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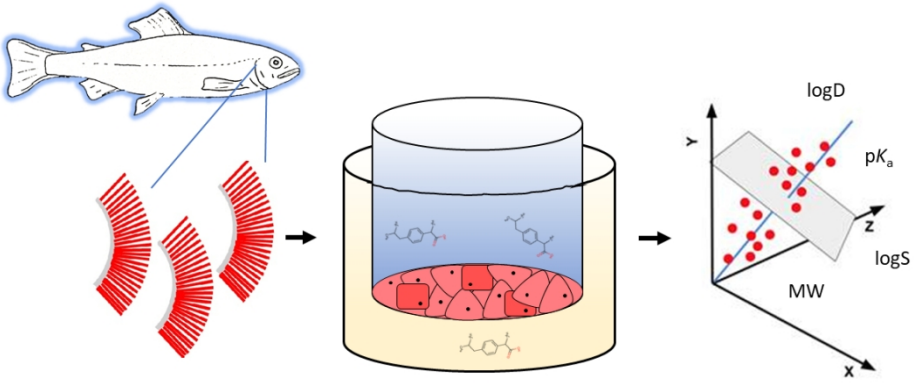

Graphic abstract

338x190mm (96 x 96 DPI)

The use of molecular descriptors to model pharmaceutical uptake by a fish primary gill cell culture epithelium

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Abstract

Modelling approaches, such as Quantitative Structure-Activity Relationships (QSARs) use molecular descriptors to predict the bioavailable properties of a compound in biota. However, these models have mainly been derived based on empirical data for lipophilic neutral compounds and may not predict the uptake of ionizable compounds. The majority of pharmaceuticals are ionizable and freshwaters can have a range of pH values that will affect speciation. In this study we assessed the uptake of 10 pharmaceuticals (acetazolamide, beclomethasone, carbamazepine, diclofenac, gemfibrozil, ibuprofen, ketoprofen, norethindrone, propranolol and warfarin) with differing modes-of action and physicochemical properties (pKa, logS, logD, logKow, molecular weight (MW) and polar surface area (PSA)) by an *in vitro* primary fish gill cell culture system (FIGCS) for 24 h in artificial freshwater. Principal component analysis (PCA) and partial least squares (PLS) regression was used to determine the molecular descriptors that influence the uptake rates. Ionizable drugs were taken up by FIGCS and a strong positive correlation was observed between logS and a negative correlation observed between pK_a, logD, MW and the uptake rate. This approach shows that models can be derived based on physicochemical properties of pharmaceuticals and using an *in vitro* gill system to predict uptake of other compounds. There is a need for a robust and validated model for gill uptake that could be used in a tiered risk assessment to prioritize compounds for experimental testing.

Introduction

Pharmaceuticals are biologically active molecules that have been detected in surface waters at ng to μg L^{-1} concentrations[1] and are widely reported in aquatic fauna[2]. The impacts associated with pharmaceutical exposure on aquatic organisms is unclear and knowledge is necessary to inform regulatory authorities and the pharmaceutical industry of compounds that may pose a risk[3].

As part of a chemical risk assessment it is necessary to determine the likelihood to bioaccumulate. A bioconcentration factor (BCF) is a measure which includes uptake (k1) and elimination rates (k2) and internal steady state concentration[4, 5]. However, the uptake process, along with metabolism, represent the largest factors of uncertainty in fish bioaccumulation models[6,7], and BCF values for individual compounds derived from *in vivo* studies can vary substantially[8]. Consequently, a novel approach to evaluating the bioavailability properties of a chemical has been proposed which utilizes non-guideline methodologies in a tiered risk assessment[8]. In this approach, *in silico* or *in vitro* data may be used in the lower tiers to assess a chemical's bioavailability; if there is enough information to classify bioaccumulation potential then a decision can be made as to whether further BCF studies are required[8].

Many mechanistic assessments of contaminant uptake are based on *in silico* models such as Quantitative Structure-Activity Relationships (QSARs), that have largely been derived from data for lipophilic neutral compounds that passively diffuse across lipid membranes and undergo little to no metabolism[6]. QSAR can include linear based estimations or more recently machine learning applications such as neural networks and tree-based learning to predict organic chemicals bioconcentration[9]. However, an estimated 77.5% of pharmaceuticals are ionizable[10]. Thus, the applicability of QSAR models developed on other contaminant classes (i.e. neutral hydrophobic contaminants) may be limited and inaccurately estimate the accumulation of pharmaceuticals. For compounds that are ionizable the acid-base

68 dissociation constant (pK_a) describes the dissociation of the drug at a given pH and influences
69 solubility, lipophilicity, permeability and protein binding[11]. In the aquatic environment, surface
70 water pH will determine chemical speciation, and this is predicted to have an influence on
71 bioavailability[12]. The typical pH of environmental water ranges between 6-9[12] although fish can
72 be found in bodies of water that are extremely acidic (pH 3)[13] and highly alkaline (pH 10.5)[14].
73 The effect of pH on the toxicity[12] and uptake/elimination of ionizable compounds in fish has
74 been demonstrated[15-17]. Recently, Bittner et al.[18] have demonstrated the impact of pH (5.5 –
75 8.6) on the uptake and toxicity of beta-blocker pharmaceuticals in zebrafish larvae; where the
76 skin is likely to be the significant route of uptake. Karlsson et al.[19] have examined the effect of
77 water and sediment pH (5.5 – 8.5 pH) on uptake of 3 pharmaceuticals uptake with a range of
78 pKa (4.01 – 9.62 pKa) in the freshwater oligochaete *Lumbriculus variegatu*s.
79
80 There is a desire towards the development of *in vitro* models to replace or supplement current
81 animal experimental procedures[20], in accordance with the replacement, reduction, and
82 refinement (3Rs) principle[21]. This is also reflected in European legislature that states non-
83 animal alternative approaches should be used in place of animal procedures wherever
84 possible. A fish gill cell culture system (FIGCS) was developed using primary fish cells that
85 has shown promise as an alternative system for whole fish chemical uptake studies[22]. FIGCS
86 maintains many of the characteristics of the *in vivo* epithelium, including the presence of
87 multiple cell types associated with transport of ions across the gills and the ability to tolerate
88 freshwater water application to the apical surface. The *in vitro* data obtained from FIGCS
89 experiments has the potential to be an important component of the lower tier in a tiered testing
90 system[8] as the gills are a primary route of uptake in fish[22].It has recently been used to
91 investigate the absorption of 7 pharmaceuticals with a similar pK_a of 8.1 to 9.6 across the gill[23].
92
93 There is a paucity in fish pharmaceutical uptake and BCF values because the tests to derive
94 these use a large number of organisms, are time consuming and expensive to conduct. In this
95 study we assessed the uptake of 10 pharmaceuticals by an *in vitro* fish gill model with differing

physiochemical properties and there were three aims. Firstly, to assess the uptake of ionizable pharmaceuticals by this fish gill epithelium. Secondly, to demonstrate how an *in vitro* epithelial model can be used to evaluate the propensity for a drug to enter a fish from the water and how this information could form part of a tiered risk assessment approach[8,24]. Thirdly, Lipsinki et al.[25] proposed that the molecular properties (molecular weight, hydrogen bond donors and acceptors and logK_{ow}) of a chemical can be used as a screening tool to determine the likelihood of absorption across a membrane, we extended this concept and used the pharmaceutical molecular descriptors and partial least squares (PLS) regression analysis to model uptake rate and identify those descriptors that influence gill uptake. We were able to show that solubility, pKa, octanol-water distribution coefficient and molecular weight are the most important descriptors driving epithelial drug uptake rates.

Materials and Methods

Fish gill cell culture system (FIGCS)

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from a local trout farm and housed in dechlorinated-aerated City of London tap water ([Na^+]=0.53mM, [Ca^{2+}]=0.92mM, [Mg^{2+}]=0.14mM, [K^+] = 0.066 mM and [NH_4^+] = 0.027 mM). Temperature was maintained at 14°C with a 14h light:10h dark cycle and fish were fed a 1% (w/v) ration of trout pellets daily.

Primary fish gill cell culture inserts were prepared in companion wells and maintained according to protocols described in Schnell et al.[22]. The transepithelial resistance (TER) was monitored daily using an epithelial tissue voltohmmeter (EVOMX) with STX-2 chopsticks (World Precision Instruments). A TER value of above 3,000Ω cm^{-2} was used as criteria for the

122 presence of a tight epithelium, as previously determined using ^{14}C-mannitol as a paracellular
123 permeability marker[23].
124
125 **Pharmaceuticals exposures and cell viability assay**
126
127 Analytical grade pharmaceuticals (purity ≥97%) from differing classes of action with differing
128 chemical properties (Table 1) were purchased from Sigma Aldrich, and included
129 acetazolamide (CAS: 59-66-5), beclomethasone (CAS: 4419-39-0), carbamazepine (CAS:
130 298-46-4), diclofenac sodium salt (CAS: 15307-79-6), gemfibrozil (CAS: 25812-30-0),
131 ibuprofen sodium salt (CAS: 31121-93-4), ketoprofen (CAS: 22071-15-4), norethindrone-19
132 (CAS: 68-22-4), propranolol hydrochloride (CAS: 318-98-9) and warfarin (CAS: 81-81-2).
133 Pharmaceutical stocks were prepared at a concentration of 1 mg mL^{-1} in methanol or ethanol
134 and stored at -80°C.
135
136 Following formation of a tight epithelium inserts were prepared for exposure by washing with
137 phosphate buffered saline. The apical freshwater (AFW) used for apical exposure was
138 prepared according to $OECD_{203}$ Test Guidelines[26] (2 mM $CaCl_2$; 0.5 mM $MgSO_4$; 0.8 mM
139 $NaHCO_3$, 77.1 µM KCl, with a measured pH 7.6) with individual pharmaceuticals added at a
140 concentration of 1 µg mL^{-1}, which is equivalent to: 450 nM acetazolamide, 245 nM
141 beclomethasone, 423 nM carbamazepine, 338 nM diclofenac, 399nM gemfibrozil, 485 nM
142 ibuprofen, 393 nM ketoprofen, 355 nM norethindrone, 386 nM propranolol, and 324 nM
143 warfarin. To expose cells 1.5 mL of exposure water was added to the apical compartment and
144 2 mL of L15 media with 5% FBS to the basal compartment. The inserts, and 1.5 mL exposure
145 water samples (T_0) were incubated at 18 °C in the dark for 24 hrs. In the case of the T_0 samples
146 this was to assess if the compounds remained stable over the 24 hrs exposure period at 18°C
147 in the absence of cells. The T_0 and the 1.5 mL apical compartment water samples after 24 hrs
148 exposure (T_{24}) were collected and stored at -80°C for further analysis. Measurements were

149 made on 4 inserts derived from 2 to 3 biological replicates, with each biological replicate
150 comprising of cells harvested from 2 fish.
151
152 To consider the adhesion of the compounds to the companion well and insert membrane
153 during 24 hrs of exposure, a cell-free experiment was performed. To assess pharmaceutical
154 toxicity, single seeded primary gill cells were grown in T75 flasks to 80% confluent, then
155 trypsinized and transferred to 96-well at a density of $1x10^5$ cells $well^{-1}$. Twenty-four hours post-
156 seeding in the 96-well plates cells were exposed to pharmaceuticals at 1 µg mL^{-1} in L15 with
157 5% FBS for 24 hours, after which a MTT viability assay 3-(4,5-dimethylthiazol-2-yl)-2,5-
158 diphenyltetrazolium bromide) was performed following methods adapted from Riss et al.[27]. pH
159 stability of the AFW with 1µg ml^{-1} of compound was measured with and without cells and found
160 to be stable over a 24 hour period.

161 **HPLC Analysis**

162
163 For HPLC analysis individual T_0 or T_{24} samples were pooled into three separate mixtures: Mix
164 A included beclomethasone, ibuprofen and warfarin, Mix B included carbamazepine,
165 diclofenac, gemfibrozil, ketoprofen and norethindrone and Mix C included acetazolamide and
166 propranolol for solid phase extraction (SPE) with Oasis HLB cartridges (200mg sorbent, 6cc).
167 Cartridges were initially conditioned with 6 mL methanol (HPLC grade) followed by 6 mL water
168 (HPLC grade) then loaded with either pooled sample Mix A, B or C. Cartridges were washed
169 with 4 mL water, dried under vacuum pressure and eluted with 6 mL methanol or stored at -
170 80°C for later elution. Samples were then dried under nitrogen at 45°C for 80 min (Biotage
171 TurboVap), reconstituted in 500 µL 90:10 (v/v) water:acetonitrile and vortexed for 2 minute
172 before transfer to amber HPLC vial for analysis.
173
174
175 Liquid chromatography was performed on an Agilent 1260 Infinity series LC system using a
176 Waters Sunfire C_{18} column (100 Å, 3.5 µm, 4.6 mm x 150 mm) at a flow rate of 0.2 mL min^{-1}

177 and injection volume of 20 µL. Mobile phase A and B consisted of HPLC grade water and
178 HPLC grade acetonitrile, respectively, with initial running conditions of 10% phase B at a
179 column temperature of 40 °C. The gradient elution was as follows; linear ramp with phase B
180 increased to 80% at 12 min, held for 13 min, then returned to initial conditions at 28 min. Total
181 run time was 40 min including a 12 min re-equilibration period. An Agilent 1290 Infinity Diode
182 Array Detector was used for detection of diclofenac and warfarin at 214 nm; carbamazepine,
183 ibuprofen and gemfibrozil at 220 nm; beclomethasone and propranolol at 230 nm;
184 norethindrone at 254 nm; ketoprofen at 263 nm and acetazolamide at 273 nm.
185
186 Method performance was assessed by matrix-matched calibration curves generated for the
187 AFW. Method linearity (5 concentrations, n=3) was determined from 0.5 -2.5 µg mL^{-1} and
188 signal to noise ratio of 3:1 and 10:1 of low concentration spiked samples was used to
189 determine the LOD and LOQ, respectively (n=6). Precision was determined using spiked
190 samples at 1 µg mL^{-1}(n=6) and accuracy was determined using spiked samples and values
191 from method linearity (n=6). Recovery was assessed by comparing spiked samples (pre-
192 extraction) to post-extract spiked samples at a concentration of 0.5, 1 or 2 µg mL^{-1} (n=3).
193
194 **Estimation of Gill Uptake Rates**
195
196 Primary gill cell culture pharmaceutical uptake was calculated based on the loss of compound
197 from the apical compartment corrected for the amount that adhered to the polystyrene plastic
198 of the companion wells and inserts without cells over 24 hrs (Equation 1).
199
200 Uptake rate (nmol cm^{-2} h^{-1}) = $(T_0\text{-}T_{24}) – (T_0^p – T_{24}^P)] / (t \times cm^2)$ Eqn. 1
201
202 Where T_0 and T_{24} represents the moles (nmoles) of drug present in the apical compartment in
203 the presence of cells at 0 and 24 hrs, respectively, and T_0^p and T_{24}^p represent the moles of
204 drug present in the apical compartment in the absence of cells at 0 h and 24 h, respectively;

t= time of the flux measurement (24 hrs) and cm^2 represents the surface area of the epithelium ($0.9\ cm^2$). Sorption controls (inserts and exposure media only) were setup to account for any losses of compound through volatilization, sorption to plastics and any other degradative processes. Insert controls showed that these processes were negligible and therefore in the presence of cells disappearance of compound is related to the uptake of the compound into the gill epithelium and transfer across into the basolateral layer over 24 hours.

Statistics and Modelling Approaches

A one-way ANOVA followed by a Tukey's post hoc test was performed to compare the uptake rates of each compound using GraphPad Prism 6.0. Modelling approaches used 6 molecular descriptors (Table 1) including; the acid dissociation constant (pK_a), the octanol water distribution coefficient at pH 7.4 (logD), the octanol-water partition coefficient ($logK_{ow}$), polar surface area (PSA) and molecular mass (M_w). The two descriptors $logK_{ow}$ and logD are both measures of hydrophobicity, but logD takes into account both neutral and ionizable species at a given pH whereas $logK_{ow}$ only takes into account the neutral fraction. Principle component analysis (PCA) and partial least squares (PLS) regression were performed using the R statistical computing language, R version 3.4.3 (freely available at https://www.r-project.org/). All scripts were written with RStudio (freely available at https://www.rstudio.com/), packages used for PCA and PLS analysis were *stats* and *plsdepot*, respectively. Full dataset used in modelling, latent variable scores, loadings, weights and cross-validation of models are given in the SI (Figure S3 and Tables S4-S7). For cross-validation of the PLS model, a leave-one-out approach was used.

Results

231 Cell viability was assessed by MTT assay and none of the pharmaceuticals at a concentration
232 of 1 μg mL^{-1} showed signs of cytotoxicity (Supporting Information (SI), Figure S1) and HPLC
233 method performance assessment is provided in the supplementary data (SI Table S1 and S2).
234
235 Pharmaceutical adhesion to the companion wells over 24 hrs was between 0.7 to 5% (data
236 not shown) and was taken into consideration when calculating uptake rates. Acetazolamide
237 uptake (0.125 ± 0.032 nmoles cm^{-2} h^{-1}) was significantly greater than beclomethasone,
238 carbamazepine, diclofenac and norethindrone (beclomethasone 0.021 ± 0.015 nmoles cm^{-2} h^{-}
239 ^{1}, carbamazepine 0.022 ± 0.004 nmoles cm^{-2} h^{-1}, norethindrone 0.024 ± 0.003 nmoles cm^{-2}
240 h^{-1}, diclofenac 0.027 ± 0.003 nmoles cm^{-2} h^{-1}) (Figure 1). The other ionizable drugs, except for
241 diclofenac , showed higher, but not significantly higher uptake rates (ibuprofen 0.072 ± 0.013
242 nmoles cm^{-2} h^{-1}, gemfibrozil 0.075 ± 0.007 nmoles cm^{-2} h^{-1}, ketoprofen 0.061 ± 0.006 nmoles
243 cm^{-2} h^{-1}, propranolol, 0.095 ± 0.026 nmoles cm^{-2} h^{-1} and warfarin 0.070 ± 0.012 nmoles cm^{-2}
244 h^{-1}) compared to the neutral drugs, beclomethasone, carbamazepine, and norethindrone
245 (Figure 1, and SI Figure S2).
246
247 Modelling of the molecular descriptors was performed using PCA analysis to identify
248 compound similarity (Fig 2). The first two principal components explained a cumulative
249 variance of 69% (PC1 = 48%, PC2 = 21%) in the descriptor space. The score plot indicates
250 that there were no apparent outliers in the dataset. Clustering of compounds was minimal but
251 was expected with the low number of cases available for modelling (n=10). The largest
252 variation in the descriptor space was observed for the compound beclomethasone (Fig. 2).
253 The variance of this case can be explained in terms of the loadings, where this compound was
254 the largest (MW = 408.92) and most hydrophobic (logD = 4.16) of all compounds that were
255 tested. From the descriptor loadings, logS and MW were negatively correlated with each other.
256 The loadings for the first latent variable also showed that logD (0.567), MW (0.505), logS (-
257 0.444) and pK_a (0.407) were more important variables than logK_{ow} (0.201) or PSA (0.146).
258

PLS was implemented to interpret molecular descriptors that influence gill uptake rates of pharmaceuticals and enable a predictive modelling approach with:

Uptake Rate (nmoles cm^{-2} h^{-1}) = 1.23E-01 + (-1.59E-03 x pK_a) + (1.53E-02 x logS) + (-7.26E-03 x logD) + (9.15E-03 x $logK_{ow}$) + (-9.48E-05 x MW) + (3.83E-04 x PSA) Eqn. 2

The adjusted correlation coefficient (R^2_{adj}) and the cross-validated R^2 (Q^2) of the PLS regression model was 0.7863 and 0.5397, respectively. No cases were observed as outliers in the PLS model determined by the Hotelling's T^2 95% confidence ellipse (data not shown). Based on the cumulative Q^2 statistic (see SI Figure S4), the optimal number of latent variables for the PLS model was two. The loadings plot (Figure 3a) indicated that logS was positively correlated with gill uptake whereas logD, pK_a and MW were negatively correlated with gill uptake. The $logK_{ow}$ and PSA descriptors were relatively less important for modelling gill uptake when compared with the previously mentioned descriptors. The use of PLS to predict gill uptake showed good performance with the mean absolute error of 0.01 ± 0.01 nmol cm^{-2} h^{-1} (MAE±SD) for all compounds tested. Larger inaccuracies in the predictions were observed for the four compounds; carbamazepine (122%), diclofenac (61%), norethindrone (35%) and propranolol (32%) (Fig 3b and c).

Discussion

The uptake rate of 10 pharmaceuticals by a fish primary gill cell culture system was assessed. From our dataset we were able to demonstrate that a PLS regression model based on the drug molecular descriptors could be developed for pharmaceutical uptake rate by this epithelium, with LogS, pK_a, logD and MW found to be the most important descriptors driving epithelial drug uptake rates.

287 The uptake rates of the compounds from the apical compartment reflect the apparent epithelial
288 permeability (P_{app}) of the compound by the FIGCS cells. The P_{app} can be expressed as an
289 equation (equation 3) and takes into consideration four factors: partitioning in the aqueous
290 boundary layer (P_{ABL}, ABL), adhesion to filter insert (P_f) and transcellular (trans) or paracellular
291 (para) transfer (P_{trans}, P_{para})[28]. The aqueous boundary layer is assumed to have a distinct
292 boundary with the bulk water adjacent to both sides of the membrane[29].
293
294 295

$$\frac{1}{P_{app}} = \frac{1}{P_{ABL}} + \frac{1}{P_f} + \frac{1}{P_{trans} + P_{para}} \qquad \text{Eqn. 3}$$

296
297 Two of the four factors can be discounted, due to the nature of the system. Firstly, adhesion
298 to plastic (P_f) and inserts was taken into consideration when calculating the uptake rate.
299 Secondly, a previous study conducted using the paracellular marker ^{14}C-mannitol determined
300 that at TER values above 3,000Ω cm^2 the FIGCS were relatively impermeable to the marker
301 and indicated that transport was via transcellular routes[23], all compounds in the current study
302 have a greater molecular weight than mannitol thus paracellular transfer (P_{para}) was negligible.
303 Thus, the uptake rates reflect partitioning in the ABL (P_{ABL}), uptake into cells and across the
304 basolateral membrane (P_{trans}) into the basolateral compartment, in addition to any potentially
305 metabolized compound efflux from the gill cells back into apical compartment.
306
307 All uptake studies were conducted in AFW and under these conditions acetazolamide (71.5%),
308 diclofenac (100.0%), gemfibrozil (99.9%), ibuprofen (99.8%), ketoprofen (99.9%), propranolol
309 (98.5%) and warfarin (99.7%) are all predicted to be ionized (% ionized in parentheses),
310 whereas beclomethasone, carbamazepine, and norethindrone are not ionized. All ionizable
311 drugs, except diclofenac, showed higher permeation into the primary gill cell epithelium when
312 compared to the neutral drugs (Figure 1). The observation that ionizable drugs are capable of
313 permeating the gill epithelium corroborates a previous study in FIGCS concerning the uptake
314 of a set of pharmaceuticals with pK_a between 8.1- 9.6[23] and a number of studies suggest that
315 ionizable compounds can be taken up by the fish gill[e.g. 15,16,18,19,23,30,31]. The uptake of 9 weakly

acidic chlorinated phenols by rainbow trout did not vary between pH 6.3 to 8.4 despite the proportion of the compounds ranging in ionization from 1 to 99 %[15], the accumulation of the weak basic diphenhydramine (pK_a 9.1) at 10 µg L^{-1} reached steady state in fathead minnow at pH 7.73 and 8.63 after ~24 h and only at pH 6.87 was accumulation greatly reduced[31] and ionizable surfactant[32], perflouroakyl acids[33] as well as phenols and carboxylic acids[34] have been observed to cross the gills of fish. However, membrane permeation may be an order of magnitude less than for the neutral form[30]. Erickson et al.,[16] developed a mechanistic model of ionized organic chemical uptake at the fish gill which expanded on an original model for unionized chemical uptake[35,36]. This new model included a factor that takes into account the ability of the fish to alter the pH adjacent to the apical membrane and thus generating a microclimate that differs to the bulk water[37]. These changes in pH at the gill surface helped to explain uptake of diphenylamine[31] and the chlorinated phenols[15,16]. However, in the current study uptake of the acidic and basic pharmaceuticals showed similar uptake rates, and if uptake is solely due to the neutral form of the drug, then pH of the culture epithelial microclimate would have to be in the region of pH 3 to ensure the weakly acidic drugs (pK_a 4 – 5.08) were unionized. It is also unlikely that uptake is solely due to the ionized form because the basic drugs show similar uptake rates (Figure 1 and [23]). In contrast to the other ionizable compounds, diclofenac is the only drug that exhibited a relatively lower uptake rate. It is unclear why this may be, but of the drugs used in the current study the structure of diclofenac is more complex containing both an amine and carboxylic acid group and lacks conformational flexibility[38] that may influence transport by the gill epithelium.

The PLS modelling approach showed that all descriptors here have an influence on the uptake rate but more of the explained variance was correlated to the logS, logD, pKa and MW descriptors. The regression model (Equation 2) showed a good potential to predict uptake rates in the FIGCS system at the tested concentration and water chemistry (r^2 = 0.786). Modelling is an important aspect to understanding fate of pharmaceuticals in the aquatic environment and these approaches are complementary to *in vitro* systems for the replacement

344 of animal testing. Comparison to *in vivo* fish uptake rates would be useful, however there are
345 a limited number of studies reporting these values. We have collated those '*steady state*'
346 plasma concentrations for 9 of the 10 pharmaceuticals in the supplemental file (SI Table S8),
347 but note that the complexity and variation in pH, exposure, species, size and temperature
348 make direct comparison to our data difficult. Furthermore, these studies do not allow us to
349 derive uptake rates and therefore are not suitable for comparison with our dataset. Predicted
350 K_1, LC_{50} and BCF values can be derived from QSAR models for fish (SI, Table S8). However,
351 a poor correlation was observed between the predicted K_1, LC_{50} and BCF and our *in vitro*
352 pharmaceutical uptake rates (Table S8), emphasizing the need for alternative models for these
353 compounds. To fully validate the model a much larger number of compounds would be
354 needed. A robust and validated model for gill uptake could then be used as a pre-screen to
355 prioritize compounds for experimental testing in a tiered approach[8]. In this scenario if a
356 compound is predicted to not be bioavailable in *in vitro* studies and other information from
357 lower tier screens support this observation, then further BCF testing in living fish may not be
358 required[8,24].
359
360 The pharmaceutical uptake rate was most strongly positively correlated to logS (Figure 2b)
361 suggesting that this physiochemical property facilitates access of the pharmaceuticals to the
362 cells and uptake. The ABL in multi-well plates is between 1,000 – 2,000 μm and forms a
363 significant diffusional barrier[39] where by the concentration in bulk solution exceeds that located
364 at the membrane surface. Increased solubility aids permeability across the ABL[29] allowing for
365 greater interaction with the membrane[40]. Carbamazepine was expected to have a higher
366 uptake based on solubility, as well as being neutral and hydrophobic, but influx rates were low
367 and carbamazepine had the largest prediction inaccuracy in the PLS regression model. The
368 reason for this is uncertain. Carbamazepine has a low BCF value in adult zebrafish (BCF_{ss} of
369 1.41 ± 7.13 L kg^{-1}) but this is likely associated with greater biotransformation capacity and
370 clearance rather than a significant reduction in uptake when compared to other pharmaceutical
371 and personal care products tested[17]. Whether the gills actively excrete carbamazepine back

into the apical water compartment remains to be determined. Carbamazepine's mode of action is promiscuous, and it interacts with different types of receptors and channels[41]. However, the main target is voltage gated Na^+ channels located on the surface of the cells[41] where it acts as a competitive inhibitor by allosteric inhibition[42]. A possibility is that in our system the drug adheres to and interacts with the surface and related channels but does not permeate into the cell.

A negative correlation of uptake rates with logD was observed. The gill membrane consists of a range of phospholipids (e.g. phosphatidylethanoilamines and phosphatidylcholine) with differing properties capable of forming electrostatic and hydrogen bonds with charged molecules. It has been shown that ionized drugs can partition into artificial lipid membranes greater than predictions based on logK_{ow}[44] and there is a positive relationship between the dipole potential in the region between the aqueous phase and the interior membrane bilayer allowing permeation of ionized compounds in these synthetic membranes[24,45,46]. This phenomenon gave rise to the pH-piston hypothesis to explain sorption of ionized drugs into artificial vesicles consisting of dioleylphosphatidylcholine, due to electrostatic interactions with acidic and basic drugs[45] and may explain how the ionized compounds are able to cross the membrane.

The PLS regression also indicated that the uptake rates were negatively correlated with MW. MW is known to play a distinct role in cellular uptake of solutes and has been used successfully to model permeability of both neutral and charged molecules, in addition to be a component of Lipinski's rule of 5 in drug discovery[43,47,48]. The LogK_{ow} and PSA accounted for some of the variance in the regression but to a much less extent than the other molecular descriptors.

The role of transport proteins in ionizable drug uptake is axiomatic[e.g. 49-51], but the extent of the role transport proteins play in drug uptake is debated. Kell and colleagues proposed that uptake is almost solely due to transport proteins[52], though this has been strongly questioned[53].

400 In our current study all flux rates were measured at concentrations that far exceed
401 environmental concentrations and it is likely that carrier mediated transport processes were
402 saturated. Here we are measuring both the passive and facilitated uptake, with passive
403 dominating and entry likely via electrostatic interactions with the phospholipid membrane of
404 the fish gill[24]. But, several organic, anion, cation or zwitterion transporters are present at the
405 gill e.g. *slco1d1*[55], OATP[56] and *slc15a2*[57], and their ability to facilitate drug uptake from the
406 water requires further understanding. An alternative explanation for the uptake of charged
407 molecules is transportation as ion pairs[58], a property that has been utilized to assist in
408 developing drug penetration for a number of epithelia, such as the ocular epithelium[59] and the
409 skin[60], but has not been considered for fish gill epithelial. Natural water contains numerous
410 potential counterions and the fish excretes ions and other charged molecules from the gill that
411 could form ion-pairs with charged drugs.
412

413 **Environmental Implications**

414 The current study shows that the FIGCS can be used to assess drug uptake by fish gills from
415 the water in accordance with previous studies[23]. It also shows that ionizable drugs are able to
416 cross the gill epithelium, but further work is required to ascertain the significance of the gill
417 microclimate at the apical membrane, ion pairing, electrostatic interactions (between the
418 ionized pharmaceutical and the membrane phospholipids) and transport proteins on ionizable
419 compound transport. A PLS regression model based on the physicochemical properties of the
420 drug was used to predict uptake rate (the model accounted for 78% of the explained variance)
421 where logS, pK_a, logD and MW were significant drivers. To fully validate the model a much
422 larger number of compounds would be needed, however, this approach shows that modelling
423 can be used to understand the uptake of pharmaceuticals by an *in vitro* epithelium system that
424 could replace whole animals in bioaccumulation studies. There is a need a robust and
425 validated model for gill uptake could then be used as a pre-screen to prioritize compounds for
426 experimental testing in a tiered risk assessment[8] where compounds that do not cross the gill
427 epithelia may not need further costly and time-consuming animal testing.

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Supporting Information.

- Cytotoxicity of the pharmaceuticals in primary gill cells (MTT assay)
- *in vivo* BCF data from literature
- QSAR predictions of K_1, LC_{50} and BCF
- HPLC method performance assessment
- Linear regression analysis of uptake rates in relation to the chemical descriptors modelling supplementary information including scores, loadings, modified weights and cross-validation for PCA and PLS.

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Table 1: Pharmaceutical molecular descriptors.

	pK_a	logS	Molecular Weight (g mol^{-1})	logD	logK_{ow}	PSA (Å^2)	% ionisation at pH 7.6
Acetazolamide	7.20	-2.36	222.24	0.23	3.48	115.04	71.5
Beclomethasone	13.85	-5.4	408.92	4.16	3.49	106.97	0
Carbamazepine	15.96	-3.2	236.27	2.28	2.28	46.33	0
Diclofenac	4.00	-4.8	296.15	1.22	1.9	52.16	100
Gemfibrozil	4.42	-4	250.33	1.40	4.77	46.53	99.9
Ibuprofen	4.91	-3.5	206.28	0.29	2.48	40.10	99.8
Ketoprofen	4.45	-4.1	254.28	0.06	0.97	54.37	99.9
Norethindrone	17.59	-4.7	298.40	2.98	3.15	37.30	0
Propranolol	9.42	-3.5	259.34	1.29	3.09	41.49	98.5
Warfarin	5.08	-3.8	308.32	0.16	0.85	63.60	99.7

Figure 1: Pharmaceutical uptake rate into the fish gill cell culture system. Values represent average of 4 inserts derived from between 4 -6 fish. Bars with differing letters are significantly different from each other when compared via a One-way ANOVA followed by a Tukey's post-hoc test, $p<0.05$.

Figure 2: PCA biplot showing the first two principal component (PC1 and PC2) loadings and scores for each molecular descriptor and case, respectively. Scores are indicated on the first axes (left and bottom, black) loadings are indicated by the second axes (right and top, red). A -acetazolamide; B – beclomethasone; C- carbamazepine; D – diclofenac; G – gemfibrozil; I – ibuprofen; K - ketoprofen; N – norethindrone; P – propranolol and W – warfarin.

Figure 3: PLS regression analysis showing (A) loadings of the first two latent variables for molecular descriptors (independent variables) and uptake rate (dependent variable) (B) predicted versus observed gill uptake rates using PLS regression model (C) raw residuals of predicted uptake rates.

656 **Figure 1**
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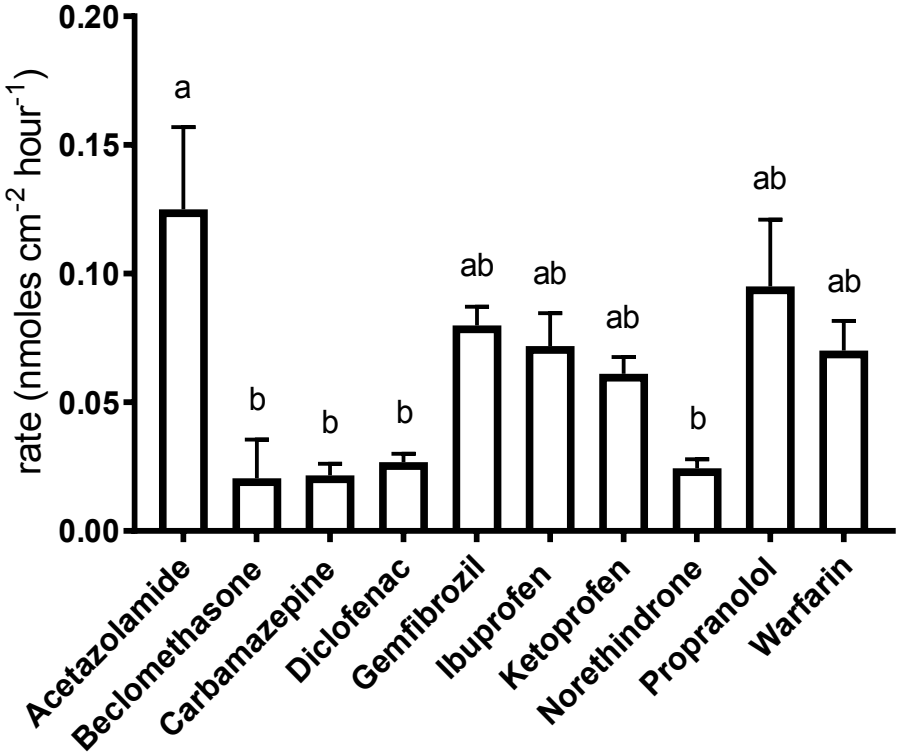

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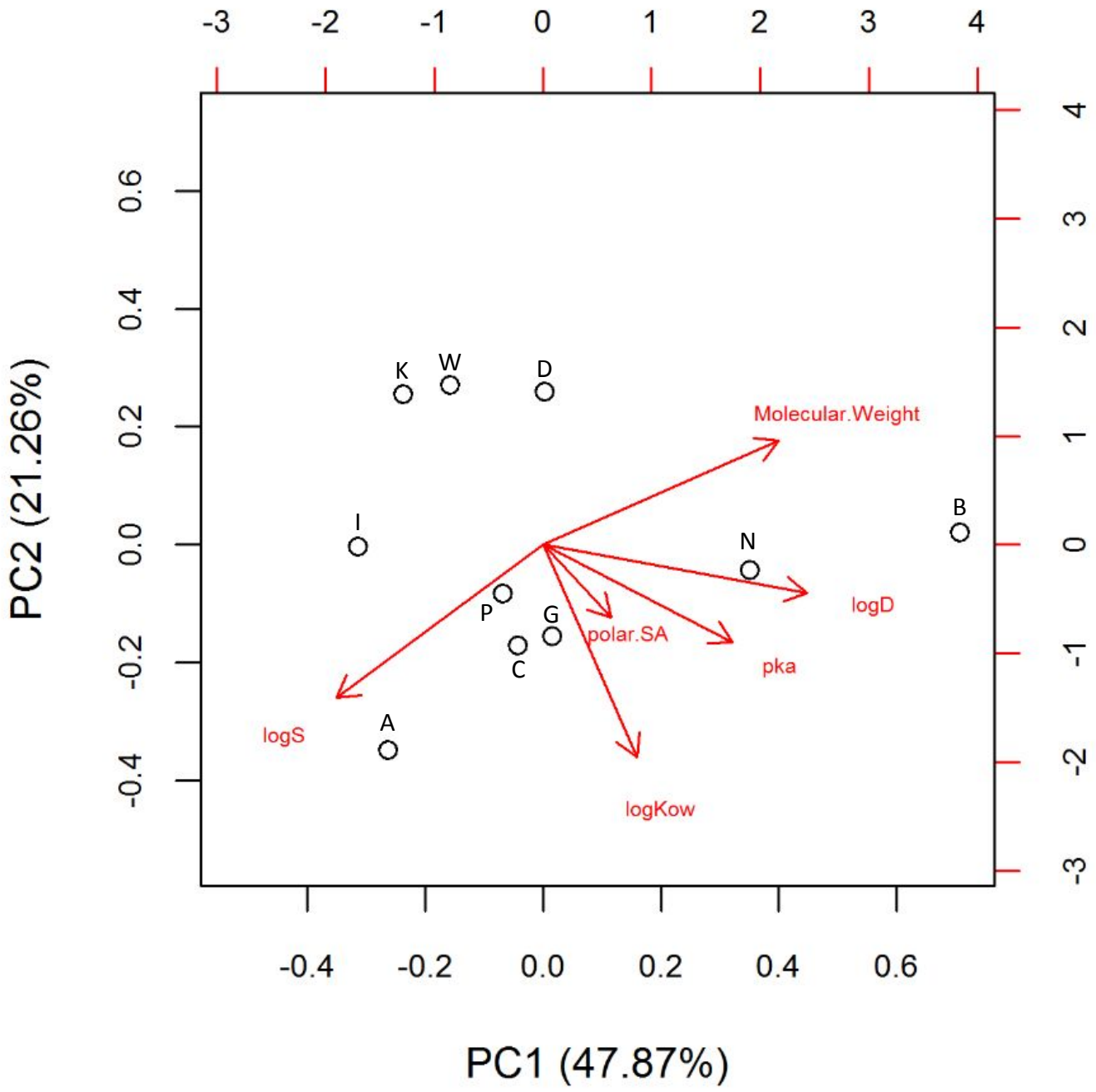
Figure 2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PC2 (21.26%)
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polar.SA
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-0.4
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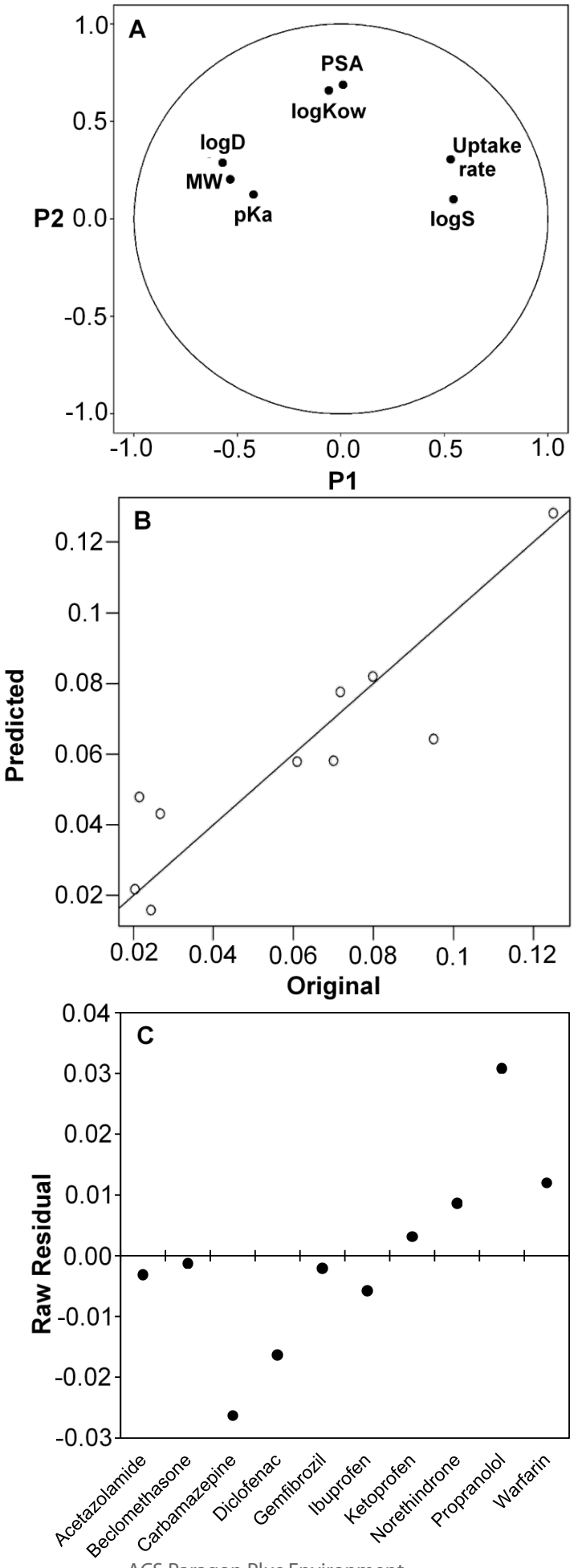
0.0
0.2
0.4
0.6
PC1 (47.87%)

Figure 3


A
PSA
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Uptake rate
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P2
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1.0
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-0.5
-1.0
B
Predicted
Original
0.02
0.04
0.06
0.08
0.1
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C
Raw Residual
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
Acetazolamide
Beclomethasone
Carbamazepine
Diclofenac
Gemfibrozil
Ibuprofen
Ketoprofen
Norethindrone
Propranolol
Warfarin