High-throughput assessment of the abiotic stability of test chemicals in *in vitro* bioassays

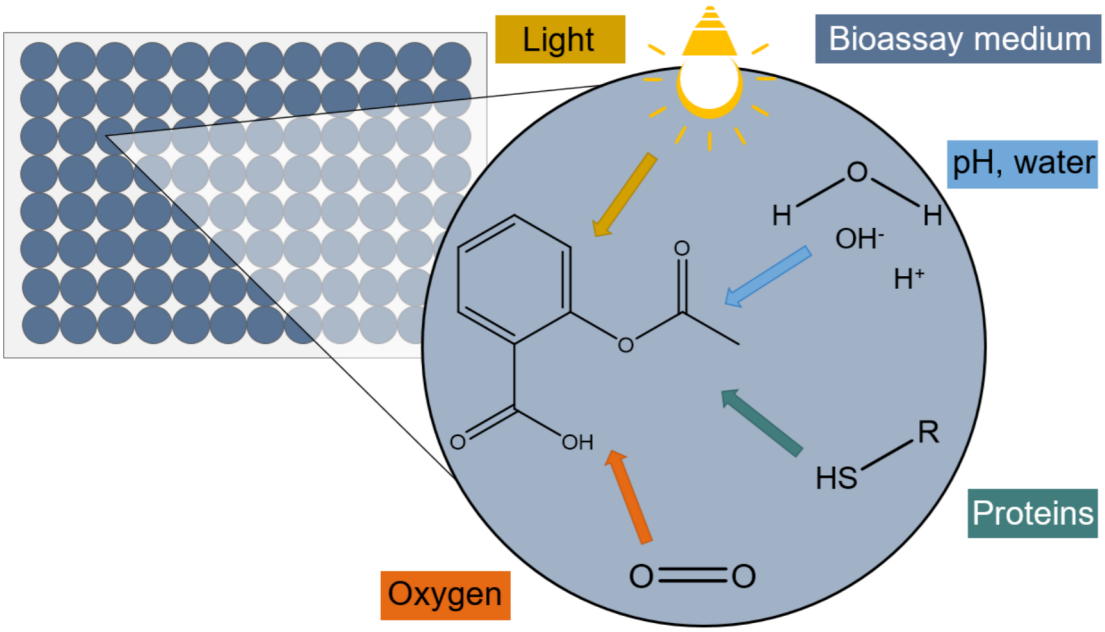
Julia Huchthausen[†], *Luise Henneberger*[†], *Sophia Mälzer*[†], *Beate Nicol*[¶], *Chris Sparham*[¶], *Beate I. Escher*[†‡*]

[†] Helmholtz Centre for Environmental Research – UFZ, Department of Cell Toxicology, Permoserstr. 15, 04318 Leipzig, Germany

[¶] Safety and Environmental Assurance Centre, Unilever, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

[‡] Eberhard Karls University Tübingen, Environmental Toxicology, Center for Applied Geoscience, 72076 Tübingen, Germany

Table of contents graphic



ABSTRACT

Abiotic stability of chemicals is not routinely tested prior to performing *in vitro* bioassays, although abiotic degradation can reduce the concentration of test chemicals leading to the formation of active or inactive transformation products, which may lead to misinterpretation of bioassay results. A high-throughput workflow was developed to measure abiotic stability of 22 test chemicals in protein-rich aqueous media under typical bioassay conditions at 37°C for 48h. These test chemicals were degradable in the environment according to a literature review. The chemicals were extracted from the exposure media at different time points using a novel 96-pin solid-phase microextraction. The conditions were varied to differentiate between various reaction mechanisms. For most hydrolyzable chemicals, pH-dependent degradation in phosphate buffered saline indicated that acid-catalyzed hydrolysis was less important than reactions with hydroxide ions. Reaction with proteins were mainly responsible for the depletion of the test chemicals in the media, which was simulated by bovine serum albumin (BSA) and

glutathione (GSH). 1,2-Benzisothiazol-3(2H)-one, 2-methyl-4-isothiazolinone and L-sulforaphane reacted almost instantaneously with GSH but not with BSA indicating that GSH is a good proxy for reactivity with electrophilic amino acids, but may overestimate the actual reaction with three-dimensional proteins. Chemicals such as hydroquinones or polyunsaturated chemicals are prone to autoxidation, but this reaction is difficult to differentiate from hydrolysis and could not be simulated by the oxidant *N*-bromosuccinimide. Photodegradation played a minor role, because cells are exposed in incubators in the dark and simulations with high light intensities did not yield realistic degradation. Stability predictions from various *in silico* prediction models for environmental conditions can give initial indications of the stability, but were not always consistent with the experimental stability in bioassays. As the presented workflow can be performed in high throughput under realistic bioassay conditions, it can be used to provide an experimental database for developing bioassay-specific stability prediction models.

Introduction

The field of human risk assessment of chemicals has undergone a paradigm shift in recent years moving away from animal testing to mechanistic *in vitro* toxicology.[1-4] New methods are being developed to enable reliable risk assessment without causing animal suffering. These so-called new approach methodologies (NAMs) also include the use of *in vitro* reporter gene bioassays, which can detect early cellular indicators for adverse outcomes in humans.[5] In various parts of the world, institutions such as the U.S. National Research Council, or the UK National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs), have worked to implement and optimize *in vitro* assays for toxicity assessment.[6, 7] Compared to conventional animal experiments, *in vitro* bioassays are more cost-effective, can be automated and, if fit-for-purpose, are ethically more acceptable.

Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) methods are used to extrapolate data from *in vitro* bioassays to the *in vivo* situation and to draw conclusions about the safety of chemicals in humans and the environment.[8] QIVIVE models rely on constant chemical exposure *in vitro* as they are typically based on the extrapolation of nominal effect concentrations *in vitro* to predicted maximum plasma concentrations *in vivo*.[9]

The dosed (nominal) concentration is the primary concentration metric used in *in vitro* toxicology.[10] Various loss processes, like volatilization, sorption to the plastic of the well plate and interactions with components of the medium or the cells, can cause the actual bioavailable concentration to deviate from the nominal concentration.[11-14] Less attention has so far been paid to abiotic transformation of the test chemicals, although it has been shown that test chemicals can react with components of the bioassay medium.[15, 16] Transformation processes may lead to a decrease in the concentration of the parent chemical over time, leading to an apparently lower effect. In addition, inactive or active transformation products can be formed, resulting in an underestimation or overestimation of the toxicity of the chemical.[16, 17] If these processes remain unnoticed, this might lead to considerable errors in QIVIVE models.[18] The stability of

chemicals in *in vitro* assays is not routinely monitored and prediction models are not tailored to bioassay conditions but rather to environmental degradation.[19-22]

The aim of this study was to develop a high throughput (HT) method to quantify degradation kinetics of chemicals in bioassay media in absence of cells to assess whether the chemical is abiotically degraded in the time course of an *in vitro* bioassay. A second aim was to decipher the transformation processes involved, which might lead to conclusions about possible transformation products. Understanding abiotic processes under *in vitro* test conditions, both qualitatively as well as quantitatively, is important as these processes have an impact on how *in vitro* assay response data can be interpreted in a QIVIVE context. In addition, the abiotic stability of the test chemicals in the environment and mechanisms of transformation were predicted with various freely available *in silico* models[19-22] to evaluate if it is possible to waive the experimental stability assessment or have a screening step before running the experimental workflow.

Experimental framework for stability testing

The framework for stability testing is depicted in Figure 1. As first step, the (pseudo) first-order degradation rate constants k and degradation half-lives ($t_{1/2}$) of all test chemicals in three different assay media were determined over the relevant time window for routine *in vitro* bioassays (48 h) used for risk assessment[23, 24] and environmental monitoring.[5]

Chemicals with $t_{1/2} \geq 100$ h can be considered abiotically stable under bioassay conditions and the *in vitro* bioassay can be performed without experimental quantification of exposure concentrations (Figure 1). For the method development and validation of the proposed workflow, however, all subsequent tests were performed for all chemicals. If $t_{1/2}$ was < 100 h, the abiotic stability should be investigated in more detail and the relevant degradation processes

identified (Figure 1). In this case the experimental quantification of exposure concentrations in the bioassay is recommended.

Four representative degradation processes in bioassay medium were evaluated: Hydrolysis, reactivity towards proteins, photodegradation, and oxidation/autoxidation. Degradation in phosphate-buffered saline (PBS) at pH 7.4, the pH of cell-based *in vitro* assays, is the core experiment, as these degradation processes also occur in all other test systems. If the $t_{1/2}$ in PBS equal those in bioassay medium, no further tests would be needed.

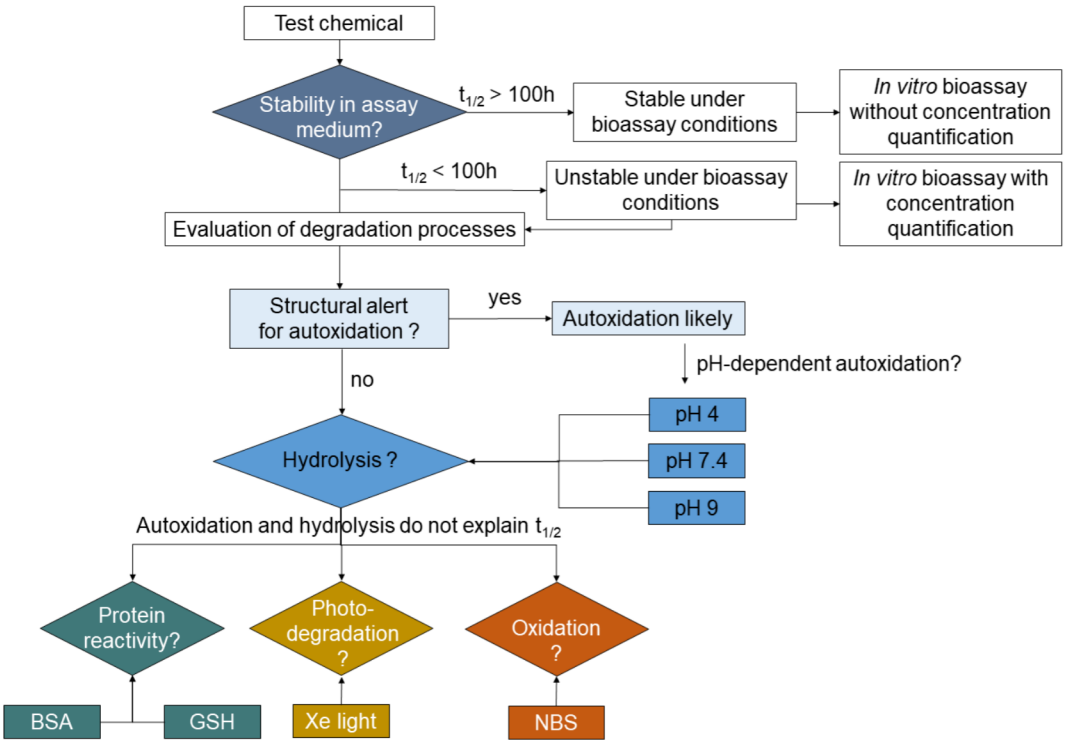


Figure 1: Framework for HT abiotic stability assessment of the test chemicals.

Autoxidation, i.e. the oxidation by oxygen in the air,[25] often catalyzed by traces of iron, cannot be experimentally distinguished from hydrolysis using the present experimental set-up but there are clear structural alerts for autoxidation such as hydroquinone moieties in polyphenols or benzohydroquinones.[16, 26] Autoxidation can also be pH-dependent.[26, 27] Hence, if such a structural alert was present in an investigated molecule, we assumed that autoxidation was the dominant mechanism over hydrolysis.

For those chemicals that were unstable in PBS, the mechanism of hydrolysis/autoxidation was assessed with pH-dependent experiments (Figure 1). Acid-catalyzed hydrolysis was assessed at pH 4 and the role of the hydroxide ion as nucleophile was determined at pH 9. This setup was based on the "Fate, Transport and Transformation Test Guideline" of the OPPTS (Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency).[28] [26]

Whenever the $t_{1/2}$ in PBS was higher than the $t_{1/2}$ in bioassay medium, additional degradation processes must have played a role (Figure 1). Reactivity towards proteins was probed with bovine serum albumin (BSA) as a surrogate for FBS, which contains mainly albumin. Since the potentially reactive amino acids might be buried due to the three-dimensional structure and folding of BSA, reduced glutathione (GSH, y-Glu-Cys-Gly), which is commonly used to simulate the reaction potential of chemicals with proteins, was also used to mimic a direct reaction with a free thiol group.

Photodegradation is highly relevant for chemicals in the environment, as they are constantly exposed to sunlight.[29, 30] In cell-based *in vitro* bioassays, photodegradation plays a minor role, as the incubation of the plates takes place in the dark. Nevertheless, the chemicals might be exposed to various light sources during the preparation of the assay. For this reason, the photodegradation potential of the test substances was investigated using a xenon test chamber (Q-SUN Xe-1, Q-LAB). The test setup was based on the OECD guideline: "Phototransformation of Chemicals in Water – Direct Photolysis".[31]

The mild oxidizing agent *N*-bromosuccinimide (NBS)[32] was used to examine the general susceptibility of the test substances to oxidation which might be an indicator for autoxidation in bioassay medium.

Using the proposed HT workflow for stability testing (Figure 1), unstable chemicals and their mechanisms of abiotic transformation can be identified under conditions that are matching those of a realistic *in vitro* bioassay, i.e., in a well plate format under identical exposure

conditions (with exception of photodegradation and oxidation). The workflow is HT because a novel solid-phase microextraction (SPME) device (Supel™ BioSPME 96-Pin Device) was used to extract the chemicals from the exposure medium, which enables the extraction of chemicals from 96-well plates in one easy process.[33] This device allowed us to upscale the experiments to a 96-well plate format, which greatly increased the throughput of the experiment.

Material and Methods

Chemicals. A set of 22 test chemicals suspected to be prone to abiotic transformation were selected for this study (Table 1). The chemical structures can be found in Table S8. All test chemicals had a purity of ≥ 90 %, were nonvolatile with water-air partitioning constants (K_{wa}) > 10000 L/L and were of moderate hydrophobicity with octanol-water partitioning constants log K_{ow} < 5 (Table S1). For all test chemicals, except for L-sulforaphane, aliquots were weighed and dissolved in methanol immediately before the experiment. The stocks were discarded after the end of the experiment. L-sulforaphane was purchased from the provider as a solution in ethanol (10 g/L) and used directly for the experiments. The content of methanol or ethanol in the test system was kept below 0.6 % for all tests.

Materials. The Supel™ BioSPME 96-Pin Devices (Sigma-Aldrich; 59683-U) had 96 polypropylene pins with a length of 24.7 mm. The tip of each pin was coated with C18 particles which were attached to the pins with polyacrylonitrile (PAN) binder. The coating length was 2.1 mm, and the average coating thickness was 12.5 µm, resulting in an approx. coating volume of 80 nL.[33] The experiments were performed in glass-coated deep-well plates (Product No. 60180-P306 and 60180-P336) from Thermo Scientific™. Oxidation experiments were performed in polystyrene deep-well plates from Labsolute (Product No. 7696548) to prevent oxidation of the glass-coating material. During incubation, the plates were sealed with adhesive

sealing film from Brand (Product No. 701367). The water for the experiments was obtained from a Milli-Q water purification system from Merck.

Table 1: Test chemicals of this study with suspected transformation processes.

Chemical	Suspected transformation process	Reference
1,2-Benzisothiazol-3(2H)-one	Reactivity towards proteins	Ref [34]
2-Methyl-4-isothiazolinone	Reactivity towards proteins	Ref [34]
6-Gingerol	Hydration – dehydration	Ref [35]
8-Gingerol	Hydration – dehydration	Ref [35]
Acetaminophen	Photodegradation	Ref [36]
Acetylsalicylic acid	Hydrolysis	Ref [37]
Amoxicillin	Hydrolysis, photodegradation	Ref,[38] Ref [39]
Andrographolide	Hydrolysis, reactivity towards proteins	Ref,[40] Ref [41]
Bendiocarb	Hydrolysis, photodegradation	Ref,[42] Ref [43]
Carbofuran	Hydrolysis, photodegradation	Ref,[44] Ref [45]
Chloramphenicol	Hydrolysis, photodegradation	Ref,[46] Ref [47]
Dinoseb	Photodegradation	Ref [48]
Furosemide	Hydrolysis, photodegradation	Ref,[49] Ref [50]
L-Sulforaphane	Hydrolysis, reactivity towards proteins	Ref,[51] Ref [52]
Malathion	Hydrolysis	Ref [53]
Oxytetracycline	Hydrolysis, photodegradation	Ref [54]
Phosmet	Photodegradation	Ref [55]
Pretilachlor	Photodegradation, reactivity towards proteins	Ref,[56] Ref [57]
Quercetin	Oxidation, photodegradation	Ref,[58] Ref [59]
Sethoxydim	Photodegradation	Ref [60]
Thiabendazole	Photodegradation	Ref [61]
Triclopyr	Photodegradation, reactivity towards proteins	Ref,[62] Ref [57]

The components of the bioassay media were purchased from Thermo Fisher Scientific. More detailed information about the chemicals and solvents used can be found in Table S2.

Chemical stability in bioassay medium. The stability of the test chemicals was tested in three different bioassay media: The AREc32 medium (10 % untreated FBS and 90 % DMEM Glutamax) had a protein content of 8.93 mL/L and a lipid content of 0.14 mL/L,[63] the GeneBLAzer medium (2 % charcoal-stripped FBS and 98 % OptiMEM) had a protein content of 4.84 mL/L and a lipid content of 0.02 mL/L,[63] and the neurobasal medium (2% B-27 Supplement, 2% GlutaMAX Supplement) had a protein content of 2.58 mL/L and a negligible lipid content.[64] All media contained 100 U/mL Penicillin and 100 µg/mL Streptomycin.

8 mL of each medium or buffer solution were spiked with the individual test chemicals. The final concentration was 5 mg/L for all test chemicals, except of 1,2-benzisothiazol-3(2H)-one (20 mg/L), 2-methyl-4-isothiazolinone (20 mg/L), acetaminophen (20 mg/L), acetylsalicylic acid (20 mg/L), amoxicillin (10 mg/L), chloramphenicol (20 mg/L), L-sulforaphane (10 mg/L), and oxytetracycline (20 mg/L). Aliquots of 600 µL per well of each medium and buffer were transferred into six glass-coated deep-well plates with one column per chemical and two rows per medium (Figure S1). One plate was preheated to 37 °C for 15 min and extracted immediately with solid-phase microextraction (SPME). The other plates were incubated at 37 °C for 2 h, 4 h, 7 h, 24 h or 48 h. After the respective incubation time, the plates were extracted with SPME. The plate for the 16 h incubation time was prepared separately by pipetting 600 µL of each medium into one well of a glass-coated deep-well plate following the pipetting scheme (Figure S1). The medium or buffer was spiked with the test chemicals directly in the plate leading to the same final concentrations as mentioned above. The plate was shaken at 1000 rpm (BioShake iQ, Quantifoil Instruments) for 5 min and then incubated at 37 °C for 16 h. The experiments were performed at least twice, and three times for chemicals that showed degradation.

Chemical stability at different pH values. The pH-driven degradation was tested in phosphate buffered saline (PBS, 137 mM NaCl, 12 mM phosphate) and three different buffer solutions, pH 4 buffer (50 mM potassium phthalate, 0.4 mM NaOH), pH 7.4 buffer (50 mM KH_2PO_4, 39.5 mM NaOH) and pH 9 buffer (50 mM KCl, 50 mM H_3BO_3, 21.3 mM NaOH). The experiments were performed in the same way as the experiments with medium. In one additional experiment, the degradation kinetics were determined for two chemicals (carbofuran and quercetin) at additional pH values covering the pH-range where degradation could be detected which was pH 6.6 to 8.6 (carbofuran) or pH 5.4 to 7.8 (quercetin) using the buffers described in Table S3.

Reactivity towards proteins. The pH of the BSA and GSH solutions in PBS was adjusted to pH 7.4 with 5 M NaOH and 5 M HCl. Since GSH is also unstable at pH 7.4 and might be oxidized rapidly by air,[65] an Ellman's test was performed to assess the stability of the GSH in the test solutions.[66] More information can be found in the supporting information (Text S2). 8 mL of the BSA or GSH solution in PBS were spiked with the individual test chemicals. 1,2-Benzisothiazol-3(2H)-one, 2-methyl-4-isothiazolinone, acetaminophen, acetylsalicylic acid, chloramphenicol and L-sulforaphane were spiked at a final concentration of 1.74×10^{-4} M. The concentration of BSA or GSH was 10 times higher (1.74×10^{-3} M). The other chemicals were spiked at a final concentration of 2.48×10^{-5} M with a BSA or GSH concentration of 2.48×10^{-4} M. As hydrolysis control, 8 mL of PBS were spiked with the corresponding concentration of the test chemicals. Aliquots of 600 µL per well were transferred into six glass-coated deep-well plates according to the pipetting scheme (Figure S1). The plates were incubated and extracted as described above. The plate for the 16 h incubation time was prepared separately as described above using the same chemical concentrations. The experiment was repeated up to three times for chemicals that showed degradation. In one additional experiment, the degradation kinetics were determined at additional GSH concentrations (1.24×10^{-3} M, 2.48×10^{-3} M, and 1.24×10^{-2} M) for two chemicals (pretilachlor and andrographolide).

Photodegradation. The photodegradation potential of the test chemicals was investigated using a xenon test chamber (Q-SUN Xe-1, Q-LAB) equipped with a Daylight-Q optical filter. 7 mL of PBS were spiked with the individual test chemicals at a final concentration of 5 mg/L for all test chemicals except of 1,2-benzisothiazol-3(2H)-one (20 mg/L), 2-methyl-4-isothiazolinone (20 mg/L), acetaminophen (20 mg/L), acetylsalicylic acid (20 mg/L), amoxicillin (10 mg/L), chloramphenicol (20 mg/L), L-sulforaphane (10 mg/L), and oxytetracycline (20 mg/L). Aliquots of 600 µL per well were transferred to six glass-coated deep-well plates according to the pipetting scheme (Figure S1). One plate was preheated for

15 min at 37 °C and extracted immediately without further incubation. The other plates were placed in the xenon test chamber and covered with a quartz glass plate to reduce volatilization of water from the samples without reducing the light intensity. The air temperature of the chamber was set at 37 °C, but the actual temperature in the sample could not be measured. The plates were incubated in three independent experiments at an irradiance at 340 nm of 0.77 W/m^2 for 1.5 h, 2 h, 3 h, 4 h, 4.5 h, 6 h or 7.5 h with full spectrum sunlight and extracted with SPME.

Oxidation. *N*-bromosuccinimide (NBS) was used as mild oxidizing reagent. In a preliminary test, 4 mL of a NBS solution in PBS was spiked with the test chemicals. 1,2-Benzisothiazol-3(2H)-one, 2-methyl-4-isothiazolinone, acetaminophen, acetylsalicylic acid, chloramphenicol and L-sulforaphane were spiked at a final concentration of 1.74×10^{-4} M. The concentration of NBS was 10 times higher (1.74×10^{-3} M). The other chemicals were spiked at a concentration of 2.48×10^{-5} M with a NBS concentrations of 2.48×10^{-4} M. 4 mL of PBS were spiked with the respective concentration of the test chemicals as hydrolysis control. Aliquots of 600 µL of each spiked solution were pipetted into three glass-coated deep-well plates according to the pipetting scheme (Figure S1). Ascorbic acid was added to each well of one plate at a concentration of 1.74×10^{-2} M or 2.48×10^{-3} M, respectively to stop the reaction. The plate was shaken at 1000 rpm (BioShake iQ, Quantifoil Instruments) for 5 min, preheated to 37 °C for 15 min and extracted with SPME. The other plates were incubated at 37 °C for 2 h or 48 h, ascorbic acid was added and the plates were extracted with SPME as described below.

Since oxidation was fast for most chemicals, additional incubation times below 2 h were tested. The plates were prepared separately by pipetting 600 µL of PBS or NBS solution into one well of a glass-coated deep-well plate following the pipetting scheme (Figure S1). The solutions were spiked with the test chemicals directly in the plate leading to the same final concentrations as mentioned above. The plates were shaken at 1000 rpm (BioShake iQ, Quantifoil Instruments) for 5 min and then incubated for 10 min, 15 min, 20 min, 30 min, 40 min, 45 min, 50 min,

60 min, 70 min or 90 min at 37 °C. After incubation, 1.74 x 10^{-2} M or 2.48 x 10^{-3} M ascorbic acid were added to each well to stop the reaction. The plates were shaken at 1000 rpm (BioShake iQ, Quantifoil Instruments) for 5 min and extracted with SPME.

Solid-phase microextraction. The plates from all stability tests were extracted using SPME immediately after spiking and after the respective incubation times. The BioSPME 96-pin device was conditioned for 20 min in a glass-coated deep-well plate containing 800 µL isopropanol per well and washed for 10 sec in a glass-coated deep-well plate with 900 µL of MilliQ water per well. It was transferred to the sample plate, attached to the plate with adhesive tape and shaken at 37 °C and 1000 rpm (BioShake iQ, Quantifoil Instruments) for 15 min. The desorption plate was prepared with 600 µL of the respective desorption solvent (Table S1) per well. The pin device was transferred to the desorption plate, attached and shaken at 1000 rpm without temperature control (BioShake iQ, Quantifoil Instruments) for 15 min. The transport time of the pin device between the plates was below 6 seconds to prevent the pin coating from drying out. After desorption, the desorption plates were stored at 4 °C until chemical analysis. Pin-water partitioning and the reproducibility of the SPME extraction is described in detail in Text S1, Figures S2 and S3 and Table S4.

Instrumental analysis. The concentration of the chemicals in the desorption solvents and in the PBS samples was quantified with a liquid chromatography instrument (Agilent 1260 Infinity II) coupled to a triple quadrupole mass spectrometer (Agilent 6420 Triple Quad). A Kinetex 1.7 µm, C18, 100 Å, LC column (50 × 2.1 mm), a BioZen 1.6 µm, Peptide PS-C18 LC column (50 × 2.1 mm), or a LunaOmega 1.6 µm, Polar C18, 100 Å, LC column (50 × 2.1 mm) were the columns used, depending on the test chemical. All LC and MS parameters can be found in the supporting information (Tables S5 and S6). PBS samples of 6-gingerol, 8-gingerol, dinoseb, phosmet and pretilachlor were diluted 1:1 with acetonitrile before measurement. Calibration standards with a concentration range of 1 – 10000 ng/mL were prepared in the

respective desorption solvent or PBS and measured with the samples. Acetonitrile blanks were measured after approx. every tenth sample.

Data evaluation. In a second-order reaction between two reactants A and B, the reaction rate depends on the concentration of the two reactants (eq. 1), where $k_{\text{second-order}}$ is the reaction rate constant.

$$\frac{\delta[A]}{\delta t} = -k_{\text{second-order}} \times [A] \times [B] \quad (1)$$

If one of the reactants is present in large excess (e.g., [B] >> [A]), its concentration remains constant over time and [B] can be combined with $k_{\text{second-order}}$ to obtain a pseudo-first-order reaction rate constant $k_{\text{pseudo first-order}}$ (eq. 2).

$$\frac{\delta[A]}{\delta t} = -k_{\text{pseudo first-order}} \times [A] \text{ with } k_{\text{pseudo first-order}} = k_{\text{second-order}} \times [B] \quad (2)$$

For all stability tests, the reaction partner [B] of the test substances was used in excess to assure that pseudo-first-order kinetics apply. The natural logarithm (ln) of the concentration in the desorption solvent after SPME (C_{des}) was plotted against the incubation time (t) to determine the degradation rate constant of the test chemicals.[67, 68] It was not necessary to convert C_{des} to the concentration in the assay, because all reactions were apparently first order. The experimental first-order rate constant k was derived from a linear regression of $\ln(C_{\text{des}})$ against t (eq. 3).

$$\ln (C_{\text{des}})_t = -k \times t \quad (3)$$

The degradation half-life ($t_{1/2}$) of the (pseudo) first-order decay constant k was calculated with eq. 4.

$$t_{1/2} = \frac{\ln(2)}{k} \quad (4)$$

***In silico* prediction of chemical stability.** Three freely available *in silico* prediction programs were used to get an indication of the stability of the test chemicals (Table 2). HYDROWIN, a model of the EPI Suite™ from the United States Environmental Protection Agency (US-EPA),

Table 2: *In silico* prediction models used for the prediction of chemical degradation.

Transforma-tion process	Provider	Module	Description
Hydrolysis	US-EPA	HYDROWIN	Aqueous hydrolysis rate constants for acid- and base-catalyzed hydrolysis and degradation half-lives for esters, carbamates, epoxides, halomethanes and selected alkyl halides.[22]
		Chemical Transformation Simulator (CTS)	Environmental and biological transformation pathways and products by comparison of the test chemical with existing reaction libraries. The abiotic hydrolysis reaction library contains 25 reaction schemes. A rank is assigned to each of the reaction schemes at pH 5, pH 7 or pH 9 at 25 °C, with which an approximate degradation half-life can be determined.[19]
Autoxidation	OECD QSAR toolbox	Autoxidation simulator	Simulates the abiotic oxidation pathways of chemicals under air or oxygen exposure at room temperature, atmospheric pressure and pH 7-9. Contains 325 structurally generalized molecular transformations, extracted from observed autoxidation pathways for 140 training set chemicals.[69, 70]
		Autoxidation simulator (alkaline conditions)	Simulates the abiotic oxidation pathways of chemicals under air or oxygen exposure at room temperature, atmospheric pressure and pH 10.2. Contains 308 structurally generalized molecular transformations, extracted from the observed autoxidation pathways of 139 training set chemicals.[69, 70]
Reactivity towards proteins		Protein binding OECD	102 reaction profiles and structural alerts for the capability of a directly acting electrophile to form covalent bonds with a protein.[71]
		Protein binding OASIS	OASIS TIMES model for skin sensitization. Contains 112 structural alerts related to interactions with (skin) proteins.[72]
		Protein binding potency Lys	Alerts for the capability of a chemical to react with lysine (Lys). The 77 structural alerts were developed based on data from the Direct Peptide Reactivity Assay (DPRA).[73-75]
		Protein binding potency Cys	Alerts for the capability of a chemical to react with cysteine (Cys). The 77 structural alerts were developed based on data from the Direct Peptide Reactivity Assay (DPRA).[73-75]

Table 2 continued: In silico prediction models used for the prediction of chemical degradation.

Transformation process	Provider	Module	Description
Reactivity towards proteins	OECD QSAR toolbox	Protein binding potency GSH	Potency of a chemical to react with proteins based on the capability to react with the thiol group of glutathione (GSH). The 137 protein binding alerts were developed based on experimental GSH RC50 values.[76]
Photo-degradation	US-EPA	Chemical Transformation Simulator (CTS)	Environmental and biological transformation pathways and products by comparison of the test chemical with existing reaction libraries. The direct photolysis reaction library contains 155 reaction schemes, but is currently unranked, so no degradation half-life can be determined.[20]

predicts aqueous hydrolysis rate constants for acid- and base-catalyzed hydrolysis and degradation half-lives.[22] The web-based Chemical Transformation Simulator (CTS),[77] developed by US-EPA, predicts different environmental and biological transformation pathways including abiotic hydrolysis[19] and photodegradation[20] and suggests possible degradation products.

The Organization for Economic Cooperation and Development (OECD) QSAR Toolbox provides QSAR-based prediction models for the prediction of adverse effects of chemicals.[21] Autoxidation of the test chemicals was predicted using two models in neutral and alkaline conditions and five different models were applied to predict the reactivity of the test chemicals towards proteins. Although the QSAR Toolbox models are called “protein binding potency” models, in this case protein binding refers to a chemical reaction, that is, the formation of irreversible covalent bonds between the test chemicals and a biological nucleophile. Possible reactions that can be predicted by the models are, for example, acylation, Michael addition, Schiff base formation, S_N2 reaction or S_NAr reaction. Reversible bonds of chemicals to proteins formed by interactions such as van der Waals forces or hydrogen bonds are not considered.

Results and Discussion

Chemical stability in bioassay media. 11 of 22 chemicals were stable in bioassay media and PBS (Figure 2) under bioassay conditions over 48h. The degradation rate constants derived from the decay curves (Figure S4) and thereof derived $t_{1/2}$ of all chemicals and media are listed in Table S7. While experiments ran over 48h, extrapolation of $t_{1/2}$ up to 100 h was possible and all chemicals with $t_{1/2} \geq 100$ h were considered stable in the respective medium or PBS.

Acetylsalicylic acid, pretilachlor, phosmet, bendiocarb and quercetin had similar $t_{1/2}$ in all media as well as PBS, indicating that hydrolysis and possibly autoxidation in the case of quercetin (Table S8) were responsible for the degradation. Phosmet showed the fastest degradation with $t_{1/2}$ below 5 h in all media. Malathion, carbofuran, amoxicillin, andrographolide and L-sulforaphane showed faster degradation in the assay media than in PBS. For these chemicals, the protein content of the medium appeared to affect the degradation rate. However, the rapid degradation of some of these chemicals in neurobasal medium was unexpected (e.g., $t_{1/2}$ malathion 4.37 h, $t_{1/2}$ andrographolide 5.01 h and $t_{1/2}$ L-sulforaphane 8.73 h). The protein content of the neurobasal medium (2.58 mL/L) was slightly below that of GeneBLAzer medium (4.84 mL/L), so the $t_{1/2}$ in GeneBLAzer and neurobasal medium were expected to be very similar. However, the $t_{1/2}$ in neurobasal medium were almost as high as those measured in AREc32 medium which has a much higher protein content (e.g., L-sulforaphane: AREc32 medium = 6.27 h and neurobasal medium = 8.73 h). It may be that the different composition of neurobasal medium influenced the degradation rate of the test chemicals, since it is an FBS-free medium. The neurobasal medium contains two supplements of undefined components that might be responsible for the accelerated degradation of the chemicals.

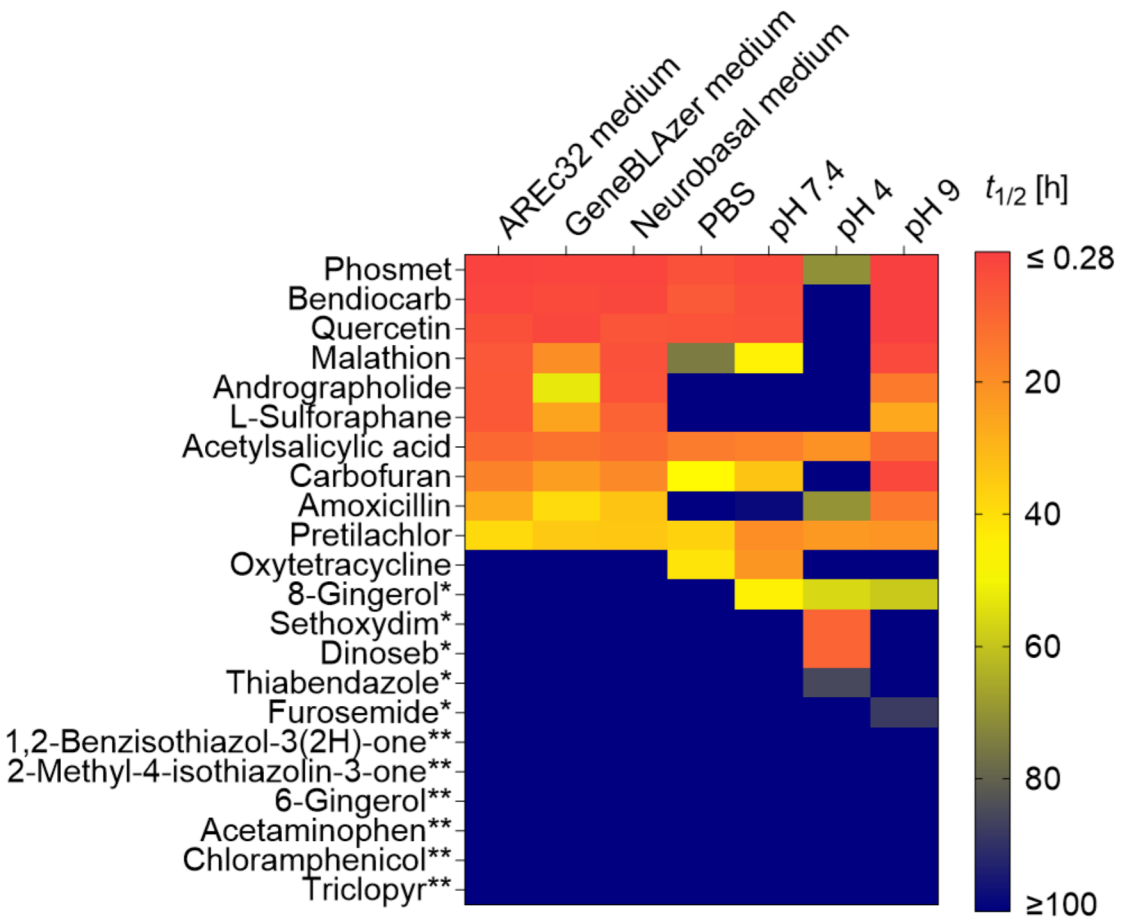


Figure 2: Stability of the test chemicals in three bioassay media, PBS and in three pH buffers. The different colors indicate the degradation half-life ($t_{1/2}$) of the chemical in the respective medium or buffer. Chemicals that were stable in all media and PBS are marked with an asterisk and chemicals that were stable in all media and buffer are marked with two asterisks.

Oxytetracycline was the only chemical that showed degradation only in PBS and not in the bioassay media. Reversible binding to proteins of the media may stabilize the structure of oxytetracycline and prevent a hydrolytic degradation.

Chemical stability at different pH values. In addition to the stability of the chemicals in cell culture media and PBS as a physiological buffer, the chemical stability was investigated at three different pH values over a maximum duration of 48 h to determine whether degradation of chemicals is more likely to be acid-catalyzed or neutral hydrolysis or might be accelerated by hydroxide ions. Autoxidation can also be pH-dependent if the redox potential of the chemical is dependent on the protonation state.[27, 78] Evaluation of the pH-dependence of degradation is

important because the pH value of the bioassay medium can change during the course of the bioassay. The degradation kinetics plots of all chemicals (Figure S5) were used to derive k and $t_{1/2}$ at the different pH values (Table S7).

Six chemicals were found to be stable at all three pH values with $t_{1/2} \geq 100$ h, and furosemide and thiabendazole showed only very slow degradation at one pH value (Figure 2). Acetylsalicylic acid, pretilachlor and 8-gingerol showed similar $t_{1/2}$ at all pH values (Figure 2), thus the degradation was independent of the hydroxide or proton concentration. For these chemicals, the nucleophile is either water or the hydrolysis is a SN1 reaction.

Most of the test chemicals (phosmet, bendiocarb, quercetin, malathion, carbofuran, andrographolide and L-sulforaphane) showed fastest degradation at pH 9 and decreasing degradation rates at pH 7.4 and pH 4. The degradation of these chemicals is apparently accelerated by hydroxide ions, since they were stable at low hydroxide concentrations (pH 4, $[OH^-] = 10^{-10}$ M) and degraded more rapidly with increasing hydroxide concentrations pH 7.4 ($[OH^-] = 10^{-6.6}$ M) and pH 9 ($[OH^-] = 10^{-5}$ M). For andrographolide and quercetin there was evidence of possible autoxidation.[69, 70, 79] For the other chemicals, it was probably a hydrolytic degradation. In this case, the overall reaction followed pseudo-first-order kinetics between the test chemicals and the hydroxide ions, despite the concentration of hydroxide ions at the highest pH values tested was not higher than the chemical concentration (e.g. [carbofuran] = 2.26×10^{-5} M and $[OH^-]$ at pH 9 = 1×10^{-5} M). The reactions took place in a buffered system to keep the pH, and thus the hydroxide ion concentration, constant throughout the duration of the test. The pH of the buffers was measured after 48 h incubation with the chemicals and showed no significant deviation from the initial pH values within 0.1 pH units.

Bendiocarb, phosmet and quercetin showed the lowest $t_{1/2}$ at pH 9 with 0.28 h (bendiocarb), 0.26 h (phosmet) and < 0.28 h (quercetin) (Table S7). The time required to perform the experiments was approx. 17 min (0.28 h). Therefore, for chemicals with very rapid

degradation, which were already degraded without additional incubation, $t_{1/2}$ < 0.28 h was reported.

For two of the test chemicals (carbofuran and quercetin), the degradation was measured from pH 6.6 to 8.6 (carbofuran) or from pH 5.4 to 7.8 (quercetin) (Table S3). The *k* increased linearly with increasing [OH^-] for carbofuran (Figure 3A) and quercetin (Figure 3B). For carbofuran, the hydroxide ion acted as a nucleophile and the measured degradation constant *k* can be broken down into hydrolysis (k_{H2O}) and reaction with OH^- (k_{OH-}) (eq. 5).

$$k = k_{H_2O} + k_{OH^-} \times [OH^-] \quad (5)$$

Thus, a linear regression of *k* plotted against [OH^-] has a slope of k_{OH-} and an intercept of k_{H2O}. Acid-catalyzed hydrolysis and reaction with water was negligible as the intercept of the linear regression of eq. 5 in Figure 3 with a k_{H2O} of 0.014 ± 0.006 h^{-1} demonstrated. The k_{OH^-} was $3.42 \times 10^4 \pm 1.69 \times 10^3$ $M^{-1}h^{-1}$.

For quercetin, which showed a structural alert for autoxidation, the reaction rate also increased linearly with increasing pH, because the autoxidation is also pH-dependent.[27] Hydroxide ions can deprotonate the transition state of this reaction, which accelerates the reaction.[26, 78] The intercept of Figure 3B was <0.01 h^{-1}, which means that protons do not play a role but k_{OH^-} was $5.58 \times 10^5 \pm 1.87 \times 10^4$ $M^{-1}h^{-1}$ for the OH^--facilitated autoxidation (eq. 6).

$$k = k_{OH^-} \times [OH^-] \quad (6)$$

Dinoseb and sethoxydim were rapidly degraded at pH 4, but had $t_{1/2} \geq 100$ h at pH 7.4 and pH 9. There is no evidence of hydrolysis of dinoseb in the literature, but the photocatalytic degradation was much faster at pH 4 than at higher pH-values.[80] Dinoseb has a acidity constant (pK_a) of 4.62[81] and the anion present at pH 7.4 and 9 is stabilized by the delocalization of the pi-electrons over the benzene ring into the electron-withdrawing nitro-substituents. For

sethoxydim, the observations are consistent with the literature, since hydrolysis of the oxime group is catalyzed by protons.[82]

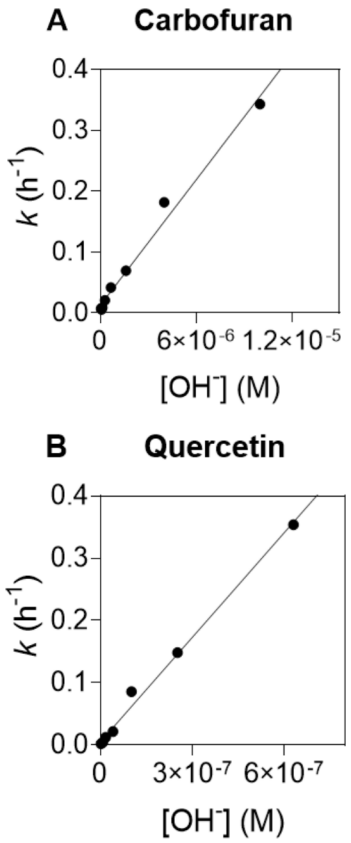


Figure 3: Experimental degradation constant (k) of (A) carbofuran and (B) quercetin plotted against the concentration of hydroxide ions [OH^-].

Oxytetracycline was the only test chemical that showed degradation only at pH 7.4. At lower or higher pH, $t_{1/2}$ was ≥ 100 h for that chemical. This observation is consistent with the literature where it has been demonstrated that oxytetracycline is hydrolyzed to apo-oxytetracycline.[54] Oxytetracycline has three acidic functions with pK_a values of 3.28, 6.68 and 12.52 and one basic amino group with pK_a of 9.00.[83] At pH 7.4, the molecule is 84% anionic and 16% zwitterionic. Differences in speciation could influence the susceptibility to hydrolytic degradation.

Reactivity towards proteins. The reactivity of the test chemicals towards proteins was tested with BSA and GSH as model nucleophiles. The pH of all solutions was adjusted to pH 7.4 and the nucleophile was always used in excess to ensure pseudo-first-order kinetics.

The reduced glutathione (GSH) was quantified with Ellman's assay.[66] The measured GSH concentration equaled the nominal concentration when measured immediately but deviated from the initial concentration by up to a factor of 10 after 48h (Figure S6). Since this observation did not occur at all concentrations, it could be an artifact. Although GSH was used in 10-fold excess to the chemical concentration, GSH could have been partially depleted after 48 h, which could slow down the reaction. The plots of degradation kinetics of the test chemicals in a GSH or BSA solution in PBS (Figure S7) were used to derive k as fit parameter and $t_{1/2}$ (Table S7).

Eight chemicals showed degradation in the GSH solution with $t_{1/2} < 100$ h, i.e., $k > 0.007$ h^{-1} (Figure 4A). For five of the chemicals (acetylsalicylic acid, andrographolide, carbofuran, malathion and pretilachlor) k in the GSH solution was higher than k measured for the PBS control, but there was only a significant difference for andrographolide and pretilachlor (unpaired t-test). There was an immediate degradation of 1,2-benzisothiazol-3(2H)-one, 2-methyl-4-isothiazolinone and L-sulforaphane, so that no k could be fitted and $t_{1/2}$ was ≤ 0.28 h for these chemicals. It is well known that isothiazolinone biocides like 1,2-benzisothiazol-3(2H)-one and 2-methyl-4-isothiazolinone can react with the cysteine residue of GSH.[84] However, there was no degradation of either chemical in BSA solution nor in the bioassay media up to 48 h of incubation. The size and three-dimensional structure of the BSA molecule may be a steric hindrance that prevents a reaction with the free cysteine, also explaining the stability of 1,2-benzisothiazol-3(2H)-one and 2-methyl-4-isothiazolinone in bioassay medium (Figure 2, Table S7). In this case, the stability test in GSH solutions did not reflect stability in the bioassay medium, so GSH should not generally be used as sole surrogate for determining reactivity towards proteins in assay medium.

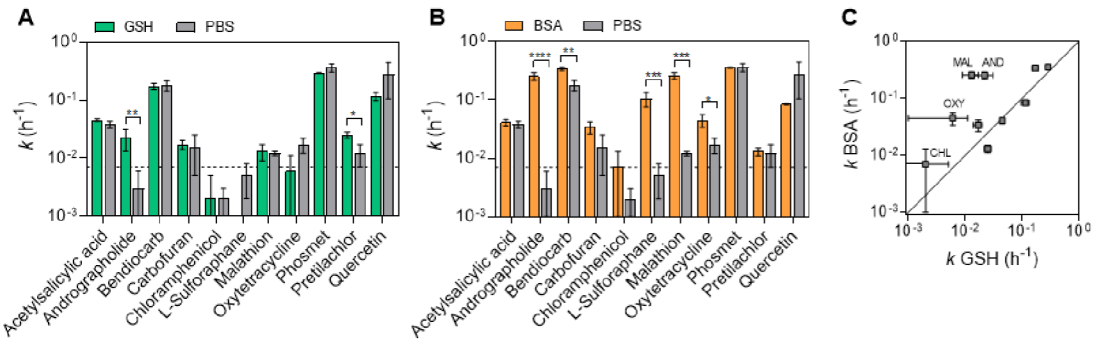


Figure 4: (A) Comparison of experimental degradation constants (k) of the test chemicals in glutathione (GSH) in phosphate-buffered saline (PBS) compared with k in PBS. (B) The k of the test chemicals in bovine serum albumin (BSA) in PBS compared with k in PBS. (C) The k in BSA solutions in PBS compared with k in GSH solutions in PBS. Only chemicals with $k > 0.007$ in at least one test solution are shown. For L-sulphoraphane no k could be measured in GSH because $t_{1/2}$ was ≤ 0.28 h. The difference between k measured in the GSH or BSA solution and in PBS was tested with an unpaired t-test (A and B). The asterisks above the columns indicate the level of significance. If no asterisks are shown, the difference was not significant. CHL: Chloramphenicol, OXY: Oxytetracycline, MAL: Malathion and AND: Andrographolide (C).

Eleven chemicals had $k > 0.007$ h^{-1} in the BSA solution (Figure 4B). The k in BSA solution was higher than k in PBS for nine of the chemicals, but the difference was significant only for andrographolide, bendiocarb, L-sulforaphane, malathion and oxytetracycline (unpaired t-test, Figure 4B). L-sulforaphane showed a very fast degradation in GSH and BSA solutions and was stable at pH 7.4 in PBS, thus, the reaction with proteins must be the main degradation pathway for this substance. This is consistent with the observation of Hanschen et al.[52] who demonstrated that L-sulforaphane can react with the thiol group of cysteine as well as with the amino group of e.g., lysine.

Bendiocarb, malathion and oxytetracycline were all hydrolyzed at pH 7.4, which makes it difficult to say whether there is an additional reaction with proteins, or whether the hydrolysis might be accelerated due to the presence of BSA. Only malathion showed faster degradation in the bioassay medium than in PBS, which also indicates a reactivity towards proteins for this chemical. Yamagishi et al.[85] recently showed that malathion can form various adducts with human serum albumin. For most chemicals, k measured in the presence of BSA or GSH did not differ greatly (Figure 4C). However, four of the test chemicals (chloramphenicol, oxytetracycline, andrographolide, malathion) showed faster degradation with BSA than with

GSH. Chloramphenicol and oxytetracycline, showed low k and high standard deviations with both nucleophiles, hampering the evaluation of this result. Malathion and andrographolide showed a significantly faster degradation in the presence of BSA compared to GSH (up to a factor of 19.9 difference). GSH has a freely accessible thiol group, but BSA contains other reactive amino acids (e.g., lysines) that can play a role in reactivity. The results obtained with both nucleophiles are generally comparable for most of the chemicals, but since some chemicals showed a significantly faster reaction with GSH than with BSA and vice versa, testing with both nucleophiles is advisable.

The degradation kinetics were measured for additional GSH concentrations for andrographolide and pretilachlor to determine the second order rate constant with GSH k_{GSH} (eq. 7). Figure 5 shows k plotted against the GSH concentration for pretilachlor (A) and andrographolide (B).

$$k = k_{H2O} + k_{GSH} \times [GSH] \quad (7)$$

For pretilachlor, k_{GSH} determined from the fit was 37.55 M^{-1} h^{-1} and k_{H2O} was 0.049 h^{-1}. This is slightly higher but in the same range as stability measured in PBS (0.022 ± 0.010 h^{-1}), because pretilachlor showed slow degradation at pH 7.4. Although pretilachlor reacted with GSH, it showed no reactivity to BSA (Figure 4B), because k in the BSA solution was higher than k_{H2O}, but the difference was not significant (unpaired t-test).

Andrographolide had a k_{GSH} of 437.90 $M^{-1}h^{-1}$ and was found to be stable at pH 7.4 with $k \leq 0.007$, so the intercept was set to 0. Michael addition is the mechanism of the second-order reaction of andrographolide with GHS.[41] Andrographolide showed an even faster reaction with BSA, which could be caused by other reactive amino acids in addition to cysteine.

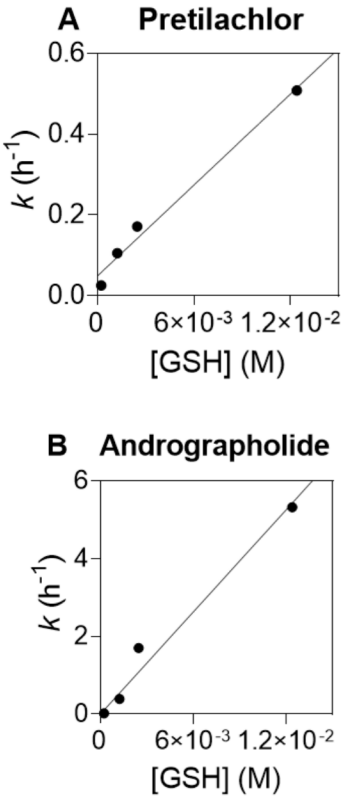


Figure 5: Experimental degradation constant (*k*) of (A) pretilachlor and (B) andrographolide plotted against the concentration of glutathione [GSH].

Photodegradation. The susceptibility of the test substances to photodegradation was tested by incubation in a xenon test chamber. The chemicals were exposed to the radiation of the lamp for up to 7.5 h, which corresponds to a multiple of the light intensity to which chemicals are normally exposed under laboratory conditions. The degradation plots of all chemicals can be found in the supporting information (Figure S8). The sample temperature could not be monitored in the xenon chamber and the samples were prone to evaporation after longer incubation. Therefore, there were volume variations in the samples from different time points. Since the volume of the desorption solution was constant, these variations should not have a large effect on the relative chemical concentration, but no kinetics for photodegradation were fitted, since these would not be comparable with the kinetics of the other test systems.

An overview of the qualitative photodegradation results for the test chemicals can be found in the supporting information (Table S7). 11 chemicals showed degradation within 7.5 h incubation in the xenon test chamber. Three of these chemicals (bendiocarb, phosmet and quercetin) showed $t_{1/2} < 7.5$ h in PBS in the dark, so the degradation of these chemicals might be caused by hydrolysis (or autoxidation) and not by photodegradation. 1,2-Benzisothiazol-3(2H)-one, chloramphenicol and furosemide, which were all stable in PBS in the dark, and oxytetracycline showed the fastest degradation in the xenon test chamber, so these chemicals are very likely to be prone to photodegradation which is also in line with the literature.[47, 50, 54, 86] All chemicals that showed fast photodegradation were found to be stable in the bioassay medium. This observation proves that for a normal use of the chemicals in the *in vitro* bioassay, photodegradation does not play a significant role, as the chemicals are not exposed to high light intensities over a longer period of time. For other *in vitro* systems such as algal toxicity, where incubation in light is necessary, these processes could play a more important role.[87]

Oxidation. Although autoxidation in the bioassay medium is possible for some chemicals, this reaction cannot be separated from the other processes and is therefore difficult to detect. Potentially comparing stability in presence and absence of antioxidants could shed more light on autoxidation. The general susceptibility for oxidation was checked by incubation of the test chemicals with the mild oxidant *N*-bromosuccinimide (NBS). NBS was used in excess to ensure a complete reaction of the chemicals. The reaction was very fast and the half-lives were lower than the process time of the experiment. The degradation plots of all chemicals can be found in the supporting information (Figure S9) and an overview over the results can be found in Table S7. 15 of the 22 test chemicals were degraded by NBS within 2 h incubation (Figure S9, Table S7). 2-Methyl-4-isothiazolinone, acetylsalicylic acid, bendiocarb, chloramphenicol, pretilachlor, thiabendazole and triclopyr were not oxidized within the total incubation time of 48 h. The observed degradation of acetylsalicylic acid, bendiocarb, and pretilachlor after 48 h

was caused by hydrolysis, since the chemical concentration in the NBS solution did not differ from the concentration in the PBS controls (Figure S9). Most of the other test chemicals were degraded rapidly (< 10 min) by NBS. The test with NBS showed that most of the test substances were principally oxidizable but oxidation does not appear to be relevant under bioassay conditions, because many chemicals that were degraded by NBS were stable in the bioassay medium (1,2-benzisothiazol-3(2H)-one, 6-gingerol, 8-gingerol, acetaminophen, dinoseb, furosemide, sethoxydim). Thus, NBS is not a good substitute to detect oxidation under bioassay conditions (autoxidation), however, reaction with NBS may indicate that these chemicals can also be oxidized within cells by metabolizing enzymes (cytochrome P450 enzymes).[88]

***In silico* predictions.** Three different *in silico* models were used to determine the susceptibility of the test chemicals to hydrolysis at different pH values, photodegradation and their reaction potential towards proteins. All models were designed for the prediction of environmental degradation processes or structural alerts for chemical reactivity. We wanted to evaluate if these models could be used to identify unstable chemicals in *in vitro* bioassays as well. The *in silico* models predicted degradation for all chemicals except thiabendazole for at least one test condition (Figure 6). More detailed information can be found in the supporting information (Table S9).

Two models (HYDROWIN and CTS) were used to predict hydrolysis of the test chemicals at three pH values. The models often provided different predictions of chemical stability, and HYDROWIN often lacked data for predicting stability at higher and lower pH. The predicted $t_{1/2}$ (Table S9) were often very high (days to years), compared to the normal duration of an *in vitro* bioassay (24 to 48 h), which was also performed at higher temperature.

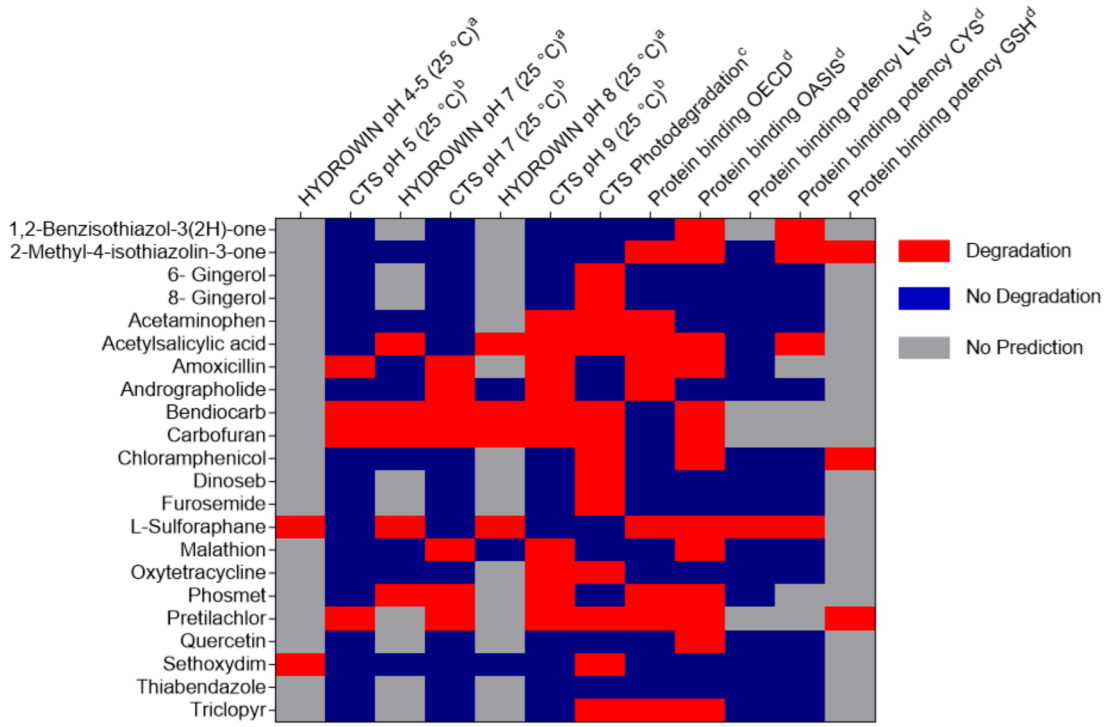


Figure 6: Overview of the *in silico* stability predictions for all test chemicals. Underlying data are given in Table S9. The *in silico* prediction models used for hydrolysis prediction also predict degradation half-lives ($t_{1/2}$). All chemicals with predicted $t_{1/2} \leq 60$ days were classified as degradable (red), those with $t_{1/2} > 60$ days as not degradable (blue). The grey boxes indicate chemicals and conditions where no prediction was possible. Only qualitative predictions were made for photodegradation and reactivity towards proteins using the QSAR toolbox. [a]HYDROWIN was accessed via EPI-Suite version 4.1.[22] [b]Chemical transformation simulator version 1.1 (CTS) was accessed via the internet (https://qed.epa.gov/cts/, 20. April 2021).[19] [c]Chemical transformation simulator version 1.1 (CTS) was accessed via the internet (https://qed.epa.gov/cts/, 20. April 2021).[20] [d]Accessed via QSAR Toolbox version 4.4.1.[71-76]

As a conservative approximation, all chemicals with predicted $t_{1/2} \leq 60$ days were considered degradable according to the persistence criterion of the European chemical regulation REACH.[89] For ten of the chemicals, both models predicted no degradation at all pH values. At pH 4 or 5, respectively, HYDROWIN predicted degradation for two chemicals (L-sulforaphane, sethoxydim) and CTS for four chemicals (amoxicillin, bendiocarb, carbofuran, pretilachlor). At pH 7, HYDROWIN predicted the degradation of five and CTS of seven chemicals and at pH 9, degradation was predicted for four chemicals by HYDROWIN and ten chemicals by CTS.

CTS was also used to predict photodegradation and 13 of the test chemicals were predicted to be prone to photodegradation. There were no $t_{1/2}$ predicted for photodegradation, so all chemicals with predicted photodegradation were considered as unstable.

Five models from the QSAR Toolbox were used for the prediction of reactivity towards proteins. The protein binding OECD model classified nine chemicals as reactive and the protein binding OASIS model classified 13 chemicals as reactive. The protein binding potency LYS model predicted degradation only for L-sulforaphane and the protein binding potency CYS model predicted degradation for 1,2-benzisothiazol-3(2H)-one, 2-methyl-4-isothiazolinone, acetylsalicylic acid and L-sulforaphane. 2-Methyl-4-isothiazolinone, chloramphenicol and pretilachlor were predicted to be unstable by the protein binding potency GSH model.

Comparison of experimentally determined stability with *in silico* predictions.

The models used for the prediction of hydrolysis or photodegradation were developed to predict these processes in the environment. The protein reactivity models are based on structural alerts and do not give any indication of reaction rates or conditions.

Since the pH of the bioassay medium is 7.4, hydrolysis at neutral pH should be a major degradation process in bioassay medium. The HYDROWIN model was able to give a prediction for 13 of the test chemicals at pH 7. Acetylsalicylic acid, bendiocarb, carbofuran, L-sulforaphane and phosmet were predicted to be degradable. All of these chemicals, except of L-sulforaphane also showed degradation in the experiment. However, two chemicals (malathion and oxytetracycline) that showed degradation at pH 7.4 in the experiment were predicted to be stable by the model. According to CTS, amoxicillin, andrographolide, bendiocarb, carbofuran, malathion, phosmet and pretilachlor were prone to degradation at pH 7. Amoxicillin and andrographolide were found to be stable in the experiment, but 8-gingerol, acetylsalicylic acid and oxytetracycline showed degradation which was not predicted by CTS. Quercetin, which also showed degradation at pH 7.4 in the experiment, was probably not

degraded hydrolytically but oxidized, which is why it was classified as stable by CTS. Table S10 compares model predictions with experimental results. For HYDROWIN, the agreement was 77 % and for CTS 76 %, respectively.

Reactivity towards proteins is a major potential degradation route for chemicals in bioassays, along with hydrolysis, since most media contain high levels of FBS or protein-rich supplements. In the experiments, six chemicals (1,2-benzisothiazol-3(2H)-one, 2-methyl-4-isothiazolinone, andrographolide, L-sulforaphane, malathion and pretilachlor) reacted with GSH and/or BSA.

The models used to predict reactivity towards proteins are normally used to get early warnings about a possible skin sensitization potential of chemicals.[73-75] Therefore, they only give structural warnings indicating potentially reactive groups of the test chemical, but do not give any indication of conditions and rate of a possible reaction. None of the models were able to identify all chemicals that showed reactivity towards proteins in the experiment. The protein binding OECD and the protein binding OASIS models gave alerts for approx. half of the chemicals. Still, both models could not identify all chemicals that showed reactivity towards proteins in the experiment. The protein binding OECD model classified 1,2-benzisothiazol-3(2H)-one and malathion as non-reactive and the protein binding OASIS model gave no alert for andrographolide. The agreement of both models with the experimental findings was 59 % respectively. 1,2-Benzisothiazol-3(2H)-one, 2-methyl-4-isothiazolinone, acetylsalicylic acid and L-sulforaphane were predicted to be reactive by the protein binding potency CYS model, but it did not give a warning for andrographolide and malathion and could not make a prediction for pretilachlor. The agreement with the experimental results was 76 % for the protein binding potency CYS model, which was the best agreement of all models predicting reactivity towards proteins. The protein binding potency GSH model had an agreement of 67 % with the experimental results and predicted reactivity for 2-methyl-4-isothiazolinone, chloramphenicol and pretilachlor and no prediction was possible for the other chemicals. L-Sulforaphane was

the only chemical classified as reactive by the protein binding LYS model. L-sulphoraphane showed degradation in the presence of BSA, but from these results it is not possible to conclude which amino acids were involved in the reaction.

Conclusion

The proposed HT workflow for the determination of the abiotic stability and characterization of degradation processes of chemicals in *in vitro* bioassays was used to evaluate the stability of 22 environmentally unstable test chemicals under bioassay conditions. Hydrolysis at pH 7.4, autoxidation (specifically for quercetin), and reactivity towards proteins were identified as the main responsible processes for the degradation of chemicals in bioassay medium. All chemicals that showed degradation in assay media were either degraded in PBS alone or showed reactivity towards proteins. The experiments showed that the abiotic stability of chemicals played a relevant role even in the relatively short time frame of *in vitro* bioassays and that stability tests are necessary to obtain reliable bioassay results.

The depicted workflow (Figure 1) suggests that first, the concentration of the test chemicals in the respective bioassay medium should be measured and compared with the initial concentration. If no reduction of the initial concentration can be detected in this test, the chemicals can be considered as abiotically stable and no further tests are necessary. However, if a reduction in chemical concentration of > 20% compared to the initial concentration is observed, the $t_{1/2}$ should be determined in the respective medium. If chemicals are not stable, the concentrations should be quantified in the bioassay and measured effect concentrations should be reported.

To identify the responsible degradation processes, the $t_{1/2}$ at pH 7.4 should be determined first, and if they are found to be higher than the corresponding $t_{1/2}$ in the medium, the reactivity towards proteins should be tested with BSA or GSH. In this case, the BioSPME

96 Pin Device can be used for a HT measurement of the relative concentration, but also other extraction techniques can be used for more hydrophilic (e.g., protein precipitation) or hydrophobic (e.g., liquid-liquid extraction) chemicals.

According to the results of the present study, tests for photodegradation are not necessary if the bioassays are performed under laboratory conditions and incubated in the dark. Tests with NBS could not mimic oxidation under bioassay conditions in the present study, but might be used to give indication of metabolic degradability.

In silico models for the prediction of hydrolysis and reactivity towards proteins, can be used in support of experimental tests to screen test chemicals for possible degradation or reactivity prior to bioassay. The models generally showed good agreement with the experimental data, but no model could predict all chemicals that showed degradation in the experiment. The models were not designed for the specific conditions in the bioassay and are therefore not sufficient on their own to evaluate the stability of chemicals in this context. Having a larger set of experimental stability data under bioassay conditions, it may be possible to develop a model capable of reliably predicting stability in the bioassay in the future, but until this is achieved, experimental stability measurements are indispensable for a reliable evaluation of the abiotic stability of chemicals and should be routinely integrated in future *in vitro* bioassay workflows.

Abiotic degradation processes that reduce the stability of the test chemical in the bioassay medium may lead to misinterpretation of the bioassay results. For example, if less active transformation products are formed, the chemical will be classified as inactive in the corresponding assay. Further research is needed to determine whether comparable degradation processes can also occur in humans. If nominal effect concentrations of unstable chemicals in *in vitro* assays are used as input parameters for QIVIVE models, the effect *in vivo* may be underestimated. QIVIVE models are usually based on the nominal concentration, which does not take into account either partitioning processes (e.g., binding to proteins or plate material)

or loss processes (abiotic degradation, metabolism, volatilization) of the test chemicals during the assay.[18, 90, 91] Differences in stability of the test chemicals between the *in vitro* bioassays and *in vivo* in humans may be a major impediment for using bioassay data for human health risk assessment.

Associated Content

Supporting Information

Additional information about the test chemicals, the plate layout of the stability tests, instrumental analysis, uptake kinetics and reproducibility of the SPME pin device, composition of additional pH buffers, GSH stability and degradation plots of all chemicals in all test systems (PDF).

Detailed results from *in silico* stability prediction models and experimental stability tests (XLSX).

Author Information

Corresponding Author

Beate I. Escher − Department of Cell Toxicology, Helmholtz Centre for Environmental Research−UFZ, DE-04318 Leipzig, Germany; Environmental Toxicology, Center for Applied Geoscience, Eberhard Karls University Tübingen, DE-72076 Tübingen, Germany; orcid.org/0000-00025304-706X; beate.escher@ufz.de

Authors

Julia Huchthausen − Department of Cell Toxicology, Helmholtz Centre for Environmental Research − UFZ, 04318 Leipzig, Germany; orcid.org/0000-0003-49161174

Luise Henneberger − Department of Cell Toxicology, Helmholtz Centre for Environmental Research−UFZ, DE04318 Leipzig, Germany; orcid.org/0000-0002-31810044

Sophia Mälzer − Department of Cell Toxicology, Helmholtz Centre for Environmental Research−UFZ, DE04318 Leipzig, Germany

Beate Nicol − Safety and Environmental Assurance Centre, Unilever, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

Chris Sparham − Safety and Environmental Assurance Centre, Unilever, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

Funding

This work was funded by Unilever.

Notes

The authors declare no competing financial interest.

Acknowledgements

The authors thank Jenny John for supporting the experiments, and Daniel Kolb from the Department of Analytical Chemistry of UFZ for providing the xenon test chamber for the photodegradation experiments. We also thank Stefan Stolte and the team from SEAC (Unilever) for reviewing the manuscript.

References

(1) Desprez, B., Dent, M., Keller, D., Klaric, M., Ouédraogo, G., Cubberley, R., Duplan, H., Eilstein, J., Ellison, C., Grégoire, S., Hewitt, N. J., Jacques-Jamin, C., Lange, D., Roe, A., Rothe, H., Blaauboer, B. J., Schepky, A., and Mahony, C. (2018) A strategy for systemic toxicity assessment based on non-animal approaches: The Cosmetics Europe Long Range Science Strategy programme. *Toxicol. In Vitro 50*, 137-146.

(2) Vinken, M., Kramer, N., Allen, T. E. H., Hoffmans, Y., Thatcher, N., Levorato, S., Traussnig, H., Schulte, S., Boobis, A., Thiel, A., and Rietjens, I. M. C. M. (2020) The use of adverse outcome pathways in the safety evaluation of food additives. *Arch. Toxicol. 94*, 959-966.

(3) Westmoreland, C., Carmichael, P., Dent, M., Fentem, J., MacKay, C., Maxwell, G., Pease, C., and Reynolds, F. (2010) Assuring safety without animal testing: Unilever's ongoing research programme to deliver novel ways to assure consumer safety. *Altex 27*, 61-65.

(4) Collins, F. S., Gray, G. M., and Bucher, J. R. (2008) Toxicology - Transforming environmental health protection. *Science 319*, 906-907.

(5) Escher, B., Neale, P., and Leusch, F. (2021) *Bioanalytical Tools in Water Quality Assessment*. IWA Publishing.

(6) Attene-Ramos, M. S., Miller, N., Huang, R., Michael, S., Itkin, M., Kavlock, R. J., Austin, C. P., Shinn, P., Simeonov, A., Tice, R. R., and Xia, M. (2013) The Tox21 robotic platform for the assessment of environmental chemicals – from vision to reality. *Drug Discov. Today 18*, 716-723.

(7) Burden, N., Chapman, K., Sewell, F., and Robinson, V. (2015) Pioneering better science through the 3Rs: an introduction to the national centre for the replacement, refinement, and reduction of animals in research (NC3Rs). *J. Am. Assoc. Lab. Anim. Sci. 54*, 198-208.

(8) Wetmore, B. A. (2015) Quantitative in vitro-to-in vivo extrapolation in a high-throughput environment. *Toxicology 332*, 94-101.

(9) Wambaugh, J. F., Hughes, M. F., Ring, C. L., MacMillan, D. K., Ford, J., Fennell, T. R., Black, S. R., Snyder, R. W., Sipes, N. S., Wetmore, B. A., Westerhout, J., Setzer, R. W., Pearce, R. G., Simmons, J. E., and Thomas, R. S. (2018) Evaluating in vitro-in vivo extrapolation of toxicokinetics. *Toxicol. Sci. 163*, 152-169.

(10) Groothuis, F. A., Heringa, M. B., Nicol, B., Hermens, J. L. M., Blaauboer, B. J., and Kramer, N. I. (2015) Dose metric considerations in in vitro assays to improve quantitative in vitro–in vivo dose extrapolations. *Toxicology 332*, 30-40.

(11) Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., and Escher, B. I. (2017) Modeling exposure in the Tox21 in vitro bioassays. *Chem. Res. Toxicol. 30*, 1197-1208.

(12) Henneberger, L., Mühlenbrink, M., Fischer, F. C., and Escher, B. I. (2019) C18-coated solid-phase microextraction fibers for the quantification of partitioning of organic acids to proteins, lipids, and cells. *Chem. Res. Toxicol. 32*, 168-178.

(13) Kramer, N. I., Krismartina, M., Rico-Rico, A., Blaauboer, B. J., and Hermens, J. L. (2012) Quantifying processes determining the free concentration of phenanthrene in basal cytotoxicity assays. *Chem. Res. Toxicol. 25*, 436-445.
(14) Kisitu, J., Hollert, H., Fisher, C., and Leist, M. (2020) Chemical Concentrations in Cell Culture Compartments (C5) - Free Concentrations. *Altex-Altern. Anim. Ex. 37*, 693-708.
(15) Hung, S., Mohan, A., Reckhow, D. A., and Godri Pollitt, K. J. (2019) Assessment of the in vitro toxicity of the disinfection byproduct 2,6-dichloro-1,4-benzoquinone and its transformed derivatives. *Chemosphere 234*, 902-908.
(16) Tentscher, P. R., Escher, B. I., Schlichting, R., König, M., Bramaz, N., Schirmer, K., and von Gunten, U. (2021) Toxic effects of substituted p-benzoquinones and hydroquinones in in vitro bioassays are altered by reactions with the cell assay medium. *Water Res. 202*, 117415.
(17) Shen, L., and Ji, H. F. (2012) The pharmacology of curcumin: is it the degradation products? *Trends Mol. Med. 18*, 138-144.
(18) Henneberger, L., Huchthausen, J., Wojtysiak, N., and Escher, B. I. (2021) Quantitative In Vitro-to-In Vivo Extrapolation: Nominal versus Freely Dissolved Concentration. *Chem. Res. Toxicol. 34*, 1175-1182.
(19) Tebes-Stevens, C., Patel, J. M., Jones, W. J., and Weber, E. J. (2017) Prediction of hydrolysis products of organic chemicals under environmental pH conditions. *Environ. Sci. Technol. 51*, 5008-5016.
(20) Yuan, C., Tebes-Stevens, C., and Weber, E. J. (2020) Reaction library to predict direct photochemical transformation products of environmental organic contaminants in sunlit aquatic systems. *Environ. Sci. Technol. 54*, 7271-7279.
(21) Dimitrov, S. D., Diderich, R., Sobanski, T., Pavlov, T. S., Chankov, G. V., Chapkanov, A. S., Karakolev, Y. H., Temelkov, S. G., Vasilev, R. A., Gerova, K. D., Kuseva, C. D., Todorova, N. D., Mehmed, A. M., Rasenberg, M., and Mekenyan, O. G. (2016) QSAR Toolbox – workflow and major functionalities. *SAR QSAR Environ. Res. 27*, 203-219.
(22) Howard, P. H., and Meylan, W. M. (1992) *Hydrolysis Rate Program*. Taylor & Francis.
(23) Villeneuve, D. L., Coady, K., Escher, B. I., Mihaich, E., Murphy, C. A., Schlekat, T., and Garcia-Reyero, N. (2019) High-throughput screening and environmental risk assessment: State of the science and emerging applications. *Environ. Toxicol. Chem. 38*, 12-26.
(24) Judson, R., Houck, K., Martin, M., Knudsen, T., Thomas, R. S., Sipes, N., Shah, I., Wambaugh, J., and Crofton, K. (2014) In Vitro and Modelling Approaches to Risk Assessment from the US Environmental Protection Agency ToxCast Programme. *Basic Clin. Pharmacol. Toxicol. 115* 69-76.
(25) Crounse, J. D., Nielsen, L. B., Jørgensen, S., Kjaergaard, H. G., and Wennberg, P. O. (2013) Autoxidation of Organic Compounds in the Atmosphere. *J. Phys. Chem. Lett. 4*, 3513-3520.
(26) Jiang, C., Garg, S., and Waite, T. D. (2015) Hydroquinone-Mediated Redox Cycling of Iron and Concomitant Oxidation of Hydroquinone in Oxic Waters under Acidic Conditions: Comparison with Iron–Natural Organic Matter Interactions. *Environ. Sci. Technol. 49*, 14076-14084.
(27) Dangles, O., Fargeix, G., and Dufour, C. (1999) One-electron oxidation of quercetin and quercetin derivatives in protic and non protic media. *J. Chem. Soc., Perkin Trans. 2*, 1387-1396.
(28) EPA. (2008) Fate, Transport and Transformation Test Guidelines: OPPTS 835.2120 Hydrolysis.
(29) Schwarzenbach, R. P., Gschwend, P. M., and Imboden, D. M. (2016) *Environmental Organic Chemistry*. Wiley.
(30) Zepp, R. G., and Cline, D. M. (1977) Rates of direct photolysis in aquatic environment. *Environ. Sci. Technol. 11*, 359-366.
(31) OECD. (2008) Test No. 316: Phototransformation of Chemicals in Water – Direct Photolysis.
(32) Subbarayappa, A., and Patoliya, P. (2007) N-Bromosuccinimide: A facile reagent for the oxidation of benzylic alcohols to aldehydes. *Synth. Commun. 37*, 1571-1577.
(33) Roy, K. S., Nazdrajić, E., Shimelis, O. I., Ross, M. J., Chen, Y., Cramer, H., and Pawliszyn, J. (2021) Optimizing a High-Throughput Solid-Phase Microextraction System to Determine the Plasma Protein Binding of Drugs in Human Plasma. *Anal. Chem. 93*, 11061-11065.
(34) Mutschler, J., Gimenez-Arnau, E., Foertsch, L., Gerberick, G. F., and Lepoittevin, J. P. (2009) Mechanistic assessment of peptide reactivity assay to predict skin allergens with Kathon (R) CG isothiazolinones. *Toxicol. In Vitro 23*, 439-446.

(35) Bhattarai, S., Tran, V. H., and Duke, C. C. (2001) The stability of gingerol and shogaol in aqueous solutions. *J. Pharm. Sci. 90*, 1658-1664.
(36) Zhou, C., Zhou, Q., and Zhang, X. (2019) Transformation of acetaminophen in natural surface water and the change of aquatic microbes. *Water Res. 148*, 133-141.
(37) Bakar, S. K., and Niazi, S. (1983) Stability of aspirin in different media. *J. Pharm. Sci. 72*, 1024-1026.
(38) Andreozzi, R., Caprio, V., Ciniglia, C., de Champdoré, M., Lo Giudice, R., Marotta, R., and Zuccato, E. (2004) Antibiotics in the environment: Occurrence in Italian STPs, fate, and preliminary assessment on algal toxicity of amoxicillin. *Environ. Sci. Technol. 38*, 6832-6838.
(39) Xu, H., Cooper, W. J., Jung, J., and Song, W. (2011) Photosensitized degradation of amoxicillin in natural organic matter isolate solutions. *Water Res. 45*, 632-638.
(40) Phattanawasin, P., Sotanaphun, U., Burana-Osot, J., and Piyapolrungroj, N. (2018) Isolation and characterization of the acid and base degradation products of andrographolide. *Pharmazie 73*, 559-562.
(41) Zhang, Z. Q., Chan, G. K. L., Li, J. L., Fong, W. F., and Cheung, H. Y. (2008) Molecular interaction between andrographolide and glutathione follows second order kinetics. *Chem. Pharm. Bull. 56*, 1229-1233.
(42) Kumar, K. S., Suvardhan, K., Rekha, D., Kiran, K., Jayaraj, B., Janardhanam, K., and Chiranjeevi, P. (2007) Development of simple and sensitive spectrophotometric method for the determination of bendiocarb in its formulations and environmental samples. *Environ. Monit. Assess. 127*, 67-72.
(43) Climent, M. J., and Miranda, M. A. (1996) Gas chromatographic-mass spectrometric study of photodegradation of carbamate pesticides. *J. Chromatogr. A 738*, 225-231.
(44) Seiber, J. N., Catahan, M. P., and Barril, C. R. (1978) Loss of carbofuran from rice paddy water: Chemical and physical factors. *J. Environ. Sci. Health, B 13*, 131-148.
(45) Campbell, S., David, M. D., Woodward, L. A., and Li, Q. X. (2004) Persistence of carbofuran in marine sand and water. *Chemosphere 54*, 1155-1161.
(46) Mitchell, S. M., Ullman, J. L., Teel, A. L., and Watts, R. J. (2015) Hydrolysis of amphenicol and macrolide antibiotics: Chloramphenicol, florfenicol, spiramycin, and tylosin. *Chemosphere 134*, 504-511.
(47) Marson, E. O., Paniagua, C. E. S., Costa-Serge, N. M., Sousa, R. M. F., Silva, G. D., Becker, R. W., Sirtori, C., Starling, M., Carvalho, S. R., and Trovó, A. G. (2021) Chemical and toxicological evaluation along with unprecedented transformation products during photolysis and heterogeneous photocatalysis of chloramphenicol in different aqueous matrices. *Environ. Sci. Pollut. Res. Int. 28*, 23582-23594.
(48) Matsuo, H., and Casida, J. E. (1970) Photodegradation of two dinitrophenolic pesticide chemicals, dinobuton and dinoseb, applied to bean leaves. *Bull. Environ. Contam. Toxicol. 5*, 72-78.
(49) Cruz, J. E., Maness, D. D., and Yakatan, G. J. (1979) Kinetics and mechanism of hydrolysis of furosemide. *Int. J. Pharm. 2*, 275-281.
(50) Bundgaard, H., Nørgaard, T., and Nielsen, N. M. (1988) Photodegradation and hydrolysis of furosemide and furosemide esters in aqueous solutions. *Int. J. Pharm. 42*, 217-224.
(51) Franklin, S. J., Dickinson, S. E., Karlage, K. L., Bowden, G. T., and Myrdal, P. B. (2014) Stability of sulforaphane for topical formulation. *Drug Dev. Ind. Pharm. 40*, 494-502.
(52) Hanschen, F. S., Brüggemann, N., Brodehl, A., Mewis, I., Schreiner, M., Rohn, S., and Kroh, L. W. (2012) Characterization of products from the reaction of glucosinolate-derived isothiocyanates with cysteine and lysine derivatives formed in either model systems or broccoli sprouts. *J. Agric. Food Chem. 60*, 7735-7745.
(53) Wolfe, N. L., Zepp, R. G., Gordon, J. A., Baughman, G. L., and Cline, D. M. (1977) Kinetics of chemical degradation of malathion in water. *Environ. Sci. Technol. 11*, 88-93.
(54) Xuan, R., Arisi, L., Wang, Q., Yates, S. R., and Biswas, K. C. (2010) Hydrolysis and photolysis of oxytetracycline in aqueous solution. *J. Environ. Sci. Health B 45*, 73-81.
(55) Sinderhauf, K., and Schwack, W. (2003) Photolysis experiments on phosmet, an organophosphorus insecticide. *J. Agric. Food Chem. 51*, 5990-5995.
(56) Choudhury, P. P., Barman, K. K., and Varshney, J. G. (2009) Photolysis of pretilachlor on soil surface. *Indian J. Weed Sci. 41*, 87-89.

(57) Clarke, E. D., Greenhow, D. T., and Adams, D. (1998) Metabolism-related assays and their application to agrochemical research: Reactivity of pesticides with glutathione and glutathione transferases. *Pestic. Sci. 54*, 385-393.
(58) Zenkevich, I. G., Eshchenko, A. Y., Makarova, S. V., Vitenberg, A. G., Dobryakov, Y. G., and Utsal, V. A. (2007) Identification of the products of oxidation of quercetin by air oxygenat ambient temperature. *Molecules 12*, 654-672.
(59) Dall'Acqua, S., Miolo, G., Innocenti, G., and Caffieri, S. (2012) The photodegradation of quercetin: Relation to oxidation. *Molecules 17*, 8898-8907.
(60) Sevilla-Morán, B., López-Goti, C., Alonso-Prados, J. L., and Sandín-España, P. (2014) Aqueous photodegradation of sethoxydim herbicide: Qtof elucidation of its by-products, mechanism and degradation pathway. *Sci. Total Environ. 472*, 842-850.
(61) Smitka, J., Lemos, A., Porel, M., Jockusch, S., Belderrain, T. R., Tesařová, E., and Da Silva, J. P. (2014) Phototransformation of benzimidazole and thiabendazole inside cucurbit[8]uril. *Photochem. Photobiol. Sci. 13*, 310-315.
(62) Mccall, P. J., and Gavit, P. D. (1986) Aqueous photolysis of triclopyr and its butoxyethyl ester and calculated environmental photodecomposition rates. *Environ. Toxicol. Chem. 5*, 879-885.
(63) Henneberger, L., Mühlenbrink, M., König, M., Schlichting, R., Fischer, F. C., and Escher, B. I. (2019) Quantification of freely dissolved effect concentrations in in vitro cell-based bioassays. *Arch. Toxicol. 93*, 2295-2305.
(64) Lee, J., Braun, G., Henneberger, L., König, M., Schlichting, R., Scholz, S., and Escher, B. I. (2021) Critical membrane concentration and mass-balance model to identify baseline cytotoxicity of hydrophobic and ionizable organic chemicals in mammalian cell lines. *Chem. Res. Toxicol. 34,* 2100-2109.
(65) Stevens, R., Stevens, L., and Price, N. C. (1983) The stabilities of various thiol compounds used in protein purifications. *Biochem. Educ. 11*, 70-70.
(66) Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys. 82*, 70-77.
(67) Frost, A. A., and Pearson, R. G. (1953) *Kinetics and Mechanism*. 2nd ed., Wiley, New York.
(68) Laidler, K. J. (1987) *Chemical Kinetics*. 3rd ed., Harper & Row, New York.
(69) Dimitrov, S., Dimitrova, G., Pavlov, T., Dimitrova, N., Patlewicz, G., Niemela, J., and Mekenyan, O. (2005) A Stepwise Approach for Defining the Applicability Domain of SAR and QSAR Models. *J. Chem. Inf. Model. 45*, 839-849.
(70) Hagvall, L., Bäcktorp, C., Norrby, P.-O., Karlberg, A.-T., and Börje, A. (2011) Experimental and Theoretical Investigations of the Autoxidation of Geranial: A Dioxolane Hydroperoxide Identified as a Skin Sensitizer. *Chem. Res. Toxicol. 24*, 1507-1515.
(71) Enoch, S. J., Ellison, C. M., Schultz, T. W., and Cronin, M. T. (2011) A review of the electrophilic reaction chemistry involved in covalent protein binding relevant to toxicity. *Crit. Rev. Toxicol. 41*, 783-802.
(72) OECD. (2017) OECD QSAR Toolbox v.4.1 Example for predicting Skin Sensitization of mixture, In *The OECD QSAR Toolbox for Grouping of chemicals into Categories*, OECD.
(73) Natsch, A., and Gfeller, H. (2008) LC-MS–based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. *Toxicol. Sci. 106*, 464-478.
(74) Jaworska, J. S., Natsch, A., Ryan, C., Strickland, J., Ashikaga, T., and Miyazawa, M. (2015) Bayesian integrated testing strategy (ITS) for skin sensitization potency assessment: a decision support system for quantitative weight of evidence and adaptive testing strategy. *Arch. Toxicol. 89*, 2355-2383.
(75) Urbisch, D., Mehling, A., Guth, K., Ramirez, T., Honarvar, N., Kolle, S., Landsiedel, R., Jaworska, J., Kern, P. S., Gerberick, F., Natsch, A., Emter, R., Ashikaga, T., Miyazawa, M., and Sakaguchi, H. (2015) Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regul. Toxicol. Pharmacol. 71*, 337-351.
(76) Schwöbel, J. A. H., Koleva, Y. K., Enoch, S. J., Bajot, F., Hewitt, M., Madden, J. C., Roberts, D. W., Schultz, T. W., and Cronin, M. T. D. (2011) Measurement and estimation of electrophilic reactivity for predictive toxicology. *Chem.l Rev. 111*, 2562-2596.
(77) EPA (2019) CTS: Chemical Transformation Simulator. 1.2.2021, from https://qed.epa.gov/cts/gentrans/.

(78) Canada, A. T., Giannella, E., Nguyen, T. D., and Mason, R. P. (1990) The production of reactive oxygen species by dietary flavonols. *Free Radic. Biol. Med. 9*, 441-449.
(79) Dangles, O., Fargeix, G., and Dufour, C. (1999) One-electron oxidation of quercetin and quercetin derivatives in protic and non protic media. *J. Chem. Soc. Perkin Trans. 2*, 1387-1395.
(80) Mir, N. A., Haque, M. M., Khan, A., Muneer, M., and Boxall, C. (2012) Photoassisted Degradation of a Herbicide Derivative, Dinoseb, in Aqueous Suspension of Titania. *Sci. World J. 2012*, 251527.
(81) Worthing, C., and Walker, S. (1983) *The Pesticide Manual - A World Compendium*. 7 ed., The Lavenham Press Limited, Lavenham, Suffolk (UK).
(82) Kalia, J., and Raines, R. T. (2008) Hydrolytic stability of hydrazones and oximes. *Angew. Chem. Int. Ed. Engl. 47*, 7523-7526.
(83) ACD/Percepta. (2015) *Build 2726, Advanced Chemistry Development, Inc., Toronto, On, Canada, http://www.acdlabs.com*.
(84) Cóllier, P. J., Ramsey, A., Waigh, R. D., Douglas, K. T., Austin, P., and Gilbert, P. (1990) Chemical reactivity of some isothiazolone biocides. *J. Appl. Bacteriol. 69*, 578-584.
(85) Yamagishi, Y., Iwase, H., and Ogra, Y. (2021) Effects of human serum albumin on post-mortem changes of malathion. *Sci. Rep. 11*, 11573.
(86) Varga, Z., Nicol, E., and Bouchonnet, S. (2020) Photodegradation of benzisothiazolinone: Identification and biological activity of degradation products. *Chemosphere 240*, 124862.
(87) Glauch, L., and Escher, B. I. (2020) The combined algae test for the evaluation of mixture toxicity in environmental samples. *Environ. Toxicol. Chem. 39*, 2496-2508.
(88) Omiecinski, C. J., Vanden Heuvel, J. P., Perdew, G. H., and Peters, J. M. (2011) Xenobiotic metabolism, disposition, and regulation by receptors: from biochemical phenomenon to predictors of major toxicities. *Toxicol. Sci. 120* S49-75.
(89) European Chemicals Agency (2017) Guidance on Information Requirements and Chemical Safety Assessment Chapter R.11: PBT/vPvB assessment.
(90) Mielke, H., Di Consiglio, E., Kreutz, R., Partosch, F., Testai, E., and Gundert-Remy, U. (2017) The importance of protein binding for the in vitro–in vivo extrapolation (IVIVE)—example of ibuprofen, a highly protein-bound substance. *Arch. Toxicol. 91*, 1663-1670.
(91) Algharably, E. A. H., Kreutz, R., and Gundert-Remy, U. (2019) Importance of in vitro conditions for modeling the in vivo dose in humans by in vitro–in vivo extrapolation (IVIVE). *Arch. Toxicol. 93,* 615-621.