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Rapid determination of polycyclic aromatic hydrocarbons (PAHs) in zebrafish eleutheroembryos as a model for the evaluation of PAH bioconcentration

S. El-Amrani [a], J. Sanz-Landaluze [a,*], J. Guinea [b], C. Cámara [a,**]

[a] Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, Ciudad Universitaria, 28040 Madrid, Spain
[b] Zf BioLabs, Ronda de Valdecarrizo 41° B. 28760, Tres Cantos, Madrid, Spain

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ABSTRACT

A simple and fast approach for a novel bioaccumulation test by exposing zebrafish eleutheroembryos to fluorene and anthracene (PAHs) at two concentration levels below 1% of their LC_{50} is presented. This alternative protocol sets an uptake period of 48 h at 26 °C for bioconcentration of the compounds tested and an additional 24 h for depuration. Two different methods to estimate bioconcentration factors (BCFs) of PAHs were used. The first consists in the ratio between concentrations of the tested compounds in the zebrafish eleutheroembryos and the exposure media when a steady state during exposure is reached (BCF_{ss}). The second employs the concentration–time profile with the use of a least-square fit to a non-linear model (BCF_k). A steady state (ss) was reached after 12 h and 22 h for fluorene and anthracene exposure, respectively. Uptake and depuration rate constants obtained were similar for the two exposure levels tested, hence toxicokinetic rate constants appeared to be independent of the exposure level. Bioconcentration factors of 1164 and 817 for fluorene exposure at 0.99 ng mL^{-1} and 7.9 ng mL^{-1} and of 2089 and 2344 for anthracene at 0.63 ng mL^{-1} and 6.1 ng mL^{-1} were assessed. The good agreement of the obtained results with those reported in the literature proves the feasibility of the proposed method for estimation of both toxicokinetic parameters and bioconcentration factors. Furthermore, this protocol, has potential to be an alternative to the Organisation for Economic Cooperation and Development (OECD) 305 method, considerably reducing time and associated costs of the test suggested by European legislation.

1. Introduction

European Union (EU) strives to protect human health and the environment through the REACH regulation, aiming accurate identification of toxicity, persistency and bioaccumulation of substances that are produced and consumed above certain limits [1,2]. Bioaccumulation, defined as the accumulation of chemicals in an organism through any route, including breathing, ingestion or direct contact, is one of the most important properties to determine the potential of danger to the environment. Unfortunately determination of bioaccumulation is complicated, and bioconcentration parameter, without intake through feeding, is most widely determined. The OECD bioconcentration test 305 [3] is the most commonly used protocol to establish bioconcentration data and is also recommended by REACH's test methods regulation [4]. Briefly, this test evaluates the accumulation of a dissolved chemical in adult fish by measuring its final concentration in both, the fish and the surrounding media after a constant response is reached (steady state). This complex method is time consuming and expensive, requiring exposure of a large number of adult fish to the compounds to be tested up to 60 days. European REACH legislation also proposes to replace animal testing wherever possible with animal free approaches (use of cell lines, microorganisms, embryos, Quantitative Structure Activity Relationship (QSAR) approximations, etc.). Thus, the development of alternative methods to establish the bioconcentration factor (BCF) of a given chemical without using adult animals and reducing the cost of the assay is mandatory.

The use of embryos and eleutheroembryos of fishes until they become free-feeding larvae are legally considered to be in vitro systems and are frequently used as alternatives to acute fish toxicity tests and other applications [5]. Zebrafish, a small tropical fish native to the rivers of India and South Asia, is an animal of great scientific interest due to its advantageous features over other vertebrate model systems [6]. The small size of larvae and adult zebrafish lower the test cost; transparent embryos allow detection of morphologic and embryonic changes and a high production and fast embryonic development facilitates fast bioaccumulation kinetics. Combined with the high genomic homology

* Corresponding author. Tel.: +3491 3944368; fax: +3491 3944329.
** Corresponding author. Tel.: +3491 3944318; fax: +3491 3944329.
E-mail addresses: jsanzlan@quim.ucm.es (J. Sanz-Landaluze), ccamara@quim.ucm.es (C. Cámara).

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with humans (over 80%), the characteristics of this fish make them ideal for laboratory use. In 2008, a workshop of experts from government, industry and academia established some key criteria for judging the reliability of alternative bioaccumulation studies, in particular (1) clear specification of test substances and investigated fish species, (2) analysis of test substances in both fish tissue and exposure medium, (3) no significant adverse effects on exposed test fish, and (4) a reported BCF test reflecting steady state conditions [7]. The quantification of chemicals in fish embryos or eleutheroembryos for BCF determination requires, even at high concentration levels, the use of high sensitive analytical methods, due to the extremely small sample size.

Knowledge of the behavior of hydrophobic organic contaminants, especially polycyclic aromatic hydrocarbons (PAHs), has significantly increased over the last decades [8]. However PAHs are ubiquitously distributed in the environment [9] and pose significant risks to the environment, organisms and human health because of their persistence and hydrophobic characteristics [10]. PAHs can be accumulated in aquatic organisms and then be transferred through the food chain [11–13]. Bioaccumulation of PAHs may cause genetic mutation in somatic cells, which eventually leads to the development of cancer [14]. The United States Environmental Protection Agency (USEPA) has categorized 16 priority PAHs for monitoring based upon their toxicity, mutagenic and carcinogenic potential. When lipophilic PAHs are accumulated by fish, some of them can affect the development, immune system, reproduction, growth, or even the survival of these organisms [15,16]. PAHs can either directly be accumulated into the body or enzymatically converted into more hydrophilic species. The resultant metabolites are usually more soluble in water and can therefore be excreted from the body [17]. However, biotranformation does not always lead to desintoxication, and the xenobiotics may also be transformed into even more toxic species than the parent compounds [18].

In this work, the OECD 305 protocol has been adapted to zebrafish eleutheroembryos, which were exposed to concentrations below 1% of their LC_{50} during a cycle of bioaccumulation and depuration experiments over 72 h. The exposure time for bioaccumulation was set to 48 h, followed by a depuration phase (exposure to a non-contaminated media) of 24 h. Samples of eleutheroembryo and surrounding media were taken at different stages which were then analyzed prior extraction by ultrasonication using acetonitrile as extractant, followed by a simple step of filtration and separation and quantification by high-performance liquid chromatography (HPLC) coupled with a fluorescence detector (FL). The aim of the present study was to develop a simple analytical method for determination of the bioconcentration factor (BCF) and toxicokinetics constants (uptake and depuration) for two priority PAHs (anthracene and fluorene) in zebrafish eleutheroembryos. The developed protocol may be used within an integrative testing strategy for BCF assessment of chemicals prior to the use of the OECD test guideline 305.

2. Experimental

2.1. Reagent and samples

Anthracene (purity 99.5%, CAS number: 120-12-7) and fluorene (purity 99.0%, CAS number: 86-73-7) were obtained from Dr. Ehrenstorfer GmbH (Germany). All solvents and reagents used were HPLC grade. Acetonitrile was obtained from LAB-SCAN (HPLC ultra gradient grade, Barcelona, Spain). Stock solutions of PAHs were stored in the dark (1–5 °C) and intermediate dilutions ($\sim$1000 ng mL^{-1}) were prepared weekly by appropriate dilution in acetonitrile. These solutions were used as calibrants, following the external calibration method (0–25 ng mL^{-1}).

Exposed zebrafish eleutheroembryos and their corresponding exposure media as well as control zebrafish were provided by ZF Biolabs (Madrid, Spain). All solutions were prepared using high-purity water with a resistivity of 18.0 M Ω cm, obtained from a Millipore ZMFQ 23004 Milli-Q water system (Bedford, MA, USA).

2.2. Instrument and apparatus

PAHs determination was carried out by liquid chromatography HPLC-system (P-4000, Thermo Electron Corporation, San Jose, USA) coupled to a fluorescence detector (model FL-3000). Separation was carried out on a 20 cm Hypersyl column (Thermo Electron Corporation), with 3 μm particle size. The mobile phase was made up of acetonitrile and water (starting at a ratio of 75:25 and finishing at 90:10) and the flow rate varied from 0.6 to 0.8 mL min^{-1}. The chromatographic column temperature was set to 30 °C. The fluorescence wavelengths used were 250 and 276 nm for excitation and 380 and 340 nm for emission, for anthracene and fluorene respectively. As BCFs should be individually determined for each compound, in an initial approach experiments for each PAHs tested were performed separately.

A Vibra cell VC × 130 ultrasonic processor (Connecticut, USA), equipped with a 3 mm diameter titanium microtip and fitted with a high-frequency generator of 130 W at 20 kHz was used to leach the PAHs from larvae in deionized water. Centrifugation was carried out in a Combi-Spin FVL-2400N centrifuge (Boeco, Germany).

2.3. Bioaccumulation experiments

An exposure media with a composition similar to that of fresh river water was prepared as follows: 16 mL of concentrated solution containing 2.9 g of $CaCl_2$, 17.2 g of NaCl, 0.76 g of KCl and 4.9 g of $MgSO_4$ per litre were diluted to 1 L with distilled water. According to the OECD 305 guideline [3], exposure conditions were as follows: dissolved oxygen $\geq$60%, 26 °C and pH 6–8.5 (before and after media renewal). About 1000 zebrafish eleutheroembryos (corresponding to the embryo since hatching until the phase of free swimming and own food uptake, about 120 h post fertilization (hpf) at 28.5 °C and 139.5 hpf at 26 °C [19,20]) were obtained at 72 hpf, just when the embryos hatched. Experiments were individually performed in three tanks (each one of 1 L of total volume): one for control (with non-contaminated media) and two containing the different concentrations of each compound to be tested. The exposure media for the bioaccumulation test was renewed after 24 h and each experiment was performed with only one PAH, as established by the OECD 305 test guideline.

The test consisted of two phases: absorption (48 h in contaminated exposure media) and depuration (24 h after replacing the exposure media by a clean, non-contaminated media). About 15–25 larvae were sampled from the tanks at different exposure times (0, 2, 4, 6, 21, 24, 45, 48, 50, 54 and 72 h) for uptake experiments to determine the concentration of the analyte absorbed and accumulated. According to the OECD 305 test, the loading rate of larvae at the beginning of the experiments ranged between 0.7 and 0.8 mg mL^{-1} (wet weight) and the mortality of larvae was lower than 20% at the end of the test. The highest nominal concentrations of the test substances (indicated in the Test OECD 305) has to be 1% of LC_{50} value and ten times lower for the second exposure concentration (whenever its quantification is possible). LC_{50} values established at 48 h, obtained from METI-NITE Japan database [21] in experiments with ricefish (*Oryzias latipes*), for anthracene and fluorene were 210 and 51.5 μg mL^{-1},

respectively. Thus, the nominal concentrations chosen to carry out the bioconcentration tests were 1.5 and 15 ng mL^{-1} for anthracene and 2 and 20 ng mL^{-1} for fluorene, considerably lower than a 1% of the LC_{50} values. Exposure solutions were changed every 24 h to fulfill the OECD 305 requirement, avoiding a fluctuation of the nominal concentration of the chemicals higher than 20% throughout the whole experiment. Subsequently, the exposure media was replaced by a clean, non-contaminated exposure media and larvae were sampled at different times during 24 h for depuration experiments.

2.4. Analysis of PAHs in exposure medium and larvae

2.4.1. Exposure solution

Three replicates of exposure medium were collected at different exposition and depuration times (0, 2, 4, 6, 12, 24, 36, 48, 52, 54, and 72 h). Volumes of 500 μL were mixed immediately with 1 mL of acetonitrile, shaken vigorously, filtered through 0.22 μm PTFE syringe filters and finally measured with HPLC-FL, using the conditions summarized in the Instrumental Section. Samples were previously spiked with 40 μL of a 500 ng mL^{-1} solution of fluorene or anthracene as internal standard.

2.4.2. Eleutheroembryos

The analytical methodology used for PAH determination in zebrafish eleutheroembryos was adapted from a previously developed methodology used for PAHs and PCBs [22,23]. Eleutheroembryos, in groups of 20, with an overall wet weight of approximately 13.3 mg, were treated with 500 μL of acetonitrile and sonicated during 40 s (using the ultrasound probe at 130 W and 20 kHz with an amplitude of 40%). Extracts were filtered through Millipore Millex- HV filters (0.22 um, Scharlab, Spain) into amber flasks and 20 μL of the extracts were injected into the chromatographic system. Acetonitrile–water mixtures were used as mobile phase at a flow-rate varying from 0.6 to 0.8 mL min^{-1}. The above specified experimental conditions were used for analysis.

2.5. Analytical data

No matrix effects, evaluated by comparing external calibration curves and standard addition methodology, were observed, hence PAH determination was carried out using a linear regression of the peak area of an external calibration with 6–9 concentration points (1–30 ng mL^{-1}) corrected with the internal standard (IS). The IS (fluorene or anthracene) was added to both the calibrants and samples (surrounding media and larvae) just after sampling. Linearity was always above $r=0.995$. Limits of detection (LOD) and quantification (LOQ) were calculated as a signal to noise ratio of 3 and 10, respectively, and from blank samples spiked with very small amount of the compound to be tested in the corresponding matrix matched sample. Analytical LODs calculated for the complete analytical method were 3 ng g^{-1} for eleutheroembryos and 0.5 ng mL^{-1} for the exposure medium.

Precision was determined using extracts obtained from matrix matched samples spiked with the corresponding PAH at different levels. Run-to-run reproducibility was evaluated by injecting six matrix matched extracts ($n=6$) within the same day, while day-to-day precision was evaluated by injecting six samples over three different days ($n=18$) and by means of the corresponding calibration graph. Relative recoveries were determined by triplicate from blank samples spiked at two different concentration levels. Recoveries of $102 \pm 2\%$ ($n=6$) and $96 \pm 4\%$ ($n=6$) for anthracene and fluorene respectively were obtained in the exposure media. The recoveries for larvae samples were $98 \pm 2\%$ ($n=6$) and $112 \pm 12\%$ ($n=6$) for anthracene and fluorene respectively.

2.6. Toxicokinetic model equations

A first-order, one-compartment bioconcentration model was used to describe uptake and elimination rates of PAHs from exposure solution and larvae [24,25]. The PAHs are absorbed by larvae at a rate characterized by the uptake rate constant (k_1) (during the uptake phase) and the elimination rate constant (k_2), as expressed in Eq. (1). Similarly, the depuration equation describes the loss of the chemicals from the larvae during depuration:

$$\frac{dC_f}{dt} = k_1 C_w - k_2 C_f \quad \text{(uptake)} \qquad \frac{dC_f}{dt} = -k_2 C_f \quad \text{(depuration)} \tag{1}$$

where C_f is the PAH concentration in fish (in ng g^{-1}, wet weight), t is the exposure time (h), k_1 is the uptake constant (L/kg dry weight/h), C_w is the concentration of the compound in the exposure media (ng mL^{-1}), and k_2 is the first order elimination rate constant (h^{-1}). Assuming that at $t=0$, the concentration of the test substance in the organism is zero and that the PAH concentration in the exposure media is constant, Eq. (2) is obtained:

$$C_f = \frac{k_1}{k_2} C_w \left(1 - e^{-k_2 t}\right) \quad \text{(uptake)} \qquad C_f = C_{f,0} e^{-k_2 t} \quad \text{(depuration)} \tag{2}$$

where $C_{f,0}$ is the PAH concentration in the organism when the depuration phase begins. k_1 and k_2 values can be determined if the experimental concentration values obtained in the bioconcentration test fit to this equation. When the equilibrium is reached (steady-state), Eq. (2) may be simplified to Eq. (3):

$$C_f / C_w = \mathrm{BCF}_k = k_1 / k_2 \tag{3}$$

This model has been widely used to calculate BCFs [26], but sometimes first order kinetics are not suitable to fit experimental data and more complex models have to be employed [27,28]. For example, when the exposure concentration of the tested compound is variable over time, the differential form of the model was used and the exposure data was set up as a step function with first order decay between the exposure exchange times (Eq. (4)):

$$C_f = \frac{k_1 C_w^0 \left(e^{-\lambda t} - e^{-k_2 t}\right)}{(k_2 - \lambda)}. \tag{4}$$

here, C_w^0 is the concentration of the compound at the beginning of the exposure and λ is the velocity for the decline of the concentration of the compound in the exposure media, while k_1 and k_2 are the uptake and elimination rate constants, respectively [29].

3. Results and discussion

3.1. Exposure media

Concentrations of anthracene and fluorene found within the exposure media along the bioaccumulation experiment are shown in Figs. 1 and 2. Estimation of the bioaccumulation factor requires, according to the OECD test 305, that the concentration of the compound tested in the exposure solution remains within a 20% of the nominal value during the experiment. To keep the concentration of PAHs constant during exposure, the solution was renewed every 24 h. Anthracene concentration during exposure

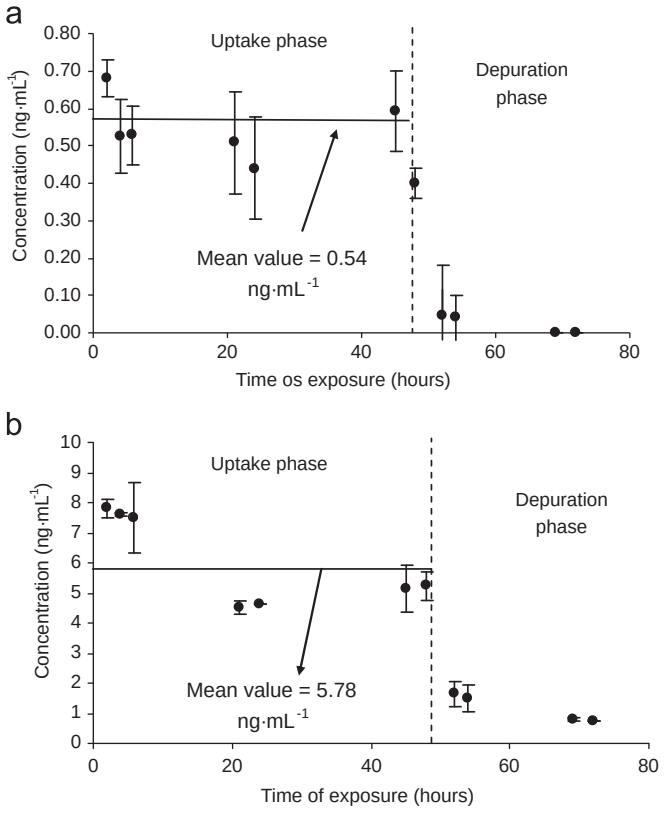

Fig. 1. Anthracene concentration (ng mL^{-1}) in the exposure medium: (a) nominal content of 1.5 ng mL^{-1} and (b) nominal content of 15 ng mL^{-1}.

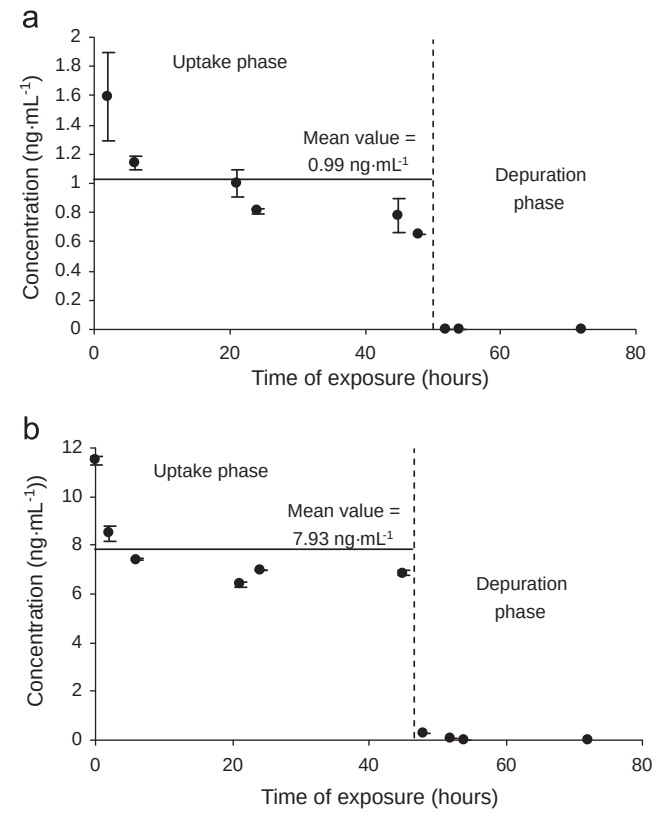

Fig. 2. Fluorene concentration (ng mL^{-1}) in the exposure medium. (a) Nominal content of 2 ng mL^{-1}; (b) nominal content of 20 ng mL^{-1}.

remained almost constant (Fig. 1) for both concentrations tested, but a continuous decrease of fluorene concentration (Fig. 2), especially acute for the lower concentration, was observed. This fact was also observed in previous bioaccumulation studies with PAHs. Landrum et al. [30], in a bioconcentration study over 700 h using elevated PAH concentrations (34–2201 ng mL^{-1}), found a variation of PAHs concentration above 50% during the first 24 h and a continuous variation of about 22%, even when the exposure media was renewed every 24 h. A continuous decrease of PAH concentration with time in seawater was also reported by Baussant et al., despite of using a continuous-flow system with closed exposure chambers and much higher PAH concentrations than those assayed in the present experiments (0.75–119 μg mL^{-1}) [31]. This type of behavior has been previously reported for other organic compounds having similar octanol/water partition coefficients [32]. This tendency was attributed to an adsorption process on the walls of the tanks. Consequently, one has to keep in mind that a strict control of PAH concentration (lower than 10 ng mL^{-1}) in an exposure media containing living organisms is quite difficult to achieve. Experimental data obtained was used for further calculation of bioaccumulation factors. PAHs presence in exposure media collected during the depuration step (exposure solution without PAHs) and in the control experiments (exposure solution without PAHs at any time) was checked but never detected.

3.2. Eleutheroembryos

Variation of PAH concentrations in eleutheroembryos over time are plotted in Figs. 3 and 4. Both PAHs were accumulated by zebrafish eleutheroembryos, but neither mortality nor significant changes in growth during their exposure at both concentration levels tested were observed. It is important to mark that a steady state after 12 and 24 h exposure for fluorene and anthracene, respectively, was reached. The accumulation rates for each PAH depend on both the exposure concentration and time. PAH concentration in eleutheroembryos significantly decreased during the depuration step, being almost negligible after 24 h, which evidences the efficient capability of the larvae to depurate these compounds.

3.3. Bioconcentration and toxicokinetics

BCF values were obtained applying two different procedures to the experimental data:

(a) Using the method defined by the OECD 305 test [3], which defines BCF_{SS} as the ratio between the concentration of the PAHs in larvae (wet weight) at the maximum time of the uptake phase (48 h) and its average concentration in the exposure solution.
(b) Using the previously described first-order one-compartment bioconcentration model, representing the variation of PAH concentration in larvae versus uptake time (Figs. 3 and 4) and by fitting data to a non-linear regression curve (Eq. (2)).

Bioaccumulation values for fluorene (whose concentration in the exposure media was not constant), was evaluated by applying Eq. (4). The multivariate non-linear regression analysis program NLREG 3.0 [33] was used for treatment of experimental data. From the best fit in terms of squared deviations, k_1 and k_2 constants were obtained from Eq. (2) and BCF_k values were obtained from Eq. (3). Table 1 shows the toxicokinetic values obtained for fluorene and anthracene with both methods: concentrations in the exposure medium (C_w) and in larvae (C_f),

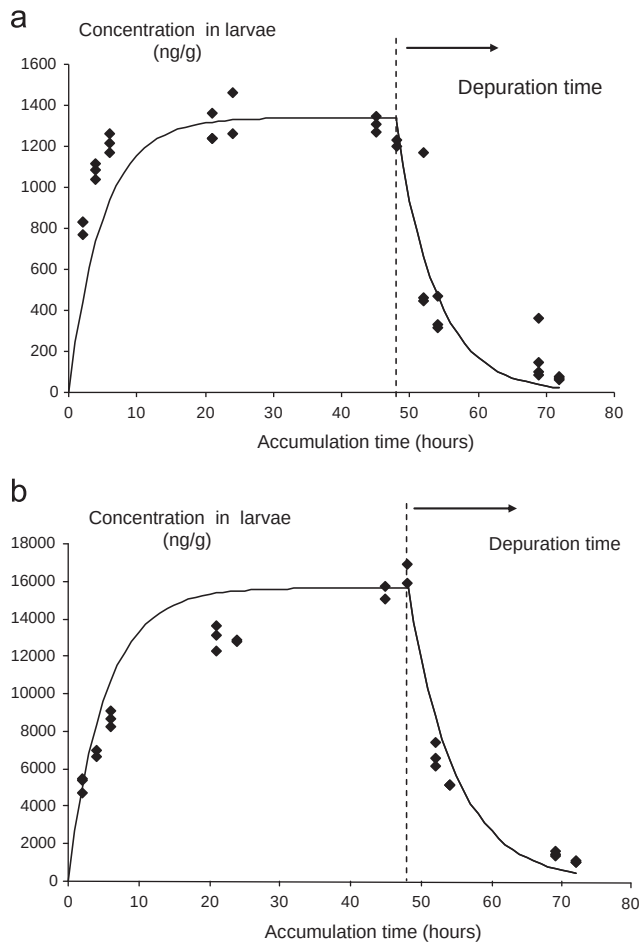

Fig. 3. Profile of anthracene accumulation in larvae in the experiments carried out at (a) nominal content of 1.5 ng mL^{-1} and (b) nominal content of 15 ng mL^{-1}. Solid circles (●) represent the experimental points and lines (—) the expected values based on model calculations.

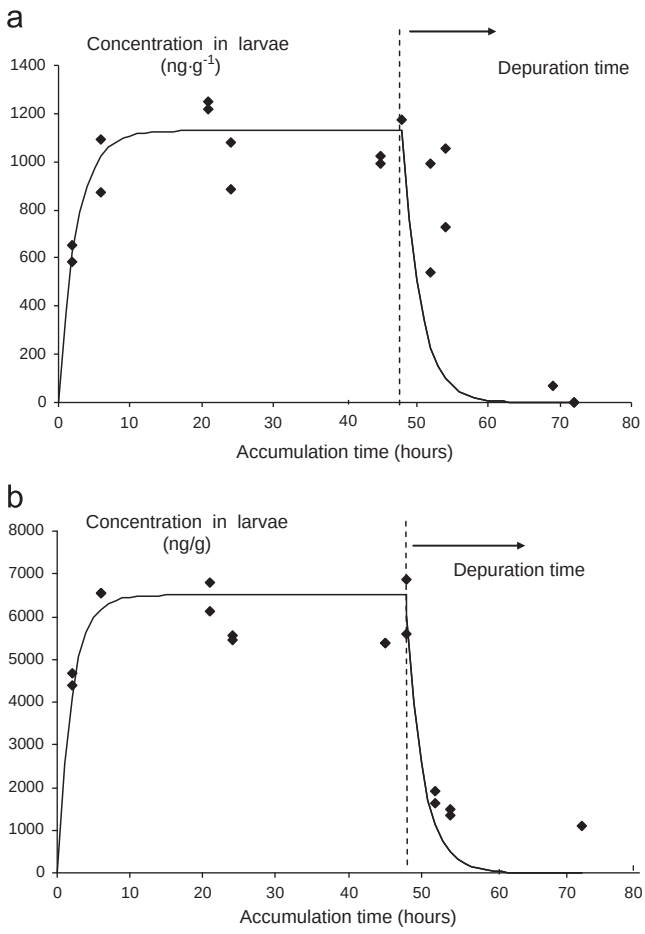

Fig. 4. Profile of fluorene accumulation in larvae in the experiments carried out at (a) nominal content of 2 ng mL^{-1} and (b) nominal content of 20 ng mL^{-1}. Solid circles (●) represent the experimental points and lines (—) the expected values based on model calculations.

uptake and depuration rate constants (k_1, $k_{2(acum)}$ and $k_{2(dep)}$ from accumulation and depuration equations respectively), and the bioconcentration factors (BCF_{ss} and BCF_k).

Toxicokinetic parameters calculated for fluorene from a time-weighted average exposure concentration (adjustment to Eq. (2)) and from an exposure concentration variable over time (Eq. (4)), were essentially identical. The constant for the decline of the compound in the exposure solution (λ) was very small and does not cause any difference on the fit (same sum of squared deviations and proportion of variance explained). This means that the impact of the variation of the concentration of the compound in the exposure solution on the overall kinetics was very small, as previously reported in other bioaccumulation experiments with PAHs [30]. The idea given by Landrum et al. to explain this behavior was that the 24 h used for renewal of the exposure solution are substantially shorter than the half-life of the compounds.

The toxicokinetic values obtained using both methods (Table 1) yielded very similar BCF_k and BCF_{ss} values for both anthracene and fluorene, mainly because both have a very rapid absorption kinetic, reaching the steady-state during the uptake phase well within the first 24 h of accumulation. The high values of log K_{ow} of these organic compounds (4.45 and 4.18 for anthracene and fluorene, respectively) could indicate that they would require longer exposure time to reach a steady state in the bioconcentration phase, as previously reported for other compounds with comparably high K_{ow} values [34]. However, the fact that the accumulation kinetics are very fast in the present experiments, allows us to conclude that the bioaccumulation experiments carried out provide more useful environmental and toxicological information than that obtained with other approaches, such as QSAR calculations.

BCFs obtained for anthracene and fluorene in the present study are in good agreement with those reported by other researchers, using several fish species (Table 2). Similar research, carried out using the OECD 305 protocol, where common carp (*Cyprinus carpio*) are exposed to 1.5 and 15 ng mL^{-1} for anthracene and 2 and 20 ng mL^{-1} for fluorene respectively, provided a log BCF value of 2.95 and 3.45 for anthracene and 2.34 and 2.91 for fluorene [21]. Other bioaccumulations data published by several authors, although none of these experiments fulfill exactly the OECD 305 guideline, are shown in Table 2 [35–41].

Previous results with phenanthrene (a PAH with the same molecular weight than anthracene), using zebrafish larvae, report a log BCF of 3.80 using the same approximation to calculate BCF_k (through a fit to the above explained first-order one-compartment bioconcentration model) [42]. However, the fact that the BCFs values were calculated on a dry weight basis, which is not recommended by the OECD 305 method, an overestimation by a factor of ten (dry weight ranges normally 20–10% of wet weight) [42,43] was achieved. It is also important to mark up that in these studies, a PAH concentration of 155 ng mL^{-1} was used, which is about ten times higher than the concentration applied in the present experiments.

Table 1
Toxicokinetic parameters and bioconcentration factors (BCF_{ss}, BCF_k) obtained from zebrafish larvae for anthracene and fluorene.

C_w	Antracene		Fluorene	
	Low concentration (0.63 ng mL^{-1})	High concentration (6.1 ng mL^{-1})	Low concentration (0.99 ng mL^{-1})	High concentration (7.93 ng mL^{-1})
C_f (ng g^{-1})	1313	15158	1139	6480
k_1 (mL ng h^{-1})	425	355	525	405
$k_{2\ (acum)}$ (ng mL^{-1})	0.2	0.14	0.4	0.5
$k_{2(dep)}$ (ng mL^{-1})	0.17	0.15	0.4	0.42
log BCF_{ss}	3.32	3.39	3.06	2.91
log BCF_k	3.33	3.37	3.11	2.91
log k_{ow}	4.45	4.18		

Table 2
Comparison between experimental BCFs obtained in this study and others found in literature.

Species of fish	Fluorene		Anthracene		Reference
	Conc. (ng mL^{-1})	Log BCF	Conc. (ng mL^{-1})	Log BCF	
Zebrafish larvae (*Danio rerio*)	2	2.77	1.5	3.31	Present
	20	2.88	15	3.34	study
Common carp (*Cyprinus carpio*)	2	2.34	1.5	2.95	21
	20	2.91	15	3.45	
Rainbow trout (*Oncorhynchus mykiss*)	19.1	2.41–2.86	50	2.81	35
			36	2.43	
			1000	2.74–2.92	
Bluegill sunfish (*Lepomis macrochirus*)			5.8	2.74–3.27	36
Bluegill sunfish (*Lepomis macrochirus*)	20	3.25			37
	86	2.84			
	47	3.25			
	175	2.3			
Fathead minnow (*Pimephales promelas*)	–	–	6	3.21–3.42	38
			11.5	3.55	
			20	2.98	
Orfe (Leuciscus idus)			26	2.95	39
Guppy (*Poecilia reticulata*)	182–883	3.34	7–40	3.86	40
Water worm (*Lumbriculus variegatus*)	50	2.60	25	3.01	41
	100	2.69	100	3.15	

It is known that a particular accumulation mechanism depends of the nature of the tested compounds. Passive diffusion across the lipid bilayer of biological membranes, as predicted by Fick's law [44], is the main process for neutral organic substances. Mechanistic models that use this approximation explain that BCFs values should be independent of the exposure concentration; in fact the OECD guideline indicates that the variation in uptake/depuration constants between the two test concentrations should be less than 20% [3,45]. Previous studies have proven that this statement is in good agreement with experimental results. Thus, it can be assumed that the variation in the concentration of anthracene in the exposure solution should not have a significant effect on the initial uptake rate, as it was demonstrated using bluegill sunfish [36]. However, the differences found between the uptake/depuration parameters in the present study and others [30] suggest that molecular diffusion is not the only mechanism involved in the accumulation of non polar organic compounds. McKim et al. [44] suggest that other mechanisms, such as flow to and after lipidic membranes or plasma binding of these xenobiotics after crossing the ephitelium, may also play an important role in bioaccumulation processes. Landrum et al. [30] found that increasing PAH concentration results in a lower uptake coefficient during exposure. The same finding was reported by Fisher et al. [46] in zebra mussel exposed to pentachlorophenol. This behavior was attributed to the narcotization effect of these contaminant compounds, which reduces swimming activity, causing decrease of the volume of contaminated water in contact with the surface of the organism and thus reducing contaminant uptake. Further, the flow until the lipidic layers is somehow slowed, which also contributes to reducing global uptake of contaminants.

Some authors have pointed out that the relatively high lipid content in larvae [42] or also, their slower metabolism compared to adult and juvenile fish may be responsible for an overestimation of BCF values when using larvae for bioaccumulation experiments [47]. Lipid content of zebrafish larvae at the end of the yolk sac stage has been reported to be near 20% of dry weight, whereas the mean lipid content of juvenile zebrafish has been found to be 11.0% of dry weight [20]. Nonetheless, literature data on this issue is rather dispersing, in particular when using different fish species without establishing strict conditions for the requirements of bioaccumulation test. Inspite of having used species in an early stage of development, the BCF values obtained in the present work do not show perceptible overestimation.

3.4. Comparison with quantitative structure–activity Relationships

As is generally accepted, accumulation of organic contaminants in larvae can be correlated with *n*-octanol/water partition coefficients of the compounds [48,49]. Table 3 shows several BCF values of zebrafish larvae determined by using the K_{ow} values of PAHs in a linear Quantitative Structure Activity Relationship (QSAR) equation, $\log BCF = a \log K_{ow} + b$. QSARs generally follow a linear path until $\log K_{ow} < 6$ [42,49–51]. The parameters a and b are the slope and the intercept of the equation, respectively. Although quite high dispersion from QSAR data was obtained, experimental BCF data from this work was found to be

Table 3
Regression values for estimating the BCF values from log K_{ow} using a linear relation log BCF$=a+b$ log K_{ow} together with the calculated values for fluorene and anthracene.

a	*b*	*n*	r^2	Life stage	Log K_{ow}		Reference
					Fluorene	Anthracene	
0.46	0.86	11	0.91	Zebrafish larvae	3.13	3.36	42
−0.7	0.85	55	0.897	Fish	2.85	3.08	49
1.33	1	71	0.95	Adult fishes	2.85	3.12	50
0.23	0.60	2393	0.52	Adult fishes	2.27	2.44	51
0.27	0.46	4119	0.38	All fish	2.19	2.32	

Log K_{ow} (octanol–water coefficient) values for anthracene and fluorene were set to 4.45 and 4.15, respectively, for the calculations.

comparable with those reported in the literature. There are some limitations for the good correlation between BCF and K_{ow}, possibly due to the metabolic biotransformation of the chemical within the organism [52,53]. Thus, an over prediction of BCF data could have been obtained, but data summarized in Table 3 does not show systematic differences between QSAR previsions and experimental data. The small difference between the depuration constant (k_2) obtained after adjustment of data to accumulation and depuration steps (Eq. (2)), previously attributed to the metabolization of the tested compounds [52], supports the theory that metabolization in larvae was not relevant. Petersen and Kristensen have also found that zebrafish does not metabolize PAHs and PCBs in the larvae stages [42]. Evidence about metabolization of larger parental PAHs, even after relatively short exposure periods at lower concentrations of these molecules [54], together with a decrease on bioavailability of these PAHs ($\log K_{ow} > 4$) was reported. This behavior was explained by the binding on suspended or dissolved particles and on sediments. The fact that the uptake coefficient rates found for large PAHs were significantly lower than those for smaller ones [36,55] evidences the future studies need to also evaluate the BCFs of these high molecular weight PAHs on the proposed zebrafish eleutheroembryo model.

4. Conclusions

This study provides the uptake and depuration values for anthracene and fluorene by zebrafish larvae. The proposed analytical methodology used is based on the use of ultrasound to assist the extraction, followed by simple filtration and separation and quantification by liquid chromatography. It allows the determination of the two PAHs tested in very small (pools of 20 eleutheroembryos corresponding to less than 0.010 g of wet weight) and complex samples (lipid content, ca. 15%), which represents a relevant analytical advance in terms of rapidity and effectiveness for analyte leaching, low solvent consumption and low hazardous residues production compared to previously reported analytical procedures.

In consequence, the OECD 305 protocol has been scaled down, avoiding the use of adult fish, thus allowing an important reduction of time (from 42 to 3 days) and cost (from 100,000 to 10,000 €) compared to conditions set in the official procedure. Inspite of the high lipid content and the lower metabolic rate in the early life stages of zebrafish, the BCFs values calculated in this study were in good agreement with those previously reported. The results obtained proved the feasibility of this 72 h uptake/depuration cycle using zebrafish eleutheroembryos to obtain valid experimental bioaccumulation data. The first-order one-compartment bioconcentration model to fit the data and to obtain toxicokinetic parameters are also adequate for final determination of the bioconcentration factors. Although future work with other compounds having different accumulation characteristics is necessary, the results obtained evidence the suitability of zebrafish eleutheroembryos as predictive vertebrate model for evaluation of both bioaccumulation and depuration of PAHs. This work is in line with European legislation requiring that non-animal testing approaches should replace the experiments with animals wherever possible, which is especially true when considering the new European chemical policy REACH, where BCFs values of some 3000 substances are required.

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