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PREDICTIVE FRAMEWORK FOR ESTIMATING EXPOSURE OF BIRDS TO PHARMACEUTICALS

Running title: Predicting exposure of birds to pharmaceuticals

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Abstract: Here we present and evaluate a framework for estimating concentrations of pharmaceuticals over time in wildlife feeding at wastewater treatment plants (WWTPs). The framework is composed of a series of predictive steps involving the estimation of pharmaceutical concentration in wastewater, accumulation into wildlife food items, uptake by wildlife with subsequent distribution into, and elimination from, tissues. As many pharmacokinetic parameters for wildlife are unavailable for the majority of drugs in use, a read-across approach was employed using either rodent or human data on absorption, distribution, metabolism and excretion (ADME). Comparison of the different steps in the framework, against experimental data for the scenario where birds are feeding on a WWTP contaminated with fluoxetine, showed that: estimated concentrations in wastewater treatment works were lower than measured concentrations; concentrations in food could be reasonably estimated if experimental bioaccumulation data are available; and that read-across from rodent data worked better than human to bird read-across. The framework provides adequate predictions of plasma concentrations and of elimination behavior in birds, but yields poor predictions of distribution in tissues. We believe the approach holds promise, but it is important that we improve our understanding of the physiological similarities and differences between wild birds and domesticated laboratory mammals used in pharmaceutical efficacy/safety trials, so that the wealth of data available can be applied more effectively in ecological risk assessments.

Keywords: Read-across, Ecological Risk Assessment, Fluoxetine, Pharmacokinetics, Wild birds

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INTRODUCTION

Over the past 15 years, there has been growing interest in the effects of active pharmaceutical ingredients (APIs; parent compound and active metabolites) on ecosystems. APIs are excreted to wastewater by patients, and subsequently can contaminate aquatic and terrestrial environments [1]. Wild birds may be exposed to pharmaceuticals when they forage on organisms in wastewater treatment plants (WWTPs), soils and surface waters [2-5]. As biologically active molecules designed to bind to and interact with a specific receptor or process in a target organism, APIs have the potential to cause effects at low concentrations in non-target species, particularly as many of the receptors have been evolutionarily conserved in wildlife [6, 7]. To date, ecotoxicological studies have largely focused on the effects of APIs on algae, invertebrates and fish [8]. In order to protect wildlife, it would be beneficial to develop an understanding of environmentally realistic exposure and potential effects of APIs in other biota such as wild birds and mammals [1, 8-10].

When registering an API in the EU and the US, there is currently no legislative requirement to consider secondary exposure, toxicity and risk to wildlife [11, 12]. Only, toxicity to plants, invertebrates (including earthworms) and fish are considered. In the EU, registration of veterinary drugs requires risk assessment of secondary exposure and hazard for wildlife [13]. The models used are based on the European Commission's Technical Guidance Document (TGD) Part II [14] for new and existing industrial compounds. This TGD approach (initially developed for metals and plant protection products) is a simple risk assessment based on daily dietary intake (mg/kg bw) relative to a toxicity reference value [14, 15]. The European Food Standards Agency (EFSA) presents a more advanced methodology than the TGD approach for calculating exposure [16], but again it is based on dietary exposure rather than internal residues (reviewed in [17]). In order to gain a greater appreciation of risk it would be beneficial to extend these diet-based models to estimate internal exposure using the wealth of data collected in laboratory mammals and humans during pre-clinical and clinical trials [12, 14, 16].

For example, risks of APIs for wildlife could also be assessed by determining whether plasma concentrations are likely to exceed therapeutic plasma concentrations. For free-living organisms, it may also be more realistic to consider accumulation over multiple low-level daily intakes rather than just a single exposure (typically done for pesticides [16]), which may under-estimate risk. Concentrations in tissues (particularly sites associated with pharmacological activity) along with diet-based assessments likely give a more robust indication of risk than diet-based toxicity reference values alone.

Accumulation of APIs over environmentally relevant foraging periods (i.e., duration a particular food source or site is used) can be assessed using pharmacokinetic equations to calculate predicted cumulative body burdens at a given elimination half-life ($t_{1/2}$, assuming first order kinetics) [18, 19]. Alternatively, the theoretical $t_{1/2}$ required to accumulate or exceed a threshold (e.g., Human Therapeutic Dose, HTD) can be back-calculated to assess the plausibility of observing such a value in wildlife Lazarus et al [19]. Using pharmacokinetic parameters such as bioavailability (the proportion of the ingested contaminant absorbed from the gastrointestinal [GI] tract) and the apparent volume of distribution (V_d, the theoretical volume required to contain the remainder of the dose at the same concentration as observed in plasma), it is possible to estimate plasma concentration for a specific cumulative body burden of an API. Furthermore, concentrations in tissues can be estimated from concentrations in plasma using tissue to plasma distribution ratios. By combining estimated tissue concentrations with $t_{1/2}$, the duration (time) that residues in tissues would remain above quantifiable levels can be estimated to inform field monitoring study design.

Pharmacokinetic parameters, relating to absorption, distribution, metabolism and excretion (ADME), required to run these predictive models have only been defined for a few APIs in wildlife (e.g., the non-steroidal anti-inflammatories diclofenac [20, 21], meloxicam [22] and ketoprofen [23]). However, it may be possible to utilize ADME data collected in studies on laboratory mammals and humans that are performed to assess the efficacy and safety of a pharmaceutical, and then to conduct cross-species extrapolation by ‘read-across’ to predict internal exposure and risk for wildlife. Similar

interspecific 'read-across' extrapolations have been demonstrated to be suitable for some classes of pharmaceuticals in fish [24-28]. The application of these approaches for assessing effects in non-target species could be valuable in identifying APIs of greatest environmental concern and for characterizing risks to free-ranging wildlife.

One API that has received considerable attention is the antidepressant fluoxetine, a commonly prescribed selective serotonin re-uptake inhibitor (SSRI). In the environment, fluoxetine is persistent, with monitoring studies often detecting it in water, sediments and aquatic organisms [29, 30]. Laboratory exposures show fluoxetine to affect behavior of aquatic invertebrates and fish at environmentally relevant concentrations (≥ 0.3 ng/L) [31-34]. To date, studies in birds on fluoxetine have been principally toxicological in nature [3, 35, 36], with most attention given to characterizing the uptake and distribution of the molecule in mammals [37-41]. In the laboratory, starling foraging behavior appears to be altered by fluoxetine at 5% of the HTD [3]. Here we present a predictive framework that used data on API use, metabolism, bioaccumulation in food items and pharmacokinetics in laboratory mammals and humans to estimate internal exposure in wildlife. We evaluate the different components of this approach using field monitoring and ADME data on fluoxetine for the scenario where starlings are feeding on invertebrates in a trickling filter bed of a WWTP.

MATERIALS AND METHODS

Predictive framework for estimating internal exposure

Dietary exposure of birds to APIs may result from foraging in a variety of environments (soils, sediments, surface waters), although greatest exposure is likely to occur during foraging at WWTPs due to the higher concentrations of APIs in these systems. A predictive framework for assessment of dietary and internal exposure of birds foraging on filter beds at WWTPs is presented in Figure 1.

The predictive framework that follows is presented in full as an editable worksheet in the Supplemental Data. The framework begins by calculating the predicted environmental concentration

(PEC) of a pharmaceutical in influent wastewater ($PEC_{wastewater}$). The PECwastwater depends upon the mass of pharmaceutical used by a population (M_{API}), the proportion of a parent compound excreted by patients (Fexcreted), the volume of wastewater emitted per capita (V), and the size of the population (Equation 1).

$$PEC_{wastewater} = \frac{MAPI \times Fexcreted}{V \times P} \quad (1)$$

The mass of a pharmaceutical used by a population can be estimated with data collected on prescribing rates in kg yr^{-1} (e.g., [42]). The proportion (≤1) of parent compound excreted by patients can be obtained from the literature or from data held by pharmaceutical companies (e.g., [43]). The product of M_{API} and Fexcreted was then divided by the product of annual per capita wastewater usage of (i.e., 73,050 L) [44] and the size of the population for which the usage data are available.

The PEC in food (PEC_{food}) is then calculated by multiplying the $PEC_{wastewater}$ by the bioaccumulation factor (BAF) derived from laboratory studies (Equation 2).

$$PEC_{food} = PEC_{wastewater} * BAF \quad (2)$$

A BAF derived for an appropriate species (e.g., earthworms) in OECD-type laboratory studies should be used in preference to predictions based on lipophilicity (e.g., [45]).

The PEC in plasma (PEC_{plasma}) of the wildlife is then calculated by adjusting cumulative body burden (CBB, measured in mg/kg body wt) of the pharmaceutical over time (e.g., concentration at peak, i.e. shortly after a meal = zenith, concentration at trough, i.e., just before a meal = nadir) for the fraction of the drug that is bioavailable (F_{bioav}, ≤1) and the apparent volume of distribution (V_d, with units of L/kg body wt) [38, 46-48] (Equations 3a and b).

$$PEC_{plasma,zenith} = \frac{CBBzenith \times Fbioav}{V_d} \quad (3a)$$

$$PEC_{plasma,nadir} = \frac{CBBnadir \times Fbioav}{V_d} \quad (3b)$$

If data for the F_{biov} or V_d are not available, the framework can be initially evaluated using values collected in laboratory mammals and humans (e.g., [38, 46]).

The CBB of an API over time is calculated for example at zenith and nadir assuming elimination by first order kinetics (Equations 4a-b) [18, 19].

$$CBB_{zenith} = (DI_{remaining\ in\ body})e^{-kt} + DI \quad \textit{Shortly after a meal} \quad (4a)$$

$$CBB_{nadir} = (DI_{remaining\ in\ body})e^{-kt} + (DI)e^{-kt} \quad \textit{Just before a meal} \quad (4b)$$

The calculation of CBB accounts for the duration of exposure (t, in days) and daily intake (DI) of contaminated food (mg/kg body wt); given by the product of mass of food eaten (M_{food}, per kg body wt) and the concentration of API in the food item (Equation 4c).

$$DI = M_{food} * PEC_{food} \quad (4c)$$

M_{food} can be derived from information on diet composition and energetics [EFSA 2009 16] and organism body weight e.g., [49] or from estimates based on observations of foraging behaviour e.g., [50, 51].

The duration of exposure is the period of time for which a bird would likely forage at a contaminated site [4, 5]. To estimate CBB over time, equations 4d.i and 4d.ii are used to obtain the fraction of daily intake remaining in the body from prior exposure ($DI_{remaining\ in\ body}$).

$$DI_{remaining\ in\ body\ d2} = (DI)e^{-kt} \qquad (4d.i)$$

$$DI_{remaining\ in\ body\ ,n} = (DI)\left[\frac{e^{-kt} - (e^{-kt})^{n}}{1 - e^{-kt}}\right] \qquad (4d.ii)$$

In equations 4d.i and 4d.ii, DI remaining in the body on day 1 is 0 (as there have been no previous exposures). DI remaining in the body on day 2 of exposure ($DI_{remaining\ in\ body\ d2}$) is given by multiplying DI (Equation 4c) by the exponential of negative kt (where k is the elimination constant and t is time in days) (Equation 4d.i). The DI remaining on day n ($DI_{remaining\ in\ body,n}$) is given by equation 4d.ii. The elimination constant, k, is calculated by dividing ln(2) by the elimination half-life (in days).

PEC in tissue (PEC_{tissue}) is then calculated as the product of PEC_{plasma} and the ratio of API measured in key tissues to plasma ($R_{tissue:plasma}$) (Equation 5).

$$PEC_{tissue} = PEC_{plasma} \times R_{tissue:plasma} \qquad (5)$$

As with F_{bioav} and V_d, it may be necessary to use information presented in the literature for $R_{tissue:plasma}$ in laboratory mammals and humans (e.g., [37,38,52]).

Post-exposure concentrations in tissue ($PEC_{post\text{-}exposure}$) are calculated from PEC_{tissue} by continuing the elimination modelling once the animal stops feeding on the contaminated source (Equation 6).

$$PEC_{post\text{-}exposure} = PEC_{tissue,final\ exposure}\ (e^{-kt})^n \qquad (6)$$

This is achieved by setting DI to 0 in equation 4a to recalculate PEC_{plasma} and PEC_{tissue} at multiple post exposure time points. The $PEC_{post\text{-}exposure}$ can be calculated for any number of days post-exposure (n).

Finally, by plotting the data and fitting an exponential trend line ($y= Ce^{-kx}$,where C is the y-axis intercept, -k is given by $\ln(2)/t_{1/2}$ [days] and x is time in days), it is possible to estimate the duration for which quantifiable residues remain in tissues (X_{LOQ}). An estimate of the time that tissue residues exceed the limit of quantification (LOQ) can be obtained by rearranging the exponential trend line (Equation 7).

$$x_{LOQ} = \frac{\ln(LOQ) - \ln(PEC_{tissue,final\ exposure})}{\ln(e^{-kt})} \qquad (7)$$

The output from Equation 7 can be used for both interpretation of risk (i.e., rapid clearance below LOQ reduces the likelihood of persistent exposure) and to help inform field monitoring (i.e., which tissues should be sampled to assess internal exposure).

Evaluation of Framework

To evaluate the predictive framework, we used laboratory and field based investigations to generate data to evaluate the different steps in the framework. Observations were then compared with predictions from the framework using input data obtained from the open literature (Table 1). A description of the validation approach is given below.

PECwastewater and PECfood

To evaluate the $PEC_{wastewater}$ and PEC_{food}, monitoring was done at four trickling filter WWTPs in Northern England in November 2013. At each site, a sample of influent was collected and placed

into a 2 L solvent rinsed amber glass bottle. Earthworms (*Eisenia fetida*) were obtained from the top 20 cm across the filter beds and placed into a clean glass jar. All samples were placed in cool bags containing ice packs and transported to the laboratory. Upon arrival, sludge was rinsed off the earthworms using deionized water. Samples were then frozen at -20°C until extraction and analysis (See Supplemental Data). To evaluate the framework equations, mean measured concentrations (MEC) in influent and earthworms were compared to PECs for influent and food.

PECplasma, PECtissue, PECpost-exposure

To evaluate the framework equations for PEC_{plasma}, PEC_{tissue} and $PEC_{post\text{-}exposure}$, an absorption, distribution and excretion study was done by exposing captive starlings to fluoxetine injected invertebrates wax moth larvae, (*Achroia grisella*), for 22 wk (see Supplemental Data or [3]). In total there were 12 males (seven fluoxetine-treated and five controls) and 12 females (five fluoxetine-treated and seven controls). Five mornings per week, the starlings were captured, hand fed one wax moth larvae and returned to their home pen [3]. Fluoxetine-treated birds received a wax moth larvae injected with 1300 ng of fluoxetine (~5% HTD) in 2.5 µL deionized water while controls received a wax moth larvae injected with 2.5 µL deionized water (see Supplemental Data). The dose contained in wax worms was quantified by HPLC (mean ± SE: 1580 ± 73 ng/larvae; see Supplemental Data or [3]). Whole blood (0.5 mL) was collected by jugular venipuncture (1 inch 25-gauge needle, 1 mL syringe) after 16 wks of treatment, 1 h after receiving a dose. Blood was immediately transferred to a lithium heparin Microtainer® (Becton Dickinson, UK) and centrifuged for 3 mins at 2000 *g*. Plasma (~150 µL) was harvested into 1.5 mL microcentrifuge tubes, held at 4°C for <1 h and then frozen at -20°C. Two hours prior to euthanasia, each of the six birds was placed into a cage lined with paper so that feces could be collected to assess concentrations of fluoxetine and metabolites. The feces from each bird were transferred into a microcentrifuge tube, and subsequently weighed, dried at 40°C, ground using a mortar and pestle, and stored in plastic bags at -20°C. Birds were euthanized by cervical dislocation at 2, 26, 50 and 74 h post-exposure (N=3 Fluoxetine and 3 Control birds per time point). From each bird,

whole brain, liver, kidney and pectoral muscle were collected, snap frozen in liquid nitrogen and transferred to glass jars held on dry ice and later stored at -80°C. Plasma samples were analyzed for fluoxetine by multiple reaction monitoring liquid chromatography triple quadrupole mass-spectrometry (MRM LC-MS-MS) and tissue samples and feces were analyzed for fluoxetine and known major metabolites by LC-MS-MS and liquid chromatography Fourier-transform ion cyclotron resonance mass spectrometry (LC-FT-ICR-MS) (see Supplemental Data). To evaluate the framework equations, mean concentrations were compared to predictions obtained from the equations.

Sensitivity and Uncertainty Analysis: The relative sensitivity of individual framework parameters was assessed by perturbing values one at a time (up and down by 10% and 90%, respectively). The output was compared to the unperturbed result, and the percentage change in framework prediction related to percentage perturbation of input parameter (R=1, directly proportional change; R<1, framework dampens down changes; R>1, framework amplifies changes in parameter). The effect of exposure time was assessed independently by running the unperturbed framework for 1, 2, 3, 7 and 365 d, respectively, and comparing the output to 22 wk.

Uncertainty in the framework was assessed by running combinations of input parameters to represent the upper and lower limits of the range and mean for i) MEC_{food}, ii) Mass of food eaten/day, 3) $t_{1/2}$. These parameters were selected based on sensitivity analysis and the availability of information on their individual uncertainties. A matrix approach was used to run the framework using 27 combinations of variables; the mean ± SE brain concentration at nadir and zenith and percent Coefficient of Variation (CV) are presented as an indication of the framework's uncertainty.

RESULTS AND DISCUSSION

Experimental data

A summary of the results of the monitoring and laboratory studies is provided in Table 2 (see Supplemental Data for data on metabolites). Only limited information is available in the literature on the occurrence of fluoxetine in the matrices we investigated. Our measured influent concentration

(1,310 ng/L) is one to two orders of magnitude greater than concentrations detected in the small number of studies that have previously reported fluoxetine in influents (typically 16.6-21.5 ng/L [53], but one study in Portugal found 105.8-157.4 ng/L [54]) . The discrepency with our data possibly reflects differences in prescribing patterns for psychoactive drugs among the countries studied.

Evaluation of predictive framework

$PEC_{wastewater}$: The $PEC_{wastewater}$ of 297 ng/L was about four times less than our mean MEC of 1,310 ng/L (range 774-1,930 ng/L, >LOQ in 6/6) in wastewater influent. Other studies have used these types of models for prediction of concentrations of APIs in wastewater (see examples in [55-58]). Ort and coworkers [55] calculated PECs for effluent dominated surface waters for eight of twelve APIs (including NSAIDs, anticonvulsants and blood pressure medicines) within 50% of MEC. Unlike our study, concentrations were typically over-estimated with the mismatch between predicted and measured concentrations suggested to be due to over estimation of the fraction excreted as parent compound and variability in flow rate. Three of the four drugs with greatest deviations were antibiotics. Such multi-fold differences between predicted and empirical observations were also found by others for antibiotics and synthetic hormones [58].

Such elementary modelling approaches are dependent upon comprehensive usage data at the catchment scale as well as reliable information on metabolism by patients and other biota, and spatial-temporal variations in flow rates [59]. While information on prescribing rates is available in some countries (e.g., NHS prescription cost analysis in the UK [42] and similar systems are in place in Denmark, Germany and Australia [59], this may over estimate consumption for some drugs. For example, patients may stop taking medication due to relief of symptoms or adverse side-effects. In western countries, the percentage of households where unused medicines are being 'hoarded' for future use ranges from 1.4 to 65% [60, 61]. For drugs available over-the-counter, prescription data significantly underestimates consumption. Metabolism by patients can also be highly variable between individuals due to factors including age, sex, health status, race and interactions with other medications,

(e.g., excretion of fluoxetine apparently ranges from 5% [62] to 24% of the dose [43]). An alternative 'inverse modelling' approach to overcome some of these difficulties has been suggested by Boxall and coworkers [59]; model input parameters are back-calculated from the MEC and used to calculate PECs in various other scenarios. Using this inverse approach, it seems unlikely that differences in metabolism of fluoxetine could account for much of the 4.4-fold underestimation of MEC that we observed (i.e., $F_{excreted}$ was already set at the upper end of the range, 0.24). The discrepancy is possibly due to underestimation of usage and over estimation of dilution in the environment.

PEC_{food}: Use of the $MEC_{wastewater}$ along with reported BAF values for earthworms from OECD-type studies with soils as a basis for estimating the PEC_{food}, a concentration of 40,348 ng/kg wet wt (range 33,274-46,898) in food was estimated. This value corresponds well with the MEC_{food} (i.e., earthworms) of 26,200 ng/kg wet wt (range 2,500-53,800 ng/kg), indicating that BAFs from standard studies with soils may be appropriate for estimating uptake into earthworms residing in WWTPs.

PEC_{plasma}: Using the fluoxetine dose administered to starlings and ADME data for rodents, the PEC_{plasma} was between 0.03 and 0.23 ng/mL (nadir-zenith). Use of human ADME data gave a range of 0.44 to 0.50 ng/mL. In the starling study, fluoxetine was not detected in the plasma (LOD = 0.15 ng/mL). It therefore appears that ADME data from laboratory rodents provide better predictions of what will occur in starlings.

The low absorption observed in starlings compared to humans could be due to differences including (i) GI tract pH, (ii) bioaccessibility, and (iii) rapid GI tract transit. Theoretically, differences in GI tract pH could affect bioavailability of a base such as fluoxetine (pKa = 10.06) [63], which would be protonated (unable to pass through biological membranes well), and therefore poorly lipid soluble in an acidic environment. Starlings have a gizzard pH in the range of 1.6-2.3, which is slightly below the average for the stomach of a satiated human (pH 2.5), but similar to that found in fasted individuals (low of pH

1.3). The pH of the avian intestine is typically around pH 6.2 compared with a value of approximately pH 7.0 in humans [64-67].

We have previously used in vitro GI tract simulations to examine bioaccessibility of fluoxetine from earthworm tissues [68], but not for the lesser wax moth larvae used in our in vivo experiments. This work found bioaccessibility of fluoxetine to be lower in birds than mammals; but the 9.6-13.7% difference cannot account for the 2-3 orders of magnitude difference between starlings and humans [68]. Additionally, a preliminary study suggested there was no difference in bioaccessibility of fluoxetine from earthworms that were injected with a solution containing the drug or those that had biologically incorporated it from soil. Wax moth larvae have higher percentage lipid content, lower percentage water content and lower mass (22% lipid and 72.4% water as reported for terrestrial arthropod larvae in [69], approximate mass ~0.2 g, range 0.14-0.32 g - measured in this study) than earthworms (9% lipid, 83.3% water, approximate mass, mean and range 0.5 g, 0.3-1.0 g [16, 70]). However, absolute lipid content (44 mg/larvae vs 45 mg/earthworm) is similar for wax moth larvae and earthworms, which would suggest that bioaccessibilities in the two organisms may also be similar. Although theoretical and unlikely, the possibility remains that absorption in starlings was very low because of poor bioaccessibility of fluoxetine contained in wax moth larvae.

Low absorption and rapid clearance of fluoxetine from starling tissues could also be due to rapid GI tract transit time. In starlings, invertebrate prey have a GI tract transit time of only 12 mins/g invertebrate and a retention time of 20 mins/g invertebrate [71]. Mean retention time of food in humans ranges from 0.7 to 4 d (mean = 2.3 d) [72]. Hypothetically, such short GI tract transit times could limit absorption of significant quantities of contaminants, conversely birds' relatively high energetic demands and lifespans might increase efficiency of contaminant absorption compared with small mammals. At the time of study design, we were unaware of these data, and suggest others who intend to investigate ADME using small passerines consider GI tract transit time when estimating the likely peak in absorption. An estimate of rapid elimination could also perhaps have been predicted from

basal metabolic rate (see Supplemental Data Figure S2). Thus, we suggest future studies investigating GI tract transit time and API bioavailability are warranted, and far simpler and more economical than measuring ADME.

*PEC_{tissue} :*As fluoxetine was not detected in plasma, we used the LOD as a basis for estimating concentrations in tissues. The PEC_{brain} was estimated at 7.35 ng/g (1.35-13.35 ng/g), PEC_{liver} was 3.0 ng/g (0.3-5.7 ng/g), PEC_{kidney} was 1.35 ng/g and PEC_{muscle} was 0.33 ng/g. Although use of rodent ADME likely estimated plasma concentration in the correct range, it appears that its distribution must be different between birds and mammals with MEC/PEC ratios of 0.41 for brain, 4.81 for liver, 5.43 for kidney, and 8.97 for muscle. Thus, distribution of fluoxetine in starling brain is much lower than for laboratory rodents, but distribution into liver, kidney and muscle is greater. As brain is the principal site for fluoxetine's mechanism of action, low distribution into this tissue could be significant for pharmacological activity and associated risk to birds [73, 74]. The poor match between MEC and PEC suggests the framework for PEC_{tissue} needs to be modified before moving onto the next stage of the framework ($PEC_{post\text{-}exposure}$). Inverse modelling needs to be applied to better estimate the distribution of fluoxetine in avian tissues compared to mammals. By dividing MEC_{tissue} by LOD_{plasma}, we can estimate $R_{brain:plasma}$, $R_{liver:plasma}$, $R_{kidney:plasma}$ and $R_{muscle:plasma}$ to be 20, 96, 49 and 20 respectively.

$PEC_{post\text{-}exposure}$: Fluoxetine was rapidly eliminated from starling tissues and detected in less than 50% of samples 26 h post-exposure (Table 2, compare values at 26 h, 50 h and 74 h with the value at 2 h). It was detected in only one of three liver samples after 50 h, which could be an outlier (possibly due to genetic variation amongst wild-caught birds). Fluoxetine was not detected in any tissue after 74 h. While data in Table 2 suggest that fecal sampling or cloacal swabs may be a non-invasive method to qualitatively assess exposure (variable rate of excretion and mass produced), destructive sampling to harvest tissues may well be the only option for quantitative purposes.

The rapid clearance from tissues indicates that we are unable to accurately estimate the elimination half-life of fluoxetine. Speculatively, we used the data in Table 2 to estimate fluoxetine $t_{1/2}$

for brain, liver, kidney and muscle at 3.1 h, 8.4 h, 8.3 h, and 10.8 h, respectively. The values are similar to the range for laboratory mammals ($t_{1/2}$ of 3.6-12.9 h), but much shorter than for humans 24-96 h [37, 41, 46]. Figures 2a-d (X-axis is time, Y-axis is fluoxetine concentration) present $MEC_{post\text{-}exposure}$ alongside $PEC_{post\text{-}exposure}$ in brain, liver, kidney and muscle for 4 d post-exposure for experimentally dosed and free-ranging starlings feeding on earthworms at WWTPs. A Y-axis reference line to represent the LOQ for each tissue is included to illustrate the time at which residues are cleared below quantifiable concentrations. In the predictive framework, we used the mid-point of the mammalian $t_{1/2}$ range (8.2 h) to calculate $PEC_{post\text{-}exposure}$, and the extremes (3.6 to 12.9 h) to illustrate variability (Figures 2a-d).

The data for the predictive framework presented in Figures 2a-d suggest fluoxetine residues would be cleared from our experimentally dosed starlings below LOQ after 36.2 h (brain), 42.9 h (liver), 31.9 h (kidney) and only 1 h (muscle). By fitting an exponential term to the in vivo data, these values are actually likely to be 15.6 h, 54.3 h, 40.8 h, and 5.7 h, respectively, which is a reasonable match for all tissues except brain. This illustrates that muscle is not a good matrix to use for monitoring purposes due to a combination of variable elimination in vivo (see Table 2) and lower sensitivity of analytical methods for this matrix. Liver and kidney are the matrices where fluoxetine is most likely to be detected, but in brain residues are cleared more than twice as fast as predicted. Applying the predictive framework to free-ranging birds, residues in liver would likely be above LOQ for 39.8 h in liver and 28.9 h in kidney (using $t_{1/2}$ of 8.2 h), but only 12.5 h in brain (using $t_{1/2}$ of 3.1 h). If the fluoxetine concentration in earthworms was actually at the lower end of the field-measured range, then residues in brain would never exceed LOQ (0.13 ng/g), while at the upper end of the MEC_{food} range for earthworms, residues would still only exceed LOQ for 17.3 h after 22 wk of sustained exposure. Therefore, if a bird moved away from feeding on the contaminated source for more than 18 h, then for all intents and purposes, its brain could be considered residue free. This narrow window of detection opportunity will certainly make destructive sampling of free-ranging birds difficult to justify.

Furthermore, the rapid clearance from brain is highly relevant, and could result in underestimation of risk.

Elimination via production of different metabolites than rodents and humans is also a potential explanation for the observed rapid clearance. We investigated production of the active metabolite norfluoxetine and six commonly produced (in humans) inactive metabolites. Norfluoxetine was detected in all tissues 2 h after the final dose (Supplemental Data), but was also eliminated rapidly with estimated $t_{1/2}$ of 5.4 h for brain, 6.7 h for kidney, 8.7 h for liver and 3.3 h for muscle. The percentage of the dose excreted as fluoxetine 2 h after the final administration is estimated at 19.0±15.9% (derived from fluoxetine concentration and mean dry fecal mass of 0.17 g produced by starlings in 2 h). Excretion as norfluoxetine accounted for approximately 0.03% of the dose, which was surprising given a mean of 25.6 ng/g in liver (1.38% of the dose/g liver). Norfluoxetine in feces was <LOD in all but two samples (Supplemental Data). Norfluoxetine must either be excreted between 2 and 26 h or further metabolized (See discussion in Supplemental Data). However, we were unable to detect any of the six inactive metabolites (commonly detected in humans taking fluoxetine), which suggests that metabolic pathways are likely very different between starlings and humans. Future ADME studies in wildlife could also benefit from an understanding of interspecies differences in protein binding, membrane transport (e.g., proteins of the cell membrane such as P-glycoprotein) and metabolic pathways (see Supplemental Data [75-77]).

If the effects on foraging behavior observed in Bean et al [3] are in fact real, then it is most likely due to subtle neurological changes that result from persistent (months) low level exposure to fluoxetine. If birds are only periodically exposed to fluoxetine (i.e., tissues are cleared for long periods during the winter and breeding season when earthworms are important in the diets of many bird species [78]), then it is less likely that up/down-regulation of receptors and endocrine related responses could be altered at brain concentrations 3 orders of magnitude below human therapeutic concentration.

Limitations and uncertainties

The relative sensitivity of the predictive framework to both increases and decreases in fluoxetine concentration in earthworms, mass of food eaten by starlings, F_{bioav} and $R_{tissue:plasma}$ was equal to 1 at both nadir and zenith (see editable framework in Supplemental Data 2). The relative sensitivity to $t_{1/2}$ and V_d differed depending on context; at nadir, changes in $t_{1/2}$ were amplified by the framework (for decreased values, R was up to 2.3, and for increased values, R was up to 2.7), whereas at zenith, the framework dampened their effect (for decreased values R was as low as 0.02, and for increased values R was as low as 0.09). An increase in V_d was dampened by the framework (R= 0.5 to 0.9), whereas a decrease in V_d was amplified by the framework (R=1.1 to 10). The output was not very sensitive to changes in exposure time due to the $t_{1/2}$ of the unperturbed model being 8.2 h. There was negligible effect of extending the modelling beyond 7 d (at 22 wk, nadir only 0.00007% lower and zenith only 0.00001% lower). Indeed, differences after 1, 2 and 3 days with concentrations after 22 wk exposure were: 1 day: nadir = -13.2%, zenith =-1.95%; 2 days: nadir = -1.73%, zenith =-0.26%; 3 days: nadir = -0.23%, zenith =-0.03%. This suggests time to stable tissue concentrations in birds is likely rapid.

There are several areas of uncertainty in the framework. First, the framework presented does not account for degradation of APIs in the WWTP [79-81], which could lead to overestimation of PEC_{food}. However, in the present study PEC_{food} and MEC_{food} for fluoxetine happened to be well matched. Second, the foraging behaviors of free-ranging birds can be quite variable; this could affect the extent to which individuals routinely ingest earthworms contaminated with pharmaceuticals. Third, in terms of pharmacokinetics it would be beneficial to know V_d and F_{bioav} for multiple APIs and how the physiologies of birds relate to laboratory mammals. Fourth, overestimation of CBB_{zenith} and underestimation of CBB_{nadir} would result from assumptions that the dose is taken up in a single meal. In reality, many species feed throughout the day and are often opportunistic (gorge themselves). Finally, once APIs are distributed into liver, some fraction may end up in bile, re-enter the intestine and blood stream, and return to the liver (entero-hepatic recirculation [82]).

Uncertainty in the framework was greater at nadir than zenith. Using the 27 different combinations of input parameters, mean ± SE brain concentration at nadir was 0.47±0.13 ng/g (CV=146.2%) and zenith was 2.70 ± 0.47 ng/g (CV= 90.5%). However, uncertainty could be reduced at nadir to, 0.58 ± 0.13 ng/g (CV = 117.1%) and at zenith to, 3.35 ± 0.39 ng/g (CV= 60.9%) by excluding fluoxetine concentrations at one of the four WWTPs where values were much lower. While the CV is relatively high, it is important to view this uncertainty analysis in perspective (i.e., describing relative change in the low ng/g to pg/g concentration range). Therapeutic effects of fluoxetine in humans and laboratory mammals are only found at µg/g levels. Thus, the uncertainty of the framework does not change the conclusions about the likelihood of adverse effects. It is important to acknowledge that this is a preliminary framework upon which we hope others can build and integrate in ecological risk assessment.

The present framework illustrates that substituting rodent fluoxetine ADME data for starling ADME data predicts low absorption and rapid elimination, but markedly overestimates distribution into brain. Nonetheless, predicted internal concentrations are still within a few ng/g of measured internal concentrations in experimentally dosed starlings. If we had compared our starling ADME to that of humans, the low absorption of fluoxetine by starlings and absence of accumulation after such an extended dosing period would have been problematic. However, using rodent ADME and inverse modelling to calculate PEC_{tissue} for starlings that were chronically dosed at 5% HTD, we would expect residue levels to be 1000 times less than the therapeutic concentration.

Data needs

In order for interspecific pharmacokinetic extrapolations to work as a predictive framework for internal API concentrations in wild birds, we must gain a better understanding of the physiological and pharmacological differences between wild birds, mammals and humans. For example, estimates of PEC_{plasma} could be improved using a combination of in silico (literature review of current knowledge on interspecific differences in GI tract physiology), in vitro (assessments of bioaccessibility [68],

CYP450 expression [77], protein binding and active transport of APIs and receptor sensitivity, see Supplemental Data for discussion) and in vivo approaches (intravenous injection compared with oral to assess bioavailability, define plasma concentrations at higher doses than environmentally relevant concentrations to exceed LOD). It would be feasible to determine absorption (compare plasma uptake curve between oral and intravenous administration) and distribution into key sites of pharmacological activity. It would be beneficial to identify and conduct these in vivo assessments in several model wild bird species that could be considered representative of key Orders of birds likely exposed to APIs (e.g., passerine, waterfowl, gallinaceous birds, corvids, gulls and even raptors). To ensure the elimination-curve can be captured, post-exposure time-points should be limited to 24 h, rather than 74 h as used herein.

Once the predictive framework is validated for one exposure pathway, the next challenge is to expand the scope of the framework to include uptake of multiple APIs in various food webs (e.g., plants/fruits/ seeds grown in soil amended with sludge, aerial insectivores above WWTPs, wading birds in estuarine sediments). Field measurements of APIs soils, sediments and aquatic environments as well as the wildlife food items that grow and develop therein, could enable estimation of BAFs. These could be applied to expand the predictive framework. It will also be important to understand the extent to which individuals rely on contaminated sources for nutrition. This could be achieved through observations [4], remote tracking methods [83] and next generation sequencing of fecal samples (e.g., [84]).

CONCLUSIONS

Dietary exposure of wild birds to APIs is plausible, but at present there is limited quantitative evidence to support this assertion, and even less for estimating hazard and risk. Gaining an improved understanding of API ADME for wildlife, and its relation to rodent and human ADME is important. This would enable the use of a wealth of information on effects of APIs gathered from pre-clinical and clinical trials for the conduct of ecological risk assessments.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability—The predictive framework is available as a Supplemental Data file and data are available from the senior author upon request (emailaddress).

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Figure 1. Schematic of predictive framework for evaluation of exposure and risk of APIs in WWTPs for wild birds. Predicted Environmental Concentrations (PEC) in influent wastewater and earthworms are used to estimate risk of therapeutic or adverse effects based on dietary intake (relative to Human Therapeutic Dose), similar to TGD approach. The TGD approach is then extended using pharmacokinetic data for laboratory mammals and humans to calculate PECs in plasma and tissues for improved risk assessment based on internal residues.

Figure 2. $PEC_{post\text{-}exposure}$ and in vivo data for fluoxetine in starling a) brain, b) liver, c) kidney and d) muscle over 4 days after the final dose. The error bars represent the minimum and maximum extent of the range, the horizontal y-reference line represents the limit of quantification (LOQ). Note the different y-axis scales.

Table 1: Parameters used in predictive modelling of internal concentration

PEC	Parameter		Value	Units	Ref
Wastewater	M	Fluoxetine usage/yr	4639.8	Kilograms per year (kg yr^{-1})	NHS 2010[42]
	$F_{excreted}$	Proportion excreted as fluoxetine	0.24	Unitless proportion	Lienert et al. 2007 [43]
	D	Dilution in the environment	3.7741×10^{12} (200 L per capita/per day Population of England 51.7 million)	liters (of water per year) (L)	Williams 2005 [44]
Food	BAF	Bioaccumulation factor including pore water	30.8 (25.4-35.8)	Unitless ratio	Carter et al. 2014[45]
Plasma	BW	Body weight of a starling	~80 (72-100)	Grams (g)	Robinson 2016 [49]
	DI	Daily intake of earthworms wet weight	15-38.5	Grams wet weight g (w/w)	Markman et al 2008 [50] East and Pottinger [51]
	T_{exp}	Duration of exposure	22	Weeks	Bean et al 2014 [3]
	$T_{1/2}$	Elimination half-life	3.6-12.9 (lab mammals) 24-96 (humans)	Hours (h), but convert to days for calculations	Vartazarmian et al 2005 [41] Caccia et al 1990 [37] Hiemke and Hartter 2000 [46]
	F_{bioav}	Fraction of the dose that is bioavailable	0.38 (lab mammals) 0.6-0.8 (humans)	Unitless proportion	Caccia et al 1990 [37] Eli Lilly 2009 [47]
	V_d	Volume of distribution	27 (lab mammals 14-100 (humans)	L/kg BW	Holladay et al 1998 [38] Hiemke and Hartter 2000 [46]
Tissue	$R_{tissue:plasma}$ ***for brain***	Ratio of fluoxetine in brain tissue relative to plasma	9-89 (lab mammals) 10-20 (humans)	Unitless ratio	Caccia et al 1990 [37], Unceta et al 2007 [40] Hiemke and Harter 2000 [46]
	$R_{tissue:plasma}$ ***for liver***	Ratio of fluoxetine in liver tissue relative to plasma	2 (lab mammals) 38 (humans)	Unitless ratio	Lefebvre et al 1990 [48] Lewis et al 2007 [52]
	$R_{tissue:plasma}$ ***for kidney***	Ratio of fluoxetine in kidney tissue relative to plasma	No data (lab mammals) 9 (humans)	Unitless ratio	Lewis et al 2007 [52]
	$R_{tissue:plasma}$ ***for muscle***	Ratio of fluoxetine in muscle tissue relative	No data (lab mammals)	Unitless ratio	

		to plasma	2.2 (humans)		Lewis et al 2007 [52]
Post-exposure	$T_{1/2}$	Elimination half-life	3.6-12.9 (lab mammals) 24-96 (humans)	Hours (h)	Vartazarmian et al 2005 [41]; Caccia et al 1990 [37] Hiemke and Hartter 2000 [46]

Table 2: Fluoxetine concentrations in influent wastewater, earthworms and starling tissues and feces after 22 weeks of treatment and in plasma after 16 weeks of treatment.

Influent ng/mL	*Eisenia fetida* (ng/g)	Time post-exposure (h)	Plasma ng/mL	Brain ng/g	Liver ng/g	Kidney ng/g	Muscle ng/g	Feces ng/g
FLUOXETINE								
1.31±0.23 (6/6)	26.20±4.70 (12/12)	2	<LOD 0/12	3.05±1.50 (3/3)	14.42±4.36 (3/3)	7.34±1.38 (3/3)	2.95-2.97* (2/3, 1<LOD)	1487.3±1223.4 (3/3)
		26	No Sample	- (3<LOD)	- 3<LOD	1.61-1.62* (2/3, 1<LOD)	1.94 (1>LOD, 2<LOD)	62.43 (1/3, 2<LOD)
		50	No Sample	- (3<LOD)	1.21 (1/3, 2<LOD)	- (3<LOD)	1.72 (1>LOD, 2<LOD)	3.47 (1>LOD, 2<LOD)
		74	No Sample	- (3<LOD)	- (3<LOD)	- (3<LOD)	- (3<LOD)	- (3<LOD)
CONTROLS								
<LOD	<LOD	2, 26, 50 and 74	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Concentrations are mean ±SE (detection frequency); * Kaplan-Meier mean calculated where fluoxetine was detected in ≥2 samples; raw data presented when detected in only one sample.

Concentrations in all tissues are given on a wet weight basis, feces on a dry weight basis

Fluoxetine limit of detection, LOD (with LOQ in parenthesis) in ng/g: influent 0.6 ng/L (2 ng/L); earthworm 0.45 ng/g (1.5 ng/g); brain 0.03 (0.15); kidney 0.03 (0.53); liver 0.07 (0.41); muscle 0.04 (2.97), feces 1.78 (5.92); plasma in ng/mL 0.15 (0.51).

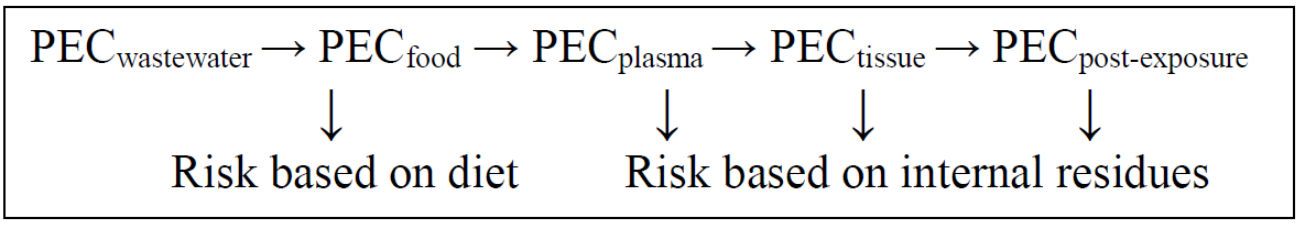


Figure 1

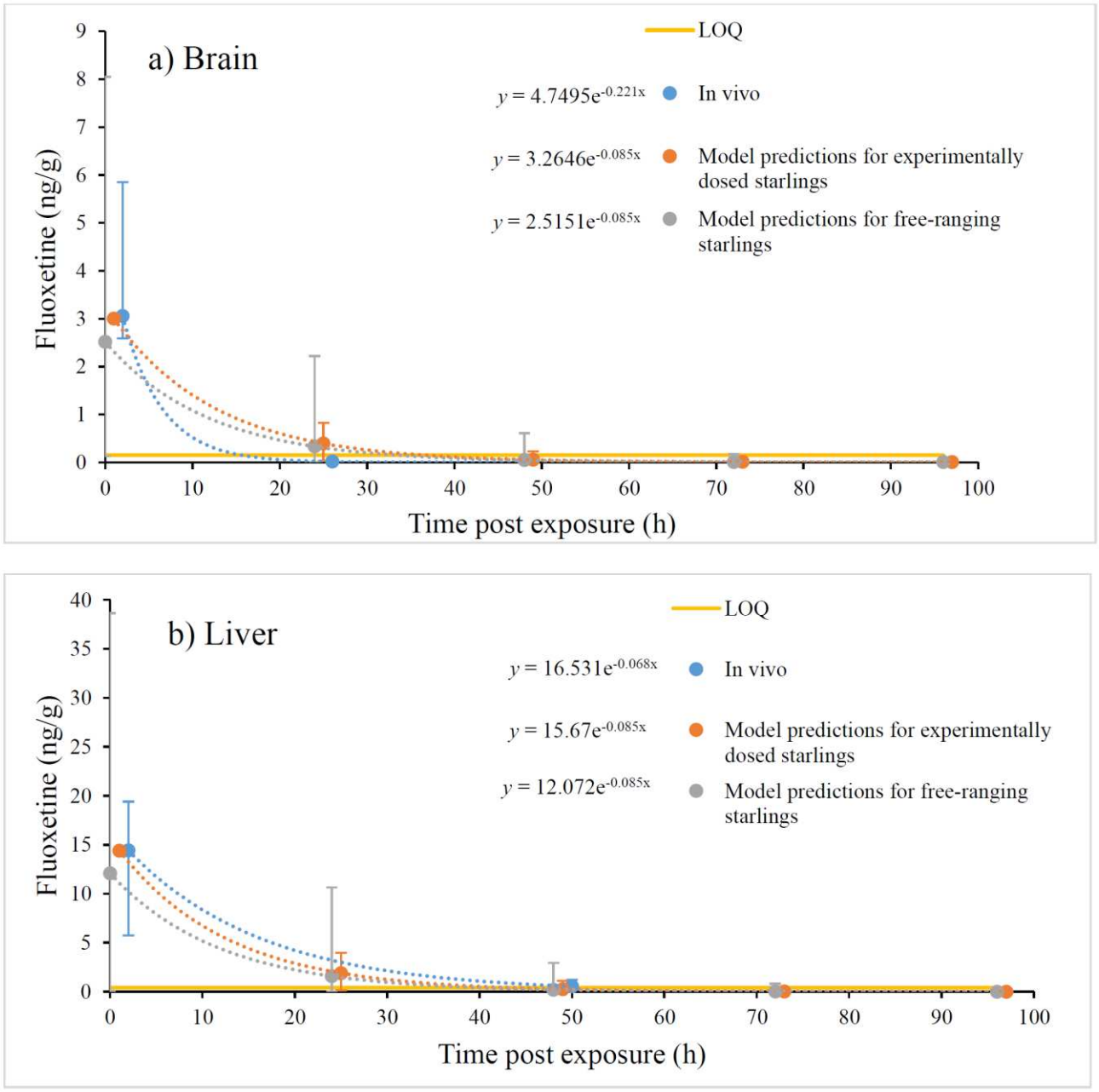
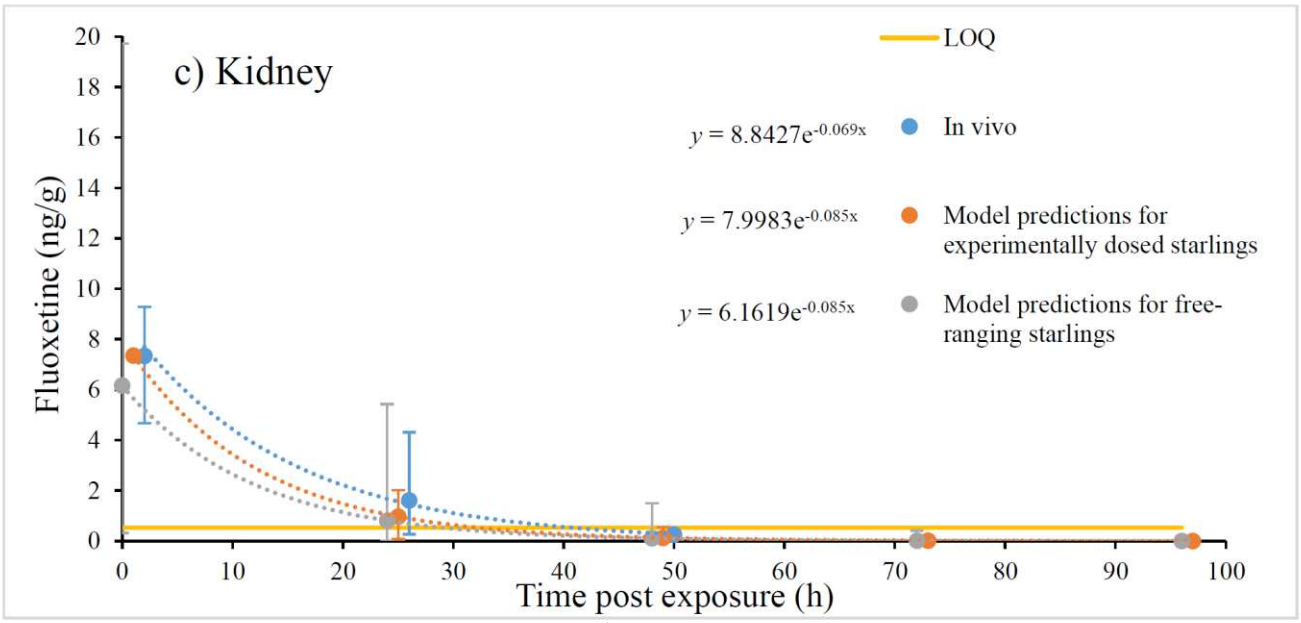


Figure 2



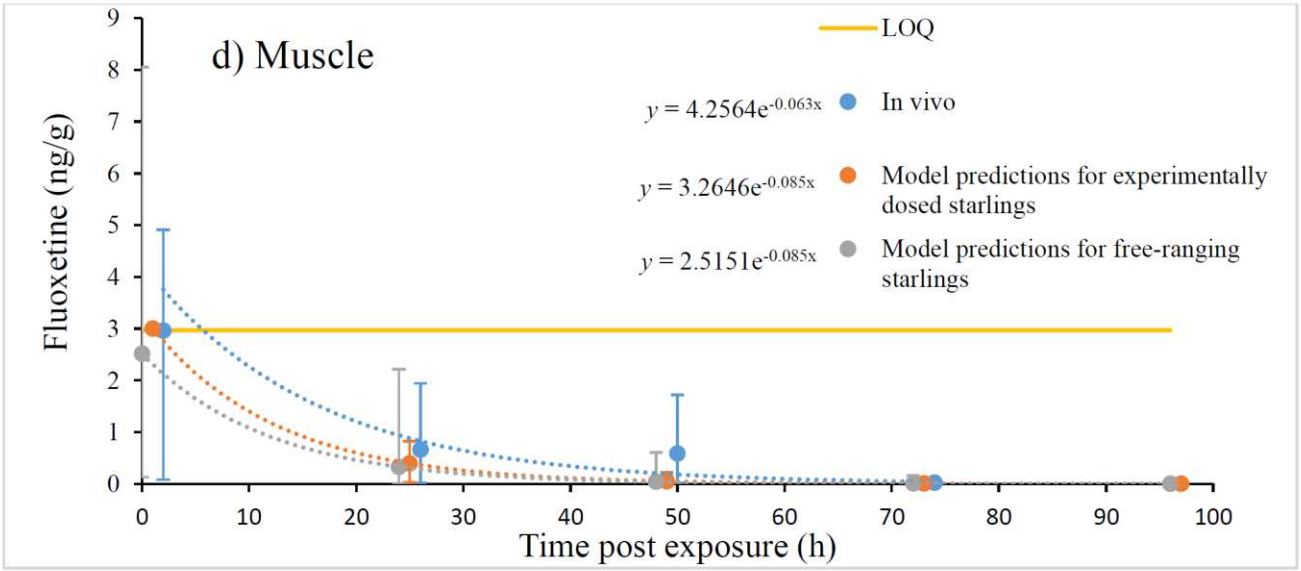


Figure 2