PBT assessment under REACH: Screening for low aquatic bioaccumulation with QSAR classifications based on physicochemical properties to replace BCF *in vivo* testing on fish

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ABSTRACT

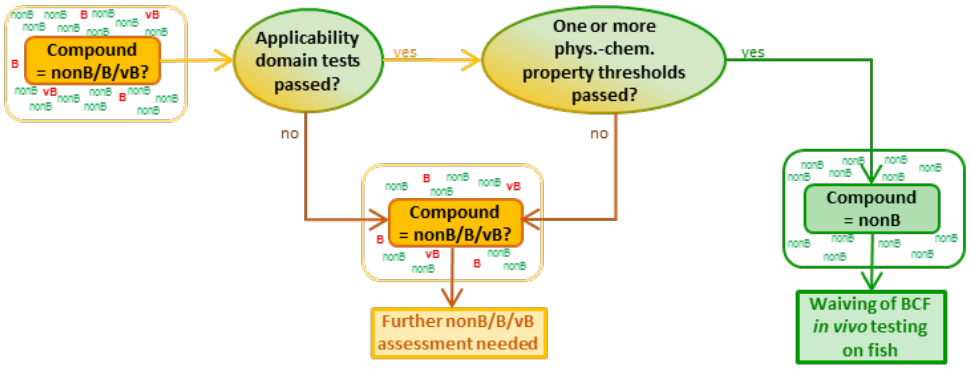
Aquatic bioconcentration factors (BCFs) are critical in PBT (persistent, bioaccumulative, toxic) and risk assessment of chemicals. High costs and use of more than 100 fish per standard BCF study (OECD 305) call for alternative methods to replace as much *in vivo* testing as possible. The BCF waiving scheme is a screening tool combining QSAR classifications based on physicochemical properties related to the distribution (hydrophobicity, ionisation), persistence (biodegradability, hydrolysis), solubility and volatility (Henry's law constant) of substances in water bodies and aquatic biota to predict substances with low aquatic bioaccumulation (nonB, BCF <2000).

The BCF waiving scheme was developed with a dataset of reliable BCFs for 998 compounds and externally validated with another 181 substances. It performs with 100% sensitivity (no false negatives), more than 50% efficacy (waiving potential), and complies with the OECD principles for valid QSARs. The chemical applicability domain of the BCF waiving scheme is given by the structures of the training set, with some compound classes explicitly excluded like organometallics, poly- and perfluorinated compounds, aromatic triphenylphosphates, surfactants. The prediction confidence of the BCF waiving scheme is based on applicability domain compliance, consensus modelling, and the structural similarity with known nonB and B/vB substances.

Compounds classified as nonB by the BCF waiving scheme are candidates for waiving of BCF *in vivo* testing on fish due to low concern with regard to the B criterion. The BCF waiving scheme supports the 3Rs with a possible reduction of more than 50% of BCF *in vivo* testing on fish. If the target chemical is outside the applicability domain of the BCF waiving scheme or not classified as nonB, further assessments with *in silico*, *in vitro* or *in vivo* methods are necessary to either confirm or reject bioaccumulative behaviour.

Keywords: BCF waiving scheme; 3Rs; octanol/water partition coefficient (log K_{ow}); (bio)degradability; consensus modelling; structural similarity with nonB/B/vB substances;

Graphical abstract



Highlights

- BCF waiving scheme to screen for absence of PBT properties
- Identification of low bioaccumulation potential based on physicochemical properties
- Reliable QSAR classifications with 100% sensitivity (no false negatives)
- Prediction confidence based on similarity with nonB and B/vB compounds
- Contribution to the 3Rs by reduction of BCF *in vivo* testing on fish by at least 50%

1. INTRODUCTION

The accumulation of chemicals in aquatic biota is of major concern for environmental hazard and risk assessment. The internal concentration of contaminants in organisms may increase by accumulation to a level that causes toxic effects, even if the external concentration remains below the critical limit. Also an exposure for a short time may produce high internal concentrations that persist in the organism much longer than in the surrounding water. Because of their elevated and lasting level in living tissues, bioaccumulative substances may evoke potentially chronic effects, not only in the organisms directly exposed but also in species at higher levels in the food chain, including humans. Bioaccumulation is therefore an important link between the pollution of surface waters and human exposure to xenobiotic substances.

National and international chemical legislations require bioaccumulation assessments mainly based on bioconcentration factors (BCFs). Within the European Union, the REACH regulation concerning the registration, evaluation, authorisation and restriction of chemicals (European Commission, 2006) requests BCF studies for chemicals produced or imported above 100 tonnes per year (Annex IX). For substances produced or imported between 10 and 100 tonnes per year, BCF studies are not required but BCFs are needed for PBT (persistent, bioaccumulative, and toxic) and vPvB (very persistent, very bioaccumulative) assessments. The criteria for bioaccumulative (B) and very bioaccumulative (vB) substances are BCFs above 2000 and 5000, respectively. Similarly, the Stockholm Convention on persistent organic pollutants (UNEP, 2015) and Environment Canada's Persistence and Bioaccumulation Regulations (CEPA, 2016) use a threshold of BCF greater than 5000 to identify bioaccumulative substances.

The standard experimental determination of BCFs according to the OECD Test Guideline 305 (Bioaccumulation in fish: aqueous and dietary exposure (OECD, 2012)) uses more than 100 fish and costs about 50000 €. Considering that measured BCFs are available for less than 5% of the tens of thousands of commercial substances that require evaluation (OECD, 2016a; Weisbrod et al., 2007), it is obvious that testing all the BCFs is neither desirable with regard to animal welfare (Directive

79 2010/63/EU on the protection of animals used for scientific purposes (European Commission, 2010))
80 nor are these tests feasible because of insufficient laboratory capacities and limited economic
81 resources. Approaches that support the 3Rs principles (replacement, refinement, and reduction of
82 animal testing (Russel and Burch, 1959)) address several options to reduce the BCF *in vivo* testing on
83 fish. Some savings are possible with reduced test design, for example, testing of only one test
84 concentration or a reduced number of sample points (OECD, 2012; OECD, 2016b; Springer et al.,
85 2008). Integrated testing and tiered assessment strategies aim to use also alternative data and non-
86 guideline methods for the evaluation of the bioaccumulation potential of chemicals in fish (de Wolf et
87 al., 2007; Lillicrap et al., 2016; Lombardo et al., 2014).
88 Screening tools can furthermore reduce BCF *in vivo* testing on fish based on the rationale that, with
89 regard to bioaccumulation assessments under REACH, substances with BCF below 2000 have low
90 testing priority because they classify as non-bioaccumulative (nonB) and, thus, they cannot be
91 PBT/vPvB. On the contrary, substances with unknown BCF may be potentially B/vB and have higher
92 testing priority to either confirm or reject bioaccumulative behaviour. Since most (>80%) chemicals
93 are nonB, reliable screening for low aquatic bioaccumulation can direct the limited resources, as
94 efficiently as possible, towards the substances with high testing priority and support to postpone or
95 waive the BCF *in vivo* testing on fish for the low priority chemicals.
96 Screening for nonB substances may be based on quantitative structure-activity relationships
97 (QSARs) describing the dependence of low aquatic bioaccumulation on structural features and
98 physicochemical properties (Nendza et al., 2013). QSAR classifications can identify nonB compounds
99 being candidates for the waiving of BCF *in vivo* testing on fish. However, waiving of BCF *in vivo*
100 testing on fish can only be accepted if the classification as nonB is plausible and reliable. If there is
101 any doubt about the classification as nonB, the chemicals should be assessed by further *in silico*, *in*
102 *vitro* and/or *in vivo* methods.
103 It is the objective of this study to improve the identification of nonB chemicals being candidates for
104 waiving BCF *in vivo* testing on fish. Based on earlier work (Nendza and Herbst, 2011; Nendza and

105 Müller, 2010), we aim for a predictive model, the BCF waiving scheme, based on QSAR classifications
106 using physicochemical properties to classify chemicals as either nonB (low testing priority) or
107 potentially B/vB (high testing priority). The BCF waiving scheme shall (1.) provide reliable nonB
108 classifications according to an external validation, (2.) perform better than the existing thresholds for
109 waiving BCF studies, and (3.) inform about prediction confidence based on applicability domain (AD)
110 compliance, consensus modelling, and the structural similarity with known nonB and B/vB substances.

111 # 2. MATERIAL AND METHODS

112 ## 2.1 Bioconcentration data

113 A training set with reliable BCF data for 998 compounds was compiled from Arnot et al. (2009),
114 CAESAR (2011), Dimitrov et al. (2005a), EURAS (2007), Fu et al. (2009), Strempel et al. (2013). The
115 data were quality controlled regarding test substance identity and chemical structures, test protocol
116 variation (e.g. exposure concentrations and pH) and represent wet-weight-based, steady-state BCF. If
117 available, BCFs determined by the kinetic method were used. The mean value was calculated in the
118 case of multiple data for the same chemical. The final dataset covers a log BCF range between -1 and
119 6, with 829 nonB (83.0%), 62 B (6.2%), and 107 vB (10.7%) compounds. The compounds are
120 chemically diverse, including industrial chemicals and pesticides, and their molecular weights range
121 between 46 and 1471 g/mol. The training set contains relevant contaminants, e.g. high production
122 volume (HPV) chemicals and priority substances under the Water Framework Directive (WFD), and
123 is detailed in the supplementary material (SI_1 Chemicals and data).

124 An external validation set with BCF data for another 181 compounds was collected from
125 EChemPortal[2]. The search criteria were ”Bioaccumulation: aquatic / sediment“ and ”Study result type:
126 experimental result“. About 5000 results were retrieved for almost 1000 chemicals. Removing
127 compounds without unique chemical structures (e.g. UVCBs) and inorganic chemicals resulted in BCF
128 and BAF values for 475 chemicals. 228 of these chemicals were included already in the training set.

[2] http://www.echemportal.org/, accessed 25.08.2016.

66 chemicals had ambiguous BCF or BAF values (e.g., it was not clear if the value referred to BCF or log BCF). The remaining 181 chemicals cover a log BCF range between 0 and 6 with 168 nonB (92.8%), 9 B (5.0%), and 4 vB (2.2%) compounds. The highest (worst case) value was used in the case of multiple data for the same substance to challenge the BCF waiving scheme. The external validation set is detailed in the supplementary material (SI_1 Chemicals and data).

2.2 Chemical domain and structural similarity

Atom centred fragments (ACFs) were used to describe the chemical structures and assess the coverage of target chemicals by the structures of the training set. The ACF method (Kühne et al., 2009) virtually decomposes molecules into structural parts with each non-hydrogen atom of the molecule acting as an ACF centre. An ACF is then defined through the atom type and the number and type of bonding neighbours. ACF-based structural similarity compares the ACFs of the target compound and the ACF pool of the training set.

The similarity of target compounds with either nonB or B/vB chemicals in the training set was assessed based on the number of common ACFs in the target compound and the most similar compounds in the respective subsets of nonB and B/vB substances (Dice, 1945; Kühne, 2007). The ratios of the 1st order ACF based averaged similarities to the 3 most similar nonB chemicals (S_{nB}) and the three most similar B/vB chemicals (S_B) were input to the index $I_{nB} = S_{nB}/S_B - T$. The threshold of $T = 1.3$ was empirically derived from the B/vB compounds as an upper limit, but as low as possible, to avoid false negative results. The index I_{nB} was evaluated only for sufficiently similar compounds with S_{nB} of at least 0.75. Positive I_{nB} indicate more similarity to nonB compounds. If I_{nB} is negative the target compound is more similar to B/vB substances. The calculation of the similarity index I_{nB} is detailed in the supplementary material (SI_2 Similarity).

2.3 Determination of physicochemical data

The physicochemical property data for the QSAR classifications of the BCF waiving scheme can be obtained by freely available *in silico* methods. Table 1 details the endpoints, the methods for their

154 determination, and the recommended data handling. The data ranges determine the physicochemical
155 properties domain of the QSAR classifications. The thresholds are the criteria values for chemicals to
156 classify as nonB (see section 3.1).

157 **Table 1.** Physicochemical properties for the QSAR classifications of the BCF waiving scheme: a
158 compound (within the AD of the BCF waiving scheme) may be considered nonB if the threshold for
159 at least one physicochemical property is fulfilled, provided that the data are determined with verified
160 methods and fit within the tested range of applicability of the endpoint.

Physicochemical property	Endpoint [units]	Methods	Data handling	Data range	Threshold
Hydrophobicity	logarithm of the octanol/water partition coefficient (log K_{ow})	KowWIN of EPI Suite (US EPA, 2012), ALOGP (Ghose et al., 1998) and XLOGP (Wang et al. 2000) of T.E.S.T. (US EPA 2016a), Chemistry Dashboard (US EPA 2016b), ChemProp fragment models (UFZ Department of Ecological Chemistry, 2016)	consensus = average of at least 3 independent values	-2.0 to >10	<3
Apparent distribution (if ionisation at pH 7 >5%)	logarithm of the distribution coefficient (log D)	dissociation at pH 7: SPARC (University of Georgia, 2011); p*K*a: ACD (Royal Society of Chemistry, 2015; log K_{ow} : average of at least 3 independent values	at pH 6 for acids: log D = log K_{ow} - log(1+10$^{(pH-pKa)}$); at pH 9 for bases: log D = log K_{ow} - log(1+10$^{(pKa-pH)}$)	-8.5 to 8.4	<3
Biodegradation	ready biodegradability	BIOWIN of EPI Suite (US EPA, 2012) or its ChemProp implementation (UFZ Department of Ecological Chemistry, 2016)		YES or NO	YES
	logarithm of the biotransformation half-life in fish (log $\tau_{1/2,bio}$ [h]) (Arnot et al., 2009)	BCFBAF of EPI Suite(US EPA, 2012) or its ChemProp implementation (UFZ Department of Ecological Chemistry, 2016)		-8.0 to 7.2	<0
Hydrolysis	logarithm of the 2nd order rate constant (log K_{hyd} [L/(mol sec)])	SPARC (University of Georgia, 2011)	minimum	-6.9 to 4	<3
	half-life in water [classes] (Kühne et al., 2007)	ChemProp (UFZ Department of Ecological Chemistry, 2016)		1 to 9	
Henry's law constant	log HLC [atm/(mol/L)]	SPARC (University of Georgia, 2011) or EPI Suite (US EPA, 2012)	minimum	-40 to 4.5	<-11
	logarithm of the air/water partition coefficient (log K_{aw})	ChemProp (UFZ Department of Ecological Chemistry, 2016)		-37 to 4.0	

161

The QSAR classifications of the BCF waiving scheme require reliable physicochemical input data. We recommend to use consensus values (average of results from multiple, independent *in silico* methods, e.g. for log K_{ow} from EPI Suite (US EPA, 2012), ChemSpider (Royal Society of Chemistry, 2015), ChemProp (UFZ Department of Ecological Chemistry, 2016), T.E.S.T. (US EPA 2016a), Chemistry Dashboard (US EPA 2016b), SPARC (University of Georgia, 2011), and valid experimental data). Since some methods perform better than others for different target chemicals, but do not generally yield superior predictions, consensus modelling may consolidate variable, possibly conflicting, *in silico* predictions. ECHA (2008) suggested obtaining predictions from at least three different methods.

The physicochemical data for the chemicals of the training set and the validation set are detailed in the supplementary material (SI_1 Chemicals and data).

2.4 Classification statistics

The results of pairwise comparisons of the predicted classifications of the chemicals of the training set and the external validation set as either nonB (low testing priority) or potentially B/vB (high testing priority) with the experimental BCF data were quantified in terms of accuracy (proportion of substances correctly classified), sensitivity (proportion of true positives (B/vB) correctly classified), specificity (proportion of true negatives (nonB) correctly classified), and efficacy (proportion of candidates for the waiving of BCF *in vivo* testing on fish):

$$Accuracy = \frac{TP + TN}{Tot} \times 100$$

$$Sensitivity = \frac{TP}{TP + FN} \times 100$$

$$Specificity = \frac{TN}{TN + FP} \times 100$$

$$Efficacy = \frac{TN}{Tot} \times 100$$

with TN: true negative, TP: true positive, FN: false negative, FP: false positive, Tot: total number of compounds.

3. RESULTS

3.1 Physicochemical properties

The BCF waiving scheme combines QSAR classifications based on physicochemical properties related to the distribution, persistence, solubility and volatility of substances in water bodies and aquatic biota. Principal component analyses (PCA) revealed characteristic combinations of physicochemical properties with two major factors explaining about 80% of the total variation in BCF data (Strempel et al. 2013). The 1st principal component is related to the stability and partitioning of compounds and the 2nd principal component summarises volatility and polarity.

3.1.1 Selection of physicochemical properties and derivation of thresholds.

The QSAR classifications proposed here use thresholds for physicochemical properties to identify nonB substances. Figure 1 shows the thresholds for selected physicochemical properties related to the chemicals of the training set. The thresholds have been derived in such a way that only nonB substances are below the threshold (left side of the graphs). Above the thresholds (right side of the graphs) are all the B and vB substances and various amounts of nonB substances. For the purpose of the BCF waiving scheme, the thresholds have been tailored to be perfectly protective (100% sensitivity) with no false negatives observed among the substances of the training set within the AD of the model, though at the cost of false positives (see section 4.2).

Distribution

Thermodynamic partitioning into non-aqueous phases, for example lipids and proteins, explains the prominence of the n-octanol/water partition coefficient log K_{ow} in BCF assessments. A hydrophobicity threshold of log K_{ow} less than 3 was adopted from the 1996 Technical Guidance Document (TGD) on risk assessment for new notified substances and existing substances (European Commission, 1996). This criterion classifies 404 (42%) of the substances[3] as nonB without false negatives (Figure 1A). A

[3] Substances of the training set within the AD, see section 3.2.

209 superlipophilicity criterion of log K_{ow} above 10 was not used due to only few substances concerned
210 and too much uncertainty in measured and calculated log K_{ow} values above 6.

211 Ionisation does not affect bioaccumulation per se but via alteration of partitioning. A threshold for
212 apparent partitioning (log D) less than 3 applies to ionogenic compounds with more than 5% ionisation
213 at pH 7 (Figure 1B). The log D is obtained at pH 6 for acids or pH 9 for bases. These pH values were
214 selected to represent the maximum ionisation of substances in natural surface waters. Among the
215 chemicals of our training set, we observed 79 acids and bases with log K_{ow} and log D below 3, and 20
216 acids and bases with log K_{ow} above 3 but log D below 3 (additional waiving candidates). Another 23
217 ionising compounds classify potentially bioaccumulative due to log K_{ow} and log D exceeding 3, like
218 pentachlorophenol and triclosan.

219 ***Persistence***

220 Stability of chemicals depending on, for example, biodegradation and hydrolysis is another
221 confounding factor of the bioconcentration potential (Schüürmann et al., 2007). The ready
222 biodegradability YES or NO classifications by BioWIN (US EPA, 2012) were analysed as a surrogate
223 of possible microbial degradation in water bodies and metabolism in fish. These predictions are very
224 conservative, the classification YES requires that the Biowin3 (ultimate survey model) result is
225 'weeks' or faster and the Biowin5 (MITI linear model) output is at least 0.5. If these conditions are not
226 satisfied, the prediction is NO (not readily biodegradable). The ready biodegradability threshold
227 classifies 129 (13%) of the substances[3] as nonB without false negatives (Figure 1C). Among the
228 compounds predicted not readily biodegradable are all the B/vB compounds. An alternative metric is
229 the biotransformation half-life in fish (Arnot et al., 2009) with a threshold of log $\tau_{1/2,bio}$ [h] less than 0,
230 indicating 124 (13%) of the substances[3] as nonB without false negatives (Figure 1D). The two
231 biodegradation criteria cover different chemical structures and together classify 221 (23%) of the
232 substances as nonB without false negatives.

233 Hydrolysis in terms of the logarithm of the 2nd order rate constant [L/(mol sec)] (SPARC (University
234 of Georgia, 2011)) and half-life class in water (Kühne et al., 2007) with a threshold of less than 3

235 correctly identify 83 (9%) of the substances[3] as nonB (Figure 1E, F). In contrast, HydroWIN (US EPA,
236 2012) estimates of acid- and base-catalysed rate constants for esters, carbamates, epoxides,
237 halomethanes, selected alkyl halides and phosphorus esters showed insufficient discriminatory power.

238 ***Solubility and volatility***

239 Individual classifications of low bioconcentration based on water solubility or vapour pressure were
240 not confirmed (Nendza and Herbst, 2011). Instead, the combination of water solubility and volatility
241 in Henry's law constant (HLC) or the air/water partition coefficient log K_{aw} indicates hydrophilic
242 substances with high water solubility relative to low volatility. A threshold of log HLC [atm/(mol/L)]
243 or log K_{aw} less than -11 allowed to identify 59 (6%) of the substances[3] as nonB without false negatives
244 (Figure 1G).
245

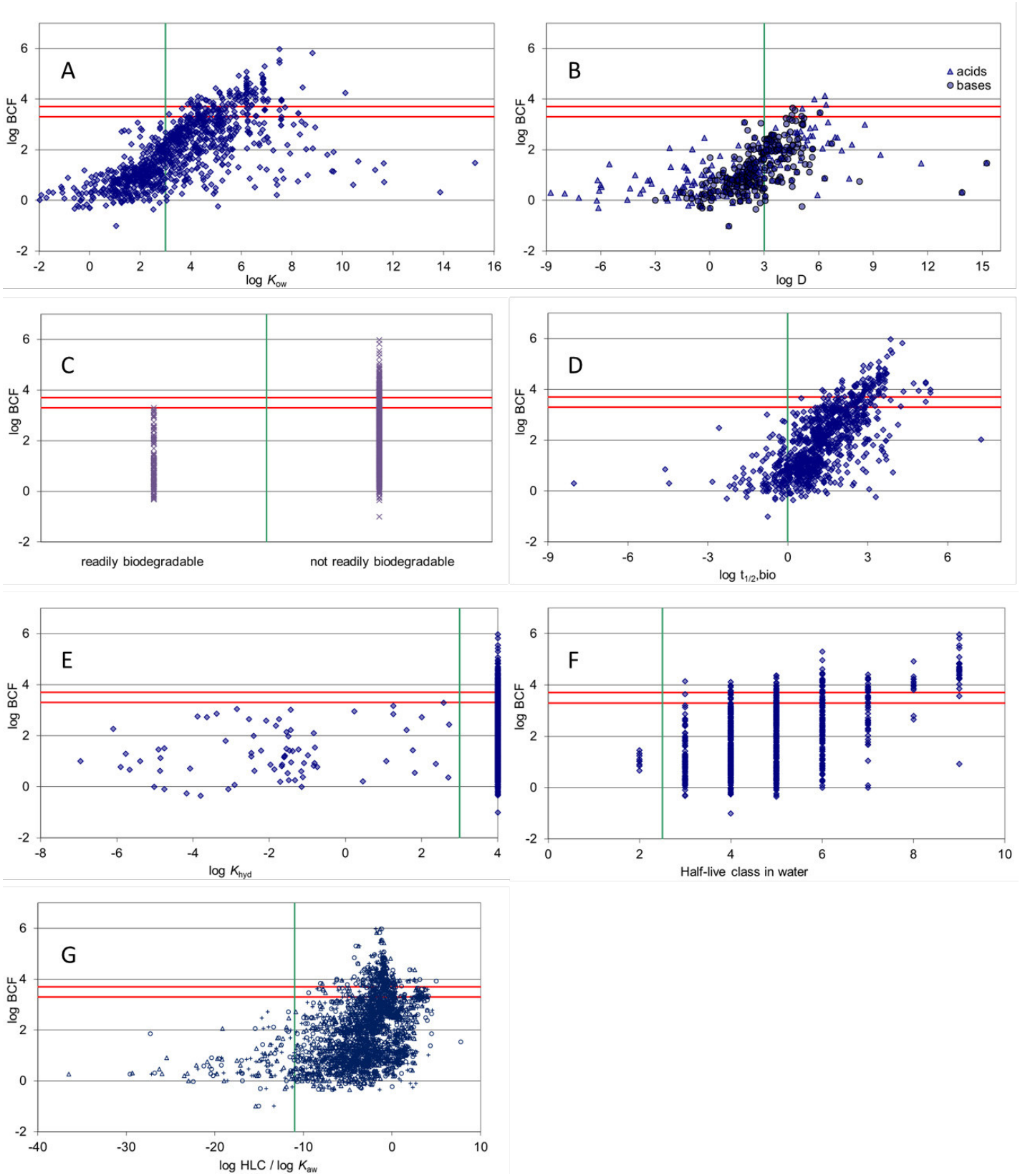


Figure 1. QSAR classifications based on physicochemical properties for the BCF waiving scheme. The thresholds (vertical green lines) discriminate between either nonB (left part of the graphs) or potentially B/vB substances (right part of the graphs). The horizontal red lines indicate B (BCF <2000) and vB (BCF <5000) criteria. A: Relationship between log BCF and log K_{ow} with a threshold of log K_{ow} <3. B: Relationship between log BCF and log D with a threshold of log D <3. C: Relationship between log BCF and ready biodegradability. D: Relationship between log BCF and log $\tau_{1/2,bio}$ [h] with a threshold of log $\tau_{1/2,bio}$ <0. E: Relationship between log BCF and log K_{hyd} with a threshold of log K_{hyd}

<3. F: Relationship between log BCF and half-life classes in water with a threshold for classes below 3. G: Relationship between log BCF and log HLC / log K_{aw} (o: log HenryWIN, +: log HLC SPARC, Δ: log K_{aw}) with a threshold of <-11.

3.2 Applicability domain

The conceptual framework of applicability domain (AD) evaluation is the assumption that similar chemicals have similar activities. It is based on the hypothesis that properties of chemicals that are similar to the training chemicals will be predicted well because the model has captured the same important features of the target and the training chemicals (Dimitrov et al. 2005b). Thus, best predictions are expected for substances included in the AD of the model while reliability decreases with increasing distance from the AD.

The chemical domain of the BCF waiving scheme is defined by two aspects based on the training set: (1.) exclusion of chemical classes with other modes of aquatic bioaccumulation causing false negative outliers, and (2.) inclusion of substances with similar chemical structures.

3.2.1 Exclusion rules

Chemicals known to accumulate in aquatic biota by modes other than thermodynamic partitioning are excluded from the AD of the BCF waiving scheme: (1.) Organometallics with log K_{ow} below 3 like methyl mercury (log K_{ow} of 0.08) and tetraethyl lead (log K_{ow} of 2.67) are known to bioaccumulate by covalent mechanisms (Iwata et al., 1997; Mason et al., 2000). The respective rule excludes compounds with Hg, Pb, and Sn attached to organic carbon. (2.) Poly- and perfluorinated compounds (PFC) are excluded because many of them are ionogenic and they are suspected to bioaccumulate in non-lipid phases (Martin et al., 2003). Their exclusion rule requires 6 or more fluorine atoms within the molecule, at least one of them attached to a non-aromatic carbon atom without hydrogen and without other monovalent non-halogen neighbours. (3.) Substances with an acyclic alkyl moiety (chain length ≥C_7) are excluded because, presumably, their uptake rates exceed the rates of the degradation processes. Their exclusion rule is triggered by an acyclic saturated chain of 7 carbon atoms and 3 additional non-aromatic carbon atoms but no heteroatom or ring in the molecule. (4.) Aromatic

triphenylphosphates bioaccumulate despite ready hydrolysis. Here, the exclusion rule is the occurrence of a phosphorus atom double bonded to an oxygen atom, and single bonded to 3 oxygen atoms that each are bonded to aromatic carbon. (5.) Surfactants were not included in the training set of the model and, thus, are excluded from its AD. Surfactants tend to absorb at biological interfaces. Their amphiphilic nature prevents thermodynamic partitioning and limits proper determination of BCF and log K_{ow}. The BCF estimates of many surfactants are below the threshold of 2000, however, dietary studies are considered to be more appropriate to cover the uptake routes and accumulation processes of surfactants. (6.) Predictions for macromolecules and polymers are excluded with a molecular weight limit of 2000 g/mol.

3.2.2 Structural AD compliance

Substances with similar chemical structures as represented by the chemicals of the training set are included in the AD of the BCF waiving scheme. The coverage of target chemicals by the structures of the training set is evaluated using two levels of ACFs (see section 2.2). ACFs were obtained as described in Kühne et al. (2009) and implemented in ChemProp (UFZ Department of Ecological Chemistry, 2016). The compounds of the BCF training set are represented by a pool of 1014 1st order ACFs and 3367 2nd order ACFs.

The AD compliance test compares the occurrence of ACFs in target compounds with the ACF pool of the entire training set. Compounds are classified as ‘in domain‘ if all the ACFs of the target compound are covered by the compounds of the training set.

3.3 BCF waiving scheme

The BCF waiving scheme combines QSAR classifications based on physicochemical properties related to mitigating factors for aquatic bioconcentration. The workflow of the BCF waiving scheme (Figure 2) starts with the assumption of unknown bioaccumulation potential (Compound = nonB/B/vB?). Prior to the QSAR classifications, the target compound is tested for compliance with the AD of the BCF waiving scheme (see section 3.2). If the target chemical is outside the AD of the

BCF waiving scheme, the status remains 'not classified' (Compound = nonB/B/vB?), meaning that further assessments are necessary. If the target chemical is within the AD of the BCF waiving scheme, the physicochemical properties of the target compound are obtained based on the methods listed in Table 1 (see section 2.3). Other methods may be used as well, provided that their predictivity has been confirmed. In the next step, the physicochemical properties of the target compound are evaluated in relation to the thresholds. If a compound passes one or more of the physicochemical property thresholds, it is classified as nonB by the BCF waiving scheme and it is a candidate for waiving of BCF *in vivo* testing on fish.

If a nonB compound does not pass one of the thresholds, it might be classified as nonB based on other physicochemical properties or remain 'not classified' (Compound = nonB/B/vB?). The latter chemicals require further assessments with *in silico*, *in vitro* or *in vivo* tools to either confirm or reject bioaccumulative behaviour.

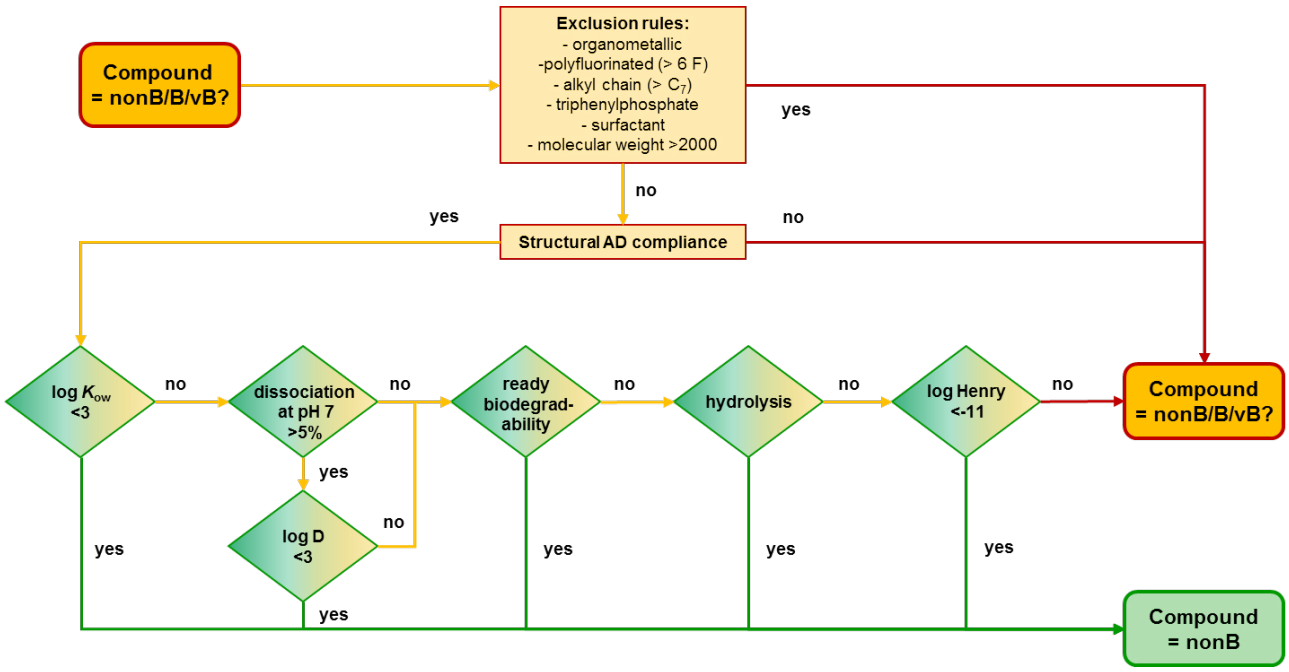


Figure 2. BCF waiving scheme combining QSAR classifications based on physicochemical properties to identify non-bioaccumulative (nonB) compounds.

Table 2 shows the performance of the BCF waiving scheme for the compounds of the training set inside the AD. Sensitivity is the most important metric for nonB classifications, looking for the B/vB

(high testing priority) compounds correctly classified. A sensitivity of 100% means that all the B/vB (high testing priority) compounds are correctly classified, i.e. no false negative classifications of B/vB compounds.

Table 2. Classification statistics of the BCF waiving scheme for the compounds of the training set.

	Accuracy	Sensitivity	Specificity	Efficacy
All compounds inside AD (n=962)	68.0%	100%	61.9%	52.0%
nonB compounds inside AD (n=808)	61.9%	---	---	61.9%
B compounds inside AD (n=55)	---	100%	---	---
vB compounds inside AD (n=99)	---	100%	---	---

---: not appropriate.

Regarding the overall performance of the BCF waiving scheme, the accuracy of 68% is the result of the perfect (100%) identification of B/vB (high testing priority) substances and the incomplete (62%) detection of nonB (low testing priority) compounds. The classification efficacy shows that 62% of all the nonB compounds inside the AD (52% of the total training set) are identified with the BCF waiving scheme and, hence, are candidates for the waiving of BCF *in vivo* testing on fish due to low concern with regard to the B criterion. For a discussion of the prediction confidence of the BCF waiving scheme see section 4.2.

3.4 Implementation of the BCF waiving scheme

The BCF waiving scheme as well as the respective AD and prediction confidence tests are available within ChemProp (UFZ Department of Ecological Chemistry, 2016) and included in the OSIRIS ITS for bioaccumulation (Lombardo et al. 2014; OSIRIS, 2011). A documentation of the ChemProp implementation of the BCF waiving scheme is available in the supplementary material (SI_3 ChemProp).

The implementation offers a partly automated scheme with evaluation of log K_{ow} (consensus model), log K_{aw} (fragment model set), biotransformation (Arnot et al., 2009), the ChemProp implementation of the EPI Suite aerobic biodegradation model (US EPA, 2012), and the half-life class in water (Kühne et al. 2007). Some properties (e.g. dissociation) cannot be calculated with ChemProp yet, they are required to be entered manually. If desired, the other properties may also be entered manually.

To support prediction confidence, the exclusion rules (exclusion of chemical classes with other modes of aquatic bioaccumulation (see section 3.2.1)) and structural AD compliance (comparison of the occurrence of ACFs in target compounds with the ACF pool of the training set (see section 3.2.2)) are checked and the similarity index I_{nB} is calculated. The number of matching criteria is shown as well as the individual comparisons to the thresholds. Also, individual AD tests for the applied QSARs are provided.

If only the automatically generated parameters can be evaluated, e.g. due to the lack of additional data, the scheme may deliver less waiving candidates. However, if one (or more) of the tested thresholds for nonB are passed by the target compound, the prediction has the same reliability as upon application of the full scheme. For the remaining 'not classified' compounds, if the similarity index I_{nB} indicates a high probability of being nonB, there is a notable chance of matching one of the remaining thresholds in order to justify waiving, and thus it might be worth the effort to look for these data elsewhere.

4. DISCUSSION

The BCF waiving scheme is a screening tool to classify nonB chemicals being candidates for the waiving of BCF *in vivo* testing on fish. The model covers bioconcentration by thermodynamic partitioning into non-aqueous phases, for example lipids, in fish while other modes of aquatic bioaccumulation are explicitly excluded.

Physicochemical properties related to the fate of chemicals in aquatic environments are a sound mechanistic basis to screen for low bioaccumulation. Partitioning and persistence relate to passive diffusion under steady-state conditions, the driver of most bioaccumulation. Dissociation of chemicals may modulate their partitioning and distribution in the environment. While individual criteria in water solubility and vapour pressure were not suitable, their combination in Henry's law constants revealed useful. Thresholds related to molecular size, assuming that membrane permeation of large molecules is limited, were not substantiated (Nendza and Müller, 2010). Rather, a modulating (smoothing) effect of molecular size on membrane permeation may exist (Dimitrov et al. 2005a; Nichols et al., 2009).

374 Lipinski's 'Rule of 5' (Lipinski et al. 1997) was found to be inadequate to identify nonB compounds
375 (Nendza and Müller, 2010). Possible reasons are key differences in the dominating processes during
376 oral absorption of pharmaceutical drugs (bulk dissolution) and the uptake of waterborne environmental
377 contaminants by aquatic organisms (continuous low-level exposure) (Gobas et al., 2006).
378 The performance of the BCF waiving scheme has been tested to provide reliable nonB classifications
379 according to an external validation (see section 4.1.1). As compared to existing thresholds for waiving
380 BCF studies, e.g. log K_{ow}, the BCF waiving scheme offers better efficacy and allows to replace more
381 BCF *in vivo* testing on fish with valid and reproducible nonB predictions without false negatives (see
382 section 4.1.2). The prediction confidence of the BCF waiving scheme is based on compliance with the
383 OECD principles for the validation of QSAR models (OECD, 2007). Applicability domain
384 considerations, consensus modelling, and structural similarity with known nonB and B/vB substances
385 inform about the reliability of predictions (see section 4.2). The compounds not positively identified
386 as nonB substances by the BCF waiving scheme require further assessments with *in silico*, *in vitro* or
387 *in vivo* methods (see section 4.3).

388 **4.1 Performance of the BCF waiving scheme**

389 **4.1.1 External validation of the BCF waiving scheme**

390 The BCF waiving scheme (Figure 2) performs well on the compounds of the training set since it was
391 developed with these data. The classification statistics (Table 2) inform about the fit of the model to
392 the training set. More than half of the substances of the training set are correctly identified by the BCF
393 waiving scheme as nonB chemicals (efficacy of 52%). The B/vB substances either are recognised
394 based on structural exclusion rules or remain 'not classified'. These compounds require further
395 assessments with *in silico*, *in vitro* or *in vivo* methods (see section 4.3). The classification statistics
396 (Table 2) do not provide measures of the predictive power of the BCF waiving scheme for other
397 chemicals, for example, in the REACH registration procedure.

The predictive power of the BCF waiving scheme to provide reliable nonB classifications was tested with an external validation. The external validation simulates the application of the BCF waiving scheme to target chemicals. The external test set contains BCF data not used for the development of the BCF waiving scheme (for details see section 2.1.). The AD compliance test delivered 116 chemicals to compare the predicted and observed classifications for the substances of the external validation set (Table 3). Based on a sensitivity of 100% (no false negatives) and a waiving potential of almost 80% (92 of the 113 nonB substances within the AD), the BCF waiving scheme is considered reliable, robust, and efficient.

Table 3. Classification statistics of the BCF waiving scheme for the compounds of the external validation set.

	TN	FN	TP	FP	Accuracy	Sensitivity	Specificity	Efficacy
All compounds inside AD (n=116)	92	0	3	21	81.9%	100.0%	81.4%	79.3%
nonB compounds inside AD (n=113)	92	0	0	21	81.4%	---	---	81.4%
B/vB compounds inside AD (n=3)	0	0	3	0	---	100.0%	---	---

---: not appropriate; for abbreviations and definitions see 2.4.

4.1.2 Comparison of the BCF waiving scheme with existing criteria

The BCF waiving scheme performs very well as compared to existing criteria with regard to reliability and efficacy. A regulatory criterion to identify nonB chemicals with the aim to waive experimental BCF studies with fish was implemented in the 1996 Technical Guidance Document (TGD) on risk assessment for new notified substances and existing substances (European Commission, 1996), stating *"… values of log K_{ow} greater than or equal to 3 indicate that the substance may bioaccumulate.*" This criterion was used to waive the BCF *in vivo* testing on fish of substances with log K_{ow} below 3 for being nonB. Figure 3 illustrates that the criterion of log K_{ow} 3 is useful and protective, avoiding BCF *in vivo* testing on fish of more than 40% of the nonB substances without false negatives. The individual log K_{ow} criterion of 3 is, however, outperformed by the BCF waiving scheme, classifying many more (at least 60%) of the nonB compounds.

Under REACH (ECHA, 2017), another log K_{ow} criterion is used: "*For the PBT and vPvB assessment a screening criterion has been established, which is log K_{ow} greater than 4.5. The assumption behind*

this is that the uptake of an organic substance is driven by its hydrophobicity. For organic substances with a log K_{ow} value below 4.5 it is assumed that the affinity for the lipids of an organism is insufficient to exceed the B criterion, i.e. a BCF value of 2000 …" Unfortunately, the latter assumption is not supported by empirical data. It can be seen from Figure 3 that a considerable number of B/vB substances (approx. 15%) have log K_{ow} between 3 and 4.5, thus, the threshold of log K_{ow} 4.5 is not protective. The B/vB compounds with log K_{ow} between 3 and 4.5 are mostly small aromatics, often with multiple halogenation. These B/vB compounds are misclassified by a screening criterion of log K_{ow} of 4.5, thus are false negatives with their bioaccumulation potential significantly underestimated. The individual log K_{ow} criterion of 4.5 is outperformed by the BCF waiving scheme with 100% sensitivity avoiding the false negative classifications.

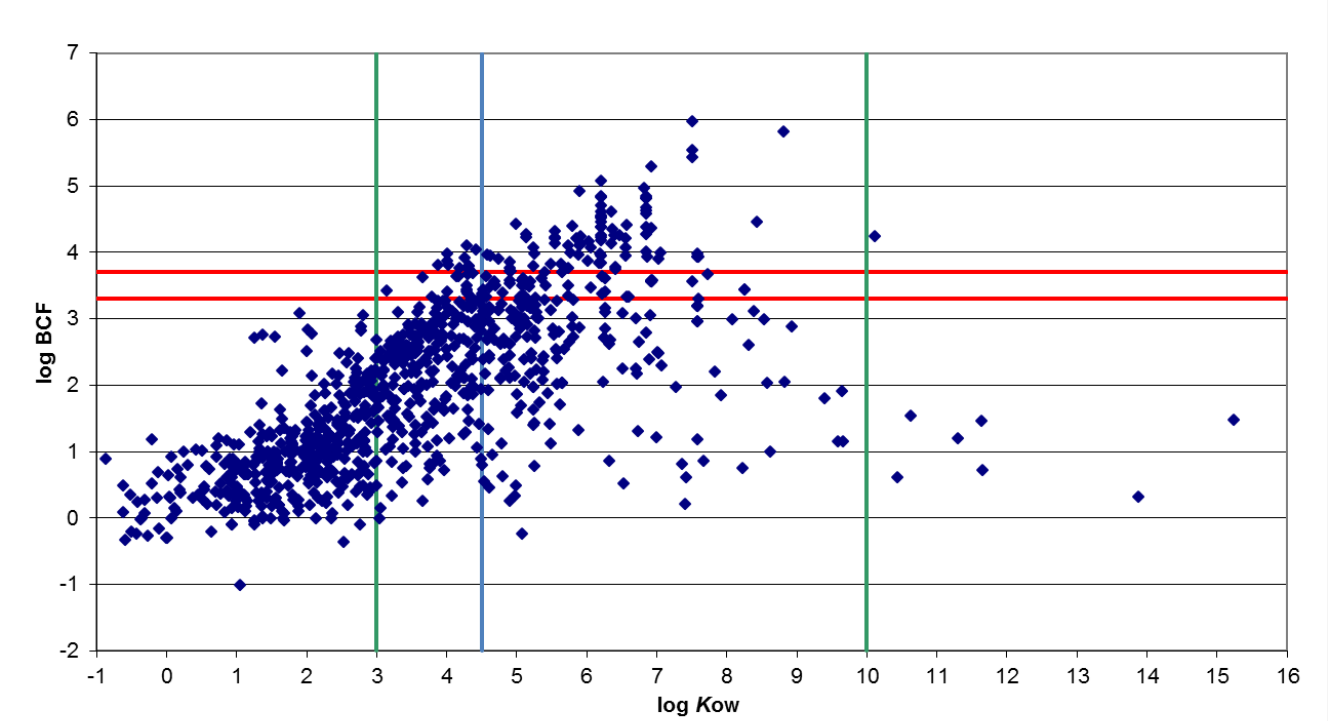


Figure 3. Empirical relationship between the log K_{ow} of chemicals and their log BCF. The horizontal lines indicate B (BCF <2000) and vB (BCF <5000) criteria. The vertical lines indicate cut-off criteria at log K_{ow} 3, 4.5 and 10, respectively.

4.2 Prediction confidence of the BCF waiving scheme

When developing the BCF waiving scheme, we focussed on safe criteria for the identification of nonB compounds being candidates for the waiving of BCF *in vivo* testing on fish, excluding false

441 negatives (sensitivity of 100%), though at the cost of false positives. The BCF waiving scheme predicts
442 as nonB rather a lower number of chemicals but with very high confidence. We prefer this approach
443 to conclude the absence of a concern as compared to models for more compounds but with lesser or
444 unknown reliability.

445 **4.2.1 Compliance of the BCF waiving scheme with the OECD principles for the validation of**
446 **QSAR models**

447 The acceptance of *in silico* predictions and alternative information in the regulatory framework, for
448 example REACH, depends on their scientific validity according to the OECD principles for the
449 validation of QSAR models (OECD, 2007). These requirements relate to a defined endpoint (see
450 section 2.1), an unambiguous algorithm (see section 3.3), a defined domain of applicability (see section
451 3.2), appropriate measures of goodness-of-fit, robustness and predictivity (see section 3.3 and 4.1.1),
452 and a mechanistic interpretation, if possible. The compliance of the BCF waiving scheme with the
453 OECD principles is presented in Table 4.

454 **Table 4.** Compliance of the BCF waiving scheme with the OECD principles (OECD, 2007).

OECD principle	BCF waiving scheme
Defined endpoint	Output of the BCF waiving scheme are nonB classifications according to REACH (European Commission, 2006) based on BCF according to OECD 305 (OECD, 2012) (BCF <2000 = nonB).
Unambiguous algorithm	The BCF waiving scheme combines QSAR classifications based on hydrophobicity (log K_{ow} <3), apparent partitioning (log D <3 if >5% ionisation at pH 7), degradability (ready biodegradability, hydrolysis), and solubility and volatility (log Henry's law constant <-11 [atm/(mol/L)]).
Defined domain of applicability	The AD of the BCF waiving scheme has been defined by structural rules (chemical classes, ACFs), excluding false negative outliers.
Appropriate measures of goodness-of-fit, robustness and predictivity	The BCF waiving scheme performs with 100% sensitivity (no false negatives) on the training set and the external validation set. Prediction confidence of the BCF waiving scheme is based on AD compliance, consensus modelling, and the structural similarity with known nonB and B/vB substances.
Mechanistic interpretation, if possible	The BCF waiving scheme combines QSAR classifications based on physicochemical properties related to the distribution, persistence, solubility and volatility of substances in water bodies and aquatic biota.

455

456 In addition to AD considerations (see section 3.2) and consensus modelling (see section 2.3), the
457 prediction confidence of the BCF waiving scheme for individual target compounds is also supported
458 by the number of physicochemical property criteria triggered, and the distance of property estimates

from thresholds: many compounds comply with more than one criterion. For example, a nonB chemical like piperazine (CAS 110-85-0, BCF <5) has log K_{ow} <3, is readily degradable, and dissociates >5% at pH 7. The intuition that classifications are more robust if target compounds meet multiple threshold criteria is supported by correlations of higher numbers of hits with decreasing mean and maximum BCF values.

Compounds with physicochemical properties far away from the threshold values are taken to be more safely classified. For example, the nonB classification of paraldehyde (CAS 123-63-7) with log K_{ow} 0.83 appears more reliable than that of o-toluidine (CAS 119-93-7) with log K_{ow} 2.95. Furthermore, a larger distance easily accommodates the margins of uncertainty of property estimates, in the case of log K_{ow} approximately 0.5 log units.

4.2.2 Structural similarity to nonB or B/vB substances

Confidence in nonB predictions increases if a target compound is more similar to the nonB subset than to the B/vB subset of our database (Kühne et al., 2007; Kühne et al., 2009). The nonB similarity index I_{nB} is based on the ratio of the similarities to nonB and B/vB substances (nonB/B ratio), respectively (see section 2.2). Positive values of I_{nB} indicate similarity to nonB compounds. If I_{nB} is negative, the target compound is more similar to B/vB substances. Common ACFs of a target compound with the chemicals in the nonB subset, but not with those in the B/vB subset, further support the confidence in nonB predictions.

Figure 4 illustrates the relationship between the log BCF values of the compounds and their similarity with nonB compounds expressed as I_{nB}. The horizontal red line at log BCF of 3.3 separates nonB (experimental BCF <2000) from B/vB compounds (experimental BCF >2000). The vertical black line separates chemicals similar to nonB compounds (right side) from chemicals more similar to B/vB compounds (left side). The B/vB compounds in our database (true positives) cluster in the upper left section of the graph, with a trend of lower I_{nB} associated with higher BCF. The lower right section collects substances that are more similar to nonB compounds (I_{nB} >0) and have BCFs below 2000 (true negatives). The empty upper right section confirms the fitness of I_{nB} to indicate nonB compounds (no

false negatives). Thus, a positive I_{nB} supports prediction confidence for the waiving of BCF tests. The lower left section indicates potential to improve the efficacy of the BCF waiving scheme. Various nonB chemicals with structural similarities to B/vB compounds but BCFs below 2000 need further parameters not yet included in the BCF waiving scheme to become candidates for non-testing.

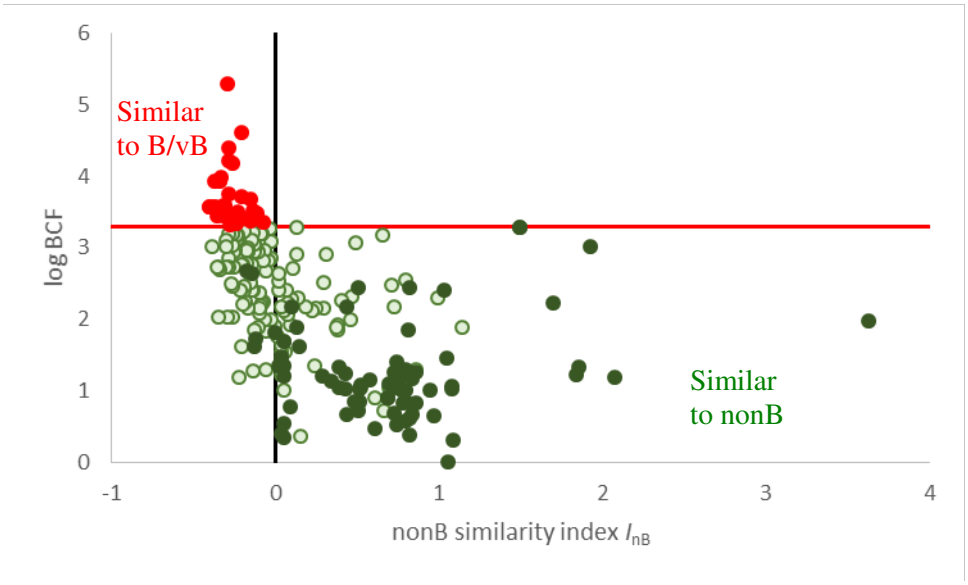


Figure 4. Similarity with nonB compounds (positive nonB similarity index $I_{nB} = S_{nB}/S_B - 1.3$ for chemicals with $S_{nB} \geq 0.75$) supports the prediction confidence in nonB classifications by the BCF waiving scheme. Symbols: dark green circles = nonB compounds identified by the BCF waiving scheme (true negatives), light green circles = nonB substances not identified by the BCF waiving scheme, red circles = B/vB compounds (true positives).

Replacing more than half of the BCF *in vivo* testing on fish with nonB predictions of the BCF waiving scheme based on valid and reproducible QSAR classifications and leaving the more difficult compounds (from a QSAR perspective) for further assessments (see section 4.3) is a considerable improvement as compared to present practice.

4.3 Assessment of chemicals not classified by the BCF waiving scheme

The assessment of compounds not positively identified as nonB substances can be done with non-testing approaches such as *in silico* methods including QSARs, read-across from similar substances or grouping and category formation. Suitable *in vitro* approaches may be partitioning assays with (artificial) membranes, parts of cells, whole cells or tissues including metabolic systems to simulate

506 the transformation in organisms (Lombardo et al. 2014; Nichols et al. 2007). Experimental screening
507 tools, for example for biodegradability in water/sediment systems (Junker et al., 2016), can further
508 improve the classifications. If *in silico* and *in vitro* methods do not allow a conclusion to be drawn
509 about the bioaccumulation potential in fish of a target chemical, *in vivo* approaches might be used.
510 This is preferable with reduced numbers of animals per test or with refined test design for the well-
511 being of test animals. Only as a last resort, a standard OECD 305 test (OECD, 2012) might be
512 conducted. A possible evaluation approach that includes the BCF waiving scheme is described in
513 Lombardo et al. (2014). It is an integrated testing strategy (ITS) that considers other *in silico* and *in*
514 *vitro* methods, and only as last resort *in vivo* tests.

515 ## 5. CONCLUSIONS

516 The BCF waiving scheme is a screening tool combining QSAR classifications based on
517 physicochemical properties related to the distribution, persistence, solubility and volatility of
518 substances in water bodies and aquatic biota. The BCF waiving scheme reliably identifies nonB
519 chemicals (sensitivity 100%: no false negatives) and supports robust decisions for waiving of
520 experimental BCF studies that are scientifically not necessary or technically not feasible. The
521 contribution to the 3Rs is a possible reduction of at least 50% of BCF *in vivo* testing on fish. Prediction
522 confidence of the BCF waiving scheme is based on AD considerations, consensus modelling, and the
523 structural similarity with known nonB and B/vB substances.

524 The remaining compounds have 'unknown bioaccumulation potential' and no conclusions are
525 possible regarding their nonB, B or vB properties. Since the remaining substances are more nonB than
526 B compounds, it shall be very clear that failure to classify as nonB by the BCF waiving scheme does
527 not mean that a substance is bioaccumulative. These chemicals require further assessments with *in*
528 *silico*, *in vitro* or *in vivo* tools to either confirm or reject bioaccumulative behaviour. Then, integrated
529 testing strategies (ITS) can provide guidance to come to a conclusion about possible concern with
530 regard to the B criterion (Lombardo et al., 2014).

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Supplementary Material

A detailed description of the training set and the external validation set (SI_1 Chemicals and data), the calculation of the similarity index I_{nB} including the ACF decomposition rules (SI_2 Similarity), and a documentation of the ChemProp implementation of the BCF waiving scheme (SI_3 ChemProp) can be found in the online version of this article, at doi: …..

Competing Interests Statement

The authors declare no competing financial interest.

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