-butyrate were present in the sample in sufficient concentrations to cause cytotoxic effects on RTL-W1 cells.[74,75] Therefore, our results require verification by different estrogenic biotests and masking of any mechanism-specific effects, such as estrogenic activity due to cytotoxicity, has to be taken into account for further investigations of biofuel-related samples. Specifically, prior to its application in a mechanism-specific biotest any test organism should be chosen very thoroughly regarding its robustness against any intermediates of the biofuel production process since acute toxic effects can differ substantially between different test organisms, as could be shown in this study with RTL-W1 cells, *S. typhimurium* and *S. saccharomyces*.

The YES assay is also known to be quite sensitive to matrix effects, such as anti-estrogenic or synergistic effects caused by the solid sample matrix.[76] Therefore, the efficiency of the solid phase extraction should be considered because complex samples, such as those investigated in the present study, might influence the results from the YES assay. Previous studies showed that in some cases an underestimation and in some cases an overestimation of the estrogenic activity can result from matrix effects. However, as could be seen in Fig. 5, the untreated substrate sample R4-S, which was tested in the highest possible concentration (62.5%), did not reveal an increased estrogenic potential in comparison to the negative control. Therefore, we postulate that any observed estrogenic activity, at least for the corn fiber derived samples R1-S and R1-E, was not due to the matrix effect but due to the effect of the pretreatment.

With these considerations, the results from the YES assay provided valuable information indicating estrogenic activity in samples CS, R1-S and R1-E. Furthermore, the susceptibility of the test organism *S. saccharomyces* to the main intermediate products of this fermentation process and thus the potential masking of any estrogenic effects, as we have shown, imply the need for a careful selection of the test organism and the extraction method in future studies, in particular involving mechanism-specific biotests.

5 Conclusion

Results from this study revealed an interesting relationship of the (eco)toxic potential with biofuel production substrate materials and pretreatment. Depending on the choice and stringency of the pretreatment for a certain biomass feedstock, toxic compounds are produced and release of these toxic compounds into the environment depends on the subsequent treatment of waste products and the handling of intermediates. However, even if no systematic release – such as the use of reactor effluent as fertilizer in agriculture – into the environment is expected, contaminations due to storage leaks or accident spills cannot be excluded. Maybe even more importantly, biotests can be utilized for the improvement of the quality and efficiency of the production process, in particular if microorganisms are involved. For example, toxic intermediates can be identified and their production can be either avoided or reduced, which would enhance the biofuel yield and contribute to process optimization. Overall, the application of ecotoxicological biotests allows a fast and early assessment of the hazard potential of biofuel-related contaminants. We identified, though, that due to the complexity of biomass-to-biofuel processing samples, necessary adaptations of biotests need to be developed specifically for these applications.

Abbreviations

AhR	Aryl hydrocarbon receptor
CPRG	Chlorophenol-red-β-galactopyranoside
DCA	3,4-Dichloroaniline
DCP	3,5-Dichlorophenol
E2	17β-Estradiol
EC_x	Effect concentration
EEQ	Estradiol equivalent quotient
ERα	Estrogen receptor α
EROD	Ethoxyresorufin-*O*-deethylase
FAME	Fatty acid methyl ester
FET	Fish embryo toxicity (test)
GHG	Green house gas
MTT	MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
NR_x	Effective concentration in the neutral red retention assay
PAH	Polycyclic aromatic hydrocarbon
PCDD/F	Dibenzo-*p*-dioxins and dibenzo-furans
RTL-W1	Rainbow trout liver-Waterloo 1
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TEQ	Toxicity equivalent quotient
YES	Yeast estrogenic screen (assay)

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