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Effects of Solvents and Dosing Procedure on Chemical Toxicity in Cell-Based *in Vitro* Assays

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Due to the implementation of new legislation, such as REACh, a dramatic increase of animal use for toxicity testing is expected and the search for alternatives is timely. Cell-based *in vitro* assays are promising alternatives. However, the behavior of chemicals in these assays is still poorly understood. We set out to quantify the exposure and associated toxicity of chemicals with different physicochemical properties toward a fish gill cell line when different solvents and procedural steps are used to introduce test chemicals to cells. Three chemicals with a range of hydrophobicity and volatility were selected and delivered in three different solvents using two common dosing procedures. Toxicity tests were coupled with chemical analysis to quantify the chemical concentrations within culture wells. The impact of solvents and dosing procedure was greatest for the most volatile and hydrophobic test chemical. We show that certain combinations of the test chemical, solvent, and procedural steps can lead to inhomogeneous distribution of the test chemical and thus differing degrees of bioavailability, resulting in quantitative differences in apparent toxicity.

Introduction

Use of animals for experimental studies, e.g., toxicity testing of chemicals, gives rise to serious ethical concerns. The development of new alternative procedures and models is therefore timely. A recently published study reveals that within the next 10 years about 54 million vertebrate animals will be needed for toxicity testing only for the compliance of REACh (*1, 2*), an EU legislation in effect since June 2007.

The search for alternatives includes the consideration of *in vitro* approaches, i.e., experimental approaches where organs, cells, or biomolecules are explored outside of the organism in a controlled environment. Among the most promising *in vitro* alternatives are cell line-based assays. A number of mammalian and nonmammalian cell lines have been established in recent years, as well as a broad range of methods to detect chemical toxicity (reviews provided in refs *3, 4)*. Since cell-based tests are commonly conducted using multiwell plates, they are compatible with automated high-throughput screening. Many replicates and even different toxicants can be applied on one plate requiring very little testing material. However, these formats also come with caveats. First, because of the small volume, even minute manipulations within culture wells, such as the addition of a test chemical, can be expected to have a significant impact on the cell culture environment but this has not yet been systematically explored. Second, the high surface to volume ratio, combined with the open nature of the systems, favor losses of test chemicals due to sorption and/or evaporation. Indeed, a few studies already showed the significant role of the Henry's law constant, H (a proxy of volatility) and/or of the octanol−water partition coefficient, K_{ow} (a proxy of hydrophobicity or lipid binding affinity of a chemical), on the outcome of *in vitro* assays (*5*−*7*).

The application of test chemicals to cell culture wells usually requires a solvent. For compounds that are well water-soluble, the solvent can be the cell culture or exposure medium. For sparingly water-soluble compounds, however, organic solvents are required to prepare appropriate dosing solutions. These bear the risk of causing an impact on cells themselves. For example, the induction of cell differentiation (*8, 9*), modulation of estrogen receptor isoforms (*10*), and mixture effects with other chemicals (*11, 12*) have been reported for the most frequently used solvent, dimethylsulfoxide (DMSO). Side-effects were likewise reported for the alcohols ethanol and methanol (MeOH) (*13, 14*). Therefore, chemical stock solutions in organic carrier solvents are generally highly concentrated so that the final volume of solvent in the culture well is kept to a minimum. One way to circumvent the addition of carrier solvent to the cell culture environment is to add the chemical in a volatile solvent, such as MeOH, and then evaporate the solvent to leave the chemical of interest behind on the culture surface (*15*). Such procedures are, however, not yet applicable to high-throughput screening so that addition of chemicals in an organic solvent still is most commonly applied.

Once the chemical stock solutions are prepared, two ways of dosing the chemical can broadly be distinguished. We call them direct and indirect dosing (Figure S1 in the Supporting Information). During direct dosing, a small volume of the chemical stock solution in a certain organic solvent is added directly into the exposure medium in the presence of cells. For indirect dosing, a dosing mixture of the exposure medium and the stock solution is prepared prior to addition to cells. The exact method of dosing is, unfortunately, often insufficiently described in the literature. Yet, a recent publication by Schnell et al. (*16*) emphasizes the importance of such details. The authors dissolved ibuprofen in DMSO and observed a 20-fold higher toxicity of this compound when dosed directly instead of indirectly. They speculated that direct dosing likely exacerbated the interaction between ibuprofen and DMSO by transitorily exposing the cells to higher concentrations of them as a result of heterogeneous mixing over the surface of the cell monolayer.

It was the goal of our study to investigate the impact of solvent and dosing procedure on the outcome of cell viability assays. We hypothesized that direct dosing leads to greater toxicity of test chemicals in two ways: first, a reduced overall

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loss of the test chemical due to fewer handling steps, and second, an enhanced chemical−cell interaction due to inhomogeneous chemical distribution where most of the chemical is immediately available to cells rather than being equally distributed in a culture well. We selected DMSO and MeOH as organic solvents in addition to an aqueous medium and expected the impact of direct dosing to be greater for DMSO than for MeOH because of the higher density of DMSO and thus an enhanced interaction with the cell monolayer. We further speculated that the impact of the solvent and dosing methods is additionally influenced by the hydrophobicity and volatility of the test chemicals. Therefore, we selected three chemicals with a range of K_{ow} and H: 1,2-dichlorobenzene (1,2-DCB), 3,4-dichloroaniline (3,4-DCA), and sodium dodecyl sulfate (SDS) (Table S1). All three chemicals are thought to act through nonspecific baseline toxicity (narcotic mode of action) thereby excluding distortion of results by different chemical modes of action. For the performance of the toxicity assay we decided to use the rainbow trout gill cell line, RTgill-W1 (*17*), as a model. The main advantage of this cell line is that the cells are able to sustain exposure in a minimal medium, L15/ex (*18, 19*), which avoids a potential overriding influence of complex medium components, particularly serum (*20*−*22*), as a medium additive. We related the measured toxicity effects to the analytically determined chemical concentration in the exposure medium within the culture well. Our study clearly shows a strong influence on toxicity by the solvent and procedural steps used to deliver the test chemicals to the cell culture wells. In accordance with our hypothesis, the influence was strongest for the most hydrophobic and volatile chemical explored.

Experimental Section

Chemicals and Cell Culture. 1,2-DCB, 3,4-DCA, and SDS were purchased from Fluka (Buchs, Switzerland). Stock solutions were prepared using either DMSO (Sigma-Aldrich, Buchs, Switzerland) or MeOH (Acros Organics, Geel, Belgium) and were 1:2 serially diluted in the respective solvent. A final solvent content of 0.5% (v/v) was chosen because this value is in the range frequently used both for indirect as well as direct dosing protocols (*16, 23, 24*). To reach the final solvent concentrations of 0.5% within the toxicity assay, the stock solutions in the organic solvents needed to be 200-times more concentrated than wished for the final exposure solutions. The exposure media, L15/ex (*19*), was used to dissolve the chemicals directly at the maximum test concentration.

The RTgill-W1 cell line used in this study was derived from gills from rainbow trout (*Oncorhynchus mykiss*) (*17*). Details for culture conditions and setup for exposures are given in the Supporting Information.

Exposure of Cells to Test Chemicals. The gill cells were exposed to the chemicals for 24 h at 19 °C in L15/ex in 24-well tissue culture plates. L15/ex is a modification of the original L15 media and contains only the salts, sodium pyruvate, and galactose (*19*). The different ways of chemical application are shown in Figure S1, and described here briefly.

Direct Dosing (Using DMSO and MeOH Stock Solutions). The L15 culture media was removed and the cells were washed once with L15/ex. Then, 1000 μL of fresh L15/ex was added to each well. Next, 5 μL of the 200-times concentrated stock solutions in DMSO or MeOH was added to the exposure media and cells using a micropipet. To properly mix the test chemical in L15/ex, the microtiter plate was gently agitated manually for about 20 s.

Indirect Dosing (Using DMSO and MeOH Stock Solutions). In a 7 mL amber glass vial (Supelco, Buchs, Switzerland), a premixture of L15/ex and the chemical was prepared. Thus, 6.7 mL of L15/ex and 33.5 μL of the 200-times concentrated stock solutions in DMSO or MeOH were added and this premixture was vigorously vortexed for 15 min. Meanwhile, the cells were washed once with L15/ex. After removing the L15/ex, 1000 μL of the premixture was added into the respective wells.

Indirect Dosing (Using L15/ex Stock Solution). The concentration of the stock solution, prepared directly in L15/ex medium, is equivalent to the highest exposure concentration. The remaining dosing mixtures were prepared by diluting the stock solution 1:2, 1:4, etc., in L15/ex. The dosing mixtures were vortexed for 15 min and afterward 1000 μL of the dosing mixtures were added onto the washed cells.

The respective pure solvent, DMSO, MeOH, or L15/ex, served as control. The same volume of solvent was added using the same application methods as for exposing the test chemical. For each concentration and control, triplicates were dosed. Each experiment was carried out at least three times on three independent days. The cells were exposed to 0, 1.56, 6.25, 12.5, 25, 50, and 100 mg/L 1,2-DCB; 0, 4.69, 9.38, 18.8, 37.5, 75, and 150 mg/L 3,4-DCA; and 0, 1.95, 7.81, 15.6, 31.3, 62.5, and 125 mg/L SDS. These concentrations are from now on referred to as nominal chemical concentrations.

Sampling of Exposure Medium for Chemical Analysis. To allow the quantification of the concentration of the test chemicals in the exposure solutions, samples from the exposure media were taken at different time points (Figure S1). For each test setup, two microtiter plates were prepared in parallel. One was used to take samples of the exposure medium at the beginning of the exposure (C_{0h}). From the second plate, samples of the exposure medium were taken after 24 h of exposure (C_{24h}). For indirect dosing, samples were taken as well from dosing mixtures (C_{DM}). Details of sample preparation can be found in the Supporting Information.

Cell Viability Assay. For the determination of chemical toxicity, a cell viability assay using the fluorescent dye AlamarBlue (Invitrogen, Basel, Switzerland), as a measure of metabolic activity (*25*), was carried out according to the procedure of Schirmer et al. (*19*); see Supporting Information for details.

Statistical Evaluation. Results are expressed as mean ± SD. All statistical analysis was performed using GraphPad Prism version 4.03. Dose−response curves were analyzed using the GraphPad Prism nonlinear regression sigmoidal dose−response curve fitting module. For comparing EC_{50} values obtained from different test setups, an unpaired student t test was applied. EC_{50} values were considered as significantly different when $P < 0.05$.

Results

Nominal versus Measured Chemical Exposure Concentrations. Regardless of dosing procedure and solvent used, all three test chemicals elicited a concentration-dependent decline in cell viability after 24 h of exposure (Figure S2, based on nominal concentrations). Yet, the location of the concentration−response curves depended on the dosing procedure, as seen clearly for the solvent DMSO (Figure S2, left column). The concentration−response curve obtained with DMSO and indirect dosing suggested a lower toxicity for all three chemicals compared to direct dosing with DMSO. But the extent of this difference varied depending on the chemical used. The greatest difference was found for the most volatile and hydrophobic chemical, 1,2-DCB. For this chemical, the concentration−response curve was also shifted to the left (higher toxicity) when dosed directly rather than indirectly with MeOH.

We hypothesized that the chemical concentrations at the beginning of the exposure are different for the two dosing methods and that these differences trigger the different degrees of toxicity. Fewer handling steps are required for

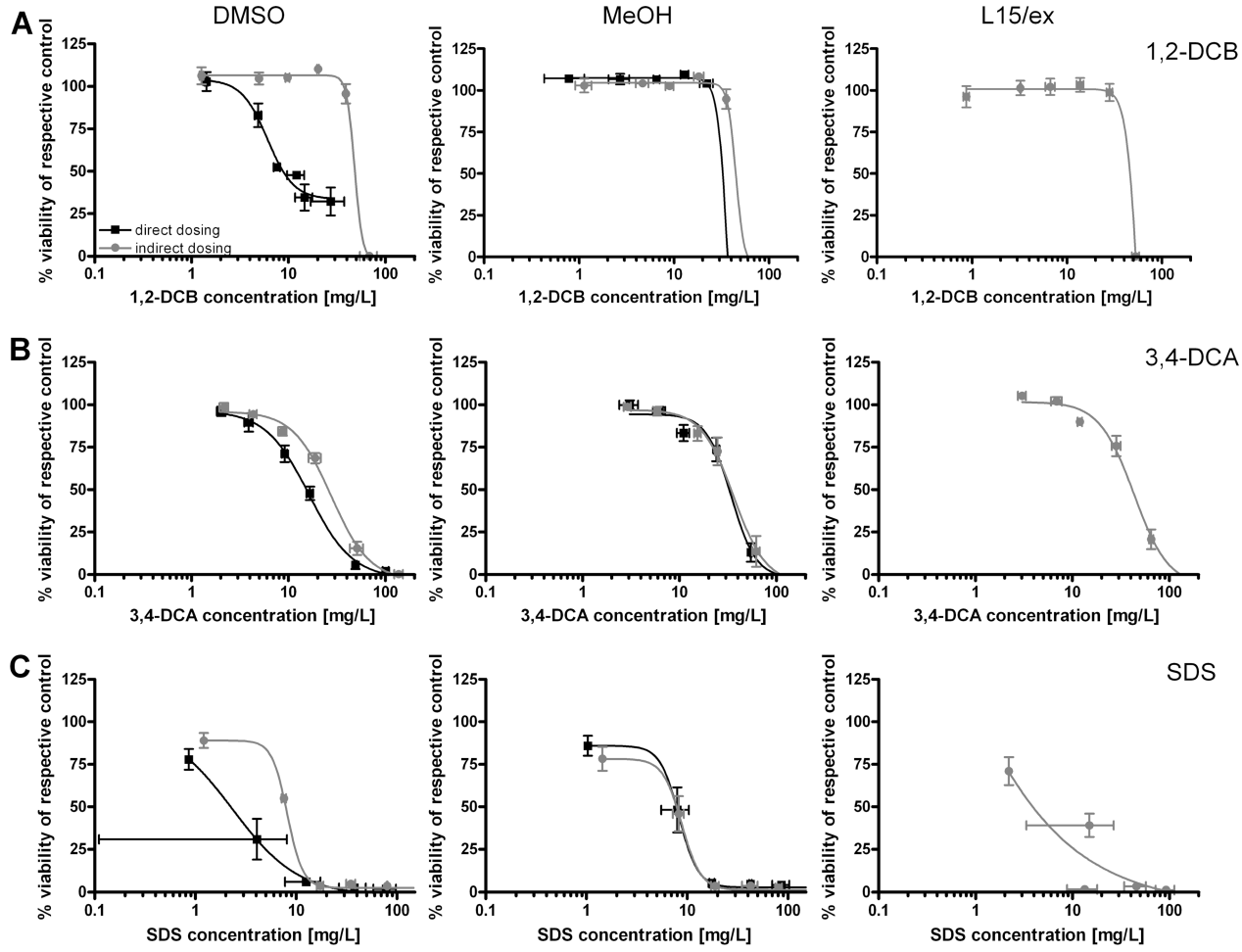

FIGURE 1. Cell viability of RTgill-W1 cells after 24 h of exposure to 1,2-DCB (A), 3,4-DCA (B), and SDS (C) decreases concentration-dependently. Test chemicals were dissolved either in DMSO (left), MeOH (middle), or L15/ex (right) and dosed directly (closed black squares) or indirectly (closed gray circles) onto the cells in two parallel sets of tissue culture plates. From one set of plates, chemical concentrations were determined at the beginning of the exposure (C_{0h}, in mg/L). From the second set of plates, cell viability was determined at the 24 h exposure time point and expressed as percentage of viability observed in respective solvent controls. Data points are means with standard deviations (SD) with the vertical lines showing the SD for cell viability ($n = 3$, for all chemicals), and the horizontal lines showing the SD for chemical analyses (C_{0h}; $n = 3$ for 1,2-DCB and 3,4-DCA and $n = 2$ for SDS).

TABLE 1. EC_{50} Values (mean ± SD) of the Test Chemicals Using Nominally Added and Measured Chemical Concentrations at the Start of the *in Vitro* Experiments (C_{0h})

	DMSO		MeOH		L15/ex
	direct	indirect	direct	indirect	indirect
			1,2-DCB ($n = 3$)		
nominal mg/L	9.0 ± 2.4[a]	85.4 ± 9.8[a]	87.2 ± 1.1[b]	76.6, ± 18.6	86.7 ± 7.2[b]
measured C_{0h} mg/L	6.0 ± 0.8[a]	68.1 ± 7.8[a]	30.7 ± 2.0[a,b]	67.2 ± 10.1[a]	68.3 ± 4.0[b]
			3,4-DCA, ($n = 3$)		
nominal, mg/L	35.2 ± 2.9[a,b]	50.0 ± 4.6[a,b]	51.0 ± 7.5[b]	52.6 ± 12.2	58.3 ± 13.7
measured C_{0h} mg/L	16.0 ± 2.5[a,b]	28.2 ± 6.4[a,b]	35.0 ± 4.4[b]	38.0 ± 11.0	46.5 ± 14.3
			SDS ($n = 3$ for nominal; $n = 2$ for measured)		
nominal mg/L	5.9 ± 2.7	8.34 ± 0.5	8.3 ± 1.0	8.2 ± 1.3	8.2 ± 0.4
measured C_{0h} mg/L	4.1 ± 5.6	8.2 ± 1.0	8.2 ± 3.6	8.5 ± 2.6	4.4 ± 3.6

[a] EC_{50} values for direct and indirect dosing are statistically different ($P < 0.05$; t test). [b] EC_{50} values based on nominal concentrations are statistically different from the EC_{50} values obtained using measured chemical concentrations C_{0h} ($P < 0.05$; t test).

direct dosing which may limit losses due to mixing and pipetting. To explore this hypothesis, chemicals were extracted from the exposure medium taken from the wells immediately after dosing. Measured quantities (C_{0h}) were used to plot all concentration−response relationships (Figure 1) and EC_{50} values were calculated from nominal and measured (C_{0h}) chemical concentrations (Table 1). Two points can be drawn from Figure 1 and Table 1. First, EC_{50} values based on measured initial chemical concentrations tended to be lower than EC_{50} values based on nominal concentrations, which indeed indicated losses during preparation and/or transfer from the stock solutions or dosing mixtures into

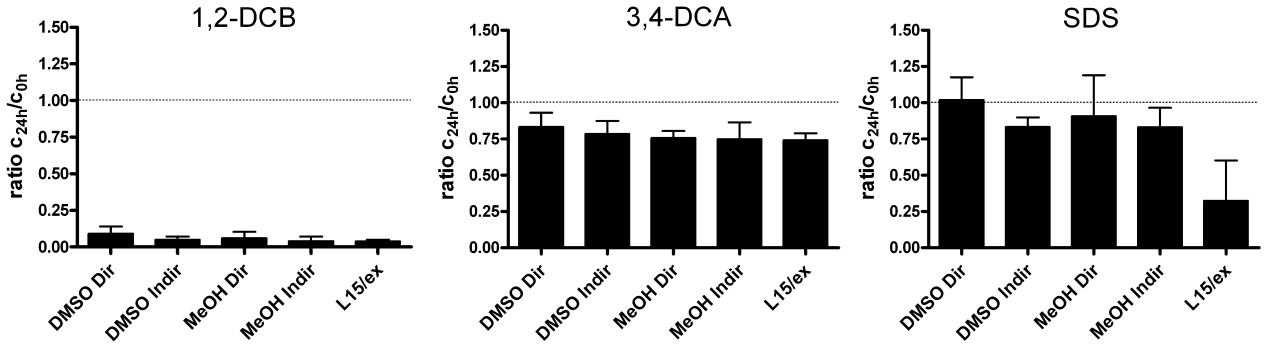

FIGURE 2. Ratio of the concentrations measured in the exposure medium at the beginning (C_{0h}) and at the end of exposure (C_{24h}) illustrates losses occurring for the test chemicals in the different exposure setups. A ratio of 1 (dashed line) indicates no loss during the 24 h of exposure while values below 1 indicate the fractions being recovered, respectively lost. Each bar represents the mean values of all tested chemical concentrations. Ratios are presented as mean ± SD of three (1,2-DCB and 3,4-DCA) and two (SDS) independent experiments. The C_{0h} values are the same as those used to plot concentration−response relationships in Figure 1. The C_{24h} was measured in the exposure medium from parallel plates used to subsequently determine cell viability.

culture wells. However, the differences could not be assigned to a particular solvent or dosing scheme. Thus, factors calculated from the respective EC_{50} values for nominal vs measured (C_{0h}) concentrations for the different solvents and dosing methods (Table 1) range from 1.1 to 2.8-fold for 1,2-DCB, from 1.3 to 2.2-fold for 3,4-DCA, and from 1 to 1.8 fold for SDS. Second, simply correcting for initial chemical concentrations (C_{0h}) in the exposure medium did not eradicate the differences observed for the different dosing procedures. In fact, the pattern of higher toxicity with direct dosing was maintained. The difference between direct and indirect dosing was most striking for the combination of 1,2-DCB and DMSO (~11-fold difference; $P < 0.05$) followed by 3,4-DCA/DMSO (~1.8-fold; $P < 0.05$), and SDS/DMSO (~1.4-fold, not statistically significant). Moreover, EC_{50} values varied significantly for direct versus indirect dosing with MeOH in the case of 1,2-DCB (~2.2-fold; $P < 0.05$) (all statistical differences for direct and indirect dosing are indicated by *a* in Table 1). These findings led us to postulate that adding a chemical with the direct dosing procedure, in particular with DMSO as a solvent, must have an impact on the cells that cannot be accounted for by measured initial chemical exposure concentrations in culture medium.

Next, exposure concentrations at the termination of exposure (C_{24h}) were quantified to investigate if these could explain the observed differences in cell viability due to solvent and dosing procedure. However, recovery of chemicals at this time point was independent of solvent and dosing procedure (Figure 2) with the exception of SDS dosed from the stock in L15/ex. Results furthermore revealed that dramatic losses of test chemicals occurred over the 24 h exposure period. For 1,2-DCB, less than 10% of the concentration measured at the onset of exposure (C_{0h}) could be recovered 24 h later. For 3,4-DCA, recovery was between 70 and 80%, whereas more than 80% was recovered for SDS except for the dosing of SDS from L15/ex stock solution. We conclude that the concentrations of chemicals after 24 h of exposure are, in contrast to the data on cell viability, independent of the solvent and dosing procedure and cannot explain the differences observed in toxicity as illustrated in Figure 1 and Table 1.

Impact of Solvent and Dosing Procedure on Chemical Recovery. To shed light on the mechanisms leading to the dependence of cell viability on solvent and dosing procedure, we compared the recovery of chemicals from the cell culture medium to the concentrations verified in the respective dosing mixtures (Figure 3). It became apparent that most if not all (between 80 to 100%) of the chemical(s) could be recovered from the exposure medium immediately after dosing by the indirect dosing method, although variability was comparatively high for SDS. Only for 1,2-DCB was recovery consistently lower than 80% (around 75%), which we attribute to its volatility (see also Figure 2). A different picture emerged for direct dosing. As can be seen most clearly for 1,2-DCB, direct dosing led to lower recoveries of this chemical from the exposure medium, which in the case of DMSO was even found to be strongly 1,2-DCB concentration dependent. The recovery from the exposure medium containing high concentrations of 1,2-DCB was lower than from medium containing low concentrations of 1,2-DCB. We therefore postulated that upon dosing 1,2-DCB directly into the well, complete and uniform dissolution of the chemical and the solvent in the exposure medium did not occur immediately. Instead, the chemical and the solvent sank to the bottom of the well and we assume that they were completely taken up by the cells. This assumption was supported by microscopic observations: when 1,2-DCB was dosed directly in DMSO, cellular morphology changed within minutes of adding the chemical. Cells retracted or rounded up which indicates cell damage and rapid cell death. When 1,2-DCB in DMSO was added in the indirect way, such rapid cell death could not be observed. Moreover, no changes in cell appearance were observed by adding DMSO alone (Figures S3, S4).

Discussion

Our study demonstrates that the outcome of cell-based toxicity assays is impacted by the solvent and procedural steps used to introduce the test chemical to the cells. At least in the case of the three chemicals investigated here, the impact is the greater the more volatile and/or hydrophobic the test chemical is. In particular we show that certain combinations of the test chemical, solvent, and procedural steps can lead to inhomogeneous distribution of the chemical, resulting in partly significant quantitative differences in toxicity.

We selected model chemicals that significantly differ in volatility and hydrophobicity (1,2-DCB > 3,4-DCA > SDS) to identify the impact of these physicochemical properties on the resulting cell viability. This approach cannot differentiate the impact of each of these properties individually but specific phenomena can be assigned to either of them.

Volatility is the likely cause of the low recovery of 1,2-DCB after 24 h of exposure in the culture wells. Thus, chemical concentrations are declining in the culture medium over time, making it difficult to link effects on cells to an exposure concentration. For the end point chosen, acute loss of cell viability (or basal cytotoxicity), we suggest that the exposure concentration present at the onset of exposure (C_{0h}) best reflects the concentration causing the effect. Future studies should focus on a time-resolved assessment of the loss

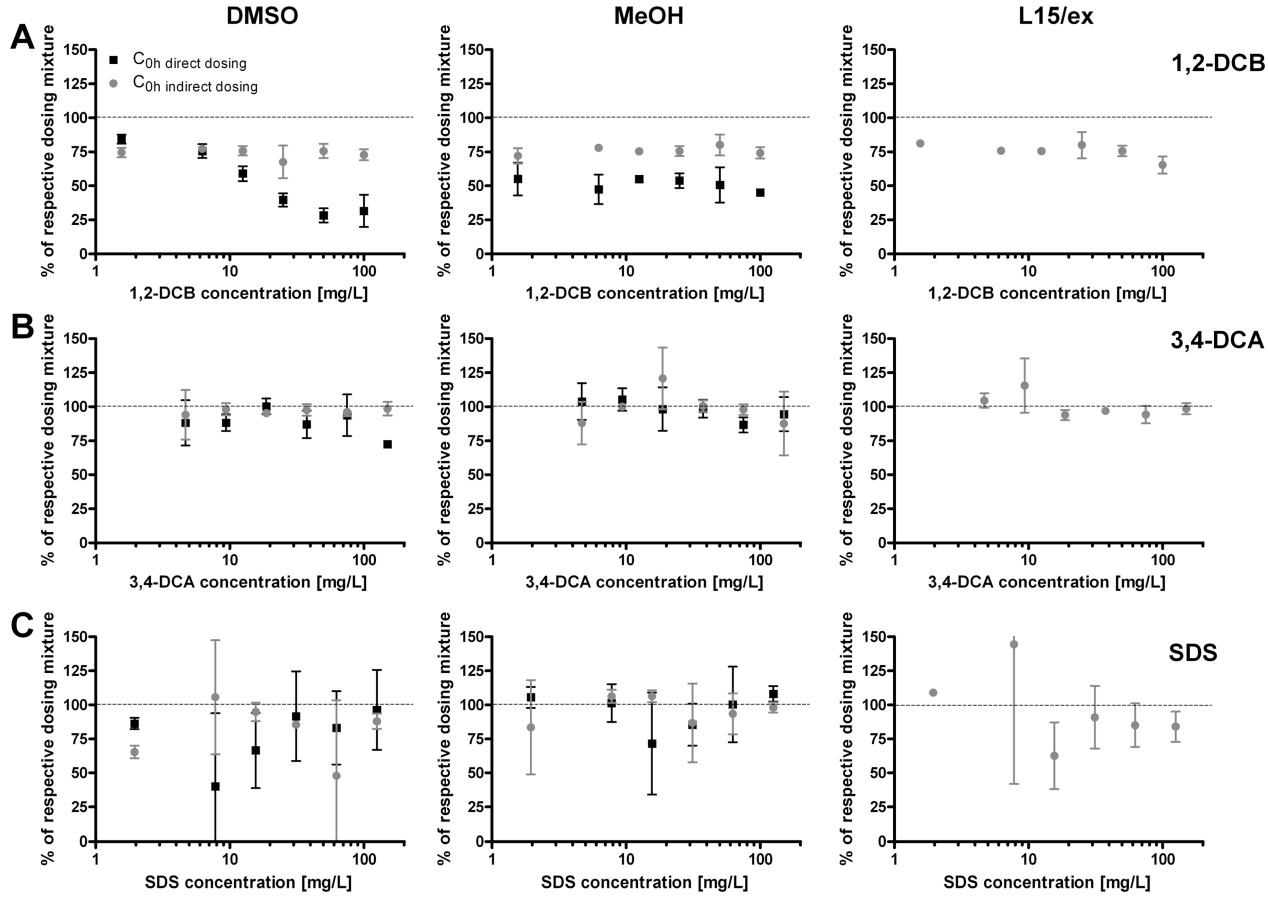

FIGURE 3. Recovery of 1,2-DCB (A), 3,4-DCA (B), and SDS (C) from the exposure medium at the immediate start of exposure (C_{0h}) for the applied nominal concentration range (in mg/L), using DMSO (left), MeOH (middle), or L15/ex (right) as solvent. For calculation of recovery, the concentrations analytically verified in the DMSO, MeOH or L15/ex dosing mixtures (C_{DM}) served as a reference point. Thus, the dashed line in each graph represents the reference, i.e., the chemical concentration in the dosing mixture (C_{DM}), which was set to 100%. The C_{0h} concentrations used for the calculations were the same as in Figures 1 and 2. Closed black squares represent recovery for the direct, and gray closed circles represent recovery for the indirect, dosing procedure. Recoveries below 100% indicate a reduced presence of the respective chemical immediately after addition to culture wells, either due to losses during transfer into the culture well or due to rapid disposition to cells and/or the culture surface. Data points are means with SD (n = 3 for 1,2-DCB and 3,4-DCA; n = 2 for SDS).

kinetics from the culture medium and its relationship to the evolution of toxicity in the cells.

Hydrophobicity, on the other hand, appears to have contributed to an inhomogeneous distribution of a chemical in the culture well when dosed directly. Extraction of 1,2-DCB from exposure medium immediately after direct dosing with DMSO or MeOH indicated a consistently lower recovery from the exposure medium compared to indirect dosing. In the case of the polar and aprotic solvent, DMSO, extraction (as percent of dosing mixture) from medium was even reciprocally dose-dependent; with higher doses, less was recovered from medium. One explanation for this observation could be precipitation of 1,2-DCB. Indeed, some precipitation was observed for the highest concentration when 1,2-DCB was added directly in DMSO or MeOH but was easily redissolved by gentle agitation. However, significant dose-dependent recovery was found only for DMSO. We assume that if precipitation affects recovery from the medium, the pattern should be similar for DMSO and MeOH. It therefore seems that hydrophobicity influences the pattern of recovery and that a higher percentage of the chemical sinks to the bottom of the well from the more concentrated DMSO stock solutions.

DMSO seems to play a special role within cell-based *in vitro* assays. This solvent yielded the most pronounced impacts on chemical distribution in a culture well and toxicological effects. Differences in EC_{50} values due to the dosing procedure were greatest for the most hydrophobic chemical tested here, 1,2-DCB. It is the hydrophobicity which most often drives the use of a carrier solvent, such as DMSO, to deliver poorly water-soluble substances into the aqueous media. A similar carrier solvent effect by DMSO has been previously reported for ibuprofen, a chemical with a log K_{ow} value of 3.97. A 10- to 20-fold higher toxicity toward a fish liver cell line was observed for direct compared to indirect dosing with the greater differences measured when serum was present in the exposure medium (*16*). The most likely explanation for the DMSO effect is a decelerated mixing of the culture medium, the test chemical, and DMSO during direct dosing. The drop of DMSO sinks and spreads immediately across the cells leading to a transitorily high solvent and chemical concentration and in this way to an instantaneous loading of the cells. A similar behavior can be proposed to explain the enhanced toxicity for 1,2-DCB in direct dosing with MeOH but is less pronounced, presumably because a homogeneous mixture between test chemical and the exposure medium is established faster with MeOH. DMSO is moreover known to enhance cell permeability and in addition the uptake of chemicals into the cells (*26, 27*).

Inhomogeneous distribution of test chemicals, which may impact the outcome of toxicity test results, is not limited to sparingly water-soluble chemicals. This was illustrated in our study for SDS, which is basically nonvolatile and well soluble in water. SDS led to highly variable results for recovery

of the chemical from the exposure mixture at the onset of exposure, which subsequently affected EC_{50} values based on measured concentrations and the quantification of losses observed within 24 h of exposure. We suspected that one cause of the observed variability is the behavior of SDS in L15/ex. Because SDS is a surfactant, it will accumulate at the interfaces, i.e. air−water, plastic wall−water, of the system (*28*).

We suggest that for exposure scenarios with heterogeneous distribution of the test chemical, cell internal concentrations need to be determined to account for the fraction of chemical that does enter the cells. Given that one determinant of the heterogeneous distribution appears to be the physicochemical characteristics of the test chemicals, an important next step would be to investigate a larger set of chemicals for their distribution in tissue culture wells to develop an alert concept that can help identify problematic chemicals up front. Few *in vitro* studies have thus far taken cell-internal concentrations into account (*15, 19, 22, 29, 30*). In a manner similar to Heringa et al. (*31*) but using cell internal rather than unbound chemical concentrations in the exposure medium, Bopp et al. (*15*) reported that the EC_{50} values between a direct dosing method with DMSO and a passive, solvent-free dosing method became nearly indistinguishable when cell internal concentrations were used.

The aim of this study was to systematically investigate how the choice of solvent and the dosing procedure impact on the outcome of cell-based *in vitro* assays for chemicals with different physicochemical properties. One area of particular relevance to this work is the establishment of alternative methods to the use of animals in the safety assessment of chemicals. We focused our investigations on a fish cell line in the context of improving fish cell line assays as surrogates for chemical toxicity to fish. However, the outcome is of relevance to cell-based assays using monolayer cultures in general. For example, to comply with the societal and scientific demand to reduce the number of animals used in toxicology, quantitative high-throughput screening (HTS) programs for cytotoxicity profiling have been started recently (*23*). The advantage of HTS is that a high number of chemicals can be analyzed in a rather short time. However, bioavailability may be a critical issue. The high number of wells on one plate, e.g., 1536, leads to a very high surface-to-volume ratio which strongly promotes sorption and evaporation may occur due to the open formats of the plates. The chemicals are dissolved and stored in DMSO in plastic multiwell plates (*24*). Dosing is conducted with the help of a robot and we assume it is direct. Our study shows that a truly quantitative analysis of cytotoxicity under these conditions requires careful analyses of the chemicals' bioavailability. We therefore suggest that the full potential of HTS for cytotoxicity or other cell-based assays can only be realized if it is coupled to high-throughput chemical extraction from exposed cells and quantitative chemical analysis.

In the absence of cell internal concentrations, we recommend to use dosing procedures which ensure a homogeneous distribution of the chemicals within the culture well. Homogenous mixing supports the establishment of equilibrium conditions, which are a prerequisite to determine the unbound chemical concentration as proposed by Gülden and Seibert (*21*) or by Heringa et al. (*31*). New models are being developed that predict cell internal concentrations in the well plate environment. For prolonged exposures with potential losses, e.g., due to evaporation, stable chemical exposures might be achieved by means of passive dosing (*32*). Regardless of these possibilities, it is of utmost importance that the applied dosing method is described in detail in each paper dealing with cell-based *in vitro* assays to avoid misleading results. When only nominally added concentrations are considered, the toxicity of the chemical will almost certainly be underestimated. This underestimation is one likely cause for the seemingly lower sensitivity of cells in *in vitro*−*in vivo* correlations (e.g., ref *4*).

Acknowledgments

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Supporting Information Available

Further experimental data as well as detailed description of experimental methods for extraction, chemical analyses, and cell viability assays. This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) European Commission Regulation (EC) No 1907/2006 of the European Parliament and of the council of 18 December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Off. J. Eur. Union* 2006, L396/1, 1−849.

(2) Hartung, T.; Rovida, C. Chemical regulators have overreached. *Nature* **2009**, *460* (7259), 1080–1081.

(3) Knight, A. Non-animal methodologies within biomedical research and toxicity testing. *ALTEX* **2008**, *25* (3), 213–231.

(4) Schirmer, K. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicology* **2006**, *224* (3), 163–183.

(5) Gülden, M.; Mörchel, S.; Tahan, S.; Seibert, H. Impact of protein binding on the availability andy cytotoxic potency of organochloride pesticides and chlorophenols *in vitro*. *Toxicology* **2002**, *175* (1−3), 201–213.

(6) Kramer, N. I.; Hermens, J. L. M.; Schirmer, K. The influence of modes of action and physicochemical properties of chemicals on the correlation between *in vitro* and acute fish toxicity data. *Toxicol. in vitro* **2009**, *23* (7), 1372–1379.

(7) Riedl, J.; Altenburger, R. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. *Chemosphere* **2007**, *67* (11), 2210–2220.

(8) Adler, S.; Pellizzer, C.; Paparella, M.; Hartung, T.; Bremer, S. The effects of solvents on embryonic stem cell differentiation. *Toxicol. in vitro* **2007**, *20* (3), 265–271.

(9) Kanebratt, K. P.; Andersson, T. B. Evaluation of HepaRG cells as an *in vitro* model for human drug metabolism studies. *Drug Metab. Dispos.* **2008**, *36* (7), 1444–1452.

(10) Mortensen, A. S.; Arukwe, A. Dimethyl sulfoxide is a potent modulator of estrogen receptor isoforms and xenoestrogen biomarker responses in primary culture of salmon hepatocytes. *Aquat. Toxicol.* **2006**, *79* (1), 99–103.

(11) Mori, M.; Wakabayashi, M. Cytotoxicity evaluation of chemicals using cultured fish cells. *Water Sci. Technol.* **2000**, *42* (7−8), 277–282.

(12) El Jay, A. Effects of organic solvents and solvent-atrazine interactions in two algae, *Chlorella vulgaris* and *Selenastrum capricornutum*. *Arch. Environ. Contam. Toxicol.* **1996**, *31* (1), 84–90.

(13) Busby, W. F.; Ackermann, J. M.; Crespi, C. L. Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on *in vitro* activities of cDNA-expressed human Cytochromes P-450. *Drug Metab. Dispos.* **1999**, *27* (2), 246–249.

(14) Etique, N.; Chardard, D.; Chesnel, A.; Flament, S.; Grillier-Vuisoz, I. Analysis of the effects of different alcohols on MCF-7 human breast cancer cells. *Ann. N.Y. Acad. Sci.* **2004**, *130*, 78–85.

(15) Bopp, S. K.; Bols, N. C.; Schirmer, K. Development of a solvent-free, solid-phase *in vitro* bioassay using vertebrate cells. *Environ. Toxicol. Chem.* **2006**, *25* (5), 1390–1398.

(16) Schnell, S.; Kawano, A.; Porte, C.; Lee, L. E. J.; Bols, N. C. Effects of ibuprofen on the viability and proliferation of rainbow trout liver cell lines and potential problems and interactions in effect assessment. *Environ. Toxicol.* **2009**, *24* (2), 157–165.

(17) Bols, N. C.; Barlian, A.; Chirinotrejo, M.; Caldwell, S. J.; Goegan, P.; Lee, L. E. J. Development of a cell-line from primary cultures of rainbow-trout, *Oncorhynchus mykiss* (Walbaum), gills. *J. Fish Dis.* **1994**, *17* (6), 601–611.
(18) Dayeh, V. R.; Schirmer, K.; Bols, N. C. Applying whole-water samples directly to fish cell cultures in order to evaluate the toxicity of industrial effluent. *Water Res.* **2002**, *36* (15), 3727–3738.
(19) Schirmer, K.; Chan, A. G. J.; Greenberg, B. M.; Dixon, D. G.; Bols, N. C. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. *Toxicol. in vitro* **1997**, *11* (1−2), 107–119.
(20) Frazier, J. M. Application of *in-vitro* systems to the prediction of *in-vivo* biokinetics. *Toxicol. in Vitro* **1995**, *9* (4), 527–536.
(21) Gülden, M.; Seibert, H. Impact of bioavailability on the correlation between *in vitro* cytotoxic and *in vivo* acute fish toxic concentrations of chemicals. *Aquat. Toxicol.* **2005**, *72* (4), 327–337.
(22) Hestermann, E. V.; Stegman, J. J.; Hahn, M. E. Serum alters the uptake and relative potencies of halogenated aromatic hydrocarbons in cell culture bioassays. *Toxicol. Sci.* **2000**, *53* (2), 316–325.
(23) Inglese, J.; Auld, D. S.; Jadhav, A.; Johnson, R. L.; Simeonov, A.; Yasgar, A.; Zheng, W.; Austin, C. P. Quantitative high-throughput screening: A titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc. Natl. Acad. Sci.* **2006**, *103* (31), 11473–11478.
(24) Xia, M.; Huang, R.; Witt, K. L.; Southall, N.; Fostel, J.; Cho, M. H.; Jadhav, A.; Smith, C. S.; Inglese, J.; Portier, C. J.; Tice, R. R.; Austin, C. P. Compound cytotoxicity profiling using quantitative high-throughput screening. *Environ. Health Perspect.* **2008**, *116* (3), 84–291.
(25) O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **2000**, *267* (17), 5421–5426.
(26) David, N. A. The pharmacology of dimethyl sulfoxide. *Annu. Rev. Pharmacol.* **1972**, *12*, 353–374.
(27) Williams, A. C.; Barry, B. W. Penetration enhancers. *Adv. Drug Delivery Rev.* **2004**, *56* (5), 603–618.
(28) Knepper, T. P.; Barceló, D.; De Voogt, P. *Analysis and Fate of Surfactants in the Aquatic Environment*, 1st ed.; Elsevier Science B.V.: Amsterdam, The Netherlands, 2003; Vol. 40, Chapter 1, pp 3−5.
(29) Yu, K. O.; Fisher, J. W.; Burton, G. A.; Tillitt, D. E. Carrier effects of dosing the H4IIE cells with 3,3′,4,4′-tetrachlorobiphenyl (PCB77) in dimethyl sulfoxide or isooctane. *Chemosphere* **1997**, *35* (4), 895–904.
(30) Bernhard, M. J.; Dyer, S. D. Fish critical cellular residues for surfactants and surfactant mixtures. *Environ. Toxicol. Chem.* **2005**, *24* (7), 1738–1744.
(31) Heringa, M. B.; Schreurs, R. H. M. M.; Busser, F.; van der Saag, P. T.; van der Burg, B.; Hermens, J. L. M. Toward more useful *in vitro* toxicity data with measured free concentrations. *Environ. Sci. Technol.* **2004**, *38* (23), 6263–6270.
(32) Smith, K. E. C.; Oostingh, G. J.; Mayer, P. Passive dosing for producing defined and constant exposure of hydrophobic organic compounds during *in vitro* toxicity tests. *Chem. Res. Toxicol.* **2010**, *23*, 55–65.

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