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Quantitation and prediction of sorptive losses during toxicity testing of polycyclic aromatic hydrocarbon (PAH) and nitrated PAH (NPAH) using polystyrene 96-well plates

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Abstract

Developing zebrafish are increasingly being used for rapid assessments of chemical toxicity, and these assays are frequently conducted in multi-well plastic plates. This study investigated the sorptive behavior of polycyclic aromatic hydrocarbons (PAHs) and nitrated PAHs (NPAHs) to uncoated 96-well polystyrene plates typically used for zebrafish (*Danio rerio*) testing. We measured the percent sorption in the presence and absence of zebrafish embryos, at two exposure concentrations, as well as using two different procedures (addition of embryos to polystyrene plates either before analyte addition, or allowing 24 hours of equilibrium between analyte addition and embryo addition to the polystyrene plates). Following exposure, the plates were extracted with hexane and analyzed using gas chromatography coupled with mass spectrometry (GC/MS). Allowing 24 hours of pre-incubation between the addition of analytes and embryos did not significantly impact the percent sorption. The percent sorption was higher for both PAHs and NPAHs at the lower exposure concentration, and sorption was lower in the presence of zebrafish embryos. A mass balance model was developed to predict the sorption to polystyrene plates, based on the PAH and NPAH mass distribution ratios between polystyrene and water. While PAH sorption was significantly correlated with subcooled liquid solubility, NPAH sorption did not correlate with any of the physical-chemical properties investigated. This indicates the need to better understand the sorptive behavior of hydrophobic analytes to plastics, and to better account for sorptive losses during toxicity testing in polystyrene plates.

Keywords

polycyclic aromatic hydrocarbon (PAH); nitrated polycyclic aromatic hydrocarbon (NPAH); polystyrene; sorption; 96-well plate

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Conflict of Interest The authors declare that there are no conflicts of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

1. Introduction

Advances in high-throughput screening capabilities have led to an increase in the popularity of plastic plates as exposure vessels for numerous toxicity-testing platforms. These plates are inexpensive and disposable, available in a wide variety of sizes and volumes to fit a range of test systems and assays, and show low toxicity to cell cultures and model organisms (Hirmann et al., 2007; Truong et al., 2014). Whole-animal systems, such as zebrafish (*Danio rerio*), are increasing in popularity and are amenable to rapid and high-throughput phenotypic screening, as well as a range of other assays (Knecht et al., 2013; Reif et al., 2015; Truong et al., 2014). However, the hydrocarbon structure and composition of the plastic plates, commonly used for cell-based and whole-animal model system testing (e.g. zebrafish embryos), can be problematic for test analytes with low water solubility and/or high hydrophobicity (Gellert and Stommel, 1999; Hirmann et al., 2007; Incardona et al., 2006; Jarema et al., 2015; Sonnack et al., 2015). The ability of plastics to sequester hydrophobic analytes from aqueous environments is advantageous in some instances, such as passive sampling technologies (Fries and Zarfl, 2012; García-Falcón et al., 2004; Kolahgar et al., 2002), and has been documented to occur with hydrophobic pollutants in the environment (Chandramouli et al., 2015; Rochman et al., 2013). However, use of plastic plates for the exposure of model systems (e.g., zebrafish embryos) to hydrophobic analytes in an aqueous media may result in the loss of these analytes from the exposure solution (Gellert and Stommel, 1999; Hirmann et al., 2007; Schreiber et al., 2008).

Attempts at modeling the sorption of hydrophobic analytes to either glass or plastic containers have often used $\log K_{ow}$, the octanol-water partition coefficient, or vapor pressure as predictive molecular characteristics, although with only moderate success (Riedl and Altenburger, 2007; Wolska et al., 2005). The conclusion from these studies is that properties such as lipophilicity or volatility are related to sorption, but cannot completely account for the sorptive losses observed (Riedl and Altenburger, 2007).

Subcooled liquid solubility, the water solubility for a hypothetical state of a subcooled liquid (Liu et al., 2013), has been used to model multi-component non-aqueous phase liquids (NAPLs), containing polycyclic aromatic hydrocarbons (PAHs), in ground water and for predicting sorption to laboratory glassware (Liu et al., 2013; Qian et al., 2011). Subcooled liquid solubility can be derived from model system or environmental properties (such as organic carbon or mineral content of the soil), but can also be calculated using thermodynamic properties for the analytes of interest (Liu et al., 2013; Peters et al., 1999, 1997). Previous modeling of chemicals in the environment, including sorption to soil and organic matter, places the focus of the model on properties of the environmental system, rather than the analytes of interest (Karickhoff et al., 1979; Su et al., 2006). However, no models currently exist for the prediction of sorptive losses during chemical exposures that utilize plastic plates.

In the event that a significant amount of analyte sorbs to the plastic plate, the available concentration to which the zebrafish (or other model system) is exposed would be reduced. This unaccounted-for error would then be propagated through any data analysis, and would result in inaccurate assessment of toxicity metrics such as the concentration at which half of

organisms show effects (EC_{50}). More accurate determination of analyte concentration to which the zebrafish are actually exposed would improve the translation of data from these high-throughput screening techniques to other systems, as well as lead to more accurate determination of potential health impacts as a result of exposure to environmental contaminants or mixtures.

The objective of this study was to determine the sorptive losses of PAHs and nitrated PAHs (NPAHs) to polystyrene 96-well plates (a common experimental format for zebrafish toxicity screening). This is the first study to compare sorptive losses in both the presence and absence of zebrafish embryos and at multiple exposure concentrations. We also compared two exposure protocols that differed in time between the addition of analytes and zebrafish embryos to the 96-well plates. The data derived herein was used to develop a predictive model that could be applied to structurally-related analytes to account for their sorptive losses to polystyrene plates.

2. Materials and Methods

2.1 Chemicals

Fluoranthene (FLA), pyrene (PYR), chrysene (CHR), benzo[a]pyrene (BaP), 3-nitrofluoranthene (3NF), 1,6-dinitropyrene (1,6DNP), and 6-nitrochrysene (6NC) were purchased from AccuStandard (New Haven, CT). 1-nitropyrene (1NP) and 6-nitrobenzo[a]pyrene (6NBaP) were purchased from Sigma-Aldrich (St. Louis, MO). All analytes were purchased as neat standards. Deuterated analytes, used as surrogates, (acenaphthene- d_{10} , fluoranthene- d_{10} , pyrene- d_{10} , benzo[a]pyrene- d_{12} , 2-nitrofluorene- d_9 , 9-nitroanthracene- d_9 , 3-nitrofluoranthene- d_9 , 1-nitropyrene- d_9 , 6-nitrochrysene- d_9) were purchased from CDN Isotopes (Point-Claire, Quebec, Canada) and Cambridge Isotope Laboratories (Andover, MA). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Ethyl acetate and hexane were purchased from Fisher Scientific (Santa Clara, CA). Flat-bottom 300 μ L total volume pre-sterilized Falcon® labware polystyrene tissue culture plates were purchased from VWR (Radnor, PA) and were not cleaned prior to use.

2.2 Zebrafish

All experiments were conducted with fertilized embryos according to Oregon State University Institutional Animal Care and Use Protocols. Embryos were collected following group spawning of adult wildtype 5D strain zebrafish as previously described (Reimers et al., 2006; Truong et al., 2010). Briefly, zebrafish embryos were dechorionated using an automated dechoriation system at approximately 4 hours post fertilization (hpf) and added to the 96-well plates via automation (Mandrell et al., 2012). For the pre-incubation protocol, the embryos were added to the plates manually. Embryos were evaluated for mortality at 24 hpf and 120 hpf to ensure adequate survival. Deceased embryos will disintegrate in the exposure media and would exhibit different partitioning behaviors than the live embryos.

2.3 Plate Exposure

The mass of individual PAH and NPAH standards were measured using an analytical balance (Mettler Toledo, Columbus, OH) and dissolved in anhydrous DMSO to create a stock solution of approximately 6 mM per analyte as a mixture. The DMSO solution was then diluted in E2 embryo media (the embryo media contained salts but no biologic materials, such as proteins) (Nusslein-Volhard and Dahm, 2002) to 10× the final exposure concentration as a mixture (in 6.4% DMSO by volume), and was utilized for the testing of both exposure procedures. The PAH and NPAH stock solution mixture and zebrafish embryos were added to the plates using our “Standard lab protocol” (Huang et al., 2010; Noyes et al., 2015; Truong et al., 2014) or a modified “Pre-incubation protocol” to investigate the degree of sorption which occurs under the test protocols:

“Standard lab protocol”: The 10× analyte mixture was added to plates that already contained embryo media (and, where appropriate, 6 hpf dechorionated zebrafish embryos) to obtain a final DMSO concentration of 0.64%.

“Pre-incubation protocol”: The 10× analyte mixture was added to the plates to obtain a DMSO concentration of 0.64%, the plates were wrapped in foil, and placed in the incubator for 24 hours, followed by addition of 6 hpf dechorionated zebrafish embryos (where appropriate).

The Pre-incubation protocol was investigated alongside the Standard lab protocol to investigate the reversibility of the sorption of the analytes to the polystyrene plates.

Final test concentrations of the individual PAHs and NPAHs were approximately 0.32 and 0.032 μM , termed “high” and “low” respectively, with 0.64% DMSO and a final liquid volume of 100 μL in each well. The nominal concentrations of each analyte in each exposure solution are given in Table S1. These concentrations were above the experimental water solubilities of chrysene and benzo[a]pyrene (by a factor of two and six, respectively) and were within the range of estimated water solubilities for the remaining PAHs and NPAHs (measured or estimated at 25°C) (US EPA, 2015). However, no visible precipitate formed during the exposures, likely because the water solubility was increased because DMSO was used at 0.64% with embryo media in the wells and the temperature was set at 28°C in the incubator. These test concentrations are also consistent with those used for zebrafish toxicity screening of PAHs and oxy-PAHs (Hawliczek et al., 2012; Knecht et al., 2013; Noyes et al., 2015). Plates were covered in Parafilm®, wrapped in aluminum foil, and placed in a dark incubator at 28 °C for five days. For both protocols and concentrations, four plates were exposed to the analytes in the presence of zebrafish embryos, and three plates were exposed to analytes in the absence of zebrafish embryos. Glass plates were also investigated initially; however, the resulting high sorptive losses led us to focus on the use of polystyrene plates (SI, Section 2).

2.4 Analyte Extraction from Exposure Plates

Each polystyrene 96-well plate contained one combination of exposure protocol, concentration, and presence or absence of zebrafish embryo making for a total of thirty-six 96-well polystyrene plates. Following the five-day exposure duration for both protocols,

plates were removed from the incubator. For each plate, the exposure solutions were removed from each of the 96-wells using glass pipettes and combined in a clean amber glass vial and archived.

Due to the solubility of polystyrene in other organic solvents, including acetone, ethyl acetate, and dichloromethane, we were restricted to the use of hexane for the plate rinses. A hexane rinse of 100 μ L was added to each of the individual 96-wells and allowed to sit in the dark for 20 min before the hexane rinse was removed from each of the 96-wells using glass pipettes and combined for each plate, for a total volume of 9.6 mL for each hexane rinse. Each plate was rinsed a total of three times and each rinse was stored and analyzed separately. One mL of each 9.6 mL hexane rinse was removed and surrogate standards were added, to account for analyte loss during sample preparation and to quantify the analyte. The extract was solvent exchanged into ethyl acetate and the volume reduced to 100 μ L. Internal standards were added to track surrogate recovery, and the extracts analyzed using gas chromatography coupled with mass spectrometry (GC/MS). Surrogate standards and internal standards were added so that the final concentration was 500 pg/ μ L.

2.5 Analyte Extraction from Zebrafish

After five days of exposure, live embryos were pooled and collected into microcentrifuge tubes ($n \approx 30$, three replicates) from each of four plates and frozen. Prior to extraction, excess exposure media was removed from the embryos, surrogate standards were added, and the embryos were extracted using liquid-liquid extraction into ethyl acetate as previously described (Goodale et al., 2013). The final extracts (250 μ L) were transferred into clean, amber glass vials, internal surrogate standards were added, and the extracts were analyzed by GC/MS. All of the analytes were detected below the limit of quantification in the zebrafish embryo extracts, therefore the amount of analyte sorbed and/or metabolized by the embryos was estimated as described in section 2.7.

2.6 GC/MS Analysis

Analysis of the hexane rinses and zebrafish extracts with GC/MS followed procedures previously described (Jariyasopit et al., 2014). Briefly, Agilent 6890 gas chromatographs coupled with Agilent 5973N mass spectrometers were operated in selected ion monitoring (SIM) and scan modes with ChemStation software (V. E.02.02.1431, Agilent Technologies). A 5%-phenyl-substituted methylpolysiloxane GC column (DB-5MS, 30 m $\times$ 0.25 mm I.D., 0.25 μ m film thickness, J&W Scientific, USA) was used for chromatographic separations. PAHs were analyzed in electron impact (EI) mode, whereas NPAHs were analyzed in negative chemical ionization (NCI) mode, with methane as the reagent gas and a programmed temperature vaporization (PTV) inlet (Gerstel, Germany). For further detailed information regarding the analytical analysis, QA/QC, ions monitored and estimated limits of detection (US EPA), see SI, Section 3.

2.7 Modeling and Statistics

We assumed that mass balance was achieved for individual PAHs and NPAHs within the individual wells of the 96-well plates (Figure 1) (Wolska et al., 2005). The physical-chemical properties used for the modeling in this study are listed in Table 1 and were

collected from the NIST web book (P.J. Lindstrom and W.G. Mallard, 2015) or estimated using the EPI Suite program (US EPA, 2015). Experimental values were used where available. However, in some cases, a lack of experimental data necessitated the use of estimated values. Subcooled liquid solubility was calculated as previously described (Mukherji et al., 1997; Peters et al., 1999) (see SI, Section 4).

Values for the Henry's Law Constants were generated using the HenryWin program in EPI Suite (US EPA, 2015), and were converted from the provided units ($\text{atm m}^3/\text{mol}$) to the dimensionless form (concentration in air/aqueous concentration), with values shown in Table 1. The dimensionless coefficients were used to estimate the percent of each analyte that was lost to the headspace of the wells (the volume of the wells occupied by air rather than aqueous exposure media) based on the ratio of aqueous volume to headspace volume.

$$mass_{\text{air}} = H_c * V_{\text{air}} * C_{\text{water, original}} \quad (\text{Equation 1})$$

The mass of analyte in the headspace is represented by $mass_{\text{air}}$, the Henry's Law constant by H_c , the volume of the headspace by V_{air} , and the original concentration of analyte in the water by $C_{\text{water, original}}$. The amount of analyte measured in the zebrafish embryos was below the limit of quantification. Therefore, we estimated the amount of each analyte sorbed and/or metabolized by the zebrafish embryos as the difference in the sorption in the presence and absence of zebrafish embryos:

$$mass_{\text{embryo}} = mass_{\text{sorption-embryo}} - mass_{\text{sorption+embryo}} \quad (\text{Equation 2})$$

The mass of analyte sorbed and/or metabolized by the embryos is represented by $mass_{\text{embryo}}$, the mass sorbed to the walls of the plate in the absence of embryos by $mass_{\text{sorption-embryo}}$, and the mass sorbed to the walls of the plate by $mass_{\text{sorption+embryo}}$. Because the analyte concentration in the aqueous exposure solutions were not measured, the analyte mass that was not accounted for by either volatilization (Equation 1), sorption to the polystyrene walls of the plate (measured), and sorption and/or metabolism by the zebrafish embryos (Equation 2) was assumed to have remained in the aqueous exposure solution:

$$mass_{\text{total}} = mass_{\text{polystyrene}} + mass_{\text{water}} + mass_{\text{embryo}} + mass_{\text{headspace}} \quad (\text{Equation 3})$$

The total mass added to the system is represented by $mass_{\text{total}}$, the mass sorbed to the polystyrene plate by $mass_{\text{polystyrene}}$, the mass of analyte remaining in the aqueous solution as $mass_{\text{water}}$, the mass of analyte sorbed and/or metabolized by the embryos as $mass_{\text{embryo}}$, and the mass of analyte in the headspace as $mass_{\text{headspace}}$. We defined a mass distribution ratio, D_{pw} (Ni et al., 2009), as the ratio of the mass of analyte sorbed to the walls of the polystyrene plate ($mass_{\text{polystyrene}}$) to the mass of analyte that was elsewhere in the system ($mass_{\text{system}}$):

$$D_{pw} = \frac{mass_{polystyrene}}{mass_{system}} \quad (\text{Equation 4})$$

Student's t-tests were used to compare analyte concentrations and exposure scenarios, with a significance cut-off of $p < 0.05$. Additionally, linear regressions between D_{pw} values and various chemical properties ($\log K_{ow}$, molecular weight, and subcooled liquid solubility) were performed using SigmaPlot 12.3.

Percent sorption was calculated as the mass of analyte sorbed to the polystyrene ($mass_{polystyrene}$) divided by the total mass dosed to the well ($mass_{total}$) multiplied by 100:

$$\% \text{ sorption} = \frac{mass_{polystyrene}}{mass_{total}} * 100 \quad (\text{Equation 5})$$

Similarly, the percent distribution in the other compartments of the system were calculated as the mass of analyte in a given compartment divided by the total mass dosed to the plate, multiplied by 100.

3. Results

3.1 Comparison of Exposure Protocols

We successfully quantified sorptive losses of all PAHs and NPAHs to the polystyrene 96-well plates using both the Standard lab protocol and the Pre-incubation protocol. For most PAHs and NPAHs, no statistically significant difference in percent sorption ($p < 0.05$) was observed between the two protocols at either of the exposure concentrations (Figure 2). The only compounds which did have a statistically significant difference were FLA and PYR at the higher concentration in the absence of zebrafish embryos, and 3NF and 1NP at the lower exposure concentration in the presence of zebrafish embryos. In the presence of zebrafish, the mean percent sorption for individual PAHs using the Standard lab protocol ranged from 1.6 to 39% and from 1.5 to 41% using the Pre-incubation protocols, while NPAHs ranged from 6.1 to 32% and 11 to 44% using the Standard lab and Pre-incubation protocols, respectively (Figure 2). In the absence of zebrafish, the mean percent sorption for individual PAHs using the Standard lab protocol ranged from 5.2 to 63% and from 4.8 to 70% using the Pre-incubation protocols, while NPAHs ranged from 28 to 69% and 28 to 83% using the Standard lab and Pre-incubation protocols, respectively (Figure 2). Because there was no statistically significant difference between the two protocols with or without zebrafish embryos, the data for the two protocols for a given concentration and presence/absence of fish were combined.

3.2 Comparison of Exposure Concentrations

Figure 3 illustrates that a statistically significant higher percent sorption was observed for most PAHs and NPAHs under the lower exposure concentration (0.032 μM) compared to the higher exposure concentration (0.32 μM). The sorption at the lower exposure concentration,

compared to the higher exposure concentration, was 7.9% higher for PAHs and 40% higher for NPAHs in the absence of zebrafish embryos, and 2.1% higher for PAHs and 9.6% higher for NPAHs in the presence of zebrafish embryos. However, the mass of each analyte sorbed at the lower exposure concentration was less than the mass sorbed at the higher exposure concentration.

3.3 Influence of Zebrafish Embryos

Figure 3 shows that the presence of zebrafish embryos significantly reduced the sorption of PAHs and NPAHs to the polystyrene plates at both exposure concentrations. The mean percent sorption of the PAHs and NPAHs was reduced by 9.6% and 25%, respectively, at the high exposure concentration and by 15% and 58%, respectively, at the lower exposure concentration. While the zebrafish embryos were collected and extracted, the amount of analyte present was below the limit of quantification, likely due to metabolism (Djomo et al., 1996).

3.4 Mass Distributions and Predictive Modeling

The PAHs and NPAHs were assumed to have reached equilibrium within each of the 96-wells, allowing us to calculate the mass balance distribution of our analytes within each of the various compartments shown in Figure 1. Table 2 shows this estimated distribution. As mentioned above, the measured percent sorption to the polystyrene wells ranged from 4.8 to 39% at the high exposure concentration, and 1.5 to 43% at the low exposure concentration. Based on the Henry's Law Constants and equation 1, less than 0.1% of each analyte was present in the headspace above the aqueous media. Based on the difference in the sorption in the presence and absence of zebrafish embryos, the percent of each analyte sorbed and/or metabolized by the zebrafish embryos (equation 2) ranged from 0.14% (FLA at the high exposure concentration) to 77% (1,6DNP at the low exposure concentration). The remainder of analyte not accounted for by either sorption to the walls of the plate, volatilization to the headspace, and sorption and/or metabolism by the zebrafish embryos was assumed to have remained in the aqueous exposure media (equation 3). From the amount (grams) of analyte sorbed to the plates and the amount remaining in the aqueous exposure solution, we calculated values of D_{pw} (equation 4). For PAHs, D_{pw} ranged from 0.05 to 0.8 and 0.016 to 1.2 under the high and low exposure concentrations, respectively. For the NPAHs, D_{pw} values ranged from 0.22 to 0.39 and 0.69 to 2.4 under the high and low exposure concentrations, respectively. While the mass of analyte sorbed to the polystyrene was measured directly, the concentration remaining in the aqueous media was estimated. This, along with the potential error associated with our estimated losses due to volatilization, as well as our assumption of sorption to and metabolism by the zebrafish embryos, must be acknowledged.

Figure 4 shows linear regressions between D_{pw} and $\log K_{ow}$, the subcooled liquid solubility, and molecular weight. Only subcooled liquid solubility was a good predictor of D_{pw} for PAHs at both high and low concentrations and none of these properties were good predictors of D_{pw} for NPAHs (Table 3).

4. Discussion

We determined that sorption to polystyrene 96-well plates during toxicity testing can be significant (1.5–91%), which is consistent with previous studies in 96-well polystyrene plates (Hirrmann et al., 2007) or in glass jars. (Wolska et al., 2005). While some analytes exhibited low sorption (e.g., FLA and PYR, sorption less than 5%), other analytes consistently showed sorption exceeding 40% (e.g., all NPAHs). Previous work by Wolska et al. with glass exposure vessels demonstrated that lower molecular weight PAHs exhibit lower sorption than higher molecular weight PAHs (Wolska et al., 2005). For example, the 2- and 3-ring PAHs and pyrene showed sorptive losses of 10% or less, whereas PAHs with 4 or more rings had sorptive losses of 40 to 70% (Wolska et al., 2005). We observed a similar pattern with the polystyrene plates, where FLA and PYR exhibited sorptive losses below 10%, while CHR, BaP, and all the NPAHs had much greater sorptive losses (up to 91%).

Diffusion into the bulk of the polystyrene, in addition to sorption on the polystyrene surface, is also possible (Schreiber et al., 2008). However, due to the structure of polystyrene as a rigid, glassy polymer with minimal void space between the polymer chains (George and Thomas, 2001), the relatively large size of the PAHs and NPAHs used in this study, and the relatively short time course of the exposure (Hopfenberg, 1978), we focused on sorptive losses to the surface of the polystyrene, rather than on diffusion into the bulk of the plastic.

We expected sorption to treated borosilicate glassware to be lower than sorption to polystyrene. This is based on previous studies, where sorption to glassware accounted for less than 10% of the total analyte. Using phenanthrene as a model analyte, Schreiber et al. noted that sorptive losses were relatively small when glass exposure vessels were used ($1.8 \pm 1.6\%$), but were much greater when polystyrene plates were used ($94 \pm 1.2\%$). Our initial experiments with 96-well glass exposure plates were abandoned because of prohibitively high sorptive losses (0.096–94%) (SI, Section 1). This was likely because the roughened surface inside the wells of the glass plates contributed to the enhanced sorptive losses due to increased surface area. Unfortunately, we were unable to source a suitable glass 96-well plate option with a smoothed surface, which would have allowed for more direct comparisons of PAH and NPAH sorptive losses to polystyrene 96-well plates. In addition, the cost of the 96-well glass plates exceeded the cost of the 96-well polystyrene plates by approximately 60 times, and the glass plates would require thorough cleaning between uses to ensure against contamination (in contrast, the polystyrene plates are disposable after a single use).

4.1 Comparison of Exposure Protocols

The majority of analytes did not have a statistically significant difference in percent sorption between the two exposure protocols (Figure 2). This indicates that sorption to the walls of the wells occurs on a fairly rapid timeframe (i.e., less than five days). This supports our assumption that equilibrium was reached within the sealed wells of the 96-well plate. The results obtained indicate that PAH and NPAH sorption to polystyrene 96-well plates is a reversible process, most likely due to adsorption on the surface of the polystyrene, rather than partitioning into the bulk polystyrene (Schreiber et al., 2008).

4.2 Comparison of Exposure Concentrations

The higher measured percent sorption at the lower exposure concentration indicates that there is potential for saturation of the polystyrene at higher analyte concentrations. It is also likely that the percent sorption would be higher for even lower exposure concentrations ($<0.032 \mu\text{M}$).

In developmental zebrafish toxicity studies, it is typical for analytes exhibiting high toxicity to be further tested at lower exposure concentrations (Knecht et al., 2013; Noyes et al., 2015). Thus, where toxic effects are observed in other studies at these very low concentrations, accounting for sorptive losses in excess of 50% is particularly important. We selected two relatively low exposure concentrations (0.32 and $0.032 \mu\text{M}$) because they were within the range of concentrations usually evaluated in the developmental embryonic zebrafish model (Knecht et al., 2013; Noyes et al., 2015). In order to attempt to extract the embryos at 120 hpf, it was necessary for the majority of the embryos to survive the full duration of the exposure, because deceased embryos will disintegrate in the exposure media and would exhibit different partitioning behaviors than the live embryos.

4.3 Influence of Zebrafish Embryos

The percent sorption was significantly lower for both PAHs and NPAHs under both exposure concentrations when zebrafish embryos were present in the polystyrene plates. Embryonic zebrafish have a high lipid content and would provide an environment favorable for the partitioning of hydrophobic analytes, such as PAHs and NPAHs, out of the aqueous exposure solution and onto the surface or into the body of the embryo, rather than onto the polystyrene plate.

While adequate survival was achieved during these experiments ($>90\%$), the concentration of analytes in the embryos was below our limit of quantification, so we were unable to directly measure the body burden. Development of the liver begins at around 48 hpf; thus, metabolism is also possible during the latter half of the five-day exposure. It is likely that the embryos metabolized the analytes following development of the liver at 48 hpf (Ober et al., 2003), leaving little of the original analytes in the zebrafish embryo. Metabolism of PAHs and NPAHs also likely contributed to the observed decrease in sorptive losses to the polystyrene plates, in the presence of zebrafish embryos. Metabolism would decrease the aqueous analyte concentration, which would impact the partitioning of the analyte between the polystyrene and aqueous media.

4.4 Mass Distributions and Predictive Modeling

We also estimated the losses of these analytes to the headspace in the sealed 96-well plates using estimated Henry's Law Constants, as volatilization is another potential source of analyte loss, and approximately two-thirds of the volume of each well of the polystyrene plate was occupied with air rather than aqueous media. Although losses due to volatilization were relatively small, less than 0.1% (Table 2), they are likely to be more significant for analytes with lower molecular weights or higher vapor pressure (Riedl and Altenburger, 2007).

Linear regressions between D_{pw} and $\log K_{ow}$, molecular weight, and the subcooled liquid solubility indicated that the subcooled liquid water solubility was a good predictor of D_{pw} for PAHs, but that none of those properties were useful predictors of D_{pw} for NPAHs (Figure 4). Parameters of statistically significant ($p < 0.05$) models are shown in Table 3. We propose several possible explanations for the lack of statistically significant correlations for the NPAHs: 1.) The lack of experimental data for NPAH physical/chemical properties inhibited the modeling capabilities, because experimental $\log K_{ow}$ values were not available for most NPAHs. Additionally, estimated values for water solubility were used in the calculations of the subcooled liquid solubility (SI, Section 2); 2.) While evidence from the literature (Wolska et al., 2005) suggests that PAHs reach equilibrium within five days, NPAHs are less well-studied and equilibrium between the polystyrene 96-well plate, zebrafish embryos, and aqueous exposure solution may require more than five days to fully establish; and 3.) We speculate NPAHs may have a different mechanism of sorption to polystyrene than PAHs due to the nature of the nitro functional group.

5. Conclusions

If multi-well plates made of polystyrene or other plastics are to continue being used as exposure vessels during toxicity screening (because of their low cost and ease-of-use), losses of hydrophobic analytes to the plastic need to be accounted for to more accurately characterize the concentration of analyte remaining in the exposure solution, for not only zebrafish toxicity testing, but for any test system, such as cell culture, which also utilizes polystyrene plates for the testing of hydrophobic environmental pollutants. One potential option currently under investigation involves the use of passive dosing to maintain the exposure concentration for the duration of the experiment, although analyte losses using phenanthrene as a model analyte are still reported to occur (Mayer et al., 1999; Smith et al., 2009; Vergauwen et al., 2015). Group exposures in glass petri dishes or tanks can also be conducted; however, this method does not allow for tracking the development of a single animal over time (Jin et al., 2015; Massarsky et al., 2015; Oliveri et al., 2015). Other potential methods for reduction of sorptive losses include pre-treatment of the plastic plates (Sonnack et al., 2015), or the use of renewal (Padilla et al., 2012; Stanley et al., 2009), rather than non-renewal assay design. However, the use of renewal systems would be costly, both from a human labor and chemical use perspective. Additionally, previous toxicity screening results for hydrophobic analytes and environmental samples may need to be re-examined with the knowledge that upwards of 50% of the analyte originally introduced to the exposure solution may have sorbed to the plastic and were no longer available to the test system. Previous experiments comparing the toxicity of hydrophobic analytes, such as PAHs, in glass and plastic exposure vessels have demonstrated that plastic exposure vessels led to an underestimation of toxicity in bacterial test systems (Gellert and Stommel, 1999; Hirmann et al., 2007). Loss of analyte due to sorption and the resulting decrease in exposure concentration would impact data analysis, as the true exposure concentrations could, in some cases, be less than half of what was added to the assay. This would have implications for downstream data analysis and determination of toxicity indicator values, such as EC_{50} , which could be used in risk assessment applications. In cases where significant sorption occurs, values such as the EC_{50} calculated based on the theoretical exposure concentration

would be higher than the true value, based on the sorption-corrected exposure concentrations, and would therefore under-estimate toxicity. This work highlights the need for further research into this ongoing research challenge.

Supplementary Material

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Figure 1.

Schematic of a single well within a sealed 96-well polystyrene plate, showing potential partitioning of a model compound (1NP) within the system. Following addition of the embryos and analyte, the wells are sealed using Parafilm® to prevent evaporative loss. Volumes of the aqueous exposure media and headspace are drawn to scale.

Figure 2.

Comparison of the Standard lab protocol and Pre-incubation protocol, at both exposure concentrations (high concentration $\approx 0.32 \mu\text{M}$, low concentration $\approx 0.032 \mu\text{M}$) in the presence and absence of zebrafish embryos for PAHs (A–D) and NPAHs (E–H) Shown as mean $\pm$ standard error. * indicates statistical significance, $p < 0.05$; $n=4$ with fish present, $n=3$ without fish present.

Figure 3.

Mean $\pm$ SE percent sorption measured for PAHs (A) and NPAHs (B) at both the high (0.32 μ M) and low (0.032 μ M) exposure concentrations. Letters indicate statistically significant difference ($p < 0.05$) against high concentration with fish (a), high concentration without fish (b), and low concentration with fish (c). $n=8$ with fish, $n=6$ without fish.

Figure 4.

Linear regressions between $\log K_{ow}$ (A, D), subcooled liquid water solubility (B, E), and molecular weight (C, F) against calculated D_{pw} values, for both PAHs (A–C) and NPAHs (D–F). Parameters of statistically significant ($p < 0.05$) models (indicated by *) are shown in Table 3.

Table 1

Abbreviations and physical-chemical properties of PAHs and NPAHs used in this study.

Analyte Name	Abbreviation	Molecular weight (g/mol)	log K_{ow}	Subcooled liquid solubility (mg/L)	Henry's Law Constant (H_C) (dimensionless)
Fluoranthene	FLA	202.3	5.16	1.4	3.4×10^{-4}
3-nitrofluoranthene	3NF	247.1	4.8*	1.1	1.3×10^{-5}
Pyrene	PYR	202.1	4.88	1.1	3.4×10^{-4}
1-nitropyrene	1NP	247.1	4.8*	0.36	1.3×10^{-6}
1,6-dinitropyrene	1,6DNP	292.3	4.6*	1.5	5.3×10^{-9}
Chrysene	CHR	228.3	5.81	0.20	2.1×10^{-4}
6-nitrochrysene	6NC	273.3	5.3*	1.3	8.1×10^{-7}
Benzo[a]pyrene	BaP	252.1	6.13	0.018	3.3×10^{-5}
6-nitrobenzo[a]pyrene	6NBaP	297.3	5.9*	0.75	1.3×10^{-7}

Log K_{ow} values are experimental (from the EPI Suite database), except where indicated by

* (estimated values were generated using EPI Suite software). Subcooled liquid solubility was calculated using a method previously described (SI, Section 4). Henry's Law Constants were estimated using the EPI Suite software.

Table 2

Estimated percent, mass (μg), and D_{pw} for each analyte in a well (Figure 1).

Analyte	% walls (μg)	% embryos (μg)	% headspace (μg)	% water (μg)	D_{pw}
High exposure concentration	FLA	5.0 (57)	0.14 (1.6)	7.1×10^{-4} (0.008)	95 1090
	3NF	12 (76)	35 (210)	2.7×10^{-3} (0.016)	53 (330)
	PYR	4.8 (67)	0.45 (6.3)	7.1×10^{-4} (0.01)	95 (1340)
	INP	14 (110)	30 (250)	2.7×10^{-4} (0.002)	56 (460)
	1,6DNP	13 (79)	31 (190)	1.1×10^{-6} (6.5×10^{-6})	56 (340)
	CHR	27 (160)	24 (150)	4.3×10^{-4} (0.003)	49 (290)
	6NC	23 (140)	19 (120)	1.6×10^{-4} (0.001)	58 (360)
	BaP	39 (220)	13 (75)	6.9×10^{-5} (0.0003)	48 (270)
Low exposure concentration	6NBaP	16 (61)	12 (45)	2.6×10^{-5} (1×10^{-4})	72 (280)
	FLA	1.5 (1.8)	3.6 (4.1)	7.1×10^{-4} (0.001)	95 (110)
	3NF	17 (11)	59 (36)	2.9×10^{-3} (0.002)	24 (15)
	PYR	3.4 (4.9)	4.5 (6.4)	7.1×10^{-4} (0.001)	92 (130)
	INP	23 (19)	57 (46)	2.7×10^{-4} (0.0002)	21 (17)
	1,6DNP	14 (8.6)	77 (47)	1.1×10^{-6} (6.5×10^{-7})	9.0 (5.5)
	CHR	39 (24)	29 (17)	4.3×10^{-4} (0.0002)	32 (19)
	6NC	43 (26)	39 (26)	1.6×10^{-4} (1×10^{-4})	18 (11)

	9NBaP	18 (6.9)	56 (6.9)	2.6×10^{-5} (1×10^{-5})	26 (9.9)	0.69
	BaP	39 (22)	25 (22)	6.9×10^{-5} (4×10^{-5})	36 (20)	1.1
	Analyte	% walls (µg)	% embryos (µg)	% headspace (µg)	% water (µg)	D_{pw}

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Table 3

Parameters for statistically significant ($p < 0.05$) models investigated for PAH (Figure 4). The y-intercept is indicated by y_0 , the independent variable by x , and the slope of the linear fit by a , $D_{pw} = y_0 + ax$. No models for NPAHs were statistically significant.

PAH Exposure concentration	y_0	a	x	p
High (0.32 μM)	0.73	-0.54	Subcooled liquid solubility	0.032
	-3.0	0.015	Molecular weight	0.011
Low (0.032 μM)	1.2	-0.95	Subcooled liquid solubility	0.035