Validation of an SH-SY5Y Cell–Based Acetylcholinesterase Inhibition Assay for Water Quality Assessment

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Abstract: The acetylcholinesterase (AChE) inhibition assay has been frequently applied for environmental monitoring to capture insecticides such as organophosphates (OTPs) and carbamates. However, natural organic matter such as dissolved organic carbon (DOC) co-extracted with solid-phase extraction from environmental samples can produce false-negative AChE inhibition in free enzyme-based AChE assays. We evaluated whether disturbance by DOC can be alleviated in a cell-based AChE assay using differentiated human neuroblastoma SH-SY5Y cells. The exposure duration was set at an optimum of 3 h considering the effects of OTPs and carbamates. Because loss to the airspace was expected for the more volatile OTPs (chlorpyrifos, diazinon, and parathion), the chemical loss in this bioassay setup was investigated using solid-phase microextraction followed by chemical analysis. The three OTPs were relatively well retained (loss <34%) during 3 h of exposure in the 384-well plate, but higher losses occurred on prolonged exposure, accompanied by slight cross-contamination of adjacent wells. Inhibition of AChE by paraoxon-ethyl was not altered in the presence of up to 68 mg/L Aldrich humic acid used as surrogate for DOC. Binary mixtures of paraoxon-ethyl and water extracts showed concentration-additive effects. These experiments confirmed that the matrix in water extracts does not disturb the assay, unlike purified enzyme-based AChE assays. The cell-based AChE assay proved to be suitable for testing water samples with effect concentrations causing 50% inhibition of AChE at relative enrichments of 0.5–10 in river water samples, which were distinctly lower than corresponding cytotoxicity, confirming the high sensitivity of the cell-based AChE inhibition assay and its relevance for water quality monitoring. Environ Toxicol Chem 2022;41:3046–3057. © 2022 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

The enzyme acetylcholinesterase (AChE; acetylcholine [ACh] acetylhydrolase, E.C. 3.1.1.7) modulates neurotransmission by breaking down the neurotransmitter ACh. In cholinergic neurons, depolarization of the presynaptic membrane triggers the release of ACh into the synaptic cleft, enabling a time-resolved transmission of excitation. Subsequent binding to the AChE receptor at postsynaptic membranes activates cholinergic or motor neurons. Inhibition of AChE, therefore, can hinder degradation of ACh and lead to overstimulation of the ACh receptor, which could disrupt neurotransmission. Acetylcholinesterase can be inhibited by diverse insecticides that have an affinity to its active site (Čolović et al., 2013; Pohanka, 2011). Depending on the interaction of the AChE inhibitors with the active sites, the insecticides bind either irreversibly or reversibly to AChE. Inhibitors of AChE have been mainly used as insecticides (Fukuto, 1990), but some are also potentially used for therapeutic purposes to treat neurological disorders such as Alzheimer’s disease (Sharma, 2019).

Insecticides that act as AChE inhibitors have raised concern because of their presence in the environment and their adverse effects on nontarget organisms. Among the neurotoxic modes of action, AChE inhibition had the highest hazard quotient in environmental monitoring from three European river basins considering the ratio of environmental concentration and effect.
concentration for the detected chemicals (Legradi et al., 2018). In addition, AChE has already been employed as biomarker of neurotoxicity, and organ homogenates collected from exposed species were used for environmental monitoring (Lionetto et al., 2011; Nunes, 2011). Alternatively, a simple enzyme-based assay (using purified enzyme) can be used to assess inhibition of AChE activity by environmental pollutants in water samples (Escher et al., 2009; Hamers et al., 2000; Macova et al., 2011; Molica et al., 2005).

Diverse AChE inhibition assays have been applied for screening the inhibitory potency of environmental chemicals or samples. High-throughput screening of inhibition of AChE activity was performed using purified enzyme, human neuroblastoma SH-SY5Y cells, and neural stem cells for chemicals in the Tox21 program (Li et al., 2021). Approximately 2.25% of the tested chemicals (187 of 8312) inhibited the AChE activity in that study. Inhibition assays based on purified AChE have been commonly applied because of low cost and simple operation (Cao et al., 2020). Despite its wide application, the enzyme-based AChE inhibition assay may be adversely impacted by natural organic matter that is co-extracted with the micropollutants during the extraction process of environmental samples. For example, matrix effects from diverse water samples were observed, and the co-extracted dissolved organic carbon (DOC) contributed to reduced sensitivity of enzyme-based AChE inhibition assays (Neale & Escher, 2013). Furthermore, cell-based AChE inhibition assays may better reflect in vivo physiology considering cellular environment and localization of AChE (Li et al., 2017, 2021). Given that localization of AChE anchored to the cell membrane or in the cytoplasm can prevent the direct interaction between DOC and AChE (Hicks et al., 2013; Thullbery et al., 2005), a cell-based assay could provide an alternative screening tool for environmental samples; but this has not been explored yet.

Loss of chemicals can occur because of partitioning of volatile chemicals from the bioassay medium to the air. Previously, a “volatility” cutoff was determined empirically. When this cutoff was applied to Tox21 high-throughput screening data, approximately 20% of the chemicals investigated in Tox21 were estimated to be partially lost during testing (Escher et al., 2019). In the typical setup of cellular assays, microplates have an air headspace, which can cause chemical partitioning to the gas phase. In bioassays, the partition constant between medium and air ($K_{\text{medium/air}}$) can serve as the best proxy to estimate loss of chemicals because it considers not only their distribution between air and water but also the retaining capacity of medium components such as proteins and lipids (Escher et al., 2019). Volatile chemicals can hinder precise quantification of effect concentrations and even can cause cross-contamination in the bioassay (Birch et al., 2019; Proença et al., 2021). Some AChE inhibitors are likely to volatilize during incubation in bioassays, and their volatility could represent a limitation in bioassays including the assessment of AChE inhibition.

The aim of the present study was to optimize a cell-based AChE inhibition assay for testing environmental samples. We explored the experimental condition using human neuroblastoma SH-SY5Y cells, which express AChE with cholinergic characteristics (de Medeiros et al., 2019). Irreversible and reversible inhibitors were considered to optimize the assay condition. Four main challenges were evaluated: (1) interference by assay medium with the assay, (2) proper exposure duration, (3) loss processes during the assay due to volatility of certain AChE inhibitors, and (4) disturbance by DOC. The assay was then applied to water extracts to demonstrate its practical applicability.

MATERIALS AND METHOD

Tested chemicals

Irreversible and reversible AChE inhibitors were tested in the present study. The irreversible AChE inhibitors included three organothiophosphates (OTPs; chlorpyrifos, diazinon, parathion) and their active metabolite organophosphates (OPs; chlorpyrifos-oxon, diazoxon, paraoxon-ethyl). Three carbamates (carbaryl, carbofuran, 3-hydroxy-carbofuran) were included as reversible AChE inhibitors. Detailed information on test chemicals (Chemical Abstracts Service number, abbreviation of name, source, and purity) is given in Supporting Information, Table S1. Methanol was used to prepare the stock solutions of the chemicals, and its final concentration in assay plates was allowed up to 1%, which did not cause any effects on cell viability and AChE activity in our assay condition. Humic acid sodium salt (Sigma-Aldrich; H16752) was used as a reference of DOC.

Cell culture and medium

Sigma-Aldrich SH-SY5Y cells (94030304) were maintained in growth medium composed of 90% Dulbecco’s modified Eagle medium (DMEM)/F12 (Gibco; 11320074) and 10% heat-inactivated fetal bovine serum (hiFBS; Gibco; 10500064) with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; 15140-122). The cells were cultured in 5% CO₂ in an incubator at 37 °C, and the passage of cells for the assay was limited from 5 to 15 to avoid senescence.

The differentiation medium consisted of Neurobasal medium with phenol red (Gibco; 21103049) and was supplemented with 2% B-27 Supplement (Gibco; 17504044), 2 mM GlutaMAX (Gibco; 35050061), as well as 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; 15140-122). For the assay, the differentiated SH-SY5Y cells were seeded, and the test chemicals were diluted in phenol red-free Neurobasal medium (Gibco; 12348017) containing the same supplement. To compare assay media, not only this Neurobasal assay medium but also other candidate assay media were considered and prepared with 99% DMEM/F12 and 1% diverse types of FBS: nontreated FBS (Gibco; 10099141), charcoal-stripped FBS (csFBS; Gibco; 12676029), dialyzed FBS (dFBS; Gibco; 26400044), and hiFBS (details above).

Cell plating and dosing

The SH-SY5Y cells were differentiated for 72 h in flasks containing differentiation medium with 10 µM all-trans retinoic acid
AChE inhibition assay

For detection, change in AChE activity was measured based on absorbance according to the method of Ellman et al. (1961). Detection mixture was prepared using acetylthiocholine iodide (ATCh iodide; Sigma-Aldrich; A5751) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich; D8130). The assay medium was removed by inverting the microplates and blotting them on a paper towel to take out as much of the medium as possible. Then, 20 µl of the detection mixture was added into each well using a multichannel pipette. The absorbance at 410 nm was measured every min for 30 min using a Tecan Infinite M1000 plate reader (Tecan). With the method of Ellman et al. (1961), the absorbance of 2-nitro-5-thiobenzoate dianion can be quantified from the reaction of DTNB and hydrolysates of ATCh.

The data were analyzed using two consecutive workflows set up in KNIME (Ver 4.4.1), and the details of the KNIME workflows can be found on GitHub (CITE-KNIME, 2022). The slope of change in absorbance over time is the enzyme velocity (v), and AChE inhibition (percentage) was calculated using Equation 1, where \( v_{\text{control}} \) and \( v \) indicate the enzyme velocity of control (nontreated cells) and test chemicals, respectively.

\[
AChE\ inhibition\% = \left(1 - \frac{v}{v_{\text{control}}}\right) \times 100\% \quad (1)
\]

Derivation of enzyme velocity and AChE inhibition was visualized for paraoxon-ethyl as an example in Supporting Information, Figure S1. Inhibition of AChE from all experimental runs was plotted together against the concentration using a log-logistic concentration–response model with fixed minimum (0%) and maximum (100%) using the drc package in R Studio, Ver 4.0.4, and the effect concentration for 50% of maximum AChE inhibition (EC50) was calculated using the ED command in the statistical computing language R (2020) (Equation 2).

\[
AChE\ inhibition\% = \frac{100}{1 + \exp[slope \times \log(\text{concentration}/\text{EC50})]} \quad (2)
\]

Prediction of potential loss of chemicals to the air

A mass balance model was applied to predict if chemicals are likely to be lost to the air (Escher et al., 2019). The physicochemical properties of the chemicals that serve as input to this model were retrieved from the linear solvation energy relationships database (Ulrich et al., 2017): protein–water partition constants \( K_{\text{protein/w}} \), liposome–water partition constants \( K_{\text{lip/w}} \), and air–water partition constants \( K_{\text{aw}} \). Bovine serum albumin (BSA) served as a surrogate for protein to derive the \( K_{\text{protein/w}} \) (Fischer et al., 2017). The input parameters regarding the composition of Neurobasal assay medium were taken from Lee et al. (2021). The partition constants and volume fractions were substituted into Equation 3 to calculate \( K_{\text{medium/air}} \). Previously, a so-called volatility cutoff, which is actually a log \( K_{\text{medium/air}} \) cutoff, was proposed at a log \( K_{\text{medium/air}} \) of 4 based on cytotoxic effects in AREc32 cells (Escher et al., 2019). The predicted \( K_{\text{medium/air}} \) of the nine AChE inhibitors were compared with this volatility cutoff to select test chemicals for further experimental volatility test.

\[
K_{\text{medium/air}} = \frac{V_{f,\text{medium}} \times K_{\text{lip/w}} + V_{f,\text{protein/medium}} \times K_{\text{BSA/w}} + V_{f,\text{water/medium}}}{K_{\text{aw}}} \quad (3)
\]

Test for loss and cross-contamination

Chemical stocks in methanol were used to prepare dilutions of the three test chemicals chlorpyrifos, diazinon, and parathion.
The final concentration of the chemicals in the plates corresponded to the highest test concentration of the AChE inhibition assay: 2.2 x 10^{-5} M for chlorpyrifos, 6.6 x 10^{-5} M for diazinon, and 3.4 x 10^{-5} M for parathion (concentration with the maximum solubility in the assay medium).

In a collagen I–coated 384-well plate, 40 μl of spiked medium per well was transferred into the plates with quadruplicates as shown in Supporting Information, Figure S2. The remaining wells were filled with 40 μl of nonspiked assay medium. The plate was covered with a lid and incubated at 37 °C and 5% CO_2 for 24 h in total. Two aliquots of 1 ml of spiked medium were incubated in closed vials under the same condition.

To quantify the initial level of chemicals, two aliquots of 30 μl of the spiked medium were transferred into high-performance liquid chromatography (HPLC) vials with insert and extracted immediately after preparation. After 3 and 24 h of incubation, 30 μl of the spiked medium from two out of the four wells of the quadruplicates and the nonspiked medium from four adjacent wells in the plates, as well as two aliquots from closed vials were collected and transferred into HPLC vials with insert for extraction.

The samples were extracted using solid-phase microextraction (SPME) for subsequent quantification of the chemical amount. The SPME method was based on protocols from previous studies of our group (Henneberger et al., 2019; Huchthausen et al., 2020), and the method parameters were adapted accordingly because of the small sample volume of the 384-well plate. Customized SPME fibers were purchased from Sigma-Aldrich and were made of nitinol wire with a coating of C18 particles embedded in polyacrylonitrile. The coating thickness was 45 μm, and the coating length was 2 mm, leading to an approximate coating volume of 69 nL. The SPME fibers were stored in methanol and conditioned in Milli-Q water for 20 min before the experiment. The fibers were transferred to the sample vials, and the vials were shaken at 37 °C and 1800 rpm (BioShake iQ; Quantifoil Instruments) for 2 h. For the chemical desorption from the fibers, aliquots of 60 μl of 90/10 (v/v) acetonitrile/Milli-Q water (chlorpyrifos) or 50/50 (v/v) acetonitrile/Milli-Q water (diazinon and parathion) were pipetted into HPLC vials with inserts. All solvents used had a purity >99%. The fibers were transferred to the vials containing the desorption solvent and incubated at 37 °C and 1800 rpm for 2 h. The fibers were removed from the vials, and the vials were stored at 4 °C until instrumental analysis.

The chemical concentration in the desorption solvent after SPME was quantified using liquid-chromatography mass-spectrometry (LC-MS). An Agilent 1260 Infinity II system was equipped with a Kinetex 1.7 μm C18, 100 Å, LC column (50 x 2.1 mm) and coupled to a triple-quadrupole MS (Agilent 6420 Triple Quad). The LC and MS parameters are given in Supporting Information, Table S2.

Relative peak area (percentage) was calculated based on an initial peak area of samples before incubation (at 0 h). Instead of converting peak area to mass using standard curves, relative peak area was used so that the data below the standard range could be included for some samples with a low concentration level, for example, samples from neighboring wells. At least two experimental runs were conducted with additional repeats in case the data from the first two runs were not comparable.

### Environmental samples

Water samples were collected from diverse small streams in Germany during rain events that were impacted by agriculture, road runoff, and combined sewer overflow, leading to a highly diverse pattern of organic pollutants. The detailed information for sampling was already described by Liess et al. (2021), and 381 chemicals were analyzed by a target screening method using LC high-resolution MS. Details of chemical analysis and detected concentrations of chemicals were published by Lee et al. (2022). Among these 85 river water samples, 13 were selected for testing in the AChE inhibition assay based on the sum of the detected concentrations of AChE inhibitors. The availability of samples (enough volume for testing) represented another selection criterion. Samples were only run once, although in a proper monitoring study, duplicate experiments would need to be performed; but the available extract volume was limited.

To calculate the sum of detected concentrations, we only considered chemicals that were classified as AChE inhibitors by the Insecticide Resistance Action Committee (2021). Non-insecticide OPs (e.g., triphenyl phosphate) typically have no or very weak AChE-inhibiting potency and were therefore not considered. The EC50 for AChE inhibition was derived as described above in units of relative enrichment factor (REF; liters of water per liters of bioassay).

### Role of DOC in the AChE inhibition assay

Humic acid and paraoxon-ethyl were exposed together for 3 h, followed by the AChE inhibition assay described above to evaluate the effects of DOC on AChE inhibition. Humic acid was serially diluted and tested up to 68 mg/L in the presence of paraoxon-ethyl at its EC50 obtained in this assay. The relative AChE inhibition level by the co-exposure of humic acid and paraoxon-ethyl was determined compared with the inhibition level triggered by the constant paraoxon-ethyl concentration.

### Mixture experiments

The toxicity of binary mixtures of chemicals or mixtures of a chemical and an extract of environmental sample were evaluated based on an isobologram approach (Altenburger et al., 1990). A fixed concentration ratio design was used, which means that stock solutions containing 10 times the EC50 of each component were mixed in ratios of 80:20, 60:40, 40:60, and 20:80 (Supporting Information, Figure S3); and these stocks with sum of concentrations in the mixture (C_{sum}) were diluted as in a single-chemical experiment to derive concentration–response curves (CRCs).

The concentration fraction of each component in the mixture (p) was calculated by Equation 4, and EC50_{mix} was
deduced from the CRCs of the mixture experiments that used $C_{\text{sum}}$ as the concentration.

$$\rho_i = \frac{C_i}{C_{\text{sum}}}$$  \hspace{1cm} (4)

The toxic units of each mixture component $i$ (TU$_i$) in Equation 5 were calculated from the experimental EC50$_{\text{mix}}$ multiplied by the $\rho_i$ and divided by the EC50 of the mixture component $i$ (EC50$_i$).

$$\text{TU}_i = \frac{\rho_i \times \text{EC50}_{\text{mix}}}{\text{EC50}_i}$$  \hspace{1cm} (5)

The binary mixture of paraoxon-ethyl and diazoxon was tested as reference with four different fixed ratios plus the components alone. While the EC50$_{\text{mix}}$ was determined individually for each experimental run because of the diverse combination of mixtures, EC50$_i$ was derived from all experimental runs together to improve the robustness of TU$_i$. The experiment was repeated two or three times.

Subsequent testing of paraoxon-ethyl and environmental samples was performed to investigate the influence of DOC on the mixture toxicity and to test if a water sample containing a complex mixture behaves in a concentration-additive way with respect to the target mode of action. Two river water samples were tested in the same isobologram design using nine or 10 different fixed concentration ratios of binary mixtures with paraoxon-ethyl. The two river water samples were selected considering following criteria: (1) no cytotoxicity accompanied by AChE inhibition, and (2) different degree of AChE inhibition potency (high and moderate). For $C_{\text{sum}}$ in the mixture of paraoxon-ethyl with environmental samples, the highest tested concentration of paraoxon-ethyl ($1.99 \times 10^{-8} \text{M}$) was set at an REF of 1 to unify their units.

RESULTS AND DISCUSSION

Type of FBS and cells used

Many cell-based AChE inhibition assays use assay medium with FBS, which can result in additional AChE in the bioassay with inconsistent composition from different species. Our assay used SH-SY5Y cells, originated from human tissue, as the source of AChE. Because FBS contains active AChE from cow (Ralston et al., 1985), FBS-containing medium can provide another source of AChE in this SH-SY5Y cell-based AChE inhibition assay. The enzymes in FBS could possibly be filtered out or inactivated by heat via additional treatment of FBS, but the type of FBS has often not been indicated clearly. Although the amino acid sequence of AChE and the corresponding enzyme structure are known to be relatively conserved between species, the sensitivity of AChE to several known AChE inhibitors was lower in cow than in horse or rat (Cohen et al., 1985; Karanth & Pope, 2003). Therefore, we compared background AChE levels from assay media containing FBS, csFBS, dFBS, and hiFBS as well as the Neurobasal assay medium to rule out the presence of external AChE and avoid any bias in AChE inhibition determination (Supporting Information, Figure S4).

The AChE activity of the medium itself (without cells) was assessed by measuring the absorbance after incubation with the known AChE inhibitor paraoxon-ethyl or methanol for 1 h. Increased absorbance from methanol-treated wells was observed for assay medium containing 1% FBS and dFBS, which indicates the presence of active AChE in the medium. For the rest of the media (DMEM/F12 with 1% csFBS or hiFBS and Neurobasal assay medium), the absorbance was comparable between paraoxon-ethyl- and methanol-treated wells and was only slightly higher than that of phosphate-buffered saline, which was used as a negative control. To prevent any possible contamination by external AChE from FBS, Neurobasal assay medium was selected as the assay medium in the present study.

Differentiated SH-SY5Y cells were used for the AChE inhibition assay because the AChE activity of the undifferentiated cells was too low and the change in absorbance too subtle to be used for analysis (data not shown). Therefore, we applied differentiated cells in our assay to increase AChE activity and the assay performance. It was reported previously that AChE activity increased 10-fold after differentiation of SH-SY5Y cells (de Medeiros et al., 2019). In addition, it was observed that AChE in undifferentiated SH-SY5Y cells was mainly localized on the neurites and distributed throughout the cytoplasm, while the majority of AChE is closely located to the nucleus for nonneuronal cells (Thullibery et al., 2005). Considering that expression of AChE along the neurites was achieved after differentiation of rat adrenal chromaffin PC-12 cells in the same study, more intense expression of AChE on the neurites and plasma membrane could be expected for differentiated SH-SY5Y cells.

To evaluate the assay quality for the optimized condition, the Z-factor (Zhang et al., 1999) was determined by comparing inhibition rate from paraoxon-ethyl-treated (100% inhibition level, positive control) and methanol-treated (negative control) measurements. The optimized condition using Neurobasal assay medium and differentiated SH-SY5Y cells gave a Z-factor of 0.81, which was above the threshold of 0.5 suggested by Zhang et al. (1999).

Change in EC50 for AChE inhibition over time

Nine AChE inhibitors were tested in differentiated SH-SY5Y cells under the optimized condition with an incubation period of 1–6 h to evaluate the changes in EC50 over time and determine an optimal exposure duration. No cytotoxic effects were observed for the dosed concentration ranges after 24-h exposure (data not shown). The inverse of EC50 was plotted to present the degree of toxicity in Figure 1, and the EC50s were derived from the CRCs in Supporting Information, Figure S5, and are given in Supporting Information, Table S3.

The EC50s of inhibitors with the same moiety (e.g., P=S moiety for OTPs; P=O for OPs) were similar to each other, which indicates comparable inhibitory potency on AChE from...
The Inhibition of AChE Increased over time with decreasing EC50 under 3-h exposure to AChE inhibitors (detailed information in Supporting Information, Table S3) and comparison with literature using a similar cell-based assay and an enzyme-based assay (Li et al., 2021).

| Chemical                  | Present study | SH-SYSY cell-based assay | Enzyme-based assay |
|---------------------------|---------------|--------------------------|--------------------|
| 3-Hydroxycarbofuran       | 0.31          | Not included             | Not included       |
| Paraoxon-ethyl            | 0.0019        | 0.1                      | 0.2                |
| Carbaryl                  | 5.8           | 30.7                     | 24.3               |
| Diazoxon                  | 0.0085        | 0.2                      | 1.8                |
| Carbofuran                | 0.047         | 0.2                      | 0.2                |
| Chlorpyrifos-oxon         | 0.00076       | 0.01                     | 0.02               |
| Parathion                 | 4.6           | 10.9                     | 24.3               |
| Diazinon                  | 4.5           | 18.6                     | 27.3               |
| Chlorpyrifos              | 5.4           | 19.9                     | 60.4               |

**TABLE 1:** Effect concentration for 50% of maximum acetylcholinesterase (AChE) inhibition after 3-h exposure to AChE inhibitors (detailed information in Supporting Information, Table S3) and comparison with literature using a similar cell-based assay and an enzyme-based assay (Li et al., 2021)

**FIGURE 1:** Effect concentration for 50% of maximum acetylcholinesterase inhibition for organothiophosphates and carbamates after 1–6-h exposure in differentiated SH-SYSY cells. EC50 = median effect concentration; AChE = acetylcholinesterase.

Loss due to partitioning of AChE inhibitors to the airspace in 384-well plates

To evaluate loss of AChE inhibitors, we predicted $K_{\text{medium/air}}$ of the nine tested AChE inhibitors based on the distribution between medium and water ($K_{\text{medium/w}}$) and the $K_{\text{aw}}$ (Table 2). The log $K_{\text{medium/air}}$ ranged from 3.59 to 9.80 for our test chemicals. The volatility cutoff in bioassays was initially defined by Escher et al. (2019), and chemicals having a log $K_{\text{medium/air}} < 4$ were considered as volatile chemicals in the assays with a 24-h exposure duration. Three OTPs, chlorpyrifos, diazinon, and parathion, had log $K_{\text{medium/air}}$ below or just above...
the defined cutoff. Therefore, we selected these three chemicals to verify the predicted loss under our test condition using chemical analysis. We also quantified the chemical losses after 24 h because this is the typical exposure duration of bioassays. For the remaining chemicals including OP metabolites and carbamates, the log $K_{\text{medium/air}}$ was clearly above the cutoff, and thus they were considered as not prone to losses to the air for the assay conditions applied.

The Initial levels (0 h) of chlorpyrifos, diazinon, and parathion were compared with the final levels in closed vials as well as with spiked and neighboring wells in 384-well plates after 3 and 24 h of incubation (Figure 2). The chemicals were incubated without cells to exclude loss due to metabolism. The amount of chemicals in closed vials was relatively stable for 24 h for all three OPs (from 85% to 100% of initial level). The constant level of chemicals in the closed system over time would mean there was minor contribution by additional loss from an abiotic process other than volatility, such as photolysis and hydrolysis (Proenca et al., 2021).

When we compared the level in the closed vials with those from spiked wells, most of the test chemicals stayed in the medium for our assay duration of 3 h: 66% of the initial level for chlorpyrifos, 83% for diazinon, and 76% for parathion. The maximum loss of 34% was observed for chlorpyrifos, which was expected from the lowest predicted $K_{\text{medium/air}}$ among the test chemicals. The loss due to partitioning to air may be considered acceptable within 3 h of exposure, but one should be cautious with more volatile chemicals whose EC50 values may be affected strongly. It is noteworthy that the observed losses in the spiked wells could also arise from sorption to the plastic.

### TABLE 2: Partition coefficients to predict loss processes from volatility in the bioassay with Neurobasal assay medium for nine acetylcholinesterase inhibitors

| Chemical          | $\log K_{\text{BSA/w}}$ | $\log K_{\text{lip/w}}$ | $\log K_{\text{medium/w}}$ | $\log K_{\text{aw}}$ | $\log K_{\text{medium/air}}$ |
|-------------------|--------------------------|-------------------------|-----------------------------|----------------------|-----------------------------|
|                   | ($L_{\text{water}}$/LBSA) | ($L_{\text{water}}$/LLip) | ($L_{\text{water}}$/Lmedium) | ($L_{\text{water}}$/Lair) | ($L_{\text{air}}$/Lmedium)  |
| 3-Hydroxycarbofuran | 0.96                     | 0.56                    | 0.01                        | -9.79                | 9.80                        |
| Paraoxon-ethyl     | 1.80                     | 1.98                    | 0.06                        | -6.70                | 6.76                        |
| Carbaryl          | 2.61                     | 2.92                    | 0.31                        | -6.26                | 6.57                        |
| Diazoxon          | 0.95                     | 0.89                    | 0.01                        | -6.42                | 6.43                        |
| Carbofuran        | 1.72                     | 1.93                    | 0.05                        | -5.94                | 5.99                        |
| Chlorpyrifos-oxon | 2.25                     | 2.53                    | 0.16                        | -5.64                | 5.80                        |
| Parathion         | 2.83                     | 3.43                    | 0.44                        | -4.65                | 5.09                        |
| Diazinon          | 2.85                     | 3.25                    | 0.45                        | -3.96                | 4.41                        |
| Chlorpyrifos      | 3.48                     | 4.30                    | 0.94                        | -2.65                | 3.59                        |

*UFZ-LSER database (Ulrich et al., 2017); pH 7.4, 37 °C.
Prediction using a mass balance model (Escher et al., 2019).

$K_{\text{BSA/w}}$ = partition constants between proteins (bovine serum albumin; BSA) and water; $K_{\text{lip/w}}$ = partition constants between lipids and water; $K_{\text{medium/w}}$ = partition constants between medium and water; $K_{\text{aw}}$ = partition constants between air and water; $K_{\text{medium/air}}$ = partition constants between medium and air.

**FIGURE 2:** Volatility test of three organophosphates in 384-well plates containing Neurobasal assay medium without cells. The chemicals at the highest tested concentration used for testing acetylcholinesterase inhibition were incubated for 3 and 24 h. Relative peak area of (A) chlorpyrifos, (B) diazinon, and (C) parathion in closed vials, spiked wells, and neighboring wells compared to the initial level at 0 h.
of microplates, but this loss process is expected to be negligible because of the high sorptive capacity of medium components (Fischer et al., 2018).

More than 50% of chlorpyrifos and diazinon were lost from the spiked wells after 24 h, which can be rationalized by their predicted $K_{\text{medium/air}}$ (Figure 3). The lower the predicted $K_{\text{medium/air}}$ was for the chemicals, the higher was the loss in the spiked wells observed after 24 h. Chlorpyrifos had the lowest log $K_{\text{medium/air}}$ of 3.59 and the biggest loss from the spiked wells among the three OPs after 3 and 24 h of incubation. Although the spiked wells of chlorpyrifos and diazinon contained chemicals at <50% of the initial level after 24 h of incubation, parathion with a log $K_{\text{medium/air}}$ of 5.09 had 71.5% detected in the spiked wells. Loss to the airspace of chemicals in the bioassay depends on $K_{\text{ow}}$ and the octanol–water partition coefficient $K_{\text{ow}}$ as shown in Figure 3 (Escher et al., 2019). The $K_{\text{ow}}$ of individual chemicals mainly determines the loss in the bioassay, and the higher the $K_{\text{ow}}$ is, the more easily chemicals partition into the headspace with some damping by the retainer capacity of the medium. Although the retaining capacity of medium was the highest for these three OPs among the nine AchE inhibitors considering higher $K_{\text{medium/air}}$ (Table 2), this had a minor contribution to alleviation of the loss to the airspace, which was confirmed by only slightly elevated $K_{\text{medium/air}}$ in simulation with the medium containing 10% FBS (Supporting Information, Figure S7).

Cross-contamination in neighboring wells was observed to a low extent for diazinon after 24 h, whose $K_{\text{medium/air}}$ is slightly above the volatility cutoff (Figure 2). The detection level in the neighboring wells was 3.5% of the initial level for diazinon, 1.3% for chlorpyrifos, and 0.5% for parathion. This trend is in line with the previous observation that chemicals with a $K_{\text{medium/air}}$ closer to volatility cutoff, that is, diazinon in the present study, caused more severe cross-contamination, whereas highly volatile chemicals were mainly lost without cross-contamination (Escher et al., 2019). Considering that a substantial amount (>60% of initial level) was retained and no cross-contamination was observed during the 3-h exposure duration of the AchE inhibition assay, the previously defined volatility cutoff is still applicable for the AchE inhibition assay; but we recommend analytically verifying the exposure concentrations when testing single chemicals that have a log $K_{\text{medium/air}}$ close to 4 and <5.

**Evaluation of the influence of DOC on the AchE inhibition assay**

During sample preparation of surface water and wastewater via solid-phase extraction (SPE), DOC is co-extracted with micropollutants from water samples (Pichon et al., 1996), while metal and inorganic ions can be well removed. Depending on the water type, 40%–70% of the DOC can be recovered in the extract after SPE (Neale & Escher, 2013). It has been shown that DOC interferes with the performance of the enzyme-based AchE assay and suppresses the effect of added AchE inhibitors (Neale & Escher, 2013). However, DOC did not disturb the performance of cell-based bioassays where the receptors of interest are at least partially inside the cells (Neale & Escher, 2014). Given that the AchE is partially located on the neurites of SH-SY5Y cells and therefore potentially in contact with the medium, we explored if this interference by DOC is also observed in the SH-SY5Y cell-based AchE inhibition assay. The inhibition level of AchE from co-exposure to DOC and paraoxon-ethyl as a reference chemical was compared with that from single exposure to paraoxon-ethyl. Aldrich humic acid was applied as representative of DOC according to Neale and Escher (2013).

Inhibition of AchE by paraoxon-ethyl was not influenced by humic acid up to 68 mg/L (Figure 4). Any experimental artifacts were prevented by removing the assay medium before detection. The inhibition level by exposure to only paraoxon-ethyl was comparable to that by co-exposure to paraoxon-ethyl and humic acid. This means that no suppressive effects were observed with humic acid. This observation is in striking contrast to what was observed in the enzyme-based AchE inhibition assay, where AchE inhibition by oxidized parathion was suppressed by humic acid at a final assay concentration as low as 2 mg/L (Neale & Escher, 2013).

The Interference by DOC has been explained by three different causes: (1) nonspecific binding of DOC to the target site, (2) experimental artifacts due to interference with measurement, and (3) sorption of micropollutants to DOC leading to low bioavailable concentration (Neale & Escher, 2014). Non-specific binding of DOC to AchE possibly interfered with inhibition by chemicals in the assay using purified AchE (Neale & Escher, 2013). However, in the cell-based assay, AchE is
Anchored to the outer cell membrane, and this might deter accessibility of humic acid to AchE. It could be that DOC is too bulky to effectively bind to anchored AchE. Charge repulsion between cell membrane and DOC, because both are negatively charged (Philippe & Schaumann, 2014), also can hinder the access of DOC to the AchE. Another possible explanation for the experimental observations arises from the tetramer structure of anchored AchE. Although two catalytic subunits of the tetramers are oriented outward, the other two subunits are toward the cell membrane (Perrier et al., 2002), and hence possibly less accessible by DOC. Some AchE is located inside the cells, and DOC cannot enter into the cells. In addition, if DOC would disturb the quantification of the enzyme activity by measurement with ATCh, for example, if the bioavailability of ATCh was reduced in the presence of DOC, one would also detect lower activity. In the SH-SYSY cell assay, the medium is removed prior to running the enzyme inhibition test with ATCh, which could protect against artifacts.

**Applicability of the cell-based AchE inhibition assay to environmental water samples**

To investigate the applicability to environmental water samples, 13 river water samples were tested in the AchE inhibition assay. Their EC50s ranged from REF 0.5 to 10; that is, the samples had to be diluted by a factor of 2 or enriched 10-fold to achieve 50% AchE inhibition. The EC50s were at least three times lower than the IC50, and cytotoxicity was determined after 24 h of exposure. Therefore, the observed AchE inhibition would be primarily caused by specific inhibition rather than by unspecific reduction due to cytotoxicity. The CRCs of the tested water samples are given for AchE inhibition and cytotoxicity in Supporting Information, Figure S8. The derived EC50s for AchE inhibition are given in Supporting Information, Table S4, together with the 24-h cytotoxicity IC50.

All 13 river water samples inhibited AchE with different effect potency (Figure 5). Higher detected concentrations of AchE inhibitors in the samples did not necessarily lead to higher AchE inhibition. For example, pirimicarb and dimethoate were detected in the sample with the highest AchE inhibition (sample b in Figure 5), but their detected concentrations were lower than the level in other samples with lower AchE inhibition potency (Supporting Information, Table S4). This indicates that there might be a contribution of AchE inhibitors below the detection limit or unknown AchE inhibitors, which demonstrates the sensitivity of our assay to capture those effects.

Two water extracts were selected for subsequent mixture experiments. To evaluate samples with different degrees of effect potency, we chose samples which showed moderate (sample a in Figure 5; EC50 = REF 3.0) and high (sample b in Figure 5; EC50 = REF 0.5) AchE inhibition.

**Mixture experiments**

We tested binary mixtures of reference chemicals and the two selected water extracts to confirm the applicability of the cell-based assay to environmental samples. Diverse mixtures with different effect concentration fractions of paraoxon-ethyl with diazoxon or water extract were tested, and their experimental toxic unit was compared with the prediction of the concentration addition model (Figure 6). Parallel log-CRCs are a prerequisite for derivation of the toxic unit (Villeneuve et al., 2000), and the slopes of the CRCs were similar within a factor of 1.4 (Supporting Information, Figure S9 and Tables S5 and S6). Detailed information for the calculation of toxic unit is given in Supporting Information, Tables S5 and S6.

The experimentally derived toxic unit agreed well with the concentration addition prediction for binary mixtures of the known AchE inhibitors paraoxon-ethyl and diazoxon (Figure 6A).

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**FIGURE 4:** Relative inhibition of acetylcholinesterase (AChE) after 3-h exposure to paraoxon-ethyl (POE) at the previously determined median effect concentration of 2 × 10^−7 M POE in the presence of variable concentrations of Aldrich humic acid (dissolved organic carbon) in the AChE inhibition assay with SH-SYSY cells. EC50 = median effect concentration; DOC = dissolved organic carbon.

**FIGURE 5:** Acetylcholinesterase inhibition (median effect concentration for 3-h exposure) and cytotoxic effects (median inhibition concentration for 24-h exposure) of 13 river water samples in differentiated SH-SYSY cells. Darker gray area indicates higher possibility of artifacts from cytotoxicity. Samples a and b were chosen for the subsequent mixture experiments. AchE = acetylcholinesterase; EC50 = median effect concentration; REF = relative enrichment factor (liters of water per liter of bioassay); IC50 = median inhibition concentration for cytotoxicity.
Because these two chemicals act through the same mode of action, no deviation is expected from the prediction. This was already demonstrated in the isobologram for oxidized diazinon and parathion in a purified AchE inhibition assay (Neale & Escher, 2013). The experimental toxic unit of water extracts mixed with single chemicals also agreed well with the prediction for concentration addition (Figure 6B,C). In the isobologram study using purified AchE, on the contrary, the experimental values highly deviated from the predictions for binary mixtures of oxidized parathion with diverse types of water samples (reverse osmosis concentrate, treated effluent, and surface water; Neale & Escher, 2013). This indicates that the AchE inhibition assay using SH-SYSY cells is not biased by binding to DOC, and hence can be applied to screening of DOC-rich environmental samples.

**CONCLUSIONS**

Limitations of bioassays for testing chemicals could be loss of chemicals during exposure, for example, by volatilization. To consider volatile loss of chemicals for experimental planning, a volatility cutoff at a log $K_{\text{medium/air}}$ of 4 was defined previously considering cytotoxic effects in various cell lines (Escher et al., 2019). While we confirmed that this cutoff is also applicable to the AchE inhibition assay with SH-SYSY cells after 3 h of exposure, we observed substantial loss and slight cross-contamination after 24 h of incubation for some chemicals whose $K_{\text{medium/air}}$ was just around the cutoff. There can be other processes that contribute to the loss of chemicals in bioassays, such as abiotic degradation and sorption to the plastic microplates. In the presence of cells, it must also be kept in mind that SH-SYSY cells are metabolically active and that a decrease in exposure concentration could occur because of cellular uptake and intracellular metabolism despite the large medium volume to cell volume ratio that would normally assure that depletion due to cell uptake and metabolism is negligible.

The OTPs were substantially less potent than OPs. Oxidation of OTPs into OPs by the metabolic activity of SH-SYSY cells needs to be investigated further to understand the role of metabolism in this cell-based AchE assay. Potentially, the metabolic activation could be boosted by addition of an external metabolizing enzyme cocktail such as S9 isolated from rat liver, as is common practice in other bioassays. In the free enzyme-based assay, the OTPs are often oxidized with N-bromosuccinimide to the corresponding OPs, but even mild oxidation can degrade other mixture components in water samples; therefore, we did not attempt chemical oxidation as part of sample preparation.

Bioanalytical tools are useful to assess the toxicity of micropollutants in environmental samples. Measurement of AchE inhibition has been considered a potential endpoint to detect certain types of neuroactive pesticides such as OPs and carbamates in environmental samples (Legradi et al., 2018). However, environmental matrices such as DOC can hinder precise assessment in bioassays using isolated enzyme because the DOC can bind chemicals and therefore reduce the bioavailability of the chemicals for the receptor—in our case, AchE—or the DOC can disturb the AchE nonspecifically (Neale & Escher, 2013). Considering that DOC can suppress AchE inhibition in assays using purified enzyme, previous testing of water extracts with isolated enzymes might have underestimated AchE inhibition in the environmental samples. Such a negative impact of co-extracted DOC had not been observed for any other cell-based assay (Neale & Escher, 2014). Consistent with these observations, our SH-SYSY cell–based assay was also unaffected by DOC and is more sensitive toward known AchE inhibitors. Therefore, it appears more suitable for environmental monitoring than any enzyme-based AchE inhibition assay.

**Supporting information**—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5490.

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Data Availability Statement—Data are available in the Supporting Information. Data, associated metadata, and calculation tools are available from the corresponding author (beate.escher@ufz.de).

REFERENCES

Altenburger, R., Bodeker, W., Faust, M., & Grimme, L. H. (1990). Evaluation of the isobilogram method for the assessment of mixtures of chemicals. Combination effect studies with pesticides in algal biostests. Ecotoxicology and Environmental Safety, 20, 98–114. https://doi.org/10.1016/0147-6513(90)90049-b

Birch, H., Kramer, N. I., & Mayer, P. (2019). Time-resolved freely dissolved concentrations of semivolatile and hydrophobic test chemicals in in vitro assays—measuring high losses and crossover by headspace solid-phase microextraction. Chemical Research in Toxicology, 32, 1780–1790. https://doi.org/10.1021/acs.chemrestox.9b00223

Cao, J., Wang, M., Yu, H., She, Y., Cao, Z., Ye, J., Abd El Aty, A. M., Hacimuftuoglu, A., Wang, J., & Lao, S. (2020). An overview on the mechanisms and applications of enzyme inhibition-based methods for determination of organophosphate and carbamate pesticides. Journal of Agricultural and Food Chemistry, 68, 7298–7315. https://doi.org/10.1021/acs.jafc.0c01962

CITE-KNIME. (2022). AChE evaluation. GitHub repository. https://github.com/CITE-KNIME/AChE_evaluation

Cohen, S. D., Williams, R. A., Killinger, J. M., & Freudenthal, R. I. (1985). Comparative sensitivity of bovine and rodent acetylcholinesterase to in vitro inhibition by organophosphate insecticides. Toxicology and Applied Pharmacology, 81, 452–459. https://doi.org/10.1016/0041-0048 (85)90416-8

Colović, M. B., Krstić, D. Z., Lazarević-Pasti, T. D., Bondzic, A. M., & Vasic, V. M. (2013). Acetylcholinesterase inhibitors: Pharmacology and toxicology. Current Neuropharmacology, 11, 315–335. https://doi.org/10.2174/1570159X11311030006

Colović, M. B., Krstić, D. Z., Ušćumlić, G. S., & Vasic, V. M. (2011). Single and simultaneous exposure of acetylcholinesterase to diazinon, chlorpyrifos and their photodegradation products. Pesticide Biochemistry and Physiology, 100, 16–22. https://doi.org/10.1016/j.pestbp.2011.01.010

de Medeiros, L. M., De Bastiani, M. A., Rico, E. P., Schonhofen, P., Pfaffenseller, B., Wollenhaupt-Aguier, B., Grun, L., Barbe-Tuana, F., Zimmer, E. R., Castro, M. A. A., Parsons, R. B., & Klamt, F. (2019). Cholinergic differentiation of human neuroblastoma SH-SYSY cell line and its potential use as an in vitro model for Alzheimer’s disease studies.

Molecular Neurobiology, 56, 7355–7367. https://doi.org/10.1007/s12035-019-1605-3

Ellman, G. L., Courtney, K. D., Andres, V. J., & Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Escher, B. I., Bramaz, N., & Ort, C. (2009). JEM spotlight: Monitoring the treatment efficiency of a full scale ozonation on a sewage treatment plant with a mode-of-action based test battery. Journal of Environmental Monitoring, 11, 1836–1846. https://doi.org/10.1039/b907093a

Escher, B. I., Glauch, L., König, M., Mayer, P., & Schlöch, R. (2019). Baseline toxicity and volatility cutoff in reporter gene assays used for high-throughput screening. Chemical Research in Toxicology, 32, 1646–1655. https://doi.org/10.1021/acs.chemrestox.9b00182

Fischer, F. C., Cirpka, O. A., Goss, K. U., Henneberger, L., & Escher, B. I. (2018). Application of experimental polystyrene partition constants and diffusion coefficients to predict the sorption of neutral organic chemicals to multiwell plates in in vivo and in vitro bioassays. Environmental Science & Technology, 52, 13511–13522. https://doi.org/10.1021/acs.est.8b04246

Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., & Escher, B. I. (2017). Modeling exposure in the Tox21 in vitro bioassays. Chemical Research in Toxicology, 30, 1197–1208. https://doi.org/10.1021/acs.chemrestox.7b00023

Fukuto, T. R. (1990). Mechanism of action of organophosphorus and carbamate insecticides. Environmental Health Perspectives, 87, 245–254. https://doi.org/10.1289/ehp.9087245

Hamers, T., Molin, K. R., Koeman, J. H., & Murch, A. J. (2000). A small-volume bioassay for quantification of the esterase inhibiting potency of mixtures of organophosphate and carbamate insecticides in rainwater: Development and optimization. Toxicological Sciences, 58, 60–67. https://doi.org/10.1093/toxsci/58.1.60

Henneberger, L., Mühlenbrink, M., Fischer, F. C., & Escher, B. I. (2019). C18-coated solid-phase microextraction fibers for the quantification of partitioning of organic acids to proteins, lipids, and cells. Chemical Research in Toxicology, 32, 168–178. https://doi.org/10.1021/acs.chemrestox.8b00249

Hicks, D. A., Makova, N. Z., Nalivaeva, N. N., & Turner, A. J. (2013). Characterization of acetylcholinesterase release from neuronal cells. Chemico-Biological Interactions, 203, 302–308. https://doi.org/10.1016/j.cbi.2012.09.019

Huchthausen, J., Mühlenbrink, M., König, M., Escher, B. I., & Henneberger, L. (2020). Experimental exposure assessment of ionizable organic chemicals in in vitro cell-based bioassays. Chemical Research in Toxicology, 33, 1845–1854. https://doi.org/10.1021/acs.chemrestox.0c00067

Insecticide Resistance Action Committee. (2021). The IRAC mode of action classification (Version 10.1). http://irac-online.org/mode-of-action/

Karanth, S., & Pope, C. (2003). In vitro inhibition of blood cholinesterase activities from horse, cow, and rat by tetrachlorvinphos. International Journal of Toxicology, 22, 429–433. https://doi.org/10.1177/109158180302200604

Lee, J., Braun, G., Henneberger, L., König, M., Schlöch, R., Scholz, S., & 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. Chemical Research in Toxicology, 34, 2100–2109. https://doi.org/10.1021/acs.chemrestox.1c00182

Lee, J., Schloch, R., König, M., Scholz, S., Krauss, M., & Escher, B. I. (2022). Monitoring mixture effects of neurotoxicants in surface water and wastewater treatment plant effluents with neurite outgrowth inhibition in SH-SYSY cells. ACS Environmental Au. Advance online publication. https://doi.org/10.1021/acsenvirocn.2c00026

Legradi, J. B., Di Paolo, C., Kraak, M. H. S., van der Geest, H. G., Schymanski, E. L., Williams, A. J., Dingemans, M. L. M., Massie, R., Brack, W., Cousin, X., Begout, M. L., van der Oost, R., Carion, A., Suarez-Ulloa, V., Silvestre, F., Escher, B. I., Engwall, M., Nilen, G., Keiter, S. H., ..., Hollert, H. (2018). An ecotoxicological view on neurotoxicity assessment. Environmental Sciences Europe, 30, Article 46. https://doi.org/10.1186/s12302-018-0179-x

Li, S., Huang, R., Solomon, S., Liu, Y., Zhao, B., Santillo, M. F., & Xia, M. (2017). Identification of acetylcholinesterase inhibitors using homogeneous cell-based assays in quantitative high-throughput screening formats. Biotechnology Journal, 12, Article 1600715. https://doi.org/10.1002/biot.201600715

Li, S., Zhao, J., Huang, R., Travers, J., Klump-Thomas, C., Yu, W., MacKerell, A. D., Jr., Sakamuru, S., Ooka, M., Xue, F., Sipes, N. S., Zimmer, E. R., Castro, M. A. A., Parsons, R. B., & Klamt, F. (2019).
Validation of an acetylcholinesterase inhibition assay—Environmental Toxicology and Chemistry, 2022;41:3046–3057

Hsieh, J. H., Ryan, K., Simeonov, A., Santillo, M. F., & Xia, M. (2021). Profiling the Tox21 chemical collection for acetylcholinesterase inhibition. Environmental Health Perspectives, 129, Article 47008. https://doi.org/10.1289/EHP6993

Liess, M., Liebmann, L., Vormeier, P., Weisner, O., Altenburger, R., Borchardt, D., Brack, W., Chatzinotas, A., Escher, B., Foit, K., Gunold, R., Henz, S., Hitzfeld, K. L., Schmitt-Jansen, M., Kamjunké, N., Kaske, O., Knillmann, S., Krauss, M., Kuster, E., Reemtsma, T. (2021). Pesticides are the dominant stressors for vulnerable insects in lowland streams. Water Research, 201, Article 117262. https://doi.org/10.1016/j.watres.2021.117262

Lionetto, M. G., Caricato, R., Calisi, A., & Schettino, T. (2011). Acetylcholinesterase inhibition as a relevant biomarker in environmental bio-monitoring: New insights and perspectives. In J. E. Visscher (Ed.), Ecotoxicology around the globe (pp. 87–115). Nova Science.

Macova, M., Toze, S., Hodgers, L., Mueller, J. F., Bartkow, M., & Escher, B. I. (2013). Coextracted dissolved organic carbon cause artefacts in cell based bioassays? Chemosphere, 108, 281–288. https://doi.org/10.1016/j.chemosphere.2014.01.053

Neale, P. A., & Escher, B. I. (2013). Biomonitoring tools for the evaluation of organic micropollutants during sewage treatment, water recycling and drinking water generation. Water Research, 45, 4238–4247. https://doi.org/10.1016/j.watres.2011.05.032

Nunes, B. (2011). The use of cholinesterases in ecotoxicology. Reviews of Environmental Contamination and Toxicology, 212, 29–59. https://doi.org/10.1007/978-1-4419-8453-1_2

Pernier, A. L., Massoulie, J., & Krejci, E. (2002). PriMA: The membrane anchor of acetylcholinesterase in the brain. Neuron, 33, 275–285. https://doi.org/10.1016/s0896-6275(01)00584-0

Philippe, A., & Schaumann, G. E. (2014). Interactions of dissolved organic matter with natural and engineered inorganic colloids: A review. Environmental Science & Technology, 48, 8946–8962. https://doi.org/10.1021/es502342r

Pichon, V., Coumes, C. C. D., Chen, L., Guenu, S., & Hennion, M. C. (1996). Simple removal of humic and fulvic acid interferences using polymeric sorbents for the simultaneous solid-phase extraction of polar acidic, neutral and basic pesticides. Journal of Chromatography A, 737, 25–33. https://doi.org/10.1016/0021-9673(95)01339-3

Pohanka, M. (2011). Cholinesterases, a target of pharmacology and toxicology. Biomedical Papers of the Medical Faculty of Palacký University in Olomouc, 155, 219–229. https://doi.org/10.5507/bp.2011.036

Proenza, S., Escher, B. I., Fischer, F. C., Fisher, C., Gregoire, S., Hewitt, N. J., Nicol, B., Paini, A., & Kramer, N. I. (2021). Effective exposure of chemicals in in vitro cell systems: A review of chemical distribution models. Toxicology In Vitro, 73, Article 105133. https://doi.org/10.1016/j.tiv.2021.105133

R: A language and environment for statistical computing. (Ver 4.0.4) [Computer software]. (2020). R Foundation for Statistical Computing.

Ralston, J. S., Rush, R. S., Doctor, B. P., & Wolfe, A. D. (1985). Acetylcholinesterase from fetal bovine serum. Purification and characterization of soluble G4 enzyme. Journal of Biological Chemistry, 260, 4312–4318.

Sharma, K. (2019). Cholinesterase inhibitors as Alzheimer’s therapeutics (review). Molecular Medicine Reports, 20, 1479–1487. https://doi.org/10.3892/mmr.2019.10374

Sogorb, M. A., Alvarez-Escalante, C., Carrera, V., & Vilanova, E. (2007). An in vitro approach for demonstrating the critical role of serum albumin in the detoxification of the carbamate carbaryl at in vivo toxicologically relevant concentrations. Archives of Toxicology, 81, 113–119. https://doi.org/10.1007/s00204-006-0142-9

Sogorb, M. A., Carrera, V., & Vilanova, E. (2004). Hydrolysis of carbaryl by human serum albumin. Archives of Toxicology, 78, 629–634. https://doi.org/10.1007/s00204-004-0584-x

Thullbery, M. D., Cox, H. D., Schule, T., Thompson, C. M., & George, K. M. (2005). Differential localization of acetylcholinesterase in neuronal and non-neuronal cells. Journal of Cellular Biochemistry, 96, 599–610. https://doi.org/10.1002/jcb.20530

Ulrich, N., Endo, S., Brown, T. N., Watanabe, N., Bronner, G., Abraham, M. H., & Goss, K.-U. (2017). UFZ-LSER database (Ver 3.2.1). Helmholtz Centre for Environmental Research-UFZ. http://www.ufz.de/lserd

Villeneuve, D. L., Blankenship, A. L., & Griesy, J. P. (2000). Derivation and application of relative potency estimates based on in vitro bioassay results. Environmental Toxicology and Chemistry, 19, 2835–2843.

Zhang, J. H., Chung, T. D., & Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. Journal of Biomolecular Screening, 4, 67–73. https://doi.org/10.1177/108705719900400206