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Reducing the number of fish in bioconcentration studies with general chemicals by reducing the number of test concentrations

Natalie Burden [a,*], Stuart Creton [b], Lennart Weltje [c], Samuel K. Maynard [d], James R. Wheeler [e]

[a] National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), Gibbs Building, 215 Euston Road, London NW1 2BE, UK
[b] Environmental Protection Authority, 215 Lambton Quay, Wellington 6011, New Zealand
[c] BASF SE, Crop Protection – Ecotoxicology, Speyerer Strasse 2, D-67117 Limburgerhof, Germany
[d] Syngenta Ltd, Product Safety, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK
[e] Dow AgroSciences, 3B Park Square, Milton Park, Abingdon, Oxfordshire OX14 4RN, UK

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ABSTRACT

Fish bioconcentration test guidelines generally require that bioconcentration factors (BCFs) are determined at two exposure concentrations. However, recent revisions to the OECD test guideline for bioconcentration testing (TG 305) provide the option to use only one exposure concentration, when justification is provided, although two concentrations may still be required for some regulatory purposes. Recently, this justification has been demonstrated for plant protection product active ingredients. To determine whether this justification has a broader validity for general chemicals, an analysis of 236 BCF studies on general chemicals was conducted. The results presented here again demonstrate that BCF values do not significantly differ between concentrations when more than one concentration is used. This relationship is particularly strong for BCFs $\geqslant$1000 L/kg, which is beneficial, since only chemicals with BCFs >2000 L/kg may require regulatory action. This analysis therefore provides a data-driven rationale for using the one test concentration approach for general chemical substances and thus could contribute to a substantial reduction in the use of fish in bioconcentration tests.

1. Introduction

Fish bioconcentration studies are used to determine the potential for substances to bioaccumulate. General chemicals are regulated in Europe under the REACH Regulation (EC) No. 1907/2006. REACH requires for chemicals produced at $\geqslant$10 tonnes per year, which are not exempted from the registration requirement, to undergo a Persistence, Bioaccumulation and Toxicity (PBT) assessment. The relevant properties are laid down in Annex XIII of the Regulation. Potential classifications are PBT or vPvB (very Persistent and very Bioaccumulative). Substances with a log octanol–water partitioning coefficient $\leqslant$4.5, whether experimentally determined or estimated by a valid Quantitative Structure Activity Relationship (QSAR), are considered not to be bioaccumulative. However, those above this screening criterion require measured data on bioconcentration in an aquatic species typically using a fish bioconcentration test (OECD, 2012), resulting in the estimation of a bioconcentration factor (BCF). A substance is considered to have met the B criterion if the BCF >2000 L/kg and vB if the BCF >5000 L/kg. Classifications of PBT or vPvB trigger an emission and risk characterisation assessment (i.e. characterisation of all emissions throughout the lifecycle of the substance), with actions that minimise exposure of humans and the environment. Fish BCF data may also be used in secondary poisoning risk assessments (i.e. fish-eating mammals or birds).

Bioconcentration tests are time and resource intensive and require large numbers of animals. The OECD flow-through fish test (test guideline [TG] 305) requires the use of at least three experimental groups (a control plus a low and high concentration exposure group), with a minimum of four fish per group sampled on at least five occasions during the uptake phase and on at least four occasions during the elimination phase – i.e. a minimum of 108 animals. In practice larger numbers (circa 150 or more) are used to allow for flexibility in case a potentially longer exposure phase is required and to allow for any incidental mortality. Under the REACH Regulation there are obligations to address animal use via data sharing, read-across and the implementation of alternative methods. Consequently, several activities to develop suitable alternative methods (Gissi et al., 2013; Scholz et al., 2013) are on-going. However, the most recent data shows that the majority of new tests on non-mammalian vertebrates required under REACH Annex IX

* Corresponding author.
E-mail address: Natalie.Burden@nc3rs.org.uk (N. Burden).

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and X (chemicals manufactured or imported at or above 100 and 1000 tonnes per annum (tpa), respectively) were carried out for bioaccumulation testing in fish (ECHA, 2014a). In the shorter term, and considering the expected high number of registrations as a result of the 2018 deadline (substances supplied at $\geqslant$1 tpa; ECHA, 2014b), a reduction in animal use may be best achieved using the OECD test guideline option to employ only one exposure group if concentration independence of the BCF can be justified. In an analysis of 55 high quality BCF studies for plant protection product active ingredients, it was demonstrated that BCF values from low and high test concentrations did not differ significantly (Creton et al., 2013), thereby justifying the use of only one test concentration and so reducing the number of test animals by one third. This paper aims to provide a similar quantitative, data-driven analysis to establish if a single test concentration is justified when testing general industrial chemicals. We hope that a robust demonstration, based on available experimental data, will lend support to a reduced animal approach for studies conducted according to OECD TG 305. This initiative originated from discussions at the ecotoxicology working group of the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

2. Materials and methods

Data were extracted from a quality assessed reference database of fish bio-concentration factors. This 'gold standard' database was developed for the CEFIC Long-range Research Initiative (project ECO7: "Development of a Reference Database: Bio-concentration Factors BCF") and is freely available at http://ambit.sourceforge.net/euras/. The version used carried the Excel file name 'CEFIC_BCF_2008-01-08.xls'. Data included had been quality assessed by applying the Klimisch reliability criteria (Klimisch et al., 1997) or by their inclusion in the METI database (the Japanese Ministry of Economy Trade and Industry) which was also deemed to be an indicator of high quality (Versonnen et al., 2008). Only data of Klimisch score 1 or 2 and METI data were included in the analyses presented here. Any data on plant protection products contained in the 'gold standard' database was removed as these have been assessed before (see below). After poor quality and truncated data (i.e. less than values) were removed, data were available for 236 chemicals employing two test concentrations. Therefore, in total there were 236 comparisons of BCF values determined from low and high exposure concentrations. All tests were conducted with carp except for two comparisons with male and female fathead minnow with Aroclor 1254 (BCF values in the separate sexes were similar so they were combined by taking the arithmetic mean). The lack of species diversity can be explained by the majority of the data used (235 of 236) being derived from the METI database and carp being the preferred Japanese test species.

The 236 data pairs (low and high concentrations) represented various chemical classes and a wide range of measured whole body BCF values (from 0.3 to 235,000 L/kg). Substances within the database identified as being ionisable according to Fu et al. (2009) were highlighted in the analysis as these chemicals may show concentration-dependent bioconcentration (Beek et al., 2000). The median fold difference between the employed low and high test concentrations was 10, as would be expected given that this is the recommended spacing factor for test concentrations in the test guidelines (OECD, 2012 and EPA, 1996).

Data were tested for normality using the Shapiro Wilk's test ($\alpha = 0.05$). As they were not normally distributed, the data were then compared using a non-parametric Wilcoxon matched pairs test ($\alpha = 0.05$). All statistical analyses were performed in GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

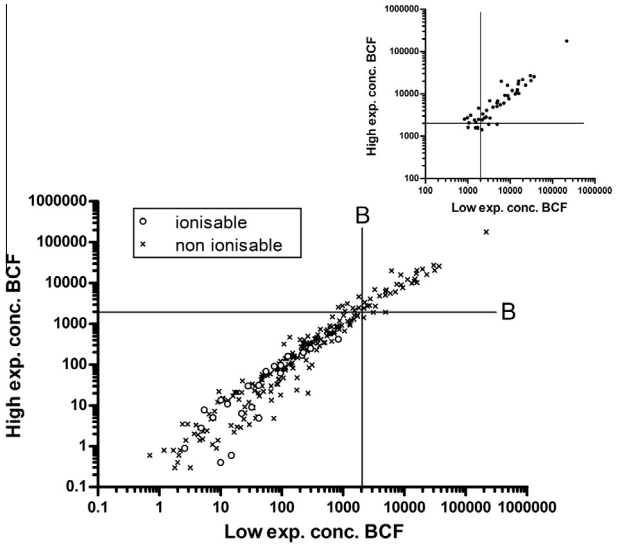

Fig. 1. Relationship between BCF values from low and high exposure concentrations (whole body). B = trigger for classifying chemicals as bioaccumulative (BCF >2000 L/kg). $n = 236$ for all substances or $n = 22$ for ionizable substances. The inset graph indicates substances likely to have a $\log K_{ow} \geqslant 4.5$ (see text).

3. Results

The relationship between low and high concentration BCF values is shown in Fig. 1. The data were not normally distributed ($p < 0.0001$; $\alpha = 0.05$; Shapiro–Wilk's normality test), and therefore the comparisons of low and high concentration BCFs were made by the Wilcoxon matched pairs test. There were no significant differences ($p = 0.0841$), confirming that there is no difference between BCF values determined using a low or a high test concentration across the dataset. For the sub-set of ionisable substances ($n = 22$) the data were also not normally distributed ($p < 0.0001$; $\alpha = 0.05$; Shapiro–Wilk's normality test), however, a significant difference ($p = 0.0386$) was observed (Wilcoxon matched pairs test). To examine the robustness of this result, the Wilcoxon matched pairs analysis was repeated for each combination of 21 data pairs, each time excluding one data pair of the full ($n = 22$) data set. This showed that the statistical result was highly dependent on single data pairs, as in 11 cases the significance between the high and low exposure concentration BCF values disappeared; most notably when one of the four highest BCF data pairs was excluded. Overall, the difference between the two BCF values was generally relatively small. To further analyse these differences they were expressed as a ratio of BCF values from low: high concentration. The median ratio was 1.10 (25% percentile 0.825; 75% percentile 1.675).

However, it is important to note that this analysis included substances with a broad range of log octanol–water partitioning coefficients; including values that would not trigger fish BCF testing ($\log K_{ow} \leqslant 4.5$). Unfortunately, the database used does not capture the $\log K_{ow}$ values. Therefore, the relationship of K_{ow} to BCF, according to Mackay (1982),[1] was used to identify those compounds with a $\log K_{ow}$ of $\geqslant 4.5$. According to Mackay's equation a BCF of $\geqslant$1518 L/kg is equivalent to a $\log K_{ow}$ of 4.5. This identified 47 substances based on the low or high concentration BCF (20% of the dataset) that likely have $\log K_{ow}$ values greater than 4.5, i.e. representative of substances that would actually trigger bioconcentration testing. Significance testing confirmed no difference between BCFs of high and low exposure concentrations (Wilcoxon matched pairs test, $p = 0.60$) when only compounds with predicted $\log K_{ow}$ values of $\geqslant 4.5$ were

[1] BCF = 0.048 * K_{ow}.